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Synthesis, Hybridization Characteristics, and Fluorescence Properties of Oligonucleotides Modified with Nucleobase-Functionalized Locked Nucleic Acid Adenosine and Cytidine Monomers

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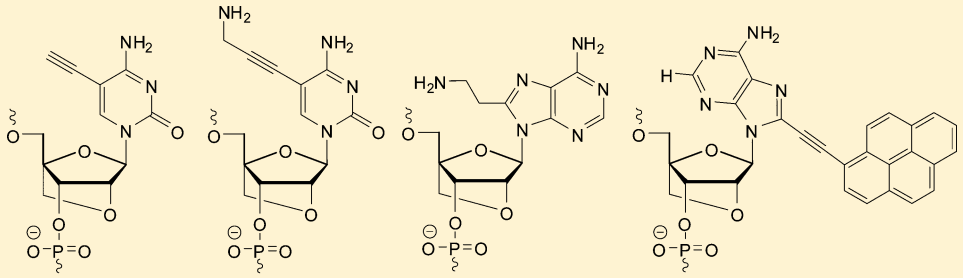
Supporting Information



ABSTRACT: Conformationally restricted nucleotides such as locked nucleic acid (LNA) are very popular as affinity-, specificity-, and stability-enhancing modifications in oligonucleotide chemistry to produce probes for nucleic acid targeting applications in molecular biology, biotechnology, and medicinal chemistry. Considerable efforts have been devoted in recent years to optimize the biophysical properties of LNA through additional modification of the sugar skeleton. We recently introduced C5-functionalization of LNA uridines as an alternative and synthetically more straightforward approach to improve the biophysical properties of LNA. In the present work, we set out to test the generality of this concept by studying the characteristics of oligonucleotides modified with four different C5-functionalized LNA cytidine and C8-functionalized LNA adenosine monomers. The results strongly suggest that C5-functionalization of LNA pyrimidines is indeed a viable approach for improving the binding affinity, target specificity, and/or enzymatic stability of LNA-modified ONs, whereas C8-functionalization of LNA adenosines is detrimental to binding affinity and specificity. These insights will impact the future design of conformationally restricted nucleotides for nucleic acid targeting applications.

INTRODUCTION

Conformationally restricted nucleotides[1,2] are widely used as affinity-, specificity-, and stability-enhancing modifications in oligonucleotides for nucleic acid targeting applications in molecular biology, biotechnology, and medicinal chemistry.[3] Locked nucleic acid (LNA) (Figure 1),[4] which was independently developed by Wengel[5] and Imanishi,[6] is one of the most promising examples of this compound class. LNA-modified oligodeoxyribonucleotides (ONs) form highly thermostable duplexes with complementary DNA/RNA; increases in thermal denaturation temperatures (T_m's) of up to 10 °C per modification relative to unmodified duplexes have been observed along with improved binding specificity. LNA has accordingly been used to alter gene expression through binding to mRNA, pre-mRNA, or miRNA, leading to the development of LNA-based drug candidates against diseases of genetic origin.[7] In view of these interesting properties, it is not surprising that a plethora of LNA analogues have been developed over the past 15 years, which aim to improve the biophysical properties of LNA.[1,2,8] These analogues have primarily focused on modification of the oxymethylene bridge spanning the C2′- and C4′-positions and/or introduction of minor-groove-oriented substituents on the bridge.

We have been exploring nucleobase functionalization of LNA as an alternative and synthetically more straightforward strategy to modulate the biophysical properties of LNA.[9] For example, we have shown that ONs modified with small C5-alkynyl-functionalized LNA uridine (LNA-U) monomers display improved affinity, specificity, and enzymatic stability relative to ONs modified with conventional LNA.[10] Moreover, we have shown that ONs modified with C5-pyrene-functionalized LNA-U monomers display more desirable photophysical properties than ONs modified with the corresponding 2′-deoxyuridine monomers, presumably as a result of more well defined placement of the fluorophore in the major groove.[10,11] Similar improvements have been observed for ONs modified with diastereomeric C5-functionalized α-L-LNA-U, which suggests that C5-functionalization of uridines is a general and synthetically straightforward approach to improve

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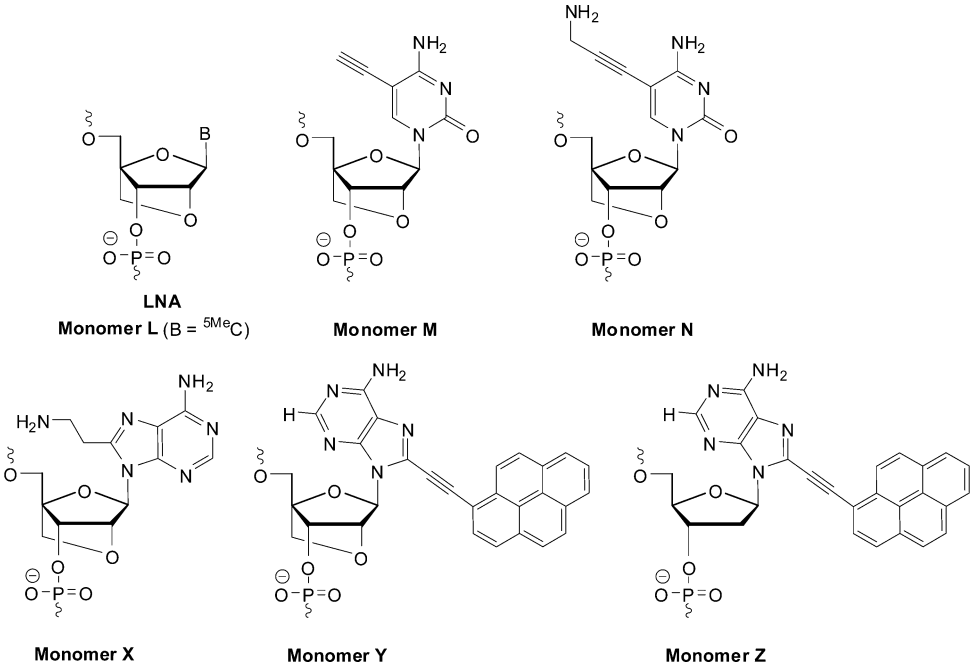

Figure 1. Structures of monomers discussed herein.

the biophysical properties of conformationally restricted nucleotide building blocks.[11,12]

Intrigued by these results and keen to test the generality of this concept across LNA nucleotides with other nucleobases, which would be important for therapeutic applications, we set out to study a series of representative C5-functionalized LNA cytidine (LNA-C) and C8-functionalized LNA adenosine (LNA-A) monomers (Figure 1). Unlike C5-functionalized pyrimidine monomers, which predictably position the substituent toward the major groove of nucleic acid duplexes,[13,14] the binding modes of C8-functionalized purines are more complex.[15] Bulky substituents promote the adoption of *syn* conformations about the glycosyl link (N1−C1′) to minimize clashes between the C8-substituent and the sugar protons, whereas a more equal distribution of *syn* and *anti* conformations is observed with medium-sized substituents. The conformational flexibility has been utilized to develop fluorophore-functionalized ON probes with interesting photophysical properties.[16] We hypothesized that the extreme pucker and conformationally restricted nature of the LNA skeleton would influence the barrier between the *syn* and *anti* conformations of C8-functionalized LNA-A monomers, resulting in building blocks with an even stronger bias for a particular conformation. Here we report the synthesis of four C5-functionalized LNA-C and C8-functionalized LNA-A phosphoramidites, their incorporation into ONs, and the characterization of the modified ONs by thermal denaturation, absorption, steady-state fluorescence, and enzymatic stability experiments.

RESULTS AND DISCUSSION

Synthesis of C5-Functionalized LNA-C Phosphoramidites. Our synthetic strategy toward target phosphoramidites **6a** and **6b** (Scheme 1) was prompted by (i) the large-scale availability of starting material **1**, which is obtained from diacetone-α-D-allose in ∼38% yield,[10] (ii) reports of successful uracil-to-cytosine transformations for closely related LNA analogues,[8k] and (iii) a desire to introduce the C5-substitutent at the latest stage possible to reduce the total number of synthetic steps. Thus, LNA C5-iodouridine **1** was first protected at the O3′-position as a *tert*-butyldimethylsilyl ether in the presence of TBDMS-Cl, *N*-methylimidazole, and iodine[17] to afford nucleoside **2** in excellent yield. The uracil-to-cytosine conversion was realized using the phosphoryl chloride/1,2,4-triazole/ammonia method[18] to give nucleoside **3** in 72% yield. Subsequent couplings of trimethylsilyl-protected acetylene and trifluoroacetyl-protected propargylamine to the C5-position of **3** under Sonogashira conditions[19] proceeded smoothly to afford nucleosides **4a** and **4b**, respectively, in excellent yields. N4-Benzoylation of **6** was followed by O3′-desilylation to furnish alcohols **5a** and **5b** in 70% and 41% yield, respectively. The low yield of **5b** was in part due to the unexpected formation of LNA pyrrolocytosine **5c** during the desilylation step (results not shown). Attempts to change the order of the reactions (i.e., conducting the Sonogashira coupling after N4-benzoylation and O3′-desilylation) also led to LNA pyrrolocytosine formation (results not shown). O3′-Phosphitylation of **5a** and **5b** using 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite and Hünig's base afforded the corresponding targets **6a** and **6b** in moderate yields.

Synthesis of C8-Functionalized LNA-A Nucleosides. Known LNA adenosine diol **7**, which is obtained in ∼25% yield from diacetone-α-D-allose,[20] was identified as a convenient starting material for the preparation of phosphoramidites **12** and **16** (Schemes 2 and 3). Treatment of **7** with molecular bromine in 1,4-dioxane and aqueous sodium acetate buffer (pH 4.5)[21] afforded nucleoside **8** in 85% yield. Subsequent O5′-dimethoxytritylation using standard conditions provided **9** in 77% yield. This was followed by protection of the exocyclic amine of the adenine moiety as an *N*,*N*-dimethylformamidine group (90% yield).[22] We found this approach to be higher-yielding and more convenient than N6-benzoylation of nucleoside **9** via a transient protection protocol[23] (72% yield over three steps; see compound **10*** in Scheme S1 in the Supporting Information). A vinyl moiety, which is converted to an aminoethyl group during standard oligonucleotide deprotection,[24] was introduced at the 8-position of **10** via Stille coupling[19] to give functionalized nucleoside **11** in

