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Stereocontrolled Solid-Phase Synthesis of Phosphate/Phosphorothioate (PO/PS) Chimeric Oligodeoxyribonucleotides on an Automated Synthesizer Using an Oxazaphospholidine–Phosphoramidite Method

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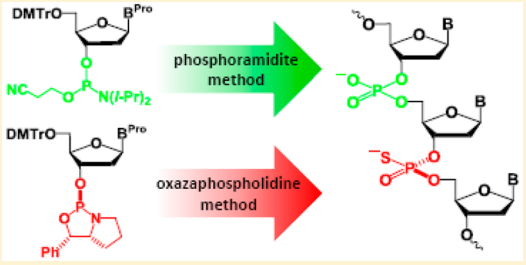
Supporting Information

ABSTRACT: Stereocontrolled solid-phase synthesis of phosphate/phosphorothioate chimeric oligodeoxyribonucleotides (PO/PS-ODNs) was achieved by integrating the conventional phosphoramidite method into a previously developed oxazaphospholidine method for the stereocontrolled synthesis of *P*-chiral oligonucleotides. *P*-Stereodefined PO/PS-ODNs with mixed sequences (up to 12-mers) were obtained in good yields and high stereoselectivities by reacting different combinations of monomers (conventional phosphoramidites/diastereopure nucleoside 3′-*O*-oxazaphospholidines), activators (ETT/CMPT), capping reagents (Pac_2O/CF_3COIm), and oxidizing/sulfurizing reagents (TBHP/POS) on an automated synthesizer. A thermal denaturation study examined the resultant diastereopure PO/PS-ODN 12-mers with three consecutive (*Rp*)- or (*Sp*)-PS-linkages at the internal or terminal regions of the molecules. We found that (*Rp*)-PO/PS-ODNs can only moderately destabilize duplexes with complementary oligoribonucleotides (ORNs) compared with their unmodified ODN counterparts (ΔT_m = −0.4 °C per modification). In contrast, (*Sp*)-PO/PS-ODNs have larger destabilizing effects (ΔT_m = −1.2 to −0.8 °C per modification). Although smaller destabilizing effects were observed when the (*Sp*)-PS-linkages were incorporated into the terminal regions of the molecule, there was a weaker correlation between the location of an incorporated (*Rp*)-PS-linkage and its destabilizing effect.



INTRODUCTION

In phosphorothioate oligodeoxyribonucleotides (PS-ODNs), a sulfur atom replaces a nonbridging oxygen atom on each phosphodiester linkage of an oligodeoxyribonucleotide (ODN). PS-ODNs are widely used as key structures in therapeutic oligonucleotides because of their enhanced stability in the presence of nucleases, cell-membrane permeability, and favorable pharmacokinetics.[1] However, PS-ODNs with fully modified phosphorothioate backbones suffer from certain therapeutic drawbacks such as cytotoxicity at high concentrations and low affinities for complementary RNAs (in comparison with unmodified ODNs).[1,2] Phosphate/phosphorothioate chimeric oligodeoxyribonucleotide (PO/PS-ODN) structures have emerged as a promising candidate for therapeutic oligonucleotides because these compounds overcome the deficiencies that are commonly observed in PS-ODNs.[2,3]

The PS-linkages in PO/PS-ODNs possess a chiral center at each phosphorus atom; certain biological and physicochemical properties such as their affinity for complementary RNAs, stability to nucleases, RNase H activity, and gene silencing potency are potentially dependent on the stereochemical configurations of these phosphorus atoms.[1d,4−9] It is therefore critical to develop an efficient method for the synthesis of *P*-stereodefined PO/PS-ODNs. Although PO/PS-ODNs with one stereodefined PS-linkage at a specific position can be effectively isolated by a chromatographic separation of diastereomixtures,[5] this method is generally inapplicable to the isolation of PO/PS-ODNs with multiple stereodefined PS-linkages. Diastereopure dimer building blocks have previously been used to incorporate multiple stereodefined PS-linkages into ODNs;[6] however, this method requires up to 32 different types of building blocks and is inapplicable to the preparation of PO/PS-ODNs with consecutive stereodefined PS-linkages. The stereocontrolled synthesis of PO/PS-ODNs with multiple stereodefined PS-linkages at either consecutive or discrete positions has been achieved with the oxathiaphospholane method,[7,10] which uses nucleoside 3′-*O*-oxathiaphospholane monomers to synthesize both unmodified PO-linkages and stereodefined PS-linkages.[10c] A recent report has demonstrated that the oxathiaphospholane method can be combined with the conventional phosphoramidite method[11] to synthesize *P*-stereodefined PO/PS-ODNs.[10e] However, the chromatographic

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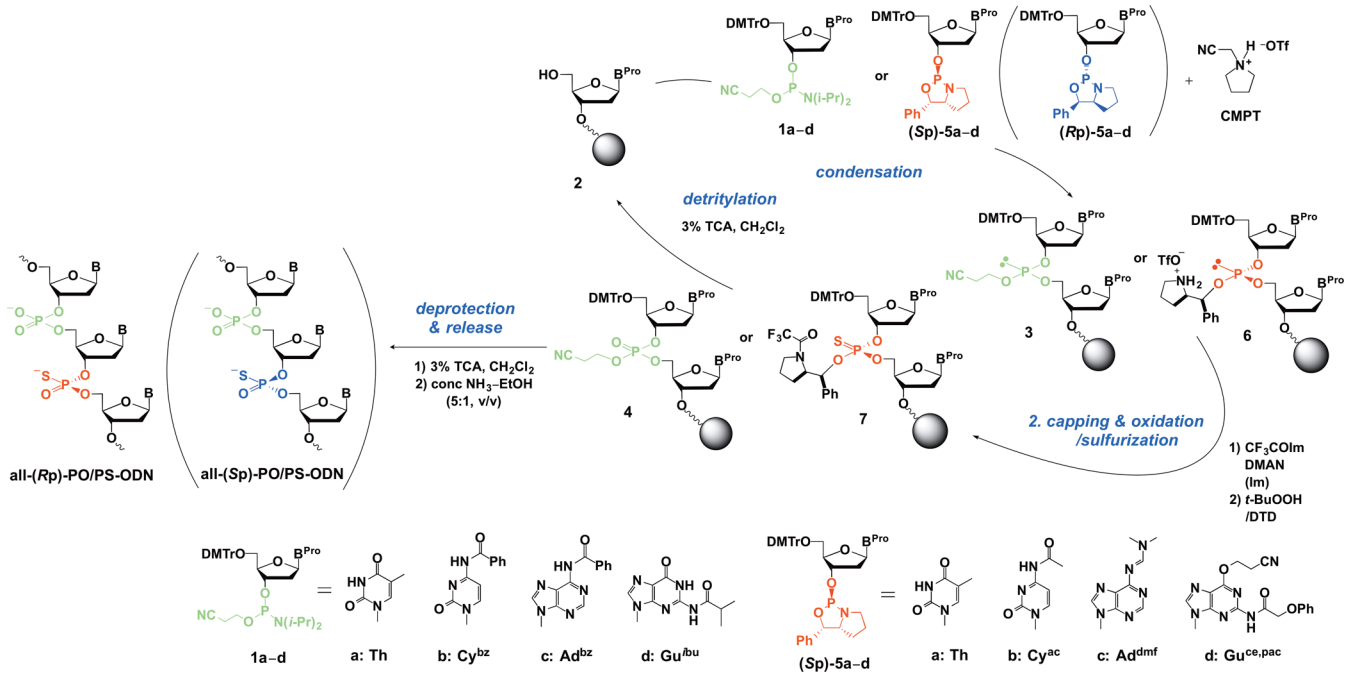
Scheme 1. Synthetic Cycle for *P*-Stereodefined PO/PS-ODNs with the Activator CMPT



separation of the (*Rp*)- and (*Sp*)-monomers from a ca. 1:1 mixture of diastereomers is required for this method.

