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An Oxazaphospholidine Approach for the Stereocontrolled Synthesis of Oligonucleoside Phosphorothioates

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Abstract: The stereocontrolled synthesis of oligodeoxyribonucleoside phosphorothioates (PS-ODNs) using nucleoside 3′-*O*-oxazaphospholidine derivatives as monomer units is described. 2-Chloro-1,3,2-oxazaphospholidine derivatives were prepared from six kinds of enantiopure 1,2-amino alcohols and used for the phosphitylation reactions of 5′-*O*-protected nucleosides. A detailed study of these reactions revealed that the diastereoselectivity of the reaction depended on the structure of the enantiopure 1,2-amino alcohol, the reaction temperature, and the amine used as a scavenger of HCl. In addition, ab initio molecular orbital calculations for the 2-chlorooxazaphospholidine derivatives were carried out to elucidate the mechanism of these diastereoselective phosphitylation reactions. The LUMO of the 2-chloro-5-phenyloxazaphospholidine derivatives on the phosphorus atom was found to be almost orthogonal to the P−Cl bond. This LUMO may be involved in the phosphitylation reactions with predominant retention of the *P*-configuration. A series of dialkyl(cyanomethyl)ammonium salts were developed and used as activators for the condensation reactions of the diastereopure nucleoside 3′-*O*-oxazaphospholidines with 3′-*O*-protected nucleosides. In the presence of the new activators, the reactions proceeded rapidly to give the corresponding dinucleoside phosphite triesters. The diastereoselectivity of the condensation reaction did not depend on the counteranion but on the structure of the dialkyl(cyanomethyl)amine. In the presence of the activator, which consists of a relatively small dialkyl(cyanomethyl)amine, the condensation proceeded with excellent diastereoselectivity. After sulfurization and deprotection, diastereopure (R_p)- and (S_p)-dinucleoside phosphorothioates were obtained in excellent yields. The present methodology was also applied to the solid-phase synthesis of stereoregulated PS-ODNs. *all*-(R_p)-$[T_{PS}]_3T$, *all*-(S_p)-$[T_{PS}]_3T$, *all*-(R_p)-d$[G_{PS}A_{PS}C_{PS}]T$, and *all*-(R_p)-$[T_{PS}]_9T$ were synthesized on a highly cross-linked polystyrene resin.

Introduction

Oligodeoxyribonucleoside phosphorothioates (PS-ODNs) have been recognized as antisense drugs, and further studies and clinical applications have also been tried for various diseases.[1] Recent in vitro studies have shown that the properties of PS-ODNs as antisense oligonucleotides, such as binding affinity to the complementary RNA, stability to nucleases, and RNase H activity, are affected by the configurations of the phosphorus atoms and that PS-ODNs with properly arranged R_p and/or S_p phosphorothioate internucleotidic linkages have much potential as antisense molecules.[2] However, a drawback in the synthesis of PS-ODNs by conventional methods is the possible formation of 2^n diastereoisomers due to the chirality of the phosphorus atoms (n is the number of internucleotidic phosphorothioate linkages);[3] almost all PS-ODNs currently used have been synthesized by nonstereoselective automated phosphoramidite methods.[4] The drawback arises from the lack of an efficient method to produce sufficient quantities of stereodefined PS-ODNs. Therefore, it is highly required to develop a method to produce stereodefined PS-ODNs with productivity comparable to that of PS-ODNs produced by the conventional phosphoramidite method.

Thus, the stereoselective syntheses of PS-ODNs have been extensively studied in recent years.[5,6] Among the studies, the oxathiaphospholane method,[5a] which has been developed by Stec et al., and the method utilizing nucleoside 3′-*O*-(3-*N*-acyl)oxazaphospholidine derivatives as monomer units, which has been developed by Beaucage et al.,[5b] afforded fully stereoregulated PS-ODNs to date. In these methods, the diastereopure monomer units are condensed with nucleosides in the presence of strong

(1) For a review, see: Levin, A. A. *Biochim. Biophys. Acta* **1999**, *1489(1)*, 69−84.
(2) (a) Koziolkiewicz, M.; Krakowiak, A.; Kwinkowski, M.; Boczkowska, M.; Stec, W. J. *Nucleic Acids Res.* **1995**, *23*, 5000−5005. (b) Koziolkiewicz, M.; Wojcik, M.; Kobylanska, A.; Karwowski, B.; Rebowska, B.; Guga, P.; Stec, W. J. *Antisense Nucl. Acid Drug Dev.* **1997**, *7*, 43−48. (c) Yu, D.; Kandimalla, E. R.; Roskey, A.; Zhao, Q.; Chen, L.; Chen, J.; Agrawal, S. *Bioorg. Med. Chem.* **2000**, *8*, 275−284 (d) Inagawa, T.; Nakashima, H.; Karwowski, B.; Guga, P.; Stec, W. J.; Takeuchi, H.; Takaku, H. *FEBS Lett.* **2002**, *528*, 48−52.
(3) Stec, W. J.; Wilk, A. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 709−722 and references therein.
(4) For a review, see: Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223−2311.
(5) (a) Stec, W. J.; Karwowski, B.; Boczkowska, M.; Guga, P.; Koziolkiewicz, M.; Sochacki, M.; Wieczorek, M. W.; Blaszczyk, J. *J. Am. Chem. Soc.* **1998**, *120*, 7156−7167. (b) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 2149−2156. (c) Lu, Y.; Just, G. *Angew. Chem., Int. Ed.* **2000**, *39*, 4521−4524.
(6) (a) Iyer, R. P.; Yu, D.; Ho, N.-H.; Tan, W.; Agrawal, S. *Tetrahedron: Asymmetry* **1995**, *6*, 1051−1054. (b) Iyer, R. P.; Guo, M.-J.; Yu, D.; Agrawal, S. *Tetrahedron Lett.* **1998**, *39*, 2491−2494. (c) Lu, Y.; Just, G. *Tetrahedron* **2001**, *57*, 1677−1687.

bases, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and *N*,*N*,*N*′,*N*′-tetramethylguanidine (TMG), as promoters with nearly complete stereospecificity. However, the diastereopure monomer units have to be prepared through the time-consumed separation of a mixture, containing essentially an equal amount of diastereomers, by column chromatography.

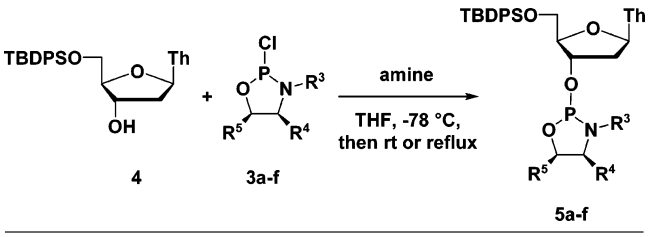
The methods utilizing nucleoside 3′-*O*-oxazaphospholidine or oxazaphosphorine derivatives derived from enantiopure amino alcohols, such as (1*R*,2*S*)-ephedrine and prolinol, as monomer units have been reported in recent years.[6] Contrary to the nucleoside 3′-*O*-(3-*N*-acyl)oxazaphospholidine derivatives, these monomer units with an alkyl group on the nitrogen atom of the oxazaphospholidine or oxazaphosphorine ring can be activated by weak acids, such as 1*H*-tetrazole, to condense with nucleosides. The most promising aspect of these methods is that diastereopure monomers can be obtained from appropriate enantiopure amino alcohols with excellent or complete diastereoselectivity. Despite this advantage, the condensation reactions with 3′-*O*-protected nucleosides are more or less nonstereospecific. The nonstereospecificity would be attributed to the repetitive attacks of a nucleophilic activator, such as 1*H*-tetrazole, on the phosphorus atom.

Under these circumstances, we developed a new class of activators, dialkyl(cyanomethyl)ammonium salts **1**, and presented a preliminary result in the previous communication.[7] In this paper, we report the results of detailed studies on the stereocontrolled synthesis of dideoxyribonucleoside phosphorothioates by an oxazaphospholidine method and on its application to a solid-phase synthesis of PS-ODNs. We also describe a possible mechanism for the diastereoselective formation of nucleoside 3′-*O*-oxazaphospholidine derivatives on the basis of ab initio molecular orbital calculations.

