Free-Radical Ring Closure to Conformationally Locked \(\alpha\-L\)-Carba-LNAs and Synthesis of Their Oligos: Nuclease Stability, Target RNA Specificity, and Elicitation of RNase H

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A new class of conformationally constrained nucleosides, \(\alpha\-L\)-ribo-carbocyclic LNA thymidine (\(\alpha\-L\)-carba-LNA-T, LNA is an abbreviation of locked nucleic acid) analogues and a novel “double-locked” \(\alpha\-L\)-ribo-configured tetracyclic thymidine (6,7\(^0\)-methylene-bridged-\(\alpha\-L\)-carba-LNA-T) in which both the sugar puckering and glycosidic torsion are simultaneously constrained, have been synthesized through a key step involving 5\(^{\text{exo}}\) free-radical intramolecular cyclization. These \(\alpha\-L\)-carba-LNA analogues have been subsequently transformed to corresponding phosphoramidites and incorporated into isosequential antisense oligonucleotides (AONs), which have then been examined for the thermal denaturation of their duplexes, nuclease stability, and RNase H recruitment capabilities. Introduction of a single 6\(^0\),7\(^0\)-substituted \(\alpha\-L\)-carba-LNA-T modification in the AON strand of AON/RNA heteroduplex led to \(T_m\) reduction by 2–3 °C as compared to the native heteroduplex, whereas the parent 2\(^\text{oxa-}\alpha\-L\)-LNA-T modification at the identical position in the AON strand has been found to lead to an increase in the \(T_m\) by 3–5 °C. This suggests that the 6\(^0\) and 7\(^0\) substitutions lead to much reduced thermal stability for the modified heteroduplex, especially the hydrophobic 7\(^0\)-methyl on \(\alpha\-L\)-carba-LNA, which is located in the major groove of the duplex. All of the AONs incorporating 6\(^0\),7\(^0\)-substituted \(\alpha\-L\)-carba-LNA-T have, however, showed considerably improved nuclease stability toward 3\(^{\text{exonuclease}}\) (SVPDE) and in human blood serum compared to the 2\(^\text{oxa-}\alpha\-L\)-LNA-T incorporated AONs. The hybrid duplexes that are formed by 6\(^0\),7\(^0\)-substituted \(\alpha\-L\)-carba-LNA-T-modified AONs with complementary RNA have been found to recruit RNase H with higher efficiency than those of the \(\beta\-D\)-LNA-T or \(\beta\-D\)-carba-LNA-T-modified counterparts. These greatly improved nuclease resistances and efficient RNase H recruitment capabilities elevate the \(\alpha\-L\)-carba-LNA-modified nucleotides into a new class of locked nucleic acids for potential RNA targeting therapeutics.
Introduction

Among the various genes of silencing technologies, the antisense strategy\(^1\) and RNA interference\(^2\) are considered to be extremely potent in terms of ensuring complete target mRNA breakdown. During the last couple of decades, exploration and examination of novel chemically modified oligonucleotides that act as potent and selective therapeutic agents have gained momentum and have led to the development of analogues which have good pharmacokinetics and minimum toxicity. In general, there are three main types of possible chemical modifications of native nucleotides, and each involves modification of either sugar, phosphodiester linkage, or nucleobase.\(^3\)

In recent years, a variety of conformationally constrained nucleotides\(^6,7\) have been synthesized and incorporated into antisense oligonucleotides (AONs) and/or siRNAs for in vitro biological studies. One particular class of compounds, so-called locked nucleic acid (LNA),\(^8\) also called BNA,\(^9,10\) is the abbreviation of bridged nucleic acid) containing a methylene linkage between the 2'-oxygen and 4'-carbon of the ribose ring, has attracted extensive interest due to their remarkable hybridization properties.\(^8\) The excellent thermodynamic features of LNA stimulated synthesis of a number of other North-type conformationally constrained nucleoside analogues, such as amino-LNA,\(^11\) β-cyclonucleoside,\(^12\) C\(^6\)-substituted LNA,\(^13,14\) BNA\(^{-}\)COC,\(^15\) BNA\(^{-}\)NC,\(^16,17\) 2'-O,4'-C-ethylene-bridged nucleic acid (ENA),\(^18,19\)aza-ENA,\(^20,21\) carba-ENA\(^22,23\) etc. All of these locked nucleoside-modified AONs have shown much better antisense properties, such as RNA affinity, nuclease resistance, and RNase H elicitation, as compared to that of the native counterpart. Some of these locked nucleotides have been tested for siRNA action and have shown remarkable silencing efficiency.\(^25,26\)

Recently, carba-LNA, carba-ENA, C\(^6\),C\(^7\)-substituted carba-LNAs, and C\(^6\),C\(^8\)-substituted carba-ENAs have been synthesized by our group through a key step involving intramolecular free-radical ring closure.\(^27–32\) The striking feature of carba-LNA and carba-ENA derivatives is that they render much better nuclease resistance for modified AONs than LNA.\(^32\) The carbocyclic ring of carba-LNA and carba-ENA also provides an effective handle for engineering new types of modifications in the minor groove, which can significantly modulate important antisense properties such as target RNA affinity, nucleic acid resistance without impairing their RNase H recruitment capabilities. Another intriguing class of conformationally constrained nucleoside, α-L-LNA, is a diastereomer of LNA\(^3,8\) referred to as β-D-LNA further in the text in order to distinguish from α-L-LNA. The α-L-LNA has also been synthesized by Wengel et al.\(^3,40\) Thermal stability of α-L-LNA-modified AON with complementary nucleic acid targets was found to be comparable to that of LNA-containing counterparts. Moreover, it has been shown that the α-L-LNA modification renders better nucleic acid resistance compared to LNA modification.\(^30\) In vitro and in vivo experiments showed that α-L-LNA-modified AONs retain the ability of RNase H elicitation and exhibit even higher gene knockdown efficacy than LNA-modified counterparts.\(^31,42\)

The striking biochemical features of α-LNA prompted us and others 43,44 to synthesize α-L-ribo-carba-LNA, in which a methylene group replaces the 2'-oxygen of α-LNA yielding a 2',4'-carbocyclic locked ring. Unlike the 2',4'-carbocyclic locked ring in carba-LNA (referred to as β-o-carba-LNA further in the text in order to distinguish from α-L-carba-LNA), which is located in the minor groove of AON/RNA duplexes, the 2',4'-carbocyclic ring in α-L-carba-LNA is located in the major groove of AON/RNA duplexes (see Figure SII 41 and 42 in Supporting Information). Hence C6',7'-substituted α-L-carba-LNAs are also very good models to study how different substitutions in the major groove modulate biophysical and biochemical properties of AON/RNA duplexes. Herein, we report a viable synthetic route to 6',7'-substituted α-L-carba-LNA thymidine derivatives (Figure 1K–M) along with a so-called “double-locked” tetra cyclic α-L-carba-LNA thymidine (Figure 1N) formed by the intramolecular addition of methylene free radical to thymine nucleobase. In order to evaluate α-L-carba-LNA nucleosides’ potential for therapeutic applications, all of these modified nucleotides have subsequently been introduced into AONs to study thermal stabilities of their duplexes with complementary RNA or DNA, as well as the 3'-exonuclease resistances, human blood serum stabilities, and RNase H recruitment capabilities in comparison with that of α-LNA and β-o-carba-LNA-modified counterparts.

Results and Discussion

1. Synthesis of Diastereomically Pure 6',7'-Substituted α-L-Carba-LNAs and 6',7'-Methylene-Bridged α-L-Carba-LNA-T. The synthetic route to the 6',7'-substituted α-L-carba-LNA thymidine phosphoramidites 16a, 16b, and 16c is shown in Schemes 1–3. The synthesis started from the known nucleoside 3,5-di-O-benzyl-4-hydroxymethyl-1,2-O-isopropylidene-β-L-ribofuranose 1,45 which was oxidized to the corresponding aldehyde 2 through Swern oxidation. The crude aldehyde was subjected to Grignard reaction with vinylmagnesium bromide to afford the key free-radical cyclization product 3a (6'R-OH) in moderate yields (32% for 3a, 29% for 3b in two steps). The configuration at C6' could not be confirmed by nuclear Overhauser effect (NOE) experiment at this stage. This could, however, be confirmed after the free-radical cyclization step (see section 2 for discussion on NMR characterization), which subsequently showed the C6'-R and S-stereochemistry for 3a and 3b, respectively. Acetylation of 3a with a mixture of acetic anhydride, acetic acid, and triflic acid gave the corresponding triacetate 4a as an amonic mixture. The crude triacetate 4a was then subjected to a modified Vorbrüggen reaction46 involving in situ silylation of the thymine and subsequent trimethylsilyl triflate mediated coupling to give exclusive α-L-ribofuranosyl thymine derivative 5a. Deacetylation of 5a using 30% methyamine in ethanol at room temperature overnight gave compound 6a quantitatively, which was treated with phenyl chlorothioformate to afford the key free-radical precursor 7a (6'-R) in high yield (86%) without any formation of 6'-O-phenoxythiocyanoethyl (PTC) product even when 2.5 equiv of phenyl chlorothiocarbonyl (PTC) product was used. We have also similarly synthesized the second free-radical precursor 7b (6'-S) from 3b (6'-S) (Scheme 1).

