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Stereocontrolled Solid-Phase Synthesis of Phosphorothioate Oligoribonucleotides Using 2′-*O*-(2-Cyanoethoxymethyl)-nucleoside 3′-*O*-Oxazaphospholidine Monomers

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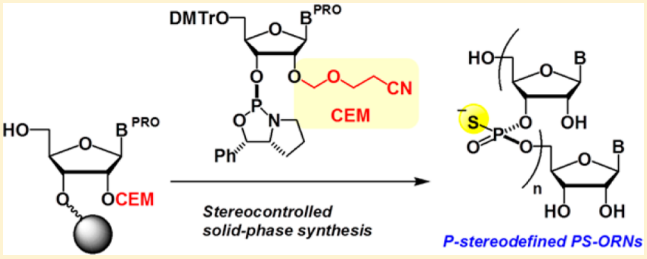
Supporting Information

ABSTRACT: A method for the synthesis of *P*-stereodefined phosphorothioate oligoribonucleotides (PS-ORNs) was developed. PS-ORNs of mixed sequence (up to 12mers) were successfully synthesized by this method with sufficient coupling efficiency (94−99%) and diastereoselectivity (≥98:2). The coupling efficiency was greatly improved by the use of 2-cyanoethoxymethyl (CEM) groups in place of the conventional TBS groups for the 2′-*O*-protection of nucleoside 3′-*O*-oxazaphospholidine monomers. The resultant diastereopure PS-ORNs allowed us to clearly demonstrate that an ORN containing an all-(*Rp*)-PS-backbone stabilizes its duplex with the complementary ORN, whereas its all-(*Sp*)-counterpart has a destabilizing effect.



INTRODUCTION

Post-transcriptional gene silencing, mediated by RNA molecules such as short interfering RNAs (siRNAs) and microRNAs (miRNAs), has been extensively studied for its therapeutic potential in treating various diseases.[1] Conversely, miRNA itself has also emerged as a potential therapeutic target owing to its susceptibility to being silenced by antisense oligonucleotides.[2] In addition, gene silencing by siRNA has become a powerful tool for functional genomics.[3] Synthetic oligoribonucleotides (ORNs) with appropriate chemical modifications are useful for these applications, especially for therapeutic purposes that require oligonucleotides with sufficient nuclease stability, cell membrane permeability, and favorable pharmacokinetic properties.[1,2] Among various chemically modified ORN analogues developed so far, phosphorothioate oligoribonucleotide (PS-ORN) is one of the most well studied analogues owing to its sequence-specific hybridizing affinity for target RNAs and water solubility, features that are comparable to those of natural ORNs, as well as its high nuclease stability and lipophilicity.[1]

A PS-ORN has chiral phosphorus atoms, and its properties are theoretically dependent on the configuration of these phosphorus atoms because it functions by interacting with chiral biomolecules such as nucleic acids and proteins.[4] For this reason, efforts have been made to develop methods for synthesizing *P*-stereodefined PS-ORNs.[4b,5] For example, ORNs containing a single stereodefined PS-linkage at a specific position have often been prepared by chromatographic separation of diastereomixtures[6] and used as probes to study the functions of the pro-*Rp* and pro-*Sp* oxygen atoms of the corresponding phosphodiesters in various RNA-related biological processes.[4] However, this method is not applicable to the preparation of ORNs with multiple stereodefined PS-linkages, which are required for therapeutic studies. Diastereopure dimer building blocks can be used to incorporate multiple stereodefined PS-linkages into ORNs; however, this method requires up to 32 types of building blocks (four types of nucleosides for each of the 3′- and 5′-nucleosides and two *P*-diastereomers) and yet cannot produce ORNs having consecutive stereodefined PS-linkages.[7a,b] *P*-Stereodefined PS-ORNs can also be synthesized using RNA polymerases, but only those with (*Rp*)-PS-linkages are available, and this method is not suitable for large-scale syntheses.[7c,8] Although chemical syntheses of *P*-stereodefined PS-ORNs using stereoselective or stereospecific reactions have also been studied, the methods reported to date suffer from low coupling efficiency or stereoselectivity.[9−11] Recently, an siRNA duplex having four consecutive (*Rp*)-PS-linkages at both ends has been synthesized using chromatographically separated diastereopure nucleoside 3′-phosphorothioate triester derivatives as monomers.[12] To the best of our knowledge, this is the only report in the literature describing the chemical synthesis of *P*-stereodefined PS-ORNs longer than 3mers with sufficient stereo-

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specificity and selectivity. However, the details of the synthesis, such as the coupling efficiency, are not described in this paper.

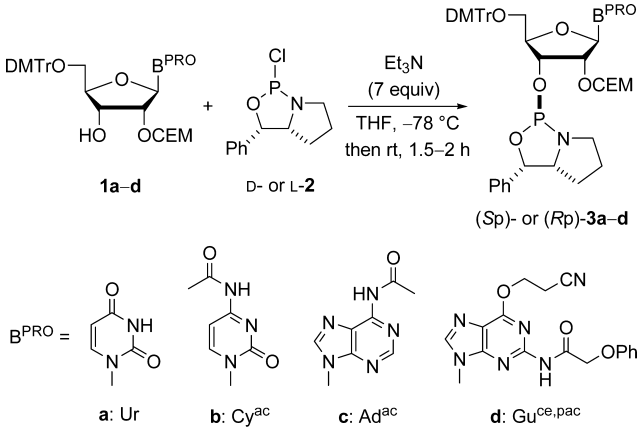
To overcome these limitations, we have recently developed a method for synthesizing *P*-stereodefined PS-ORNs using diastereopure 2′-*O*-TBS-protected nucleoside 3′-*O*-(1,3,2-oxazaphospholidine) monomer units.[13] Using this method, we have synthesized all-(*Rp*)- and all-(*Sp*)-PS-U_{10} with good coupling yields (97−99%) and stereoselectivity (≥96:4) and have shown that the duplex of the former with the complementary ORN (rA_{10}) was slightly more stable than the unmodified duplex (ΔT_m = ca. +0.4 °C per modification). On the other hand, hybridization was not observed above 4 °C for the all-(*Sp*)-PS-U_{10} and rA_{10}. However, attempts to synthesize *P*-stereodefined PS-ORNs of mixed sequence for a thermal denaturing study have revealed that the coupling efficiency was lower than that observed for the synthesis of PS-U_{10}, and only short oligomers up to 4mers were accessible in satisfactory yields. To solve this problem, we sought to develop new monomers with improved reactivity. Recently, significant efforts have been devoted to the development of new methods for synthesizing ORNs owing to their growing demand in biological and medical studies, and a variety of new protecting groups for the 2′-OH of ribonucleosides have been developed.[14−16] Among these new protecting groups, we selected the 2-cyanoethoxymethyl (CEM)[16] group because the replacement of the conventional 2′-*O*-TBS group by this new, less bulky protecting group has been demonstrated to greatly improve the reactivity of the phosphoramidite monomers, and very long ORNs (110−170mers) were efficiently synthesized.[16b,c] It is also notable that CEM groups can be removed under mild conditions using TBAF. In this paper, we describe the development of novel 2′-*O*-CEM-protected oxazaphospholidine monomers, their application to the stereocontrolled synthesis of PS-ORNs, and a thermal denaturing study on the duplexes of the resultant PS-ORNs.

RESULTS AND DISCUSSION

Stereoselective Synthesis of 2′-*O*-CEM-Protected 3′-*O*-Oxazaphospholidine Monomers 3a−d. Table 1 summarizes the synthesis of the 2′-*O*-CEM-protected nucleoside 3′-*O*-oxazaphospholidine monomers **3a−d**. The 2′-*O*-CEM-protected nucleosides having a free 3′-OH (**1a−d**)[16] were allowed to react with 2-chlorooxazaphospholidine derivatives D- and L-**2**, which were synthesized from D- and L-proline, respectively.[17] The reactions proceeded with complete stereoselectivity, and only the *trans*-isomers were generated as was the case for their 2′-deoxy and 2′-*O*-TBS counterparts.[13,17] Because the 2′-*O*-CEM-monomers were less stable on silica gel than the 2′-*O*-TBS-protected oxazaphospholidine monomers, 3-aminopropyl-functionalized silica gel was used for purification.[18] As a result, the complete set of diastereopure monomer units required for the synthesis of the *P*-stereodefined PS-ORNs of mixed sequence were successfully isolated in modest to good yields (Table 1), though partial decomposition of the monomers was observed even with the 3-aminopropyl-functionalized silica gel. The stability of monomers varied with the *P*-configuration and nucleobase; the (*Sp*)- and pyrimidine monomers were more stable than the (*Rp*)- and purine counterparts, respectively.