We have previously developed a method for synthesizing stereocontrolled *P*-chiral oligonucleotides using nucleoside 3′-*O*-oxazaphospholidine derivatives as monomers (the oxazaphospholidine method).[12] The oxazaphospholidine monomers can be stereoselectively synthesized from enantiopure 1,2-amino alcohols as chiral auxiliaries with d.r. > 99:1. Various *P*-chiral oligonucleotides such as PS-ODNs can be stereoselectively synthesized from these monomers. In this study, we developed a novel method for the stereocontrolled synthesis of PO/PS-ODNs by combining the oxazaphospholidine method with the conventional phosphoramidite method. During the course of our study, Wan et al. reported that our oxazaphospholidine method could be combined with the phosphoramidite method to synthesize "gapmer" antisense oligonucleotides with stereodefined PS-linkages located at the internal gap regions.[13] In this paper, we describe the stereocontrolled synthesis of PO/PS-ODNs with mixed sequences (up to 12-mers) that contain three consecutive (*Rp*)- or (*Sp*)-PS-linkages at either the internal or terminal regions using the oxazaphospholidine−phosphoramidite method. A thermal denaturation study of the resultant diastereopure PO/PS-ODNs revealed the varying regional effects of the stereodefined PS-linkages on the duplex formation of ODNs with complementary ORNs.

RESULTS AND DISCUSSION

Stereocontrolled Solid-Phase Synthesis of PO/PS-ODNs Using *N*-(Cyanomethyl)pyrrolidinium Triflate (CMPT) as an Activator in Both Phosphoramidite and Oxazaphospholidine Cycles. We first attempted to synthesize *P*-stereodefined PO/PS-ODNs by incorporating the phosphoramidite monomers into the synthetic cycle of the oxazaphospholidine method (Scheme 1). The commercially available **1a−d** or the oxazaphospholidine monomers **5a−d** prepared according to ref 12c are condensed with the 5′-OH of a nucleoside **2** that is anchored to a highly cross-linked polystyrene (HCP). This is accomplished in the presence of the activator *N*-(cyanomethyl)pyrrolidinium triflate (CMPT)[12b] for both types of monomers to generate the phosphite intermediate **3** or **6**. The unreacted 5′-OH and the secondary amino group of the resultant phosphite intermediates are capped with 1-(trifluoroacetyl)imidazole and 1,8-bis(dimethylamino)naphthalene (DMAN).[12c] The phosphorus atoms of **3** and **6** are then oxidized and sulfurized with *tert*-butyl hydroperoxide (TBHP)[14] and *N*,*N*′-dimethylthiuram disulfide (DTD),[15] respectively, to give the corresponding phosphate and phosphorothioate triesters **4** and **7**. Finally, the resulting molecules are treated with 3% trichloroacetic acid (TCA) in CH_2Cl_2 to remove the 5′-*O*-(4,4′-dimethoxytrithyl) (DMTr) group. After this cycle is repeated, the resultant oligomers are deprotected and cleaved from the solid support with a blend of concentrated aqueous NH_3 and EtOH (5:1, v/v) at 55 °C to yield the *P*-stereodefined PO/PS-ODNs.

We initially investigated the application of phosphoramidite monomers to this cycle by synthesizing unmodified ODNs. However, $DMTr^+$ assays showed that the coupling efficiency was over 100% when the chain was elongated; this unusual phenomenon was further examined by the synthesis of unmodified ODN 5-mers d(NNNNT) (N = T, C, A, G) **8**−**11**. The $DMTr^+$ assay was used to compare the results for the third and fourth couplings. These unusual increases were dependent on the number of thymidine residues in the ODNs, as shown in Table 1. The value of the $DMTr^+$ assay increased to 173% for homothymidylate **8** (entry 1) and remained within the range of 110%−120% for ODNs **9**−**11** that possessed only one thymidine residue (entries 2−4). The $DMTr^+$ assay increased by 10%−20% for every thymidine residue; this was attributed to the O^4-phosphitylation of the thymine bases. Although the *O*-phosphitylation of thymine and guanine can occur during the synthesis of nucleic acids,[16a,b] the resultant *O*-phosphites cannot survive under conventional coupling conditions because of attack by highly nucleophilic activators

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Table 1. Solid-Phase Synthesis of Unmodified ODN 5-mers 8−11 Using CMPT as the Activator

			coupling yield (%)[a]	
entry	PO/PS-ODN[a]	Im (M)	third	fourth
1	d(TTTTT) **8**	0	143	173
2	d(CCCCT) **9**	0	116	115
3	d(AAAAT) **10**	0	117	120
4	d(GGGGT) **11**	0	114	110
5	d(TTTTT) **8**	0.05	128	141
6	d(TTTTT) **8**	0.1	106	110
7	d(TTTTT) **8**	0.2	101	99
8	d(TTTTT) **8**	0.3	99	100

[a]Determined by $DMTr^+$ assay.

(e.g., 1*H*-tetrazole). We reasoned that the low nucleophilicity of CMPT enabled the O^4-phosphites in this study to survive.[16c] To confirm the presence of the O^4-phosphite, we performed ^{31}P NMR analysis on the condensation reaction of **1a** with 3′-*O*-DMTr-thymidine **12** in the presence of CMPT (Scheme 2). The spectrum showed minor signals around δ 130 ppm, which were in the same range as the signals of O^6-phosphitylguanines reported in the literature.[16b] In addition, intense signals around δ 140 ppm corresponded to the phosphite triester, which indicated that O^4-phosphites were present as minor products.[17]

To eliminate byproducts, 0.05−0.3 M imidazole was added to the capping reagents. We expected the reagent to behave as a nucleophile and remove the O^4-phosphites.[18] Entries 5−8 in Table 1 show that the O^4-phosphites were completely removed in the presence of 0.2−0.3 M imidazole. These results encouraged us to synthesize homothymidylate 8-mers with one or two stereodefined PS-linkages (**14a**, **14b**, and **15** in Table 2) based on the synthetic strategy shown in Scheme 1. The O^4-phosphites were removed by adding 0.3 M imidazole to the capping reagents. TBHP was selected as the oxidizing agent on the basis of previous reports in the literature that demonstrated its compatibility with phosphorothioate triesters.[19,20] $DMTr^+$ assays revealed that the average coupling yields were 98%−99%. Reversed-phase HPLC (RP-HPLC) analyses showed that PO/PS-ODNs had been synthesized in a highly stereocontrolled manner without any significant byproducts (Figure 1) and that the targeted products were isolated in diastereopure forms (Figure 2). The diastereopurity of **15** was also confirmed by enzymatic digestion using an experimental process that is described below.

Stereocontrolled Solid-Phase Synthesis of PO/PS-ODNs Using Different Activators in the Phosphoramidite and Oxazaphospholidine Cycles. The previous section described the successful syntheses of *P*-stereodefined PO/PS-ODNs via CMPT-promoted condensation of the phosphoramidite and oxazaphospholidine monomers and cleavage of the O^4-phosphites with imidazole. However, in the case of ODNs that contained multiple thymines, we had anticipated that the byproducts of O^4-phosphitylation of the thymine bases would not be completely eliminated with imidazole. We thus investigated the synthesis of *P*-stereodefined PO/PS-ODNs with a conventional, highly nucleophilic azole as the activator to prevent O^4-phosphitylation of the phosphoramidite monomers. This method is depicted in Scheme 3, in which two different synthetic cycles employ 5-ethylthio-1*H*-tetrazole (ETT)[21] and CMPT as activators for the phosphoramidite and oxazaphospholidine monomers, respectively. We had predicted that the O^4-phosphites on the thymine residues would decompose during the condensation step with ETT. The "PO cycle" follows the same steps as the conventional phosphoramidite method: (1) condensation of the phosphoramidites **1a−d** with the 5′-OH of a nucleoside on the solid-support **2** in the presence of ETT to produce phosphite triester intermediates **3**; (2) capping of the unreacted 5′-OH with Pac_2O and *N*-methylimidazole (NMI), followed by oxidation of the phosphite triesters with TBHP to phosphate triesters **4**; and (3) 5′-detritylation by 3% TCA to regenerate 5′-OH. The "PS cycle," developed for the stereocontrolled synthesis of PS-ODNs,[12c] consists of the following three steps: (1) condensation of the diastereopure nucleoside 3′-*O*-oxazaphospholidine monomers (*Rp*)- or (*Sp*)-**5a−d** with **2** in the presence of CMPT to produce stereospecific phosphite triesters **6**; (2) capping of the unreacted 5′-OH and the secondary amino group of the resultant phosphite intermediates **6** with CF_3COIm and NMI, followed by transformation into the phosphorothioate triester intermediates **7** via sulfurization with 3-phenyl-1,2,4-dithiazoline-5-one (POS);[22] and (3) 5′-detritylation. *P*-Stereodefined PO/PS-ODNs are synthesized by switching between these two cycles. The deprotection and release of the resultant oligomers from the solid support are accomplished by treatment with a blend of concentrated aqueous NH_3 and EtOH (5:1, v/v). *P*-Stereodefined PO/PS-ODNs are then obtained from purification by RP-HPLC.

