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Solid-Phase Synthesis of *P*-Boronated Oligonucleotides by the *H*-Boranophosphonate Method

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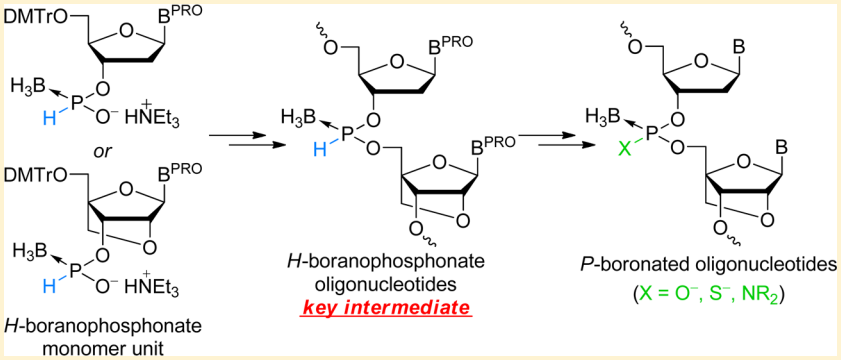
Supporting Information



ABSTRACT: Recently, *P*-boronated oligonucleotides have been attracting much attention as potential therapeutic oligonucleotides. In this study, we developed *H*-boranophosphonate oligonucleotide bearing a borano group and hydrogen atom on the internucleotidic phosphorus and demonstrated that this novel *P*-boronated oligonucleotide is a versatile precursor to various *P*-boronated oligonucleotides such as boranophosphate, boranophosphorothioate, and boranophosphoramidate. The method was also applicable to the synthesis of a locked nucleic acid-modified boranophosphate oligonucleotide, which exhibited a dramatically enhanced affinity to complementary oligonucleotides.

INTRODUCTION

Chemical modifications of the internucleotidic phosphate linkages of oligonucleotides have been widely used for the stabilization of unmodified natural oligonucleotides, which are inherently labile to nucleases, especially for therapeutic purposes.[1] Such modifications have also been used to improve their cellular uptake for the same purposes.[2] Phosphorothioate oligonucleotides (PS-ODNs) modified with sulfur have been particularly studied:[3] two therapeutic oligonucleotides have been approved, and many of them are in clinical trials.[4] However, they suffer from some drawbacks such as nonspecific binding to proteins and toxicity, which has brought forth the need for novel P-modified oligonucleotides that are more suitable for therapeutic use.[3]

Recently, P-boronated oligonucleotides have attracted much attention as potential therapeutic oligonucleotides because lower toxicity than that of their phosphorothioate counterparts is expected.[5] Moreover, their stability to nucleases is even higher than that of PS-ODNs, and high potency for gene suppression has been demonstrated.[5b,6] Furthermore, they are also potential ^{10}B carriers for boron neutron capture therapy for cancer treatment;[6] however, synthetically available P-boronated oligonucleotides are still very limited, despite intensive efforts by many researchers.[7−10] To find potent therapeutic oligonucleotides, the diversity of P-boronated oligonucleotides that are synthetically available needs to be expanded.

The methods for the synthesis of P-boronated oligonucleotides to date have generally been developed on an individual basis; synthesizing different kinds of P-boronated oligonucleotides requires different methods and different sets of monomer units.[7−10] In this study, rather than following the conventional strategies, we developed a novel type of P-boronated oligonucleotide, H-boranophosphonate oligonucleotide, with the expectation that it could be used as a versatile precursor to various P-boronated oligonucleotides via substitutions of the hydrogen atoms of its H−P→BH_3 backbone (Scheme 1). A similar strategy has been used for the synthesis of P-modified oligonucleotides using H-phosphonate precursors.[11] In fact, the diversity of P-modified oligonucleotides has been greatly expanded since the development of H-phosphonate oligonucleotides. Although H-boranophosphonate diesters cannot be

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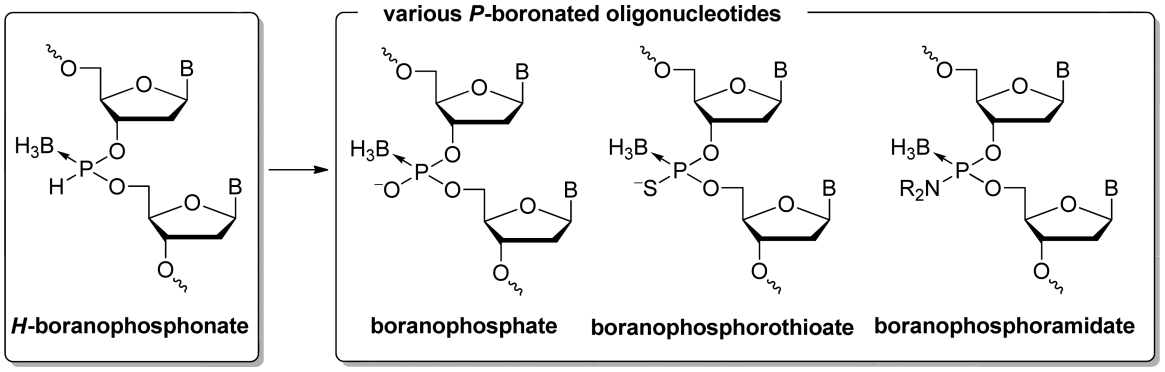
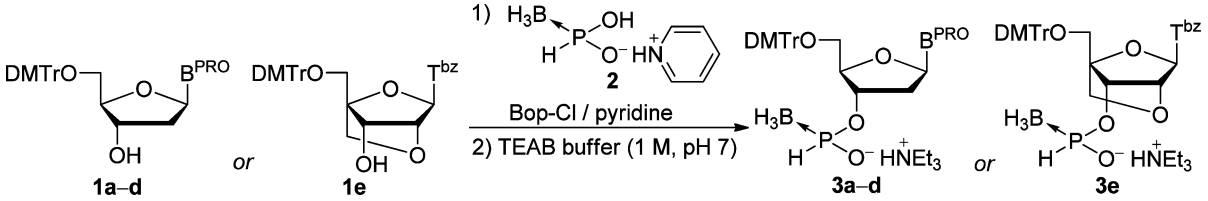
Scheme 1. Synthesis of Diverse *P*-Boronated Oligonucleotides Using *H*-Boranophosphonate Oligonucleotides As Platforms[a]



[a]B = Nucleobase.

Table 1. Synthesis of 3′-*H*-Boranophosphonate Monomers 3a–e[a]



entry	**1** (B^{PRO})	reagents and conditions	yield of **3** [%]
1	**a** (T^{bz})	**2** (1.2 equiv), Bop-Cl (1.2 equiv) rt, 1 h	95
2	**b** (A^{bz})	**2** (2.0 equiv), Bop-Cl (2.0 equiv), 0 °C to rt, 25 min	72
3	**c** (C^{ibu})	**2** (2.0 equiv), Bop-Cl (2.0 equiv), 0 °C to rt, 30 min	60
4	**d** ($G^{ce,ibu}$)	**2** (2.0 equiv), Bop-Cl (2.0 equiv), 0 °C to rt, 15 min	74
5	**e** (T^{bz})	**2** (2.0 equiv), Bop-Cl (2.0 equiv), 0 °C to rt, 20 min	75

[a]B^{PRO} = Protected nucleobase; T^{bz} = N^3-benzoylthymin-1-yl; A^{bz} = N^6-benzoyladenin-9-yl; C^{ibu} = N^4-isobutyrylcytosin-1-yl; $G^{ce,\,ibu}$ = O^6-cyanoethyl-N^2-isobutyrylguanin-9-yl; Bop-Cl = bis(2-oxo-3-oxazolidinyl)phosphinic chloride; TEAB = triethylammonium bicarbonate; DMTr = 4,4′-dimethoxytrityl.

derivatized via tautomerization to the tricoordinate forms, as in the case of their H-phosphonate counterparts, it has been demonstrated independently by Montchamp et al.[12] and our group[13] that the P–H function of H-boranophosphonate diesters can be modified by deprotonation and subsequent reactions with electrophiles. The chemistry of secondary phosphine–borane complexes and their analogues[14] was also used for the development of our novel strategy.

