C5-Alkynyl-Functionalized α -L-LNA: Synthesis, Thermal Denaturation Experiments and Enzymatic Stability

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S Supporting Information



ABSTRACT: Major efforts are currently being devoted to improving the binding affinity, target specificity, and enzymatic stability of oligonucleotides used for nucleic acid targeting applications in molecular biology, biotechnology, and medicinal chemistry. One of the most popular strategies toward this end has been to introduce additional modifications to the sugar ring of affinity-inducing conformationally restricted nucleotide building blocks such as locked nucleic acid (LNA). In the preceding article in this issue, we introduced a different strategy toward this end, i.e., C5-functionalization of LNA uridines. In the present article, we extend this strategy to α -L-LNA: i.e., one of the most interesting diastereomers of LNA. α -L-LNA uridine monomers that are conjugated to small C5-alkynyl substituents induce significant improvements in target affinity, binding specificity, and enzymatic stability relative to conventional α -L-LNA. The results from the back-to-back articles therefore suggest that C5-functionalization of pyrimidines is a general and synthetically straightforward approach to modulate biophysical properties of oligonucleotides modified with LNA or other conformationally restricted monomers.

INTRODUCTION

Significant efforts have been devoted to the development of conformationally restricted nucleotides.¹⁻³ Oligonucleotides that are modified with such building blocks often display high affinity toward nucleic acid targets and are accordingly used for a variety of applications in molecular biology, biotechnology, and medicinal chemistry.⁴ Locked nucleic acid (LNA),⁵ which is also known as bridged nucleic acid (BNA),⁸ is one of the most promising members of this compound class, as it displays some of the highest affinities toward complementary DNA/RNA targets reported to date (increases in duplex thermal denaturation temperatures, $T_{\rm m}$'s, of up to +10 °C per modification have been observed). One of the diastereoisomers of LNA, i.e., α -L-LNA (α -L-ribo configuration; Figure 1) displays similar hybridization characteristics⁹ and lower hepatotoxicity^{10a} and has accordingly been studied as a potential modification for antisense, antigene, and decoy oligonucleotides.¹⁰ The interesting properties of α -L-LNA have spurred the development of many analogues, all of which have focused on further improving the biophysical

properties of α -L-LNA through modification or expansion of the oxymethylene bridge spanning the C2' and C4' positions of α -L-LNA and/or introduction of small branching substituents onto the conformationally restricted furanose skeleton.^{10a,11}

Our continued interest in LNA chemistry and C5-functionalized pyrimidine DNA building blocks^{4c,12} prompted us to pursue the synthesis of C5-alkynyl-functionalized LNA uridine (U) monomers.¹³ We hypothesized that C5 substituents could be used to modulate the characteristics of LNA pyrimidines. Our preliminary results on a small set of C5-alkynylfunctionalized LNA-U were promising and supported this hypothesis.¹³ Thus, oligodeoxyribonucleotides (ONs) modified with LNA-U monomers that are conjugated to small alkynes display increased target affinity and binding specificity along with moderately improved protection against 3'-exonucleases. ONs modified with large C5-functionalized LNA-U monomers display very high enzymatic stability, albeit at the expense of

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Figure 1. Structures of nucleotide monomers studied herein.





"Abbreviations: BSA, N,O-bis(trimethylsilyl)acetamide; U, uracil-1-yl; CAN, ceric ammonium nitrate; DMTr, 4,4'-dimethoxytrityl.

target affinity. Motivated by these results, we set out to (i) study a greater number of C5-functionalized LNA-U monomers¹⁴ and (ii) explore if this strategy for modulation of biophysical properties can be applied to other conformationally restricted nucleotides.

Here, we present the synthesis of six different C5-alkynylfunctionalized α -L-LNA-U phosphoramidites, their incorporation into ONs, and the characterization of these modified ONs via thermal denaturation, enzymatic stability, and steadystate fluorescence emission experiments. These monomers were selected to ensure a representation of C5 substituents with different sizes and polarities (Figure 1) and to facilitate direct comparison with corresponding C5-alkynyl-functionalized LNA-U.¹⁴

RESULTS AND DISCUSSION

Synthesis of \alpha-L-LNA Key Intermediate 9. Our synthetic route to C5-functionalized α -L-LNA-U phosphoramidites **11S**-**Y** is inspired by the optimized routes to LNA¹⁵ and α -L-LNA,^{9b} as well as our recent synthesis of C5-alkynyl-functionalized LNA nucleotides.^{13,14} Thus, fully protected glycosyl donor **1**, which is obtained from diacetone- α -D-glucose in six steps and ~30% overall yield,^{9b,16} was used as a starting material (Scheme 1). One-pot glycosylation of **1** with persilylated uracil under Vorbrüggen conditions afforded nucleoside **2** in 85% yield via anchimeric assistance. Treatment of **2** with hydrogen chloride in methanol afforded O2'-deacetylated nucleoside **3** in 97% yield. We found these conditions to be preferable to the use of cold dilute methanolic ammonia,^{9b} which results in the formation of small amounts of xylo-LNA byproducts. Subsequent O2'-mesylation of **3**

Scheme 2. Synthesis of C5-Alkynyl-Functionalized α -L-LNA-U Phosphoramidites 11S-Y^a



^{*a*}Abbreviation: PCl-reagent, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

Table 1. Thermal Denaturation Data for C5-Alkynyl-Functionalized α -L-LNA and Reference Strands against Complementary DNA/RNA^{*a*}

			$\Delta T_{\rm m}/{ m mod}$ (°C)						
ON	duplex	B =	L	S	v	W	х	Y	Z
B1	5′-GTG A <u>b</u> a TGC		+4.0	+7.5	+7.0	+9.5	+1.0	-2.5	-1.0
D2	3'-CAC TAT ACG								
D1	5'-GTG ATA TGC		+2.0	+3.5	+4.0	+5.5	-0.5	-10.0	-10.5
B2	3'-CAC <u>B</u> AT ACG								
D1	5'-GTG ATA TGC		+8.5	+8.5	+7.0	+9.5	+0.5	-2.0	±0.0
B3	3'-CAC TA <u>B</u> ACG								
D1	5'-GTG ATA TGC		+4.0	+4.0	+4.0	+6.5	nd	nd	+0.5
B4	3'-CAC <u>B</u> A <u>B</u> ACG								
B1	5'-GTG ABA TGC		+9.5	+11.0	+8.5	+12.5	+3.5	+1.0	+1.5
R2	3'-CAC UAU ACG								
R1	5'-GUG AUA UGC		+4.0	+5.0	+5.0	+7.0	+2.5	+0.5	-8.5
B2	3'-CAC <u>B</u> AT ACG								
R1	5'-GUG AUA UGC		+9.0	+9.0	+8.5	+12.0	+2.5	-0.5	+2.5
B3	3'-CAC TA <u>B</u> ACG								
D.							1	1	
KI	S'-GUG AUA UGC		+5.5	+6.0	+6.0	+7.5	nd	nd	+2.0
B4	3'-CAC BAB ACG								

 ${}^{a}\Delta T_{\rm m}$ = change in $T_{\rm m}$ values relative to unmodified reference duplexes D1:D2 ($T_{\rm m} \equiv 29.5 \,^{\circ}\text{C}$), D1:R2 ($T_{\rm m} \equiv 27.0 \,^{\circ}\text{C}$), and D2:R1 ($T_{\rm m} \equiv 27.0 \,^{\circ}\text{C}$). $T_{\rm m}$ values are determined as the first-derivative maximum of denaturation curves ($A_{260} \,$ vs T) recorded in medium salt phosphate buffer ([Na⁺] = 110 mM, [Cl⁻] = 100 mM, pH 7.0 (NaH₂PO₄/Na₂HPO₄)), using 1.0 μ M of each strand. $T_{\rm m}$ values are averages of at least two measurements within 1.0 $^{\circ}$ C. See Figure 1 for structures of monomers. nd = not determined. Data for L1–L4 were previously reported in ref 11b.

provided activated nucleoside **4** in 98% yield. Treatment of **4** with aqueous sodium hydroxide resulted in a cascade reaction,¹⁷ i.e., formation of an O2,O2'-anhydronucleoside, hydrolysis of the anhydronucleoside, and ring formation via intramolecular nucleophilic displacement, to afford α -L-LNA nucleoside **5** in quantitative yield. Subsequent protecting group manipulations entailing nucleophilic substitution of the O5'-mesylate of **5** (98%), O5'-debenzoylation of **6** (95%), and O3'-debenzylation of **7** (85%), using catalytic transfer hydrogenation conditions known to minimize undesired uracil C5–C6 reduction (formic acid and Pd(OH)₂/C),¹⁸ proceeded smoothly to afford diol **8**. Next, a reaction sequence entailing

O3',O5'-diacetylation, C5-iodination, O3',O5'-deacylation, and O5'-dimethoxytritylation converted diol **8** into key intermediate **9** in high yield without purification of intermediates. Direct C5-iodination of **8** and subsequent O5'-dimethoxytritylation to directly afford key intermediate **9** in only two steps is also possible but less attractive, due to lower overall yield and more complicated purification (see Scheme S1, Supporting Information). Hence, key intermediate **9** is obtained in ~45% overall yield and four chromatographic purification steps from glycosyl donor **1** (Scheme 1).

Synthesis of C5-Alkynyl-Functionalized α -L-LNA Phosphoramidites. Sonogashira reactions¹⁹ between key

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Table 2. Discrimination of Mismatched DNA/RNA Targets by Singly Modified C5-Alkynyl-Functionalized α -L-LNA and Reference ONs^a

		DNA: 3'-CAC T <u>B</u> T ACG			RNA: 3'-CAC U <u>B</u> U ACG				
		$T_{\rm m}$		ΔT_{m}		$T_{\rm m}$		ΔT_{m}	
ON	sequence	A	С	G	Т	Α	С	G	U
D1	5'-GTG ATA TGC	29.5	-16.5	-8.0	-15.5	27.0	<-17.0	-4.5	<-17.0
L1	5'-GTG ALA TGC	33.5	-23.5	-13.5	-17.5	36.5	-22.5	-8.0	-22.5
S1	5'-GTG ASA TGC	37.0	-24.0	-17.0	-23.0	38.0	-27.0	-11.0	-25.0
V1	5'-GTG AVA TGC	36.5	-23.5	-15.5	-22.0	35.5	-21.0	-8.5	-21.0
W1	5'-GTG AWA TGC	39.0	-25.0	-17.0	-22.5	39.5	-23.5	-11.0	-24.5
X1	5'-GTG AXA TGC	30.5	-18.0	-15.5	-18.5	30.5	-15.0	-7.0	-20.0
Y1	5'-GTG AYA TGC	27.0	<-17.0	<-17.0	<-17.0	28.0	<-18.0	-11.0	<-18.0
Z1	5'-GTG AZA TGC	28.5	-13.5	-13.5	-8.5	28.5	<-18.5	-13.0	<-18.5

^{*a*}For experimental conditions and sequences see Table 1. ΔT_m = change in T_m value relative to fully matched ON:DNA or ON:RNA duplex (**<u>B</u>** = A). Data for **L1** previously reported in reference 11b.