We first synthesized four different types of PO/PS-ODN 8-mers (**16a**, **16b**, **17a**, and **17b**) that possessed either an (*Rp*)- or an (*Sp*)-PS-linkage (Table 3, entries 1−4). RP-HPLC analyses showed that all the 8-mers were produced with high coupling efficiency and diastereopurity.[17] PO/PS-ODNs were isolated by RP-HPLC in good yields (29%−62%). We were further motivated to synthesize PO/PS-ODN 12-mers (**18a**, **18b**, **19a**, **19b**, **20a**, and **20b**) with three consecutive (*Sp*)- or (*Rp*)-PS-linkages at the internal or terminal regions of the molecule (Table 3, entries 5−10). The RP-HPLC profiles of the crude products (Figure 3) showed that ODNs were efficiently synthesized with a high stereospecificity. The desired 12-mers **18−20** were isolated as major products with good yields (28%−48%) (Figure 4). MALDI-TOF-MS analysis of the

Scheme 2. O^4-Phosphitylation of Thymine in the Presence of CMPT

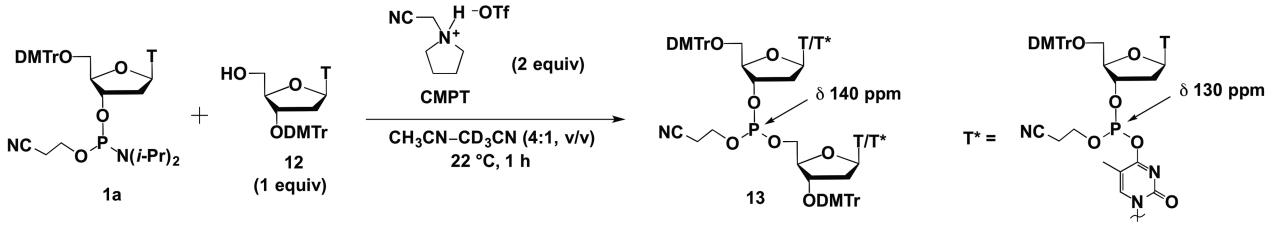

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Table 2. *P*-Stereodefined PO/PS-ODNs 14 and 15, Which Were Synthesized According to Scheme 1

entry	PO/PS-ODN[a]	O.D.[b]	isolated yield (%)	MALDI-TOF-MS calcd.	MALDI-TOF-MS found
1	(*Rp*)-d(TTTT$_S$TTTT) **14a**	20	60	2385.4	2386.0
2	(*Sp*)-d(TTTT$_S$TTTT) **14b**	20	60	2385.4	2386.2
3	all-(*Sp*)-d(TTTTTT$_S$T$_S$T) **15**	18	54	2401.4	2400.0

[a]Subscript "S" = phosphorothioate diester. [b]Overall materials (O.D. = optical density units) measured at 260 nm UV absorption.

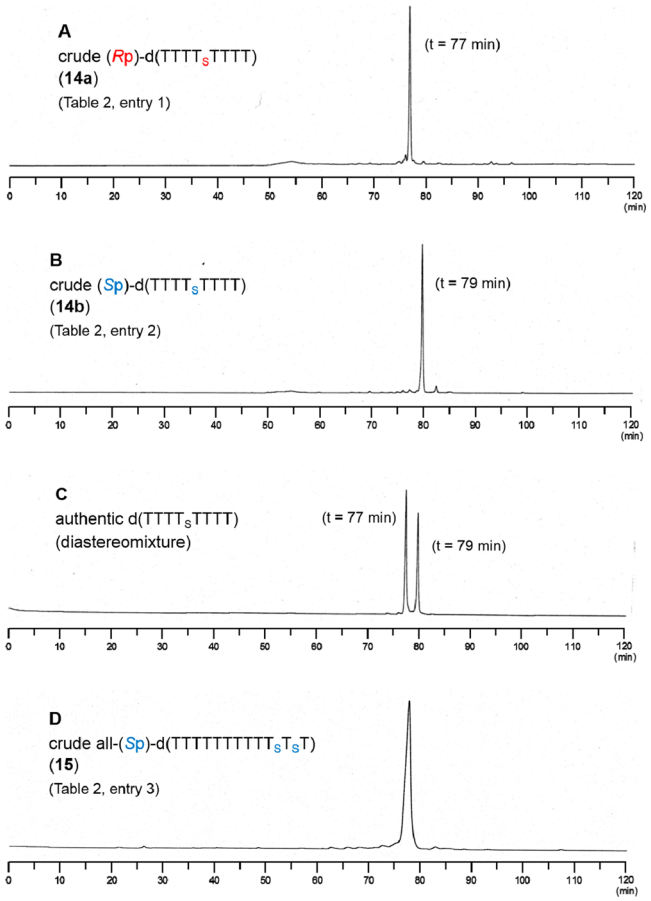

Figure 1. RP-HPLC profiles of crude *P*-stereodefined PO/PS-ODNs: (A) **14a**; (B) **14b**; (C) (*Rp*/*Sp*)-d(TTTT$_S$TTTT), the product of a nonstereoselective synthesis (shown as a reference); and (D) **15**. RP-HPLC was performed with a linear gradient of 0%−20% CH_3CN in 0.1 M TEAA buffer (pH 7.0) at 30 °C for 60 min at a rate of 0.5 mL/min.

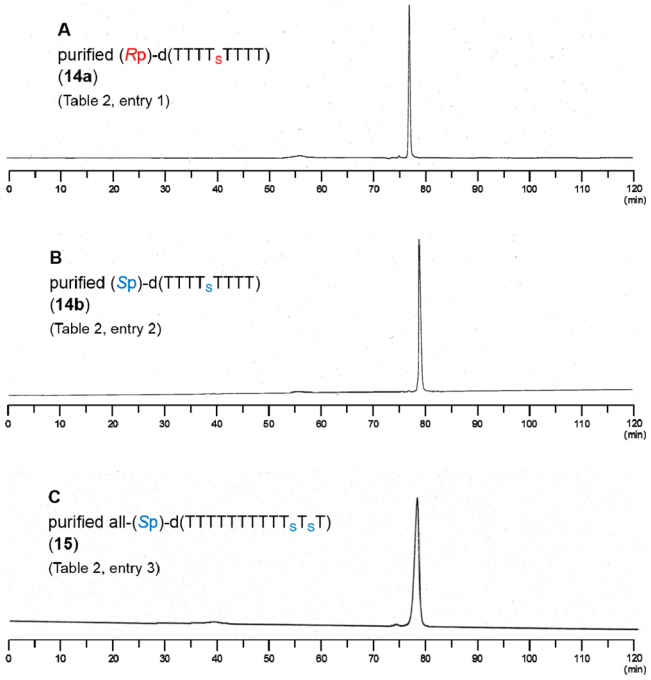

Figure 2. RP-HPLC profiles of purified *P*-stereodefined PO/PS-ODNs: (A) **14a**, (B) **14b**, and (C) **15**. RP-HPLC was performed with a linear gradient of 0%−20% CH_3CN in 0.1 M TEAA buffer (pH 7.0) at 30 °C for 60 min at a rate of 0.5 mL/min.

resultant oligomers showed that none of the PS-linkages were desulfurized during the synthetic process.[17]