RESULTS AND DISCUSSION

Synthesis of 3′-*H*-Boranophosphonate Monomers 3a–e. Table 1 summarizes the synthesis of the 2′-deoxyribonucleoside 3′-*H*-boranophosphonate monomers **3a–d** and locked nucleic acid (LNA)[15] thymidine 3′-*H*-boranophosphonate monomer **3e**. 2′-Deoxythymidine monomer **3a** was obtained in 95% from the thymidine derivative bearing the 3′-OH **1a** and pyridinium *H*-boranophosphonate **2** according to the procedure described in the literature.[13a] 2′-Deoxyadenosine, cytosine, guanosine, and LNA thymidine monomers **3b–e** were synthesized by the method for the synthesis of **3a** with some modifications.

Solid-Phase Synthesis of *PBX*-ODNs. Scheme 2 shows the synthesis of *P*-boronated oligodeoxyribonucleotides bearing oxygen, sulfur,[16,17] or 2-morpholinoethylamino[18] as the substituent X on the phosphorus atoms (*PBX*-ODNs) *via* *H*-boranophosphonate oligodeoxyribonucleotides (*PBH*-ODNs). The monomers **3a–e** were condensed with the 5′-OH of nucleosides or oligonucleotides on a controlled-pore glass (CPG) in the presence of 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (MNTP)[19] and 2,6-lutidine, and the 5′-end was deprotected by 3% dichloroacetic acid (DCA). Released dimethoxytrityl (DMTr) cations were reduced by Et_3SiH, because they would otherwise cause side reactions with the internucleotidic BH_3 groups.[20] The *PBH*-ODN chains were elongated by this cycle, and the modification of the phosphorus atoms and subsequent deprotection afforded *PBX*-ODNs.

First, *PBX*-ODN 2mers were synthesized using this cycle to optimize reaction conditions for the synthesis of oligomers. The monomers **3a–d** were condensed with the 5′-OH of N^3-benzoylthymidine anchored to a CPG *via* a succinate linker using MNTP and 2,6-lutidine to afford the *PBH*-ODN 2mers on a solid support (Scheme 2, **5**, n = 0). Subsequently, 5′-detritylation, *P*-modification (oxidation, sulfurization, or amination), deprotection, and cleavage of the linker afforded the desired *PBX*-ODN 2mers. *P*-Oxidation of the *PBH*-ODN 2mers on a solid support was performed *via* oxidative *P*-chlorination with CCl_4 and subsequent nucleophilic substitution by H_2O in the presence of *i*-Pr_2NEt (Scheme 3, oxidation).[21] *N*,*N*′-Dimethylthiuram disulfide (DTD)[22] and *i*-Pr_2NEt were used for the *P*-sulfurization step (Scheme 3, sulfurization). The introduction of a 2-morpholinoethylamino group onto the phosphorus atom was performed *via* oxidative *P*-chlorination with CCl_4 and subsequent reaction with 4-(2-

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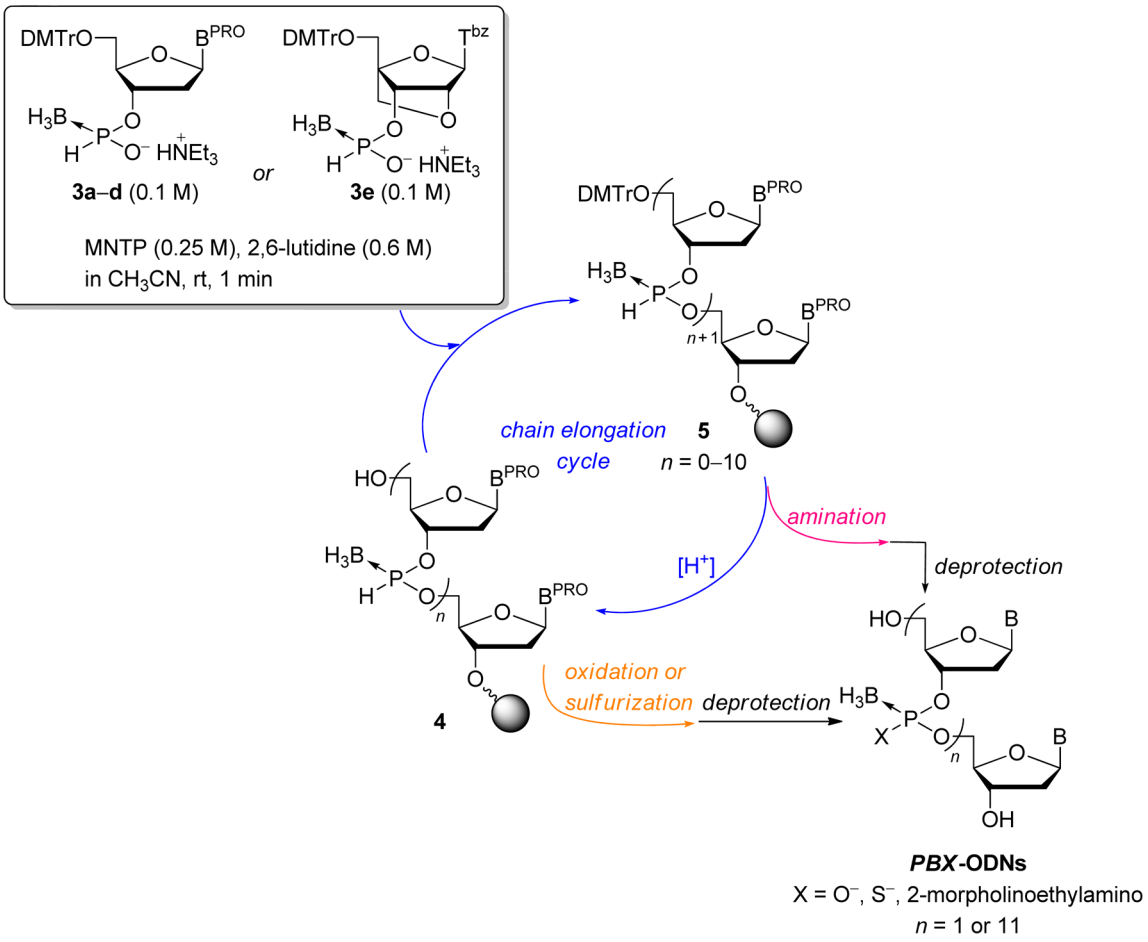
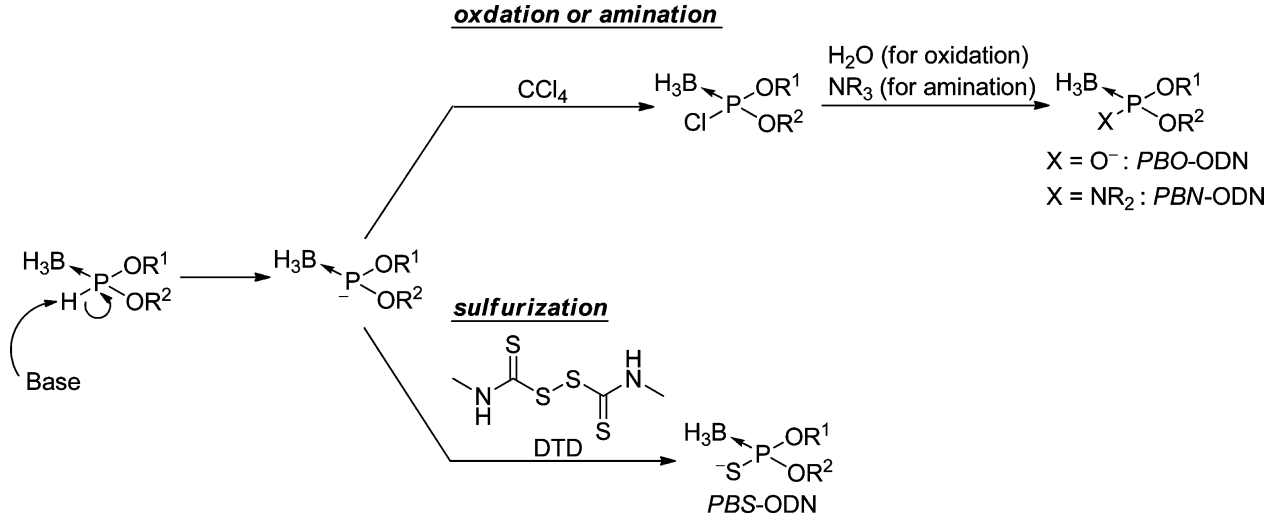
Scheme 2. Solid-Phase Synthesis of *PBX*-ODNs using *PBH*-ODNs 4 and 5 As Precursors[a]



