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

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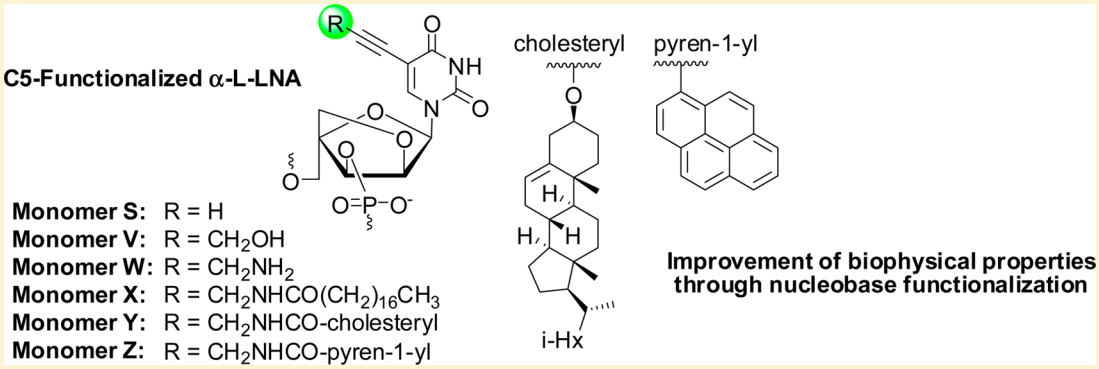
Supporting Information



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),[5−7] 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_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 characteristics[9] 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-alkynyl-functionalized 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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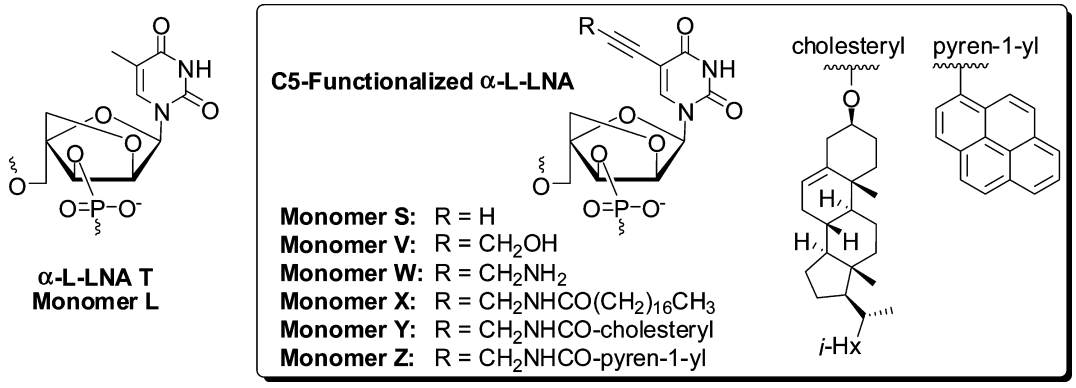
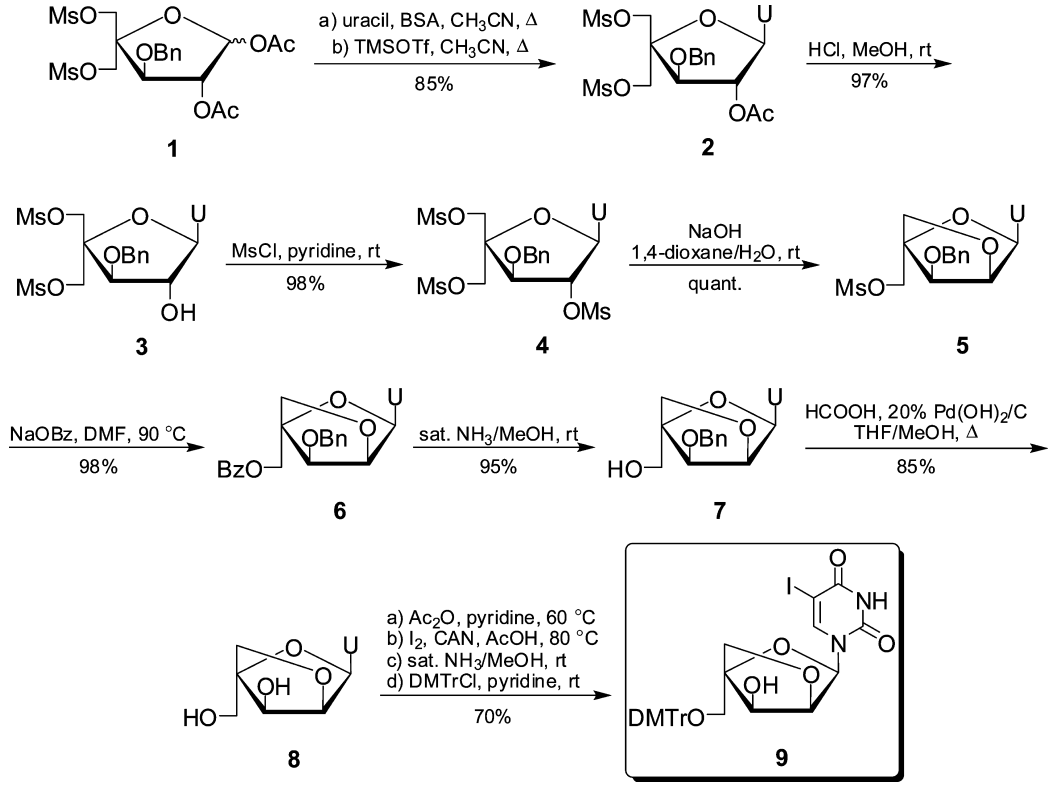