Stereocontrolled Solid-Phase Synthesis of PS-ORNs. Next, we investigated the manual solid-phase synthesis of PS-ORN 2mers by using the monomers described above (Table 2). Uridine anchored to a controlled-pore glass (CPG) support via a succinate linker (**4**) was condensed with each of the monomers in the presence of *N*-(cyanomethyl)pyrrolidinium triflate (CMPT) that we developed for the stereospecific condensation of oxazaphospholidine monomers.[19] The resultant dinucleoside phosphite intermediates were then sulfurized with *N*,*N*′-dimethylthiuram disulfide (DTD)[20] to give dinucleoside phosphorothioate triester intermediates. Finally, the 5′-*O*-(4,4′-dimethoxytrityl) (DMTr) group was removed by treat-

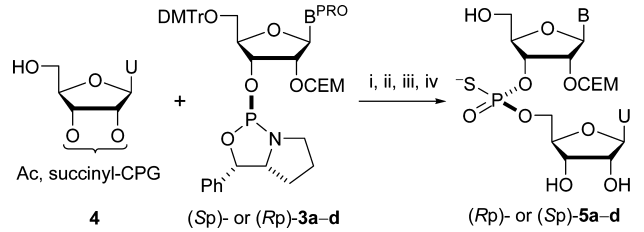
Table 1. Synthesis of 2′-*O*-CEM-Nucleoside 3′-*O*-Oxazaphospholidine Monomers 3a−d



entry	product	B^{PRO}	yield (%)	*trans*:*cis*[a]
1	(*Sp*)-**3a**	U	71	>99:1
2	(*Sp*)-**3b**	C^{ac}	75	>99:1
3	(*Sp*)-**3c**	A^{ac}	51	>99:1
4	(*Sp*)-**3d**	$G^{ce,pac}$	54	>99:1
5	(*Rp*)-**3a**	U	62	>99:1
6	(*Rp*)-**3b**	C^{ac}	53	>99:1
7	(*Rp*)-**3c**	A^{ac}	49	>99:1
8	(*Rp*)-**3d**	$G^{ce,pac}$	42	>99:1

[a]Determined by ^{31}P NMR.

Table 2. Stereocontrolled Solid-Phase Synthesis of 2′-*O*-CEM-Protected Dinucleoside Phosphorothioates N_SU 5a−d[a,b]



entry	monomer	product		yield (%)[c]	*Rp*:*Sp*[c]
1	(*Sp*)-**3a**	(*Rp*)-U_SU	(*Rp*)-**5a**	96	>99:1
2	(*Sp*)-**3b**	(*Rp*)-C_SU	(*Rp*)-**5b**	95	>99:1
3	(*Sp*)-**3c**	(*Rp*)-A_SU	(*Rp*)-**5c**	97	>99:1
4	(*Sp*)-**3d**	(*Rp*)-G_SU	(*Rp*)-**5d**	96	>99:1
5	(*Rp*)-**3a**	(*Sp*)-U_SU	(*Sp*)-**5a**	98	>1:99
6	(*Rp*)-**3b**	(*Sp*)-C_SU	(*Sp*)-**5b**	96	>1:99
7	(*Rp*)-**3c**	(*Sp*)-A_SU	(*Sp*)-**5c**	95	>1:99
8	(*Rp*)-**3d**	(*Sp*)-G_SU	(*Sp*)-**5d**	97	>1:99

[a]Reagents and conditions: (i) (*Sp*)- or (*Rp*)-**3a−d** (0.13 M, 40 equiv), CMPT (1 M, 300 equiv), CH_3CN, rt, 5 min; (ii) DTD (0.3 M, 120 equiv), CH_3CN, rt, 10 min; (iii) 3% DCA, CH_2Cl_2, rt, 4 × 15 s; (iv) conc NH_3 aq/ EtOH (3:1, v/v), rt, 3 h. [b]Subscript S denotes PS-linkage. [c]Determined by RP-HPLC.

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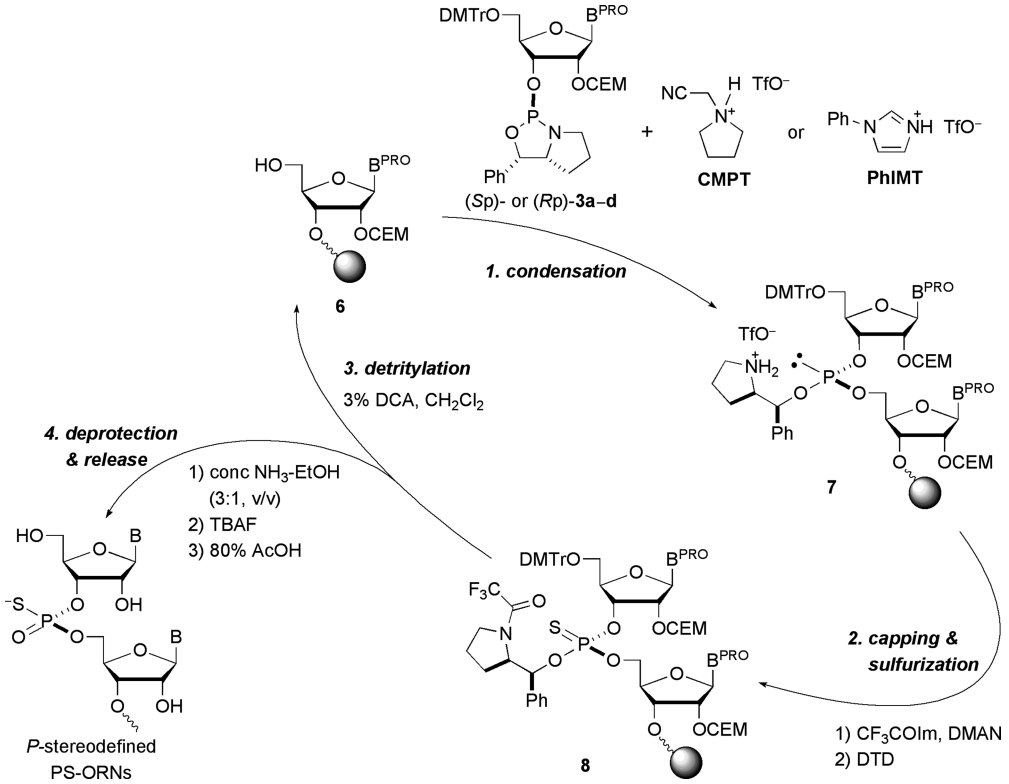
Scheme 1. Synthetic Cycle for *P*-Stereodefined PS-ORNs



ment with 3% dichloroacetic acid (DCA) in CH_2Cl_2, and the oligomer on the solid support was then treated with concentrated aqueous ammonia to deprotect the base and the PS-linkage and cleave the linker. HPLC analysis of the resultant crude 2′-*O*-CEM-protected dinucleoside phosphorothioates (*Rp*)- and (*Sp*)-**5a–d**[21] showed that the efficiency of the cycle (95–98%) and diastereopurity of the products (>99:1) were good enough for the synthesis of oligomers (Table 2).

Encouraged by these results, we extended this method to the synthesis of oligomers. The synthetic cycle is shown in Scheme 1. The cycle consists of the following steps: (1) chain elongation by a condensation reaction between the 5′-OH of a nucleoside or a PS-ORN on a solid support and one of the oxazaphospholidine monomers (**3a–d**) in the presence of an activator, (2) capping of any unreacted 5′-OH as well as the liberated secondary amino group of intermediate **7** using trifluoroacetylimidazole (CF_3COIm) and 1,8-bis-(dimethylamino)naphthalene (DMAN)[13,17] and the subsequent *P*-sulfurization with DTD, and (3) 5′-*O*-detritylation with 3% DCA. The capping step was added to the method shown in Table 2 in order to facilitate the purification of the products as well as to protect the secondary amino group derived from the chiral auxiliary. Trifluoroacetylation is used for capping in place of the conventional acetylation because the *N*-acetylated chiral auxiliaries would be too stable to be removed from the product.[13,17] After repeating the cycle to assemble the desired oligomer, the solid support is treated with a mixture of concentrated aqueous ammonia and ethanol in order to remove the protecting groups and chiral auxiliaries from the nucleobases and PS-linkages, respectively, and to cleave the succinate linker. The resultant PS-ORN bearing the 5′-*O*-DMTr and 2′-*O*-CEM protecting groups is then treated with a solution of TBAF in DMSO containing 0.5 vol % CH_3NO_2, a scavenger of acrylonitrile to deprotect the 2′-*O*-CEM groups.[16] Subsequent purification by RP-HPLC (DMTr-on and -off) affords the desired *P*-stereodefined PS-ORN.

First, four kinds of PS-ORN 4mers, all-(*Rp*)-PS-U_4 (**9a**), all-(*Sp*)-PS-U_4 (**9b**), all-(*Rp*)-PS-CAGU (**9c**), and all-(*Sp*)-CAGU (**9d**) (Table 3, entries 1–5), were manually synthesized, and the efficiency of the synthesis was evaluated from the average coupling yields and the RP-HPLC profiles of the resulting PS-ORNs[21] (2′-*O*-CEM-on–5′-*O*-DMTr-off and fully deprotected). The results showed that the efficiency of the synthesis was greatly improved by employing the 2′-*O*-CEM protection. Thus, the desired PS-ORNs **9a–d** were obtained in the average coupling yields of 94–98% when the coupling time was extended to 10–15 min (entries 2–5) from that used for the synthesis of 2mers (5 min). In contrast, we have previously reported that the average coupling yields were 67–90% when the same oligomers were synthesized by using the 2′-*O*-TBS-protected oxazaphospholidine monomers under similar conditions with a longer coupling time (20 min).[13] This improvement can be attributed to the enhanced reactivity of the new monomers bearing a less bulky CEM group as was the case with the phosphoramidite monomers.[16] Furthermore, the HPLC analyses showed that the 2′-*O*-CEM-protecting groups were removed from the resultant 4mers by treatment with TBAF virtually quantitatively without observable side reactions.[21]