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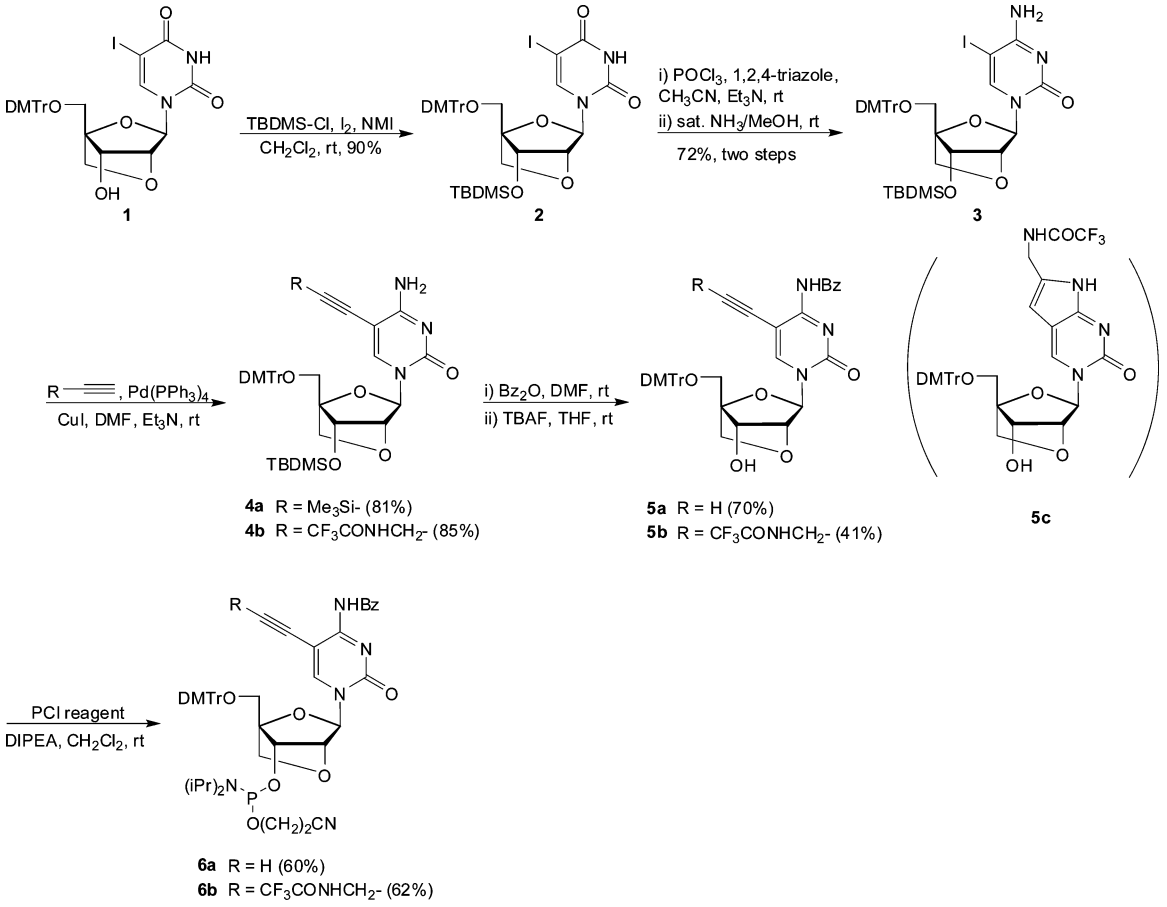
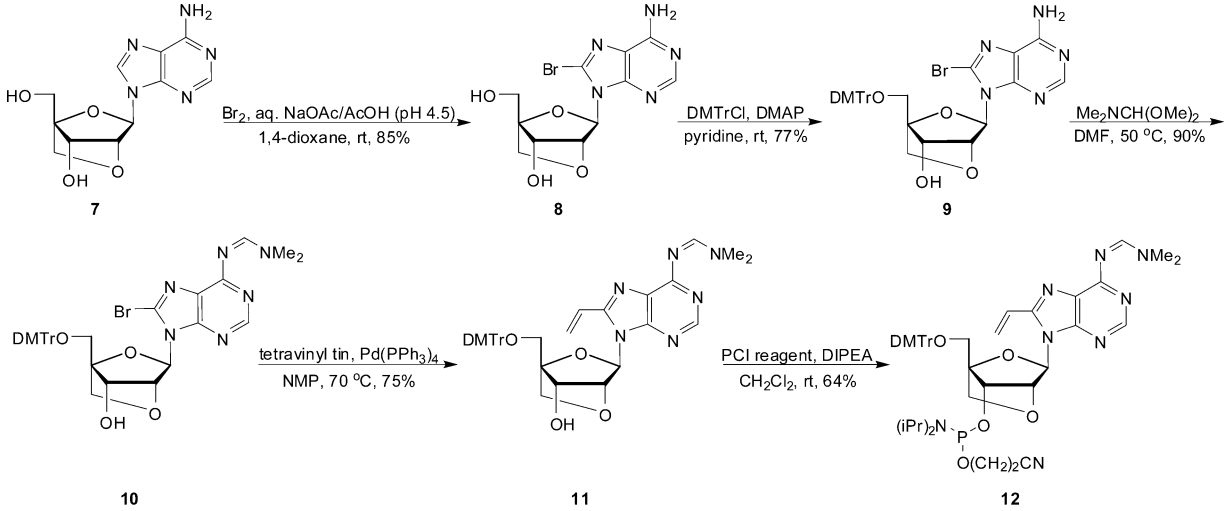
Scheme 1. Synthesis of C5-Functionalized LNA Cytidine Phosphoramidites 6a and 6b[a]



[a]Abbreviations: NMI = *N*-methylimidazole; PCl = 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite; DIPEA = *N*,*N*′-diisopropylethylamine.

Scheme 2. Synthesis of C8-Vinyl LNA Adenosine Phosphoramidite 12[a]



[a]Abbreviations: DMTrCl = 4,4′-dimethoxytrityl chloride; NMP = *N*-methylpyrrolidone; PCl reagent = 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite; DIPEA = *N*,*N*′-diisopropylethylamine.

75% yield. Subsequent O3′-phosphitylation using 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite afforded the target phosphoramidite **12** in 64% yield.

The reaction sequence had to be modified for the synthesis of C8-pyrene-functionalized LNA adenosine phosphoramidite **16** (Scheme 3) as the Sonogashira reaction between C8-brominated nucleoside **10** and 1-acetylenepyrene was sluggish and did not proceed to completion, presumably because of steric interference from the O5′-DMTr group. Instead, unprotected C8-bromo LNA adenosine **8** proved to be a viable substrate for Sonogashira coupling with 1-acetylenepyrene, as nucleoside **13** was obtained in 61% yield. Subsequent O5′-dimethoxytritylation (80%),

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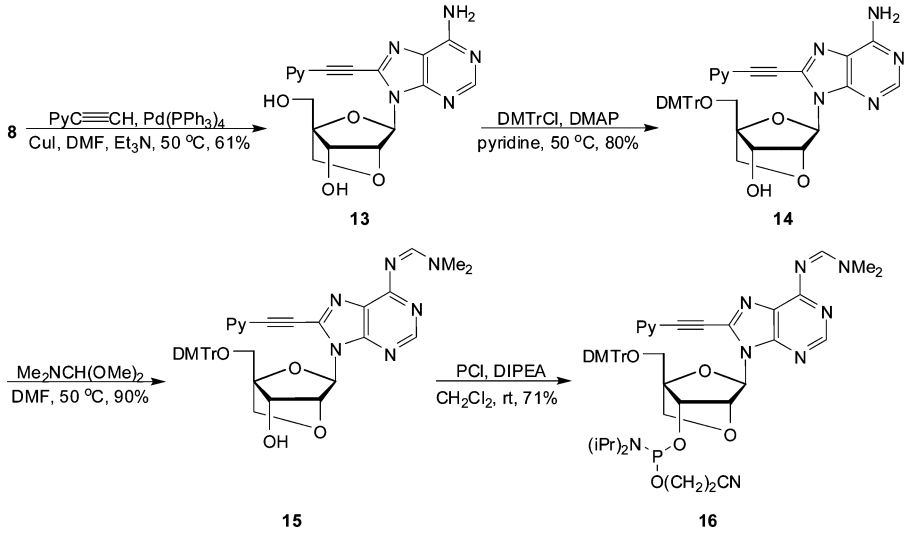
Scheme 3. Synthesis of C8-Pyrene-Functionalized LNA Adenosine Phosphoramidite 16[a]



[a]Abbreviations: DMTrCl = 4,4′-dimethoxytrityl chloride; PCl reagent = 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite; DIPEA = *N*,*N*′-diisopropylethylamine.

N6-protection (90%), and O3′-phosphitylation (64%) finally provided phosphoramidite **16**.

Conformational Analysis of C8-Functionalized LNA-A Nucleosides. Rotating-frame nuclear Overhauser effect correlation spectroscopy (ROESY) spectra of selected nucleosides were recorded to examine whether C8-functionalization influences the *syn*−*anti* equilibrium about the glycosyl link. Bulky C8-substituents are known to shift this equilibrium toward an increasing *syn* preference in 2′-deoxyribonucleosides as a result of steric repulsion between the C8-substituent and the sugar ring.[15] However, these shifts are accompanied by changes in the sugar pucker, which likely are more difficult with the conformationally restricted sugar skeleton of LNA.

The ROESY spectra of C8-bromo- and C8-vinyl-substituted LNA-A nucleosides **10** and **11** offer little evidence of significant *syn* populations (Figures S1 and S2 in the Supporting Information). Most notably, there is an absence of cross-peaks corresponding to through-space interactions between H2 and any of the sugar protons. In fact, the only sign that nucleoside **11** adopts *syn* conformations is a through-space coupling between H1′ and the penultimate proton of the vinyl group. In contrast, the ROESY spectrum of nucleoside **13** displays numerous cross-peaks consistent with *syn* conformations (Figures S3 and S4 in the Supporting Information), including through-space interactions between (i) H2 and H2′/H3′/5′-OH and (ii) pyrene protons and H1′/H2′/H5″/3′-OH.

ON Synthesis. Phosphoramidites **6a** and **6b** were used in machine-assisted solid-phase DNA synthesis to incorporate monomers **M** and **N** into 9-mer mixed-sequence ONs, while phosphoramidites **12** and **16** were used to incorporate monomers **X** and **Y** into 13-mer mixed-sequence ONs. To examine whether the LNA skeleton has any influence on the position of C8-substituents, we also synthesized ONs modified with the DNA analogue of monomer **Y** (i.e., monomer **Z**; Figure 1). The following hand-coupling conditions (activator, coupling time, coupling yield) were used for monomer **M** (4,5-dicyanoimidazole, 15 min, ∼95%), monomer **N** (pyridinium hydrochloride, 15 min, ∼90%), monomers **X** and **Y** (5-(ethylthio)-1*H*-tetrazole, 20 min, ∼95%), and monomer **Z** (5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole, 20 min, ∼95%). Suitable activators were identified through initial screening of common activators (results not shown). The compositions and purities of all of the modified ONs were ascertained by MALDI MS analysis (Tables S1 and S2 in the Supporting Information) and ion-pair reversed-phase HPLC, respectively.

Thermal Denaturation Studies—Experimental Setup. Thermal denaturation temperatures of duplexes between modified ONs and DNA/RNA targets were evaluated by UV−vis thermal denaturation experiments performed in a medium-salt phosphate buffer ($[Na^+]$ = 110 mM). All of the denaturation curves displayed monophasic sigmoidal transitions (Figures S5 and S6 in the Supporting Information).

Binding Affinities/Specificities of ONs Modified with C5-Functionalized LNA-C Monomers. ONs modified with C5-ethynyl LNA-C monomer **M** or C5-aminopropynyl LNA-C monomer **N** display markedly increased affinity toward complementary DNA and RNA targets relative to unmodified ONs (ΔT_m between +5.5 and +10.0 °C; Table 1); larger increases are observed with RNA targets. The stabilizing effects of monomers **M** and **N** are additive, as evidenced by the similar ΔT_m per modification values observed for triply modified **B4** and singly modified **B1**−**B3**. Remarkably, the duplex between triply modified **N4** and complementary RNA exhibits an absolute T_m of 69 °C, which is 33 °C higher than that of the corresponding unmodified duplex. Interestingly, **M**- and **N**-modified ONs display similar or slightly higher affinities toward DNA/RNA targets than the corresponding ONs modified with conventional LNA 5-methylcytidine (^{5Me}C) monomer **L**, which reinforces our recent observations with C5-functionalized LNA-U.[10] Most likely, the stabilizing properties of monomer **N** are the result of favorable stacking and electrostatic interactions, in a similar manner as previously suggested for C5-aminopropynyl-modified DNA monomers.[14e,h]

The binding specificities of singly modified **B2** and triply modified **B4** were evaluated against DNA/RNA targets with centrally mismatched nucleotides (Table 2). **M**- and **N**-modified ONs discriminate mismatched targets very efficiently, as evidenced by the large drops in T_m for the mismatched duplexes. However, comparison with ONs modified with conventional LNA-C monomers reveals that the C5-substituents of monomers

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Table 1. T_m's of Duplexes between L/M/N-Modified ONs and Complementary DNA or RNA[a]

			T_m (ΔT_m/mod)/°C					
			DNA: 3′-CGT AGA GTG			RNA: 3′-CGU AGA GUG		
ON	sequence	B =	L	M	N	L	M	N
B1	5′-G**B**A TCT CAC		40.0 (+5.0)	40.5 (+5.5)	42.5 (+7.5)	46.0 (+10.0)	45.0 (+9.0)	46.0 (+10.0)
B2	5′-GCA T**B**T CAC		41.0 (+6.0)	40.5 (+5.5)	43.0 (+8.0)	44.0 (+8.0)	45.0 (+9.0)	44.5 (+8.5)
B3	5′-GCA TCT **B**AC		42.0 (+7.0)	41.5 (+6.5)	42.0 (+7.0)	44.0 (+8.0)	45.0 (+9.0)	44.0 (+8.0)
B4	5′-G**B**A T**B**T **B**AC		53.0 (+6.0)	53.0 (+6.0)	60.0 (+8.3)	64.0 (+9.3)	63.5 (+9.2)	69.0 (+11.0)

[a]T_m was determined as the maximum of the first derivative of the denaturation curve (A_{260} vs T) recorded in T_m buffer ([Na^+] = 110 mM, [Cl^-] = 100 mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4)) with each strand at 1.0 μM. T_m's are averages of at least two measurements within 1.0 °C. "ΔT_m/mod" is the change in T_m per incorporation relative to the unmodified reference duplex. The T_m's of the unmodified DNA:DNA and DNA:RNA duplexes are 35 and 36 °C, respectively.