The free-radical cyclization was carried out in refluxing anhydrous toluene with Bu3SnH, using AIBN as the initiator, which was added dropwise in order to avoid the side reactions. Cyclization of 7a (6'-R) took place with high stereoselectivity

![Image](https://example.com/image.png)

**FIGURE 1.** Structures of β-o-LNA (A),7–9 β-o-carba-LNA (B),27–29 α-LNA (C),33–37 6'–Me-α-LNA (D),44 2’-amino-α-LNA (E) and derivatives (F–H),40 tetra cyclic 2’-amino-α-LNA (I),39 7’-methylene-α-L-carba-LNA (J); This nucleoside was reported in a recent patent,45 but it was not characterized, not even partially, by either 1H or 13C NMR. Instead, only 31P NMR of the corresponding phosphoramidite was supplied, which in fact could be the same for any nucleoside phosphoramidite). 6’,7’-Substituted α-L-carba-LNA derivatives (K–M), along with a novel double-locked α-L-carba-LNA analogue: 6’,7’-methylene-bridged α-L-carba-LNA-T (N).

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to give α-t-carba-LNA nucleoside 8a (6′R-OH, 7′S-CH₃) in good yield (57%, Scheme 2). Under an identical condition, cyclization of 7b (6′′-S), on the other hand, gave α-t-carba-LNA nucleoside 8b (6′′S-OH, 7′′S-CH₃) in 43% yield along with an unexpected product 8c in 14% yield (Scheme 2). The mechanism of the free-radical cyclization reaction for the formation of 8a, 8b, and 8c will be discussed in section 3.

In an effort to remove the 6′S-OH in compound 8b by radical deoxygenation strategy, we attempted to esterify the 6′-OH using phenyl chlorothioformate but failed. This suggested that 6′S-OH is much more inert than the 2′-OH group in the pentose sugar moiety. Transformation of OH to (methylthio)thiocarbonate has been proven to be an efficient solution to remove an inert hydroxyl group by radical deoxy-
genation.⁴⁷ Thus, 8b was treated with CS₂ and MeI along with NaH as a base at 0 °C for 4 h, giving the radical precur-
sor 9 in 64% yield. Then, compound 9 was subjected to the standard Barton–McCombie deoxygenation⁴⁸ in the presence of Bu₃SnH and AIBN to furnish 7′S-CH₃-α-t-carba-
LNA nucleoside 10 in 42% yield plus an unexpected bicyclo-
[2.2.1]-2′,6′-methylene-bridged hexopyranosyl nucleoside 11 in 7% yield (Scheme 2). The radical rearrangement process⁴⁹ that leads to 11 will be discussed in section 3.

In order to convert 8a/b to the corresponding phosphor-
amidites for solid-supported DNA synthesis, the 6′-OH

group of 8a and 8b must be protected (Scheme 3). Thus compounds 8a and 8b were treated with p-toluoyl chloride in dry pyridine to give compounds 12a and 12b in 73 and 71% yield, respectively. Then compounds 12a, 12b, and 10 were subjected to debenzylation using 20% Pd(OH)$_2$/C and ammonia formate followed by selectively protecting the 5'-OH with DMT, giving 13a, 13b, and 13c, respectively. It should be noted that debenzylation of 12b should be limited to 20 min, and a prolongation of reaction time led to cleavage of a 6'-O-p-toluoyl group by the attack of the primary 5'-OH group. 29 In order to invert the configuration of 3'-OH, compounds 13a, 13b, and 13c were oxidized with Dess–Martin periodinane 30–32 followed by the reduction with sodium borohydride in ethanol to give 15a, 15b, and 15c, respectively, in 57–71% yields. The complete inversion of 3'-OH is facilitated by the carbocyclic ring and 7'-Me on the top side of the furan sugar, which leads to significant steric hindrance, thus promoting exclusive attack of the hydride from the bottom of the furanose sugar ring. Compounds 15a, 15b, and 15c were then phosphorylated with 2-cyanoethyl-N,N-diisopropylphosphorinochloridite under standard conditions, 27–29 giving phosphoramidites 16a, 16b, and 16c, respectively, as a diastereomeric mixture in 63–71% yield.

The unexpected product 8c obtained during free-radical cyclization of 7b was also transformed to corresponding phosphoramidite 22 (Scheme 4). Thus, 8c was treated with CS$_2$, Mel, and NaH, leading to the successful acquisition of radical precursor 17 in 69% yield, followed by the Barton–McCombie deoxygenation 48 affording product 18 in high yield (95%).

Debenzylation of 18 using 20% Pd(OH)$_2$/C and ammonium formate, followed by 5'-O-dimethoxytritylation smoothly gave 19 (72% in two steps). Since oxidation of 19 with Dess–Martin periodinane failed to give the corresponding ketone, another mild oxidizing reagent known as TPAP (tetra-n-propylammonium perruthenate) was used. 53 Therefore, compound 19 was treated with catalytic amount of TPAP along with the stoichiometric oxidant NMO (N-methylmorpholine-N-oxide) at room temperature to afford 20, which was directly subjected to the reduction with sodium borohydride at −20 °C, giving products 21 (22% in two steps) plus recovery of starting material 19 (19% in two steps). Subsequently, the phosphorylation of 21 was performed with 2-cyanoethyl N,N-diisopropylphosphoramidochloridite under standard conditions 27–29 to furnish 22 (73% yield) as a diastereomeric mixture.

2. NMR Characterization of Key Intermediates Involved in the Synthesis of α-L-Carba-LNA Analogues. All of the key carbocyclic nucleoside intermediates have been characterized by 1H, 13C, COSY, 3H–1H HMBC, long-range 1H–13C correlation (HMBC) NMR experiments as well as by mass spectroscopy (see Supporting Information).

The formation of bicyclic systems in compounds 8a, 8b, 11, and the tetracyclic system in compound 8c was confirmed by HMBC and COSY experiments (discussed in details in part II of Supporting Information).

The orientation of substituents in the carbocyclic moiety of compound 8a and 8b was determined by 1D NOE experiments (Figure 2) as well as from the vicinal coupling constants evaluation. For compound 8a, irradiation of H6 of thymine led to NOE enhancement for H7 (2.6%) ($d_{\text{H6-H7}} \approx 2.5$ Å) and for 6'-OH (0.9%) ($d_{\text{H6'-OH}} \approx 3.2$ Å, but none for 7'-CH$_3$ and H6 (d$_{\text{H6-7'-CH$_3$}} \approx 4.2$ Å, d$_{\text{H6-H6'}} \approx 4.6$ Å), whereas irradiation of 7'-CH$_3$ led to NOE enhancement for H6 (1.7%) ($d_{\text{H6-H7'-CH$_3$}} \approx 2.5$ Å) and H3' (1.7%) ($d_{\text{H3'-H7'-CH$_3$}} \approx 2.4$ Å), but none for 6'-OH ($d_{\text{6'-OH}} \approx 3.9$ Å), strongly suggesting that C6' is in 6'R configuration, and C7' is in 7'S configuration. In addition, the trans disposition of H6' and H7' was also in agreement with the small coupling constant.

for $^3J_{\beta,\gamma}$ (3.6 Hz, hence dihedral angle H6'–C6'–C7'–H7' ≈ 232° according to Karplus equation). As for compound 8b, irradiation of H6 of the thymine group led to NOE enhancement for H7' (1.4%) ($d_{H6'-H7'} ≈ 2.8$ Å) and H6' (2.7%) ($d_{H6'-H7'} ≈ 2.5$ Å), but none for $^7$-CH3 and $^6$-OH ($d_{H6'-CH3} ≈ 4.3$ Å, $d_{H6'-OH} ≈ 4.4$ Å), whereas irradiation of $^7$'-CH1 leads to NOE enhancement for H3' (1.6%) ($d_{H3'-CH1} ≈ 2.4$ Å), but none for H6' ($d_{H6'-CH1} ≈ 3.8$ Å), suggesting that C6' is in 6'S configuration, and C7' is in 7'S configuration. Furthermore, the large coupling constant for $^3J_{\beta,\gamma}$ (8.5 Hz) corresponding to the dihedral angle of H6'–C6'–C7'–H7' ≈ 26° also suggested a cis disposition of H6' and H7' in 8b.

The stereochemistry of compound 11 was also determined by 1D NOE experiment (Figure 2). Therefore, selective irradiation of H4' led to distinct NOE enhancement for H3' (3.8%) ($d_{H4'-H3'} ≈ 2.3$ Å), and irradiation of $^7$'-CH1 led to NOE enhancement for H3' (1.2%) ($d_{H4'-CH1} ≈ 2.4$ Å) and H4' (1.4%) ($d_{H4'-CH1} ≈ 2.4$ Å), respectively, which suggested that H3' and H4' are cis oriented and they are on the same face as that of $^7$'-CH1. The observation that irradiation of H1' led to NOE enhancement for H5', S' (1.1%) ($d_{H1'-H5'} ≈ 2.8$ Å) but none for H3' ($d_{H1'-H3'} ≈ 3.7$ Å) and H4' ($d_{H1'-H4'} ≈ 4.2$ Å) suggested that H5', S' are located on the face close to H1'. Hence, both C3' and C4' are in R configuration for compound 11 (Figure 2).

For compound 8c, in which four new chiral centers have been formed during a single free-radical cyclization step, the configuration of every chiral center was also well determined by 1D NOE experiments (see Figure 2). Selective irradiation of H6 in the thymine moiety led to strong NOE enhancement for H6' (4.9%) ($d_{H6'-H6'} ≈ 2.3$ Å) and H8' (2.4%) ($d_{H6'-H8'} ≈ 2.4$ Å), but none for $^6$-OH and H7' ($d_{H6'-H7'} ≈ 4.1$ Å, $d_{H6'-H7'} ≈ 3.8$ Å), unequivocally suggesting that C6' is in 6'S configuration, C7' is in 7'S configuration, and C6 is in 6'S configuration, respectively. The trans disposition of H6' and H7' was also consistent with the coupling constant ($^3J_{\beta,\gamma} = 2.0$ Hz, with dihedral angle H6'–C6'–C7'–H7' ≈ 116°).