Next, we applied the optimized reaction conditions for the synthesis of 4mers to the synthesis of 12mers (entries 6–12). As shown in entries 6 and 7, all-(*Rp*)-PS-U_{12} (**10a**) and all-(*Sp*)-PS-U_{12} (**10b**) were manually synthesized in excellent coupling yields under these conditions, although the isolated yields were rather low owing to partial loss of the product during a double purification procedure by RP-HPLC (DMTr-on and -off) (Figures 1 and 2, A and B). However, attempts to synthesize all-(*Rp*)-PS-$(CAGU)_3$ (**10c**) and all-(*Sp*)-PS-

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Table 3. Stereocontrolled Solid-Phase Synthesis of PS-ORN 4−12mers

entry	product		activator	coupling time (min)	coupling yield (%)[a,b]	isolated yield (%)
1	All-(*Rp*)-PS-U_4	**9a**	CMPT	5	91	
2	All-(*Rp*)-PS-U_4	**9a**	CMPT	10	98	
3	All-(*Sp*)-PS-U_4	**9b**	CMPT	10	96	
4	All-(*Rp*)-PS-CAGU	**9c**	CMPT	15	98	
5	All-(*Sp*)-PS-CAGU	**9d**	CMPT	15	94	
6	All-(*Rp*)-PS-U_{12}	**10a**	CMPT	15	99	12
7	All-(*Sp*)-PS-U_{12}	**10b**	CMPT	15	99	14
8	All-(*Rp*)-PS-$(CAGU)_3$	**10c**	CMPT	15	90 (68)	
9	All-(*Rp*)-PS-$(CAGU)_3$	**10c**	CMPT	15	92 (75[c])	
10	All-(*Sp*)-PS-$(CAGU)_3$	**10d**	CMPT	15	93 (80)	
11[d]	All-(*Rp*)-PS-$(CAGU)_3$	**10c**	PhIMT	15	94 (86)	6
12	All-(*Sp*)-PS-$(CAGU)_3$	**10d**	PhIMT	15	97 (92)	10

[a]Average coupling yield. [b]Average coupling yields of C^{ac}-monomers are given in parentheses. [c]Double coupling. [d]Synthesized on automated DNA synthesizer.

$(CAGU)_3$ (**10d**) under the same conditions resulted in lower average coupling yields (entries 8−10). The $DMTr^+$ assay showed that the coupling yields of the C^{ac}-monomers were particularly lower than those of the other monomers. Double coupling of the C^{ac}-monomer improved the yield only slightly (entry 9). The low coupling yield might be due to the relatively high basicity of cytosine, which could be initially protonated and thus hamper the protonation of its oxazaphospholidine moiety. To solve this problem, we employed *N*-phenylimidazolium triflate (PhIMT)[22] as the activator for the synthesis of **10c** and **10d**. As expected, the coupling yield of the C^{ac}-monomer improved, and the desired oligomers of mixed sequence (**10c** and **10d**) were isolated (Table 3, entries 11 and 12) (Figures 1 and 2, C and D). The diastereoselectivity of the coupling reaction using PhIMT (98:2 for *Rp*; >99:1 for *Sp*) was determined to be comparable or slightly lower than that obtained using CMPT by independently synthesizing (*Rp*)-U_SU and (*Sp*)-U_SU.[21] We also applied the method to an automated synthesis. The synthesis of all-(*Rp*)-PS-$(CAGU)_3$ (**10c**) was performed on an automated DNA synthesizer to demonstrate the applicability of the method to automation. The desired all-(*Rp*)-PS-$(CAGU)_3$ (**10c**) was obtained with average coupling yield of 94% as shown in entry 11. All-(*Sp*)-PS-$(CAGU)_3$ (**10d**) was also synthesized on an automated synthesizer with similar efficiency (av. 95%) to that obtained by a manual solid-phase synthesis.[21]

***P*-Configurational Assignment of *P*-Stereodefined PS-ORNs by Enzymatic Digestion.** Isolated PS-ORN 12mers **10a−d** were treated with snake venom phosphodiesterase[23] (svPDE) (*Rp*-specific) and nuclease P1[24] (nP1) (*Sp*-specific) for configurational assignments. After being incubated with svPDE or nP1 for 16 h at 37 °C, the PS-ORNs were analyzed by RP-HPLC.[21] All-(*Sp*)-PS-U_{10} (**10b**) and all-(*Sp*)-PS-$(CAGU)_3$ (**10d**) were completely digested with nP1, whereas only ca. 34% of **10b** and ca. 31% of **10d** were digested with svPDE. On the other hand, complete digestion was observed when all-(*Rp*)-PS-U_{10} (**10a**) and all-(*Rp*)-PS-$(CAGU)_3$ (**10c**) were treated with svPDE, whereas ca. 32% of **10a** and ca. 78% of **10c** were digested with nP1. The partial digestion is probably owing to the lower specificity of the enzymes.[23b,25] These experiments confirmed that the stereochemistry of the PS-ORNs synthesized using the new 2′-*O*-CEM oxazaphospholidine monomers was as expected, that is, (*Rp*)- and (*Sp*)-PS-linkages were formed from the (*Sp*)- and (*Rp*)-monomers, respectively.

Hybridization Properties of the *P*-Stereodefined PS-ORNs. Finally, the hybridization properties of the *P*-stereodefined all-(*Rp*)-PS-$(CAGU)_3$ (**10c**) and all-(*Sp*)-PS-$(CAGU)_3$ (**10d**) were studied. A UV denaturing study was carried out with four kinds of duplexes consisting of **10c** and **10d**, stereo-random-PS-$(CAGU)_3$, or an unmodified $(CAGU)_3$ and the complementary ORNs. As shown in Figure 3, the T_m value of the duplex containing of **10c** was slightly higher than that with unmodified $(CAGU)_3$ (ΔT_m = +3.7 °C). The stabilization effect of an (*Rp*)-PS-linkage (ΔT_m = ca. +0.4 °C per modification) was virtually the same as that obtained previously with all-(*Rp*)-PS-U_{10}.[13] In sharp contrast, the T_m values obtained with all-(*Sp*)-PS-$(CAGU)_3$ (**10d**) and stereo-random-PS-$(CAGU)_3$ were much lower than that of the unmodified duplex (ΔT_m = −10.8 and −4.2 °C, respectively). We have also shown the destabilizing effect of (*Sp*)-PS-linkages previously by using all-(*Sp*)-PS-U_{10}; however, no distinct T_m value was obtained.[13] Thus, the destabilizing effect of (*Sp*)- and stereo-random PS-linkages was evaluated more precisely in the current study using *P*-stereodefined PS-ORNs of mixed sequence.

■ CONCLUSION

In conclusion, we successfully developed a method for synthesizing *P*-stereodefined PS-ORNs of mixed sequence by employing 2′-*O*-CEM protection in the oxazaphospholidine method. The coupling efficiency of the 2′-*O*-CEM-protected oxazaphospholidine monomers was greater than that of the 2′-*O*-TBS-protected monomers. The resultant *P*-stereodefined PS-ORNs of mixed sequence enabled us to demonstrate that a PS-ORN−ORN duplex was stabilized by incorporating (*Rp*)-PS-linkages, whereas it was largely destabilized with (*Sp*)- or stereo-random-PS-linkages. The expanded availability of *P*-stereodefined PS-ORNs should promote their use in therapeutic studies. Furthermore, the stabilizing effect of (*Rp*)-PS-linkages can support the design of miRNA-based drug candidates, which require a much higher hybridization potential to target RNAs than that needed for siRNA.[2]

■ EXPERIMENTAL SECTION

***O^6*-Cyanoethyl-*N^2*-phenoxyacetyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(2-cyanoethoxymethyl)-guanosine 11.** N^2-Phenoxyacetyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-2′-*O*-(2-cyanoethoxymethyl)-guanosine (11.18 g, 14.9 mmol) was dried by repeated coevaporations with dry toluene and dissolved in dry CH_2Cl_2 (100 mL) under argon. DMAP (0.092 g, 0.75 mmol), triethylamine (8.3 mL, 60 mmol), and 2-mesitylenesulfonyl chloride (3.92 g, 17.9 mmol) were successively added, and the mixture was stirred for 30 min at rt. A saturated $NaHCO_3$ aqueous solution (50 mL) was then added. The organic layer was separated and washed with saturated $NaHCO_3$ aqueous solutions (2 × 50 mL). The washings were combined and extracted with $CHCl_3$ (2 × 30 mL). The organic