Table 2. Discrimination of Mismatched DNA/RNA Targets by ONs B2 and B4[a]

			DNA: 3′-CGT A**B**A GTG				RNA: 3′-CGU A**B**A GUG			
			T_m/°C	ΔT_m/°C			T_m/°C	ΔT_m/°C		
ON	sequence	B =	G	A	C	T	G	A	C	U
D1	5′-GCA TCT CAC		**35.0**	< −25.0	< −25.0	−21.5	**36.0**	−23.5	< −26.0	< −26.0
L2	5′-GCA T**L**T CAC		**41.0**	−25.0	−28.0	−24.5	**44.0**	−25.0	−26.0	−26.0
L4	5′-G**L**A T**L**T **L**AC		**53.0**	−27.0	−29.0	−25.0	**64.0**	−24.0	−29.0	−27.0
M2	5′-GCA T**M**T CAC		**40.5**	−24.5	−25.0	−22.0	**45.0**	−21.0	−28.0	−26.0
M4	5′-G**M**A T**M**T **M**AC		**53.0**	−22.0	−25.0	−21.0	**63.5**	−18.5	−26.0	−22.0
N2	5′-GCA T**N**T CAC		**43.0**	−24.5	−26.5	−23.5	**44.5**	−19.0	−28.0	−23.0
N4	5′-G**N**A T**N**T **N**AC		**60.0**	−22.0	−29.0	−24.0	**69.0**	−19.0	−24.0	−22.0

[a]For experimental conditions, see Table 1. ΔT_m is the change in T_m relative to the fully matched ON:DNA or ON:RNA duplex (**B** = G).

Table 3. T_m's of Duplexes between Centrally Modified ONs and Complementary or Singly Mismatched DNA Targets[a]

			T_m (ΔT_m)/°C	mismatch ΔT_m/°C		
ON	sequence	B =	T	A	C	G
D5	5′-GCGTT AAA TTGCG		48.5	−11.0	−12.0	−9.0
D6	5′-GCGTT CAC TTGCG		55.0	−9.5	−13.5	−4.5
D7	5′-GCGTT GAG TTGCG		55.5	−8.0	−10.0	−8.0
D8	5′-GCGTT TAT TTGCG		48.5	−10.0	−14.0	−5.0
X5	5′-GCGTT A**X**A TTGCG		47.0 (−1.5)	−5.0	−10.0	−11.0
X6	5′-GCGTT C**X**C TTGCG		50.0 (−5.0)	−5.0	−11.0	−8.0
X7	5′-GCGTT G**X**G TTGCG		56.0 (+0.5)	−6.0	−7.0	−12.0
X8	5′-GCGTT T**X**T TTGCG		44.0 (−4.5)	−12.0	−8.0	−7.0
Y5	5′-GCGTT A**Y**A TTGCG		47.0 (−1.5)	−1.0	+1.0	−1.0
Y6	5′-GCGTT C**Y**C TTGCG		49.0 (−6.0)	−2.0	−5.0	−1.0
Y7	5′-GCGTT G**Y**G TTGCG		52.0 (−3.5)	+0.5	+1.0	−3.5
Y8	5′-GCGTT T**Y**T TTGCG		44.5 (−4.0)	−2.0	−2.0	+2.0
Z5	5′-GCGTT A**Z**A TTGCG		46.5 (−2.0)	−0.5	+1.5	−1.0
Z6	5′-GCGTT C**Z**C TTGCG		47.0 (−8.0)	−1.0	−5.0	−0.5
Z7	5′-GCGTT G**Z**G TTGCG		53.5 (−2.0)	−2.0	±0.0	−5.5
Z8	5′-GCGTT T**Z**T TTGCG		44.5 (−2.0)	−2.5	−1.5	+0.5

[a]For experimental conditions, see Table 1. "Mismatch ΔT_m" is the change in T_m relative to the fully matched duplex (**B** = T). DNA targets: 3′-CGCAA T**B**T AACGC (for **B5**), 3′-CGCAA G**B**G AACGC (for **B6**), 3′- CGCAA C**B**C AACGC (for **B7**), and 3′- CGCAA A**B**A AACGC (for **B8**). For the structures of monomers **X**–**Z**, see Figure 1.

M and **N** do not induce additional improvements in binding specificity.

Binding Affinities/Specificities of ONs Modified with C8-Functionalized LNA-A Monomers. ONs modified with C8-functionalized LNA-A monomer **X** or **Y** generally display lower affinities toward complementary DNA than control ONs (ΔT_m between −6.0 and +0.5 °C for **X5**–**X8** and **Y5**–**Y8**; Table 3). Duplex destabilization is more pronounced when the monomers are flanked by pyrimidines, most likely because of less efficient stacking with the smaller nucleobases (compare the ΔT_m's for **B5** and **B7** with those for **B6** and **B8**, respectively; Table 3). Interestingly, only minor differences between the T_m's of **X**- and **Y**-modified DNA duplexes are observed despite the very different natures of the C8-substituents. It is also noteworthy that DNA duplexes involving ONs modified with LNA-based monomer **Y** or DNA-based monomer **Z** display similar T_m's, which indicates that the LNA skeleton exerts little influence on the position of the destabilizing structural elements.

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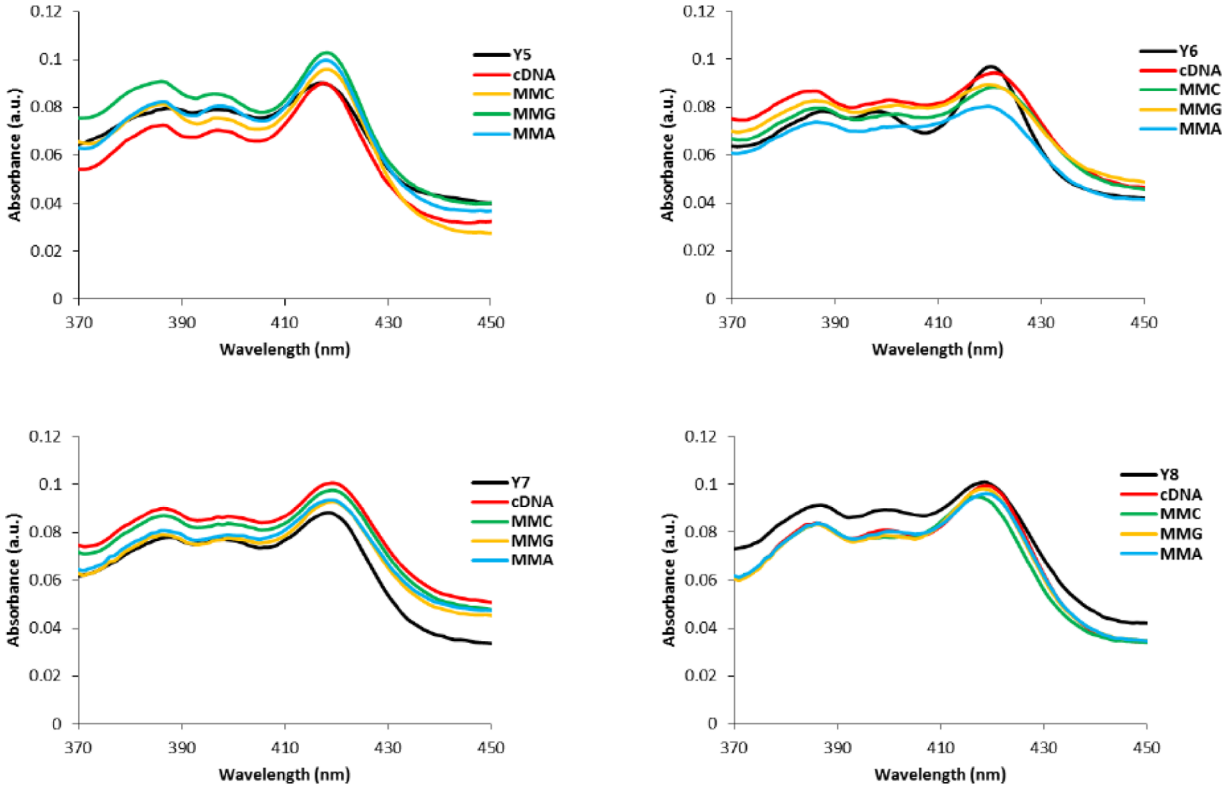

Figure 2. UV−vis absorption spectra of single-stranded **Y5**−**Y8** and the corresponding duplexes with complementary DNA (cDNA) or centrally mismatched DNA (MMB, where B is the central mismatched nucleotide). Spectra were recorded in T_m buffer at T = 5 °C using each strand at 1.0 μM concentration.

The binding specificities of **X**/**Y**/**Z**-modified ONs were determined by performing thermal denaturation experiments using DNA targets with mismatched nucleotides opposite the modified monomer (Table 3). As expected, unmodified duplexes with mismatched base pairs display significantly lower T_m's than fully complementary duplexes. ONs modified with C8-aminoethyl-functionalized LNA-A monomer **X** display less efficient discrimination of DNA targets with centrally mismatched dA or dC nucleotides than unmodified ONs but improved discrimination of targets with mismatched dG nucleotides (compare the ΔT_m's for mismatched DNA duplexes involving **D5**−**D8** and **X5**−**X8**; Table 3). The latter observation is interesting since the dA moiety of mismatched dA:dG base pairs is known to have a preference for *syn* conformations in certain sequence contexts.[15d,25] We speculate that the adoption of *syn* conformations is energetically unfavorable for C8-aminoethyl LNA-A monomer **X**, leading to more destabilized and thus better discriminated **X**:dG mismatches.

Conversely, **Y**-modified ONs display poor binding specificity (compare the ΔT_m's for mismatched DNA duplexes involving **Y5**−**Y8** and **D5**−**D8**; Table 3), which is indicative of a preference for *syn* conformations and (partial) intercalation of the pyrene moiety, as intercalating moieties are known to reduce binding specificity.[26] Only very minor differences in binding specificities are observed for **Y**- and **Z**-modified ONs.

Photophysical Characterization of ONs Modified with C8-Pyrene-Functionalized LNA-A Monomer Y. To gain additional insight into the binding mode of the pyrene moiety of monomer **Y**, absorption and steady-state fluorescence emission spectra of **Y**-modified ONs were recorded in the absence or presence of complementary or centrally mismatched DNA targets. Single-stranded probes **Y5**−**Y8** and the corresponding duplexes with complementary/mismatched DNA targets have very similar UV−vis absorption spectra, including a well-defined absorption maximum at ∼420 nm as well as shoulders at ∼385 and ∼400 nm (Figure 2). The absence of major hybridization-induced shifts in the pyrene absorption maxima[27] ($\Delta\lambda$ between −2 and +1 nm; Table S3 in the Supporting Information) suggests that the pyrene moiety is in a similar microenvironment in the single-stranded and double-stranded states. This is in agreement with the preference for *syn* conformations of nucleoside **13**, which would place the pyrene moiety of monomer **Y** in close contact with flanking nucleobases regardless of the hybridization state. The most notable difference between **Y**- and **Z**-modified ONs/duplexes is that the pyrene absorption maxima of single-stranded **Z**-modified ONs are blue-shifted by 1−3 nm, which indicates weaker pyrene−nucleobase interactions in the single-stranded state (Table S3 and Figure S7 in the Supporting Information). Hence, subtle hybridization-induced bathochromic shifts are observed for **Z**-modified ONs ($\Delta\lambda$ = −1 to +4 nm; Table S3).