Results and Discussion

Synthesis of Oxazaphospholidine Monomer Units. In the present strategy, enantiopure amino alcohols have to work as chiral auxiliaries for the diastereoselective synthesis of monomer units and also as protecting groups for internucleotidic linkages. This means that these amino alcohols must be effective chiral auxiliaries and be easily removed without the epimerization of the resultant PS-ODN. In addition, both enantiomers of the amino alcohols should be available for the formations of both R_p and S_p internucleotidic phosphorothioate linkages. From these viewpoints, enantiopure 1,2-amino alcohols are considered to be suitable candidates, since it has been reported that the reactions of 5′-*O*-protected nucleosides with 2-chloro-1,3,2-oxazaphospholidines derived from some enantiopure 1,2-amino alcohols afford the *trans*-nucleoside 3′-*O*-oxazaphospholidine monomers diastereoselectively[6a,b] and that 2-aminoethyl moieties derived from 1,2-amino alcohols can be removed without epimerization of the resultant phosphorothioates.[5b] Thus, we selected six kinds of enantiopure 1,2-amino alcohols (**2a**−**f**), both enantiomers of which can be easily obtained,[8] and carried out an extensive study on the synthesis of nucleoside 3′-*O*-oxazaphospholidine monomer units. 2-Chlorooxazaphospholidine derivatives **3a**−**f** were synthesized from **2a**−**f** and phosphorus trichloride according to the procedure described in the literature,[6a] and allowed to react with 5′-*O*-(*tert*-butyldiphenylsilyl)thymidine [5′-*O*-(TBDPS)thymidine] (**4**) under various reaction conditions to prepare the nucleoside 3′-*O*-oxazaphospholidine derivatives **5a**−**f** (Table 1). Excellent diastereoselectivity was achieved when **3a**,**c** were used, although the suitable reaction conditions were different; in the case of **3a**, the reaction under kinetic conditions (see the Experimental Section) resulted in excellent diastereoselectivity (*trans*-**5a**:*cis*-**5a** = 95:5, entry 1),[8] and in the case of **3c**, the reaction under thermodynamic conditions (see the Experimental Section) gave the best result (*trans*-**5c**:*cis*-**5c** = 97:3, entry 10). Among the resultant compounds, diastereopure *trans*-**5a**−**d** were very easily isolated by simple silica gel column chromatography in modest to excellent yields, and used as the monomer units for a study on the condensation reactions with 3′-*O*-protected nucleosides (entries 1, 7, 10, and 11).

Table 1. Synthesis of 5′-*O*-(TBDPS)thymidine 3′-*O*-Oxazaphospholidines **5a**−**f**



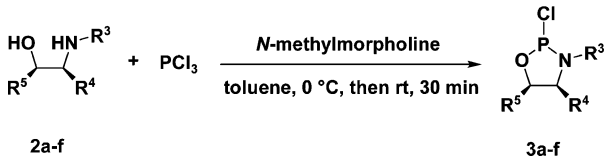
entry	**5**	R^3	R^4	R^5	amine	reaction conditions	*trans*:*cis*	isolated yield[a,b] (%)
1	**a**	Me	H	Ph	Et_3N	rt,[c] 30 min	95:5	77
2					Et_3N	reflux, 1 h	59:41	
3					*i*-Pr_2NEt	rt, 30 min	88:12	
4	**b**	Me	Me	Ph	Et_3N	rt, 30 min	70:30	
5					Et_3N	rt, 12 h	72:28	
6					Et_3N	reflux, 1 h	87:13	
7					*i*-Pr_2NEt	rt, 30 min	82:18	64
8	**c**	Me	Ph	Ph	Et_3N	rt, 30 min	58:42	
9					*i*-Pr_2NEt	rt, 30 min	92:8	
10					*i*-Pr_2NEt	reflux, 1 h	97:3	96
11	**d**	*i*-Pr	H	Ph	Et_3N	rt, 30 min	93:7	64
12					Et_3N	reflux, 1 h	67:33	
13	**e**	Me	H	*i*-Pr	Et_3N	rt, 30 min	85:15	
14					Et_3N	reflux, 1 h	64:38	
15					*i*-Pr_2NEt	rt, 30 min	71:29	
16	**f**	Me	*i*-Pr	H	Et_3N	rt, 30 min	20:80	
17					Et_3N	reflux, 1 h	76:24	
18					*i*-Pr_2NEt	rt, 30 min	44:56	

[a] Diastereopure *trans*-**5a**−**d** were isolated by silica gel column chromatography. [b] Isolation of *trans*-**5** was not attempted in entries 2−6, 8, 9, and 12−18. [c] rt = room temperature.

Stereochemistry of the Diastereoselective Phosphitylation Reactions. The results summarized in Table 1 show that the diastereoselectivity for the formation of **5** dramatically varied with the structure of the phosphitylating agent **3**, the reaction temperature, and the amine used as a scavenger of HCl. For example, *cis*-**5f** was mainly obtained under kinetic conditions (*trans*-**5f**:*cis*-**5f** = 20:80, entry 16), while *trans*-**5f** was the major isomer under thermodynamic conditions (*trans*-**5f**:*cis*-**5f** = 76:24, entry 17). This result is in good agreement with our assumption that the *trans*-isomer, which may be thermodynamically more stable than the corresponding *cis*-isomer because of the steric repulsion between the chlorine atom and the substituent (R^4) on the oxazaphospholidine ring, would be generated preferentially. In contrast, **5a** was obtained with excellent diastereoselectivity under kinetic conditions (*trans*-**5a**:*cis*-**5a** =

(7) Oka, N.; Wada, T.; Saigo, K. *J. Am. Chem. Soc.* **2002**, *124*, 4962−4963.
(8) See the Supporting Information.

Table 2. Synthesis of 2-Chloro-1,3,2-oxazaphospholidine Derivatives **3a**−**f**



2a-f 3a-f

entry	5	R^3	R^4	R^5	^{31}P chemical shifts of the crude **3a**–**f**[a]	isolated yield[b,c] (%)
1	**a**	Me	H	Ph	172.6 (172.4, 171.4)[d]	65
2	**b**	Me	Me	Ph	171.4 (171.5)	52
3	**c**	Me	H	Ph	171.8[e]	
4	**d**	*i*-Pr	Me	Ph	170.7[f]	
5	**e**	Me	H	*i*-Pr	173.1 (173.7, 172.5)	60
6	**f**	Me	*i*-Pr	H	176.8 (176.7)	70

[a] In parentheses, the ^{31}P chemical shifts of distilled **3** are given. [b] **3a**,**b**,**e**,**f** were purified by distillation under reduced pressure. [c] **3c**,**d** were not purified. [d] Small broad signals (ca. 5%) were observed at around 165 ppm. [e] A small broad signal (ca. 5%) was observed at 175.4 ppm. [f] The obviously broad signal indicated that the product was a mixture of the *trans*- and *cis*-isomers.

Table 3. Stereocourse of the Phosphitylations under Kinetic Conditions

3	stereocourse	**5**
3a,**d**,**e** (mixture)		**5a**,**d**,**e** (*trans*-rich)
3b,**c** (*trans*-rich)	retention	**5b**,**c** (*trans*-rich)
3f (*trans*-rich)	inversion	**5f** (*cis*-rich)

95:5, entry 1), whereas thermodynamic conditions gave **5a** with miserable diastereoselectivity (*trans*-**5a**:*cis*-**5a** = 59:41, entry 2). This phenomenon cannot be explained by our assumption. Thus, we tried to elucidate the mechanism of the present diastereoselective phosphitylation reaction.

At first, the *P*-configurations of **3a**−**f** were justly determined to make the stereocourses of the reactions clear. ^{31}P NMR spectra of crude **3a**−**f**, containing a slight amount of the HCl salt produced during the synthesis of **3**, fundamentally showed a broad signal in a range of 180−170 ppm (Table 2). In contrast, in the ^{31}P and ^{1}H NMR spectra of the distilled **3a**,**e**, the signals of the phosphorus atom and the proton at the 5-position in the oxazaphospholidine ring were split into two broad singlets (ca. 1:1, entries 1 and 5). These observations indicate that **3a**,**e** are a ca. 1:1 mixture of the *trans*- and *cis*-isomers, and that Cl^- quickly and repetitively attacked the phosphorus atom. However, when the concentration of Cl^- was reduced by distillation, the rate of the isomerization between the *trans*- and *cis*-isomers decreased to be observed individually by NMR spectroscopies. Similarly, **3d** was assumed to be a mixture of *trans*- and *cis*-isomers because the signal of the 5-H in the oxazaphospholidine ring of **3d** was obviously broad, even though the signal was not split distinctly into two singlets.[8] This explanation is strongly supported by the fact that the corresponding NMR signals coalesced to a broad singlet again upon addition of a small amount of *N*-methylmorpholinium chloride to the solutions of distilled **3a**,**e**. On the other hand, **3b**,**c**,**f** were considered to exist as a single diastereomer on the basis of their ^{1}H and ^{31}P NMR spectra.

Combining the results of the phosphitylations and the elucidation of the stereochemistry of the phosphitylating agents **3**, the stereocourses of the phosphitylation reactions, carried out under kinetic conditions, can be summarized as shown in Table 3. Despite **3a** being a mixture of *trans*- and *cis*-isomers, excellent diastereoselectivity was achieved when **3a** was used as a phosphitylating agent as shown in Table 1 (*trans*-**5a**:*cis*-**5a** = 95:5). To explain this anomalous phenomenon of the diastereoenrichment from ca. 1:1 to 95:5, we carried out ab initio MO calculations for **3a**.

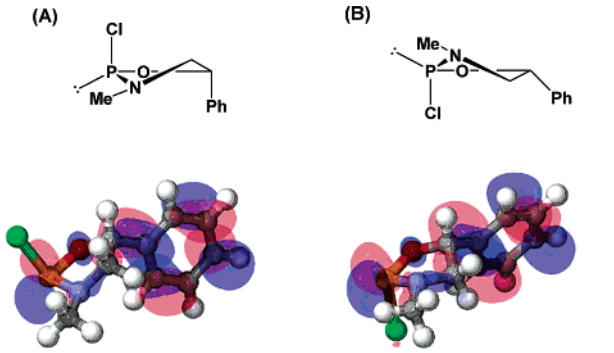


Figure 1. LUMO of the 2-chloro-1,3,2-oxazaphospholidines (A) *trans*-**3a** and (B) *cis*-**3a** calculated at the HF/6-31G* level.