[a]Detritylation: 3% dichloroacetic acid (DCA) in CH_2Cl_2–Et_3SiH (1:1, v/v), rt, 30 s; oxidation: *i*-Pr_2NEt, CCl_4, H_2O in CH_3CN, rt, 30 min; sulfurization: *i*-Pr_2NEt, *N*,*N*′-dimethylthiuram disulfide (DTD) in CH_3CN, rt, 1 h; amination: CCl_4, 4-(2-aminoethyl)morpholine in CH_3CN, rt, 30 min.

Scheme 3. Plausible Mechanisms of Conversions



aminoethyl)morpholine (Scheme 3, amination).[18,21] Reversed-phase HPLC (RP-HPLC) analysis of the crude mixtures showed that the desired *PBX*-ODN 2mers were obtained in 94–98% yields without any side reactions (Table 2).[23] Notably, the boranophosphate (*PBO*) and boranophosphorothioate (*PBS*) diesters were susceptible to $DMTr^+$, even in the presence of Et_3SiH.[20] Therefore, as shown in Scheme 2, the 5′-detritylation step had to be performed prior to the *P*-modification (oxidation or sulfurization) step for the synthesis of the *PBO* and *PBS*-ODN 2mers. In contrast, the

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Table 2. Solid-Phase Synthesis of *PBO*, *PBS*, and *PBN*-ODN 2mers

entry	monomer (B^{PRO})	*P*-modification	product[a]	yield [%][b]
1	**3a** (T^{bz})	oxidation	$T_{PBO}T$ (**6a**)	96
2	**3b** (A^{bz})	oxidation	$dA_{PBO}T$ (**6b**)	96
3	**3c** (C^{ibu})	oxidation	$dC_{PBO}T$ (**6c**)	95
4	**3d** ($G^{ce,\ ibu}$)	oxidation	$dG_{PBO}T$ (**6d**)	97
5	**3a** (T^{bz})	sulfurization	$dT_{PBS}T$ (**7a**)	98
6	**3b** (A^{bz})	sulfurization	$dA_{PBS}T$ (**7b**)	96
7	**3c** (C^{ibu})	sulfurization	$dC_{PBS}T$ (**7c**)	97
8	**3d** ($G^{ce,\ ibu}$)	sulfurization	$dG_{PBS}T$ (**7d**)	98
9	**3a** (T^{bz})	amination[c]	$T_{PBN}T$ (**8**)	96
10	**3a** (T^{bz})	amination[d]	$T_{PBN}T$ (**8**)	94

[a]Subscript 'PBO', 'PBS', and 'PBN' = boranophosphate diester, boranophosphorothioate diester, and *N*-(2-morpholinoethyl) boranophosphoramidate diester, respectively. [b]Determined by RP-HPLC. [c]Amination was performed prior to 5′-detritylation. [d]Amination was performed after 5′-detritylation.

boranophosphoramidate linkage of the *PBN*-ODN was less susceptible to $DMTr^+$; thus, the amination step for the synthesis of the *PBN*-ODN 2mer could be performed either before or after the 5′-detritylation, although a slightly better yield was obtained when the amination was performed prior to the 5′-detritylation (Table 2, entry 9 vs entry 10).

Next, using the optimized conditions, we synthesized *PBX*-ODN 12mers **9**–**14** (Table 3) bearing backbones thoroughly

Table 3. *PBX*-ODN 12mers 9–14 Synthesized *via* *PBH*-ODNs

PBX-ODN	yield [%][b]
$T(_{PBO}T)_{11}$ (**9**)	28
$T(_{PBS}T)_{11}$ (**10**)	31
$T(_{PBN}T)_{11}$ (**11**)	10
$G_{PBO}C_{PBO}A_{PBO}T_{PBO}T_{PBO}G_{PBO}G_{PBO}T_{PBO}A_{PBO}T_{PBO}T_{PBO}C$ (**12**)	44
$G_{PBS}C_{PBS}A_{PBS}T_{PBS}T_{PBS}G_{PBS}G_{PBS}T_{PBS}A_{PBS}T_{PBS}T_{PBS}C$ (**13**)	16
$G_{PBO}C_{PBO}A_{PBO}\mathbf{T^L}_{PBO}\mathbf{T^L}_{PBO}G_{PBO}G_{PBO}\mathbf{T^L}_{PBO}A_{PBO}\mathbf{T^L}_{PBO}\mathbf{T^L}_{PBO}C$ (**14**)[a]	7

[a]$\mathbf{T^L}$ = LNA thymidine. [b]Isolated yield.

modified with boranophosphate diesters (*PBO*-ODNs **9**, **12**, **14**), boranophosphorothioate diesters (*PBS*-ODNs **10**, **13**), or *N*-(2-morpholinoethyl)boranophosphoramidate diesters (*PBN*-ODN **11**) according to Scheme 2. An antisense sequence against apoB protein mRNA[24] (5′-GCA TTG GTA TTC-3′) was chosen for the *PBX*-ODNs **12**–**14**. We also synthesized a *PBO*-ODN wherein all the thymidine 3′-boranophosphate moieties were replaced with LNA thymidine 3′-boranophosphates (^{LNA}*PBO*-ODN **14**) by using LNA thymidine monomer **3e**.[25]