Enzymatic Digestion of *P*-Stereodefined PO/PS-ODNs. PO/PS-ODNs (**15**, **18a**, **18b**, **19a**, **19b**, **20a**, and **20b**) were digested by nuclease P1[23] (nP1) (*Sp*-specific) and snake venom phosphodiesterase (svPDE)[24] (*Rp*-specific) to confirm the stereochemical configurations and diastereopurities of the products. PO/PS-ODNs were analyzed by RP-HPLC after an incubation period of 12 h at 37 °C with nP1 or svPDE (Figures 6 and 7). To reduce any complications within RP-HPLC analyses, alkaline phosphatase was also added to the digestion mixtures to ensure that 5′-phosphorylated PS-d(TTT) or PS-d(CAGT) would not be generated (**19a**/**20a** + nP1 and **15**/**19b**/**20b** + svPDE). Figure 5A and Figure 6D−F show that all-(*Sp*)-PO/PS-ODNs (**15**, **18b**, **19b**, and **20b**) were completely digested with nP1. All-(*Rp*)-PS-d(CAGT) remained undigested when all-(*Rp*)-PO/PS-ODNs (**18a**, **19a**, and **20a**) were treated with nP1 (+ alkaline phosphatase) (Figures 6A−C). The ratios of the digestion products obtained from **19a** and **20a** (four kinds of 2′-deoxyribonucleosides and all-(*Rp*)-PS-d(CAGT)) were in agreement with the calculated values (dC:dG:dT:dA:all-(*Rp*)-d(CAGT) = 1.97:2.28:2.00:1.96:1.00 from **19a** (Figure 6B) and 1.95:2.23:2.01:1.88:1.00 from **20a** (Figure 6C), respectively). Conversely, complete digestion was observed when all-(*Rp*)-PO/PS-ODNs (**18a**, **19a**, and **20a**) were treated with svPDE (Figures 7A−C), whereas all-(*Sp*)-PS-d(TTT) and all-(*Sp*)-PS-d(CAGT) remained undigested in the mixtures of all-(*Sp*)-PO/PS-ODNs (**15**, **18b**, **19b**, and **20b**) with svPDE (+ alkaline phosphatase) (Figure 5B and Figure 7D−F). The ratios of hydrolysates obtained from **15**, **19b**, and **20b** were also in agreement with the calculated values (dT:all-(*Sp*)-d(T_ST_ST) = 4.81:1.00 from **15** (Figure 5B), dC:dG:dT:dA:all-(*Sp*)-d(CAGT) = 1.84:2.15:1.94:1.88:1.00 from **19b** (Figure 7B), and 1.67:1.93:1.74:1.67:1.00 from **20b** (Figure 7C), respectively). These experiments verified that the proper stereochemical configurations were obtained for the syntheses of PO/PS-ODNs with oxazaphospholidine monomers **5a**−**d** since (*Rp*)- and (*Sp*)-PS-linkages were formed from the (*Sp*)- and (*Rp*)-monomers, respectively.[12c] The diastereopurities of PO/PS-ODNs were also confirmed.

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Scheme 3. Synthetic Cycles for *P*-Stereodefined PO/PS-ODNs Using ETT and CMPT as Activators

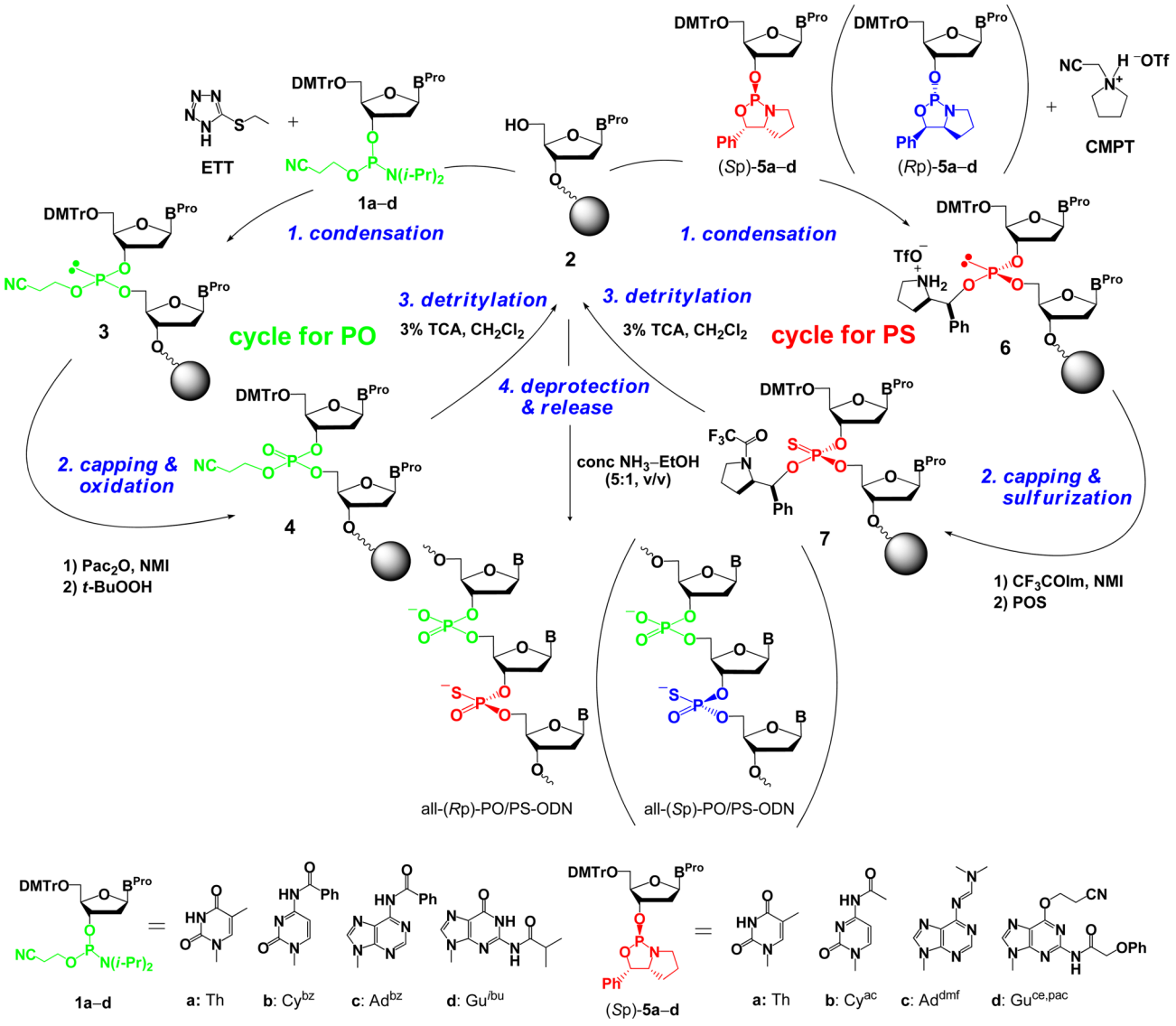

Table 3. *P*-Stereodefined PO/PS-ODN 8–12-mers 16–20 That Were Synthesized According to Scheme 3

entry	PO/PS-ODN[a]	O.D.[b]	isolated yield (%)	MALDI-TOF-MS calcd.	MALDI-TOF-MS found
1	(*Rp*)-d(CAGT$_S$CAGT) **16a**	27	62	2423.4	2424.2
2	(*Sp*)-d(CAGT$_S$CAGT) **16b**	26	59	2423.4	2425.2
3	(*Rp*)-d(CG$_S$GCCGCC) **17a**	10	29	2385.4	2386.2
4	(*Sp*)-d(CG$_S$GCCGCC) **17b**	11	33	2385.4	2386.1
5	all-(*Rp*)-d(C$_S$A$_S$G$_S$TCAGTCAGT) **18a**	21	31	3690.6	3692.4
6	all-(*Sp*)-d(C$_S$A$_S$G$_S$TCAGTCAGT) **18b**	29	44	3690.6	3692.7
7	all-(*Rp*)-d(CAGTC$_S$A$_S$G$_S$TCAGT) **19a**	19	28	3690.6	3692.4
8	all-(*Sp*)-d(CAGTC$_S$A$_S$G$_S$TCAGT) **19b**	31	47	3690.6	3692.6
9	all-(*Rp*)-d(CAGTCAGTC$_S$A$_S$G$_S$T) **20a**	17	26	3690.6	3692.6
10	all-(*Sp*)-d(CAGTCAGTC$_S$A$_S$G$_S$T) **20b**	32	48	3690.6	3692.6

[a]Subscript "S" = phosphorothioate diester. [b]Overall materials (O.D. = optical density units) measured at 260 nm UV absorption.