The steady-state fluorescence emission spectra (λ_{ex} = 385 nm, T = 5 °C) of duplexes between **Y5**−**Y8** and complementary or centrally mismatched DNA targets exhibit a broad emission maximum centered at ∼460 nm, which is indicative[16a] of strong electronic interactions between the pyrene and adenine moieties (Figure 3). Up to 2-fold increases in fluorescence intensity are observed upon hybridization of **Y5** or **Y8** with DNA targets, whereas hybridization of **Y6** or **Y7** with DNA results in subtle decreases in fluorescence intensity. The nature of the nucleotide opposite the modification does not appear to have a major influence on the fluorescence properties. The different trends of **Y5**/**Y8** and **Y6**/**Y7**, in which A/T and C/G base pairs flank monomer **Y**, respectively, strongly suggest that monomer **Y**

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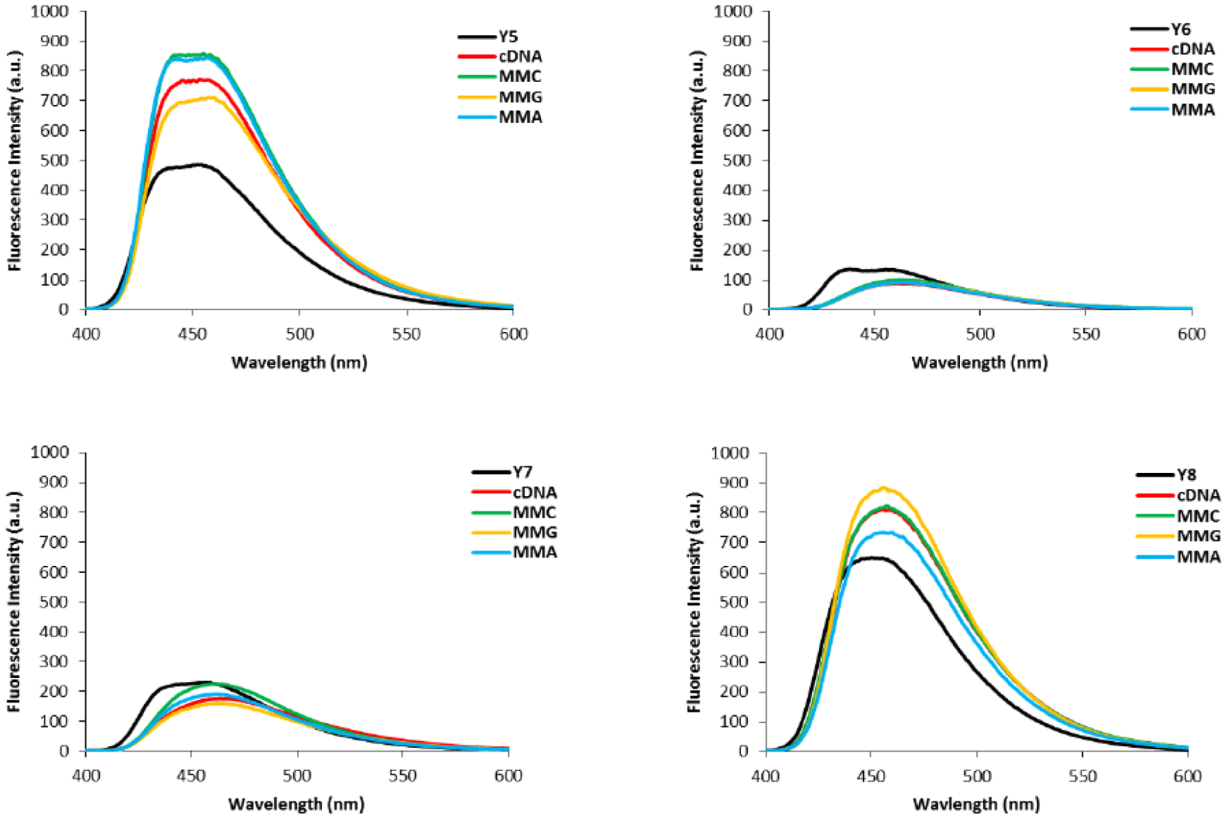

Figure 3. Steady-state fluorescence emission spectra of single-stranded **Y5**–**Y8** and the corresponding duplexes with complementary (cDNA) or centrally mismatched DNA (MMB, where B is the central mismatched nucleotide). Spectra were recorded in T_m buffer at T = 5 °C using each strand at 1.0 μM concentration; λ_{ex} = 385 nm. Please note that different Y-axis scales are used.

predominantly adopts *syn* conformations leading to intercalation of pyrene and nucleobase-mediated quenching[11,16b,28] of the pyrene fluorescence. Thus, in duplexes involving **Y5** or **Y8**, the pyrene moiety is near weakly quenching A/T base pairs, while it is near strongly quenching C/G base pairs in duplexes involving **Y6** or **Y7**. Additional support for this hypothesis comes from the fact that the fluorescence intensities of duplexes involving **Y6** and **Y7** are very low. Similar trends are seen for **Z**-modified duplexes, again suggesting that the LNA skeleton only has a minor influence on the position of the C8-fluorophore (Figure S8 in the Supporting Information).

3′-Exonuclease Stability of ONs Modified with C5-Functionalized LNA-C Monomers. Prompted by the interesting hybridization characteristics of **M**- and **N**-modified ONs, we set out to determine the stability of singly modified **M3** and **N3** against the 3′-exonuclease snake venom phosphodiesterase (SVPDE) by monitoring the change in absorbance at 260 nm for the ONs (Figure 4). As expected, unmodified DNA strand **D1** is rapidly cleaved, whereas conventional LNA **L3** exhibits moderate resistance against degradation by SVPDE (~70% cleavage within ~50 min). Gratifyingly, **M3** and **N3** are considerably more resistant toward degradation by SVPDE (~50% and ~30% cleavage within ~50 min, respectively), presumably since the C5-substituent interferes with SVPDE activity. These results are in agreement with our observations for the C5-alkynyl-functionalized LNA-U series,[10] which suggests that conjugation of alkynes to the C5-position of LNA pyrimidines is a general approach for improving the enzymatic stability of LNA-modified ONs.

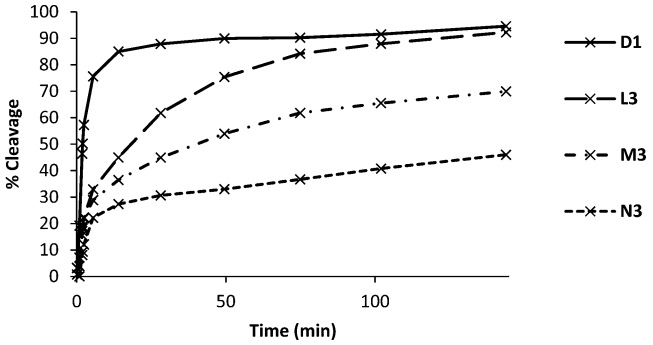

Figure 4. 3′-Exonuclease (SVPDE) degradation of singly modified **B3** and reference strands. Nuclease degradation studies were performed in magnesium buffer (50 mM Tris·HCl, 10 mM Mg^{2+}, pH 9.0) using 3.3 μM ONs and 0.03 unit of SVPDE.

CONCLUSION

Taken together with the results from our recent reports,[10,35] the current study demonstrates that attachment of alkynes to the C5-position of LNA pyrimidines is a straightforward approach for improving the binding affinity, target specificity, and/or enzymatic stability of LNA-modified ONs. This strategy extends to α-L-LNA and presumably most other conformationally restricted pyrimidines.[12] In contrast, C8-functionalization of LNA adenosines is detrimental to the binding affinity and specificity, demonstrating that the beneficial effects of the LNA skeleton do not supersede the destabilizing effect of the C8-substituent. Similar effects have been observed upon C5-functionalization of LNA uridines[10] and N2-functionalization of 2′-amino-LNA thymidines[29] with large hydrophobic moieties.

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In summary, C5-functionalized LNA pyrimidines are interesting affinity-, specificity-, and stability-enhancing modifications that can be used by themselves or alongside other chemically modified nucleotides for the development of enabling tools for nucleic acid targeting applications in molecular biology, biotechnology, and medicinal chemistry.

EXPERIMENTAL SECTION

(1*R*,3*R*,4*R*,7*S*)-7-(*tert*-Butyldimethylsilyloxy)-1-(4,4′-dimethoxytrityloxymethyl)-3-(5-iodouracil-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (2). To a solution of nucleoside **1**[10] (3.20 g, 4.67 mmol), I_2 (3.50 g, 14.0 mmol), and *N*-methylimidazole (1.2 mL, 14.0 mmol) in anhydrous CH_2Cl_2 (50 mL) was added TBDMS-Cl (1 M in CH_2Cl_2, 5.6 mL, 5.60 mmol). The reaction mixture was stirred at rt for 4 h, whereupon it was diluted with CH_2Cl_2 (100 mL) and sat. aq. sodium thiosulfate (100 mL). The layers were separated, and the organic layer was washed with sat. aq. sodium thiosulfate (2 × 200 mL). The combined aqueous phases were then extracted with CH_2Cl_2 (100 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness to afford a residue that was purified by column chromatography (0−40% v/v EtOAc in petroleum ether) to afford nucleoside **2** (3.30 g, 90%) as a slightly yellow solid material. R_f = 0.5 (40% v/v EtOAc in petroleum ether); ESI-HRMS *m*/*z* 821.1731 ([M + Na]$^+$, $C_{37}H_{43}IN_2O_8Si{\cdot}Na^+$, calcd 821.1726); ^{1}H NMR (DMSO-d_6) δ 11.74 (s, 1H, ex, NH), 8.08 (s, 1H, H6), 7.42−7.45 (m, 2H, Ar), 7.21−7.36 (m, 7H, Ar), 6.89 (d, 4H, *J* = 8.5 Hz, Ar), 5.48 (s, 1H, H1′), 4.27 (s, 2H, H2′, H3′), 3.72−3.76 (m, 7H, 2 × CH_3O, H5″), 3.65−3.68 (d, 1H, *J* = 8.0 Hz, H5″), 3.33−3.36 (d, 1H, *J* = 11.0 Hz, H5′), 3.21−3.24 (d, 1H, *J* = 11.0 Hz, H5′), 0.71 (s, 9H, Me_3C), 0.04 (s, 3H, CH_3Si), −0.03 (s, 3H, CH_3Si); ^{13}C NMR (DMSO-d_6) δ 160.7, 158.11, 158.10, 149.8, 144.5, 143.0 (C6), 135.20, 135.17, 129.6 (Ar), 129.5 (Ar), 127.9 (Ar), 127.5 (Ar), 126.7 (Ar), 113.3 (Ar), 113.2 (Ar), 87.6, 87.2 (C1′), 85.6, 78.5 (C2′), 71.6 (C5″), 70.4 (C3′), 69.0, 58.5 (C5′), 55.0 (CH_3O), 25.3 (Me_3C), 17.3, −4.9 (CH_3Si), −5.4 (CH_3Si).