Hybridization Properties of *PBX*-ODNs. Finally, the hybridization properties of *PBX*-ODN 12mers **9**–**14** with complementary ODNs and oligoribonucleotides (ORNs) were examined. Melting temperatures (T_m) for the duplexes of the homothymidylate 12mers **9**–**11** with the complementary dA_{12} and rA_{12} could not be determined.[23] In contrast, the 12mers containing all four nucleobases **12**–**14** formed duplexes with the complementary ODN and ORN (Table 4, Figures 1 and 2). On the basis of the T_m values of 2′-deoxy ODNs (Table 4, entries 1–4), the order of duplex stability is natural ODN **15**, *PS*-ODN **16**, *PBO*-ODN **12**, and *PBS*-ODN **13**. These results suggest that the hydrophobic and sterically hindered groups

Table 4. T_m Values of the Duplexes of ODNs with Complementary ODN and ORN

		complementary strand	
		ODN[b]	ORN[c]
entry	oligonucleotide[a]	T_m [°C] (ΔT_m [°C], ΔT_m/mod. [°C])	T_m [°C] (ΔT_m [°C], ΔT_m/mod. [°C])
	0.1 M NaCl, 10 mM NaH_2PO_4–Na_2HPO_4, pH 7.0		
1	natural ODN (**15**)	42.7	42.0
2	*PS*-ODN (**16**)	31.3 (-11.4^d, -1.0^e)	32.2 (-9.8^d, -0.9^e)
3	*PBO*-ODN (**12**)	25.9 (-16.8^d, -1.5^e)	29.9 (-12.1^d, -1.1^e)
4	*PBS*-ODN (**13**)	15.7 (-27.0^d, -2.5^e)	25.0 (-17.0^d, -1.5^e)
5	^{LNA}*PO*-ODN (**17**)	60.1 ($+17.4^d$, $+3.5^f$)	69.0 ($+27.0^d$, $+5.4^f$)
6	^{LNA}*PS*-ODN (**18**)	54.6 (-5.5^g, -0.5^h)	64.2 (-4.8^g, -0.4^h)
7	^{LNA}*PBO*-ODN (**14**)	52.1 (-8.0^g, -0.7^h)	63.9 (-5.1^g, -0.5^h)

[a]Natural ODN **15**: $G_{PO}C_{PO}A_{PO}T_{PO}T_{PO}G_{PO}G_{PO}T_{PO}A_{PO}T_{PO}T_{PO}C$; *PS*-ODN **16**: $G_{PS}C_{PS}A_{PS}T_{PS}T_{PS}G_{PS}G_{PS}T_{PS}A_{PS}T_{PS}T_{PS}C$; ^{LNA}*PO*-ODN **17**: $G_{PO}C_{PO}A_{PO}\mathbf{T^L}_{PO}\mathbf{T^L}_{PO}G_{PO}G_{PO}\mathbf{T^L}_{PO}A_{PO}\mathbf{T^L}_{PO}\mathbf{T^L}_{PO}C$; ^{LNA}*PS*-ODN **18**: $G_{PS}C_{PS}A_{PS}\mathbf{T^L}_{PS}\mathbf{T^L}_{PS}G_{PS}G_{PS}\mathbf{T^L}_{PS}A_{PS}\mathbf{T^L}_{PS}\mathbf{T^L}_{PS}C$. [b]3′-CGT AAC CAT AAG-5′. [c]3′-CGU AAC CAU AAG-5′. [d]Difference from the T_m of natural ODN/ODN (ORN). [e]Difference of the T_m values per *PS*, *PBO*, or *PBS* linkage. [f]Difference of the T_m values per LNA thymidine. [g]Difference from the T_m of ^{LNA}*PO*-ODN/ODN (ORN). [h]Difference of the T_m values per *PS* or *PBO* linkage.

destabilize the duplex. However, notably, the *PBX*-ODN 12mers **12** and **13** formed more stable duplexes with ORN than with ODN, indicating that the *PBX*-backbones generally favor the duplex formation with complementary ORNs rather than ODNs, which would be advantageous for antisense strategy.[10b,26]

The duplex stability of ^{LNA}*PBO*-ODN **14** was comparable with that of ^{LNA}*PS*-ODN **18** (Table 4, entry 6 vs 7). Although the T_m value of ^{LNA}*PBO*-ODN **14** was lower than that of ^{LNA}*PO*-ODN **17**, the difference of the T_m values was 5.1–8.0 °C (Table 4, entry 5 vs 7). On the other hand, the difference of the T_m values of 2′-deoxy natural ODN **15** and *PBO*-ODN **12** was 12.1–16.8 °C (Table 4, entry 1 vs 3). These results suggest that the incorporation of LNA can make up for the reduction of the duplex stability caused by the BH_3 group. Thus, because ^{LNA}*PBO*-ODN **14** showed a significantly enhanced affinity to the complementary strands, it has a great potency as an antisense therapeutic oligonucleotide.

■ CONCLUSION

In conclusion, we developed *H*-boranophosphonate oligonucleotide and demonstrated its versatility as a precursor to various *PBX*-ODNs. By using the *H*-boranophosphonate method, incorporation of LNA to the oligomers can be performed easily. Therefore, the present approach is efficient for the synthesis of versatile antisense molecules. The *P*-boronated oligonucleotides have a chirality center at the phosphorus atom and therefore are obtained as a mixture of diastereomers. Thus, the stereoregulated synthesis of *P*-boronated oligonucleotides is in progress by our group.[10c] From the hybridization studies, although the *PBX*-backbones destabilized the duplexes with complementary ODN and ORN, it was fully complemented by incorporating LNA-modified boranophosphate motifs. Further study toward the expansion of synthetically available *P*-boronated oligonucleotides and the evaluation of their physicochemical and biological properties is in progress.

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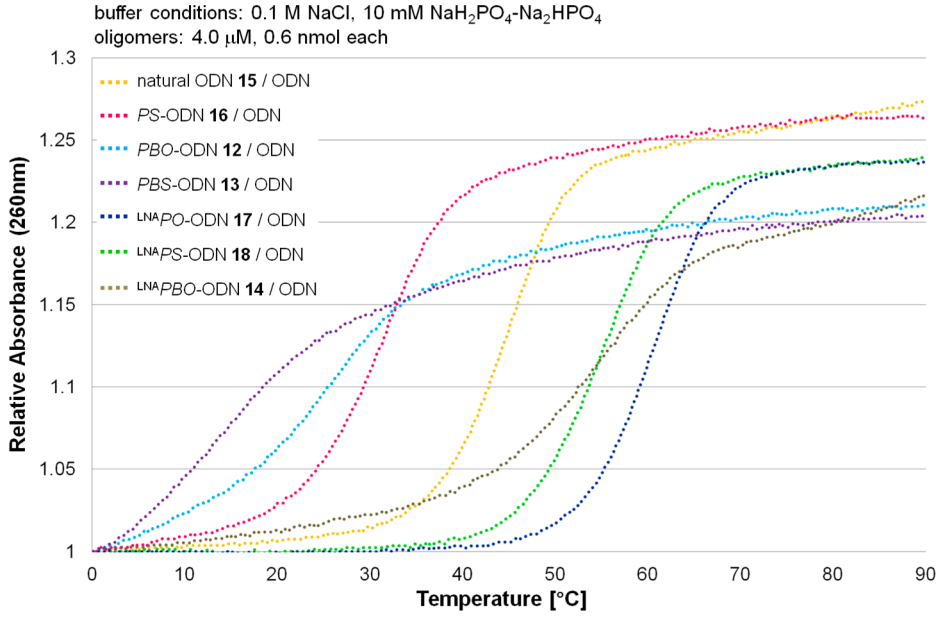

Figure 1. UV melting curves of the duplex of ODNs with complementary ODN.