Hybridization Properties of *P*-Stereodefined PO/PS-ODNs with Complementary ORN. The hybridization properties of the *P*-stereodefined PO/PS-ODN 12-mers (**18a**, **18b**, **19a**, **19b**, **20a**, and **20b**) with a complementary ORN were studied with a UV-melting experiment that used unmodified d(CAGT)$_3$ **21** as the standard (Figure 8 and Table 4).

The T_m values for the PO/PS-ODN duplexes are shown in Table 4. Duplexes with (*Rp*)-PS-linkages (**18a**, **19a**, and **20a**)

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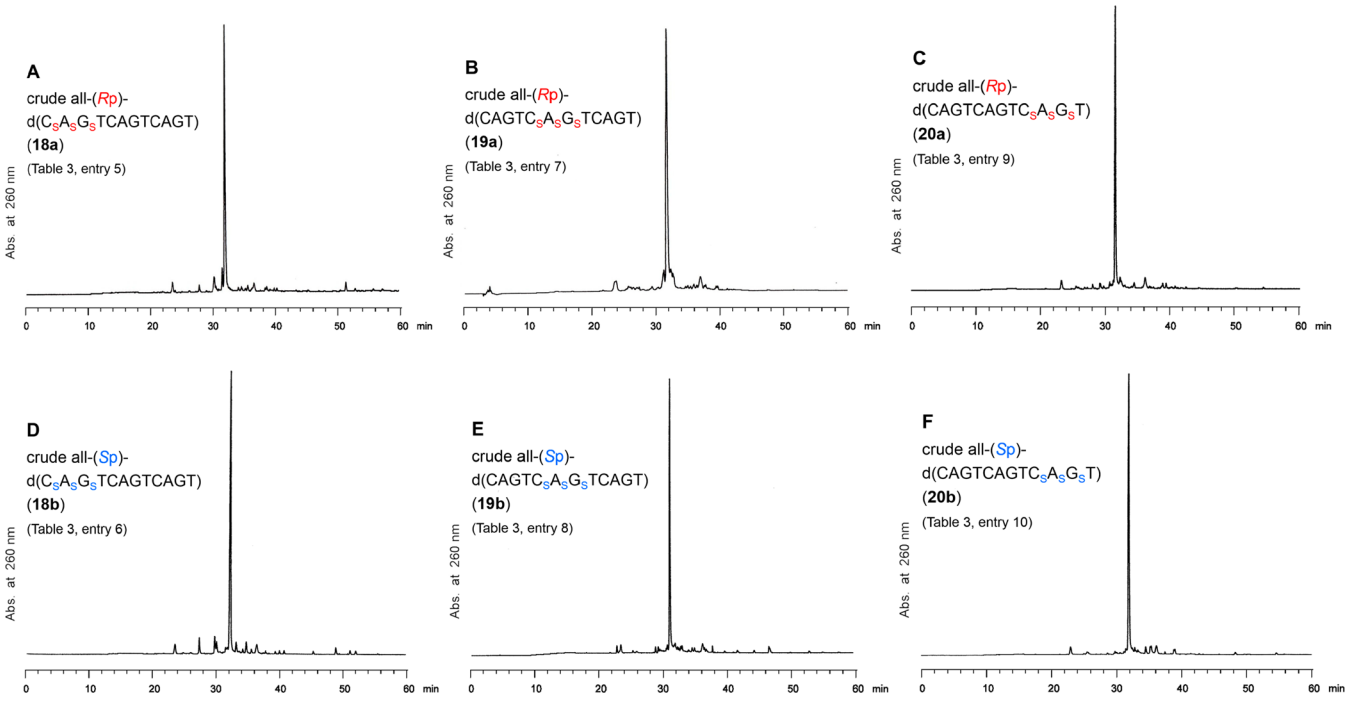

Figure 3. RP-HPLC profiles of crude PO/PS-ODN 12-mers: (A) **18a**, (B) **19a**, (C) **20a**, (D) **18b**, (E) **19b**, and (F) **20b**. RP-HPLC was performed with a linear gradient of 0%−30% CH_3CN in 0.1 M TEAA buffer (pH 7.0) over 60 min at 30 °C at a rate of 0.5 mL/min.

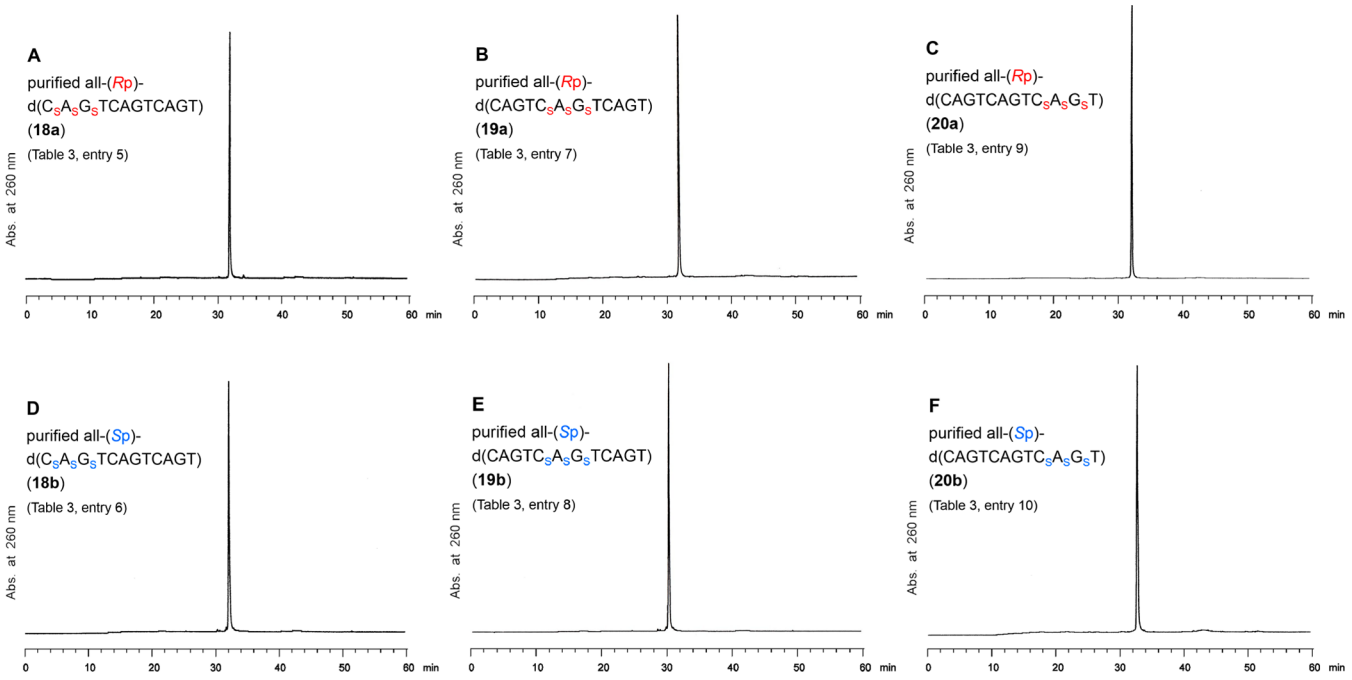

Figure 4. RP-HPLC profiles of purified PO/PS-ODN 12-mers: (A) **18a**, (B) **19a**, (C) **20a**, (D) **18b**, (E) **19b**, and (F) **20b**. RP-HPLC was performed with a linear gradient of 0%−30% CH_3CN in 0.1 M TEAA buffer (pH 7.0) over 60 min at 30 °C at a rate of 0.5 mL/min.

exhibited moderately lower values of T_m than unmodified d(CAGT)$_3$ **21** (ΔT_m = −1.4 to −1.3 °C or −0.4 °C/PS-linkage); their all-(*Sp*)-counterparts (**18b**, **19b**, and **20b**) showed even lower values (ΔT_m = −3.5 to −2.4 °C or −1.2 to −0.8 °C/PS-linkage). The decrease in T_m for each modification [(*Rp*)- or (*Sp*)-PS-linkage] was very similar to results reported for fully modified PS-ODNs.[7b] We discovered that the destabilizing effects of (*Sp*)-PS-linkages were dependent on their location within the molecule. There was a smaller decrease in T_m when these linkages were incorporated into the terminal regions of the molecule (entries 3 and 7 rather than entry 5 in Table 4). In contrast, the effects of the (*Rp*)-PS-linkages were not strongly dependent on their location within the molecule (entries 2, 4, and 6).