Figure 1. Structures of nucleotide monomers studied herein.

Scheme 1. Synthesis of Key Intermediate 9[a]



[a]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 monomers[14] 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-alkynyl-functionalized α-L-LNA-U phosphoramidites, their incorporation into ONs, and the characterization of these modified ONs via thermal denaturation, enzymatic stability, and steady-state 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 α-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 LNA[15] 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**

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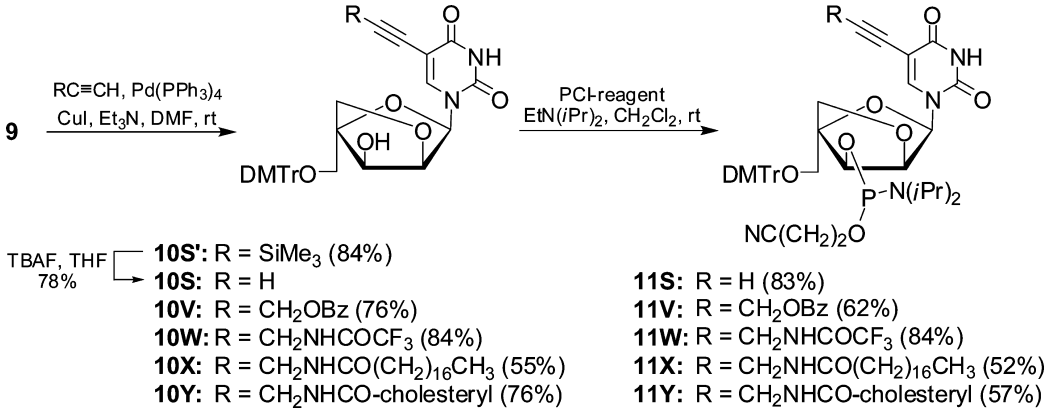
Scheme 2. Synthesis of C5-Alkynyl-Functionalized α-L-LNA-U Phosphoramidites 11S−Y[a]



[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]

			ΔT_m/mod (°C)						
ON	duplex	B =	L	S	V	W	X	Y	Z
B1	5′-GTG A**B**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 **B**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**B** ACG								
D1	5′-GTG ATA TGC		+4.0	+4.0	+4.0	+6.5	nd	nd	+0.5
B4	3′-CAC **B**A**B** ACG								
B1	5′-GTG A**B**A 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 **B**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**B** ACG								
R1	5′-GUG AUA UGC		+5.5	+6.0	+6.0	+7.5	nd	nd	+2.0
B4	3′-CAC **B**A**B** ACG								

[a]ΔT_m = change in T_m values relative to unmodified reference duplexes **D1**:**D2** ($T_m \equiv 29.5$ °C), **D1**:**R2** ($T_m \equiv 27.0$ °C), and **D2**:**R1** ($T_m \equiv 27.0$ °C). T_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 (NaH_2PO_4/Na_2HPO_4)), using 1.0 μM of each strand. T_m values are averages of at least two measurements within 1.0 °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 reactions[19] 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**B**T ACG				RNA: 3′-CAC U**B**U ACG			
		T_m	ΔT_m			T_m	ΔT_m		
ON	sequence	**A**	C	G	T	**A**	C	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 (**B** = 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**B**A TGC				RNA: 5′-GUG A**B**A UGC			
		T_m	ΔT_m			T_m	ΔT_m		
ON	sequence	**T**	A	C	G	**U**	A	C	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]For 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 alkynes[20] provided C5-alkynyl-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 52−84% yield.