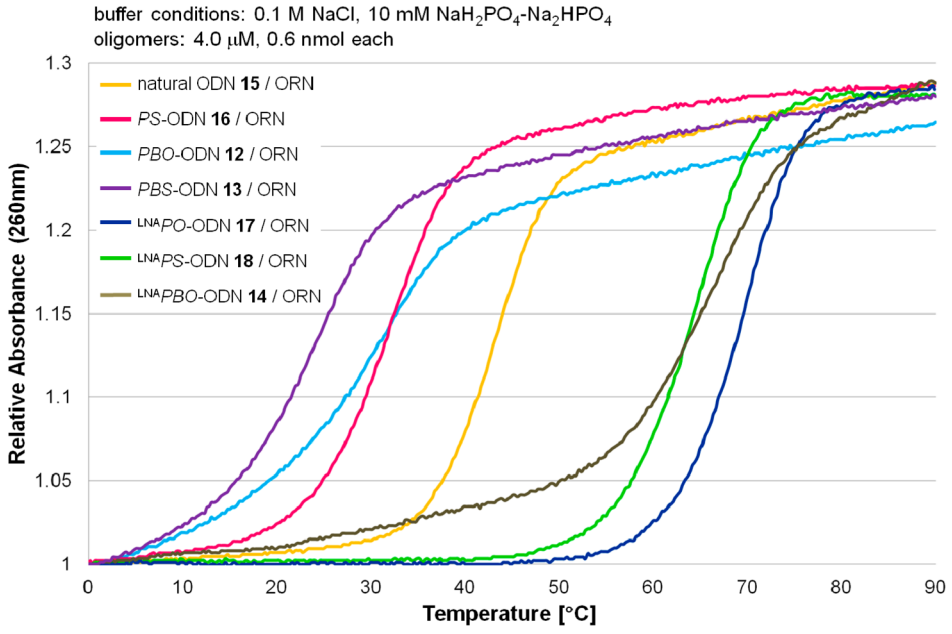

Figure 2. UV melting curves of ODNs with complementary ORN.

EXPERIMENAL SECTION

Triethylammonium 5′-*O*-Dimethoxytrityl-N^6-benzoyladenosine 3′-*H*-Boranophosphonate As a Mixture of (*S*p)- and (*R*p)-Diastereomers (3b). 5′-*O*-DMTr-N^6-benzoyladenosine **1b** (0.66 g, 1.0 mmol) and pyridinium *H*-boranophosphonate **2** (0.30 g, 2.0 mmol) were dried individually by repeated coevaporation with dry pyridine (3 × 3 mL for **1b** and 5 × 3 mL for **2**) and dissolved together in dry pyridine (10 mL) at 0 °C under argon. Bis(2-oxo-3-oxazolidinyl)phosphinic chloride (Bop-Cl) (0.51 g, 2.0 mmol) was added, and the mixture was stirred for 25 min at rt. The mixture was diluted with CH_2Cl_2 (50 mL) and washed with 1 M triethylammonium bicarbonate (TEAB) buffer (pH 7) (3 × 50 mL). The washings were combined and back-extracted with CH_2Cl_2 (2 × 150 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was then purified by silica gel column chromatography [3.7 × 4.8 cm, 28 g of silica gel (spherical, neutral, 40−50 μm), ethyl acetate−MeOH−Et_3N (99:1:1, v/v/v) to CH_2Cl_2−MeOH−Et_3N (99.5:0.5:1−99:1:1, v/v/v)]. The fractions containing **3b** were combined and concentrated under reduced pressure. The residue was dissolved in ethyl acetate (100 mL) and washed with 1 M TEAB buffer (100 mL). The washings were combined and back-extracted with ethyl acetate (100 mL). The organic layers were combined, dried over $MgSO_4$, filtered, concentrated under reduced pressure to afford **3b** (0.61 g, 0.72 mmol, 72%) as a colorless foam. ^{1}H NMR ($CDCl_3$) δ 9.09 (br s, 1H), 8.71, 8.70 (s, s, 1H), 8.23, 8.18 (s, s, 1H), 8.04−8.00 (m, 2H), 7.63−7.58 (m, 1H), 7.54−7.49 (m, 2H), 7.42−7.38 (m, 2H), 7.31−7.16 (m, 7H), 7.31 (br d, $^1J_{PH}$ = 389 Hz, 1H), 6.81−6.76 (m, 4H), 6.61−6.55 (m, 1H), 5.13−5.02 (m, 1H), 4.49−4.42 (m, 1H), 3.77, 3.77 (s, s, 6H), 3.43, 3.42 (s, s, 2H), 3.01−2.75 (m, 9H), 1.22 (t, J = 7.4 Hz, 11H), 1.03−0.08 (br, 3H); ^{13}C NMR ($CDCl_3$) δ 164.7, 158.3, 158.3, 152.1, 151.4, 151.3, 149.3, 144.4, 144.3, 141.5, 141.3, 135.5, 135.4, 135.4, 133.5, 133.5, 132.6, 129.9, 129.8, 128.6, 127.9, 127.9, 127.7, 126.7, 123.4, 113.0, 112.9, 86.3, 86.2, 86.0, 85.9, 85.6, 85.6, 84.6, 84.5,

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76.8, 76.7, 75.5, 75.5, 63.4, 63.2, 55.0, 45.1, 40.1, 39.0, 8.5; ^{31}P NMR ($CDCl_3$) δ 105.6−102.4 (m). ESI-HRMS: Calcd for $C_{38}H_{38}BN_5O_7P^-$ $[M - H]^-$ 718.2607, found 718.2615.