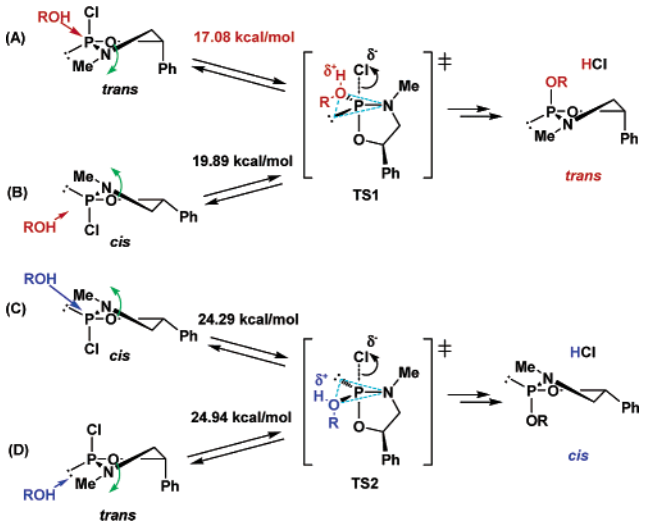


Figure 2. Possible pathways and the activation energies for the reactions of *trans*-**3a** and *cis*-**3a** with an alcohol.

The optimized geometries and the LUMOs of *trans*-**3a** and *cis*-**3a** were calculated at the HF/6-31G* level (Figure 1). The calculations surprisingly showed that the LUMO on the phosphorus atom of **3a** is almost orthogonal to the P−Cl bond, even though such an unoccupied molecular orbital (UMO), orthogonal to the P−Cl bond, usually has higher energy than the LUMO. This unique LUMO of **3a** is considered to be stabilized by the interaction of the UMO orthogonal to the P−Cl bond with the UMO of the phenyl group at the 5-position in the oxazaphospholidine ring. The resultant LUMO would induce the nucleophilic attack of an alcohol from the backside of the P−N or P−O bond. Thus, four reaction pathways via trigonal bipyramidal transition states would be possible (Figures 2 and 3). The activation energy of each pathway was then calculated at the B3LYP/6-31G*//HF/6-31G* level, using methanol as a nucleophile in place of the nucleoside **4** (Figure 2). The calculations showed that the activation energy of pathway A, which gives the *trans*-isomer with *retention of configuration* by the nucleophilic attack of methanol from the backside of the P−N bond, is the smallest (17.08 kcal/mol). Taking into account the existence of fast equilibrium between *trans*-**3a** and

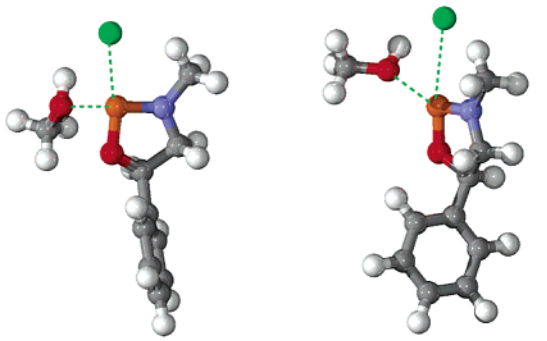


Figure 3. Transition-state structures for the reaction of **3a** with methanol calculated at the HF/6-31G* level.

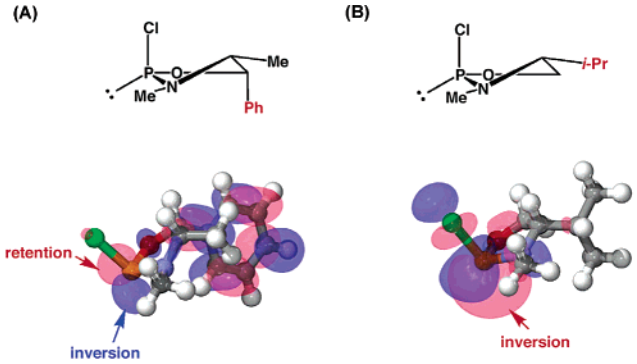


Figure 4. LUMO of the 2-chloro-1,3,2-oxazaphospholidines (A) **3b** and (B) **3f** calculated at the HF/6-31G* level.

cis-**3a**, which is promoted and accelerated by a HCl salt formed during the condensation, *trans*-**5a** would be produced predominantly from a mixture of *trans*-**3a** and *cis*-**3a**. This explanation is consistent with the experimental result.

The new theoretical model can also be applied for the explanation of the diastereoselectivity of the reaction of **3b** with **4**. The calculation indicated that the LUMO on the phosphorus atom of **3b** is almost orthogonal to the P−Cl bond in analogy with **3a** (Figure 4A). Since **3b** exists completely in a *trans* form and since there is no equilibrium between the *trans*- and *cis*-isomers, two reaction pathways, analogous to pathways A and D in the case of **3a** (Figure 2), would be considered. The activation energies for these pathways were then calculated at the B3LYP/6-31G*//HF/6-31G* level. The activation energy for giving *trans*-**5b** is found to be smaller by 2.87 kcal/mol than that for giving *cis*-**5b**. Therefore, the pathway giving *trans*-**5b** would be more favorable. However, *cis*-**5b** may be formed to some extent because the difference between the activation energies in not enough to prevent the reaction through the pathway analogous to pathway D in Figure 2. As a result, the condensation of **3b** with **4** gave a mixture of *trans*-**5b** and *cis*-**5b** in a ratio of 70:30.

The predominant inversion of the *P*-configuration in the reaction of **3f** with **4** can also be explained on the basis of the new model. In the case of **3f**, there is a large LUMO on the backside of the P−Cl bond in analogy with the common trivalent organophosphorus compounds having a leaving group (Figure 4B). Thus, the reaction of **3f** with an alcohol would be most likely to proceed through an S_N2 mechanism with inversion of configuration; this is consistent with the experimental result (*trans*-**5f**:*cis*-**5f** = 20:80).

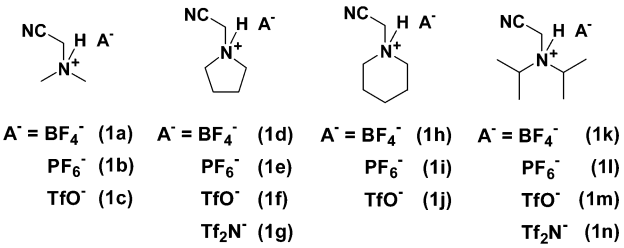


Figure 5. Dialkyl(cyanomethyl)ammonium salts **1a**−**n**.

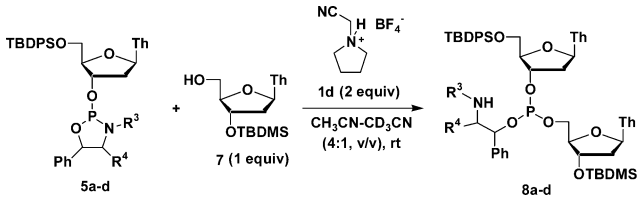
Development of a New Class of Activators. For a new class of activators it is essential to promote the condensation of diastereopure nucleoside 3′-*O*-oxazaphospholidine monomers with a nucleoside without any loss of the diastereopurity. However, the loss of diastereopurity would be unavoidable in the presence of a conventional, nucleophilic activator such as 1*H*-tetrazole, due to its repetitive attacks on the phosphorus atom. Thus, we tried to devise a novel type of activator to solve the problem; an ideal activator should consist of less nucleophilic components and can activate a phosphoramidite only by the protonation of the nitrogen atom of the phosphoramidite with a strong proton-donating ability. Strong acids, however, tend to protonate the phosphorus atom of a phosphoramidite to deactivate the P−N bond.[9] In addition, in the presence of a strong acid, some side reactions, such as the cleavage of a 5′-*O*-DMTr group used as a protecting group, would occur.

Then, we focused our attention on ammonium salts as activators,[10] since the acidity and nucleophilicity of the components can be precisely tuned by using the combinations of various amines and acids. From some candidates, dialkyl(cyanomethyl)amines **6a**−**d** were selected as amino components, since their protonated forms have a little stronger acidity than the conventional activator 1*H*-tetrazole [pK_{BH^+} of *N*-(cyanomethyl)piperidine (**6c**), 4.55; pK_a of 1*H*-tetrazole, 4.90].[11] As acidic components, four kinds of acids, which generate a less nucleophilic conjugate anion, were tested.[9,12] All of the new activators could be easily prepared by simple mixing of an *N*-(cyanomethyl)amine with an acid and had good solubility in CH_3CN (>1 M). Compounds **1a**,**d**,**e**,**i** (Figure 5) were very deliquescent and had to be handled in an inert atmosphere. On the other hand, **1f**,**j**,**m** (Figure 5) were a little deliquescent and could be handled in air, and **1b**,**c**,**g**,**h**,**k**,**l**,**n** (Figure 5) were not deliquescent. Using **1a**−**n**, an extensive study on the condensation of the diastereopure nucleoside 3′-*O*-oxazaphospholidine monomers **5** with a nucleoside was carried out.