Table 3. Discrimination of Mismatched DNA/RNA Targets by Doubly Modified C5-Alkynyl-Functionalized α -L-LNA^a

			DNA: 5'-GTG A <u>B</u> A TGC			RNA: 5'-GUG A <u>B</u> A UGC			
		$T_{\rm m}$		$\Delta T_{\rm m}$		$T_{\rm m}$		$\Delta T_{\rm m}$	
ON	sequence	Т	A	С	G	U	А	С	G
D2	3'-CAC TAT ACG	29.5	<-19.5	-16.5	-7.5	27.0	-16.0	-16.0	-11.0
L4	3'-CAC LAL ACG	37.0	-23.0	-18.0	-17.0	38.0	-17.5	-19.0	-14.0
S4	3'-CAC SAS ACG	37.0	-18.0	-16.5	-12.5	39.0	-15.0	-17.0	-13.5
V4	3'-CAC VAV ACG	37.5	-23.0	-15.5	-12.0	38.5	-14.0	-15.5	-14.0
W4	3'-CAC WAW ACG	42.0	-21.5	-21.5	-14.5	41.5	-14.5	-16.5	-12.5
Z4	3'-CAC ZAZ ACG	30.5	<-20.5	-14.5	<-20.5	30.5	-15.5	<-20.5	-16.0
a _r	1 1 1		TT 1 1 AT	1 . 7	7 1 1.··	· C 11	. 1 1 1 1	(D T)	

^aFor experimental conditions and sequences see Table 1. ΔT_m = change in T_m value relative to fully matched duplexes (**B** = T).

intermediate **9** and different terminal alkynes²⁰ provided C5alkynyl-functionalized LNA uridines **10** in moderate to excellent yield (Scheme 2). Desilylation of **10S**' using TBAF afforded **10S** in 78% yield. Finally, O3'-phosphitylation of nucleosides **10** using 2-cyanoethyl-N,N'-diisopropylchlorophosphoramidite provided target phosphoramidites **11S**-**11Y** in S2-84% yield.

Structural Verification of α -L-LNA Nucleosides. As expected,^{9b} the ¹H NMR signals of H1', H2', and H3' of the α -L-LNA nucleosides appear as singlets or narrow doublets (J < 2 Hz),²¹ since the torsion angles defined by H1'-C1'-C2'_-H2' and H2'-C2'-C3'-H3' are restricted to +*gauche* and -*gauche* conformations, respectively. Moreover, the ROESY spectrum of α -L-LNA diol 8 exhibits through-space couplings between (i) H6 and H5", (ii) H1', H2' and H3', and (iii) H5' and H3', whereas no through-space coupling between H2' and H6 is observed (Figure S1, Supporting Information). These observations are fully consistent with the proposed stereochemical configuration.

Incorporation of C5-Alkynyl-Functionalized α -L-LNA **Monomers into ONs.** Novel phosphoramidites 11S-Y (and the known phosphoramidite of monomer Z²²) were used to incorporate monomers S-Z into 9-mer mixed-sequence ONs via machine-assisted DNA synthesis. Standard procedures were applied except for the use of extended hand-coupling times during incorporation of C5-alkynyl-functionalized α -L-LNA monomers (generally 15 min with 4,5-dicyanoimidazole as an activator—see the Experimental Section). The composition and purity of all modified ONs was verified by MALDI-MS (Table S1, Supporting Information) and ion-pair reversedphase HPLC, respectively. Unmodified reference DNA and RNA strands are denoted D1/D2 and R1/R2, respectively, while ONs containing a single incorporation of a modified nucleotide in the 5'-GTG ABA TGC context are denoted S1, V1, W1, and so on. Similar conventions are used for ONs in the B2–B4 series.

Thermal Denaturation Studies. The thermostabilities of duplexes between singly or doubly modified 9-mer ONs and DNA/RNA complements were determined by thermal denaturation experiments conducted in medium salt phosphate buffer ($[Na^+] = 110 \text{ mM}$). Thermal denaturation temperatures of modified duplexes are discussed relative to unmodified reference duplexes unless otherwise mentioned (Table 1).

As previously noted,^{11b} ONs modified with conventional α -L-LNA-T monomer L form very thermostable duplexes, especially with RNA targets, although considerable sequence variation is observed ($\Delta T_{\rm m}$ = +2.5 to +9.5 °C, Table 1). ONs modified with α -L-LNA uridines that are conjugated to small alkynes at the C5 position generally result in the formation of even more thermostable duplexes (compare $\Delta T_{\rm m}$ values for S/ V/W-modified ONs with L-modified ONs, Table 1). The effect is most pronounced with W-modified ONs, which result in additional duplex stabilization on the order of 1.0-5.5 °C relative to ONs modified with conventional α -L-LNA-T. We initially attributed these effects to improved base stacking (larger aromatic surface area due to extended conjugation) and reduced electrostatic repulsion (partially positively charged aminopropynyl shielding negatively charged strands).²³ However, analysis of thermodynamic parameters for the formed duplexes indicates that the structural underpinnings accounting for these results are more complex (vide infra). In contrast, α -L-LNA-U monomers that are conjugated to large hydrophobic entities reduce duplex thermostability relative to conventional α -L-LNA, presumably due to unfavorable steric interactions

Table 4. Thermodynamic Parameter	s for Formation of Duplexes Modifie	l with Select C5-Functionaliz	ed α -L-LNA Monomers"
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			+complementary 1	DNA	+complementary RNA					
ON	sequence	$\Delta G^{298} \left[\Delta \Delta G^{298} ight] $ (kJ/mol)	$\Delta H [\Delta \Delta H]$ (kJ/mol)	$\begin{array}{c} -T^{298}\Delta S \left[\Delta (T^{298}\Delta S)\right] \\ (\text{kJ/mol}) \end{array}$	$\Delta G^{298} \begin{bmatrix} \Delta \Delta G^{298} \end{bmatrix}$ (kJ/mol)	$\Delta H \left[\Delta \Delta H\right]$ (kJ/mol)	$\begin{array}{c} -T^{298}\Delta S \left[\Delta (T^{298}\Delta S)\right] \\ (\text{kJ/mol}) \end{array}$			
D1	5'-GTG ATA TGC	-42	-314	271	-36	-278	241			
D2	3'-CAC TAT ACG	-42	-314	271	-39	-293	254			
L1	5′-GTG A <u>L</u> A TGC	-49 [-7]	-349 [-35]	300 [+29]	-48 [-12]	-308 [-30]	260 [+19]			
L2	3'-CAC <u>L</u> AT ACG	-45 [-3]	-311 [+3]	266 [-5]	-44 [-5]	-306 [-13]	262 [+8]			
L3	3'-CAC TA <u>L</u> ACG	-48 [-6]	-302 [+12]	254 [-17]	-46 [-7]	-295 [-2]	249 [-5]			
S 1	5′-GTG A <u>s</u> a tgc	-49 [-7]	-333 [-19]	284 [+13]	-47 [-11]	-290 [-12]	243 [+2]			
S2	3'-CAC <u>s</u> at acg	-45 [-3]	-276 [+38]	231 [-40]	-45 [-6]	-299 [-6]	254 [±0]			
\$3	3'-CAC TA <u>s</u> ACG	-48 [-6]	-325 [-11]	275 [+4]	-48 [-9]	-318 [-25]	270 [+16]			
V1	5′-GTG A <u>v</u> a tgc	-51 [-9]	-406 [-92]	354 [+83]	-50 [-14]	-302 [-24]	252 [+11]			
V2	3'-CAC <u>V</u> AT ACG	-49 [-7]	-391 [-77]	342 [+71]	-46 [-7]	-340 [-47]	293 [+39]			
V3	3'-CAC TA <u>V</u> ACG	-51 [-9]	-343 [-29]	292 [+21]	-49 [-10]	-289 [+4]	240 [-14]			
W1	5'-GTG A <u>w</u> a TGC	-49 [-7]	-317 [-3]	267 [-4]	-50 [-14]	-302 [-24]	252 [+11]			
W2	3'-CAC <u>W</u> AT ACG	-46 [-4]	-312 [+2]	266 [-5]	-46 [-7]	-340 [-47]	293 [+39]			
W3	3'-CAC TA <u>W</u> ACG	-49 [-7]	-271 [+43]	222 [-49]	-49 [-10]	-289 [+4]	240 [-14]			
X1	5'-GTG A <u>X</u> A TGC	-44 [-2]	-304 [+10]	260 [-11]	-44 [-8]	-319 [-41]	275 [+34]			
X2	3'-CAC XAT ACG	-41 [-3]	-294 [+20]	253 [-18]	-41 [-2]	-245 [+48]	204 [-50]			
X3	3'-CAC TA <u>X</u> ACG	-43 [-1]	-287 [+27]	244 [-27]	-42 [-3]	-283 [+10]	241 [-13]			
Y1	5'-GTG A <u>y</u> a tgc	-39 [+3]	-313 [+1]	274 [+3]	-40 [-4]	-313 [-35]	273 [+32]			
Y2	3'-CAC <u>Y</u> AT ACG	-36 [+6]	-305 [+9]	269 [-2]	-40 [-1]	-325 [-32]	285 [+31]			
¥3	3'-CAC TA <u>Y</u> ACG	-39 [+3]	-324 [-10]	285 [-14]	-39 [±0]	-310 [-17]	271 [+17]			
^a Daran	Parameters were determined from thermal denaturation curves which were recorded as described in Table 1. $\Delta \Lambda G^{298}$ $\Delta \Delta H$ and $\Lambda (T^{298} \Delta S)$ are									

"Parameters were determined from thermal denaturation curves, which were recorded as described in Table 1. $\Delta\Delta G^{298}$, $\Delta\Delta H$, and $\Delta (T^{298}\Delta S)$ are calculated relative to reference duplexes D1:D2, D1:R2, and D2:R1.

and/or disruption of the hydration sphere in the major groove (note $\Delta T_{\rm m}$ values for X/Y/Z-modified ONs; Table 1). Nevertheless, many of the X/Y/Z-modified duplexes display thermostabilities similar to those of the unmodified reference duplexes.