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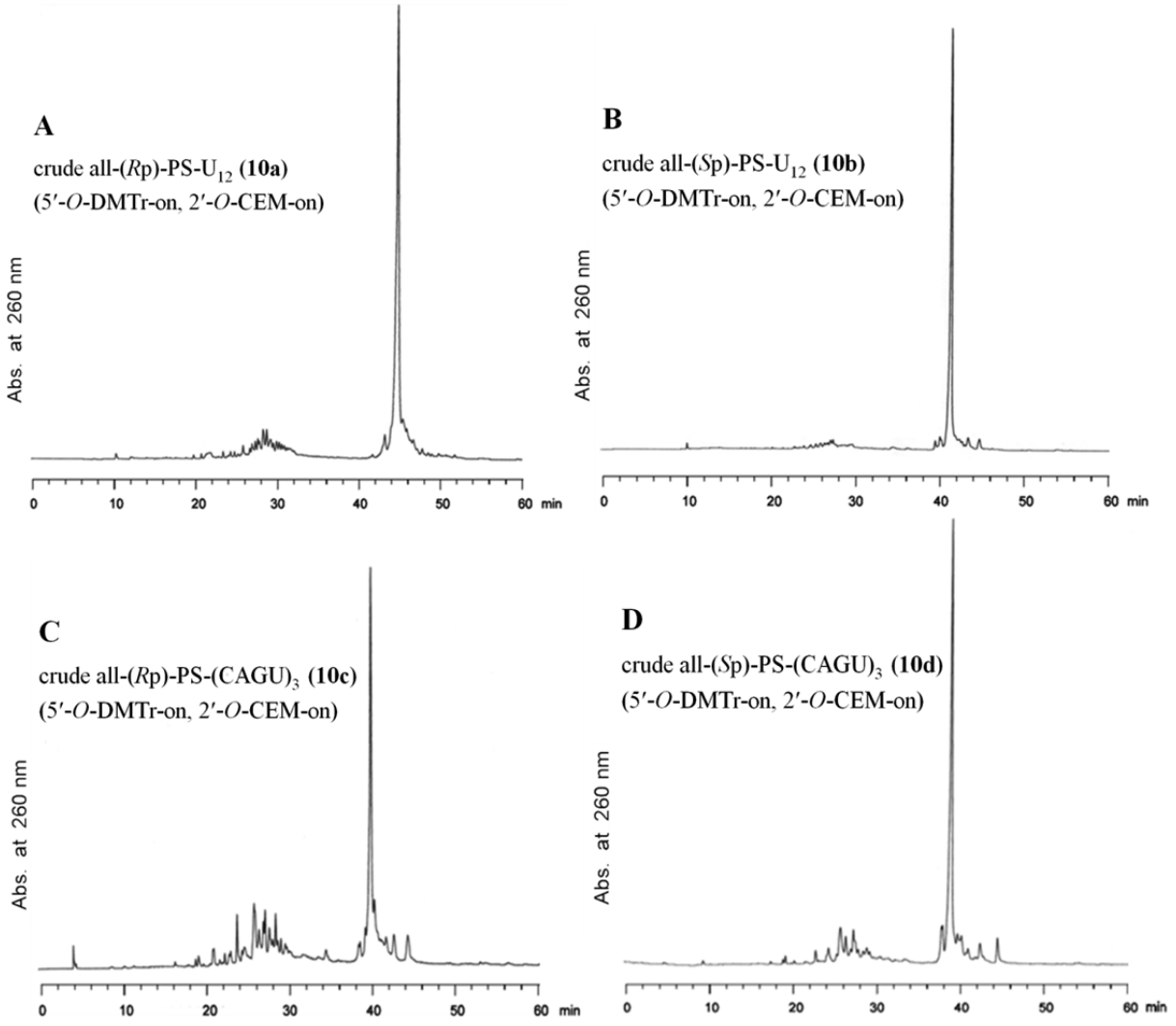

Figure 1. RP-HPLC profiles of PS-ORNs bearing 5′-*O*-DMTr and 2′-*O*-CEM groups: (A) crude all-(*R*p)-PS-U_{12} (**10a**); (B) crude all-(*S*p)-PS-U_{12} (**10b**); (C) crude all-(*R*p)-PS-$(CAGU)_3$ (**10c**); (D) crude all-(*S*p)-PS-$(CAGU)_3$ (**10d**). RP-HPLC was performed with a linear gradient of 0−60% CH_3CN in 0.1 M TEAA buffer (pH 7.0) over 60 min at 30 °C and a rate of 0.5 mL/min.

layers were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was dried by repeated coevaporations with dry toluene and dissolved in dry CH_2Cl_2 (100 mL) under argon. *N*-Methylpyrrolidine (15.9 mL, 149 mmol) was added at 0 °C, and the mixture was stirred for 40 min at the same temperature. 2-Cyanoethanol (10.1 mL, 149 mmol) and DBU (3.3 mL, 22 mmol) were then added, and the mixture was further stirred for 25 min at 0 °C. A 1.0 M KH_2PO_4 aqueous solution (50 mL) was then added, and the organic layer was separated and washed with 1.0 M KH_2PO_4 aqueous solutions (2 × 50 mL). The washings were combined and extracted with $CHCl_3$ (2 × 30 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography [ethyl acetate−hexane (50:50, v/v to 100:0, v/v)]. The fractions containing **11** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford **11** (6.20 g, 7.8 mmol, 52%) as a pale yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.92 (1H, brs, 2-NH), 8.31 (1H, s, 8-H), 7.40−7.38 (2H, dd, J = 8.0, 8.0 Hz, 3-H of Pac), 7.11−6.98 (3H, m, 2,4-H of Pac), 6.16 (1H, s, 1′-H), 5.20 (1H, d, J = 7.2 Hz, OCH_2O of CEM), 5.10 (1H, d, J = 7.2 Hz, OCH_2O of CEM), 4.85−4.78 (2H, m, O^6-OCH_2CH_2CN), 4.69 (2H, s, CH_2 of Pac), 4.53 (1H, dd, J = 4.2, 9.3 Hz, 2′-H), 4.35−4.17 (3H, m, 3′-H, 5′-H), 4.08−4.01 (2H, m, 4′-H, OCH_2CH_2CN, of CEM), 3.92−3.78 (1H, m, OCH_2CH_2CN, of CEM), 3.02 (2H, t, J = 6.8 Hz, O^6-OCH_2CH_2CN), 2.66−2.61 (2H, t, J = 6.6 Hz, OCH_2CH_2CN of CEM), 1.12−0.95 (28H, m, *i*-Pr). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.7, 159.6, 156.9, 152.0, 150.8, 139.8, 129.9, 122.6, 119.0, 117.8, 116.8, 114.9, 94.7, 88.4, 81.5, 78.6, 68.4, 67.9, 63.1, 61.7, 59.4, 18.8, 18.1, 17.5, 17.3, 17.3, 17.3, 17.1, 17.0, 16.9, 16.8, 13.3, 13.0, 12.9, 12.6. ESI-HRMS: m/z calcd for $C_{37}H_{54}N_7O_9Si_2^+$ [$(M + H)^+$] 796.3516, found 796.3527.

***O*^{6}-Cyanoethyl-*N*^{2}-phenoxyacetyl-2′-*O*-(2-cyanoethoxymethyl)-guanosine 12.** Compound **11** (1.92 g, 2.4 mmol) was dried by repeated coevaporations with dry toluene and dissolved in dry THF (5.0 mL) under argon. $Et_3N{\cdot}3HF$ (0.40 mL, 2.4 mmol) was added at 35 °C, and the mixture was stirred for 2 h at the same temperature. The mixture was then diluted with $CHCl_3$ (50 mL) and washed with saturated $NaHCO_3$ aqueous solutions (3 × 30 mL). The washings were combined and extracted with $CHCl_3$ (2 × 20 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography [$CHCl_3$−MeOH (100:3, v/v)] to afford **12** (0.86 g, 1.6 mmol, 64%) as a pale yellow foam. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.73 (1H, brs, 2-NH), 8.57 (1H, s, 8-H), 7.33−7.28 (2H, dd, 7.8, 7.8 Hz, 3-H of Pac), 6.96−6.93 (3H, m, 2,4-H of Pac), 6.09 (1H, d, J = 6.0 Hz, 1′-H), 5.38 (1H, d, J = 5.1 Hz, 3′-OH), 5.11−4.98 (3H, m, 5′-OH, OCH_2O of CEM), 4.78−4.65 (5H, m, 2′-H, O^6-OCH_2CH_2CN, CH_2 of Pac), 4.36 (1H, dd, J = 3.6, 4.7 Hz, 3′-H), 3.98 (1H, d, J = 3.6 Hz, 4′-H), 3.72−3.51 (3H, m, 5′-H,

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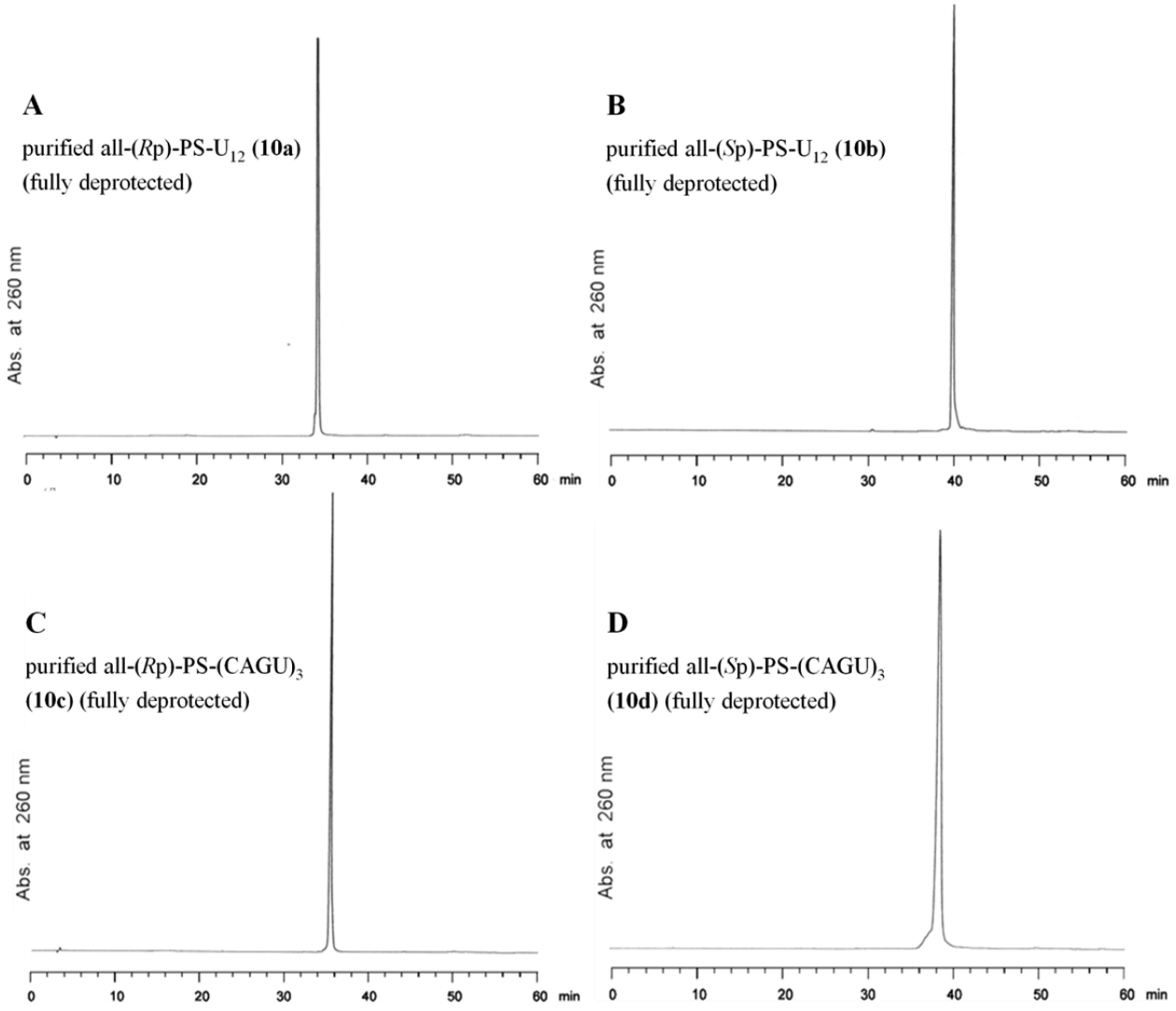