In addition, selective irradiation of H8'' of 19 (having the same carbon skeleton as 8c) led to strong NOE enhancement for H5' (5.2%) ($d_{H5'-H8''} ≈ 2.3$ Å) and for H2' (1.2%) ($d_{H2'-H8''} ≈ 2.8$ Å), but none for 5-CH3 group ($d_{H5-CH3} ≈ 3.8$ Å), suggesting that C5 is in 5'S configuration.

The configuration of 3'-OH in compounds 15a/b/c and 21 was determined by 1D NOE experiment. Irradiation of H3' showed 1.3, 0.6, and 0.5% NOE enhancements for H1' ($d_{H3'-H1'} ≈ 2.3$ Å), H2' ($d_{H3'-H2'} ≈ 2.7$ Å), and H5', respectively, in compound 15a (Figure SII.12 in Supporting Information), 3.2 and 1.9% NOE enhancements for H1' and H5', respectively, in compound 21 (Figure SII.11). Irradiation of H1' led to 1.7 and 1.2% enhancement for H2' ($d_{H1'-H2'} ≈ 2.3$ Å) and H3' ($d_{H1'-H3'} ≈ 2.5$ Å), respectively, in 15b (Figure SII.13) and 2.5 and 2.0% NOE enhancement for H2' and H3', respectively, in compound 15c (Figure SII.14). These observations thus unequivocally indicated that 3'-OH's are in pseudoequatorial positions, while H3' and H1' are in pseudaxial positions in compounds 15a/b/c and 21.

### 3. Mechanism of the Free-Radical Cyclization Reaction and Radical Rearrangement

It is known that the 5-hexenyl intramolecular ring-closure reaction undergoes preferentially 1,5-ring closure (endo mode) over 1,6-ring closure (exo mode), yielding the thermodynamically less stable 5-exo-cyclization product. The previous studies of 5-hexenyl and 6-heptenyl cyclization of the substituted β-d-carba-LNA and β-d-carba-ENA analogues in our lab have also suggested that the formation of bicyclic product is going predominantly via exo mode ring closure over endo mode. After treatment of 7a with Bu3SnH and AIBN, the C2' radical is supposed to be generated (TS1 in Scheme 5A), which should be capable of attacking the C=C double bond from both the “top” and “bottom” faces by the 5-exo cyclization pathway, resulting into two plausible intermediates, TS2 and TS3 (see Scheme 5A). The optimized structures have shown that in the TS2 state, the thymine moiety,
developing 7'-CH$_2^*$ radical and 6'-OH are all occupying the axial positions. The steric hindrance between them makes TS2 much more unstable than TS3 because, in the TS3 state the thymine moiety, developing 7'-CH$_2^*$ radical and 6'-OH are occupying the axial, equatorial, and axial positions, respectively (Scheme 5A). This comparison may explain why the exclusive formation of cyclic product 8a (6'R,7'S) has been observed.

Similarly, cyclization of 7b can proceed through intermediates TS5 and TS6 (Scheme 5B). In TS5, the cis orientation of 6'-OH (eq) and 7'-CH$_2^*$ radical (eq) is favored because of steric hindrance, but the orientation between thymine moiety (ax) and 6'-OH (eq) as well as 7'-CH$_2^*$ radical (eq) is favored. On the other hand, in TS4, the trans orientation of 6'-OH (eq) and 7'-CH$_2^*$ radical (ax) is favored, but 1,3-diaxial disposition of thymine (ax) and 7'-CH$_2^*$ radical (ax) is unfavorable. Taking together, cyclization of 7b through both TS5 and TS6 is possible to give product 8b and 8c with TS5 predominating since the products 8b and 8c were obtained in a ratio of 3:1. The formation of minor product 8c starting from TS6 can be easily understood as follows: First, in TS6, the primary –CH$_2^*$ is not stable and will be intramolecularly trapped by the double bond of the thymine moiety before being quenched by Bu$_3$SnH to give intermediate TS7. Then, the newly formed radical center at C5 of the
SCHEME 5. Mechanism of the Formation of 8a, 8b, 8c, and 11 by Intramolecular Free-Radical Cyclization or Rearrangement

Steric hindrance makes this pathway improbable.

Theoretically, C2' radical attack to the C=C from both faces are possible.

thymine moiety was reduced by Bu₃SnH from the less sterically hindered face, giving chiral C5 in S-configuration. Hence, tetracyclic nucleoside 8c was obtained through aza-Michael addition of the 2'-amino to C6 position of thymine. Moreover, the formed 2'-aminothymine 8c was supposed to have gone through a similar mechanism. Thus after treatment of compound 9 with Bu₃SnH and AIBN in refluxing toluene, 6'-C6 bond and 6'-OH of β,δ-carba-LNA nucleoside 9 was putatively formed (Scheme 5C), which can be reduced by Bu₃SnH directly to give product 10. On the other hand, the 6'-C6 bond could also lead to scission of the C4'-O4' bond to give a new C6'=C4' double bond. As a result, the 4'C radical was transformed to 4'O' radicals. Then the 4'O radical attacked the C6'=C4' radical again, resulting in formation of O4'-C6' bond and the rearrangement of 4'O' radical to 4'C, which was reacted with Bu₃SnH by the less hindered face to furnish hepxopyranosyl nucleoside 11.

4. Synthesis and Purification of α-1-Carba-LNA Derivatives and α-1-LNA-Modified AONs. The phosphoramidites 16a/b/c and 22 as well as α-1-LNA monomers were incorporated as monosubstitutions, but at four different sites in a 15-mer DNA sequence on an automated RNA/DNA synthesizer. With exception of specially designed fast deprotecting reagents and cycles were utilized to synthesize the DNA oligos targeted to coding region of SV 40 large T antigen. The sequences, modification site, and structures of modifications are shown in Table 1. All of the modified building blocks gave modest values measured as the maximum of the first derivative of the melting curve (ΔT_m) in medium salt buffer (60 mM Tris-HCl at pH 7.5, 60 mM KCl, 0.8 mM MgCl₂) with temperature range of 20 to 65 °C using 1 μM concentrations of the two complementary strands. The value of ΔT_m gives the average of two or three independent measurements. If the error of the first two measurements exceeded ±0.3 °C, the third measurement was carried out to confirm if the error is indeed within ±0.3 °C. ΔT_m values were obtained by comparing the T_m values of AONs 2–21 with that of native AON 1. The ΔT_m values obtained here is the average value for four AONs incorporated with the same compound at four different modification sites. The RNA selectivity ΔΔT_m was calculated by this equation: ΔΔT_m = ΔT_m of AON/RNA − ΔT_m of AON/DNA. ΔT_m of AON1/RNA and AON1/DNA are adopted for T_m comparison; therefore, their ΔT_m values are set to 0.

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<th>Major groove Average ΔT (ave)</th>
<th>With DNA ΔT_m</th>
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<td>+2.8</td>
<td></td>
</tr>
<tr>
<td>AON11</td>
<td>6'-C6'-H</td>
<td>5'-d (CTT CAT TTT TCC TCT)</td>
<td>+5.0</td>
<td>+1.8</td>
<td>+1.8</td>
<td>+2.8</td>
<td></td>
</tr>
<tr>
<td>AON12</td>
<td>6'-C6'-H</td>
<td>5'-d (CTT CAT TTT TCC TCT)</td>
<td>+5.5</td>
<td>+0.9</td>
<td>+0.9</td>
<td>+2.2</td>
<td></td>
</tr>
<tr>
<td>AON13</td>
<td>6'-C6'-H</td>
<td>5'-d (CTT CAT TTT TCC TCT)</td>
<td>+3.8</td>
<td>+2.2</td>
<td>+2.2</td>
<td>+2.2</td>
<td></td>
</tr>
<tr>
<td>AON14</td>
<td>6'-C6'-H</td>
<td>5'-d (CTT CAT TTT TCC TCT)</td>
<td>-7.6</td>
<td>-6.9</td>
<td>-6.9</td>
<td>-4.4</td>
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<tr>
<td>AON15</td>
<td>6'-C6'-OH</td>
<td>5'-d (CTT CAT TTT TCC TCT)</td>
<td>-12.6</td>
<td>-16.3</td>
<td>-16.3</td>
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<tr>
<td>AON16</td>
<td>6'-C6'-H</td>
<td>5'-d (CTT CAT TTT TCC TCT)</td>
<td>-13.8</td>
<td>-18.2</td>
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<tr>
<td>AON17</td>
<td>6'-C6'-H</td>
<td>5'-d (CTT CAT TTT TCC TCT)</td>
<td>-13.9</td>
<td>-16.2</td>
<td>-16.2</td>
<td>-16.2</td>
<td></td>
</tr>
</tbody>
</table>

*Molecular weights of all antisense sequences are confirmed by MALDI-TOF mass spectrum (see Table SII.2 in Supporting Information). A = native adeninyl, C = cytosinyl, T = thyminyl, 'T' indicates α-1-carba-LNA or α-1-LNA-modified thymidime monomer with specified structure. T_m values measured as the maximum of the first derivative of the melting curve (ΔT_m vs T) in medium salt buffer (60 mM Tris-HCl at pH 7.5, 60 mM KCl, 0.8 mM MgCl₂) with temperature range of 20 to 65 °C using 1 μM concentrations of the two complementary strands. The value of ΔT_m is calculated by this equation: ΔT_m = ΔT_m of AON/RNA − ΔT_m of AON/DNA. ΔT_m of AON1/RNA and AON1/DNA are adopted for T_m comparison; therefore, their ΔT_m values are set to 0.
total coupling yield (20 to 45%). The reduction of coupling yield probably originates from close proximity of the carbocyclic or heterocyclic ring fused on the upper face of the sugar moiety as well as the 6′ and 7′ substituents to the 3′-phosphate, thereby interfering with the coupling and reducing coupling efficiency to some extent. Cleavage from the solid phase support and deprotection steps were carried out by treating the solid support with 33% aqueous ammonia at rt for 12 h (for AONs 10–21) or at 55 °C for 72 h (deprotection of Tol group used for protection 6′-OH in AONs 2–9 needs a longer deprotection time), followed by purification through 20% denatured PAGE, and confirmation of structural integrity using MALDI-TOF mass spectroscopy (see Table SII.3 in Supporting Information).