The binding specificities of ONs with a single central modification (**B1** series) were evaluated against centrally mismatched DNA/RNA targets (Table 2). As previously reported,^{11b} ONs modified with conventional α -L-LNA monomer L display significantly improved binding specificity relative to the unmodified reference strand, as evidenced by the greater drops in $T_{\rm m}$ values of mismatched duplexes (compare $\Delta T_{\rm m}$ values for L1 and D1, Table 2). Interestingly, the high-affinity ONs S1/V1/W1 display similar or slightly improved binding specificity in comparison to conventional α -L-LNA L1 (compare $\Delta T_{\rm m}$ values for S1/V1/W1 and L1, Table 2), whereas improvements are less pronounced for ONs modified with hydrophobic C5-functionalized α -L-LNA-U monomers (compare $\Delta T_{\rm m}$'s for X1/Y1/Z1 and L1, Table 2).

The binding specificities of ONs with two next-nearest neighbor modifications (**B4** series) were determined using DNA/RNA targets with a mismatched nucleotide opposite the central 2'-deoxyriboadenosine (Table 3). α -L-LNA-T modified L4 displays improved binding specificity relative to the unmodified reference **D2**. Unlike the observations in the **B1** series, ONs modified with C5-alkynyl-functionalized monomers do not result in additional improvements in mismatch discrimination (compare ΔT_m values for the **B4** series, Table 3). This suggests that C5-alkynyl-functionalized α -L-LNA

should be designed in a manner that places likely singlenucleotide polymorphism (SNP) sites directly opposite the modified nucleotide for optimal thermal discrimination of singly mismatched targets.

Thermodynamic Parameters for Duplex Formation. Thermodynamic parameters for formation of duplexes modified with C5-functionalized α -L-LNA-U monomers were derived from thermal denaturation curves via curve fitting.²⁴ In agreement with the $T_{\rm m}$ data, duplexes modified with conventional α -L-LNA thymidines are 3-12 kJ/mol more stable than unmodified reference duplexes (see $\Delta\Delta G^{298}$ values for L1–L3, Table 4). Duplexes that are modified with α -L-LNA-U monomers conjugated to small alkynes display duplex stabilities comparable to or slightly higher than those of duplexes modified with conventional α -L-LNA-U monomers, while X/Y-modified duplexes are less stable (compare $\Delta\Delta G^{298}$ values for S/V/W series and X/Y series vs L series, Table 4).

The underlying structural underpinnings accounting for the additional stabilization of S/V/W-modified duplexes are not as clear as those for the corresponding C5-alkynyl-functionalized LNA, which are stabilized by more favorable enthalpy, a phenomenon that we ascribed to improved base stacking.¹⁴ For example, the additional stabilization of V-modified DNA duplexes is enthalpic in nature, whereas the situation is more ambiguous with V-modified DNA:RNA duplexes (compare $\Delta\Delta H$ and $\Delta(T^{298}\Delta S)$ values for V vs L series, Table 4). In contrast, S/W-modified duplexes are generally stabilized by more favorable entropy (compare $\Delta\Delta H$ and $\Delta(T^{298}\Delta S)$ values for S/W series vs L series, Table 4). The lower stability of DNA

duplexes modified with hydrophobic monomers X and Y is due to unfavorable enthalpic contributions, whereas Y-modified duplexes with RNA are destabilized by unfavorable entropic contributions. Additional studies are clearly needed to fully delineate the underlying reasons that govern these observations. However, the mechanisms through which the C5-alkynyl substituents exert their influence on duplex thermostability appear to be different between LNA-U and α -L-LNA-U nucleotides. This is not necessarily surprising, since incorporation of LNA and α -L-LNA nucleosides is known to have different effects on global duplex geometries, i.e., LNA nucleotides tune duplexes toward more RNA-like geometries,²⁵ whereas α -L-LNA nucleotides leave duplexes globally unperturbed.²⁶

3'-Exonuclease Stability of C5-Alkynyl-Functionalized α -L-LNA. Encouraged by the promising hybridization properties of S- and W-modified ONs, we set out to study their stability in the presence of snake venom phosphodiesterase (SVPDE), a 3'-exonuclease (Figure 2). As expected,



Figure 2. 3'-Exonuclease degradation of singly (top, 3'-CAC <u>B</u>AT ACG) and doubly modified (bottom, 3'-CAC <u>B</u>A<u>B</u> ACG) C5-functionalized α -L-LNA and reference strands. Nuclease degradation studies were conducted in magnesium buffer (50 mM Tris-HCl, 10 mM Mg²⁺, pH 9.0) using [ON] = 3.3 μ M and 0.03 U of snake venom phosphodiesterase.

unmodified **D2** is degraded rapidly (>95% degradation after 15 min), while conventional α -L-LNA **L1** displays moderate resistance against SVPDE-mediated degradation (~95% degradation after 2 h). Interestingly, C5-ethynyl- and C5aminopropynyl-functionalized α -L-LNA **S1** and **W1** confer additional protection against SVPDE (<80% and <60% degradation after 2 h, respectively), which strongly suggests that the C5 substituents interfere with SVPDE's mode of action. Expectedly, these trends are more pronounced with doubly modified ONs (**B4** series). Thus, considerable amounts of conventional **L4** and C5-ethynyl-functionalized α -L-LNA **S4** remain after 2 h (<70% and <40% cleavage, respectively). It is noteworthy that C5-aminopropynyl-functionalized α -L-LNA **W4**, following a brief period of degradation of the 1–3 nucleotides closest to the 3'-end, is inert to further degradation.

Fluorescence Properties of Z-Modified ONs. Steadystate fluorescence emission spectra of Z-modified ONs and the corresponding duplexes with complementary or mismatched DNA targets were recorded to evaluate the diagnostic potential of these probes. Hybridization of singly Z-modified ONs with complementary DNA/RNA generally results in significantly increased fluorescence emission and the formation of duplexes with two broad emission maxima at ~388 and ~401 nm (Figures 3 and Figures S2 and S3 (Supporting Information)).



Figure 3. Steady-state fluorescence emission spectra of single-stranded **Z1** and corresponding duplexes with complementary or mismatched DNA/RNA strands (mismatched nucleotide opposite to modification in parentheses). Conditions: λ_{ex} 344 nm, T = 5 °C, each oligonucleotide used in 1 μ M concentration. Note that different axis scales are used.

In contrast, hybridization of doubly modified Z4 with DNA/ RNA complements results in decreased monomer emission along with increased excimer emission ($\lambda_{em} \sim 510$ nm, Figures S4 and S5 (Supporting Information)), which is consistent with the formation of pyrene–pyrene dimers in the major groove.^{12c,27}

Interestingly, the fluorescence intensity of **Z1** is sensitive to the nature of the nucleotide opposite to the modification; hybridization with matched DNA/RNA targets results in the formation of highly fluorescent duplexes, whereas incubation with centrally mismatched targets results in much lower fluorescence intensities (Figure 3). Presumably this is due to different positioning of the pyrene moiety in matched vs mismatched duplexes in a similar manner as proposed for the corresponding DNA analogue of monomer Z.²⁸ According to this hypothesis, the pyrene moiety is directed into the nonquenching environment of the major groove in matched duplexes, whereas it is intercalating in mismatched duplexes, leading to nucleobase-mediated quenching²⁹ of pyrene fluorescence. In contrast, hybridization of doubly modified Z4 with centrally mismatched DNA/RNA targets results in a less intense excimer signal but more pronounced monomer emission (Figures S4 and S5 (Supporting Information)). This suggests that the presence of mismatches in the vicinity of two Z monomers positioned as next-nearest neighbors perturbs pyrene-pyrene stacking in a manner similar to that observed with other pyrene array forming probes.^{12a,30}

We have recently studied the fluorescent properties of longer Z-modified probes in detail.²² In comparison to ONs modified with the analogous DNA monomer,²⁸ Z-modified probes (i) display slightly larger increases in fluorescence intensity upon hybridization with complementary DNA, (ii) result in formation of more brightly fluorescent duplexes, and (iii) discriminate single-nucleotide polymorphisms more efficiently in AT-rich sequence contexts. In summary, Z-modified ONs are interesting probes for the discrimination of single-nucleotide polymorphisms for applications in nucleic acid diagnostics.

CONCLUSION

Attachment of small alkynyl entities (ethynyl, hydroxypropynyl, aminopropynyl) to the C5 position of α -L-LNA uridines significantly increases the target affinity, binding specificity, and enzymatic stability of oligonucleotides modified with these building blocks in comparison to conventional α -L-LNA uridines. On the other hand, attachment of larger alkynyl groups (derivatives of stearic acid, cholesterol, and pyrene) counteracts the stabilization provided by the extended conjugation. Suitably designed C5-functionalized α -L-LNA uridines are therefore interesting oligonucleotide modifications for nucleic acid targeting applications in molecular biology, biotechnology, and medicinal chemistry. The results from the back-to-back articles strongly suggest that C5 functionalization of pyrimidines is a general and synthetically convenient approach for improving the pharmacodynamic properties of oligonucleotides modified with LNA or other conformationally restricted monomers.