Figure 2. RP-HPLC profiles of fully deprotected and purified PS-ORNs: (A) all-(*R*p)-PS-U_{12} (**10a**); (B) all-(*S*p)-PS-U_{12} (**10b**); (C) all-(*R*p)-PS-$(CAGU)_3$ (**10c**); (D) purified all-(*S*p)-PS-$(CAGU)_3$ (**10d**). RP-HPLC was performed with a linear gradient of 0−30% CH_3CN in 0.1 M TEAA buffer (pH 7.0) over 60 min at 30 °C and a rate of 0.5 mL/min.

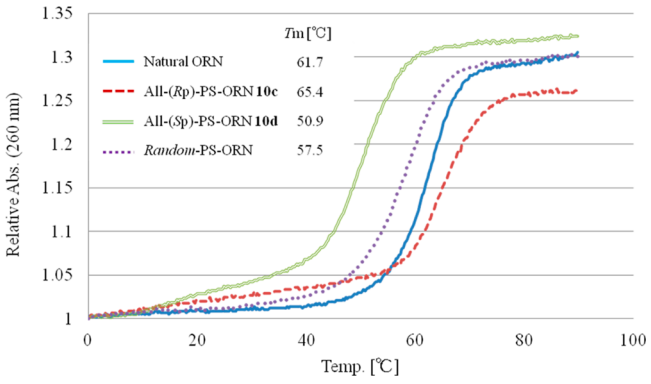

Figure 3. UV melting curves of PS-$(CAGU)_3$-PO-$(ACUG)_3$.

OCH_2CH_2CN, of CEM), 3.47−3.25 (1H, m, OCH_2CH_2CN of CEM), 3.22−3.16 (2H, t, J = 5.9 Hz, O^6-OCH_2CH_2CN), 2.66−2.50 (2H, m, OCH_2CH_2CN of CEM). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.1, 160.0, 156.8, 152.1, 150.6, 142.6, 129.8, 122.5, 119.6, 117.6, 116.9, 114.8, 95.7, 88.4, 86.9, 79.9, 70.8, 67.5, 63.3, 62.4, 61.9, 18.7, 18.0. ESI-HRMS: m/z calcd for $C_{25}H_{28}N_7O_8^+$ $[(M + H)^+]$ 554.1994, found 554.1996.

***O*^{6}-Cyanoethyl-*N*^{2}-phenoxyacetyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-cyanoethoxymethyl)-guanosine 1d.** Compound **12** (0.86 g, 1.6 mmol) was dried by repeated coevaporations with dry pyridine and dissolved in dry pyridine (5.0 mL) under argon. DMTrCl (0.80 g, 2.4 mmol) was added at 0 °C, and the mixture was stirred for 9 h at rt. MeOH (5 mL) was then added, and the mixture was concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ (50 mL), and the solution was washed with saturated $NaHCO_3$ aqueous solutions (3 × 30 mL). The washings were combined and extracted with $CHCl_3$ (3 × 20 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography [ethyl acetate−hexane−pyridine (50:50:0.5, v/v/v to 100:0:0.5, v/v/v)] to afford **1d** (1.21 g, 1.4 mmol, 91%) as a pale yellow foam. ^1H NMR (300 MHz, $CDCl_3$) δ 8.84 (1H, brs, 2-NH), 8.10 (1H, s, 8-H), 7.42−6.99 (14H, m, 2-H of *p*-An, Ph of Pac and DMTr), 6.79 (4H, d, J = 8.1 Hz, 3-H of *p*-An), 6.24 (1H, d, J = 3.9 Hz, 1′-H), 5.03, 4.96 (2H, 2d, J = 7.1 Hz, OCH_2O of CEM), 4.90−4.79 (3H, m, 2′-H, O^6-OCH_2CH_2CN), 4.68−4.61 (3H, m, 3′-H, CH_2 of Pac), 4.26 (1H, d, J = 3.3 Hz, 4′-H), 3.78−3.65 (8H, m, OMe, OCH_2CH_2CN of CEM), 3.49 (2H, d, J = 3.9 Hz, 5′-H), 3.02 (2H, t, J = 6.6 Hz, O^6-OCH_2CH_2CN), 2.62 (1H, d, J = 6.3 Hz, 3′-OH), 2.50 (2H, t, J = 6.2 Hz, OCH_2CH_2CN of CEM). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.7, 159.7, 158.5, 156.9, 152.8, 150.8, 149.7, 144.4, 140.9,

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135.5, 135.4, 130.0, 129.8, 128.1, 127.9, 127.0, 122.5, 118.7, 117.6, 116.8, 114.9, 113.1, 95.6, 87.0, 86.6, 83.9, 80.2, 70.2, 67.7, 63.4, 63.1, 61.7, 55.2, 18.8, 18.0. ESI-HRMS: m/z calcd for $C_{46}H_{46}N_7O_{10}^+$ [(M + H)$^+$] 856.3301, found 836.3300.

(*Sp*)-U-Monomer [(*Sp*)-3a]. A Typical Procedure for the Synthesis of 3a–d. 5′-*O*-DMTr-2′-*O*-CEM-uridine (0.94 g, 1.5 mmol) was dried by repeated coevaporations with dry pyridine and dry toluene and dissolved in freshly distilled THF (5.0 mL) under argon. Triethylamine (1.5 mL, 10.5 mmol) and a 0.5 M solution of the 2-chloro-1,3,2-oxazaphospholidine derivative D-**2** in freshly distilled THF (9.3 mL, 4.7 mmol) were successively added at −78 °C, and the mixture was stirred for 1.5 h at rt. The mixture was then diluted with $CHCl_3$ (400 mL) and washed with a saturated $NaHCO_3$ aqueous solutions (3 × 100 mL). The washings were combined and extracted with $CHCl_3$ (2 × 30 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 [NH silica gel, hexane–ethyl acetate–triethylamine (20:10:0.03 to 10:20:0.03, v/v/v)]. The fractions containing (*Sp*)-**3a** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford (*Sp*)-**3a** (0.89 g, 1.1 mmol, 71%) as a colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (1H, brs, 3-NH), 8.11 (1H, d, J = 8.4 Hz, 6-H), 7.41–7.16 (14H, m, 5″-Ph, 2-H of *p*-An, Ph of DMTr), 6.85 (4H, dd, J = 2.1, 9.0 Hz, 3-H of *p*-An), 5.98 (1H, d, J = 1.5 Hz, 1′-H), 5.79 (1H, d, J = 6.3 Hz, 5″-H), 5.20 (1H, d, J = 8.4 Hz, 5-H), 4.89–4.81 (3H, m, 3′-H, OCH_2O of CEM), 4.32–4.25 (2H, m, 2′-H, 4′-H), 3.87–3.68 (10H, m, 5′-H, 4″-H, OCH_2CH_2CN, OMe), 3.54–3.41 (2H, m, 5′-H, 6″-H), 3.14–3.03 (1H, m, 6″-H), 2.47 (2H, ddd, J = 1.8, 6.0, 6.0 Hz, OCH_2CH_2CN), 1.68–1.59 (2H, m, 7″-H), 1.28–1.17 (1H, m, 8″-H), 1.01–0.88 (1H, m, 8″-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.0, 158.7, 158.7, 150.1, 144.0, 139.8, 137.7 (d, $^3J_{PC}$ = 4.0 Hz), 135.0, 134.8, 130.3, 129.0, 128.4, 128.3, 128.2, 128.0, 127.7, 127.2, 125.3, 117.9, 113.2, 113.2, 102.2, 94.6, 88.4, 87.1, 82.3 (d, $^2J_{PC}$ = 9.5 Hz), 81.8 (d, $^3J_{PC}$ = 4.3 Hz), 78.5, 69.3 (d, $^2J_{PC}$ = 15.5 Hz), 67.3 (d, $^2J_{PC}$ = 3.2 Hz), 62.9, 60.2, 55.2, 47.0 (d, $^2J_{PC}$ = 34.4 Hz), 27.9, 25.9 (d, $^3J_{PC}$ = 3.4 Hz), 18.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 159.4. ESI-HRMS: m/z calcd for $C_{47}H_{51}N_5O_{10}P^+$ [(M + H)$^+$] 835.3103, found 835.3104.