Structural Verification of α-L-LNA Nucleosides. As expected,[9b] the ^{1}H 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 **Z**[22]) 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 reversed-phase 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 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 (ΔT_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 ΔT_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

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Table 4. Thermodynamic Parameters for Formation of Duplexes Modified with Select C5-Functionalized α-L-LNA Monomers[a]

		+complementary DNA			+complementary RNA		
ON	sequence	ΔG^{298} [$\Delta\Delta G^{298}$] (kJ/mol)	ΔH [$\Delta\Delta H$] (kJ/mol)	$-T^{298}\Delta S$ [$\Delta(T^{298}\Delta S)$] (kJ/mol)	ΔG^{298} [$\Delta\Delta G^{298}$] (kJ/mol)	ΔH [$\Delta\Delta H$] (kJ/mol)	$-T^{298}\Delta S$ [$\Delta(T^{298}\Delta S)$] (kJ/mol)
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**L**A TGC	−49 [−7]	−349 [−35]	300 [+29]	−48 [−12]	−308 [−30]	260 [+19]
L2	3′-CAC **L**AT ACG	−45 [−3]	−311 [+3]	266 [−5]	−44 [−5]	−306 [−13]	262 [+8]
L3	3′-CAC TA**L** ACG	−48 [−6]	−302 [+12]	254 [−17]	−46 [−7]	−295 [−2]	249 [−5]
S1	5′-GTG A**S**A TGC	−49 [−7]	−333 [−19]	284 [+13]	−47 [−11]	−290 [−12]	243 [+2]
S2	3′-CAC **S**AT ACG	−45 [−3]	−276 [+38]	231 [−40]	−45 [−6]	−299 [−6]	254 [±0]
S3	3′-CAC TA**S** ACG	−48 [−6]	−325 [−11]	275 [+4]	−48 [−9]	−318 [−25]	270 [+16]
V1	5′-GTG A**V**A TGC	−51 [−9]	−406 [−92]	354 [+83]	−50 [−14]	−302 [−24]	252 [+11]
V2	3′-CAC **V**AT ACG	−49 [−7]	−391 [−77]	342 [+71]	−46 [−7]	−340 [−47]	293 [+39]
V3	3′-CAC TA**V** ACG	−51 [−9]	−343 [−29]	292 [+21]	−49 [−10]	−289 [+4]	240 [−14]
W1	5′-GTG A**W**A TGC	−49 [−7]	−317 [−3]	267 [−4]	−50 [−14]	−302 [−24]	252 [+11]
W2	3′-CAC **W**AT ACG	−46 [−4]	−312 [+2]	266 [−5]	−46 [−7]	−340 [−47]	293 [+39]
W3	3′-CAC TA**W** ACG	−49 [−7]	−271 [+43]	222 [−49]	−49 [−10]	−289 [+4]	240 [−14]
X1	5′-GTG A**X**A TGC	−44 [−2]	−304 [+10]	260 [−11]	−44 [−8]	−319 [−41]	275 [+34]
X2	3′-CAC **X**AT ACG	−41 [−3]	−294 [+20]	253 [−18]	−41 [−2]	−245 [+48]	204 [−50]
X3	3′-CAC TA**X** ACG	−43 [−1]	−287 [+27]	244 [−27]	−42 [−3]	−283 [+10]	241 [−13]
Y1	5′-GTG A**Y**A TGC	−39 [+3]	−313 [+1]	274 [+3]	−40 [−4]	−313 [−35]	273 [+32]
Y2	3′-CAC **Y**AT ACG	−36 [+6]	−305 [+9]	269 [−2]	−40 [−1]	−325 [−32]	285 [+31]
Y3	3′-CAC TA**Y** ACG	−39 [+3]	−324 [−10]	285 [−14]	−39 [±0]	−310 [−17]	271 [+17]

[a]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 ΔT_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_m values of mismatched duplexes (compare ΔT_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 ΔT_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 ΔT_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 single-nucleotide 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_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

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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,