(1*R*,3*R*,4*R*,7*S*)-7-(*tert*-Butyldimethylsilyloxy)-1-(4,4′-dimethoxytrityloxymethyl)-3-(5-iodocytosin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (3). To a cold stirred suspension of 1,2,4-triazole (4.60 g, 66.3 mmol) in anhydrous acetonitrile (25 mL) was added freshly distilled $POCl_3$ (1.5 mL, 15.6 mmol). The mixture was stirred at ∼0 °C for 15 min, whereupon anhydrous triethylamine (11.0 mL, 79.2 mmol) was added. After another 30 min of stirring at 0 °C, a solution of nucleoside **2** (1.60 g, 2.00 mmol) in anhydrous acetonitrile (45 mL) was added. The reaction mixture was stirred at rt for 3 h, at which point the solvents were removed under reduced pressure. The resulting residue was taken up in EtOAc (100 mL) and water (100 mL). The layers were separated, and the organic layer was washed with sat. aq. $NaHCO_3$ (2 × 100 mL). The combined aqueous layers were then extracted with EtOAc (100 mL). The combined organic phases were dried (Na_2SO_4) and evaporated to dryness to afford a solid material that was dissolved in sat. methanolic ammonia (150 mL). The reaction mixture was stirred at rt for ∼12 h, at which point the solvent was evaporated off and the resulting residue was purified by column chromatography (0−2% MeOH in CH_2Cl_2) to afford **3** (1.15 g, 72%) as a slightly yellow solid material. R_f = 0.4 (2% MeOH in CH_2Cl_2); ESI-HRMS *m*/*z* 820.1895 ([M + Na]$^+$, $C_{37}H_{44}IN_3O_7Si{\cdot}Na^+$, calcd 820.1885); ^{1}H NMR (DMSO-d_6) δ 8.06 (s, 1H, H6), 7.91 (br s, 1H, ex, NH), 7.43−7.46 (m, 2H, Ar), 7.21−7.35 (m, 7H, Ar), 6.89−6.92 (m, 4H, Ar), 6.68 (br s, 1H, ex, NH), 5.47 (s, 1H, H1′), 4.26 (s, 1H, H2′), 4.20 (s, 1H, H3′), 3.72−3.75 (m, 7H, 2 × CH_3O, H5″), 3.64−3.66 (d, 1H, *J* = 8.0 Hz, H5″), 3.34−3.37 (d, 1H, *J* = 11.0 Hz, H5′), 3.18−3.21 (d, 1H, *J* = 11.0 Hz, H5′), 0.71 (s, 9H, Me_3C), 0.00 (s, 3H, CH_3Si), −0.06 (s, 3H, CH_3Si); ^{13}C NMR (DMSO-d_6) δ 164.0, 158.1, 153.5, 145.4 (C6), 144.5, 135.3, 135.2, 129.6 (Ar), 129.5 (Ar), 127.9 (Ar), 127.5 (Ar), 126.7 (Ar), 113.28 (Ar), 113.25 (Ar), 87.5 (C1′), 87.4, 85.6, 78.5 (C2′), 71.5 (C5″), 70.3 (C3′), 58.6 (C5′), 56.7, 55.0 (CH_3O), 25.3 (Me_3C), 17.4, −5.0 (CH_3Si), −5.3 (CH_3Si).

(1*R*,3*R*,4*R*,7*S*)-7-(*tert*-Butyldimethylsilyloxy)-1-(4,4′-dimethoxytrityloxymethyl)-3-[5-(trimethylsilylethynyl)cytosin-1-yl]-2,5-dioxabicyclo[2.2.1]heptane (4a). Nucleoside **3** (0.50 g, 0.63 mmol), $Pd(PPh_3)_4$ (72 mg, 0.06 mmol), CuI (24 mg, 0.13 mmol), and trimethylsilylacetylene (0.25 mL, 1.76 mmol) were added to anhydrous DMF (10 mL), and the reaction chamber was degassed and placed under an argon atmosphere. To this was added anhydrous Et_3N (0.35 mL, 2.51 mmol), and the reaction mixture was stirred at rt for ∼12 h, at which point the solvent was evaporated off. The resulting residue was taken up in EtOAc (100 mL) and washed with brine (2 × 50 mL) and sat. aq. $NaHCO_3$ (50 mL). The combined aqueous layers were then extracted with EtOAc (100 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness, and the resulting residue was purified by column chromatography (0−5% v/v MeOH in CH_2Cl_2) to afford nucleoside **4a** (0.39 g, 81%) as a pale-yellow solid material. R_f = 0.4 (5% v/v MeOH in CH_2Cl_2); ESI-HRMS *m*/*z* 790.3310 ([M + Na]$^+$, $C_{42}H_{53}N_3O_7Si_2{\cdot}Na^+$, calcd 790.3314); ^{1}H NMR (DMSO-d_6) δ 8.00 (s, 1H, H6), 7.86 (br s, 1H, ex, NH), 7.41−7.45 (m, 2H, Ar), 7.20−7.27 (m, 7H, Ar), 6.86−6.90 (m, 4H, Ar), 6.68 (br s, 1H, ex, NH), 5.43 (s, 1H, H1′), 4.28 (s, 1H, H2′), 4.21 (s, 1H, H3′), 3.75−3.78 (d, 1H, *J* = 7.5 Hz, H5″), 3.72−3.73 (2s, 6H, CH_3O), 3.69−3.71 (d, 1H, *J* = 7.5 Hz, H5″), 3.30−3.36 (m, 2H, H5′), 0.73 (s, 9H, Me_3C), −0.01 (s, 3H, CH_3Si), −0.04 (s, 9H, Me_3Si), −0.07 (s, 3H, CH_3Si); ^{13}C NMR (DMSO-d_6) δ 164.2, 158.10, 158.07, 152.9, 144.5, 143.6 (C6), 135.3, 135.1, 129.7 (Ar), 129.4 (Ar), 127.8 (Ar), 127.5 (Ar), 126.6 (Ar), 113.18 (Ar), 113.15 (Ar), 99.7, 96.4, 89.7, 87.6 (C1′), 87.5, 85.5, 78.3 (C2′), 71.5 (C5″), 70.4 (C3′), 58.6 (C5′), 55.0 (CH_3O), 25.3 (Me_3C), 17.4, −0.6 (Me_3Si), −4.9 (CH_3Si), −5.3 (CH_3Si).

(1*R*,3*R*,4*R*,7*S*)-7-(*tert*-Butyldimethylsilyloxy)-1-(4,4′-dimethoxytrityloxymethyl)-3-[5-(3-trifluoroacetylaminopropyn-1-yl)-cytosin-1-yl]-2,5-dioxabicyclo[2.2.1]heptane (4b). Nucleoside **3** (0.50 g, 0.63 mmol), $Pd(PPh_3)_4$ (72 mg, 0.06 mmol), CuI (24 mg, 0.13 mmol), and 2,2,2-trifluoro-*N*-(2-propynyl)acetamide[30] (0.33 mL, 1.88 mmol) were added to anhydrous DMF (10.0 mL), and the reaction chamber was degassed and placed under an argon atmosphere. To this was added anhydrous Et_3N (0.35 mL, 2.51 mmol), and the reaction mixture was stirred at rt for ∼12 h, at which point the solvent was evaporated off. The resulting residue was taken up in EtOAc (100 mL) and washed with brine (2 × 50 mL) and sat. aq. $NaHCO_3$ (50 mL). The combined aqueous layers were 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 column chromatography (0−7% v/v MeOH in CH_2Cl_2) to afford nucleoside **4b** (0.44 g, 85%) as a pale-yellow solid material. R_f = 0.5 (7% v/v MeOH in CH_2Cl_2); ESI-HRMS *m*/*z* 843.3040 ([M + Na]$^+$, $C_{42}H_{47}F_3N_4O_8Si{\cdot}Na^+$, calcd 843.3007); ^{1}H NMR (DMSO-d_6) δ 9.84 (t, 1H, ex, *J* = 4.7 Hz, $NHCH_2$), 7.95 (br s, 2H, 1 ex, H2, NH_2), 7.40−7.44 (m, 2H, Ar), 7.21−7.34 (m, 7H, Ar), 6.88−6.92 (d, 4H, *J* = 8.5 Hz, Ar), 6.84 (br s, 1H, ex, NH_2), 5.50 (s, 1H, H1′), 4.20 (s, 1H, H2′), 4.14−4.19 (m, 2H, H3′, CH_2NH), 4.06−4.12 (dd, 1H, *J* = 17.8 Hz, 4.7 Hz, CH_2NH), 3.77−3.80 (d, 1H, *J* = 8.0 Hz, H5″), 3.74 (br s, 6H, CH_3O), 3.69−3.72 (d, 1H, *J* = 8.0 Hz, H5″), 3.40−3.44 (d, 1H, *J* = 11.0 Hz, H5′), 3.30−3.34 (d, 1H, *J* = 11.0 Hz, H5′), 0.70 (s, 9H, Me_3C), −0.02 (s, 3H, CH_3Si), −0.08 (s, 3H, CH_3Si); ^{13}C NMR (DMSO-d_6) δ 164.6, 158.2, 158.1, 155.9 (q, *J* = 36.5 Hz, $-COCF_3$), 153.0, 144.7, 142.6 (C6), 135.2, 134.7, 129.8 (Ar), 129.5 (Ar), 127.8 (Ar), 127.4 (Ar), 126.6 (Ar), 115.7 (q, *J* = 286 Hz, CF_3CO), 113.3 (Ar), 113.2 (Ar), 89.7, 88.9, 87.5 (C1′), 87.3, 85.5, 78.6 (C2′), 75.6, 71.5 (C5″), 70.3 (C3′), 58.6 (C5′), 55.0 (CH_3O), 29.9 (CH_2NH), 25.3 (Me_3C), 17.4, −5.0 (CH_3Si), −5.4 (CH_3Si); ^{19}F NMR (DMSO-d_6) δ −74.8.

(1*R*,3*R*,4*R*,7*S*)-3-[4-*N*-Benzoyl-5-ethynylcytosin-1-yl]-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]-heptane (5a). To a solution of nucleoside **4a** (0.73 g, 0.95 mmol) in anhydrous DMF (14.0 mL) was added benzoic anhydride (0.47 g, 2.10 mmol). The reaction mixture was stirred at rt for 24 h, at which point it was concentrated to near dryness, diluted with EtOAc (100 mL), and washed with brine (2 × 50 mL). The aqueous layer was back-extracted with EtOAc (100 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness, and the resulting residue (∼0.73 g) was dissolved in THF (20 mL). TBAF (1 M in THF, 1.45 mL, 1.45 mmol) was added, and the reaction mixture was stirred at rt for ∼3.5 h, at which point EtOAc (100 mL) was added. The organic layer was washed with brine (2 × 50 mL) and H_2O (50 mL). The aqueous layer was back-extracted with EtOAc (100 mL). The combined organic

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layers were dried (Na_2SO_4) and evaporated to dryness, and the resulting residue was purified by column chromatography (0−5% v/v MeOH in CH_2Cl_2) to afford nucleoside **5a** (0.46 g, 70% over two steps) as a slightly yellow solid material. R_f = 0.3 (7% v/v MeOH in CH_2Cl_2); ESI-HRMS *m/z* 708.2294 ($[M + Na]^+$, $C_{40}H_{35}N_3O_8{\cdot}Na^+$, calcd 708.2316); ^{1}H NMR[31] (DMSO-d_6) δ 12.78 (br s, 0.5H, ex, NH_A), 10.81 (br s, 0.5H, ex, NH_B), 7.9−8.3 (broad signal, 3H, Ar, H6), 7.60−7.65 (broad signal, 1H, Ar), 7.50−7.55 (m, 2H, Ar), 7.42−7.46 (m, 2H, Ar), 7.30−7.36 (m, 6H, Ar), 7.22−7.26 (m, 1H, Ar), 6.91 (d, 4H, *J* = 9.0 Hz, Ar), 5.73 (br s, 1H, ex, 3′-OH), 5.54 (br s, 1H, H1′), 4.32 (s, 1H, H2′), 4.08−4.10 (2s, 2H, H3′, HC≡C), 3.78−3.83 (m, 2H, H5″), 3.75 (br s, 6H, CH_3O), 3.48−3.52 (1H, d, *J* = 11.0 Hz, H5′), 3.30−3.33 (1H, d, *J* = 11.0 Hz, H5′, partial overlap with H_2O signal); ^{13}C NMR (DMSO-d_6) δ 158.1, 144.6, 135.3, 135.2, 132.7 (Ar), 129.7 (Ar), 129.6 (Ar), 128.4 (Ar), 127.9 (Ar), 127.6 (Ar), 126.7 (Ar), 113.3 (Ar), 87.8 (C1′), 85.7, 78.6 (C2′), 75.6, 71.4 (C5″), 69.3 (C3′), 58.9 (C5′), 55.0 (CH_3O).