Triethylammonium 5′-*O*-Dimethoxytrityl-*N*^{4}-isobutyrylcytidine 3′-*H*-Boranophosphonate As a Mixture of (*Sp*)- and (*Rp*)-Diastereomers (3c). Compound **3c** was obtained in 60% (0.48 g, 0.60 mmol) from 5′-*O*-DMTr-*N*^{4}-isobutyrylcytidine **1c** (0.60 g, 1.0 mmol) and **2** (0.30 g, 2.0 mmol) as a colorless foam in a manner similar to the synthesis of **3b**, except for the reaction time (30 min) and the conditions for silica gel column chromatography [3.7 × 5.3 cm, 30 g of silica gel (spherical, neutral, 40−50 μm), ethyl acetate−MeOH−Et_3N (99:1:0.5, v/v/v) to CH_2Cl_2−MeOH−Et_3N (99:1:0.5 to 99:1:1, v/v/v)]. ^{1}H NMR ($CDCl_3$) δ 8.28, 8.26 (s, s, 1H), 8.20, 8.17 (s, s, 1H), 7.41−7.37 (m, 2H), 7.33−7.23 (m, 7H), 7.28 (br d, $^1J_{PH}$ = 394 Hz, 1H), 7.15−7.08 (m, 1H), 6.87−6.83 (m, 4H), 6.29, 6.22 (t, J = 6.0 Hz, t, J = 5.7 Hz, 1H), 5.09−5.01, 4.87−4.79 (m, m, 1H), 4.40−4.30 (m, 1H), 3.81, 3.80 (s, s, 6H), 3.46−3.44 (m, 2H), 2.95−2.87 (m, 8H), 2,57, 2.56, (sept, J = 6.9 Hz, sept, J = 6.9 Hz, 1H), 2.40−2.26 (m, 1H), 1.25−1.20 (m, 16.5H), 1.04−0.07 (br, 3H); ^{13}C NMR ($CDCl_3$) δ 177.0, 176.9, 162.3, 162.2, 158.4, 154.9, 154.9, 144.4, 144.4, 144.0, 143.9, 135.3, 135.3, 135.1, 135.1, 129.9, 129.9, 129.8, 128.0, 127.9, 127.8, 127.8, 126.9, 113.1, 113.1, 96.1, 96.0, 87.0, 86.8, 86.7, 86.6, 85.7, 85.6, 85.5, 85.4, 76.3, 76.1, 73.5, 73.4, 62.5, 62.0, 55.1, 45.2, 41.3, 40.7, 36.3, 36.2, 19.0, 18.8, 8.5; ^{31}P NMR ($CDCl_3$) δ 107.1−102.2 (m). ESI-HRMS: Calcd for $C_{34}H_{40}BN_3O_8P^-$ $[M - H]^-$ 660.2652, found 660.2653.

Triethylammonium 5′-*O*-Dimethoxytrityl-*O*^{6}-cyanoethyl-*N*^{2}-isobutyrylguanosine 3′-*H*-Boranophosphonate As a Mixture of (*Sp*)- and (*Rp*)-Diastereomers (3d). Compound **3d** was obtained in 74% (0.66 g, 0.74 mmol) from 5′-*O*-dimethoxytrityl-*O*^{6}-cyanoethyl-*N*^{2}-isobutyrylguanosine **1d** (0.67 g, 1.0 mmol) and **2** (0.30 g, 2.0 mmol) as a colorless foam in a manner similar to the synthesis of **3b**, except for the reaction time (15 min) and the conditions for silica gel column chromatography [3.7 × 4.8 cm, 28 g of silica gel (spherical, neutral, 40−50 μm), ethyl acetate−MeOH−Et_3N (99:1:1, v/v/v) to CH_2Cl_2−MeOH−Et_3N (99:1:1, v/v/v)]. ^{1}H NMR ($CDCl_3$) δ 8.01, 7.98 (s, s, 1H), 7.93, 7.90 (s, s, 1H), 7.42−7.39 (m, 2H), 7.28 (br d, $^1J_{PH}$ = 390 Hz, 1H), 7.31−7.17 (m, 7H), 6.79−6.74 (m, 4H), 6.44−6.38 (m, 1H), 5.23−5.11 (m, 1H), 4.80, 4.79 (t, J = 6.6 Hz, t, J = 6.6 Hz, 2H), 4.40−4.35 (m, 1H), 3.77, 3.77 (s, s, 6H), 3.38, 3.36 (s, s, 2H), 3.06, 3.04 (t, J = 6.6 Hz, t, J = 6.6 Hz, 2H), 3.01−2.83 (m, 9H), 2.76−2.66 (m, 2H), 1.23−1.14 (m, 18H), 1.02−0.07 (br, 3H); ^{13}C NMR ($CDCl_3$) δ 175.4, 159.4, 158.3, 152.7, 152.6, 151.5, 151.5, 144.5, 144.4, 140.8, 140.7, 135.6, 135.5, 135.5, 135.5, 129.8, 127.9, 127.7, 127.6, 126.7, 118.0, 118.0, 116.9, 112.9, 112.9, 86.2, 86.1, 85.7, 85.6, 85.4, 85.3, 84.4, 84.3, 76.1, 76.0, 75.2, 75.1, 63.4, 63.3, 61.4, 55.0, 45.2, 39.6, 38.8, 35.6, 29.5, 29.1, 19.1, 19.0, 17.9, 8.5; ^{31}P NMR ($CDCl_3$) 105.1−102.1 (m). ESI-HRMS: Calcd for $C_{38}H_{43}BN_6O_8P^-$ $[M - H]^-$ 753.2979, found 753.2986 .

Triethylammonium (1*R*,3*R*,4*R*,7*S*)-3-(*N*^{3}-Benzoylthymin-1-yl)-1-dimethoxytrityloxymethyl-2,5-dioxabicyclo[2.2.1]-heptane-7-*H*-boranophosphonate As a Mixture of (*Sp*)- and (*Rp*)-Diastereomers (3e). Compound **3e** was obtained in 75% (0.65 g, 0.75 mmol) from (1*R*,3*R*,4*R*,7*S*)-3-(*N*^{3}-benzoylthymin-1-yl)-1-dimethoxytrityloxymethyl-7-hydroxy-2,5-dioxabicyclo[2.2.1]heptanes **1e** (0.69 g, 1.0 mmol) and **2** (0.30 g, 2.0 mmol) as a colorless foam in a manner similar to the synthesis of **3b**, except for the reaction time (20 min) and the conditions for silica gel column chromatography [3.7 × 15.8 cm, 85 g of silica gel (spherical, neutral, 63-210 μm), ethyl acetate−hexane−Et_3N (67:33:0.5, v/v/v) to CH_2Cl_2−MeOH−Et_3N (99.5:0.5:0.5, v/v/v)]. ^{1}H NMR ($CDCl_3$) δ 8.00−7.93 (m, 2H), 7.83, 7.81 (d, J = 1.2 Hz, d, J = 0.9 Hz, 1H), 7.67−7.61 (m, 1H), 7.53−7.46 (m, 4H), 7.41−7.30 (m, 6H), 7.43, 7.36 (br d, $^1J_{PH}$ = 385 Hz, br d, $^1J_{PH}$ = 410 Hz, 1H), 7.24−7.21 (m, 1H), 6.89−6.85 (m, 4H), 5.63 (s, 1H), 4.92, 4.66 (d, J = 6.6 Hz, d, J = 6.6 Hz, 1H), 4.67, 4.63 (s, s, 1H), 4.00−3.72 (m, 8H), 3.57−3.44 (m, 2H), 2.90 (q, J = 7.2 Hz, 7.5H), 1.68, 1.66 (d, J = 0.9 Hz, d, J = 0.9 Hz, 3H), 1.21 (t, J = 7.2 Hz, 11H), 1.01−0.07 (br, 3H); ^{13}C NMR ($CDCl_3$) δ 168.8, 162.8, 158.5, 148.7, 148.7, 144.4, 144.2, 135.3, 135.3, 135.1, 135.0, 134.9, 134.4, 134.3, 131.3, 131.2, 130.5, 130.4, 130.0, 129.9, 129.8, 129.1, 129.1, 128.0, 128.0, 127.9, 127.8, 126.9, 113.3, 113.2, 110.3, 110.3, 88.1, 88.0, 87.9, 87.4, 86.5, 78.6, 78.5, 74.6, 74.4, 72.2, 72.1, 71.5, 57.8, 57.5, 55.1, 45.3, 12.6, 12.5, 8.4; ^{31}P NMR ($CDCl_3$) δ 107.5 (m). ESI-HRMS: Calcd for $C_{39}H_{39}BN_2O_{10}P^-$ $[M - H]^-$ 737.2441, found 737.2443.