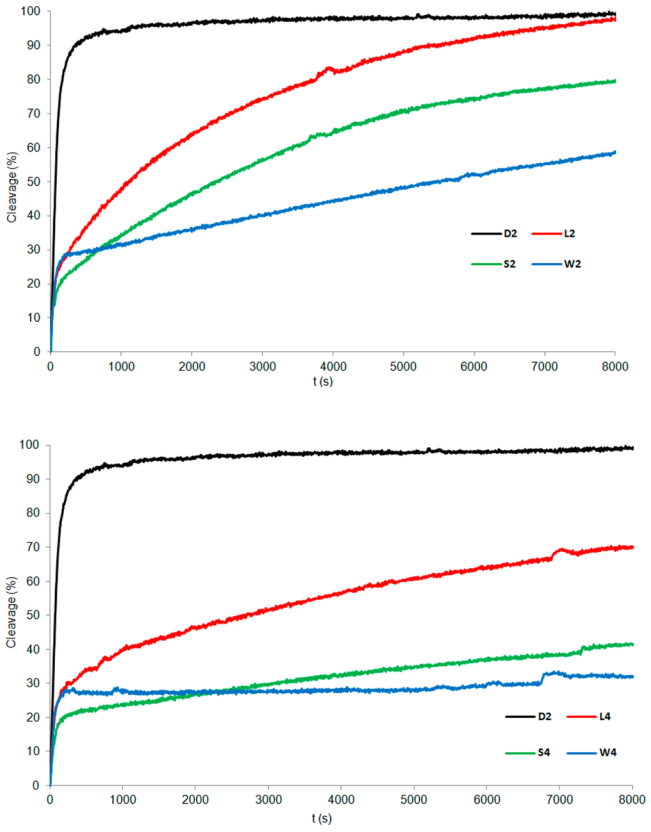

Figure 2. 3′-Exonuclease degradation of singly (top, 3′-CAC **B**AT ACG) and doubly modified (bottom, 3′-CAC **B**A**B** ACG) C5-functionalized α-L-LNA and reference strands. Nuclease degradation studies were conducted in magnesium buffer (50 mM Tris-HCl, 10 mM Mg^{2+}, pH 9.0) using [ON] = 3.3 μM and 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 C5-aminopropynyl-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. Steady-state 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)).

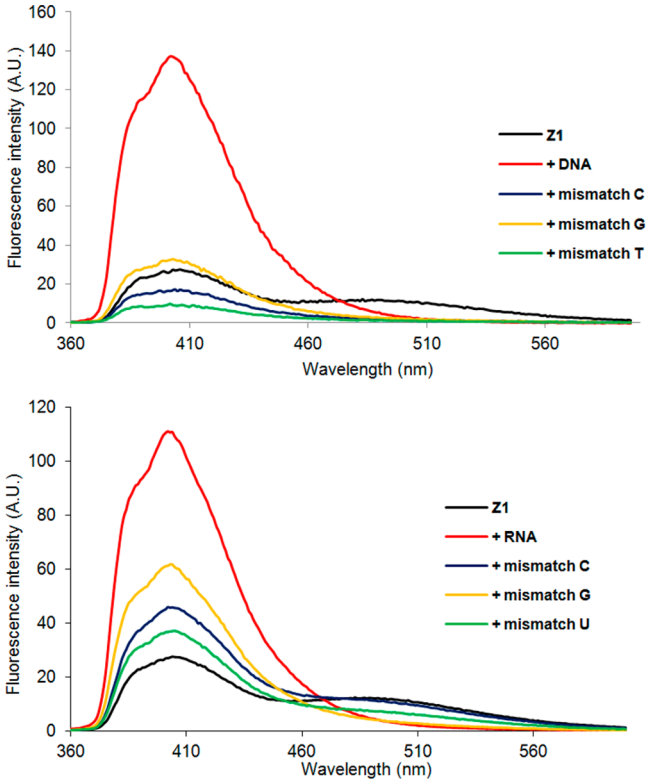

Figure 3. Steady-state fluorescence emission spectra of single-stranded **Z1** and corresponding duplexes with complementary or mismatched DNA/RNA strands (mismatched nucleotide opposite to modification in parentheses). Conditions: λ_{ex} 344 nm, T = 5 °C, each oligonucleotide used in 1 μM concentration.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

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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 quenching[29] 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-L-*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 CH_3CN (100 mL) and resuspended in anhydrous CH_3CN (150 mL). To this was added *N*,*O*-bis(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 $NaHCO_3$ (2 × 100 mL) and brine (100 mL). The aqueous phase was back-extracted with EtOAc (100 mL), and the combined organic layers were dried (Na_2SO_4) 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 CH_2Cl_2, v/v); FAB-HRMS *m*/*z* 563.0998 ($[M + H]^+$, $C_{21}H_{25}N_2O_{12}S_2{\cdot}H^+$, calcd 563.1005); ^{1}H 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, CH_2Ph), 4.65−4.68 (d, 1H, *J* = 12.0 Hz, CH_2Ph), 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, CH_3SO_2), 3.20 (s, 3H, CH_3SO_2), 2.02 (s, 3H, CH_3); ^{13}C 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 (CH_2Ph), 68.3 (C5″), 67.9 (C5′), 36.7 (CH_3SO_2), 36.6 (CH_3SO_2), 20.3 (CH_3).