EXPERIMENTAL SECTION

1-[2-O-Acetyl-3-O-benzyl-5-O-(methanesulfonyl)-4-C-(methanesulfonyloxymethyl)- α - ι -threo-pentofuranosyl]uracil (2). Glycosyl donor 1 (6.10 g, 12.0 mmol) and uracil (2.70 g, 24.0 mmol) were coevaporated with anhydrous CH₃CN (100 mL) and resuspended in anhydrous CH₃CN (150 mL). To this was added N,Obis(trimethylsilyl)acetamide (BSA; 10.4 mL, 41.9 mmol), and the solution was refluxed until homogeneous. After the mixture was cooled to room temperature, trimethylsilyl triflate (TMSOTf, 5.5 mL, 29.9 mmol) was added and the reaction mixture was refluxed for 28 h, whereupon it was concentrated to near dryness. The resulting residue was taken up in EtOAc (200 mL), and the organic phase was washed with saturated aqueous NaHCO₃ ($2 \times 100 \text{ mL}$) and brine (100 mL). The aqueous phase was back-extracted with EtOAc (100 mL), and the combined organic layers were dried (Na2SO4) and evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0–2% MeOH in CH_2Cl_2 , v/v) to afford nucleoside 2 (4.80 g, 85%) as a white foam: $R_f = 0.5$ (2% MeOH in CH₂Cl₂, v/v); FAB-HRMS m/z 563.0998 ([M + H]⁺, C₂₁H₂₅N₂O₁₂S₂·H⁺, calcd 563.1005); ¹H NMR (DMSO- d_6) δ 11.40 (d, 1H, ex, J = 2.0 Hz, NH), 7.76 (d, 1H, J = 8.0 Hz, H6), 7.33–7.39 (m, 5H, Ph), 6.09 (d, 1H, J = 6.0 Hz, H1'), 5.69 (dd, 1H, J = 8.0 Hz, 2.0 Hz, H5), 5.54 (t, 1H, J = 6.0 Hz, H2'), 4.68–4.71 (d, 1H, J = 12.0 Hz, CH₂Ph), 4.65–4.68 (d, 1H, J = 12.0 Hz, CH₂Ph), 4.58–4.62 (d, 1H, J = 11.0 Hz, H5'), 4.39–4.47 (m, 4H, H3', H5', 2 × H5"), 3.27 (s, 3H, CH₃SO₂), 3.20 (s, 3H, CH₃SO₂), 2.02 (s, 3H, CH₃); ¹³C NMR (DMSO- d_6) δ 169.6, 162.7, 150.4, 140.2 (C6), 137.1, 128.3 (Ar), 127.91 (Ar), 127.86 (Ar), 102.7 (C5), 84.6 (C1'), 81.8, 80.7 (C3'), 77.3 (C2'), 72.5 (CH₂Ph), 68.3 (C5"), 67.9 (C5'), 36.7 (CH₃SO₂), 36.6 (CH₃SO₂), 20.3 (CH₃).

1-[3-O-Benzyl-5-O-(methanesulfonyl)-4-C-(methanesulfonyloxymethyl)- α -L-threo-pentofuranosyl]uracil (3). Method A. A 1 M solution of HCl in MeOH (50 mL) was added to a solution of nucleoside 2 (2.81 g, 5.00 mmol) in MeOH (30 mL). and after the reaction mixture was stirred at room temperature for 24 h, the solvent was evaporated off. The resulting residue was dissolved in CH₂Cl₂ (100 mL), and the organic phase was washed with saturated aqueous NaHCO₃ (2 \times 100 mL). The aqueous phase was then back-extracted with CH_2Cl_2 (2 × 50 mL). The combined organic phase was dried (Na_2SO_4) and evaporated to dryness to afford analytically pure nucleoside 3 (2.52 g, 97%) as a white solid material: $R_f = 0.4$ (4%) MeOH in CH₂Cl₂, v/v); FAB-HRMS m/z 521.0900 ([M + H]⁺, $C_{19}H_{24}N_2O_{11}S_2 \cdot H^+$, calcd 521.0894); ¹H NMR (DMSO- d_{δ}) δ 11.42 (d, 1H, ex, J = 2.0 Hz, NH), 7.76 (d, 1H, J = 8.0 Hz, H6), 7.29-7.40 (m, 5H, Ph), 6.12 (d, 1H, ex, J = 5.0 Hz, 2'-OH), 5.92 (d, 1H, J = 7.5Hz, H1'), 5.68 (dd, 1H, J = 8.0 Hz, 2.0 Hz, H5), 4.74-4.77 (d, 1H, J = 12.0 Hz, CH₂Ph), 4.67–4.69 (d, 1H, J = 12.0 Hz, CH₂Ph), 4.33–4.52 $(m, 5H, H2', 2 \times H5', 2 \times H5''), 4.19-4.21 (d, 1H, J = 6.5 Hz, H3'),$ 3.23 (s, 3H, CH₃SO₂), 3.17 (s, 3H, CH₃SO₂); ¹³C NMR (DMSO-d₆) δ 162.8, 150.8, 140.4 (C6), 137.5, 128.2 (Ar), 127.7 (Ar), 127.6 (Ar), 102.5 (C5), 86.0 (C1'), 82.8 (C3'), 80.9, 75.7 (C2'), 72.3 (CH₂Ph), 68.9 (C5"), 68.2 (C5'), 36.7 (CH₃SO₂), 36.6 (CH₃SO₂).

Method B. To a solution of nucleoside 2 (1.50 g, 2.66 mmol) in MeOH (50 mL) was added saturated methanolic ammonia (50 mL). The solution was stirred for 2 h at room temperature, whereupon solvents were evaporated off. The resulting residue was purified by silica gel column chromatography (0–3% MeOH in CH_2Cl_2 , v/v) to afford nucleoside 3 (1.11 g, 80%) as a white solid material with physical data as reported above.

1-[3-O-Benzyl-2,5-O-bis(methanesulfonyl)-4-C-(methanesulfonyloxymethyl)-α-L-threo-pentofuranosyl]uracil (4). Nucleoside 3 (10.0 g, 19.2 mmol) was coevaporated with anhydrous pyridine $(2 \times 75 \text{ mL})$ and redissolved in anhydrous pyridine (120 mL). To this was added methanesulfonyl chloride (MsCl, 1.8 mL, 23.3 mmol), and the reaction mixture was stirred for 4 h at room temperature, whereupon it was poured into saturated aqueous NaHCO₃ (200 mL) and extracted with CH_2Cl_2 (2 × 200 mL). The organic phase was dried (Na₂SO₄) and concentrated to dryness to afford analytically pure nucleoside 4 (11.3 g, 98%) as a slightly brown solid material: $R_f = 0.4$ (2% MeOH in CH₂Cl₂, v/v); FAB-HRMS m/z 599.0675 ([M + H]⁺, $C_{20}H_{26}N_2O_{13}S_3 \cdot H^+$, calcd 599.0670); ¹H NMR (DMSO- d_6) δ 11.48 (d, 1H, ex, J = 2.0 Hz, NH), 7.77 (d, 1H, J = 8.0 Hz, H6), 7.32-7.43 (m, 5H, Ph), 6.25 (d, 1H, J = 7.5 Hz, H1'), 5.70 (dd, 1H, J = 8.0 Hz, 2.0 Hz, H5), 5.54 (t, 1H, J = 7.0 Hz, H2'), 4.72 (s, 2H, CH₂Ph), 4.58-4.64 (2d, 2H, J = 11.0 Hz, J = 7.0 Hz, H5', H3), 4.41-4.49 (m, 3H, H5', $2 \times H5''$), 3.28 (s, 3H, CH₃), 3.25 (s, 3H, CH₃), 3.18 (s, 3H, CH₃); ¹³C NMR (DMSO- d_6) δ 162.7, 150.5, 140.3 (C6), 136.8, 128.3 (Ar), 128.1 (Ar), 128.0 (Ar), 102.8 (C5), 83.6 (C1'), 81.1 (C2'), 81.0, 80.7 (C3'), 73.0 (CH₂Ph), 68.5 (C5"), 67.7 (C5'), 37.8 (CH₃SO₂) 36.7 (CH₃SO₂), 36.6 (CH₃SO₂).

(15,3*R*,45,7*R*)-7-Benzyloxy-1-methanesulfonyloxymethyl-3-(uracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (5). To a solution of nucleoside 4 (9.87 g, 16.5 mmol) in 1,4-dioxane/H₂O (60 mL, 1/1, v/ v) was added aqueous NaOH (2 M, 50 mL, 0.10 mol). After it was stirred at room temperature for 4 h, the reaction mixture was neutralized by 10% aqueous AcOH and diluted with EtOAc (300 mL) and the phases were separated. The organic phase was washed with saturated aqueous NaHCO₃ (100 mL) and H₂O (100 mL), dried (Na₂SO₄), and evaporated to dryness to afford nucleoside 5 (7.00 g, quantitative) as a slightly brown solid material, which was used in the next step without further purification: $R_f = 0.5$ (80% EtOAc in petroleum ether, v/v); FAB-HRMS *m/z* 425.1018 ([M + H]⁺, C₁₈H₂₀N₂O₈S·H⁺, calcd 425.1013); ¹H NMR (DMSO-*d*₆) δ 11.39 (br s, 1H, ex, NH), 7.80 (d, 1H, *J* = 8.0 Hz, H6), 7.35–7.41 (m, 5H, Ph), 5.97 (s, 1H, H1'), 5.63 (d, 1H, *J* = 8.0 Hz, 1.2 Hz, H5), 4.55–4.73 (m, SH, 2 × CH₂Ph, H2', 2 × H5'), 4.50 (s, 1H, H3'), 4.04–4.08 (d, 1H, *J* = 8.5 Hz, H5"), 3.99–4.03 (d, 1H, *J* = 8.5 Hz, H5"), 3.23 (s, 3H, CH₃); ¹³C NMR (DMSO-*d*₆) δ 163.0, 150.2, 140.3 (C6), 137.5, 128.3 (Ar), 127.7 (Ar), 127.5 (Ar), 100.6 (C5), 86.7, 86.6 (C1'), 79.2 (C3'), 76.4 (C2'), 71.9 (C5"), 71.2 (CH₂Ph), 65.4 (C5') 36.9 (CH₃SO₂). A trace impurity of 1,4-dioxane was identified at 66.3 ppm in the ¹³C NMR spectrum.³¹