5. Thermal Denaturation Studies. The $T_m$ values of duplexes formed by AONs 2–21 with the complementary RNA or DNA have been measured and compared with that of the native counterpart (Table 1). Just as in previous reports, 33–36 one α-1-LNA (type IV) modification resulted in roughly 4.4 °C increase in $T_m$ for the AON/RNA hybrid. On the contrary, 7′-R-Me-α-1-carba-LNA (type III) led to $T_m$ decrease around 3 °C modification. Type I and type II modifications, just like the type III modification, also led to $T_m$ decrease by around 3 °C modification. This observation indicates that 6′-OH in 6′-S-OH-7′-R-Me-α-1-carba-LNA (type I) and 6′-R-OH-7′-R-Me-α-1-carba-LNA (type II) exert no obvious effect on $T_m$ for the AON/RNA hybrid regardless of their chirality. Hence, the drop in $T_m$ caused by types I, II, and III modification could be due to steric clash of the hydrophobic 7′-R-methyl group, which points toward vicinal 3′-phosphate ($d(\text{7′-S-methyl-3′}) \approx 4.1 \text{ Å}$), thereby impairing the AON/RNA thermal stability by perturbing the hydration pattern 58–63 and other stereoelectronic interactions in the major groove of the DNA/RNA duplex. Similarly declined $T_m$ results were also observed in the 2′-N-ethyl and acetyl functionalized 2′-amino-α-1-LNA-modified AONs, which showed $3\text{−}10\text{ °C}$ decrease (sequence dependent) in the thermal stability toward complementary RNA 46 once again suggesting that the introduction of a bulky hydrophobic group in position pointing at 3′-phosphate in the major groove of the AON/RNA hybrid can cause substantial negative effects on the duplexes stability.

It is noteworthy that the 6′,7′-substituted β-1-carba-LNA derivatives were found in our previous studies 27–29 to lead to increased $T_m$, by 2–4 °C depending on different substitutions at 6′ and 7′ positions. This should be compared to the observation made in the present study showing a $T_m$ drop of 2–3 °C for 6′,7′-substituted α-1-carba-LNA. This significant difference in thermal stabilities of β-1-carba-LNA vis-à-vis α-1-carba-LNA-modified duplexes hints that the substitutions on the carbocyclic ring of α-1-carba-LNA located in the major groove of DNA/RNA duplex have significantly destabilized the duplex, while the modifications of the carbocyclic ring of β-1-carba-LNA located in the minor groove lead to stabilization of the duplexes. We have also found that all the α-1-LNA and α-1-carba-LNA derivatives are RNA selective since $\Delta T_m (\Delta T_m = \Delta T_m(\text{ave}))$ of AON/RNA $\Delta T_m(\text{ave})$ of AON/DNA values were found in the range of 1.6–2.8 °C (Table 1).

Incorporation of the hyperconstrained 6′,7′-methylene-bridged-α-1-carba-LNA thymidine (type V) into 15-mer oligonucleotides led to dramatic decrease in thermal affinity toward both complementary RNA and DNA ($T_m$ dropped 7–14 °C with RNA, and dropped 7–18 °C with DNA; see AON 18–21 in Table 1). This result was quite similar to the previous observations that AONs modified by 5′S,6′R-configured tetracyclic “locked LNA” also exhibit very low affinity toward complementary RNA and DNA. 39 It was speculated that the loss of aromaticity in nucleobase resulted in the increased steric bulk of the nucleobase moiety (from planar to tetrahedral geometry at C5 and C6 position), thereby destabilizing the duplex owing to perturbation of the base stacking of modified nucleic acid with neighboring base pair to some extent due to energetically unfavorable intranucleotide interaction. Alternatively, as a consequence of constrained glycosidic torsion angle of type V, the nucleobase participating in hydrogen bonding might not be disposed optimally for efficient Watson–Crick base pairing, therefore, also impairing the duplexes stability to some extent.

6. Circular Dichroism Analysis. 6.1. CD of AON−RNA Duplex: CD spectra were recorded to evaluate the overall conformation of single modified AON/DNA and AON/RNA duplexes compared to the native DNA/DNA, RNA/RNA, and DNA/RNA duplexes (see Figure 3). As shown in Figure 3A, all of the single modified AON/RNA hybrids exhibited CD profiles intermediate between the native A-type RNA/RNA and B-type DNA/DNA duplexes, but resemble the natural AON 1/RNA hybrid, which suggested that the single modification with types I, II, III, IV, and V in AONs 4/8/12/16/20 did not cause much conformational perturbation for AON/RNA duplex compared to the native AON 1/RNA duplex. Hence, types I, II, III, IV, and V modified AON/RNAs could be good substrates for RNase H digestion. Especially, we found that the hyperconstrained tetracyclic 6′,7′-methylene-bridged α-1-carba-LNA-Thy (type V) modification led to less conformational perturbation for AON/RNA hybrid than type I, II, III, and IV modifications. As discussed in section 5, the conformationally constrained glycosidic torsion angle of type V modification could however impair its ability to form efficient Watson–Crick base pair with adenine in the opposite strand, thus this type V modified AON strand might perturb the driving force for conformation change necessary in the modified AON strand for forming thermodynamically stable duplex with the target opposite RNA strand.

6.2. CD of AON−DNA Duplex: The duplexes of AONs 4/8/12/16 with complementary DNA also showed relatively similar global helical conformation to the native DNA/DNA homoduplex (shown in Figure 3B). However, the CD profile for the duplex of type V modified AON 20 with complementary DNA was found to obviously shift to shorter wavelength (blue shift) with more intense positive Cotton peak around 276 nm compared with the native AON 1/DNA duplex. Hence, it seems a single type V modification can significantly perturb the global conformation of AON/DNA duplex.
were measured. Thus the selected AONs were labeled at the 5'-end with 32P-ATP/kinase and then incubated with SVPDE in containing medium or in various eukaryotic cell lines, and modifications located at 3'-terminus can significantly contribute to the nuclease resistance of an oligonucleotide. The stabilities of the newly synthesized AONs (AON 1, 2, 6, 10, 14, 18) with a single modification (types I, II, III, IV, V) at position T13 (position 3 from 5'-end) toward 3'-exonuclease (phosphodiesterase I) were measured. Thus the selected AONs were labeled at the 5'-end with 32P-ATP/kinase and then incubated with SVPDE [SVPDE 6.7 ng/μL, AON 3 μM, 100 mM Tris-HCl (pH 8.0), 15 mM MgCl2, total volume 30 μL] at 21 °C. Aliquots were taken out at appropriate time intervals and analyzed by 20% denaturing PAGE. The gel pictures were obtained upon autoradiography and are shown in Figure SII.44 in Supporting Information.

The native AON was completely degraded in ~10 min under present conditions. All of the other modified AONs containing α-1-carba-LNA derivatives showed considerably improved 3'-exonuclease resistance to a variable extent. Because the AONs were singly modified at position T13, the phosphate P14 (see the structure in Figure 4A for phosphate (P) numbering) was considerably resistant to 3'-exonuclease cleavage. Moreover, T13 modification also improves the stability of P13 phosphate to some extent. Therefore, two bands referring to 14-mer and 13-mer oligos could be observed on the PAGE pictures (Figure SII.44). However, once the P13 was cleaved, AONs were degraded to the monomer blocks quickly, and hence no bands referring to 12-mer to dimer oligos could be observed.

Total percentages of intergraded 14-mer and 13-mer AONs were plotted against time points to give SVPDE digestion curve for each selected AON in Figure 4B, and pseudo-first-order reaction rates (Figure 4B and Table SII.2) were obtained by fitting the curves to single-exponential decay functions. A comparison of digestion rates of AONs with different types of modifications exhibited the following results and implications:

7.1. Relative 3'-Exonucleolytic Stabilities of α-1-Carba-LNA-Modified AONs. The stabilities of AONs 2, 6, 10, 14, 18 toward SVPDE incubation decreased in following order: 6,7'-methylene-bridged α-1-carba-LNA-T (type V) modified AON 18 ($k = 0.0040 \pm 0.0012 \text{ min}^{-1}$) > 7'R-methyl-α-1-carba-LNA (type III) modified AON 10 ($k = 0.0068 \pm 0.0009 \text{ min}^{-1}$) > 6'R-hydroxy-7'S-methyl-α-1-carba-LNA (type II) modified AON 6 ($k = 0.0076 \pm 0.0018 \text{ min}^{-1}$) > 6'S-hydroxy-7'S-methyl-α-1-carba-LNA (type I) modified AON 2 ($k = 0.0125 \pm 0.0007 \text{ min}^{-1}$) > α-1-LNA (type IV) modified AON 14 ($k = 0.0777 \pm 0.0072 \text{ min}^{-1}$) > β-δ-LNA (type VI) incorporated AON ($k = 0.5331 \pm 0.1800 \text{ min}^{-1}$). Hence, α-1-carba-LNA analogue modified AONs have been found to be 3'-exonucleolytically more stable than parent α-1-LNA and β-δ-LNA-modified counterparts. Another strategy, namely, phosphorothioate backbone modification, is also popular to produce AONs with high nucleolytic stability, but this type of modification, on the other hand, can lead to extensive cellular toxicity and side effects. Hence, α-1-carba-LNA-modified AONs with phosphodiester linkage probably will show better pharmacologic properties than AONs containing both α-1 (or β-δ)-LNA and phosphorothioate backbone modifications.