Effect of the Oxazaphospholidine Ring Structure on the Diastereoselective Condensation. To investigate the effect of the oxazaphospholidine ring structure on the diastereoselectivity of the condensation, diastereopure 5′-*O*-(TBDPS)thymidine 3′-*O*-oxazaphospholidine derivatives **5a**−**d** were allowed to react

(9) (a) Nurminen, E. J.; Mattinen, J. K.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **2001**, *11*, 2159−2165. (b) Nurminen, E. J.; Mattinen, J. K.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **2000**, *11*, 2238−2240.
(10) Various ammonium salts have already been employed as activators for the phosphoramidite method. However, these activators consist of nucleophilic components, and would cause *P*-epimerization of the monomer unit. (a) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859−1862. (b) Arnold, L.; Tocik, Z.; Bradkova, E.; Hostomsky, Z.; Paces, V.; Smrt, J. *Collect. Czech. Chem. Commun.* **1989**, *54*, 523−532. (c) Hayakawa, Y.; Kawai, R.; Hirata, A.; Sugimoto, J.; Kataoka, M.; Sakakura, A.; Hirose, M.; Noyori, R. *J. Am. Chem. Soc.* **2001**, *123*, 8165−8176.
(11) *Lange's Handbook of Chemistry*, 15th ed.; McGraw-Hill: New York, 1999.
(12) Nifantyev, E. E.; Gratchev, M. K.; Burmistrov, S. Y.; Antipin, M. Y.; Struchkov, Y. T. *Phosphorus Sulfur* **1992**, *70*, 159−174.

Table 4. Condensation of **5a**−**d** with **7** in the Presence of **1d**



entry	**5**	R^3	R^4	configuration[a]	reaction time	dr of **8**[b,c]
1	(S_p)-**5a**	Me	H	(2*S*,5*S*)	<5 min	>99:1 (140.1)
2	(R_p)-**5a**	Me	H	(2*R*,5*R*)	<5 min	96:4 (140.1, 138.1)
3	(R_p)-**5b**	Me	Me	(2*R*,4*S*,5*R*)	<15 min	95:5 (139.8, 137.4)
4	(R_p)-**5c**	Me	Ph	(2*R*,4*S*,5*R*)	5 h	93:7 (140.4, 138.0)
5	(R_p)-**5d**	*i*-Pr	H	(2*R*,5*R*)	<5 min	98:2 (140.9, 138.5)

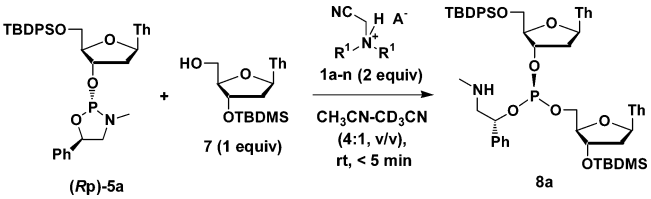
[a] The configuration of the oxazaphospholidine ring. [b] The dr was determined by ^{31}P NMR. [c] In parentheses, the ^{31}P chemical shifts of **8** are given.

with 3′-*O*-(*tert*-butyldimethylsilyl)thymidine [3′-*O*-(TBDMS)-thymidine] (**7**) in the presence of **1d**, and the reactions were monitored by ^{31}P NMR spectroscopy. The results are summarized in Table 4. The rate of condensation strongly depended on the substituent R^4 in the oxazaphospholidine ring. In the cases of **5a**,**d** (R^4 = H), the condensations were completed within 5 min, whereas the reaction of **5b** (R^4 = Me) was completed within 15 min and the reaction of **5c** (R^4 = Ph) required 5 h to complete. This dependence on the substituent R^4 can be attributed to the bulkiness and the electron-withdrawing character of the substituent R;[4] the large and/or electron-withdrawing substituent R^4 on the oxazaphospholidine ring would retard the N^3-protonation of the oxazaphospholidine derivatives to diminish the reaction rate. On the contrary, the reaction rate was not affected by the bulkiness of the substituent R^3 at the 3-position in the oxazaphospholidine ring (entry 5); the reaction of **5d** (R^3 = *i*-Pr) was completed within 5 min, which is comparable to the reaction time of **5a** (R^3 = Me). The N^3-*i*-Pr group would not retard the N^3-protonation because of the free rotation of the N^3−*i*-Pr bond and/or because of the stronger electron-donating ability of the isopropyl group than the methyl group.

The diastereoselectivity of the reaction of (R_p)-**5a** depended on the substituent R;[4] **5** having a less bulky substituent as the R^4 showed better diastereoselectivity. As a result, **5a** (R^4 = H) gave the best result. The monomers, derived from (*R*)- and (*S*)-2-methylamino-1-phenylethanol, gave different results; the reaction of (S_p)-**5a** with **7** afforded only one diastereomer (entry 1). This means that the combination of (S_p)-**5a** and **7** is matching whereas the combination of (R_p)-**5a** and **7** is mismatching for the present diastereoselective reaction activated by **1d**.

Effect of the Activators on the Diastereoselective Condensation. Next, the effect of the activators on the condensation reaction was determined. Diastereopure (R_p)-**5a** was allowed to condense with **7** in the presence of a series of the new activators **1a**−**n**, and the reactions were monitored by ^{31}P NMR spectroscopy. The results are summarized in Table 5. All of the reactions were completed within 5 min. The diastereoselectivity of the reaction was little dependent on the counteranion of the activators, probably because of their lower nucleophilicity. On the other hand, the structure of the *N*-(cyanomethyl)amines, which play an important role in the protonation of the N^3 of the monomer, was found to affect the

Table 5. Condensation of (R_p)-**5a** with **7** in the Presence of **1a**−**n**



entry	**1**	R^1	A^-	(S_p)-**8a**:(R_p)-**8a**[a]
1	**a**	CH_3	BF_4^-	>99:1 (140.1)
2	**b**		PF_6^-	>99:1 (140.3)
3	**c**		TfO^-	>99:1 (139.9)
4	**d**	$(CH_2)_4$	BF_4^-	96:4 (140.1, 138.1)
5	**e**		PF_6^-	>99:1 (140.3)
6	**f**		TfO^-	>99:1 (139.8)
7	**f**[b]		TfO^-	96:4 (139.9, 138.2)
8	**g**		Tf_2N^-	98:2 (140.4, 138.3)
9	**h**	$(CH_2)_5$	BF_4^-	96:4 (140.0, 138.1)
10	**i**		PF_6^-	97:3 (140.2, 138.2)
11	**j**		TfO^-	99:1 (139.9, 138.1)
12	**k**	*i*-Pr	BF_4^-	55:45 (140.0, 138.1)
13	**l**		PF_6^-	56:44 (140.2, 138.2)
14	**m**		TfO^-	39:61 (139.8, 138.2)
15	**n**		Tf_2N^-	34:66 (140.3, 138.3)

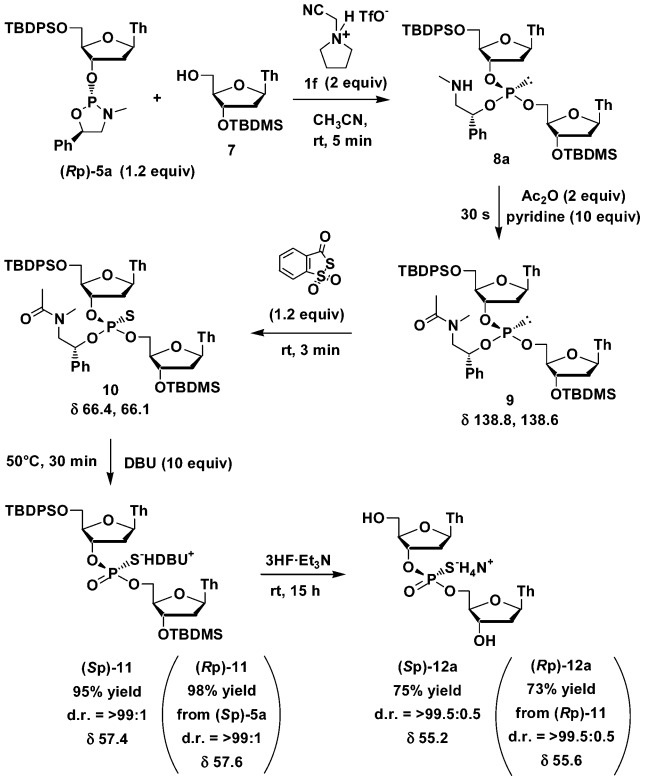
[a] In parentheses, the ^{31}P chemical shifts of **8a** are given. [b] (R_p)-**5a** contaminated with 4 mol % of the corresponding *cis*-isomer [(2*S*,5*R*)-**5a**] was used as a monomer unit.

diastereoselectivity; the diastereoselectivity varied in the following order: *N*-(cyanomethyl)dimethylamine ≥ *N*-(cyanomethyl)pyrrolidine ≥ *N*-(cyanomethyl)piperidine ≫ *N*-(cyanomethyl)diisopropylamine. In the presence of **1a**−**c**,**e**,**f**, the condensations proceeded with complete diastereoselectivity to afford only one diastereoisomer (entries 1−3, 5, and 6). In contrast, the diastereoselectivities dramatically decreased when **1k**−**n** were used as activators (entries 12−15). From the viewpoints of easy preparation and handling, sufficient solubility in CH_3CN, and satisfactory diastereoselectivity, **1f** was used in the following studies.