(1S,3R,4S,7R)-1-Benzoyloxymethyl-7-benzyloxy-3-(uracil-1yl)-2,5-dioxabicyclo[2.2.1]heptane (6). To a solution of nucleoside 5 (7.00 g, 16.5 mmol) in anhydrous DMF (300 mL) was added NaOBz (7.00 g, 48.6 mmol), and the reaction mixture was stirred at 90 °C for 20 h, whereupon it was cooled to room temperature and poured into ice-cold water (500 mL). The solution was extracted with EtOAc (2 \times 300 mL) and the organic phase washed with H2O (2 \times 150 mL) and brine (100 mL). The organic phase was dried (Na_2SO_4) and concentrated to dryness to afford analytically pure nucleoside 6 (7.24 g, 98%) as a slightly brown solid material: $R_f = 0.6$ (80% EtOAc in petroleum ether, v/v); FAB-HRMS m/z 451.1505 ([M + H]⁺, $C_{24}H_{22}N_2O_7 \cdot H^+$, calcd 451.1500); ¹H NMR (DMSO- d_6) δ 11.38 (br s, 1H, ex, NH), 7.98 (dd, 2H, J = 8.5 Hz, 1.0 Hz, Ar), 7.82 (d, 1H, J = 8.0 Hz, H6), 7.66–7.71 (t, 1H, J = 7.5 Hz, Ar), 7.51–7.55 (m, 2H, Ar), 7.25-7.38 (m, 5H, Ar), 6.00 (s, 1H, H1'), 5.62 (dd, 1H, J = 8.0 Hz, 8.0 Hz, 1.5 Hz, H5), 4.66–4.76 (m, 3H, $2 \times CH_2Ph$, H5'), 4.62 (s, 1H, H2'), 4.59 (s, 1H, H3'), 4.54–4.58 (d, 1H, J = 12.5 Hz, H5'), 4.14-4.17 (d, 1H, J = 8.5 Hz, H5"), 4.07-4.10 (d, 1H, J = 8.5 Hz, H5"); ¹³C NMR (DMSO- d_6) δ 165.2, 163.1, 150.3, 140.5 (C6), 137.6, 133.5 (Ar), 129.4 (Ar), 129.1, 128.7 (Ar), 128.2 (Ar), 127.6 (Ar), 127.5 (Ar), 100.5 (C5), 87.0, 86.6 (C1'), 79.3 (C3'), 76.3 (C2'), 72.1 (C5"), 71.1 (CH₂Ph), 59.8 (C5').

(1R,3R,4S,7R)-7-Benzvloxy-1-hydroxymethyl-3-(uracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (7). Method A. To a solution of nucleoside 6 (5.00 g, 11.1 mmol) dissolved in MeOH (100 mL) was added saturated methanolic ammonia (100 mL). The reaction mixture was stirred at room temperature for 14 h in a sealed flask. The solvent was then evaporated and the resulting residue purified by silica gel column chromatography (0-7% MeOH in CH2Cl2v/v) to afford nucleoside 7 (3.66 g, 95%) as a white solid material: $R_f = 0.4$ (7%) MeOH in CH₂Cl₂, v/v); FAB-HRMS m/z 347.1230 ([M + H]⁺, $C_{17}H_{18}N_2O_6 \cdot H^+$, calcd 347.1243); ¹H NMR (DMSO- d_6) δ 11.36 (s, 1H, ex, NH), 7.80 (d, 1H, J = 8.0 Hz, H6), 7.30-7.40 (m, 5H, Ph), 5.88 (s, 1H, H1'), 5.62 (d, 1H, J = 8.0 Hz, H5), 5.05 (t, 1H, ex, J = 5.5 Hz, 5'-OH), 4.63-4.71 (2d, 2H, J = 12.0 Hz, CH_2Ph), 4.52 (s, 1H, H2'), 4.33 (s, 1H, H3'), 3.92–3.98 (2d, 2H, J = 8.5 Hz, H5"), 3.73– 3.78 (m, 2H, 2 × H5'); ¹³C NMR (DMSO- d_6) δ 163.1, 150.3, 140.4 (C6), 137.9, 128.3 (Ar), 127.6 (Ar), 127.4 (Ar), 100.3 (C5), 90.1 (C4'), 86.5 (C1'), 79.3 (C3'), 76.3 (C2'), 72.4 (C5"), 71.1 (CH₂Ph), 57.1 (C5').

Method B. To a solution of nucleoside 6 (1.50 g, 3.33 mmol) in THF/H₂O (25 mL, 1/1, v/v) was added aqueous NaOH (2 M, 10.0 mL, 20.0 mmol), and the reaction mixture was stirred at room temperature for 4 h, whereupon it was carefully neutralized with 10% aqueous AcOH at 0 °C and diluted with EtOAc (50 mL). The organic phase was washed with saturated aqueous NaHCO₃ (50 mL) and the combined aqueous phase extracted with EtOAc (2 × 50 mL). The combined organic layers were dried (Na₂SO₄) and concentrated to dryness to afford nucleoside 7 (1.05 g, 91%) as a slightly brown solid.

(1*R*,3*R*,4*S*,7*R*)-7-Hydroxy-1-hydroxymethyl-3-(uracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (8). To a solution of nucleoside 7 (1.50 g, 4.32 mmol) in THF/MeOH (100 mL, 9/1, v/v) were added $Pd(OH)_2/C$ (20 wt %, 0.60 g) and 88% aqueous formic acid (2.3 mL, 61.1 mmol) from a freshly opened bottle. The reaction mixture was refluxed for 24 h, whereupon it was cooled to room temperature. The catalyst was filtered off and washed with excess MeOH, and the combined filtrates were concentrated to dryness. The resulting crude residue was purified by silica gel column chromatography (0–16% MeOH in CH₂Cl₂, v/v) to afford nucleoside **8** (0.94 g, 85%) as a white solid material: $R_f = 0.4$ (15% MeOH in CH₂Cl₂, v/v); FAB-HRMS *m*/*z* 257.0760 ([M + H]⁺, C₁₀H₁₂N₂O₆·H⁺, calcd 257.0768); ¹H NMR (DMSO-*d*₆) δ 11.34 (s, 1H, ex, NH), 7.80 (d, 1H, *J* = 8.0 Hz, H6), 5.87 (s, 1H, H1'), 5.85 (d, ex, *J* = 4.0 Hz, 3'-OH), 5.63 (d, 1H, *J* = 8.0 Hz, H5), 4.93 (t, ex, *J* = 5.5 Hz, 5'-OH), 4.27 (d, 1H, *J* = 4.0 Hz, H3'), 4.21 (s, 1H, H2'), 3.88–3.94 (2d, 2H, *J* = 8.5 Hz, HS"), 3.73 (d, 2H, *J* = 5.5 Hz, 2 × H5'); ¹³C NMR (DMSO-*d*₆) δ 163.2, 150.3, 140.4 (C6), 100.2 (C5), 90.9, 86.5 (C1'), 78.7 (C2'), 72.5 (C3'), 71.9 (C5"), 57.4 (C5').

(15,3*R*,45,7*R*)-1-(4,4'-Dimethoxytrityloxymethyl)-7-hydroxy-3-(5-iodoracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (9). Ac₂O (0.21 mL, 2.20 mmol) was added to a solution of nucleoside 8 (0.25 g, 1.00 mmol) in anhydrous pyridine (10 mL) and the reaction mixture was stirred at 60 °C for 14 h. After it was cooled to room temperature, the reaction mixture was diluted with saturated aqueous NaHCO₃ (30 mL) and CH₂Cl₂ (30 mL) and the phases were separated. The organic phase was washed with saturated aqueous NaHCO₃ (20 mL) and the combined aqueous phase back-extracted with CH₂Cl₂ (2 × 20 mL). The combined organic layers were dried (Na₂SO₄), evaporated to dryness, and coevaporated with toluene/ absolute EtOH (2 × 30 mL, 1/2, v/v). The resulting residue, tentatively assigned as the O3',O5'-diacetylated nucleoside, was used in the next step without further purification ($R_{\rm f}$ = 0.5 (2% MeOH in CH₂Cl₂, v/v); FAB-MS *m*/z 341 ([M + H]⁺)).

To a solution of the crude O3',O5'-diacetylated α -L-LNA uridine in glacial AcOH (10 mL) were added iodine (160 mg, 0.62 mmol) and ceric ammonium nitrate (CAN, 235 mg, 0.50 mmol), and the reaction mixture was stirred at 80 °C for 50 min. After it was cooled to room temperature, the reaction mixture was evaporated to dryness and taken up in CH₂Cl₂ (50 mL). The organic phase was washed with saturated aqueous NaHCO₃ (2 × 20 mL) and H₂O (20 mL). The combined aqueous phase was back-extracted with CH₂Cl₂ (2 × 20 mL). The combined organic layers were dried (Na₂SO₄) and evaporated to dryness. The resulting residue, tentatively assigned as the C5-iodo-O3',O5'-diacetylated nucleoside, was used in the next step without further purification ($R_{\rm f}$ = 0.5 (3% MeOH in CH₂Cl₂, v/v); FAB-HRMS *m*/*z* 466.9966 ([M + H]⁺, C₁₄H₁₅IN₂O₈·H⁺, calcd 466.9946)).

The crude C5-iodo-O3',O5'-diacetylated nucleoside was dissolved in saturated methanolic ammonia (30 mL) and stirred in a sealed flask at room temperature for 12 h. The reaction mixture was evaporated to dryness, affording a residue that was tentatively assigned as the C5iodo α -L-LNA diol and used in the next step without further purification ($R_f = 0.4$ (15% MeOH in CH₂Cl₂, v/v); FAB-HRMS m/z382.9735 ([M + H]⁺, C₁₀H₁₁IN₂O₆·H⁺, calcd 382.9740)).