7.2. Effect of 7'-Me Substitution on 3'-Exonucleolytic Stability. We have found that 7'R-methyl-α-1-carba-LNA (type III) modified AON 10 is about 10 times more stable than α-1-LNA (type IV) modified AON 14, which suggests that the replacement of the 2'-O- with hydrophobic methyl-methylene function in α-1-LNA can render significantly positive effects on the nuclease resistance.

7.3. Effect of 6'R-OH Substitution (Pointing away from 3'-Phosphate) on 3'-Exonucleolytic Stability. C6'-OH substitution on the α-1-carba-LNA can modulate the stability of modified AONs to different extent depending on the stereochemical orientation of the hydroxyl group. 6'R-Hydroxyl substitution led to virtually no effect since 6'R-hydroxy-7'S-methyl-α-1-carba-LNA (type II) modified AON 6 have shown very similar overall stability with the 7'R-methyl-α-1-carba-LNA (type III) modified AON 10. We have also found the 6'R-OH substitution can improve the nuclease resistance for the vicinal 5'-phosphate as can be seen from the

![Figure 3](image-url)
band corresponding to 13-mer oligo which was observed in PAGE for type II modified AON while the 13-mer band was not present for type I modified AON and type III modified AON (Figure SII.44 in Supporting Information).

Effectively the $R'_6$-OH protects the $5'_0$-phosphate by interfering with binding of SVPDE to this phosphate.$^{32}$ Indeed the $R'_6$-OH in $R'_6$-hydroxyl-$7'S$-methyl-$\alpha$-L-carba-LNA (type II) has been found located relatively close to the $5'_0$-phosphate ($d_{5'_0-OH-SVPDE}$ $\approx$ 4.0 Å).

7.4. Effect of $6'S$-OH Substitution (Pointing at $3'_0$-Phosphate) on $3'_0$-Exonucleolytic Stability. Previous study$^{32}$ has shown that in $\beta$-D-carba-LNA $6'$-OH substitution can remarkably reduce $3'_0$-exonucleolytic stability of modified AONs if this OH points at the vicinal $3'_0$-phosphate probably because of its assistance in the departure of $3'_0$-oxyanion during SVPDE mediated $3'_0$O$-P$ bond scission.$^{32}$ Similar conclusions can be reached in the present investigation as $6'S$-hydroxyl substitution [OH group is also pointing at the vicinal $3'_0$-phosphate] in $6'S$-hydroxyl-$7'S$-methyl-$\alpha$-L-carba-LNA (type I) leads, upon incorporation into modified AON 2, to significantly less $3'_0$-exonucleolytic stable duplex than the duplex incorporating the $7'R$-methyl-$\alpha$-L-carba-LNA (type III) modification (AON 10).

7.5. Effect of Tetracyclic System on $3'_0$-Exonucleolytic Stability. $6,7'R$-Methylene-bridged $\alpha$-L-carba-LNA-T (type V) modified AON 18 showed better $3'_0$-exonucleolytic resistance than type I, II, and III modified AONs. It can be concluded that the extra six-membered ring of $6,7'$-methylene-bridged $\alpha$-L-carba-LNA-T (type V), which lies above the pentose sugar, gave slightly higher nuclease resistance compared to other $\alpha$-L-carba-LNA derivatives (types I, II, and III).

Comparison of $3'_0$-exonucleolytic resistance of $\alpha$-L-carba-LNA-modified AONs with that of $\beta$-D-LNA and $\beta$-D-carba-LNA-modified counterparts has also been carried out. Corresponding PAGE, $3'_0$-exonuclease (SVPDE) promoted digestion plot and discussion can be found in part II of Supporting Information.

**FIGURE 4.** (A) Molecular structure of T13 modified AONs. The full sequence is $3'_0$-d (CTT$^{13}$CTT TTT TAC TTC$^{32}P$)-$5'_0$. (B) Amount of remaining initial oligonucleotide (taken 14-mer and 13-mer together in the calculation of percentage remaining) during $3'_0$-exonuclease (SVPDE) promoted digestion. The digestion conditions were used as follows: AON 3 μM ($5'_0$-end$^{32}P$-labeled with specific activity 80 000 cpm), 100 mM Tris-HCl (pH 8.0), 15 mM MgCl$_2$, SVPDE 6.7 ng/μL, reaction temperature 21°C, total reaction volume 30 μL.
8. Relationship between 3′-Exonuclease Stability and Solvation Free Energy for α-1, Carba-LNA Derivatives and Parent α-1-LNA. The solvation properties of modified AON affect the relative access of the water molecule to the scissile phosphate, therefore giving variable extent of 3′-exonuclease resistance. In order to check the relationship between solvation capability and nucleic stability of AONs with different modifications, we have analyzed the solvation free energy of α-1-carba-LNA derivatives (types I, II, III) and R-LNA (type IV) (Table SII.1) employing Baron and Cossi’s implementation of polarizable conductor CPCM model\(^{65}\) of solvation on the \(ab\) \(initio\) optimized (HF,6-31G** basis set) gas phase molecular geometries. The solvation free energies obtained have clearly demonstrated relative hydrophilic nature of different 2′,4′-constrained modifications in α-1-carba-LNA and α-1-LNA. The energy of solvation of α-1-carba-LNA derivatives (types I, II, III) and α-1-LNA (type IV) decreases in the following order: 7′R-methyl-α-1-carba-LNA (type III, \(\sim 3.04\) kcal mol\(^{-1}\)) > 6′R-hydroxyl-7′S-methyl-α-1-carba-LNA (type II, \(\sim 4.30\) kcal mol\(^{-1}\)) > 6′S-hydroxyl-7′S-methyl-α-1-carba-LNA (type I, \(\sim 4.35\) kcal mol\(^{-1}\)) > α-1-LNA (type IV, \(\sim 8.51\) kcal mol\(^{-1}\)). This trend shows the α-1-carba-LNA derivatives are not as well solvated as the parent α-1-LNA, which suggests the scissile phosphate of α-1-LNA is relatively more solvated as compared to that of α-1-carba-LNA derivatives. The magnitudes of 3′-exonuclease stability of these α-1,2′,4′-constrained nucleoside modified AONs have also shown the following decreasing trend: 7′R-methyl-α-1-carba-LNA (type III, \(k = 0.0068 \pm 0.0009\) min\(^{-1}\)) > 6′R-hydroxyl-7′S-methyl-α-1-carba-LNA (type II, \(k = 0.0076 \pm 0.0018\) min\(^{-1}\)) > 6′S-hydroxyl-7′S-methyl-α-1-carba-LNA (type I, \(k = 0.0125 \pm 0.0007\) min\(^{-1}\)) > α-1-LNA (type IV, \(k = 0.0777 \pm 0.0072\) min\(^{-1}\)), which is fully consistent with the results from solvation free energy calculation. This corroborated our original hypothesis that the hydration around a scissile phosphate was most probably critical for the nuclease-promoted hydrolysis.\(^{27}\)

9. Stability of Functionalized α-1, Carba-LNA-Modified AONs in Human Blood Serum. The newly synthesized AONs with a single modification types I, II, III, and IV at position T13 (position 3 from 3′-end) were assayed for stability in human blood serum. The AONs (5′-end \(^{32}\)P-labeled) were incubated with human blood serum (male, type AB) for up to 48 h at 21 °C, and aliquots were taken out at regular time intervals and then analyzed by 20% denaturing PAGE. The gel pictures obtained by autoradiography are shown in Figure SII.43. Due to the presence of alkaline phosphatase in blood serum that gradually removes the 5′-end \(^{32}\)P-label, it was impossible to obtain accurate degradation rate for each AON by quantifying the gel picture. Visual comparison of the gel pictures have shown that 6′S-hydroxyl-7′S-methyl-α-1-carba-LNA (type I) modified AON 2 and 7′R-methyl-α-1-carba-LNA (type III) modified AON 10 can be sustained in blood serum for more than 48 h. These modifications result in more stable AONs compared to 6′R-hydroxyl-7′S-methyl-α-1-carba-LNA (type II) modified AON 6 and 6′S-methylene-bridged α-1-carba-LNA-T (type V) modified AON 18, which is sustained for 36 h in blood serum. The α-1-LNA (type IV) modified AON 14 exhibited the least stability in human blood serum. The order of relative stabilities of AONs in human blood serum has been found to be similar to that observed upon treatment by 3′-exonucleases.

10. RNase H Digestion Studies of Functionalized α-1, Carba-LNA-Modified AON/RNA Duplexes. It has been reported that RNase H binds in the minor groove of the DNA/RNA hybrid.\(^{66,67}\) Hence α-1-carba-LNA modifications are supposed to have relatively minor effect on the RNase H recruitment than β-D-carba-LNA modification because the 2′,4′-carbocyclic ring in the former is located in the minor groove but the carbocyclic ring in the latter is located in the minor groove of the AON/RNA hybrid.