(1*R*,3*R*,4*R*,7*S*)-3-[4-*N*-Benzoyl-5-(3-trifluoroacetylaminopropyn-1-yl)cytosin-1-yl]-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptane (5b). Benzoic anhydride (0.30 g, 1.34 mmol) was added to a solution of nucleoside **4b** (0.50 g, 0.61 mmol) in anhydrous DMF (10.0 mL), and the reaction mixture was stirred at rt for 24 h, at which point it was evaporated to near dryness. The resulting residue was taken up in ethyl acetate (100 mL) and washed with brine (2 × 50 mL), and the aqueous layer was back-extracted with EtOAc (100 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness, and the resulting residue (0.41 g) was dissolved in THF (12 mL). To this was added TBAF (1 M in THF, 0.17 mL, 0.57 mmol), and the reaction mixture was stirred at rt for ∼3.5 h. At this point, EtOAc (100 mL) was added, and the organic layer was washed with brine (2 × 50 mL) and H_2O (50 mL). The aqueous layer was back-extracted with EtOAc (100 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness, and the resulting residue was purified by column chromatography (0−40% v/v EtOAc in petroleum ether) to afford nucleoside **5b** (0.20 g, 41% yield) as a slightly yellow solid material. R_f = 0.5 (40% v/v EtOAc in petroleum ether); ESI-HRMS *m/z* 833.2415 ($[M + Na]^+$, $C_{43}H_{37}F_3N_4O_9{\cdot}Na^+$, calcd 833.2405); ^{1}H NMR (DMSO-d_6) δ 12.86 (br s, 0.5H, ex, NH), 10.46 (br s, 0.5H, ex, NH), 9.92 (br s, 1H, ex, NH), 7.9−8.2 (broad signal, 3H, Ar, H6), 7.59−7.64 (t, 1H, *J* = 7.0 Hz, Ar), 7.24−7.50 (m, 11H, Ar), 6.90−6.95 (m, 4H, Ar), 6.91−6.92 (d, 2H, *J* = 2.5 Hz, Ar), 5.77 (br s, 1H, 3′-OH), 5.50 (s, 1H, H1′), 4.32 (s, 1H, H2′), 3.94−4.09 (m, 3H, H3′, CH_2NH), 3.84 (br s, 2H, H5″), 3.75 (s, 6H, CH_3O), 3.59−3.62 (d, 1H, *J* = 11.0 Hz, H5′), 3.29−3.32 (d, 1H, H5′, overlap with H_2O signal); ^{13}C NMR (DMSO-d_6) δ 158.12, 158.08, 155.9 (q, *J* = 35.6 Hz, $COCF_3$), 144.7, 135.4, 134.9, 132.6 (Ar), 129.8 (Ar), 129.6 (Ar), 128.3 (Ar), 127.9 (Ar), 127.5 (Ar), 126.7 (Ar), 115.7 (q, *J* = 286 Hz, CF_3CO), 113.3 (Ar), 113.2 (Ar), 87.8 (C1′),[32] 87.5, 85.6, 78.5 (C2′), 74.8, 71.4 (C5″), 69.5 (C3′), 59.0 (C5′), 55.0 (CH_3O), 29.4 (CH_2NH); ^{19}F NMR (DMSO-d_6) δ −74.8.

(1*R*,3*R*,4*R*,7*S*)-3-[4-*N*-Benzoyl-5-ethynylcytosin-1-yl]-7-[2-cyanoethoxy(diisopropylamino)phosphinoxy]-1-(4,4′-dimethoxytrityloxymethyl)-2,5-dioxabicyclo[2.2.1]heptane (6a). Nucleoside 5**a** (0.45 g, 0.65 mmol) was dried through coevaporation with anhydrous 1,2-dichloroethane (3 × 25 mL) and dissolved in anhydrous CH_2Cl_2 (12 mL). To this were added anhydrous *N*,*N*′-diisopropylethylamine (0.45 mL, 2.60 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.32 mL, 1.42 mmol), and the reaction mixture was stirred at rt for ∼3.5 h. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with 5% aq. $NaHCO_3$ (2 × 25 mL), and the combined aqueous layers were back-extracted with CH_2Cl_2 (50 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness, and the resulting residue was purified by column chromatography (0−4% v/v MeOH in CH_2Cl_2) and subsequent trituration from CH_2Cl_2 and petroleum ether to provide phosphoramidite **6a** (0.34 g, 60%) as a yellow foam. R_f = 0.7 (3% v/v MeOH in CH_2Cl_2); ESI-HRMS *m/z* 908.3429 ($[M + Na]^+$, $C_{49}H_{52}N_5O_9P{\cdot}Na^+$, calcd 908.3395); ^{31}P NMR ($CDCl_3$) δ 149.9, 149.5.

(1*R*,3*R*,4*R*,7*S*)-3-[4-*N*-Benzoyl-5-(3-trifluoroacetylaminopropyn-1-yl)cytosin-1-yl]-7-[2-cyanoethoxy(diisopropylamino)-phosphinoxy]-1-(4,4′-dimethoxytrityloxymethyl)-2,5-dioxabicyclo[2.2.1]heptane (6b). Nucleoside **5b** (0.25 g, 0.33 mmol) was dried through coevaporation with anhydrous 1,2-dichloroethane (2 × 10 mL) and dissolved in anhydrous CH_2Cl_2 (8 mL). To this were added anhydrous *N*,*N*′-diisopropylethylamine (0.23 mL, 1.33 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.15 mL, 0.67 mmol), and the reaction mixture was stirred at rt for ∼3.5 h. The reaction mixture was diluted with CH_2Cl_2 (25 mL) and washed with 5% aq. $NaHCO_3$ (2 × 10 mL), and the combined aqueous layers were back-extracted with CH_2Cl_2 (25 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness, and the resulting residue was purified by column chromatography (0−4% v/v MeOH in CH_2Cl_2) and subsequent trituration from CH_2Cl_2 and petroleum ether to provide phosphoramidite **6b** (210 mg, 62%) as a yellow foam. R_f = 0.7 (4% v/v MeOH in CH_2Cl_2); ESI-HRMS *m/z* 1033.3516 ($[M + Na]^+$, $C_{52}H_{54}F_3N_6O_{10}P{\cdot}Na^+$, calcd 1033.3483); ^{31}P NMR ($CDCl_3$) δ 150.0, 149.3.

(1*S*,3*R*,4*R*,7*S*)-3-(8-Bromoadenin-9-yl)-7-hydroxy-1-hydroxymethyl-2,5-dioxabicyclo[2.2.1]heptane (8). A solution of Br_2 (0.28 mL, 5.07 mmol) in 1,4-dioxane (20 mL) was added dropwise to a solution of known diol **7**[8a] (1.13 g, 4.05 mmol) in 1,4-dioxane (12 mL) and 0.5 M aq. sodium acetate buffer (23.2 mL, pH 4.5). The reaction mixture was stirred at rt overnight, at which point sat. aq. $Na_2S_2O_3$ was added until the red color from bromine disappeared. The solution was then carefully neutralized using 0.5 M aq. NaOH. The mixture was concentrated to approximately half volume and allowed to stand at ∼5 °C overnight. The resulting precipitate was filtered off and washed with a cold solution of H_2O and 1,4-dioxane (1:1 v/v) to obtain C8-brominated LNA nucleoside **8** (1.23 g, 85%) as a slightly pale yellow solid material. R_f = 0.7 (10% v/v MeOH in CH_2Cl_2); ESI-HRMS *m/z* 379.9965 ($[M + Na]^+$, $C_{11}H_{12}BrN_5O_4{\cdot}Na^+$, calcd 379.9965); ^{1}H NMR (DMSO-d_6) δ 8.12 (s, 1H, H2), 7.43 (br s, 2H, ex, NH_2), 5.76 (s, 1H, H1′), 5.74 (d, 1H, ex, *J* = 4.3 Hz, 3′-OH), 4.95 (t, 1H, ex, *J* = 6.0 Hz, 5′-OH), 4.83 (s, 1H, H2′), 4.69 (d, 1H, *J* = 4.3 Hz, H3′), 3.95−3.97 (d, 1H, *J* = 8.0 Hz, H5″), 3.76−3.78 (d, 1H, *J* = 8.0 Hz, H5″), 3.72 (d, 2H, *J* = 6.0 Hz, H5′); ^{13}C NMR (DMSO-d_6) δ 154.9, 152.4 (C2), 149.9, 126.4, 119.0, 88.5, 87.5 (C1′), 79.4 (C2′), 71.9 (C3′), 71.8 (C5″), 57.1 (C5′).

(1*R*,3*R*,4*R*,7*S*)-3-(8-Bromoadenin-9-yl)-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptane (9). Nucleoside **8** (0.50 g, 1.40 mmol) was coevaporated with anhydrous pyridine (10 mL) and redissolved in anhydrous pyridine (15 mL). DMAP (10 mg, 0.35 mmol) and 4,4′-dimethoxytrityl chloride (0.62 g, 1.82 mmol) were added to this solution, and the reaction mixture was stirred at rt for 6 h. Methanol (∼1.25 mL) was added, and the solvents were evaporated off to furnish a crude material that was partitioned between CH_2Cl_2 (∼50 mL) and aq. $NaHCO_3$ (25 mL). The phases were separated, and the organic phase was washed with aq. $NaHCO_3$ (2 × 30 mL). The aqueous layer was back-extracted with CH_2Cl_2 (30 mL), and the combined organic layers were dried ($NaSO_4$) and concentrated to dryness. The resulting residue was coevaporated with toluene/abs. EtOH (2 × 100 mL, 1:2 v/v) and purified by silica gel column chromatography (0−5% v/v MeOH in $CHCl_3$) to provide nucleoside **9** (0.71 g, 77%) as a yellow solid material. R_f = 0.4 (5% v/v MeOH in CH_2Cl_2); MALDI-HRMS *m/z* 682.1277 ($[M + Na]^+$, $C_{32}H_{30}BrN_5O_6{\cdot}Na^+$, calcd 682.1272); ^{1}H NMR (DMSO-d_6) δ 8.11 (s, 1H, H2), 7.43 (bs, 2H, ex, NH_2), 7.33−7.35 (d, 2H, Ar), 7.18−7.27 (m, 7H, Ar), 6.82−6.86 (m, 4H, Ar), 5.85 (s, 1H, H1′), 5.78 (d, 1H, ex, *J* = 5.0 Hz, 3′-OH), 5.05 (s, 1H, H2′), 4.64 (d, 1H, *J* = 5.0 Hz, H3′), 3.97−4.02 (2d, 2H, *J* = 8.0 Hz, H5″), 3.73 (s, 3H, CH_3O), 3.72 (s, 3H, CH_3O), 3.30−3.33 (d, 1H, *J* = 11.0 Hz, H5′, partial overlap with H_2O), 3.21−3.24 (d, 1H, *J* = 11.0 Hz, H5′); ^{13}C NMR (DMSO-d_6) δ 158.0, 154.9, 152.5 (C2), 149.9, 144.7, 135.4, 135.3, 129.7 (Ar), 129.6 (Ar), 127.7 (Ar), 127.6 (Ar), 126.7, 126.5 (Ar), 118.9, 113.1 (Ar), 87.1 (C1′), 86.6, 85.2, 78.8 (C2′), 72.7 (C3′), 72.0 (C5″), 60.1 (C5′), 55.0 (CH_3O).

(1*R*,3*R*,4*R*,7*S*)-3-(8-Bromo-6-*N*-[(dimethylamino)methylene]-adenin-9-yl)-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptane (10). *N*,*N*-Dimethylformamide dimethyl acetal (0.17 mL, 1.20 mmol) was added to a solution of nucleoside **9** (0.32 g, 0.50 mmol) in anhydrous DMF (5.0 mL), and the reaction mixture was stirred for 5 h at 50 °C. All of the volatile components were removed, and the resulting residue was taken up in ethyl acetate (15 mL) and subsequently washed with brine (2 × 25 mL) and saturated aq. $NaHCO_3$ (25 mL). The organic layer was dried (Na_2SO_4) and evaporated

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to dryness, and the resulting residue was purified by silica gel column chromatography (0−5% v/v MeOH in CH_2Cl_2) to furnish nucleoside **10** (0.31 g, 90%) as a pale-yellow solid material. R_f = 0.5 (5% v/v MeOH in CH_2Cl_2); MALDI-HRMS *m/z* 715.1848 ($[M + H]^+$, $C_{35}H_{35}BrN_6O_6{\cdot}H^+$, calcd 715.1874); ^{1}H NMR (DMSO-d_6) δ 8.87 (s, 1H, CH(NMe_2)), 8.37 (s, 1H, H2), 7.33−7.36 (m, 2H, Ar), 7.18−7.28 (m, 7H, Ar), 6.82−6.86 (m, 4H, Ar), 5.88 (s, 1H, H1′), 5.80 (d, 1H, ex, *J* = 5.0 Hz, 3′-OH), 5.08 (s, 1H, H2′), 4.65 (d, 1H, *J* = 5.0 Hz, H3′), 3.98−4.04 (2d, *J* = 7.5 Hz, H5″), 3.72 (br s, 6H, CH_3O), 3.32−3.34 (d, 1H, *J* = 11.0 Hz, H5′), 3.21−3.24 (d, 1H, *J* = 11.0 Hz, H5′), 3.20 (s, 3H, NCH_3), 3.13 (s, 3H, NCH_3); ^{13}C NMR (DMSO-d_6) δ 158.00, 157.97, 157.7 (CH(NMe_2)), 151.7 (C2), 144.6, 135.4, 135.3, 129.7 (Ar), 129.6 (Ar), 129.3, 127.7 (Ar), 127.63 (Ar), 126.58 (Ar), 125.4, 113.1 (Ar), 87.1 (C1′), 86.6, 85.2, 78.9 (C2′), 72.8 (C3′), 72.0 (C5″), 60.2 (C5′), 55.0 (CH_3O), 40.7 (NCH_3), 34.6 (NCH_3).