Conversion to (R_p)- and (S_p)-Dithymidine Phosphorothioates. Diastereopure dinucleoside phosphite **8a**, obtained by the condensation of (R_p)-**5a** (1.2 equiv) with **7** in the presence of **1f**, was treated with acetic anhydride and pyridine for the acetylation of the methylamino function, which was essential to prevent side reactions, and then sulfurized by the Beaucage reagent (Scheme 1).[13] Although a pair of signals was observed in the ^{31}P NMR spectra of **9** and **10**, the phenomenon would be attributable to the existence of acetamide rotamers.[5b] The acetylated chiral auxiliary could be removed by treatment with 10 equiv of DBU for 30 min at 50 °C to afford 5′-*O*- and 3′-*O*-silylated dithymidine phosphorothioate **11** in excellent yield without any epimerization.[7] Alternatively, the chiral auxiliary was found to be removed by treatment with 25% NH_3–pyridine for 30 min at 55 °C.[5b] The diastereomer ratio (dr) of **11** was determined to be >99:1 by ^{31}P NMR spectroscopy. Finally, the 5′-*O*- and 3′-*O*-silyl groups were removed by treatment with 3HF·Et_3N[14] to afford fully deprotected dimer **12a**. The resultant dimer was almost diastereopure (dr = >99.5:0.5), and the *P*-configuration of the dimer was found to be S_p by RP-HPLC

(13) Iyer, R. P.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 1253−1254.
(14) Gasparutto, D.; Livache, T.; Bazin, H.; Duplaa, A.-M.; Guy, A.; Khorlin, A.; Molko, D.; Roget, A.; Teoule, R. *Nucleic Acids Res.* **1992**, *20*, 5159−5166.

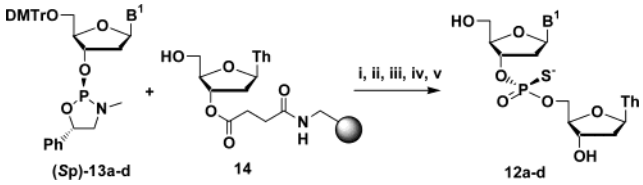
Scheme 1. Synthesis of (R_p)- and (S_p)-Dithymidine Phosphorothioates (R_p)-**12a** and (S_p)-**12a**



analysis.[15] Diastereopure (R_p)-**12a** could also be obtained from (S_p)-**5a** according to the same procedure. The exclusive production of (R_p)-**12a** and (S_p)-**12a** from (S_p)-**5a** and (R_p)-**5a**, respectively, in the presence of **1f** strongly indicates that the condensation of **5a** with **7** in the presence of **1f** proceeds with complete inversion of configuration at the phosphorus atom, upon assuming that the sulfurization by the Beaucage reagent and the removal of the chiral auxiliary by DBU treatment proceed with retention of configuration.

Solid-Phase Synthesis. The results of the solution-phase synthesis indicate that the present method would be sufficiently applicable to the solid-phase synthesis of PS-ODNs since the condensation proceeded rapidly with excellent diastereoselectivity. Then, we tried the solid-phase synthesis of PS-ODNs by the present method. Diastereopure 5′-*O*-(DMTr)nucleoside 3′-*O*-oxazaphospholidine monomers **13a**−**d** could be synthesized in 62−75% isolated yields in a manner similar to the procedure for the preparation of **5**; the phosphitylation reactions giving **13a**−**d** were compeleted within 30 min with good diastereoselectivity in a range of 93:7 to 96:4.[7,8,16] At first, dithymidine phosphorothioate was synthesized on the conventional solid supports, a controlled pore glass (CPG) and a highly cross-linked polystyrene (HCP),[17] using (S_p)-**13a** and **1f** (Table 6). The condensation on the HCP proceeded with excellent diastereoselectivity, whereas the diastereoselectivity of the condensation on the CPG was extremely low (Table 6, entries 1 and 2).[18] Thus, the HCP was selected as a solid support. However, the diastereoselectivity must be further improved for the efficient

Table 6. Solid-Phase Synthesis of Dinucleoside Phosphorothioates **12a**−**d**[a]



entry	solid support	**13**	B^1	concn of **13** (M)	reaction time (min)	(R_p)-**12**:(S_p)-**12**
1	CPG	**a**	Th	0.1	5	64:36
2	HCP	**a**	Th	0.1[b]	5	97:3
3	HCP	**a**	Th	0.2[c]	5	98:2
4	HCP	**a**	Th	0.2	1.5	99:1
5[d]	HCP	**a**	Th	0.2	1.5	2:98
6	HCP	**b**	Ad^{bz}	0.2	1.5	97:3
7[e]	HCP	**c**	Cy^{bz}	0.2	1.5	99:1
8	HCP	**d**	$Gu^{ce,ibu}$	0.2	1.5	98:2
9[f]	HCP	**a**	Th	0.2	1.5	99:1
10[g]	HCP	**a**	Th	0.2	1.5	96:4

[a] Reagents and conditions: (i) **1f**, CH_3CN, rt; (ii) Ac_2O, *N*-methylimidazole, THF, rt, 30 s; (iii) Beaucage reagent, CH_3CN, rt, 60 s; (iv) 3% TCA, CH_2Cl_2, rt; (v) 25% NH_3(aq)−pyridine (9:1, v/v), 55 °C. [b] A 10 μmol sample of (S_p)-**13a** and 50 μmol of **1f** were used for the condensation with **14** (0.5 μmol). [c] A 20 μmol sample of (S_p)-**13a** and 50 μmol of **1f** were used for the condensation with **14** (0.5 μmol). [d] (R_p)-**13a** (*trans*:*cis* = 99:1) was used as a monomer unit. [e] A 1.0 M solution of **1f** in CH_3CN was used for the condensation since in the case of using 0.5 M **1f** the coupling efficiency was relatively low (ca. 90%). [f] After the condensation step, the resultant phosphite triester intermediate was treated with a 0.5 M solution of **1f** in CH_3CN for 5 min. [g] (S_p)-**13a** was treated with a 0.5 M solution of **1f** in CH_3CN for 5 min prior to being added to **14**.

synthesis of PS-ODNs. We then considered that the loss of the diastereopurity during the synthesis would be attributed to the epimerization of the monomer unit (S_p)-**13a** by acidic **1f** prior to the condensation, because a large excess amount of **1f** was used in the solid-phase synthesis. On the basis of this consideration, we carried out the condensation upon increasing the concentration of (S_p)-**13a** to accelerate the condensation and shortening the condensation time to prevent the epimerization. In fact, the diastereoselectivity was improved upon these modifications (entries 3 and 4). Under the optimized conditions shown in entry 4, the dinucleoside phosphorothioates could be synthesized with excellent diastereoselectivity (98:2 to 99:1) and average coupling yields (98 to >99%, entries 4−8).

Our assumption that the epimerization of the monomer unit (S_p)-**13a** is the main factor for the deterioration of the diastereopurity was also confirmed by the following reactions. When the dithymidine phosphite intermediate, obtained by the condensation of (S_p)-**13a** with **14**, was treated with a 0.5 M solution of **1f** in CH_3CN for 5 min, no deterioration of the diastereoselectivity of **12a** was observed (entry 9). On the contrary, when (S_p)-**13a** was preactivated with a 0.5 M solution of **1f** in CH_3CN for 5 min and then allowed to condense with **14**, the reaction afforded **12a** with a little lower diastereoselectivity (96:4, entry 10). These results strongly indicate that

(15) (R_p)-$T_{PS}T$ eluted faster than (S_p)-$T_{PS}T$ in RP-HPLC. Wada, T.; Kobayashi, N.; Mori, T.; Sekine, M. *Nucleosides Nucleotides* **1998**, *17* (1−3), 351−364.
(16) B^1 = Th (**13a**); B^1 = Ad^{bz} (**13b**); B^1 = Cy^{bz} (**13c**); B^1 = $Gu^{ce,ibu}$ (**13d**).
(17) McCollum, C.; Andrus, A. *Tetradedron Lett.* **1991**, *32*, 4069−4072.
(18) The relatively low coupling efficiency and the extremely low diastereoselectivity in the case of using the CPG may be attributed to the hydrophilic nature of the CPG due to the silanol groups on the surface. The hydrophilic CPG tends to retain water which hydrolyzes the monomer unit to reduce the concentration of the monomer unit during the coupling step.[17] Nucleophilic silanols also reduce the concentration of the monomer unit.[17] A sufficiently high concentration of the monomer unit is necessary for an excellent diastereoselectivity as well as for an excellent coupling yield as described below.