(*Sp*)-C^{ac}-Monomer [(*Sp*)-3b]. Crude (*Sp*)-**3b** was synthesized from 5′-*O*-DMTr-2′-*O*-CEM-*N*^{4}-acetylcytidine **1b** (0.67 g, 1.0 mmol) following the typical procedure described above and purified by silica gel column chromatography [NH silica gel, toluene–ethyl acetate–triethylamine (10:20:0.03, v/v/v)]. The fractions containing (*Sp*)-**3b** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford (*Sp*)-**3b** (0.66 g, 0.75 mmol, 75%) as a pale yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.07 (1H, brs, 4-NH), 8.61 (1H, d, J = 7.5 Hz, 6-H), 7.44–7.16 (14H, m, 5″-Ph, 2-H of *p*-An, Ph of DMTr), 6.99 (1H, d, J = 7.5 Hz, 5-H), 6.86 (4H, dd, J = 1.8, 8.7 Hz, 3-H of *p*-An), 5.98 (1H, s, 1′-H), 5.78 (1H, d, J = 6.3 Hz, 5″-H), 5.04 (1H, d, J = 6.9 Hz, OCH_2O of CEM), 4.95 (1H, d, J = 6.9 Hz, OCH_2O of CEM), 4.78 (1H, ddd, J = 4.8, 9.3, 9.3 Hz, 3′-H), 4.34–4.27 (2H, m, 2′-H, 4′-H), 3.88–3.73 (10H, m, 5′-H, 4″-H, OCH_2CH_2CN, OMe), 3.56–3.39 (2H, m, 5′-H, 6″-H), 3.12–3.00 (1H, m, 6″-H), 2.49 (2H, t, J = 6.9 Hz, OCH_2CH_2CN), 2.24 (3H, s, Ac), 1.78–1.58 (2H, m, 7″-H), 1.28–1.11 (1H, m, 8″-H), 0.99–0.86 (1H, m, 8″-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.2, 162.6, 158.7, 154.8, 144.7, 144.0, 137.8 (d, $^3J_{PC}$ = 4.3 Hz), 135.1, 135.0, 135.0, 130.3, 128.3, 128.3, 128.0, 127.6, 127.2, 125.3, 118.0, 113.2, 113.2, 113.2, 96.5, 94.6, 90.0, 87.1, 82.4 (d, $^2J_{PC}$ = 9.5 Hz), 81.3 (d, $^3J_{PC}$ = 4.0 Hz), 78.6, 68.6 (d, $^2J_{PC}$ = 14.9 Hz), 67.2 (d, $^2J_{PC}$ = 3.2 Hz), 63.0, 59.7, 55.2, 46.9 (d, $^2J_{PC}$ = 35.4 Hz), 29.7, 27.9, 25.9 (d, $^3J_{PC}$ = 3.8 Hz), 24.9, 18.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 159.0. ESI-HRMS: m/z calcd for $C_{47}H_{51}N_5O_{10}P^+$ [(M + H)$^+$] 876.3368, found 876.3365.

(*Sp*)-A^{ac}-Monomer [(*Sp*)-3c]. Crude (*Sp*)-**3c** was synthesized from 5′-*O*-DMTr-2′-*O*-CEM-*N*^{6}-acetyladenosine **1c** (0.70 g, 1.0 mmol) following the typical procedure described above and purified by silica gel column chromatography [NH silica gel, toluene–ethyl acetate–triethylamine (50:50:0.1, v/v/v)]. The fractions containing (*Sp*)-**3c** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford (*Sp*)-**3c** (0.46 g, 0.51 mmol, 51%) as a colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.60 (1H, s, 2-H), 8.51 (1H, brs, 6-NH), 8.21 (1H, s, 8-H), 7.42–7.22 (14H, m, 5″-Ph, 2-H of *p*-An, Ph of DMTr), 6.81 (4H, d, J = 6.9 Hz, 3-H of *p*-An), 6.22 (1H, d, J = 5.1 Hz, 1′-H), 5.79 (1H, d, J = 6.6 Hz, 5″-H), 5.07–4.98 (2H, m, 2′-H, 3′-H), 4.84 (1H, d, J = 7.2 Hz, OCH_2O of CEM), 4.76 (1H, d, J = 7.2 Hz, OCH_2O of CEM), 4.36 (1H, dd, J = 3.9, 8.1 Hz, 4′-H), 3.87 (1H, ddd, J = 6.0, 10.5, 10.5 Hz, 4″-H), 3.78 (6H, s, OMe), 3.64–3.44 (4H, m, 5′-H, 6″-H, OCH_2CH_2CN), 3.40 (1H, dd, J = 3.9, 10.8 Hz, 5′-H), 3.17–3.06 (1H, m, 6″-H), 2.61 (3H, s, Ac), 2.37 (2H, t, J = 6.3 Hz, OCH_2CH_2CN), 1.72–1.56 (2H, m, 7″-H), 1.28–1.19 (1H, m, 8″-H), 1.02–0.92 (1H, m, 8″-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 158.6, 152.4, 151.0, 149.1, 144.3, 141.7, 138.0 (d, $^3J_{PC}$ = 4.0 Hz), 135.5, 135.4, 130.16, 128.3, 128.2, 127.9, 127.6, 127.0, 126.6, 126.0, 125.4, 122.2, 117.5, 113.1, 94.9, 87.1, 86.7, 83.6 (d, $^3J_{PC}$ = 3.7 Hz), 82.4 (d, $^2J_{PC}$ = 9.5 Hz), 78.0, 70.7 (d, $^2J_{PC}$ = 12.0 Hz), 67.3 (d, $^2J_{PC}$ = 3.2 Hz), 62.9, 62.2, 55.2, 53.1, 50.7, 47.1 (d, $^2J_{PC}$ = 34.7 Hz), 39.6, 27.9, 26.6, 26.0, 25.8 (d, $^3J_{PC}$ = 18.6 Hz), 20.6, 18.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 156.1. ESI-HRMS: m/z calcd for $C_{48}H_{51}N_7O_9P^+$ [(M + H)$^+$] 900.3480, found 900.3479.

(*Sp*)-G^{ce,pac}-Monomer [(*Sp*)-3d]. Crude (*Sp*)-**3d** was synthesized from 5′-*O*-DMTr-2′-*O*-CEM-*O*^{6}-cyanoethyl-*N*^{2}-phenoxyacetylguanosine **1d** (1.28 g, 1.5 mmol) following the typical procedure described above and purified by silica gel column chromatography [NH silica gel, hexane–ethyl acetate–triethylamine (30:10:0.04 to 10:20:0.03, v/v/v)]. The fractions containing (*Sp*)-**3d** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford (*Sp*)-**3d** (0.87 g, 0.82 mmol, 54%) as a colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.77 (1H, brs, 2-NH), 8.13 (1H, s, 8-H), 7.45–6.98 (19H, m, 5″-Ph, 2-H of *p*-An, Ph of DMTr, Ph of Pac), 6.79 (4H, d, J = 6.6 Hz, 3-H of *p*-An), 6.23 (1H, d, J = 4.8 Hz, 1′-H), 5.76 (1H, d, J = 6.6 Hz, 5″-H), 4.99–4.75 (6H, m, 2′-H, 3′-H, OCH_2O of CEM, *O*^{6}-OCH_2CH_2CN), 4.62 (2H, s, CH_2 of Pac), 4.39 (1H, d, J = 3.3 Hz, 4′-H), 3.85 (1H, ddd, J = 6.0, 10.4, 10.4 Hz, 4″-H), 3.77 (6H, s, OMe), 3.62–3.42 (5H, m, 5′-H, 6″-H, OCH_2CH_2CN of CEM), 3.19–3.03 (3H, m, 6″-H, *O*^{6}-OCH_2CH_2CN), 2.34 (2H, t, J = 6.3 Hz, OCH_2CH_2CN of CEM), 1.75–1.59 (2H, m, 7″-H), 1.28–1.17 (1H, m, 8″-H), 1.01–0.89 (1H, m, 8″-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.7, 159.7, 158.6, 157.0, 153.0, 151.0, 144.3, 140.8, 137.9 (d, $^3J_{PC}$ = 3.8 Hz), 135.4, 135.4, 130.1, 129.8, 128.5, 128.3, 128.2, 127.9, 127.6, 127.0, 125.4, 122.4, 118.6, 117.6, 116.8, 114.9, 113.2, 94.8, 86.8, 86.5, 83.8 (d, $^3J_{PC}$ = 3.7 Hz), 82.3 (d, $^2J_{PC}$ = 9.5 Hz), 78.0, 70.6 (d, $^2J_{PC}$ = 12.9 Hz), 67.7, 67.3 (d, $^2J_{PC}$ = 3.2 Hz), 63.0, 62.6, 61.8, 55.2, 47.1 (d, $^2J_{PC}$ = 34.9 Hz), 27.9, 25.9 (d, $^3J_{PC}$ = 3.5 Hz), 18.6, 18.0. ^{31}P NMR (121 MHz, $CDCl_3$) δ 157.5. ESI-HRMS: m/z calcd for $C_{57}H_{58}N_8O_{11}P^+$ [(M + H)$^+$] 1061.3957, found 1061.3977.