(1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-3-(6-*N*-benzoyl-8-bromoadenin-9-yl)-7-hydroxy-2,5-dioxabicyclo-[2.2.1]heptane (10*). Nucleoside **9** (0.25 g, 0.38 mmol) was dried through coevaporation with pyridine (2 × 10 mL) and redissolved in anhydrous pyridine (5 mL). To this was added trimethylchlorosilane (0.14 mL, 1.14 mmol), and the reaction mixture was allowed to stir for 30 min at rt. At this point, BzCl (0.22 mL, 1.90 mmol) was added, and the reaction mixture was stirred at rt for 5 h. The reaction mixture was then cooled to 0 °C, and water (∼1 mL) was added. After 15 min of stirring, aq. NH_3 (29%, 5.0 mL) was added, and the suspension was stirred at rt for 30 min. The mixture was evaporated to near dryness, and the resulting material was taken up in CH_2Cl_2 (25 mL) and washed with 5% aq. $NaHCO_3$ (2 × 10 mL). The organic layer was evaporated to dryness, and the resulting residue was purified using silica gel column chromatography (0−5% v/v MeOH in CH_2Cl_2) to obtain nucleoside **10*** (210 mg, 72%) as a pale-brown solid material. R_f = 0.7 (5% v/v MeOH in CH_2Cl_2); ESI-HRMS: *m/z* 786.1534 ($[M + Na]^+$, $C_{39}H_{34}BrN_5O_7{\cdot}Na^+$, calcd 786.1539); ^{1}H NMR (DMSO-d_6) δ 11.26 (s, 1H, ex, NH), 8.70 (s, 1H, H2), 8.02−8.05 (d, 2H, *J* = 7.0 Hz, Ar), 7.63−7.67 (t, 1H, *J* = 7.0 Hz, Ar), 7.53−7.58 (d, 2H, *J* = 7.0 Hz, Ar), 7.34−7.38 (m, 2H, Ar), 7.18−7.30 (m, 7H, Ar), 6.84−6.88 (m, 4H, Ar), 5.95 (s, 1H, H1′), 5.87 (d, 1H, ex, *J* = 5.0 Hz, 3′-OH), 5.16 (s, 1H, H2′), 4.62 (d, 1H, *J* = 5.0 Hz, H3′), 4.06−4.08 (d, 1H, *J* = 8.0 Hz, H5″), 4.02−4.05 (d, 1H, *J* = 8.0 Hz, H5″), 3.72 (br s, 6H, CH_3O), 3.38−3.42 (d, 1H, *J* = 11.0 Hz, H5′), 3.22−3.25 (d, 1H, *J* = 11.0 Hz, H5′); ^{13}C NMR (DMSO-d_6) δ 165.6, 158.0, 152.4, 151.5 (C2), 149.2, 144.7, 135.5, 135.3, 133.1, 132.5 (Ar), 132.1, 129.7 (Ar), 129.6 (Ar), 128.5 (Ar), 128.4 (Ar), 127.8 (Ar), 127.6 (Ar), 126.6 (Ar), 125.4, 113.2 (Ar), 87.3 (C1′), 86.9, 85.2, 78.9 (C2′), 72.7 (C3′), 72.1 (C5″), 60.3 (C5′), 55.0 (CH_3O).

(1*R*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-3-(6-*N*-(dimethylamino)methylene-8-vinyladenin-9-yl)-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptane (11). Nucleoside **10** (0.30 g, 0.42 mmol), $Pd(PPh_3)_4$ (49 mg, 0.04 mmol), and tetravinyltin (0.17 mL, 0.92 mmol) were added to anhydrous *N*-methylpyrrolidone (5.0 mL), and the mixture was degassed and placed under argon. The reaction mixture was stirred at 70 °C for 5 h, at which point EtOAc (15 mL) and 5% aq. $NaHCO_3$ (20 mL) were added. The phases were separated, and the organic phase was washed with sat. aq. $NaHCO_3$ (20 mL). The aqueous phase was back-extracted with ethyl acetate (2 × 20 mL), and the combined organic phases were dried (Na_2SO_4) and evaporated to near dryness. The resulting crude material was purified by column chromatography (0−5% v/v MeOH in CH_2Cl_2) to afford nucleoside **11** (0.21 g, 75%) as an off-white solid material. R_f = 0.5 (5% v/v MeOH in CH_2Cl_2); MALDI-HRMS *m/z* 663.2930 ($[M + H]^+$, $C_{37}H_{38}N_6O_6{\cdot}H^+$, calcd 663.2926); ^{1}H NMR (DMSO-d_6) δ 8.91 (s, 1H, CH(NMe_2)), 8.37 (s, 1H, H2), 7.32−7.35 (m, 2H, Ar), 7.18−7.26 (m, 7H, Ar), 7.14 (dd, 1H, *J* = 17.2 Hz, 11.0 Hz, CH═CH_2), 6.80−6.85 (2d, 4H, *J* = 7.0 Hz, Ar), 6.47 (dd, 1H, *J* = 17.2 Hz, 2.0 Hz, CH_2═CH_{trans}), 6.07 (s, 1H, H1′), 5.79 (d, 1H, ex, *J* = 5.0 Hz, 3′-OH), 5.72 (dd, 1H, *J* = 11.0 Hz, 2.0 Hz, CH_2═CH_{cis}), 5.25 (s, 1H, H2′), 4.35 (d, 1H, *J* = 5.0 Hz, H3′), 4.02−4.04 (d, 1H, *J* = 8.0 Hz, H5″), 3.97−3.99 (d, 1H, *J* = 8.0 Hz, H5″), 3.72 (br s, 6H, CH_3O), 3.28−3.31 (d, 1H, H5′, overlap with H_2O), 3.20−3.23 (d, 1H, *J* = 11.0 Hz, H5′), 3.20 (s, 3H, NCH_3), 3.14 (s, 3H, NCH_3); ^{13}C NMR (DMSO-d_6) δ 158.7, 158.0, 157.7 (CH(NMe_2)), 151.4 (C2), 151.2, 149.2, 144.6, 135.4, 135.2, 129.62 (Ar), 129.59 (Ar), 127.7 (Ar), 127.6 (Ar), 126.6 (Ar), 124.84 (CH═CH_2), 124.81, 122.9 (CH_2═CH), 113.1, 86.5, 85.9 (C1′), 85.2, 78.8 (C2′), 72.6 (C3′), 72.0 (C5″), 60.1 (C5′), 54.9 (CH_3O), 40.6 (CH_3N), 34.6 (CH_3N).

(1*R*,3*R*,4*R*,7*S*)-7-[2-Cyanoethoxy(diisopropylamino)-phosphinoxy]-1-(4,4′-dimethoxytrityloxymethyl)-3-(6-*N*-(dimethylamino)methylene-8-vinyladenin-9-yl)-2,5-dioxabicyclo[2.2.1]heptane (12). Nucleoside **11** (200 mg, 0.30 mmol) was dried through coevaporation with anhydrous 1,2-dichloroethane (2 × 10 mL) and redissolved in anhydrous CH_2Cl_2 (8 mL). To this were added anhydrous *N*,*N*′-diisopropylethylamine (0.21 mL, 1.21 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.15 mL, 0.66 mmol), and the reaction mixture was stirred at rt for 3.5 h. The reaction mixture was diluted with CH_2Cl_2 (25 mL) and washed with 5% aq. $NaHCO_3$ (2 × 10 mL), and the combined aqueous phase was 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 (0−4% v/v MeOH in CH_2Cl_2) and subsequent trituration from CH_2Cl_2 and petroleum ether to provide phosphoramidite **12** (165 mg, 64%) as a white foam. R_f = 0.5 (4% v/v MeOH in CH_2Cl_2); MALDI-HRMS *m/z* 863.4039 ($[M + H]^+$, $C_{46}H_{55}N_8O_7P{\cdot}H^+$, calcd 863.4004); ^{31}P NMR ($CDCl_3$) δ 150.4, 150.0.

(1*S*,3*R*,4*R*,7*S*)-7-Hydroxy-1-hydroxymethyl-3-[8-(2-(1-pyrenyl)ethynyl)adenin-9-yl]-2,5-dioxabicyclo[2.2.1]heptane (13). Nucleoside **8** (0.40 g, 1.18 mmol), $Pd(PPh_3)_4$ (130 mg, 0.11 mmol), CuI (48 mg, 0.22 mmol), and 1-ethynylpyrene[33] (0.56 g, 2.46 mmol) were added to anhydrous DMF (8.0 mL), and the resulting mixture was degassed and placed under argon. To this was added anhydrous Et_3N (0.66 mL, 4.72 mmol), and the reaction mixture was stirred at 50 °C for 6 h, whereupon the solvents were evaporated off. The resulting residue was taken up in EtOAc (15 mL), dried (Na_2SO_4), evaporated to dryness, and purified by silica gel column chromatography (0−10% v/v MeOH in CH_2Cl_2) to obtain nucleoside **13** (0.34 g, 61%) as a bright-yellow solid material. R_f = 0.6 (10% v/v MeOH in CH_2Cl_2); MALDI-HRMS *m/z* 526.1514 ($[M + Na]^+$, $C_{29}H_{21}N_5O_4{\cdot}Na^+$, calcd 526.1486); ^{1}H NMR (DMSO-d_6) δ 8.73 (d, 1H, *J* = 9.0 Hz, Ar), 8.15−8.46 (m, 9H, Ar, H2), 7.61 (br s, 2H, ex, NH_2), 6.25 (s, 1H, H1′), 5.83 (d, 1H, ex, *J* = 5.0 Hz, 3′-OH), 5.02 (t, 1H, ex, *J* = 5.5 Hz, 5′-OH), 4.97 (s, 1H, H2′), 4.85 (d, 1H, *J* = 5.0 Hz, H3′), 4.12−4.14 (d, 1H, *J* = 8.0 Hz, H5″), 4.00−4.02 (d, 1H, *J* = 8.0 Hz, H5″), 3.84 (d, 2H, *J* = 6.0 Hz, H5′); ^{13}C NMR (DMSO-d_6) δ 156.0, 153.6 (C2), 148.5, 133.2, 132.0, 131.9, 130.7, 130.4, 129.9 (Ar), 129.5 (Ar), 129.2 (Ar), 127.2 (Ar), 127.0 (Ar), 126.5 (Ar), 126.4 (Ar), 125.0 (Ar), 124.5 (Ar), 123.5, 123.1, 119.2, 114.1, 94.0, 88.6, 86.9 (C1′), 84.6, 79.6 (C2′), 72.1 (C5″), 72.0 (C3′), 57.4 (C5′).