The crude C5-iodo α -L-LNA diol was dried through coevaporation with anhydrous pyridine (10 mL) and redissolved in anhydrous pyridine (10 mL). To this was added 4,4'-dimethoxytrityl chloride (DMTrCl, 0.40 g, 1.20 mmol) and the reaction mixture was stirred at room temperature for 16 h, whereupon it was diluted with saturated aqueous NaHCO₃ (20 mL) and CH₂Cl₂ (25 mL). The phases were separated, and the organic phase was washed with saturated aqueous NaHCO₃ (20 mL). The aqueous phase was back-extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layers were dried (Na₂SO₄), evaporated to near dryness, and coevaporated with toluene/absolute EtOH (2×30 mL, 1/2, v/v). The resulting residue was purified by silica gel column chromatography (0-4.5% MeOH in CH_2Cl_2 , v/v) to afford nucleoside 9 (0.48 g, 70%, over four steps) as a slightly yellow solid material: $R_f = 0.5$ (5% MeOH in CH₂Cl₂, v/v); FAB-HRMS m/z 684.0980 ([M]⁺, C₃₁H₂₉IN₂O₈⁺, calcd 684.0969); ¹H NMR (DMSO-d₆) δ 11.78 (s, 1H, ex, NH), 8.16 (s, 1H, H6), 7.23-7.43 (m, 9H, Ar), 6.92 (d, 4H, J = 8.5 Hz, Ar), 5.92 (d, 1H, ex, J = 4.5Hz, 3'-OH), 5.89 (s, 1H, H1'), 4.35 (d, 1H, J = 4.5 Hz, H3'), 4.27 (s, 1H, H2'), 3.98-4.02 (d, 1H, J = 8.5 Hz, H5"), 3.92-3.96 (d, 1H, J = 8.5 Hz, H5"), 3.75 (s, 6H, $2 \times CH_2O$), 3.34–3.37 (d, 1H, J = 10.5 Hz, H5'), 3.28-3.31 (d, 1H, J = 10.5 Hz, H5' - partial overlap with H₂O); $^{13}\mathrm{C}$ NMR (DMSO- $d_6)$ δ 160.6, 158.134, 158.126, 149.9, 144.7, 144.3 (C6), 135.2, 135.1, 129.64, 129.62 (Ar), 127.9 (Ar), 127.5 (Ar), 126.7

(Ar), 113.3 (Ar), 89.3, 87.2 (C1'), 85.3, 78.8 (C2'), 72.9 (C3'), 72.3 (C5"), 67.7, 60.0 (C5'), 55.0 (CH₃O).

Representative Protocol for Sonogashira Coupling Reactions (10S'–Z). The key intermediate 9, $Pd(PPh_3)_4$, CuI, and alkyne were added to anhydrous DMF (quantities and volumes specified below), and the reaction chamber was degassed and placed under an argon atmosphere. To this was added Et_3N , and the reaction mixture was stirred in the dark at room temperature (unless otherwise mentioned) for 6–12 h, whereupon the solvents were evaporated. The resulting residue was dissolved in EtOAc (100 mL), and the organic phase was washed with brine (2 × 50 mL) and saturated aqueous NaHCO₃ (50 mL). The combined aqueous phase was back-extracted with EtOAc (100 mL). The combined organic phase was dried (Na₂SO₄) and evaporated to dryness and the resulting residue purified by silica gel column chromatography (0–5% MeOH in CH_2Cl_2 , v/v) to afford the desired product.

(1S.3R.4S.7R)-1-(4.4'-Dimethoxytrityloxymethyl)-7-hydroxy-3-[5-(trimethylsilylethynyl)uracil-1-yl]-2,5-dioxabicyclo[2.2.1]heptane (105'). Nucleoside 9 (0.68 g, 1.00 mmol), Pd(PPh₃)₄ (120 mg, 0.10 mmol), CuI (40 mg, 0.20 mmol), trimethylsilylacetylene (0.42 mL, 3.00 mmol), and Et₃N (0.60 mL, 4.27 mmol) in anhydrous DMF (10 mL) were reacted as described in the representative Sonogashira protocol, and the mixture was stirred at room temperature for 12 h. After work-up and purification, nucleoside 10S' (0.55 g, 84%) was obtained as a slightly brown solid material: $R_f = 0.5$ (5% MeOH in CH₂Cl₂, v/v); FAB-HRMS m/z 655.2462 ([M + H]⁺, C₃₆H₃₈N₂O₈Si-H⁺, calcd 655.2476); ¹H NMR (DMSO- d_6) δ 11.75 (s, 1H, ex, NH), 7.93 (s, 1H, H6), 7.21-7.43 (m, 9H, Ar), 6.90 (d, 4H, J = 8.5 Hz, Ar), 5.94 (d, 1H, ex, J = 4.5 Hz, 3'-OH), 5.92 (s, 1H, H1'), 4.38 (d, 1H, J = 4.5 Hz, H3'), 4.26 (s, 1H, H2'), 3.99-4.02 (d, 1H, J = 8.5 Hz, H5"), 3.93-3.96 (d, 1H, J = 8.5 Hz, H5"), 3.74 (s, 6H, 2 × CH₃O), 3.34 (s, 2H, H5'), 0.21 (s, 9H, Me₃Si); ¹³C NMR (DMSO-d₆) δ 161.3, 158.1, 149.1, 144.6, 143.7 (C6), 135.2, 135.1, 129.7 (Ar), 129.6 (Ar), 127.8 (Ar), 127.6 (Ar), 126.7 (Ar), 113.2 (Ar), 97.8, 97.1, 96.9, 89.4, 87.3 (C1'), 85.3, 78.6 (C2'), 72.7 (C3'), 72.3 (C5"), 60.0 (C5'), 55.0 (CH₃O), -0.12 (Me₃Si).

(1S,3R,4S,7R)-1-(4,4'-Dimethoxytrityloxymethyl)-3-(5-ethynyluracil-1-yl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptane (10S). TBAF in THF (1M, 1.2 mL, 1.2 mmol) was added to a solution of nucleoside 10S' (0.53 g, 0.81 mmol) in THF (20 mL) and the reaction mixture was stirred at room temperature for 2 h. EtOAc (50 mL) was added, and the solution was washed with brine $(2 \times 30 \text{ mL})$ and H₂O (30 mL). The combined aqueous phase was back-extracted with EtOAc (30 mL). The combined organic layer was dried (Na2SO4) and concentrated to dryness and the resulting residue purified by silica column chromatography (0-5% MeOH in CH2Cl2, v/v) to afford nucleoside 10S (0.37 g, 78%) as a light brown solid material: $R_f = 0.5$ (5% MeOH in CH₂Cl₂, v/v); ESI-HRMS m/z 605.1918 ([M + Na]⁺, $C_{33}H_{30}N_2O_8 \cdot Na^+$, calcd 605.1894); ¹H NMR (DMSO- d_6) δ 11.74 (s, 1H, ex, NH), 8.03 (s, 1H, H6), 7.23-7.43 (m, 9H, Ar), 6.90 (d, 4H, J = 8.5 Hz, Ar), 5.90-5.96 (m, 2H, 1ex, H1', 3'-OH), 4.40 (s, 1H, H3'), 4.28 (s, 1H, H2'), 4.18 (s, 1H, HC≡C), 4.01–4.04 (d, 1H, J = 8.5 Hz, H5"), 3.91-3.94 (d, 1H, J = 8.5 Hz, H5"), 3.75 (s, 6H, $2 \times CH_3O$), 3.32 (br s, 2H, H5'); ¹³C NMR (DMSO- d_6) δ 161.6, 158.1, 149.2, 144.7, 143.7 (C6), 135.2, 135.1, 129.7 (Ar), 128.9 (Ar), 127.8 (Ar), 127.6 (Ar), 126.7 (Ar), 113.2 (Ar), 96.4, 89.4, 87.3 (C1'), 85.3, 83.4 (HC≡C), 78.7 (C2'), 76.3, 72.8 (C3'), 72.2 (C5"), 59.8 (C5'), 55.0 (CH_3O) .

(15,3*R*,45,7*R*)-3-[5-(3-Benzoyloxypropyn-1-yl)uracil-1-yl]-1-(4,4'dimethoxytrityloxymethyl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptane (**10V**). Nucleoside 9 (0.50 g, 0.73 mmol), Pd(PPh₃)₄ (90 mg, 0.07 mmol), CuI (30 mg, 0.14 mmol), prop-2-ynyl benzoate³² (180 mg, 1.12 mmol), and Et₃N (0.40 mL, 2.84 mmol) in anhydrous DMF (10 mL) were reacted as described in the representative Sonogashira protocol, and the mixture was stirred at room temperature for 12 h. After workup and purification, nucleoside **10V** (0.40 g, 76%) was obtained as a slightly brown solid material: $R_f = 0.5$ (5% MeOH in CH₂Cl₂, v/v); ESI-HRMS *m*/*z* 739.2234 ([M + Na]⁺, C₄₁H₃₆N₂O₁₀. Na⁺, calcd 739.2262); ¹H NMR (DMSO-*d*₆) δ 11.79 (s, 1H, ex, NH), 8.03 (s, 1H, H6), 7.98 (dd, 2H, *J* = 8.3 Hz, 1.2 Hz, Ar), 7.65–7.69 (td, 1H, J = 7.5 Hz, 1.2 Hz, Ar), 7.50–7.54 (m, 2H, Ar), 7.17–7.44 (m, 9H, Ar), 6.90 (d, 4H, J = 9.0 Hz, Ar), 5.94 (br s, 2H, 1 ex, H1', 3'-OH), 5.20 (s, 2H, CH₂OBz), 4.42 (d, 1H, J = 4.5 Hz, H3'), 4.28 (s, 1H, H2'), 4.01–4.04 (d, 1H, J = 8.0 Hz, H5"), 3.90–3.94 (d, 1H, J = 8.0 Hz, H5"), 3.73 (s, 6H, 2 × OCH₃), 3.29 (s, 2H, H5'); ¹³C NMR (DMSO- d_6) δ 165.0, 161.4, 158.1, 149.2, 144.7, 144.0 (C6), 135.2, 135.1, 133.6 (Ar), 129.68 (Ar), 129.66 (Ar), 129.2 (Ar), 129.0, 128.8 (Ar), 127.8 (Ar), 127.5 (Ar), 126.6 (Ar), 113.2 (Ar), 96.1, 89.4, 87.3 (C1'), 86.6, 85.3, 79.2, 78.6 (C2'), 72.7 (C3'), 72.2 (C5"), 59.7 (C5'), 55.0 (CH₃O), 53.2 (CH₂OBz).