(*Rp*)-U-Monomer [(*Rp*)-3a]. Crude (*Rp*)-**3a** was synthesized from 5′-*O*-DMTr-2′-*O*-CEM-uridine **1a** (0.63 g, 1.0 mmol) following the typical procedure described above and purified by silica gel column chromatography [NH silica gel, toluene–ethyl acetate–triethylamine (60:40:0.1, v/v/v)]. The fractions containing (*Rp*)-**3a** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford (*Rp*)-**3a** (0.52 g, 0.62 mmol, 62%) as a colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.98 (1H, brs, 3-NH), 8.07 (1H, d, J = 8.1 Hz, 6-H), 7.41–7.19 (14H, m, 5″-Ph, 2-H of *p*-An, Ph of DMTr), 6.79 (4H, dd, J = 8.1, 8.1 Hz, 3-H of *p*-An), 5.95 (1H, d, J = 1.2 Hz, 1′-H), 5.75 (1H, d, J = 6.6 Hz, 5″-H), 5.16 (1H, d, J = 8.1 Hz, 5-H), 5.01, 4.94 (2H, 2d, J = 7.2 Hz, OCH_2O of CEM), 4.89 (1H, ddd, J = 6.9, 8.4, 8.4 Hz, 3′-H), 4.35 (1H, dd, J = 1.2, 4.8 Hz, 2′-H), 4.21 (1H, d, J = 8.1 Hz, 4′-H), 3.94–3.87 (3H, m, 4″-H, OCH_2CH_2CN), 3.77, 3.74 (6H, 2s, OMe), 3.63–3.52 (3H, m, 5′-H, 6″-H), 3.09–3.03 (1H, m, 6″-H), 2.67 (2H, ddd, J = 2.7, 6.5, 6.5 Hz, OCH_2CH_2CN), 1.65–1.56 (2H, m, 7″-H), 1.25–1.19 (1H, m, 8″-H), 1.03–0.91 (1H, m, 8″-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.0, 158.7, 158.6, 150.1, 144.3, 140.0, 138.0 (d, $^3J_{PC}$ = 4.0 Hz), 135.0, 130.3, 130.1, 128.3, 128.2, 128.0, 127.6, 127.2, 125.5, 117.9, 113.2,

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113.2, 102.1, 94.6, 88.5, 87.1, 82.6 (d, $^2J_{PC}$ = 9.6 Hz), 81.8, 78.4 (d, $^3J_{PC}$ = 3.2 Hz), 69.4 (d, $^2J_{PC}$ = 14.4 Hz), 67.3 (d, $^2J_{PC}$ = 3.2 Hz), 63.1, 60.3, 55.2, 55.2, 47.1 (d, $^2J_{PC}$ = 34.7 Hz), 28.1, 26.0 (d, $^3J_{PC}$ = 3.7 Hz), 18.7. ^{31}P NMR (121 MHz, $CDCl_3$) δ 158.2. ESI-HRMS: m/z calcd for $C_{45}H_{48}N_4O_{10}P^+$ [(M + H)$^+$] 835.3103, found 835.3104.

(*Rp*)-C^{ac}-Monomer [(*Rp*)-3b]. Crude (*Rp*)-**3b** was synthesized from 5′-*O*-DMTr-2′-*O*-CEM-*N*^{4}-acetylcytidine **1b** (1.01 g, 1.5 mmol) following the typical procedure described above and purified by silica gel column chromatography [NH silica gel, hexane−ethyl acetate−triethylamine (20:10:0.03 to 10:30:0.03, v/v/v)]. The fractions containing (*Rp*)-**3b** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford (*Rp*)-**3b** (0.69 g, 0.79 mmol, 53%) as a pale yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.39 (1H, brs, 4-NH), 8.54 (1H, d, J = 7.5 Hz, 6-H), 7.42−7.16 (14H, m, 5″-Ph, 2-H of *p*-An, Ph of DMTr), 6.88 (1H, d, J = 7.5 Hz, 5-H), 6.84−6.77 (4H, dd, J = 6.0, 9.0 Hz, 3-H of *p*-An), 5.94 (1H, s, 1′-H), 5.72 (1H, d, J = 6.3 Hz, 5″-H), 5.15 (1H, d, J = 6.9 Hz, OCH_2O of CEM), 4.98, (1H, d, J = 6.9 Hz, OCH_2O of CEM), 4.84 (1H, ddd, J = 4.8, 9.3, 9.3 Hz, 3′-H), 4.33−4.26 (2H, m, 2′-H, 4′-H), 3.96−3.86 (3H, m, 4″-H, OCH_2CH_2CN), 3.77, 3.75 (6H, 2s, OMe), 3.69−3.50 (3H, m, 5′-H, 6″-H), 3.15−3.04 (1H, m, 6″-H), 2.78−2.59 (2H, m, OCH_2CH_2CN), 2.24 (3H, s, Ac), 1.67−1.51 (2H, m, 7″-H), 1.28−1.17 (1H, m, 8″-H), 1.01−0.93 (1H, m, 8″-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 162.6, 158.7, 158.6, 154.9, 144.8, 144.2, 138.0 (d, $^3J_{PC}$ = 4.0 Hz), 135.1, 130.3, 130.1, 128.3, 128.2, 128.0, 127.6, 127.2, 125.5, 118.1, 113.2, 98.4, 96.4, 94.4, 90.1, 87.1, 82.6 (d, $^2J_{PC}$ = 9.5 Hz), 81.4, 78.2, 68.8 (d, $^2J_{PC}$ = 14.6 Hz), 67.3 (d, $^2J_{PC}$ = 3.5 Hz), 63.0, 59.9, 55.2, 47.1 (d, $^2J_{PC}$ = 34.7 Hz), 28.0, 25.9 (d, $^3J_{PC}$ = 3.5 Hz), 24.9, 18.7. ^{31}P NMR (121 MHz, $CDCl_3$) δ 158.0. ESI-HRMS: m/z calcd for $C_{47}H_{51}N_4O_{10}P^+$ [(M + H)$^+$] 876.3368, found 876.3367.

(*Rp*)-A^{ac}-Monomer [(*Rp*)-3c]. Crude (*Rp*)-**3c** was synthesized from 5′-*O*-DMTr-2′-*O*-CEM-*N*^{6}-acetyladenosine **1c** (0.70 g, 1.0 mmol) following the typical procedure described above and purified by silica gel column chromatography [NH silica gel, toluene−ethyl acetate−triethylamine (20:10:0.03, v/v/v)]. The fractions containing (*Rp*)-**3c** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford (*Rp*)-**3c** (0.44 g, 0.49 mmol, 49%) as a colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.64 (1H, brs, 6-NH), 8.61 (1H, s, 2-H), 8.26 (1H, s, 8-H), 7.45−7.16 (14H, m, 5″-Ph, 2-H of *p*-An, Ph of DMTr), 6.76 (4H, d, J = 9.0 Hz, 3-H of *p*-An), 6.24 (1H, d, J = 4.8 Hz, 1′-H), 5.77 (1H, d, J = 6.3 Hz, 5″-H), 5.06−4.95 (2H, m, 2′-H, 3′-H), 4.90, 4.84 (2H, 2d, J = 7.2 Hz, OCH_2O of CEM), 4.36 (1H, dd, J = 3.0, 6.0 Hz, 4′-H), 3.88 (1H, ddd, J = 6.3, 10.5, 10.5 Hz, 4″-H), 3.81−3.51 (10H, m, 5′-H, 6″-H, OMe, OCH_2CH_2CN), 3.41 (1H, dd, J = 3.6, 10.5 Hz, 5′-H), 3.18−3.06 (1H, m, 6″-H), 2.60 (3H, s, Ac), 2.49 (2H, t, J = 6.3 Hz, OCH_2CH_2CN), 1.69−1.59 (2H, m, 7″-H), 1.28−1.18 (1H, m, 8″-H), 0.98−0.91 (1H, m, 8″-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 158.5, 158.5, 152.3, 150.9, 149.2, 144.4, 141.9, 138.0 (d, $^3J_{PC}$ = 3.7 Hz), 135.5, 135.4, 130.1, 130.1, 128.3, 128.2, 127.8, 127.6, 126.9, 125.5, 122.2, 117.5, 113.1, 95.2, 87.3, 86.7, 83.6, 82.8 (d, $^2J_{PC}$ = 10.0 Hz), 78.0, 71.0 (d, $^2J_{PC}$ = 10.6 Hz), 67.3 (d, $^2J_{PC}$ = 3.2 Hz), 63.1, 62.4, 55.2, 47.0 (d, $^2J_{PC}$ = 34.9 Hz), 28.1, 25.8 (d, $^3J_{PC}$ = 18.1 Hz), 18.6. ^{31}P NMR (121 MHz, $CDCl_3$) δ 157.0. ESI-HRMS: m/z calcd for $C_{48}H_{51}N_7O_9P^+$ [(M + H)$^+$] 900.3480, found 900.3480.