A General Procedure for the Manual Solid-Phase Synthesis of *PBH*-ODN 2mers (for the synthesis of *PBO*- and *PBS*-ODN 2mers). CPG loaded with 5′-*O*-DMTr-*N*^{3}-benzoylthymidine *via* a succinate linker (0.5 μmol) was treated with 3% dichloroacetic acid (DCA) in dry CH_2Cl_2 (4 × 15 s), washed with dry CH_2Cl_2 and CH_3CN, and then dried *in vacuo* for 10 min. Condensation reaction of the corresponding 2′-deoxyribonucleoside 3′-*H*-boranophosphonate monomer units **3a−d** (0.1 M) was carried out in the presence of 1,3-dimethyl-2-(3-nitro-1,2,4-triazol-1-yl)-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (MNTP) (0.25 M) and 2,6-lutidine (0.6 M) in dry CH_3CN for 1 min under argon. The solid support was then washed with dry CH_3CN and CH_2Cl_2. The 5′-*O*-DMTr group of the resultant *PBH*-ODN 2mer was removed by treatment with 3% DCA in CH_2Cl_2−Et_3SiH (1:1,v/v) (6 × 5 s), and the solid support was washed with dry CH_2Cl_2 and CH_3CN and dried *in vacuo* for 10 min. The resultant *PBH*-ODN 2mer was oxidized or sulfurized as described below.

Synthesis of *PBO*-ODN 2mers (6a−d) (Table 2, entries 1−4). *PBH*-ODN 2mer generated on the CPG as above was oxidized with *i*-Pr_2NEt (0.3 M), CCl_4 (1 M) and H_2O (0.5 M) in dry CH_3CN at rt for 30 min under argon, and the CPG was successively washed with dry CH_3CN and CH_2Cl_2. The CPG was then treated with 25% NH_3 aq−EtOH (3:1, v/v) at 30 °C for 3 h (for $T_{PBO}T$ **6a**), at 30 °C for 12 h (for $A_{PBO}T$ **6b** and $C_{PBO}T$ **6c**), or at 50 °C for 12 h (for $G_{PBO}T$ **6d**), filtered, and washed with EtOH (4 mL). The filtrate and washings were combined and concentrated to dryness under reduced pressure, and the residue was analyzed by RP-HPLC and MALDI-TOF MS. RP-HPLC was performed with a linear gradient of 0−30% CH_3CN in 0.1 M triethylammonium acetate (TEAA) buffer (pH 7) for 60 min at 30 °C at a flow rate of 0.5 mL/min using a column of C_{18}. MALDI-TOF MS: Calcd for $T_{PBO}T$ (**6a**) $[M - H]^-$ 543.17, found 543.51. Calcd for $dA_{PBO}T$ (**6b**) $[M - H]^-$ 552.18, found 552.21. Calcd for $dC_{PBO}T$ (**6c**) $[M - H]^-$ 528.17, found 528.10. Calcd for $dG_{PBO}T$ (**6d**) $[M - H]^-$ 568.17, found 568.10.

Synthesis of *PBS*-ODN 2mers (7a−d) (Table 2, entries 5−8). *PBH*-ODN 2mer generated on CPG as above was sulfurized with *i*-Pr_2NEt (1 M) and dimethylthiuram disulfide (DTD) (0.3 M) in dry CH_3CN at rt for 1 h under argon, and the CPG was successively washed with dry CH_3CN and CH_2Cl_2. The CPG was then treated with 25% NH_3 aq at 30 °C for 3 h (for $T_{PBS}T$ **7a**), at 30 °C for 12 h (for $A_{PBO}T$ **7b** and $C_{PBO}T$ **7c**), or at 50 °C for 12 h (for $G_{PBO}T$ **7d**), filtered, and washed with EtOH (1 mL). The filtrate and washings were combined and concentrated to dryness under reduced pressure, and the residue was analyzed by RP-HPLC and MALDI-TOF MS. RP-HPLC was performed with a linear gradient of 0−30% CH_3CN in 0.1 M TEAA buffer (pH 7) for 60 min at 30 °C at a flow rate of 0.5 mL/min using a column of C_{18}. MALDI-TOF MS: Calcd for $T_{PBS}T$ (**7a**) $[M - H]^-$ 559.14, found 559.45. Calcd for $dA_{PBS}T$ (**7b**) $[M - H]^-$ 568.16, found 568.68. Calcd for $dC_{PBS}T$ (**7c**) $[M - H]^-$ 544.14, found 544.09. Calcd for $dG_{PBS}T$ (**7d**) $[M - H]^-$ 584.15, found 584.53.

Synthesis of $T_{PBN}T$ (8) (Table 2, entry 10). 5′-*O*-DMTr-$T_{PBH}T$ (Scheme 2, **5**, n = 0) was synthesized on the CPG as described above. The $T_{PBH}T$ was treated with CCl_4 (1 M) and 4-(2-aminoethyl)-morpholine (0.5 M) in dry CH_3CN at rt for 30 min under argon, and the CPG was then successively washed with dry CH_3CN and CH_2Cl_2. The 5′-*O*-DMTr group was removed by treatment with 3% DCA in CH_2Cl_2−Et_3SiH (1:1, v/v) (6 × 5 s), and the CPG was washed with dry CH_2Cl_2. The CPG was then treated with 25% NH_3 aq−EtOH (3:1, v/v) at 30 °C for 1 h, filtered, and washed with EtOH (4 × 1 mL). The filtrate and washings were combined and concentrated to dryness under reduced pressure, and the residue was analyzed by RP-HPLC and MALDI-TOF MS. RP-HPLC was performed with a linear gradient of 0−45% CH_3CN in 0.1 M TEAA buffer (pH 7) for 60 min at 30 °C at a flow rate of 0.5 mL/min using a column of C_{18}. MALDI-TOF MS: Calcd for $[M + H]^+$ 657.28, found 657.31.