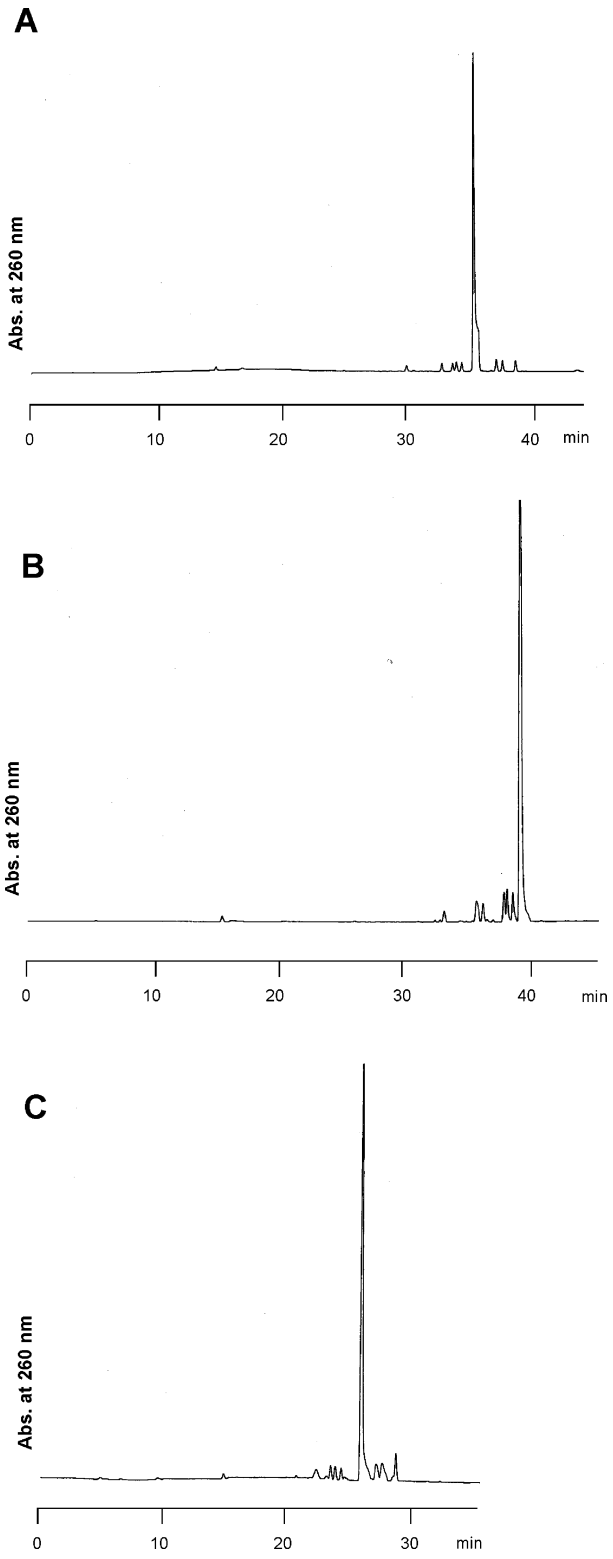


Figure 6. Reversed-phase HPLC profiles of the crude tetramers: (A) **15**; (B) **16**; (C) **17**. RP-HPLC was performed with a linear gradient of 0−20% acetonitrile in 0.1 M ammonium acetate buffer (pH 7.0) at 50 °C for 60 min at a rate of 0.5 mL/min.

(S_p)-**13a** is partially epimerized by acidic **1f** prior to the condensation with **14**; the condensation should be carried out in as a short time as possible with a high concentration of (S_p)-**13a** for achieving excellent diastereoselectivity.

Under the optimized conditions, *all*-(R_p)-[T_{PS}]$_3$T (**15**), *all*-(S_p)-[T_{PS}]$_3$T (**16**), *all*-(R_p)-d[$C_{PS}A_{PS}G_{PS}$]T (**17**), and *all*-(R_p)-[T_{PS}]$_9$T (**18**) were synthesized manually on an HCP resin. The average coupling yields were estimated to be 98% in the cases of tetramers and 97% in the case of a decamer by $DMTr^+$ assay. RP-HPLC profiles of the crude **15**−**18** and purified **18** are shown in Figures 6 and 7. The yields and the average diastereoselectivities of **15**−**17** were determined on the basis of the RP-HPLC profiles to be 80% (**15**), 78% (**16**), and 75% (**17**) (yields) and >97% (**15**), >95% (**16**), and >94% (**17**) (selectivities) (Figure 6). **18** was isolated by RP-HPLC in 35% yield (Figure 7B), and its diastereopurity was estimated to be >96% on the basis of the main peak area of the RP-HPLC chromatogram of the products produced by the incubation of the purified **18** with nuclease P1 for 1 h.[19]

15, **17**, and purified **18** were completely digested by incubation with snake venom phosphodiesterase for 1 h at 37 °C,[20] and crude **16** was completely digested by incubation with nuclease P1 for 1 h at 37 °C. These results indicate that the present method gave fully *P*-stereoregulated PS-ODNs as the main products.

Conclusion

We have developed a new efficient method for the highly diastereoselective formation of internucleotidic phosphorothioate linkages. The present new oxazaphospholidine method using a new activator (**1f)** enabled us to produce stereoregulated PS-ODNs. This method is expected to be applied to an automated solid-phase synthesis directly, since the steps of this method are identical to those used in the conventional automated phosphoramidite method. This is a great advantage from a viewpoint of the large-scale production of stereoregulated PS-ODNs. In addition, this method has possibilities to produce other stereoregulated backbone-modified DNA analogues. This strategy is also applicable to the synthesis of chimeric PO/PS-ODNs containing stereodefined phosphorothioate linkages upon switching the oxidation/sulfurization steps.

Additionally, new mechanistic aspects of diastereoselective formation of 2-alkoxyoxazaphospholidine derivatives could be proposed on the basis of ab initio MO calculations. The information would facilitate the stereoselective synthesis of a wide variety of chiral phosphorus compounds, which have been reported to be useful for many purposes[21] as well as the precursors of the DNA analogues.[5b,6,7]

Experimental Section

General Information. ^{1}H NMR spectra were obtained at 300 MHz on a Varian MERCURY 300 spectrometer with tetramethylsilane (TMS) as an internal standard in $CDCl_3$, with TMS as an external standard in CD_3CN, and with 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid sodium salt (DSS) as an external standard in D_2O. ^{13}C NMR spectra were obtained at 75.45 MHz on a Varian MERCURY 300 spectrometer with $CDCl_3$ as an internal standard (δ 77.0) in $CDCl_3$, with TMS as an external standard in CD_3CN, and with DSS as an external standard in D_2O. ^{31}P NMR spectra were obtained at 121.5 MHz on a Varian MERCURY 300 spectrometer using 85% H_3PO_4 as an external standard. IR spectra were recorded on a JASCO FT/IR-480 Plus spectrophotometer. UV/vis spectra were recorded on a JASCO V-550 UV/vis spectrophotometer. Melting points were measured using a YAMATO MP-21 and are

(19) Potter, B. V. L.; Connolly, B. A.; Eckstein, F. *Biochemistry* **1983**, *22*, 1369−1377.
(20) Bryant, F. R.; Benkovic, S. J. *Biochemistry* **1979**, *18*, 2825−2828.
(21) For a review, see: Kolodiazhnyi, O. I. *Tetrahedron: Asymmetry* **1998**, *9*, 1279−1332.

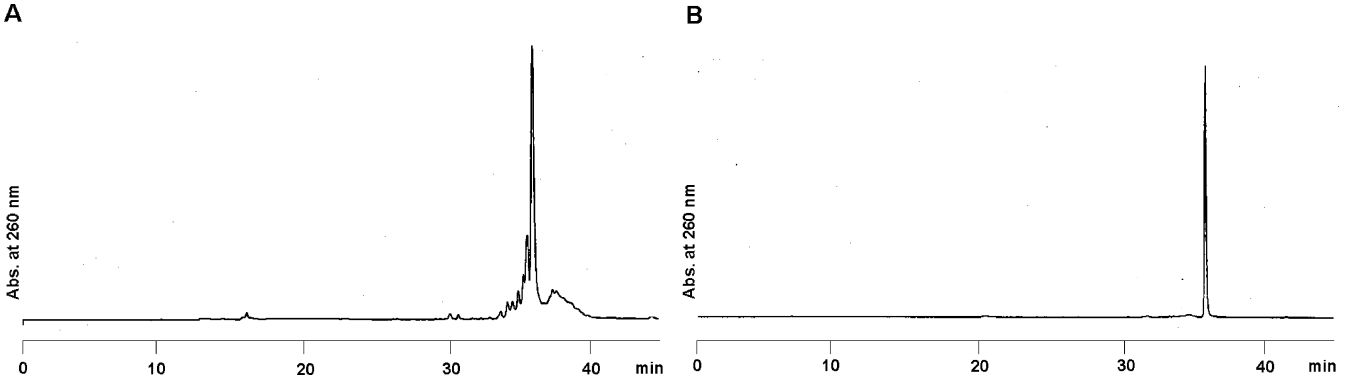


Figure 7. Reversed-phase HPLC profiles: (A) crude **18**; (B) **18** purified by reversed-phase HPLC. RP-HPLC was performed with a linear gradient of 0−20% acetonitrile in 0.1 M ammonium acetate buffer (pH 7.0) at 50 °C for 60 min at a rate of 0.5 mL/min.