■ CONCLUSIONS

We have developed a novel method for the production of *P*-stereodefined PO/PS-ODNs on an automated synthesizer. The

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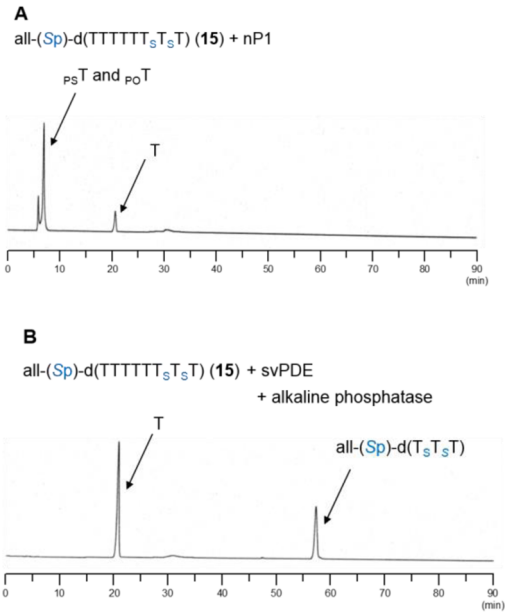

Figure 5. RP-HPLC profiles of reaction mixtures obtained through the digestion of purified **15** with (A) nP1 and (B) svPDE and alkaline phosphatase. RP-HPLC was performed with a linear gradient of 0%−20% CH_3CN in 0.1 M TEAA buffer (pH 7.0) over 90 min at 50 °C at a rate of 0.5 mL/min.

conventional phosphoramidite method was integrated into the oxazaphospholidine method that we had previously developed for the stereocontrolled synthesis of *P*-chiral oligonucleotides. *P*-Stereodefined PO/PS-ODNs that possess multiple PS-linkages at targeted positions were efficiently synthesized with a high degree of stereoselectivity. A thermal denaturation study compared the T_m values for an unmodified ODN−ORN duplex with those for duplexes formed from all-(*Rp*)-PO/PS-ODNs and the complementary ORN; the T_m decreased by 0.4 °C for every modification. Conversely, the T_m values of their all-(*Sp*)-counterparts were lower than the unmodified standard by 0.8 to 1.2 °C for every modification. The destabilizing effect of the (*Sp*)-PS-linkages was smaller when they were located in the terminal regions of the molecule. The effects of the (*Rp*)-PS-linkages were largely independent of their locations within the molecule.

EXPERIMENTAL SECTION

A General Procedure for the Stereocontrolled Solid-Phase Synthesis of PO/PS-ODNs that Uses CMPT as an Activator for Both Phosphoramidite and Oxazaphospholidine Monomers (14a, 14b, and 15). The automated solid-phase synthesis of *P*-stereodefined PO/PS-ODNs (**14a**, **14b**, and **15**) with 5′-*O*-DMTr-thymidine-loaded HCP (0.5 μmol) was performed according to the procedure given in Table 5. The chain was elongated by repeating steps 1−8 in Table 5, and the 5′-*O*-DMTr group was subsequently removed with 3% TCA in CH_2Cl_2. The deprotection of both the nucleobases and the PO/PS-linkages, along with the cleavage of the linker, was accomplished with a 1 mL blend of 25% NH_3 aqueous solution and EtOH (5:1, v/v) for 12−48 h at 55 °C. The resultant crude products were analyzed or purified by RP-HPLC. The fractions that contained the targeted PO/PS-ODN were collected and lyophilized. Overall materials (O.D. = optical density units, weight (triethylammonium salt)) and isolated yields were determined by UV quantitation at 260 nm. (*Rp*)-d(TTTTSTTTT) **14a**, 20 O.D., 0.93 mg, 60% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{80}H_{104}N_{16}O_{53}P_7S^-$ [(M − H)$^-$] 2385.4, found 2386.0. (*Sp*)-d(TTTTSTTTT) **14b**, 20 O.D., 0.93 mg, 60% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{80}H_{104}N_{16}O_{53}P_7S^-$ [(M − H)$^-$] 2385.4, found 2386.2. All-(*Sp*)-d(TTTTTTSTST) **15**, 18 O.D., 0.84 mg, 54% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{80}H_{104}N_{16}O_{53}P_7S^-$ [(M − H)$^-$] 2401.4, found 2400.0.

A General Procedure for the Stereocontrolled Solid-Phase Synthesis of PO/PS-ODNs that Uses ETT and CMPT as Activators (16−20a,b). The automated solid-phase synthesis of *P*-

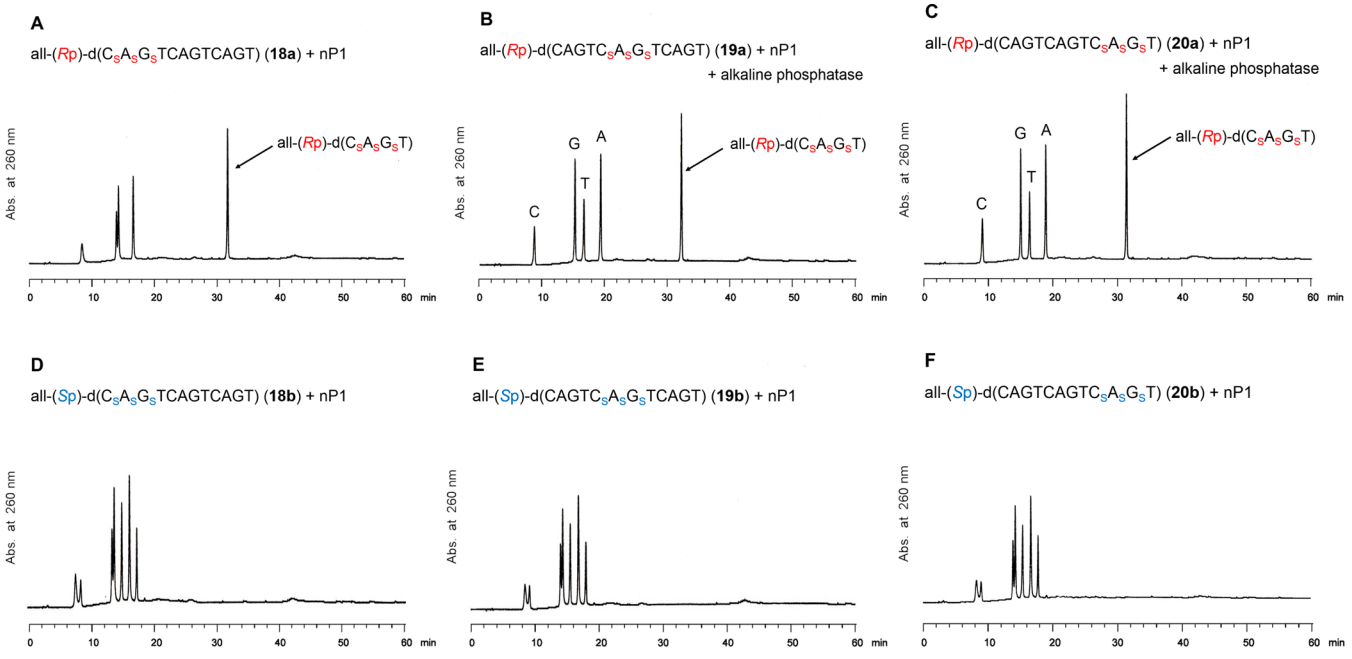

Figure 6. RP-HPLC profiles of reaction mixtures obtained through the digestion of purified all-(*Rp*)- and all-(*Sp*)-PO/PS-ODN 12-mers with nP1: (A) **18a** with nP1, (B) **19a** with nP1 and alkaline phosphatase, (C) **20a** with nP1 and alkaline phosphatase, (D) **18b** with nP1, (E) **19b** with nP1, and (F) **20b** with nP1; RP-HPLC was performed with a linear gradient of 0%−30% CH_3CN in 0.1 M TEAA buffer (pH 7.0) over 60 min at 30 °C at a rate of 0.5 mL/min.