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Synthesis of 2′-Deoxy *PBO* and *PBS*-ODN 12mers. CPG loaded with 5′-*O*-DMTr-N^3-benzoylthymidine or 5′-*O*-DMTr-N^4-isobutyrylcytidine (0.5 μmol) *via* a succinate linker was treated with 3% DCA in dry CH_2Cl_2 (4 × 15 s), washed with dry CH_2Cl_2 and CH_3CN, and dried *in vacuo* for 10 min. Chain elongation was performed by repeating 11 times the following steps (a) and (b). (a) Condensation of the corresponding nucleoside 3′-*H*-boranophosphonate monomer units **3a–d** (0.1 M) in the presence of MNTP (0.25 M) and 2,6-lutidine (0.6 M) in dry CH_3CN for 1 min under argon and subsequent washing with dry CH_3CN and CH_2Cl_2. (b) Removal of 5′-*O*-DMTr group by treatment with 3% DCA in CH_2Cl_2–Et_3SiH (1:1, v/v) (6 × 5 s), subsequent washing with dry CH_2Cl_2 and CH_3CN, and drying *in vacuo* for 5 min. The resultant *PBH*-ODN 12mers were oxidized or sulfurized as described above. After the conversions, the CPG was treated with 25% NH_3 aq–EtOH (3:1, v/v) at 30 °C for 12 h (for $T(_{PBO}T)_{11}$ (**9**)), 25% NH_3 aq at 30 °C for 12 h (for $T(_{PBS}T)_{11}$ (**10**)), 25% NH_3 aq–EtOH (3:1, v/v) at 50 °C for 12 h (for *PBO*-ODN 12mer (5′-GCA TTG GTA TTC-3′) (**12**)) and 25% NH_3 aq at 50 °C for 12 h (for *PBS*-ODN 12mer (5′-GCA TTG GTA TTC-3′) (**13**)). Then, the CPG was filtered and washed with EtOH (4 × 1 mL). The filtrate and washings were combined and concentrated to dryness under reduced pressure. The residue was dissolved in H_2O (4 mL) and washed with $CHCl_3$ (6 × 500 μL). The aqueous layer was analyzed by RP-HPLC and MALDI-TOF MS. RP-HPLC analysis was performed with a linear gradient of 0–60% CH_3CN in 0.1 M TEAA buffer (pH 7) for 60 min at 30 °C at a flow rate of 0.5 mL/min using a column of C_{18}. Purification by RP-HPLC was performed with a linear gradient of 8–44% CH_3CN in 0.1 M TEAA buffer (pH 7) for 90 min at rt at a flow rate of 1 mL/min using a column of C_{18}. Isolated yields: 28% ($T(_{PBO}T)_{11}$ (**9**)); 31% ($T(_{PBS}T)_{11}$ (**10**)); 44% (*PBO*-ODN 12mer (5′-GCA TTG GTA TTC-3′) (**12**)); 16% (*PBS*-ODN 12mer (5′-GCA TTG GTA TTC-3′) (**13**)). MALDI-TOF MS: Calcd for $T(_{PBO}T)_{11}$ (**9**) $[M - H]^-$ 3564.01, found 3564.17; Calcd for $T(_{PBS}T)_{11}$ (**10**) $[M - H]^-$ 3739.75, found 3739.05; Calcd for *PBO*-ODN 12mer (5′-GCA TTG GTA TTC-3′) (**12**) $[M - H]^-$ 3627.05, found 3626.82; Calcd for *PBS*-ODN 12mer (5′-GCA TTG GTA TTC-3′) (**13**) $[M - H]^-$ 3802.80, found 3801.52.

Synthesis of ^{LNA}*PBO*-ODN 12mer (5′-GCA T^LT^LG GT^LA T^LT^LC-3′) (14). ^{LNA}*PBH*-ODN 12mer (5′-GCA $\mathbf{T^LT^L}$G $\mathbf{GT^L}$A $\mathbf{T^LT^L}$C-3′) synthesized on CPG as above was oxidized[25] with Et_3N (0.1 M) in CCl_4–2,6-lutidine–H_2O (5:12.5:1, v/v/v) at rt for 1 h under argon, and the CPG was successively washed with dry CH_3CN and CH_2Cl_2. The CPG was then treated with 25% NH_3 aq–EtOH (3:1, v/v) at 50 °C for 12 h, filtered, and washed with EtOH (4 × 1 mL). The filtrate and washings were combined and concentrated to dryness under reduced pressure. The residue was dissolved in H_2O (4 mL) and washed with $CHCl_3$ (6 × 500 μL). The aqueous layer was then analyzed by RP-HPLC, ion-exchange HPLC (IE-HPLC) and MALDI-TOF MS. RP-HPLC analysis was performed with a linear gradient of 0–60% CH_3CN in 0.1 M TEAA buffer (pH 7) for 60 min at 30 °C at a flow rate of 0.5 mL/min using a column of C_{18}. IE-HPLC analysis and purification were performed with a linear gradient of 0–1 M NaCl, 50–25% CH_3CN in 10 mM Tris–HCl buffer (pH 8) for 20 min at rt at a flow rate of 0.8 mL/min using a column of quaternary ammonium anion exchange resin. Fractions containing **14** (see Supporting Information, page S10, *Figure S9* (b), fraction D) were combined and concentrated to dryness under reduced pressure and desalted with a short cartridge of C_{18}. The cartridge was washed with H_2O (6 × 5 mL), and then the desired product was eluted with 50% CH_3CN. Isolated yield: 7%. MALDI-TOF MS: Calcd for $[M - H]^-$ 3767.02, found 3767.06.

$T(_{PBN}T)_{11}$ (11). 5′-*O*-DMTr-$T(_{PBH}T)_{11}$ (Scheme 2, **5**, n = 10) synthesized by repeating condensation and detritylation was aminated as well as $T_{PBN}T$ **8**. The 5′-*O*-DMTr group was removed by treatment with 3% DCA in CH_2Cl_2–Et_3SiH (1:1, v/v) (6 × 5 s) and washed with dry CH_2Cl_2. The CPG was treated with 25% NH_3 aq–EtOH (3:1, v/v) at 30 °C for 1 h, filtered, and washed with EtOH (4 × 1 mL). The filtrate and washings were combined and concentrated to dryness under reduced pressure. The residue was then analyzed by RP-HPLC and MALDI-TOF MS. RP-HPLC analysis and purification were performed with a linear gradient of 0–60% CH_3CN in 0.1 M TEAA buffer (pH 7) for 60 min at 30 °C at a flow rate of 0.5 mL/min using a column of C_{18}. Isolated yield: 10%. MALDI-TOF MS: Calcd for $[M + H]^+$ 4799.12, found 4799.43.

Thermal Denaturation Studies (for natural, *PS*, *PBO*, *PBS*, ^{LNA}*PO*, ^{LNA} *PS*, and ^{LNA}*PBO*-ODN). Pairs of complementary strands (1:1 molar ratio, 4.0 μM, 0.6 nmol each) were dissolved in 10 mM NaH_2PO_4–Na_2HPO_4 buffer solutions (pH 7.0) containing 0.1 or 1 M NaCl. The solutions were heated for 10 min at 90 °C or for 15 min at 95 °C and cooled to 0 °C at a rate of −0.5 °C/min, and then left at 0 °C for 1 h. Denaturation studies were carried out in a 1 cm path length quartz cell. UV absorbance values (260 nm) were recorded at a rate of 0.5 °C/min from 0 to 90 °C or 100 °C.

Thermal Denaturation Studies (for *PBN*-ODN). Pairs of complementary strands (1:1 molar ratio, 4.0 μM, 0.6 nmol each) were dissolved in (a) 10 mM NaH_2PO_4–Na_2HPO_4 buffer solutions (pH 7.0) containing 0, 0.1, or 1 M NaCl, (b) 10 mM NaH_2PO_4–Na_2HPO_4 (pH 5.8) without NaCl or (c) 10 mM NaH_2PO_4 (pH 5.0) without NaCl. The solutions were heated for 10 min at 90 °C and cooled to 0 °C at a rate of −0.5 °C/min, and then left at 0 °C for 1 h. Denaturation studies were carried out in a 1 cm path length quartz cell. UV absorbance values (260 nm) were recorded at a rate of 0.5 °C/min from 0 to 90 °C.

■ ASSOCIATED CONTENT

Supporting Information

^1H, ^{13}C, and ^{31}P NMR spectra, HPLC profiles, and UV melting curves. 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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