uncorrected. MALDI-TOF-MS spectrometry was carried out on an Applied Biosystems Voyager-DE STR spectrometer. Thin-layer chromatography (TLC) was performed on TLC plates coated with silica gel 60 F_{254} (Merck, No. 5715). Silica gel column chromatography was carried out using silica gel 60N (63−210 μm). Reversed-phase HPLC was carried out using a μBondasphere 5 μm C18 column (100 Å, 3.9 mm × 150 mm) (Waters). Organic solvents were purified and dried by the appropriate procedure. Aminomethylated highly cross-linked polystyrene was purchased from Perkin-Elmer Applied Biosystems.[17] Manual solid-phase synthesis was performed by using a glass filter (10 mm × 50 mm) with a stopper at the top and a stopcock at the bottom as a reaction vessel. Snake venom phoshodiesterase (svPDE) was purchased from Sigma, and nuclease P1 was purchased from Yamasa. Stereorandom PS-ODNs were purchased from Amersham Biosciences.

Ab Initio Calculations. Ab initio molecular orbital calculations were carried out using the Gaussian 98[22] and Spartan'02[23,24] programs on a Silicon Graphics Inc. OCTANE workstation. Geometry optimizations were carried out at the HF/6-31G* level. In some cases, single-point energy calculations were carried out at the B3LYP/6-31G* level.

***N*-(Cyanomethyl)pyrrolidinium Trifluoromethanesulfonate (1f).** Trifluoromethanesulfonic acid (1.77 mL, 20 mmol) was added dropwise to a stirred solution of *N*-(cyanomethyl)pyrrolidine (2.20 g, 20 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C. The mixture was then allowed to warm to rt, and diluted with dry Et_2O (40 mL). The resultant precipitate was collected by filtration, washed with dry Et_2O (3 × 5 mL), and dried under vacuum to afford **1f** (5.20 g, 20 mmol) as a white crystalline solid. Mp: 67.0−67.5 °C. IR (KBr): ν_{max} 2996, 2841, 2651, 2477, 2347, 2282, 1637, 1462, 1437, 1269, 1228, 1168, 1033, 985, 911, 849, 761, 641 cm^{-1}. ^1H NMR (300 MHz, CD_3CN): δ 8.14 (br, 1H), 4.28 (s, 2H), 3.48 (br, 4H), 2.09 (m, 4H). ^{13}C NMR (75 MHz, CD_3CN): δ 121.5 (q, $^1J_{CF}$ = 320 Hz), 112.6, 56.2, 42.3, 23.9. Anal. Calcd for $C_7H_{11}F_3N_2O_3S$: C, 32.31; H, 4.26; N, 10.76. Found: C, 32.31; H, 4.22; N, 10.81.

1a−**e**,**g**−**n** were synthesized in a similar manner. The experimental details and characterization data for **1a**−**e**,**g**−**n** are given in the Supporting Information.

Preparation of (5*R*)-2-Chloro-3-methyl-5-phenyl-1,3,2-oxazaphospholidine [(5*R*)-3a]. A Typical Procedure for the Synthesis of 3a−**f.** (*R*)-**2a** (756 mg, 5.0 mmol) was dried by repeated coevaporations with dry toluene and dissolved in dry toluene (2.5 mL). *N*-Methylmorpholine (1.10 mL, 10 mmol) was added to the solution, and the mixture was added dropwise to a stirred solution of phosphorus trichloride (436 μL, 5.0 mmol) in dry toluene (2.5 mL) at 0 °C under argon. The mixture was allowed to warm to rt and stirred for 30 min. The resultant salt was removed by filtration at −78 °C under argon, and the filtrate was concentrated under reduced pressure to afford crude (5*R*)-**3a** (1.07 g, 5.0 mmol) as a pale yellow oil. This material was used without further purification for the phosphitylation of nucleosides. ^{13}C NMR (75 MHz, $CDCl_3$): δ 138.1, 128.5, 128.5, 126.5, 85.4 (br), 56.3 (d, $^2J_{PC}$ = 6.6 Hz), 31.0 (d, $^2J_{PC}$ = 13.0 Hz). Crude (5*S*)-**3a** and **3b**−**f** were obtained according to the same procedure. Further purification conditions and the characterization data for (5*S*)-**3a** and **3b**−**f** are given below. Crude (5*R*)-**3a** could be distilled under reduced pressure to afford (5*R*)-**3a** (2.10 g, 9.7 mmol) as a colorless liquid (bp 81−82 °C, 0.2 mmHg) from 2.27 g (15 mmol) of (*R*)-**2a** as a starting material. The ^1H and ^{31}P NMR spectra of crude and distilled (5*R*)-**3a** are given in the Supporting Information.

(5*S*)-2-Chloro-3-methyl-5-phenyl-1,3,2-oxazaphospholidine [(5*S*)-3a]. Crude (5*S*)-**3a** was also obtained quantitatively from (*S*)-**2a** and could be used for the phosphitylation of nucleosides without further purification. Crude (5*S*)-**3a** could also be distilled under reduced pressure to afford (5*S*)-**3a** (2.22 g, 12 mmol) as a colorless liquid, using 3.02 g (20 mmol) of (*S*)-**2a** as a starting material. The physical data were identical to those of (5*R*)-**3a**.

(4*S*,5*R*)-2-Chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine (3b).[6a] **3b** (356 mg, 1.6 mmol) was obtained as a colorless liquid from the crude product by Kugelrhor bulb-to-bulb distillation (170 °C, 0.4 mmHg), using (1*R*,2*S*)-ephedrine (**2b**) (496 mg, 3.0 mmol) as a starting material. ^1H NMR (300 MHz, $CDCl_3$): δ 7.40−7.23 (m, 5H), 5.85 (d, $^3J_{HH}$ = 6.3 Hz, 1H), 3.72−3.61 (m, 1H), 2.72 (d, $^3J_{PH}$ = 15.6 Hz, 3H), 0.72 (d, $^3J_{HH}$ = 4.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 136.4 (d, $^3J_{PC}$ = 2.6 Hz), 128.3, 128.3, 126.9, 87.7 (d, $^2J_{PC}$ = 8.9 Hz), 57.7 (br), 29.0 (d, $^2J_{PC}$ = 13.8 Hz), 14.4. ^{31}P NMR (121 MHz, $CDCl_3$): δ 171.5.

(4*S*,5*R*)-2-Chloro-3-methyl-4,5-diphenyl-1,3,2-oxazaphospholidine (3c). Crude **3c** (3.17 g, 10 mmol) was obtained as a pale yellow solid from (1*R*,2*S*)-2-methylamino-1,2-diphenylethanol (**2c**) (2.27 g, 10 mmol). ^1H NMR (300 MHz, $CDCl_3$): δ 7.08−7.05 (m, 6H), 6.91−6.81 (m, 4H), 6.15 (d, $^3J_{HH}$ = 8.3 Hz, 1H), 4.64 (dd, $^3J_{HH}$ = 8.3 Hz,

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$^3J_{PH}$ = 4.2 Hz, 1H), 2.64 (d, $^3J_{PH}$ = 15.3 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 135.8 (d, $^3J_{PC}$ = 3.2 Hz), 134.5 (d, $^3J_{PC}$ = 5.4 Hz), 128.2, 127.9, 127.7, 127.6, 127.5, 126.5, 88.0 (d, $^2J_{PC}$ = 8.6 Hz), 68.1 (d, $^2J_{PC}$ = 6.3 Hz), 29.6 (d, $^2J_{PC}$ = 14.1 Hz). ^{31}P NMR (121 MHz, $CDCl_3$): δ 171.7.

(5*R*)-2-Chloro-3-isopropyl-5-phenyl-1,3,2-oxazaphospholidine (3d). Crude **3d** (1.22 g, 5.0 mmol) was obtained as a pale yellow liquid from (*R*)-2-isopropylamino-1-phenylethanol (**2d**) (896 mg, 5.0 mmol). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.45−7.34 (m, 5H), 5.94−5.24 (br, 1H), 3.62−3.47 (m, 1H), 3.18 (dd, $^2J_{HH}$ = 18.3 Hz,$^3J_{HH}$ = 9.3 Hz, 1H), 1.35 (d, $^3J_{HH}$ = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 138.5 (br), 128.6, 128.5, 126.5 (br), 84.7 (br), 51.3 (br), 47.3 (d, $^2J_{PC}$ = 10.6 Hz), 22.0 (d, $^3J_{PC}$ = 8.9 Hz), 21.8 (d, $^3J_{PC}$ = 9.5 Hz). ^{31}P NMR (121 MHz, $CDCl_3$): δ 170.6 (br).