(1*S*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-[8-(2-(1-pyrenyl)ethynyl)adenin-9-yl]-2,5-dioxabicyclo-[2.2.1]heptane (14). Diol **13** (0.30 g, 0.59 mmol) was coevaporated with anhydrous pyridine (2 × 10 mL) and redissolved in anhydrous pyridine (10 mL). To this were added 4,4′-dimethoxytrityl chloride (0.26 g, 0.77 mmol) and DMAP (18 mg, 0.15 mmol), and the reaction mixture was stirred at ∼50 °C for 6 h, whereupon it was diluted with sat. aq. $NaHCO_3$ (20 mL) and CH_2Cl_2 (25 mL). The phases were separated, and the organic phase was washed with sat. aq. $NaHCO_3$ (20 mL). The aqueous phase was back-extracted with CH_2Cl_2 (2 × 20 mL), and 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 crude material was purified by silica gel column chromatography (0−5% v/v MeOH in CH_2Cl_2) to afford nucleoside **14** (0.38 g, 80%) as a slightly yellow solid material. R_f = 0.5 (5% v/v MeOH in CH_2Cl_2); MALDI-HRMS *m/z* 828.2828 ($[M + Na]^+$, $C_{50}H_{39}N_5O_6{\cdot}Na^+$, calcd 828.2793); ^{1}H NMR (DMSO-d_6) δ 8.89 (d, 1H, *J* = 9.5 Hz, Ar), 8.15−8.49 (m, 9H, Ar, H2), 7.60 (br s, 2H, ex, NH_2), 7.26−7.28 (m, 2H, Ar), 6.99−7.15 (m, 7H, Ar), 6.53−6.59 (2d, 4H, *J* = 9.0 Hz, Ar), 6.37 (s, 1H, H1′), 5.89 (d, 1H, ex, *J* = 5.0 Hz, 3′-OH), 5.21 (s, 1H, H2′), 4.61 (d, 1H, *J* = 5.0 Hz, H3′), 4.38−4.41 (d, 1H, *J* = 8.0 Hz, H5″), 4.17−4.20 (d, 1H, *J* = 8.0 Hz, H5″), 3.50 (s, 3H, CH_3O), 3.46 (s, 3H, CH_3O), 3.42−3.45 (d, 1H, *J* = 11.0 Hz, H5′), 3.19−3.22 (d, 1H, *J* = 11.0 Hz, H5′); ^{13}C NMR (DMSO-d_6) δ 157.8, 157.7, 156.0, 153.7 (C2), 148.4, 144.4, 135.5, 135.1, 133.3, 131.93, 131.90, 130.7, 130.4, 129.8 (Ar), 129.6 (Ar), 129.42 (Ar), 129.37 (Ar), 129.1 (Ar), 127.6 (Ar), 127.5 (Ar), 127.1 (Ar),

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126.9 (Ar), 126.4 (Ar), 125.0 (Ar), 124.4 (Ar), 123.5, 123.1, 118.9, 114.4, 112.85 (Ar), 112.83 (Ar), 93.8, 86.7, 86.6 (C1′), 85.2, 85.0, 78.9 (C2′), 72.9 (C3′), 72.5 (C5″), 60.9 (C5′), 54.68 (CH_3O), 54.66 (CH_3O).

(1*S*,3*R*,4*R*,7*S*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-3-[6-*N*-(dimethylamino)methylene-8-(2-(1-pyrenyl)ethynyl)-adenin-9-yl]-2,5-dioxabicyclo[2.2.1]heptane (15). *N*,*N*-Dimethylformamide dimethyl acetal (0.11 mL, 0.82 mmol) was added to a solution of nucleoside **14** (0.33 g, 0.41 mmol) in anhydrous DMF (10 mL), and the reaction mixture was stirred at 50 °C for 5 h. At this point, all of the volatile components were removed, and the resulting residue was taken up in ethyl acetate (15 mL). The organic phase was washed with brine (2 × 25 mL) and sat. aq. $NaHCO_3$ (25 mL), dried (Na_2SO_4), and evaporated to dryness. The resulting residue was purified by silica gel column chromatography (0−5% v/v MeOH in CH_2Cl_2) to obtain nucleoside **15** (0.32 g, 90%) as a bright-yellow solid material. R_f = 0.6 (6% v/v MeOH in CH_2Cl_2); MALDI-HRMS *m*/*z* 883.3243 ([M + Na]$^+$, $C_{53}H_{44}N_6O_6{\cdot}Na^+$, calcd 883.3215); ^{1}H NMR (DMSO-d_6) δ 8.94 (s, 1H, CH(NMe_2)), 8.91 (d, 1H, *J* = 9.0 Hz, Ar), 8.15−8.48 (m, 9H, Ar, H2), 7.25−7.29 (m, 2H, Ar), 6.98−7.16 (m, 7H, Ar), 6.57 (d, 2H, *J* = 9.0 Hz, Ar), 6.54 (d, 2H, *J* = 9.0 Hz, Ar), 6.40 (s, 1H, H1′), 5.90 (d, 1H, ex, *J* = 5.0 Hz, 3′-OH), 5.25 (s, 1H, H2′), 4.61 (d, 1H, *J* = 5.0 Hz, H3′), 4.41−4.43 (d, 1H, *J* = 7.5 Hz, H5″), 4.19−4.21 (d, 1H, *J* = 7.5 Hz, H5″), 3.49 (s, 3H, CH_3O), 3.47 (s, 3H, CH_3O), 3.42−3.45 (d, 1H, *J* = 11.0 Hz, H5′), 3.24 (s, 3H, NCH_3), 3.18−3.22 (m, 4H, H5′, NCH_3); ^{13}C NMR (DMSO-d_6) δ 159.1, 157.81 (CH(NMe_2)), 157.76, 157.69, 152.7 (C2), 150.3, 144.4, 135.6, 135.2, 135.1, 132.03, 131.97, 130.6, 130.4, 129.9 (Ar), 129.6 (Ar), 129.4 (Ar), 129.3 (Ar), 129.2 (Ar), 127.6 (Ar), 127.5 (Ar), 127.1 (Ar), 126.9 (Ar), 126.46 (Ar), 126.43 (Ar), 126.40 (Ar), 125.4, 125.0 (Ar), 124.4 (Ar), 123.5, 123.1, 114.3, 112.9 (Ar), 112.8 (Ar), 94.4, 86.7, 86.6 (C1′), 85.2, 85.1, 79.0 (C2′), 72.9 (C3′), 72.6 (C5″), 60.9 (C5′), 54.7 (CH_3O), 40.8 (NCH_3), 34.7 (NCH_3).

(1*S*,3*R*,4*R*,7*S*)-7-[2-Cyanoethoxy(diisopropylamino)-phosphinoxy]-1-(4,4′-dimethoxytrityloxymethyl)-3-[6-*N*-(dimethylamino)methylene-8-(2-(1-pyrenyl)ethynyl)adenin-9-yl]-2,5-dioxabicyclo[2.2.1]heptane (16). Nucleoside **15** (0.32 g, 0.37 mmol) was dried through coevaporation with anhydrous 1,2-dichloroethane (2 × 10 mL) and dissolved in anhydrous CH_2Cl_2 (8 mL). To this were added anhydrous *N*,*N*′-diisopropylethylamine (0.26 mL, 1.50 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (0.18 mL, 0.82 mmol), and the reaction mixture was stirred at rt for 3.5 h. The reaction mixture was diluted with CH_2Cl_2 (25 mL) and washed with 5% aq. $NaHCO_3$ (2 × 10 mL), and the combined aqueous phases were 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 (0−4% v/v MeOH in CH_2Cl_2) and subsequent trituration from CH_2Cl_2 and petroleum ether to provide phosphoramidite **16** (0.28 g, 71%) as a yellow foam. R_f = 0.5 (4% v/v MeOH in CH_2Cl_2); MALDI-HRMS *m*/*z* 1083.4299 ([M + Na]$^+$, $C_{62}H_{61}N_8O_7P{\cdot}Na^+$, calcd 1083.4293); ^{31}P NMR ($CDCl_3$) δ 150.0, 149.8.

Synthesis and Purification of ONs. Synthesis of modified ONs was performed with a DNA synthesizer on a 0.2 μmol scale using succinyl-linked long-chain alkylamine controlled-pore glass (LCAA-CPG) columns with a pore size of 500 Å. Standard protocols for incorporation of DNA phosphoramidites (A^{Bz}, C^{Bz}, G^{DMF}, and T) were used. A ∼50-fold molar excess of modified phosphoramidite in anhydrous CH_3CN (0.05 M, phosphoramidites **6a** and **6b**) or anhydrous CH_2Cl_2 (0.05 M, phosphoramidites **12** and **16** and the DNA analogue of **16**[34]) was used along with extended oxidation (45 s) and the following hand-coupling conditions (activator, coupling time, coupling yield) for monomer **M** (0.25 M 4,5-dicyanoimidazole in CH_3CN, 15 min, ∼95%), monomer **N** (0.25 M pyridinium hydrochloride in CH_3CN, 15 min, ∼90%), monomers **X** and **Y** (0.25 M 5-(ethylthio)-1*H*-tetrazole in CH_3CN, 20 min, ∼95%), and monomer **Z** (0.25 M 5-[3,5-bis-(trifluoromethyl)phenyl]-1*H*-tetrazole in CH_3CN, 20 min, ∼95%). Cleavage from the solid support and removal of nucleobase protecting groups was realized using 32% aq. ammonia (55 °C, ∼18 h). ONs were purified (DMTr-on) by ion-pair reversed-phase HPLC (XTerra MS C18 column) using a 0.05 mM triethylammonium acetate buffer−25% (v/v) water/acetonitrile gradient. Purified ONs were detritylated using 80% aq. AcOH (∼20 min) and precipitated from NaOAc/$NaClO_4$/acetone (−18 °C, 12−16 h). The identities of the synthesized ONs were verified through MALDI MS analysis performed in positive ion mode on a quadrupole time-of-flight tandem mass spectrometer using anthranilic acid as a matrix (Tables S1 and S2 in the Supporting Information), while their purities (>80%) were verified by RP-HPLC running in analytical mode.

Thermal Denaturation Experiments. ON concentrations were estimated using the following extinction coefficients for DNA (OD/μmol): G (12.01), A (15.20), T (8.40), C (7.05); for RNA (OD/μmol): G (13.70), A (15.40), U (10.00), C (9.00), pyrene (22.4).[26c] The strands constituting a given duplex were mixed and annealed. Thermal denaturation temperatures of duplexes (1.0 μM final concentration of each strand) were determined on a temperature-controlled UV−vis spectrophotometer using quartz optical cells with 1.0 cm path lengths. T_m was determined as the maximum of the first derivative of the thermal denaturation curve (A_{260} vs *T*) recorded in medium-salt phosphate buffer (100 mM NaCl, 0.1 mM EDTA, pH 7.0 adjusted with 10 mM NaH_2PO_4 and 5 mM Na_2HPO_4). The temperature of the denaturation experiments ranged from at least 15 °C below T_m to 20 °C above T_m (although not below 5 °C). A temperature ramp of 0.5 °C/min was used in all of the experiments. The reported T_m's are averages of two experiments within ±1.0 °C.

Absorption Spectroscopy. UV−vis absorption spectra were recorded at 5 °C using the same samples and instrumentation as in the thermal denaturation experiments.

Fluorescence Spectroscopy. Steady-state fluorescence emission spectra were recorded in non-deoxygenated thermal denaturation buffer (each strand 1.0 μM) using an excitation wavelength (λ_{ex}) of 385 nm, an excitation slit width of 5.0 nm, an emission slit width of 5.0 nm, and a scan speed of 600 nm/min. The experiments were performed at a temperature of ∼5 °C.

Exonuclease Studies. The changes in absorbance at 260 nm as functions of time were monitored for solutions of ONs (3.3 μM) in magnesium buffer (600 μL, 50 mM Tris·HCl, 10 mM $MgCl_2$, pH 9.0) at 37 °C to which snake venom phosphordiesterase (SVPDE, Worthington Biochemical Corporation) dissolved in H_2O (1.3 μL, 0.52 μg, 0.03 unit) had been added. Rate constants were determined from plots of $-\ln(1 - C)$ versus time obtained for the initial stages of degradation, where *C* denotes the fraction of degraded oligonucleotide (Figure S9 and Table S4 in the Supporting Information).

■ ASSOCIATED CONTENT

Supporting Information

General experimental section; additional synthetic details; NMR spectra for new compounds; ROESY spectra for **10**/**11**/**13**; MS data for new modified ONs; representative T_m curves; and additional UV−vis absorption, steady-state fluorescence emission, and 3′-exonuclease degradation 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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(31) ^{1}H NMR signals of H6 and the benzoyl group are very broad and/or split up as a result of slow interconversion (relative to the NMR time scale) between the two rotameric benzamide forms.
(32) The assignments of the ^{13}C NMR signals at 87.8 and 87.5 ppm are interchangeable.
(33) Wu, W.; Wu, W.; Ji, S.; Guo, H.; Zhao, J. *Eur. J. Inorg. Chem.* **2010**, 4470−4482.
(34) Our synthesis of this phosphoramidite will be presented elsewhere.
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