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 CH_2Cl_2 (100 mL), and the organic phase was washed with saturated aqueous $NaHCO_3$ (2 × 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 CH_2Cl_2, v/v); FAB-HRMS *m*/*z* 521.0900 ($[M + H]^+$, $C_{19}H_{24}N_2O_{11}S_2{\cdot}H^+$, calcd 521.0894); ^{1}H NMR (DMSO-d_6) 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* = 7.5 Hz, H1′), 5.68 (dd, 1H, *J* = 8.0 Hz, 2.0 Hz, H5), 4.74−4.77 (d, 1H, *J* = 12.0 Hz, CH_2Ph), 4.67−4.69 (d, 1H, *J* = 12.0 Hz, CH_2Ph), 4.33−4.52 (m, 5H, H2′, 2 × H5′, 2 × H5″), 4.19−4.21 (d, 1H, *J* = 6.5 Hz, H3′), 3.23 (s, 3H, CH_3SO_2), 3.17 (s, 3H, CH_3SO_2); ^{13}C NMR (DMSO-d_6) 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 (CH_2Ph), 68.9 (C5″), 68.2 (C5′), 36.7 (CH_3SO_2), 36.6 (CH_3SO_2).

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 × 75 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 $NaHCO_3$ (200 mL) and extracted with CH_2Cl_2 (2 × 200 mL). The organic phase was dried (Na_2SO_4) 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 CH_2Cl_2, v/v); FAB-HRMS *m*/*z* 599.0675 ($[M + H]^+$, $C_{20}H_{26}N_2O_{13}S_3{\cdot}H^+$, calcd 599.0670); ^{1}H 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, CH_2Ph), 4.58−4.64 (2d, 2H, *J* = 11.0 Hz, *J* = 7.0 Hz, H5′, H3), 4.41−4.49 (m, 3H, H5′, 2 × H5″), 3.28 (s, 3H, CH_3), 3.25 (s, 3H, CH_3), 3.18 (s, 3H, CH_3); ^{13}C 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 (CH_2Ph), 68.5 (C5″), 67.7 (C5′), 37.8 (CH_3SO_2) 36.7 (CH_3SO_2), 36.6 (CH_3SO_2).

(1*S*,3*R*,4*S*,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/H_2O (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 $NaHCO_3$ (100 mL) and H_2O (100 mL), dried (Na_2SO_4), and evaporated to dryness to afford nucleoside **5** (7.00 g,

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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]$^+$, $C_{18}H_{20}N_2O_8S{\cdot}H^+$, calcd 425.1013); ^{1}H 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, 5H, 2 × CH_2Ph, 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, CH_3); ^{13}C 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 (CH_2Ph), 65.4 (C5′) 36.9 (CH_3SO_2). A trace impurity of 1,4-dioxane was identified at 66.3 ppm in the ^{13}C NMR spectrum.[31]

(1*S*,3*R*,4*S*,7*R*)-1-Benzoyloxymethyl-7-benzyloxy-3-(uracil-1-yl)-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 × 300 mL) and the organic phase washed with H_2O (2 × 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); ^{1}H 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 × 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″); ^{13}C 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 (CH_2Ph), 59.8 (C5′).

(1*R*,3*R*,4*S*,7*R*)-7-Benzyloxy-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 CH_2Cl_2,v/v) to afford nucleoside **7** (3.66 g, 95%) as a white solid material: R_f = 0.4 (7% MeOH in CH_2Cl_2, v/v); FAB-HRMS m/z 347.1230 ([M + H]$^+$, $C_{17}H_{18}N_2O_6{\cdot}H^+$, calcd 347.1243); ^{1}H 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′); ^{13}C 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 (CH_2Ph), 57.1 (C5′).

Method B. To a solution of nucleoside **6** (1.50 g, 3.33 mmol) in THF/H_2O (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 $NaHCO_3$ (50 mL) and the combined aqueous phase extracted with EtOAc (2 × 50 mL). The combined organic layers were dried (Na_2SO_4) 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 CH_2Cl_2, v/v) to afford nucleoside **8** (0.94 g, 85%) as a white solid material: R_f = 0.4 (15% MeOH in CH_2Cl_2, v/v); FAB-HRMS m/z 257.0760 ([M + H]$^+$, $C_{10}H_{12}N_2O_6{\cdot}H^+$, calcd 257.0768); ^{1}H 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, H5″), 3.73 (d, 2H, J = 5.5 Hz, 2 × H5′); ^{13}C 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′).