Hence, the RNase H recruitment study of four newly synthesized α-1-carba-LNA derivative modified AONs (containing a single type I, II, III, or V modification) as well as the parent α-1-LNA-modified AON (type IV) has been carried out and compared with the native AON 1 as well as with the β-D-carba-LNA-modified AONs.\(^{27–29}\)

The gel pictures in Figure SII.37–39 have shown that all the modified AONs 2–21 RNA hybrids are good substrates for RNase H. For type I, II, III, and IV modified AON/RNA duplexes, the cleavage patterns have been found to be very similar and independent of the nature of the modification but dependent on the site of modification. As shown in Figure S5A, the cleavage activity of RNase H was suppressed within a stretch of a 5 base pairs region that starts from the modification site toward the 3′-end in the RNA strand. In addition, the original preferred A8 cleavage site also shifted to the edges of suppressed region if A8 is included within this 5 bp suppressed area. Hence, the observed RNase H cleavage patterns for type I, II, III, and IV modified AON/RNA duplexes were found to be very closely similar to the previous studies of β-D-carba-LNA,\(^{27–29}\) β-D-carba-ENA,\(^{27,28}\) and β-D-aza-ENA,\(^{20,21}\) modified AON/RNA hybrids, suggesting that RNase H does not make any distinction on whether the 2′,4′-carbocyclic ring is located in the minor or the major groove, it renders very similar cleavage pattern. However, it is interesting to note that tetracyclic 6,7′-methylene-bridged α-1-carba-LNA (type V) modified AON 19/RNA and AON 20/RNA hybrids showed different cleavage patterns compared to the type I, II, III, and IV modified AON/RNA duplexes at the same position: a stretch of 3 bp region was observed for RNase H cleavage suppression in AON 19/RNA duplex, whereas a stretch of 5 bp suppressed region was also observed in AON 20/RNA duplex but with different preferred cleavage site and suppressed stretch region (see Figure 5B). This result hints that type V modification led to relatively less conformational perturbation for AON/RNA hybrid than type I, II, III, and IV modifications, which is consistent with the result obtained by CD study.

All the α-1,carba-LNA derivative modified AON/RNA duplexes were degraded by RNase H with comparable cleavage rates as digestion of native AON/RNA hybrid (Figure 6). Remarkably, the cleavage rates of types III (7′R-methyl-α-1-carba-LNA) and IV (α-1-LNA) modified AON/RNA duplex were even 2 times higher than cleavage of...
the native counterpart. Given that β-D-carba-LNA-modified AON/RNA duplexes generally showed less RNase H digestion efficiency than the native counterpart,28,29 we could arrive at the conclusion that R-L-carba-LNA modifications led to much less effect on RNase H elicitation than β-D-carba-LNA modification. This is because of the fact that the stereochemical location of the hydrophobic 2\,2\,0,4\,4\,0-carbocyclic ring in β-D-carba-LNA is in the minor groove, which retards the RNase H binding in the minor groove, but, in contrast, that is not the case for the R-L-carba-LNA since its 2\,2\,0,4\,4\,0-carbocyclic ring is located in the major groove.

Conclusion

1. Herein, we report convenient synthetic routes toward the α-L-carba-LNA thymidine derivatives. The synthesis of these novel α-L-ribo-configured conformationally constrained carbocyclic analogues has been achieved by employing free-radical scavenging by a tethered olefin in an intramolecular reaction as a key step. Various NMR experiments, including 1H, 13C, 1H−1H COSY, one-bond 1H−13C correlation (HMQC), and long-range 1H−13C HMBC have been carried out to characterize all synthesized compounds unambiguously. The relative chirality of substituents in the key intermediates was determined by 1D NOE and was also corroborated by the 1JHH coupling constants obtained from H−H homo or double decoupling experiments.

2. The thermal denaturation study of duplexes formed by AON containing a single α-L-carba-LNA derivatives with complementary RNA revealed that \( T_m \) of 6',7'-substituted α-L-carba-LNA-modified AON/RNA is about 3 °C lower than that of the native AON/RNA and 7 °C lower than that of the parent α-L-LNA-modified counterpart. This suggests that the hydration perturbation in the major groove caused by the substitution of α-L-carba-LNA results in more negative effects on duplex thermal stability compared to the substitution of β-D-carba-LNA derivatives in minor groove.

3. Substituted α-L-carba-LNA-modified AONs have been found in presence of both snake venom phosphodiesterase and human blood serum to be more stable than the parent α-L-LNA and β-D-LNA-modified counterparts.

4. α-L-Carba-LNA derivatives and α-L-LNA-modified AON/RNA duplexes have shown similar RNase H digestion pattern as the β-D-carba-LNA-modified counterparts, but better digestion efficiency. Especially, the cleavage rates of types III (7'-O-methyl-α-L-carba-LNA) and IV (α-L-LNA) modified AON/RNA duplex were, in some sequences, even 2 times faster than cleavage rates of the native counterpart, suggesting the α-L-carba-LNA modification in which the 2',4'-carbocyclic ring is located in the major groove can lead to a positive effect on the RNase H digestion efficiency of AON/RNA.
Implication. The obvious merit of introduction of α-L-carba-LNA derivatives into AONs is that they can improve nuclease resistance while rendering positive effect on the RNase H digestion. Unfortunately, all four newly synthesized α-L-carba-LNA derivatives in the present study led to lower RNA affinity (around \(-3^\circ\)C/modification compared to native counterpart). As discussed in section 5, the decrease in the RNA affinity probably originates from the steric clash with the hydrophobic 7′-methyl group in α-L-carba-LNA, which points toward vicinal 3′-phosphate, thereby impairing the AON/RNA thermal stability by perturbing the hydration pattern in the major groove of the DNA/RNA duplex. Work is in progress to replace 7′-methyl group with less steric and less hydrophobic hydrogen atom as in parent α-L-carba-LNA [structure (O) in Figure 1] which upon introduction into AONs should hopefully lead to a more effective antisense therapeutic candidate, thereby will further add to the value of α-L-carba-LNA as a modified nucleoside in the AON or siRNA. We anticipate that this parent α-L-carba-LNA should have (1) higher target RNA affinity, just like α-L-LNA, (2) significantly increased enzymatic stability compared to α-L-LNA, β-D-LNA, and β-L-carba-LNA derivatives.

Experimental Section

3,5-Di-O-benzyl-4-C-(1R-hydroxymethyl)-1,2-O-isopropylidene-β-L-ribofuranose (3a) and 3,5-Di-O-benzyl-4-C-(1S-hydroxymethyl)-1,2-O-isopropylidene-β-L-ribofuranose (3b). Oxalyl chloride (2.84 mL, 32 mmol) was added to the precooled dichloromethane (92 mL) at \(-78^\circ\)C. Then DMSO (3.90 mL, 54 mmol) in dichloromethane (8 mL) was added dropwise to the solution over 30 min. After stirring for 20 min, a solution of 1 (5.20 g, 13.0 mmol) in dichloromethane (24 mL) was added dropwise over about 20 min, and the mixture was kept stirring at \(-78^\circ\)C for another 30 min. DIPEA (16 mL) was added to this cooled mixture. The reaction solution was then allowed to warm to room temperature and stirred for 1 h whereupon water (10 mL) was added. The organic layer was separated, washed with water (50 mL) and brine (50 mL), dried over MgSO₄, and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel (0–20% ethyl acetate in cyclohexane, v/v) to give 3a (1.80 g, 32%) and 3b (1.61 g, 29%) as colorless oil.

FIGURE 6. Bar plots of the observed cleavage rates of the RNase H1 promoted degradation of RNA in AON 1-21/RNA hybrid duplexes.
$J_{\text{gem}} = 11.5$ Hz, CH$_2$Bn), 4.52 (1H, d, $J_{\text{gem}} = 11.5$ Hz, CH$_2$Bn), 4.48 (1H, m, H6), 4.45 (1H, m, H2), 4.05 (1H, d, $J_{\text{gem}} = 5.0$ Hz, H3), 3.99 (1H, d, $J_{\text{gem}} = 11.0$ Hz, H2), 3.74 (1H, d, $J_{\text{gem}} = 11.0$ Hz, H3), 2.74 (s, DABCO), 1.43 (3H, s, CH$_3$), 1.23 (3H, s, CH$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 137.2 (Bn), 135.6 (Bn), 133.7 (C7), 127.3--126.5 (aromatic), 116.0 (C8), 112.4 (isopropyl), 103.3 (C1), 87.7 (C4), 77.8 (C2), 76.3 (C3), 76.2 (C6), 71.6 (CH$_2$Bn), 69.2 (C5), 45.9 (DABCO), 25.7 (CH$_3$), 25.6 (3CH$_3$); MALDI-TOF m/z [M + Na]$^+$, found 449.199, calculated 449.197; 396.3 (C6), 136.4 (aromatic), 135.8 (aromatic), 133.8 (C7), 127.6--126.9 (aromatic), 119.6 (C8), 110.0 (C5), 97.4 (C1), 89.2 (C4), 74.2 (C3), 73.2 (CH$_2$Bn), 72.1 (CH$_2$Bn), 71.9 (C2), 70.6 (C5), 11.2 (5-CH$_3$); MALDI-TOF m/z [M + Na]$^+$, found 517.200, calculated 517.199. 