(*Rp*)-G^{ce,pac}-Monomer [(*Rp*)-3d]. Crude (*Rp*)-**3d** was synthesized from 5′-*O*-DMTr-2′-*O*-CEM-*O*^{6}-cyanoethyl-*N*^{2}-phenoxyacetylguanosine **1d** (0.86 g, 1.0 mmol) following the typical procedure described above and purified by silica gel column chromatography [NH silica gel, toluene−ethyl acetate−triethylamine (80:20:0.1, v/v/v)]. The fractions containing (*Rp*)-**3d** were collected, washed with a saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure to afford (*Rp*)-**3d** (0.45 g, 0.42 mmol, 42%) as a colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.79 (1H, brs, 2-NH), 8.10 (1H, s, 8-H), 7.42−7.00 (19H, m, 5″-Ph, 2-H of *p*-An, Ph of DMTr, Ph of Pac), 6.76 (4H, d, J = 6.6 Hz, 3-H of *p*-An), 6.23 (1H, d, J = 5.1 Hz, 1′-H), 5.74 (1H, d, J = 6.3 Hz, 5″-H), 5.01−4.82 (6H, m, 2′-H, 3′-H, OCH_2O of CEM, *O*^{6}-OCH_2CH_2CN), 4.63 (2H, s, CH_2 of Pac), 4.35 (1H, d, J = 2.7 Hz, 4′-H), 3.90−3.42 (12H, m, 5′-H, 4″-H, 6″-H, OMe, OCH_2CH_2CN), 3.19−3.00 (3H, m, 6″-H, *O*^{6}-OCH_2CH_2CN), 2.48 (2H, t, J = 6.3 Hz, OCH_2CH_2CN of CEM), 1.73−1.59 (2H, m, 7″-H), 1.29−1.12 (1H, m, 8″-H), 1.01−0.87 (1H, m, 8″-H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.7, 159.7, 158.5, 158.5, 157.0, 153.0, 151.0, 144.4, 140.8, 138.0 (d, $^3J_{PC}$ = 4.1 Hz), 135.5, 135.4, 130.1, 130.0, 129.8, 128.2, 128.1, 127.9, 127.6, 127.0, 125.5, 122.4, 118.7, 117.6, 116.8, 114.9, 113.1, 95.1, 86.7, 86.6, 83.7, 82.7 (d, $^2J_{PC}$ = 9.5 Hz), 78.2 (d, $^3J_{PC}$ = 3.5 Hz), 71.1 (d, $^2J_{PC}$ = 12.0 Hz), 67.7, 67.3 (d, $^2J_{PC}$ = 3.2 Hz), 63.2, 62.6, 61.8, 55.2, 47.0 (d, $^2J_{PC}$ = 34.7 Hz), 28.0, 25.9 (d, $^3J_{PC}$ = 3.5 Hz), 18.6, 18.0. ^{31}P NMR (121 MHz, $CDCl_3$) δ 156.8. ESI-HRMS: m/z calcd for $C_{57}H_{58}N_8O_{11}P^+$ [(M + H)$^+$] 1061.3957, found 1061.3964.

A General Procedure for Solid-Phase Synthesis of PS-ORNs. Manual solid-phase synthesis of *P*-stereodefined PS-ORNs was performed according to the procedure given in Table 4 by using 5′-*O*-DMTr-uridine-loaded HCP or CPG (0.5 μmol). Automated solid-phase synthesis (**10c,d**) was performed according to the procedure given in Table 5 by using 5′-*O*-DMTr-uridine-loaded HCP (0.25 μmol). After the chain elongation by repeating steps 1−8 in Table 4 or 5, the 5′-*O*-DMTr group was removed by treatment with 3% DCA in CH_2Cl_2 except for those isolated ones, for which the DMTr group was left as a purification handle. The deprotection of the bases and the PS-linkages and the cleavage of the linker were performed by treatment with a 25% NH_3 aqueous solution−EtOH (3:1, v/v) (6 mL) for 3 h (for 2mers), 12 h (for 4mers), or 48 h (for 12mers) at rt. The resultant crude products were analyzed (2−4mers) or purified (12mers) by RP-HPLC. Fractions containing the desired PS-ORN 12mers (2′-*O*-CEM-on−5′-*O*-DMTr-on) were collected and lyophilized. The residue was then treated with a 0.5 M TBAF solution in dry DMSO containing 0.5% CH_3NO_2 (400 μL) for 5 h at rt and diluted with a 0.1 M TEAA buffer solution (pH 7.0) (40 mL). The mixture was purified with a Sep-pak C18 cartridge. The Sep-pak was washed twice with a 0.1 M TEAA buffer solution (pH 7.0) (5.0 mL) to remove DMSO, TBAF,

Table 4. Procedure for Manual Solid-Phase Synthesis of PS-ORNs

step	operation	reagents and solvents	time
1	detritylation	3% DCA in CH_2Cl_2	4 × 15 s
2	washing	(i) CH_2Cl_2, (ii) dry CH_3CN, (iii) drying *in vacuo*	
3	condensation	0.13 M monomer **3a−d**, 1 M activator, dry CH_3CN	5, 10, or 15 min
4	washing	(i) dry CH_3CN, (ii) drying *in vacuo*	
5	capping	0.5 M CF_3COIm and 1 M DMAN in dry THF	30 s
6	washing	(i) dry THF, (ii) dry CH_3CN, (iii) drying *in vacuo*	
7	sulfurization	0.3 M DTD in dry CH_3CN	10 min
8	washing	(i) dry CH_3CN, (ii) drying *in vacuo*	

Table 5. Procedure for Automated Solid-Phase Synthesis of PS-ORNs

step	operation	reagents and solvents	time
1	detritylation	3% DCA in CH_2Cl_2	49 s
2	washing	dry CH_3CN	
3	condensation	0.15 M monomer **3a−d**, 1 M activator, dry CH_3CN	15 min
4	washing	dry CH_3CN	
5	capping	0.5 M CF_3COIm and 1 M DMAN in dry THF	30 s
6	washing	dry CH_3CN	
7	sulfurization	0.3 M DTD in dry CH_3CN	6 min
8	washing	dry CH_3CN	

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and CH_3NO_2, and then the desired PS-ORNs were eluted with 80% CH_3CN and lyophilized. The residue was then treated with an 80% AcOH aqueous solution (500 μL) for 1 h at rt and diluted with a 2 M TEAA buffer solution (pH 7.0) (20 mL). The mixture was desalted with a Sep-pak C18 cartridge. The Sep-pak was washed twice with a 0.1 M TEAA buffer solution (pH 7.0) (5.0 mL), and then the product was eluted with 40% CH_3CN. The eluate was concentrated under reduced pressure and purified by RP-HPLC to afford the desired PS-ORNs. Isolated yields were determined by UV quantitation at 260 nm. All-(*Rp*)-PS-U_{12} **10a**, 12% isolated yield, MALDI-TOF MS: *m*/*z* calcd for $C_{108}H_{132}N_{24}O_{83}P_{11}S_{11}^-$ [(M − H)$^-$] 3785.09, found 3788.62. All-(*Sp*)-PS-U_{12} **10b**, 14% isolated yield, MALDI-TOF MS: *m*/*z* calcd for $C_{108}H_{132}N_{24}O_{83}P_{11}S_{11}^-$ [(M − H)$^-$] 3785.09, found 3789.25. All-(*Rp*)-PS-$(CAGU)_3$ **10c**, 6% isolated yield, MALDI-TOF MS: *m*/*z* calcd for $C_{114}H_{141}N_{45}O_{71}P_{11}S_{11}^-$ [(M − H)$^-$] 3968.29, found 3971.19. All-(*Sp*)-PS-$(CAGU)_3$ **10d**, 10% isolated yield, MALDI-TOF MS: *m*/*z* calcd for $C_{114}H_{141}N_{45}O_{71}P_{11}S_{11}^-$ [(M − H)$^-$] 3968.29, found 3972.53.

Enzymatic Digestion of *P*-Stereodefined PS-ORNs. *Digestion with svPDE.* An aqueous solution (20 μL, pH 8.5) containing a purified PS-ORN (**10a**−**d**) (1.0 nmol), svPDE (0.1×10^{-3} unit), 100 mM Tris-HCl, and 15 mM $MgCl_2$ was incubated for 16 h at 37 °C. The mixture was then diluted with 0.1 M TEAA buffer (pH 7.0) (80 μL), heated for 1 min at 100 °C to inactivate the enzyme, filtrated, and analyzed by RP-HPLC.

Digestion with nP1. An aqueous solution (20 μL, pH 7.2) containing a purified PS-ORN (**10a**−**d**) (1.0 nmol), nuclease P1 (1 unit), 50 mM CH_3COONa, and 1 mM $ZnCl_2$ was incubated for 16 h at 37 °C. The mixture was then diluted with 0.1 M TEAA buffer (pH 7.0) (80 μL), heated at 100 °C for 1 min to inactivate the enzyme, filtrated, and analyzed by RP-HPLC.

Thermal Denaturating Experiment. An aqueous solution (200 μL, pH 7.0) containing a 1:1 ratio of complementary ORNs (0.45 nmol each), 10 mM phosphate, 100 mM NaCl, and 0.1 mM EDTA was deaerated for 10 min under reduced pressure. An aliquot (165 μL) was placed in a 1 cm path length quartz cell. The solution was then heated at a rate of 5 °C/min from rt to 90 °C, kept for 10 min at 90 °C, and cooled at a rate of −2 °C/min from 90 to 0 °C to hybridize the ORNs. After being kept for 90 min at 0 °C, the solution was gradually heated for denaturating experiments. UV absorbance values (260 nm) were recorded at intervals of 0.5 °C while the temperature was ramped at a rate of 0.5 °C/min from 0 to 90 °C under Ar.

■ 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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NOTE ADDED AFTER ASAP PUBLICATION

There were errors in two of the chemical formulas in the version published ASAP August 29, 2012. The correct version reposted September 5, 2012.