(1*S*,3*R*,4*S*,7*R*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-(5-iodoracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (9). Ac_2O (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 $NaHCO_3$ (30 mL) and CH_2Cl_2 (30 mL) and the phases were separated. The organic phase was washed with saturated aqueous $NaHCO_3$ (20 mL) and the combined aqueous phase back-extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layers were dried (Na_2SO_4), 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_f = 0.5 (2% MeOH in CH_2Cl_2, 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 CH_2Cl_2 (50 mL). The organic phase was washed with saturated aqueous $NaHCO_3$ (2 × 20 mL) and H_2O (20 mL). The combined aqueous phase was back-extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layers were dried (Na_2SO_4) 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_f = 0.5 (3% MeOH in CH_2Cl_2, v/v); FAB-HRMS m/z 466.9966 ([M + H]$^+$, $C_{14}H_{15}IN_2O_8{\cdot}H^+$, 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 C5-iodo α-L-LNA diol and used in the next step without further purification (R_f = 0.4 (15% MeOH in CH_2Cl_2, v/v); FAB-HRMS m/z 382.9735 ([M + H]$^+$, $C_{10}H_{11}IN_2O_6{\cdot}H^+$, 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 $NaHCO_3$ (20 mL) and CH_2Cl_2 (25 mL). The phases were separated, and the organic phase was washed with saturated aqueous $NaHCO_3$ (20 mL). The aqueous phase was back-extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layers were dried (Na_2SO_4), 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 CH_2Cl_2, v/v); FAB-HRMS m/z 684.0980 ([M]$^+$, $C_{31}H_{29}IN_2O_8^+$, calcd 684.0969); ^{1}H NMR (DMSO-d_6) 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 = 4.5 Hz, 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 × CH_3O), 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 H_2O); ^{13}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

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(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 (CH_3O).

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 $NaHCO_3$ (50 mL). The combined aqueous phase was back-extracted with EtOAc (100 mL). The combined organic phase was dried (Na_2SO_4) 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 (**10S′**).* Nucleoside **9** (0.68 g, 1.00 mmol), $Pd(PPh_3)_4$ (120 mg, 0.10 mmol), CuI (40 mg, 0.20 mmol), trimethylsilylacetylene (0.42 mL, 3.00 mmol), and Et_3N (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 CH_2Cl_2, v/v); FAB-HRMS *m/z* 655.2462 ($[M + H]^+$, $C_{36}H_{38}N_2O_8Si\cdot H^+$, calcd 655.2476); ^{1}H 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 × CH_3O), 3.34 (s, 2H, H5′), 0.21 (s, 9H, Me_3Si); ^{13}C NMR (DMSO-d_6) 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 (CH_3O), −0.12 (Me_3Si).

*(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 × 30 mL) and H_2O (30 mL). The combined aqueous phase was back-extracted with EtOAc (30 mL). The combined organic layer was dried (Na_2SO_4) and concentrated to dryness and the resulting residue purified by silica column chromatography (0−5% MeOH in CH_2Cl_2, v/v) to afford nucleoside **10S** (0.37 g, 78%) as a light brown solid material: R_f = 0.5 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS *m/z* 605.1918 ($[M + Na]^+$, $C_{33}H_{30}N_2O_8\cdot Na^+$, calcd 605.1894); ^{1}H 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 × CH_3O), 3.32 (br s, 2H, H5′); ^{13}C 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).

*(1S,3R,4S,7R)-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), $Pd(PPh_3)_4$ (90 mg, 0.07 mmol), CuI (30 mg, 0.14 mmol), prop-2-ynyl benzoate[32] (180 mg, 1.12 mmol), and Et_3N (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 CH_2Cl_2, v/v); ESI-HRMS *m/z* 739.2234 ($[M + Na]^+$, $C_{41}H_{36}N_2O_{10}\cdot Na^+$, calcd 739.2262); ^{1}H 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, CH_2OBz), 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 × OCH_3), 3.29 (s, 2H, H5′); ^{13}C 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 (CH_3O), 53.2 (CH_2OBz).

*(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), $Pd(PPh_3)_4$ (90 mg, 0.07 mmol), CuI (30 mg, 0.14 mmol), 2,2,2-trifluoro-*N*-(prop-2-ynyl)acetamide[33] (180 mg, 1.46 mmol), and Et_3N (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_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, $NHCH_2$), 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′, CH_2NH), 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 × OCH_3), 3.28−3.32 (m, 2H, H5′, overlap with H_2O); ^{13}C NMR (DMSO-d_6) δ 161.5, 158.1, 156.0 (q, *J* = 36.3 Hz, $COCF_3$) 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, CF_3), 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 (CH_3O), 29.5 (CH_2NH).