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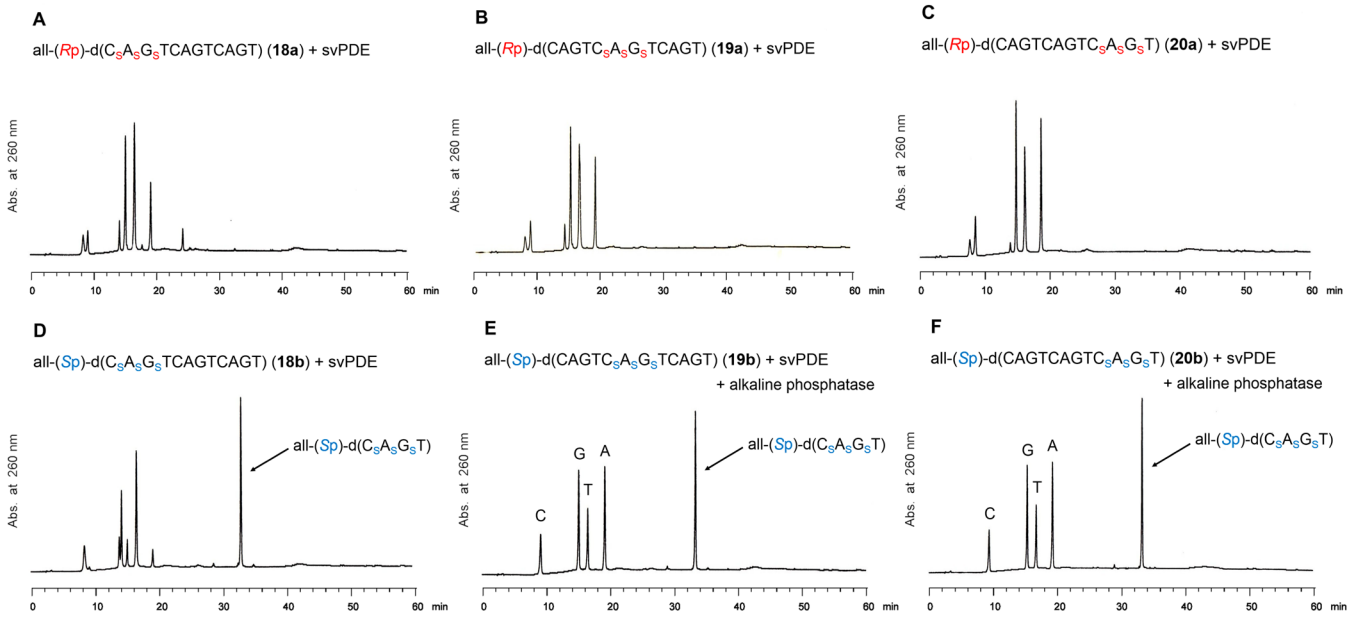

Figure 7. RP-HPLC profiles of reaction mixtures obtained through the digestion of purified all-(*Rp*)- and all-(*Sp*)-PO/PS-ODN 12-mers with svPDE: (A) **18a** with svPDE, (B) **19a** with svPDE, (C) **20a** with svPDE, (D) **18b** with svPDE, (E) **19b** with svPDE and alkaline phosphatase, and (F) **20b** with svPDE and alkaline phosphatase. RP-HPLC was performed with a linear gradient of 0%−30% CH_3CN in 0.1 M TEAA buffer (pH 7.0) over 60 min at 30 °C at a rate of 0.5 mL/min.

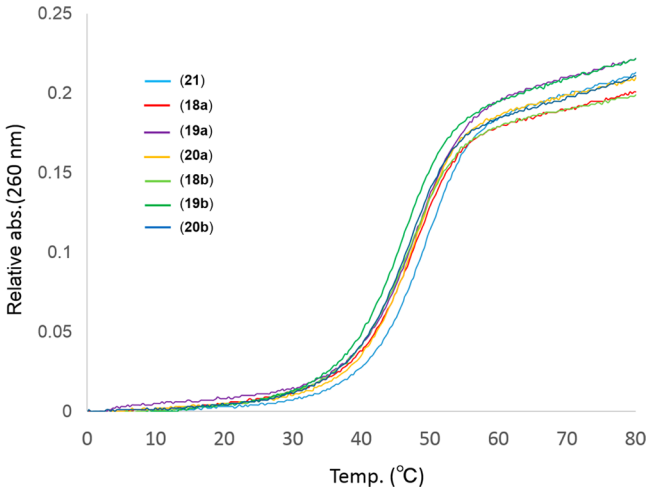

Figure 8. UV-melting curves for the duplexes of *P*-stereodefined PO/PS-ODN 12-mers with r(ACUG)$_3$. The buffer conditions were 10 mM sodium phosphate (pH 7.0) and 100 mM NaCl.

stereodefined PO/PS-ODNs with 5′-*O*-DMTr-thymidine-loaded HCP (0.5 μmol) was performed according to the procedure given in Table 6. The chain was elongated by repeating steps 1−8 in Table 6, and the 5′-*O*-DMTr group was subsequently removed with 3% TCA in CH_2Cl_2. The deprotection of both the nucleobases and the PO/PS-linkages, along with the cleavage of the linker, was accomplished with a 6 mL blend of 25% NH_3 aqueous solution and EtOH (5:1, v/v) for 12 h at 30 °C. The resultant crude products were purified by RP-HPLC. Fractions that contained the targeted PO/PS-ODN were collected and lyophilized. Overall materials (O.D. = optical density units) and isolated yields were determined by UV quantitation at 260 nm.[25] (*Rp*)-d(CAGT$_S$CAGT) **16a**, 27 O.D., 0.97 mg, 62% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{78}H_{98}N_{30}O_{45}P_7S^-$ [(M − H)$^-$] 2423.4, found 2424.2. (*Sp*)-d(CAGT$_S$CAGT) **16b**, 26 O.D., 0.92 mg, 59% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{78}H_{98}N_{30}O_{45}P_7S^-$ [(M − H)$^-$] 2423.4, found 2425.2. (*Rp*)-d(CG$_S$GCCGCC) **17a**, 10 O.D., 0.49 mg, 29% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{75}H_{96}N_{30}O_{45}P_7S^-$ [(M − H)$^-$] 2385.4, found 2386.2. (*Sp*)-d(CG$_S$GCCGCC) **17b**, 11 O.D., 0.51 mg, 33% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{75}H_{96}N_{30}O_{45}P_7S^-$ [(M − H)$^-$] 2385.4, found 2386.1. All-(*Rp*)-d($C_SA_SG_S$TCAGTCAGT) **18a**, 21 O.D., 0.75 mg, 31% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{117}H_{147}N_{45}O_{67}P_{11}S_3^-$ [(M − H)$^-$] 3690.6, found 3692.4. All-(*Sp*)-d($C_SA_SG_S$TCAGTCAGT) **18b**, 29 O.D., 1.06

Table 4. T_m-Values for the Duplexes of *P*-Stereodefined PO/PS-ODN 12-mers with r(ACUG)$_3$

entry	ODN	PO/PS-ODN/r(ACUG)$_3$	
		T_m (°C)[a]	ΔT_m (°C)[b]
1	unmodified d(CAGT)$_3$ **21**	45.3 ± 0.3	–
2	all-(*Rp*)-d($C_SA_SG_S$TCAGTCAGT) **18a**	44.0 ± 0.3	−1.3
3	all-(*Sp*)-d($C_SA_SG_S$TCAGTCAGT) **18b**	42.9 ± 0.3	−2.4
4	all-(*Rp*)-d(CAGTC$_S$A$_S$G$_S$TCAGT) **19a**	44.0 ± 0.1	−1.3
5	all-(*Sp*)-d(CAGTC$_S$A$_S$G$_S$TCAGT) **19b**	41.8 ± 0.1	−3.5
6	all-(*Rp*)-d(CAGTCAGTC$_S$A$_S$G$_S$T) **20a**	43.9 ± 0.1	−1.4
7	all-(*Sp*)-d(CAGTCAGTC$_S$A$_S$G$_S$T) **20b**	42.8 ± 0.1	−2.5

[a]Average values of the experiment in triplicate. [b]The difference in T_m relative to unmodified d(CAGT)$_3$ **21**.