(1S,3R,4S,7R)-1-(4,4'-Dimethoxytrityloxymethyl)-7-hydroxy-3-[5-(3-trifluoroacetylaminopropyn-1-yl)uracil-1-yl]-2,5-dioxabicyclo-[2.2.1]heptane (10W). Nucleoside 9 (0.50 g, 0.73 mmol), Pd(PPh₃)₄ (90 mg, 0.07 mmol), CuI (30 mg, 0.14 mmol), 2,2,2-trifluoro-N-(prop-2-ynyl)acetamide³³ (180 mg, 1.46 mmol), and Et₃N (0.4 mL, 2.84 mmol) in anhydrous DMF (10 mL) were reacted as described in the representative Sonogashira protocol and stirred at room temperature for 12 h. After workup and purification, nucleoside 10W (0.43 g, 84%) was obtained as a slightly brown solid material: $R_{\rm f}$ = 0.5 (5% MeOH in CH_2Cl_2 , v/v); MALDI-HRMS m/z 730.1976 $([M + Na]^+, C_{36}H_{32}F_3N_3O_9 \cdot Na^+, calcd 730.1983); {}^{1}H NMR (DMSO$ d_6) δ 11.76 (s, 1H, ex, NH(U)), 10.04 (t, ex, 1H, J = 5.5 Hz, NHCH₂), 7.94 (s, 1H, H6), 7.21–7.43 (m, 9H, Ar), 6.91 (d, 4H, J = 9.0 Hz, Ar), 5.94-5.96 (m, 2H, 1ex, H1', 3'-OH), 4.44 (d, 1H, J = 4.0 Hz, H3'), 4.25-4.29 (m, 3H, H2', CH₂NH), 3.98-4.00 (d, 1H, J = 8.5 Hz, H5"), 3.92-3.95 (d, 1H, J = 8.5 Hz, H5"), 3.74 (s, 6H, $2 \times OCH_3$), 3.28–3.32 (m, 2H, H5', overlap with H₂O); ^{13}C NMR (DMSO-d_6) δ 161.5, 158.1, 156.0 (q, J = 36.3 Hz, COCF₃) 149.2, 144.7, 143.4 (C6), 135.2, 135.1, 129.72 (Ar), 129.69 (Ar), 127.8 (Ar), 127.6 (Ar), 126.7 (Ar), 115.7 (q, J = 287 Hz, CF₃), 113.2 (Ar), 96.5, 89.4, 87.3, 87.1 (C1'), 85.3, 78.6 (C2'), 75.4, 72.7 (C3'), 72.3 (C5"), 59.7 (C5'), 55.0 (CH₃O), 29.5 (CH₂NH).

(1S,3R,4S,7R)-1-(4,4'-Dimethoxytrityloxymethyl)-7-hydroxy-3-[5-(3-octadecanoylaminopropyn-1-yl)uracil-1-ylj-2,5-dioxabicyclo-[2.2.1]heptane (10X). Nucleoside 9 (0.34 g, 0.50 mmol), Pd(PPh₃)₄ (60 mg, 0.05 mmol), CuI (20 mg, 0.10 mmol), N-(prop-2ynyl)stearamide, 14 (0.28 g, 1.00 mmol) and Et_3N (0.30 mL, 2.13 mmol) in anhydrous DMF (10 mL) were reacted as described in the representative Sonogashira protocol, and the mixture was stirred at 40 °C for 6 h. After workup and purification, nucleoside 10X (0.24 g, 55%) was obtained as a brown solid material: $R_{\rm f} = 0.5$ (5% MeOH in CH₂Cl₂, v/v); ESI-HRMS m/z 900.4813 ([M + Na]⁺, C₅₂H₆₇N₃O₉· Na⁺, calcd 900.4770); ¹H NMR (CDCl₃) δ 7.89 (s, 1H, H6), 7.24-7.46 (m, 9H, Ar), 6.87 (d, 4H, J = 9.0 Hz, Ar), 5.97 (s, 1H, H1'), 5.88 $(t, ex, 1H, J = 5.0 Hz, NHCH_2), 4.55 (s, 1H, H2'), 4.48 (s, 1H, H3'),$ 4.27-4.29 (m, 2H, CH₂NH), 4.10-4.13 (d, 1H, J = 9.0 Hz, H5"), 3.97-4.01 (d, 1H, J = 9.0 Hz, H5"), 3.81 (s, 6H, $2 \times CH_3O$), 3.50-3.57 (2d, 2H, J = 11.0 Hz, H5'), 2.16-2.19 (m, 2H, CH₂CONH),1.60–1.64 (m, 2H, CH₂CH₂CONH), 1.20–1.25 (m, 28H, 14 × CH₂), 0.89 (t, 3H, J = 7.0 Hz, CH₃CH₂); ¹³C NMR (CDCl₃) δ 172.8, 161.6, 158.77, 158.76, 149.0, 144.3, 142.7 (C6), 135.23, 135.21, 130.04 (Ar), 130.03, (Ar), 128.04 (Ar), 127.98 (Ar), 127.1 (Ar), 113.4 (Ar), 98.5, 89.8, 89.6, 88.0 (C1'), 86.7, 78.9 (C2'), 74.5, 74.3 (C3'), 72.8 (C5"), 59.5 (C5'), 55.3 (CH₃O), 36.5 (CH₂CONH), 31.9 (CH₂), 30.1 (CH₂NH), 29.69 (CH₂), 29.68 (CH₂), 29.66 (CH₂), 29.65 (CH₂), 29.63 (CH₂), 29.5 (CH₂), 29.353 (CH₂), 29.345 (CH₂), 29.33 (CH₂), 25.5 (CH₂CH₂CONH), 22.7 (CH₂), 14.1 (CH₃).

(15, 3*R*, 45, 7*R*)-3-[5-(3-Cholesterylcarbonylaminopropyn-1-yl)uracil-1-yl]-1-(4, 4' -dimethoxytrityloxymethyl)-7-hydroxy-2, 5dioxabicyclo[2.2.1]heptane (10Y). Nucleoside 9 (0.34 g, 0.50 mmol), Pd(PPh₃)₄ (60 mg, 0.05 mmol), CuI (20 mg, 0.10 mmol), cholesterylprop-2-ynyl-carbamate amine³⁴ (0.47 g, 1.00 mmol), and Et₃N (0.30 mL, 2.13 mmol) in anhydrous DMF (8 mL) were reacted as described in the representative Sonogashira protocol and the reaction mixture was stirred at room temperature for 12 h. After workup and purification, nucleoside 10Y (0.39 g, 76%) was obtained as a slightly yellow solid material: $R_f = 0.5$ (5% MeOH in CH₂Cl₂, v/ v); ESI-HRMS *m*/z 1046.5533 ([M + Na]⁺, C₆2H₇₇N₃O₁₀·Na⁺, calcd 1046.5501); ¹H NMR (CDCl₃) δ 8.92 (bs, 1H, ex, NH(U)), 7.89 (s, 1H, H6), 7.23–7.46 (m, 9H, Ar), 6.87 (d, 4H, J = 9.0 Hz, Ar), 5.98 (s,

1H, H1'), 5.36 (d, 1H, J = 5.0 Hz, HC=C-Chol), 5.12 (t, ex, 1H, J =5.0 Hz, NHCH₂), 4.47-4.56 (m, 3H, H2', HC-O-Chol, H3'), 4.20 (d, 2H, I = 5.0 Hz, CH₂NH), 4.11-4.15 (d, 1H, I = 9.0 Hz, H5"), 3.98-4.02 (d, 1H, J = 9.0 Hz, H5"), 3.81 (s, 6H, 2 × CH₃O), 3.50–3.58 (2d, 2H, J = 11.0 Hz, H5'), 0.87–2.36 (m, 40H, Chol), 0.69 (s, 3H, CH₃-Chol); ¹³C NMR (CDCl₃) δ 161.6, 158.74, 158.73, 149.0, 144.3, 142.7 (C6), 139.8, 135.3, 132.1 (Ar), 132.0 (Ar), 130.04 (Ar), 130.03 (Ar), 128.5 (Ar), 128.4 (Ar), 128.02 (Ar), 128.00 (Ar), 127.1 (Ar), 122.5 (CH=C-chol), 113.4 (Ar), 98.6, 89.9, 88.0 (C1'), 86.6, 78.9 (C2'), 74.9 (CH-O-chol), 74.4, 74.3 (C3'),³⁵ 72.8 (C5"), 59.5 (C5'), 56.7 (CH-chol), 56.2 (CH-chol), 55.2 (CH₃O), 50.0 (CH-chol), 42.3, 39.8 (CH₂-chol), 39.5 (CH₂-chol), 38.5 (CH₂-chol), 37.0 (CH₂-chol), 36.6, 36.2 (CH₂-chol), 35.8 (CH-chol), 31.9 (CH-chol), 31.8 (CH₂NH and CH₂-chol overlap), 28.2 (CH₂-chol), 28.1 (CH₂-chol), 28.0 (CH-chol), 24.3 (CH2-chol), 23.8 (CH2-chol), 22.8 (CH2-chol), 22.5 (CH₃-chol), 21.0 (CH₂-chol), 19.3 (CH₃-chol), 18.7 (CH₃-chol), 11.9 (CH₂-chol).

Representative Protocol for Phosphitylation. Unless otherwise mentioned, the following protocol was used. Alcohol 10 was dried through coevaporation with anhydrous 1,2-dichloroethane (2×10) mL) and dissolved in anhydrous CH₂Cl₂. To this solution was added anhydrous EtN(iPr)₂ (DIPEA) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (PCl-reagent), and the reaction mixture was stirred at room temperature until analytical TLC showed full conversion of the starting material (2-3 h) (quantities and volumes specified below). The reaction mixture was diluted with CH_2Cl_2 (25) mL) and washed with 5% aqueous NaHCO₃ (2 \times 10 mL) and the combined aqueous phase back-extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were dried (Na₂SO₄) and evaporated to dryness, and the resulting residue was purified by silica gel column chromatography (0-3% MeOH in CH_2Cl_2 , v/v) and subsequently triturated from CH2Cl2 and petroleum ether to provide phosphoramidite 11.