*(1S,3R,4S,7R)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-[5-(3-octadecanoylaminopropyn-1-yl)uracil-1-yl]-2,5-dioxabicyclo-[2.2.1]heptane (**10X**).* Nucleoside **9** (0.34 g, 0.50 mmol), $Pd(PPh_3)_4$ (60 mg, 0.05 mmol), CuI (20 mg, 0.10 mmol), *N*-(prop-2-ynyl)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_f = 0.5 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS *m/z* 900.4813 ($[M + Na]^+$, $C_{52}H_{67}N_3O_9\cdot Na^+$, calcd 900.4770); ^{1}H NMR ($CDCl_3$) 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, CH_2NH), 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 × CH_3O), 3.50−3.57 (2d, 2H, *J* = 11.0 Hz, H5′), 2.16−2.19 (m, 2H, CH_2CONH), 1.60−1.64 (m, 2H, CH_2CH_2CONH), 1.20−1.25 (m, 28H, 14 × CH_2), 0.89 (t, 3H, *J* = 7.0 Hz, CH_3CH_2); ^{13}C NMR ($CDCl_3$) 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 (CH_3O), 36.5 (CH_2CONH), 31.9 (CH_2), 30.1 (CH_2NH), 29.69 (CH_2), 29.68 (CH_2), 29.66 (CH_2), 29.65 (CH_2), 29.63 (CH_2), 29.5 (CH_2), 29.353 (CH_2), 29.345 (CH_2), 29.33 (CH_2), 25.5 (CH_2CH_2CONH), 22.7 (CH_2), 14.1 (CH_3).

*(1S,3R,4S,7R)-3-[5-(3-Cholesterylcarbonylaminopropyn-1-yl)-uracil-1-yl]-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptane (**10Y**).* Nucleoside **9** (0.34 g, 0.50 mmol), $Pd(PPh_3)_4$ (60 mg, 0.05 mmol), CuI (20 mg, 0.10 mmol), cholesterylprop-2-ynyl-carbamate amine[34] (0.47 g, 1.00 mmol), and Et_3N (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 CH_2Cl_2, v/v); ESI-HRMS *m/z* 1046.5533 ($[M + Na]^+$, $C_{62}H_{77}N_3O_{10}\cdot Na^+$, calcd 1046.5501); ^{1}H NMR ($CDCl_3$) 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,

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1H, H1′), 5.36 (d, 1H, J = 5.0 Hz, HC═C-Chol), 5.12 (t, ex, 1H, J = 5.0 Hz, $NHCH_2$), 4.47−4.56 (m, 3H, H2′, HC-O-Chol, H3′), 4.20 (d, 2H, J = 5.0 Hz, CH_2NH), 4.11−4.15 (d, 1H, J = 9.0 Hz, H5″), 3.98−4.02 (d, 1H, J = 9.0 Hz, H5″), 3.81 (s, 6H, 2 × CH_3O), 3.50−3.58 (2d, 2H, J = 11.0 Hz, H5′), 0.87−2.36 (m, 40H, Chol), 0.69 (s, 3H, CH_3-Chol); ^{13}C NMR ($CDCl_3$) 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 (CH_3O), 50.0 (CH-chol), 42.3, 39.8 (CH_2-chol), 39.5 (CH_2-chol), 38.5 (CH_2−chol), 37.0 (CH_2−chol), 36.6, 36.2 (CH_2−chol), 35.8 (CH-chol), 31.9 (CH-chol), 31.8 (CH_2NH and CH_2-chol overlap), 28.2 (CH_2-chol), 28.1 (CH_2-chol), 28.0 (CH-chol), 24.3 (CH_2-chol), 23.8 (CH_2-chol), 22.8 (CH_3-chol), 22.5 (CH_3-chol), 21.0 (CH_2-chol), 19.3 (CH_3-chol), 18.7 (CH_3-chol), 11.9 (CH_3-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 CH_2Cl_2. 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 $NaHCO_3$ (2 × 10 mL) and the combined aqueous phase back-extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layers were dried (Na_2SO_4) 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 CH_2Cl_2 and petroleum ether to provide phosphoramidite **11**.