1-[3,5-Di-O-benzyl-4-C-(1R-hydroxylallyl)-6-hydroxyl-2-O-phenoxylthiocardionyl-α-ribofuranosyl]thymine (7a). Compound 6a (872 mg, 1.70 mmol) was coevaoporized twice with anhydrous pyridine and dissolved in the same solvent (38 mL). The solution was cooled by ice bath, and then phenyl chloroformate (0.29 mL, 2.10 mmol) was added dropwise, while temperature was maintained at 0°C during addition. After 3 h of stirring at room temperature, pyridine was recovered under reduced pressure. The residue was dissolved in dichloromethane (50 mL) and washed with saturated solution of NaHCO$_3$ (20 mL). The organic layer was separated, dried over MgSO$_4$, and evaporated to furnish crude product 3a as a white solid: 1H NMR (500 MHz, CDCl$_3$) δ 8.16 (1H, br s, NH), 7.30--7.17 (13H, m, aromatic), 6.86 (2H, d, $J = 8.0$ Hz, aromatic), 5.94 (1H, app t, H2), 5.81 (1H, d, $J_{\text{gem}} = 6.0$ Hz, H1), 5.70 (1H, m, H7), 5.46 (1H, d, $J = 17.0$ Hz, H8'), 5.17 (1H, d, $J = 10.5$ Hz, H8'), 4.63 (1H, d, $J_{\text{gem}} = 6.5$ Hz, H3), 4.59 (1H, d, $J_{\text{gem}} = 5.5$ Hz, H6), 4.56 (1H, d, $J_{\text{gem}} = 12.0$ Hz, CH$_2$Bn), 4.55 (1H, d, $J_{\text{gem}} = 12.0$ Hz, CH$_2$Bn), 4.44 (1H, d, $J_{\text{gem}} = 12.0$ Hz, CH$_2$Bn), 3.73 (1H, d, $J_{\text{gem}} = 11.0$ Hz, H5'), 3.52 (1H, d, $J_{\text{gem}} = 17.0$ Hz, H5), 3.29 (1H, br s, 6-CH$_3$), 1.85 (3H, s, 5-CH$_3$); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 193.2 (C=O, acetyl), 162.8 (C4), 149.5 (C2), 131.3 (C7), 128.6 (aromatic), 120.6 (aromatic), 123.9 (aromatic), 119.6 (C8), 110.4 (C5), 89.4 (C4), 89.3 (C3), 80.0 (C2), 73.8 (C3), 73.7 (CH$_2$Bn), 72.9 (CH$_2$Bn), 71.9 (C6), 69.0 (C5), 11.4 (5-CH$_3$); MALDI-TOF m/z [M + Na]$^+$, found 563.197, calculated 565.193. 

(1,3,5,4,5,5,6,7,8,5)-7-Benzoxyl-1-benzoxymethyl-6-hydroxy-5-methyl-3-thymin-1-yl-2-oxabicyclo[2.2.1]heptane (8a). Compound 6a (794 mg, 1.55 mmol) was dissolved in 140 mL of anhydrous toluene that was purged with N$_2$ for half an hour. The mixture was heated to reflux and Bu$_3$SnH (0.41 mL in 12 mL of 1,2-dimethoxyethane, 155 mmol) and AIBN (293 mg in 24 mL of anhydrous toluene, 1.55 mmol) were added dropwise in four portions over 4 h. Then the refluxing was continued for another 1 h. Solvent was evaporated, and the residue was purified by column chromatography on silica gel (20--40% ethyl acetate in cyclohexane, v/v) to afford 8a (820 mg, 57%) as a white solid: 1H NMR (600 MHz, CDCl$_3$) δ 8.63 (1H, br s, NH), 8.16 (1H, s, H6), 7.30--7.18 (10H, m, Bn), 6.16 (1H, d, $J_{\text{gem}} = 2.4$ Hz, H1'), 4.63 (1H, d, $J_{\text{gem}} = 12.0$ Hz, CH$_2$Bn), 4.62 (1H, d, $J_{\text{gem}} = 12.0$ Hz, CH$_2$Bn), 4.45 (1H, d, $J_{\text{gem}} = 12.0$ Hz, CH$_2$Bn), 3.86 (1H, d, $J_{\text{gem}} = 1.5$ Hz, H3), 3.81 (1H, m, H6), 3.51 (1H, d, $J_{\text{gem}} = 10.2$ Hz, H5'), 2.83 (1H, s, 6-CH$_3$), 2.63 (1H, m, H2'), 1.86 (3H, s, 5-CH$_3$), 1.56 (3H, s, H7), 0.95 (3H, d, $J_{\text{gem}} = 7.8$ Hz, 4CH$_2$Bn); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 163.1 (C4), 149.3 (C2), 137.8 (C6), 136.2 (aromatic), 136.1 (aromatic), 127.6--126.6 (aromatic), 107.6 (C5), 88.6 (C1'), 88.1 (C4'), 80.4 (C3'), 78.9 (C6'), 73.1 (CH$_2$Bn), 70.8 (CH$_2$Bn), 68.1 (C5'), 47.8 (C2'), 32.3 (C7'), 25.9 (cyclohexane, coming from chromatography elution
(1S,3R,4S,5S,6S,7S)-7-Benzylxlo-1-benzyloxymethyl-6-hydroxyl-5-methyl-3-(thymin-1-yl)-2-oxacyclo[2.2.1]heptane (8b) and (1R,6S,7S,9R,10S,11S,12S,13S)-12-Benzylxlo-11-benzyloxymethyl-2,4-diaza-10-hydroxy-6-methyltetraacyclo[9.2.1.0^1.3.0^1.5]tetradecane-3,5-dione (8c).

Compound 7b (3.0 g, 4.76 mmol) was dissolved in 410 mL of anhydrous toluene that was purged with \( \text{N}_2 \) for 30 min prior to use. The mixture was heated to reflux and Bu\( \text{Sn} \)H (1.26 mL in 44 mL anhydrous toluene, 4.76 mmol) and AIBN (899 mg in 92 mL of anhydrous toluene, 4.76 mmol) were added dropwise in four portions over 4 h. Then the refluxing was continued for another 1 h. Solvent was evaporated, and the residue was purified by column chromatography on silica gel (20–40% ethyl acetate in cyclohexane, \( \nu/v \) to \( \eta/ \nu \)) to give 8b (986 mg, 43%) and 8c (360 mg, 14%) as white solids.

8b: \( ^1\text{H} \) NMR (500 MHz, CDCl\( _3 \)) \( \delta \) 8.74 (1H, s, H6), 7.30 (12H, m, aromatic), 84.5 (C4), 152.7 (C2), 136.8 (aromatic), 136.3 (aromatic), 127.6 (aromatic), 127.0 (aromatic), 74.4 (C6), 72.9 (C6), 71.1 (C13), 62.4 (C5), 48.2 (C2), 31.9 (C7), 20.7 (Tol-CH3), 17.4 (76-C3), 11.0 (5-CH3); MALDI-TOF m/z \([\text{M}+\text{Na}]^+\), found 579.62, calcld 579.57.

(1S,3R,4S,5S,6R,7S)-1-(4,4'-Dimethoxytritylomethyl)-7-hydroxy-5-methyl-6-(4-methylbenzozate)-3-(thymin-1-yl)-2-oxacyclo[2.2.1]heptane (13a).

To a solution of compound 12a (252 mg, 0.420 mmol) in anhydrous methanol (10 mL) were added 20% \( \text{Pd(OH)}_2 \)/C (315 mg) and ammonium formate (1.60 g, 25.5 mmol), and the mixture was refluxed for 2 h. The suspension was filtered over Celite, and the organic phase was evaporated in vacuo to give crude 12a, which was coevaporated twice with anhydrous pyridine and dissolved in the same solvent (6 mL). 4,4'-Dimethoxytrityl chloride (282 mg, 0.84 mmol) was added, and the mixture was stirred overnight at room temperature. Then solvent was removed, and the residue was diluted with dichloromethane (10 mL), washed with aqueous saturated NaHCO\( _3 \) solution (10 mL), and dried over MgSO\( _4 \). After evaporation of solvent, the residue was subjected to short column chromatography on silica gel (0.3–1.2% methanol in dichloromethane containing 1% pyridine, \( \nu/v \)) to afford 13a (225 mg, 75%): \( ^1\text{H} \) NMR (500 MHz, CDCl\( _3 \) + DABCO) \( \delta \) 7.73 (1H, s, H6), 7.71 (2H, m, aromatic), 7.33–7.08 (11H, m, aromatic), 6.68 (4H, aromatic), 6.14 (1H, d, \( J_{H_1,H_{16}} = 2.0 \text{ Hz} \)), 1.49 (1H, d, \( J_{H_6,H_{16}} = 4.0 \text{ Hz} \)), 1.47 (1H, d, \( J_{H_6,H_{16}} = 1.0 \text{ Hz} \)), 3.62 (1H, s, CH3), 3.59 (3H, s, CH3), 3.48 (1H, d, \( J_{H_6,H_{16}} = 10.0 \text{ Hz} \)), 2.76 (1H, d, \( J_{H_6,H_{16}} = 7.5 \text{ Hz} \)), 2.49 (1H, d, \( J_{H_6,H_{16}} = 3.0 \text{ Hz} \)), 2.0 (1H, d, \( J_{H_6,H_{16}} = 3.0 \text{ Hz} \)), 1.35 (1H, d, \( J_{H_6,H_{16}} = 3.0 \text{ Hz} \)), 1.32 (1H, d, \( J_{H_6,H_{16}} = 3.0 \text{ Hz} \)), 1.26 (3H, s, CH3), 1.26 (3H, s, CH3), 1.25 (3H, s, CH3), 1.23 (3H, s, CH3), 1.2 (3H, s, CH3), 1.5 (3H, s, CH3).