(15, 3*R*, 45, 7*R*)-7-[2-Cyanoethoxy(diisopropylamino)phosphinoxy]-1-(4,4'-dimethoxytrityloxymethyl)-3-(5-ethynyluracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (115). Nucleoside 10S (0.35 g 0.60 mmol), DIPEA (0.50 mL, 2.88 mmol), and PCI-reagent (0.20 mL, 0.87 mmol) in anhydrous CH₂Cl₂ (10 mL) were reacted, worked up, and purified as described in the representative phosphitylation protocol to provide 11S (0.39 g, 83%) as a white foam: $R_{\rm f}$ = 0.5 (2% MeOH in CH₂Cl₂, v/v); ESI-HRMS *m*/*z* 805.2943 ([M + Na]⁺, C₄₂H₄₇N₄O₉P·Na⁺, calcd 805.2958); ³¹P NMR (CDCl₃) δ 150.2, 149.9.

(15,3R,45,7R)-3-[5-(3-Benzoyloxypropyn-1-yl)uracil-1-yl]-7-[2cyanoethoxy(diisopropylamino)phosphinoxy]-1-(4,4'-dimethoxytrityloxymethyl)-2,5-dioxabicyclo[2.2.1]heptane (11V). Nucleoside **10V** (0.35 g, 0.49 mmol) was coevaporated with anhydrous 1,2dichloroethane (2 × 7 mL) and redissolved in anhydrous CH₂Cl₂ (7 mL). To this solution were added DIPEA (425 μ L, 2.44 mmol) and Nmethylimidazole (31 μ L, 0.39 mmol), followed by dropwise addition of the PCI-reagent (220 μ L, 0.98 mmol). The reaction mixture was stirred at room temperature for 3 h, whereupon it was evaporated to near dryness. The resulting residue was purified by silica gel column chromatography (0–3% MeOH in CH₂Cl₂) and subsequently triturated from CH₂Cl₂ and petroleum ether to provide **11V** (0.28 g, 62%) as a white foam: $R_{\rm f}$ = 0.5 (3% MeOH in CH₂Cl₂); ESI-HRMS m/z 939.3332 ([M + Na]⁺, C₅₀H₅₃N₄O₁₁P·Na⁺, calcd 939.3341); ³¹P NMR (CDCl₃) δ 150.1, 149.8.

(15,3*R*,45,7*R*)-7-[2-Cyanoethoxy(diisopropylamino)phosphinoxy]-1-(4,4'-dimethoxytrityloxymethyl)-3-[5-(3-trifluoroacetylaminopropyn-1-yl)uracil-1-yl]-2,5-dioxabicyclo[2.2.1]heptane (11W). Nucleoside 10W (0.25 g 0.35 mmol), DIPEA (0.30 mL, 1.7 mmol), and PCl-reagent (0.10 mL, 0.45 mmol) in anhydrous CH₂Cl₂ (10 mL) were reacted, worked up, and purified as described in the representative phosphitylation protocol to provide 11W (0.27 g, 84%) as a white foam: $R_f = 0.5$ (2% MeOH in CH₂Cl₂, v/v); ESI-HRMS m/ z 930.3068 ([M + Na]⁺, C₄₅H₄₉F₃N₅O₁₀P·Na⁺, calcd 930.3080); ³¹P NMR (CDCl₃) δ 150.2, 149.9.

(1 S, 3R, 4 S, 7R)-7-[2-Cy a noethoxy (diisopropylamino)phosphinoxy]-1-(4,4'-dimethoxytrityloxymethyl)-3-[5-(3-octadecanoylaminopropyn-1-yl)uracil-1-yl]-2,5-dioxabicyclo[2.2.1]heptane (11X). Nucleoside 10X (170 mg, 0.19 mmol), DIPEA (145 μ L, 0.83 mmol), and PCl-reagent (61 μ L, 0.27 mmol) in anhydrous CH₂Cl₂ (2 mL) were mixed and reacted as described in the representative phosphitylation protocol. After it was stirred at room temperature for 3 h, the mixture was diluted with EtOAc (20 mL) and washed with H₂O (2 × 30 mL). The organic phase was dried (Na₂SO₄) and evaporated to dryness, and the resulting residue was purified by silica gel column chromatography (0–70% EtOAc in petroleum ether, v/v) and subsequently triturated from CH₂Cl₂ and petroleum ether to provide 11X (107 mg, 52%) as a white foam: R_f = 0.4 (5% MeOH in CH₂Cl₂, v/v); ESI-HRMS *m*/z 1100.5859 ([M + Na]⁺, C₆₁H₈₄N₅O₁₀P·Na⁺, calcd 1100.5848); ³¹P NMR (CDCl₃) δ 150.2, 149.9.

(15,3*R*,45,7*R*)-3-[5-(3-Cholesterylcarbonylaminopropyn-1-yl)uracil-1-yl]-7-[2-cyanoethoxy(diisopropylamino)phosphinoxy]-1-(4,4'-dimethoxytrityloxymethyl)-2,5-dioxabicyclo[2.2.1]heptane (11Y). Nucleoside 10Y (150 mg, 0.15 mmol), DIPEA (105 μL, 0.59 mmol), and PCI-reagent (50 μL, 0.21 mmol) in anhydrous CH₂Cl₂ (1.5 mL) were mixed and reacted as described in the representative phosphitylation protocol. After it was stirred at room temperature for 3 h, the reaction mixture was diluted with EtOAc (20 mL) and washed with H₂O (2 × 30 mL). The organic layer was dried (Na₂SO₄) and evaporated to dryness, the resulting residue was purified by silica gel column chromatography (0–70% EtOAc in petroleum ether, v/v); subsequent trituration from CH₂Cl₂ and petroleum ether provided 11Y (102 mg, 57%) as a white foam: $R_{\rm f}$ = 0.4 (5% MeOH in CH₂Cl₂, v/v); ESI-HRMS *m*/z 1246.6519 ([M + Na]⁺, C₇₁H₉₄N₅O₁₁P·Na⁺, calcd 1246.6579); ³¹P NMR (CDCl₃) δ 150.2, 149.9.

Alternative Route to (1S,3R,4S,7R)-1-(4,4'-Dimethoxytrityloxymethyl)-7-hydroxy-3-(5-iodoracil-1-yl)-2,5-dioxabicyclo-[2.2.1]heptane (9). Nucleoside 12³⁶ (200 mg, 0.52 mmol) was coevaporated with anhydrous pyridine (10 mL) and redissolved in anhydrous pyridine (10 mL). To this was added 4,4'-dimethoxytrityl chloride (DMTrCl, 230 mg, 0.68 mmol), and the reaction mixture was stirred at room temperature for 16 h. At this point, saturated aqueous NaHCO₃ (20 mL) and CH₂Cl₂ (25 mL) were added and the phases were separated. The organic phase was washed with saturated aqueous NaHCO₃ (20 mL). The combined aqueous phase was back-extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layer was dried (Na₂SO₄), concentrated to near dryness, and coevaporated with toluene/absolute EtOH (2 \times 30 mL, 1/2, v/v). The resulting crude product was purified by silica gel column chromatography (0-4.5% MeOH in CH₂Cl₂, v/v) to afford nucleoside 9 (250 mg, 70%) as a light yellow solid material.

(1R.3R.4S.7R)-7-Hvdroxy-1-hvdroxymethyl-3-(5-iodoracil-1yl)-2,5-dioxabicyclo[2.2.1]heptane (12). To a solution of nucleoside 8 (200 mg, 0.78 mmol) in glacial AcOH (10 mL) were added iodine (119 mg, 0.47 mmol) and ceric ammonium nitrate (213 mg, 0.39 mmol), and the reaction mixture was stirred at 80 °C for 50 min. After it was cooled to room temperature, the mixture was evaporated to dryness and the resulting residue purified by silica gel column chromatography (0–16% $MeOH/CH_2Cl_2$, v/v) to afford 12^{36} (240 mg, 80%) as a white solid material: $R_f = 0.4$ (15% MeOH in CH₂Cl₂, v/v); FAB-HRMS m/z 382.9735 ([M + H]⁺, C₁₀H₁₁IN₂O₆·H⁺, calcd 382.9740); ¹H NMR (DMSO- d_6) δ 11.76 (s, 1H, ex, NH), 8.09 (s, 1H, H6), 5.88 (d, 1H, ex, J = 4.5 Hz, 3'-OH), 5.84 (s, 1H, H1'), 4.97 (t, 1H, ex, J = 5.4 Hz, 5'-OH), 4.25 (d, 1H, J = 4.5 Hz, H3'), 4.23 (s, 1H, H2'), 3.93-3.96 (d, 1H, J = 8.5 Hz, H5"), 3.78-3.81 (d, 1H, J = 8.5 Hz, H5"), 3.70–3.77 (m, 2H, 2 × H5'); ¹³C NMR (DMSO- d_6) δ 160.5, 149.9, 144.2 (C6), 91.2, 87.1 (C1'), 78.7 (C2'), 72.4 (C3'), 72.0 (C5"), 67.7, 57.5 (C5').

Synthesis of Oligodeoxyribonucleotides and Biophysical Characterization Studies. Unmodified DNA and RNA strands were obtained from commercial suppliers and used without further purification. L1–4 were prepared and characterized with respect to identity (MALDI-MS) and purity (>80%, ion-pair reverse-phase HPLC) in a previous study.^{11b} ONs modified with C5-alkynyl-functionalized α -L-LNA monomers were synthesized, purified, structurally characterized, and utilized in biophysical experiments essentially as described for the corresponding C5-alkynyl-function

alized LNA in the preceding article.¹⁴ The following hand-coupling conditions (coupling time; activator; phosphoramidite solvent) were used for incorporation of monomers L-Z into ONs: monomers S/V/W/X (15 min; 0.25 M 4,5-dicyanoimidazole in CH₃CN; CH₃CN), monomer Y (15 min; 0.25 M 5-[3,5-bis(trifluoromethyl)phenyl]-1H-tetrazole³⁷ in CH₃CN; CH₃CN), and monomer Z (30 min; 0.25 M 4,5-dicyanoimidazole in CH₃CN; CH₂Cl₂).

ASSOCIATED CONTENT

Supporting Information

Text and figures giving general experimental details, NMR spectra for all new compounds, ROESY spectra for compound 8, MS data for all new modified ONs, and additional fluorescence data. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

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(35) Signals of CH-O-chol and C3' are interchangeable.

(36) For the structure of nucleoside **12**, see Scheme S1 (Supporting Information).

(37) Initial screening revealed that this activator results in higher stepwise coupling yields than the normal 4,5-dicyanoimidazole activator.