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Table 5. Procedure for the Automated Solid-Phase Synthesis of PO/PS-ODNs with CMPT as an Activator

		cycle for PO		cycle for PS	
step	operation	reagents	time	reagents	time
1	detritylation	3% (w/v) TCA in CH_2Cl_2	49 s	3% (w/v) TCA in CH_2Cl_2	49 s
2	washing	dry CH_3CN	–	dry CH_3CN	–
3	condensation	0.1 M monomer **1a** and 0.5 M CMPT in dry CH_3CN	5 min	0.1 M monomer **5a** and 0.5 M CMPT in dry CH_3CN	5 min
4	washing	dry CH_3CN	–	dry CH_3CN	–
5	capping	0.5 M CF_3COIm, 1.0 M DMAN, and 0.3 M Im in dry THF	15 s	0.5 M CF_3COIm, 1.0 M DMAN, and 0.3 M Im in dry THF	15 s
6	washing	dry CH_3CN	–	dry CH_3CN	–
7	oxidation/sulfurization	1 M *t*-BuOOH in dry toluene	2 min	0.3 M DTD in dry CH_3CN	6 min
8	washing	dry CH_3CN	–	dry CH_3CN	–

Table 6. Procedure for the Automated Solid-Phase Synthesis of PO/PS-ODNs with ETT and CMPT as Activators

		cycle for PO		cycle for PS	
step	operation	reagents	time	reagents	time
1	detritylation	3% (w/v) TCA in CH_2Cl_2	12 s	3% (w/v) TCA in CH_2Cl_2	12 s
2	washing	dry CH_3CN	–	dry CH_3CN	–
3	condensation	0.1 M monomer **1a–d** and 0.25 M ETT in dry CH_3CN	30 s	0.1 M monomer **5a–d** and 0.5 M CMPT in dry CH_3CN	5 min
4	washing	dry CH_3CN	–	dry CH_3CN	–
5	capping	Pac_2O and 16% (v/v) NMI in dry THF	40 s	0.5 M CF_3COIm and 16% (v/v) NMI in dry THF	40 s
6	washing	dry CH_3CN	–	dry CH_3CN	–
7	oxidation/sulfurization	1 M *t*-BuOOH in dry toluene	30 s	0.3 M POS in dry CH_3CN	8 min
8	washing	dry CH_3CN	–	dry CH_3CN	–

mg, 44% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{117}H_{147}N_{45}O_{67}P_{11}S_3^-$ [(M − H)$^-$] 3690.6, found 3692.7. All-(*Rp*)-d(CAGTC$_S$A$_S$G$_S$TCAGT) **19a**, 19 O.D., 0.67 mg, 28% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{117}H_{147}N_{45}O_{67}P_{11}S_3^-$ [(M − H)$^-$] 3690.6, found 3692.4. All-(*Sp*)-d(CAGTC$_S$A$_S$G$_S$TCAGT) **19b**, 31 O.D., 1.13 mg, 47% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{117}H_{147}N_{45}O_{67}P_{11}S_3^-$ [(M − H)$^-$] 3690.6, found 3692.6. All-(*Rp*)-d(CAGTCAGTC$_S$A$_S$G$_S$T) **20a**, 17 O.D., 0.62 mg, 26% yield, MALDI-TOF MS: *m*/*z* calcd for $C_{117}H_{147}N_{45}O_{67}P_{11}S_3^-$ [(M − H)$^-$] 3690.6, found 3692.6. All-(*Sp*)-d(CAGTCAGTC$_S$A$_S$G$_S$T) **20b**, 32 O.D., 1.15 mg, 48% yield, MALDI-TOF-MS: *m*/*z* calcd for $C_{117}H_{147}N_{45}O_{67}P_{11}S_3^-$ [(M − H)$^-$] 3690.6, found 3692.6.

Enzymatic Digestion of *P*-Stereodefined PO/PS-ODNs. *Digestion of PO/PS-ODN (**15**) with nP1.* An aqueous solution (200 μL, pH 7.2) comprising purified **15** (2.0 nmol), nuclease P1 from *Penicillium citrinum* (4 units), 100 mM Tris–HCl, and 1 mM $ZnCl_2$ was incubated for 12 h at 37 °C. The mixture was heated at 100 °C for 1 min to inactivate the enzyme. The solution was then filtered and analyzed by RP-HPLC.

*Digestion of PO/PS-ODNs (**18a**, **18b**, **19b**, and **20b**) with nP1.* An aqueous solution (100 μL, pH 7.2) comprising a purified PO/PS-ODN (1.0 nmol), nuclease P1 from *Penicillium citrinum* (2 units), 100 mM Tris–HCl, and 1 mM $ZnCl_2$ was incubated for 12 h at 37 °C. The mixture was diluted with 0.1 M TEAA buffer (100 μL, pH 7.0) and heated at 100 °C for 1 min to inactivate the enzyme. The solution was then filtered and analyzed by RP-HPLC.

*Digestion of PO/PS-ODNs (**19a** and **20a**) with nP1.* An aqueous solution (100 μL, pH 7.2) comprising purified PO/PS-ODN (1.0 nmol), nuclease P1 from *Penicillium citrinum* (2 units), 100 mM Tris–HCl, alkaline phosphatase from calf intestine (0.4 units), and 1 mM $ZnCl_2$ was incubated for 12 h at 37 °C. The mixture was diluted with 0.1 M TEAA buffer (100 μL, pH 7.0) and heated at 100 °C for 1 min to inactivate the enzymes. The solution was then filtered and analyzed by RP-HPLC.

*Digestion of PO/PS-ODN (**15**) with svPDE.* An aqueous solution (200 μL, pH 8.6) comprising a purified PO/PS-ODN (2.0 nmol), svPDE from *Crotalus adamanteus* (8.0×10^{-3} units), alkaline phosphatase from calf intestine (0.8 units), 100 mM Tris–HCl, and 15 mM $MgCl_2$ was incubated for 12 h at 37 °C. The mixture was then heated for 1 min at 100 °C to inactivate the enzymes. The solution was then filtered and analyzed by RP-HPLC.

*Digestion of PO/PS-ODNs (**18a**, **18b**, **19a**, and **20a**) with svPDE.* An aqueous solution (100 μL, pH 8.6) comprising purified PO/PS-ODN (1.0 nmol), svPDE from *Crotalus adamanteus* (4.0×10^{-3} units), 100 mM Tris–HCl, and 15 mM $MgCl_2$ was incubated for 12 h at 37 °C. The mixture was diluted with 0.1 M TEAA buffer (100 μL, pH 7.0) and heated for 1 min at 100 °C to inactivate the enzyme. The solution was then filtered and analyzed by RP-HPLC.

*Digestion of PO/PS-ODNs (**19b** and **20b**) with svPDE.* An aqueous solution (100 μL, pH 8.6) comprising purified PO/PS-ODN (1.0 nmol), svPDE from *Crotalus adamanteus* (4.0×10^{-3} units), alkaline phosphatase from calf intestine (0.4 units), 100 mM Tris–HCl, and 15 mM $MgCl_2$ was incubated for 12 h at 37 °C. The mixture was diluted with 0.1 M TEAA buffer (100 μL, pH 7.0) and heated for 1 min at 100 °C to inactivate the enzymes. The solution was then filtered and analyzed by RP-HPLC.

Thermal Denaturation Study. An aqueous solution (150 μL, pH 7.0) comprising a 1:1 ratio of ODN (**18–21**) and r(ACUG)$_3$ (0.30 nmol each), 10 mM phosphate, and 100 mM NaCl was heated from rt to 90 °C. The solution was maintained at 90 °C for 10 min and then cooled at a rate of −1 °C/min from 90 to 10 °C to hybridize the oligomers. The solution was kept at 0 °C for 30 min and then gradually heated for denaturation experiments. The UV absorbance values (at 260 nm) were recorded at 0.5 °C intervals, while the temperature was ramped at a rate of 0.5 °C/min from 0 to 80 °C under Ar.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b02793.

^{31}P NMR analyses of O^4-phosphitylation and desulfurization, HPLC profiles, and MALDI-TOF-MS spectra (PDF)

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Notes

The authors declare no competing financial interest.

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