By short column chromatography on silica gel (0.3–1.2% methanol in dichloromethane containing 1% pyridine, \( \nu/v \)) to afford 3a (285 mg, 60% mol) was coevaporated with anhydrous pyridine twice and dissolved in the same solvent (10 mL). The mixture was cooled with an ice bath, and 4-methyl benzyl chloride (0.12 mL, 0.90 mmol) was added dropwise to this precooled solution. The mixture was allowed to stir at room temperature for 6 h. Pyridine was recovered under reduced pressure, and the residue was dissolved in dichloromethane (10 mL). The obtained solution was washed with saturated NaHCO\( _3 \) aqueous solution (10 mL), dried over MgSO\( _4 \), and concentrated under reduced pressure to give crude product, which was subjected to short column chromatography on silica gel (15–30% ethyl acetate in cyclohexane, \( \nu/v \)) to afford 13a (260 mg, 73%): \( ^1\text{H} \) NMR (500 MHz, CDCl\( _3 \)) \( \delta \) 8.69 (1H, br s, NH), 7.82 (1H, s, H6), 7.80 (2H, m, aromatic), 7.29–7.10 (12H, m, aromatic), 6.06 (1H, d, \( J_{H_6,H_{16}} = 2.5 \text{ Hz} \)), 4.91 (1H, d, \( J_{H_6,H_{16}} = 4.0 \text{ Hz} \)), 4.67 (1H, d, \( J_{H_6,H_{16}} = 12.0 \text{ Hz} \)), 4.49 (1H, d, \( J_{H_6,H_{16}} = 12.0 \text{ Hz} \)), 4.48 (1H, d, \( J_{H_6,H_{16}} = 12.0 \text{ Hz} \)), 4.48 (1H, d, \( J_{H_6,H_{16}} = 12.0 \text{ Hz} \)), 3.70 (1H, d, \( J_{H_6,H_{16}} = 9.5 \text{ Hz} \)), 3.62 (1H, d, \( J_{H_6,H_{16}} = 9.5 \text{ Hz} \)), 2.79 (1H, s, H2), 2.36 (3H, s, Tol-CH3), 1.65 (1H, m, \( J_{H_7,H_8} = 3.0 \text{ Hz} \)), 1.46 (3H, s, 5-CH3), 1.20 (3H, d, \( J_{H_3,H_7} = 7.0 \text{ Hz} \)), 1.20 (3H, d, \( J_{H_3,H_7} = 7.0 \text{ Hz} \)), 1.10 (5-CH3); MALDI-TOF m/z \([\text{M}+\text{Na}]^+\), found 851.20, calcld 851.03.
(2H, dd, J_{gem} = 10.5 Hz, 10.5 Hz, H5, \ imagin', 2.75 (s, DABCO), 2.36 (1H, s, Tol-CH3), 1.77 (1H, m, H7), 1.51 (1H, s, 5-CH3), 1.39 (3H, d, J_{pr-Pac} = 7.0 Hz - 7'-CH3, 134.3 (aromatic), 134.2 (aromatic), 129.0 - 124.3 (aromatic), 112.2 (aromatic), 108.0 (C5), 87.7 (C4), 86.2 (C1), 85.8 (OCPB, 81.7 (C6), 77.2 (C3'), 58.9 (C5'), 54.1 (CH3-O), 48.9 (C7), 45.6 (DABCO), 34.3 (C7), 20.7 (Tol-CH3), 174 (T-CH3), 11.0 (5-CH3); MALDI-TOF m/z [M + Na]^+ found 747.281, calecd 747.279.

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2 Bn), 4.65 (1H, d, J_{gem} = 11.5 Hz, H3), 2.02 (1H, dd, J = 4.0 Hz, H2)', 2.52 (1H, m, J_{gem} = 10.0 Hz, H1', J_{H2-C5} = 1.5 Hz, H'), 2.06 (1H, m, J_{H2-C5} = 4.0 Hz, J_{gem} = 7.0 Hz, H7'), 1.88 (3H, s, 5'-CH3), 0.92 (3H, d, J_{gem} = 7.0 Hz - 7'-CH3, 131C NMR (125 MHz, CDCl3) δ 162.8 (C4), 148.8 (C2), 137.5 (aromatic), 137.0 (aromatic), 135.1 (C6), 127.4 - 126.5 (aromatic), 108.0 (C5), 83.1 (C1), 81.6 (C6'), 73.6 (C3'), 72.5 (CH2-Bn), 70.5 (CH3-Bn), 64.9 (C5'), 47.7 (C2'), 40.8 (C4'), 36.4 (C7'), 11.7 (5'-CH3), 8.9 (7'-CH3); MALDI-TOF m/z [M + H]^+ found 463.219, calecd 463.223.

General Procedure for Phosphoramidite Synthesis. To a solution of substrate (1 equiv) in dry dichloromethane were added DIPEA (6 equiv) and 2-cyanoethyl N,N-diisopropyl phosphoramidochloride (3 equiv) dropwise in an ice bath. The reaction was allowed to warm to rt and stirred at this temperature for 3 h. After being quenched with methanol, the mixture was diluted with dichloromethane, washed with saturated NaHCO3 solution, dried over MgSO4, and concentrated. The residue was subjected to short column chromatography on silica gel to give phosphoramidite, which was first precipitated in 1-n-hexane and then dried over P2O5 on vacuum for 3 days before it was used for DNA synthesis.

Oligonucleotide Synthesis and Purification. All AONs were synthesized using an automated DNA/RNA synthesizer based on phosphoramidite chemistry. For native A, G, and C building blocks, fast deprotection phosphoramidites (Ac for C, Bz-Pac for G, Bz for A) were used. Standard DNA synthesis reagents and cycle were used except that 0.25 M 5-[3,5-bis(trifluoro-methyl)phenyl]-1H-tetrazole (activator 42) was used as the activator and TsCl as the cap A. For incorporation of modified nucleotides, extended coupling time (10 min comparing to 5 min for native nucleotides) was used. For AONs 1 - 9, the deprotections were carried out in 33% aqueous NH3 for 72 h at 55 °C (the longer deprotection times were used to ensure complete removal of the Tol group). Other oligo (AONs 1 - 10) were deprotected at room temperature by treatment with 33% aqueous NH3 for 12 h. After deprotection, all crude oligos were purified by denaturing PAGE (20% polyacrylamide with 7 M urea), extracted with 0.3 M NaOAc, and desalted with a C-18 reverse phase cartridge to give AONs in >99% purity, and correct masses have been obtained by MALDI-TOF mass spectroscopy for each of them.

RNA was also synthesized by a solid-supported phosphoramidite approach based on the 2'-O-TEG strategy.66,67

UV Melting Experiments. Determination of the Tm 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 MgCl2. Absorbance was measured at 260 nm in the temperature range from 20 to 65 °C using a UV spectrophotometer equipped with a Peltier temperature programmer with the heating rate of 1 °C/min. Prior to measurements, the samples (1 μM of AON and 1 μM complementary DNA or RNA mixture) were preannealed by heating to 80 °C for 5 min followed by slow cooling to 21 °C and 30 min equilibration at this temperature. The value of Tm is the average of two or three independent measurements. If error of the first two measurements is > ±0.3 °C, the third measurement was carried out to check if the error is indeed within ±0.3 °C; otherwise, it is repeated.

CD Spectroscopy. CD spectra were recorded from 300 to 200 nm in 0.2 cm path length cuvettes. Spectra were obtained with an AON/RNA or AON/DNA duplex concentration of 10 μM in


60 mM Tris-HCl (pH 7.5), 60 mM KCl, 0.8 mM MgCl₂. All spectra were measured at 20 °C, and each spectrum is an average of five experiments from which the CD value of the buffer was subtracted.

32P Labeling of Oligonucleotides. The oligoribonucleotides and oligodeoxyribonucleotides were 5'-end-labeled with 32P using T4 polynucleotide kinase, [γ-32P]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.

SVPDE Degradation Studies. Stability of the AONs toward 3'-exonucleases was tested using phosphodiesterase I from Crotalus adamanteus (obtained from USB Corporation, Cleveland, OH). All reactions were performed at 3 μM DNA concentration (5'-end 32P-labeled with specific activity 80 000 cpm) in 100 mM Tris-HCl (pH 8.0) and 15 mM MgCl₂ at 21 °C. An exonuclease concentration of 6.7 ng/μL was used for digestion of oligonucleotides. Total reaction volume was 30 μL. Aliquots (3 μL) were taken at proper time points and quenched by addition of 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]. Reaction progress was monitored by 20% denaturing (7 M urea) PAGE and autoradiography.

Stability Studies in Human Blood Serum. AONs at 2 μM concentration (5'-end 32P-labeled with specific activity 80 000 cpm) were incubated in 10 μL of human blood serum (male AB, obtained from Sigma-Aldrich) at 21 °C (total reaction volume was 36 μL). Aliquots (3 μL) were taken at proper time points and quenched with 4 μL of stop 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 gel electrophoresis and visualized by autoradiography.

RNase H Digestion Assay. Target 0.1 μM RNA (specific activity 80 000 cpm) and AON (2 μM) were incubated in a buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, and 0.1 mM DTT at 21 °C in the presence of 0.01 U E. coli RNase H (obtained from USB Corporation, Cleveland, OH). 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 5, 10, 15, 30, and 60 min, and the reactions were terminated by mixing with stop solution [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. Pseudo-first-order reaction rates could be obtained by fitting the digestion curves to single-exponential decay functions.

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Supporting Information Available: 1H, 13C, 31P, DEPT, COSY, HMQC, HMBC of α-L-carba-LNA derivatives (part I); 1D NOE spectra, 1H homo or double decoupling spectra of key intermediates 5a, 5b, 8a, 8b, 10, 11, 19, 21, 15a, 15b, and 15c (part II); autoradiograms of 20% denaturing PAGE as well as degradation curves, showing the cleavage kinetics of target RNA in AON/RNA hybrids by E. coli RNase H1; synthesis and NMR characterization of intermediates 5b, 6b, 7b, 12b, 13b, 15b, 13c, 15e, 17, 18, 19, 21 as well as the characterization for final amidites 16a, 16b, 16c, and 22 (part II); chemical models of AON/RNA hybrids containing one α-L-carba-LNA or β-D-carba-LNA modification (part II). This material is available free of charge via the Internet at http://pubs.acs.org.