*(1S,3R,4S,7R)-7-[2-Cyanoethoxy(diisopropylamino)-phosphinoxy]-1-(4,4′-dimethoxytrityloxymethyl)-3-(5-ethynyluracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (**11S**).* Nucleoside **10S** (0.35 g 0.60 mmol), DIPEA (0.50 mL, 2.88 mmol), and PCl-reagent (0.20 mL, 0.87 mmol) in anhydrous CH_2Cl_2 (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_f = 0.5 (2% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 805.2943 ([M + Na]$^+$, $C_{42}H_{47}N_4O_9P{\cdot}Na^+$, calcd 805.2958); ^{31}P NMR ($CDCl_3$) δ 150.2, 149.9.

*(1S,3R,4S,7R)-3-[5-(3-Benzoyloxypropyn-1-yl)uracil-1-yl]-7-[2-cyanoethoxy(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 1,2-dichloroethane (2 × 7 mL) and redissolved in anhydrous CH_2Cl_2 (7 mL). To this solution were added DIPEA (425 μL, 2.44 mmol) and *N*-methylimidazole (31 μL, 0.39 mmol), followed by dropwise addition of the PCl-reagent (220 μL, 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 CH_2Cl_2) and subsequently triturated from CH_2Cl_2 and petroleum ether to provide **11V** (0.28 g, 62%) as a white foam: R_f = 0.5 (3% MeOH in CH_2Cl_2); ESI-HRMS m/z 939.3332 ([M + Na]$^+$, $C_{50}H_{53}N_4O_{11}P{\cdot}Na^+$, calcd 939.3341); ^{31}P NMR ($CDCl_3$) δ 150.1, 149.8.

*(1S,3R,4S,7R)-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 CH_2Cl_2 (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 CH_2Cl_2, v/v); ESI-HRMS m/z 930.3068 ([M + Na]$^+$, $C_{45}H_{49}F_3N_5O_{10}P{\cdot}Na^+$, calcd 930.3080); ^{31}P NMR ($CDCl_3$) δ 150.2, 149.9.

*(1S,3R,4S,7R)-7-[2-Cyanoethoxy(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 μL, 0.83 mmol), and PCl-reagent (61 μL, 0.27 mmol) in anhydrous CH_2Cl_2 (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 H_2O (2 × 30 mL). The organic phase was dried (Na_2SO_4) 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 CH_2Cl_2 and petroleum ether to provide **11X** (107 mg, 52%) as a white foam: R_f = 0.4 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 1100.5859 ([M + Na]$^+$, $C_{61}H_{84}N_5O_{10}P{\cdot}Na^+$, calcd 1100.5848); ^{31}P NMR ($CDCl_3$) δ 150.2, 149.9.

*(1S,3R,4S,7R)-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 PCl-reagent (50 μL, 0.21 mmol) in anhydrous CH_2Cl_2 (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 H_2O (2 × 30 mL). The organic layer was dried (Na_2SO_4) 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 CH_2Cl_2 and petroleum ether provided **11Y** (102 mg, 57%) as a white foam: R_f = 0.4 (5% MeOH in CH_2Cl_2, v/v); ESI-HRMS m/z 1246.6519 ([M + Na]$^+$, $C_{71}H_{94}N_5O_{11}P{\cdot}Na^+$, calcd 1246.6579); ^{31}P NMR ($CDCl_3$) δ 150.2, 149.9.

Alternative Route to (1*S*,3*R*,4*S*,7*R*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-(5-iodoracil-1-yl)-2,5-dioxabicyclo-[2.2.1]heptane (9). Nucleoside **12**[36] (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 $NaHCO_3$ (20 mL) and CH_2Cl_2 (25 mL) were added and the phases were separated. The organic phase was washed with saturated aqueous $NaHCO_3$ (20 mL). The combined aqueous phase was back-extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layer was dried (Na_2SO_4), concentrated to near dryness, and coevaporated with toluene/absolute EtOH (2 × 30 mL, 1/2, v/v). The resulting crude product was purified by silica gel column chromatography (0−4.5% MeOH in CH_2Cl_2, v/v) to afford nucleoside **9** (250 mg, 70%) as a light yellow solid material.

(1*R*,3*R*,4*S*,7*R*)-7-Hydroxy-1-hydroxymethyl-3-(5-iodoracil-1-yl)-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 CH_2Cl_2, v/v); FAB-HRMS m/z 382.9735 ([M + H]$^+$, $C_{10}H_{11}IN_2O_6{\cdot}H^+$, calcd 382.9740); ^{1}H 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′); ^{13}C 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-

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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 CH_3CN; CH_3CN), monomer **Y** (15 min; 0.25 M 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole[37] in CH_3CN; CH_3CN), and monomer **Z** (30 min; 0.25 M 4,5-dicyanoimidazole in CH_3CN; CH_2Cl_2).

■ 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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Notes

The authors declare no competing financial interest.

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(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.