(5*R*)-2-Chloro-3-methyl-5-isopropyl-1,3,2-oxazaphospholidine (3e). **3e** (1.09 g, 6.0 mmol) was obtained as a colorless liquid from the crude product by bulb-to-bulb distillation (80 °C, 0.3 mmHg), using (*R*)-1-methylamino-3-methyl-2-butanol (**2e**) (1.17 g, 10 mmol) as a starting material. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.68, 4.18 (br, br, 1H), 3.16, 2.95 (m, m, 2H), 2.72 (d, $^3J_{PH}$ = 15.0 Hz, 3H), 2.01, 1.90 (br, br, 1H), 1.06−0.99 (m, 3H), 0.94 (d, $^3J_{HH}$ = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 90.7, 88.7 (br, br), 53.0, 51.8 (br, br), 32.9 (br), 31.1 (d, $^2J_{PC}$ = 14.3 Hz), 18.3, 18.2 (br, br). ^{31}P NMR (121 MHz, $CDCl_3$): δ 173.7, 172.5 (br, br).

(4*S*)-2-Chloro-3-methyl-4-isopropyl-1,3,2-oxazaphospholidine (3f). **3f** (1.27 g, 7.0 mmol) was obtained as a colorless liquid from the crude product by bulb-to-bulb distillation (80 °C, 0.4 mmHg), using (*S*)-2-methylamino-3-methyl-1-butanol (**2f**) (1.17 g, 10 mmol) as a starting material. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.57 (d, $^2J_{HH}$ = 8.7 Hz, $^3J_{HH}$ = 8.7 Hz, 1H), 4.21 (m, 1H), 3.25 (m, 1H), 2.68 (d, $^3J_{PH}$ = 16.2 Hz, 3H), 2.03 (m, 1H), 0.92 (d, $^3J_{HH}$ = 7.2 Hz, 3H), 0.84 (d, $^3J_{HH}$ = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 71.5 (d, $^2J_{PC}$ = 9.2 Hz), 63.5 (br), 29.1 (d, $^2J_{PC}$ = 14.1 Hz), 26.7 (d, $^3J_{PC}$ = 4.9 Hz), 19.3, 14.5. ^{31}P NMR (121 MHz, $CDCl_3$): δ 176.8.

Preparation of 5′-*O*-(*tert*-Butyldiphenylsilyl)-3′-*O*-[(2*R*,5*R*)-3-methyl-5-phenyl-1,3,2-oxazaphospholidin-2-yl]thymidine [(R_p)-5a]. A Typical Procedure for the Synthesis of 5a,b,d−f and 13a−d. 4 (721 mg, 1.5 mmol) was dried by repeated coevaporations with dry pyridine and dry toluene, and then dissolved in dry THF (7.5 mL). Et_3N (1.05 mL, 7.5 mmol) was added, and the mixture was cooled to −78 °C. A 0.22 M solution of (5*R*)-**3a** in dry THF (7.50 mL, 1.65 mmol) was added dropwise via a syringe, and then the mixture was allowed to warm to rt and stirred for 30 min under argon. Saturated $NaHCO_3$ aqueous solution (75 mL) and $CHCl_3$ (75 mL) were added to the mixture, and the organic layer was separated and washed with saturated $NaHCO_3$ aqueous solution (2 × 75 mL). The combined aqueous layers were back-extracted with $CHCl_3$ (2 × 75 mL). The combined organic layers were then dried over Na_2SO_4, filtered, and concentrated to dryness to give the crude product. ^{31}P NMR (121 MHz, $CDCl_3$): δ 144.1 (95%, *trans*-**5a**), 143.2 (5%, *cis*-**5a**). The diastereomer ratio was essentially the same as that obtained by the reaction performed for 5 min at −78 °C. The crude product was applied to a column of silica gel (2.5 × 14 cm, 40 g of silica gel). The product was eluted with hexanes−ethyl acetate−triethylamine (50:50:2, v/v/v). The fractions containing (R_p)-**5a** were combined, washed with saturated $NaHCO_3$ aqueous solution (100 mL), dried over Na_2SO_4, filtered, and concentrated to dryness to afford (R_p)-**5a** (765 mg, 1.2 mmol) as a colorless foam. ^{31}P NMR (121 MHz, $CDCl_3$): δ 144.6. FAB-HRMS: *m*/*z* calcd for $C_{35}H_{43}N_3O_6PSi^+$ (M + H$^+$) 660.2659, found 660.2658. The R_f value [hexanes−ethyl acetate (50:50, v/v)] of (R_p)-**5a** on the silica gel TLC plate was 0.51. The minor *cis*-isomer was eluted a little faster than (R_p)-**5a**. Since the amount of the *cis*-isomer was very small, it could be easily removed by silica gel column chromatography, though the R_f values were close to each other. The purification conditions and characterization data for **5a,b,d−f** and **13a−d** are given in the Supporting Information.

5′-*O*-(*tert*-Butyldiphenylsilyl)-3′-*O*-[(2*R*,4*S*,5*R*)-3-methyl-4,5-diphenyl-1,3,2-oxazaphospholidin-2-yl]thymidine (5c). 4 (961 mg, 2.0 mmol) was dried by repeated coevaporations with dry pyridine and dry toluene, and then dissolved in dry THF (10 mL). *i*-Pr_2NEt (1.70 mL, 10 mmol) was added, and the mixture was cooled to −78 °C. A 0.22 M solution of **3c** in dry THF (10 mL, 2.2 mmol) was added dropwise via a syringe, and then the mixture was allowed to warm to rt, and then refluxed for 1 h. The additional heating did not affect the diastereomer ratio of the crude product. The mixture was then cooled to rt, saturated $NaHCO_3$ aqueous solution (75 mL) and $CHCl_3$ (75 mL) were added to the mixture, and the organic layer was separated and washed with saturated $NaHCO_3$ aqueous solution (2 × 75 mL). The combined aqueous layers were back-extracted with $CHCl_3$ (2 × 75 mL). The combined organic layers were then dried over Na_2SO_4, filtered, and concentrated to dryness to give the crude product. ^{31}P NMR (121 MHz, $CDCl_3$): δ 150.6 (3%, *cis*-**5c**), 144.2 (97%, *trans*-**5c**). Purification of the crude product by silica gel column chromatography (2 × 40 cm, 50 g of silica gel, hexanes−ethyl acetate−triethylamine, 67:33:2 and then 0:100:2, v/v/v) afforded **5c** (1.41 g, 1.9 mmol) as a colorless foam. ^{31}P NMR (121 MHz, $CDCl_3$): δ 142.3. FAB-HRMS: *m*/*z* calcd for $C_{41}H_{47}N_3O_6PSi^+$ (M + H$^+$) 736.2972, found 736.2975.

Monitoring the Condensation of (R_p)-5a with 7 in the Presence of 1d by ^{31}P NMR Spectroscopy. A Typical Procedure for Monitoring the Condensation of 5a−d with 7 in the Presence of 1d, and (R_p)-5a with 7 in the Presence of 1a−n. (R_p)-**5a** (33.0 mg, 50 μmol) and **7** (17.8 mg, 50 μmol) were dried over P_2O_5 under high vacuum for 12 h in an NMR sample tube. A 0.25 M solution of **1d** (400 μL, 100 μmol) in CH_3CN, dried over MS 3A for 8 h, and dry CD_3CN (100 μL) were added to the tube under argon. After 3 min, the data accumulation was started. The diastereomer ratio was estimated on the basis of the integration of the resonance signals.

(S_p)-1,8-Diazabicyclo[5.4.0]undec-7-enium 5′-*O*-(*tert*-Butyldiphenylsilyl)thymidin-3′-yl 3′-*O*-(*tert*-Butyldimethylsilyl)thymidin-5′-yl Phosphorothioate [(S_p)-11]. A Typical Procedure for the Synthesis of (R_p)-11 and (S_p)-11. (R_p)-**5a** (39.6 mg, 60 μmol) and **7** (17.8 mg, 50 μmol) were dried over P_2O_5 for 12 h in an NMR sample tube, and a 0.2 M solution of **1f** in CH_3CN (500 μL, 100 μmol), dried over MS 3A for 8 h, was added at rt under argon. After 5 min, dry pyridine (43.0 μL, 500 μmol) and Ac_2O (14.9 μL, 100 μmol) were added. After an additional 30 s, 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (12.0 mg, 60 μmol) was added. After an additional 3 min, 1,8-diazabicyclo[5.4.0]-undec-7-ene (74.6 μL, 500 μmol) was added, and the reaction mixture was heated for 30 min at 50 °C. The mixture was then allowed to cool to rt, diluted with $CHCl_3$ (3 mL), and washed with 0.2 M phosphate buffer (pH 7.0, 3 mL). The aqueous layers were back-extracted with $CHCl_3$ (2 × 3 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was purified by PTLC [CH_2Cl_2−MeOH−Et_3N (99:1:0.5, v/v/v, × 2, 96:4:0.5, v/v/v, × 2)]. The product was dissolved in $CHCl_3$ (3 mL), washed with 0.2 M 1,8-diazabicyclo[5.4.0]undec-7-enium bicarbonate buffer (3 mL), and back-extracted with $CHCl_3$ (2 × 3 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated to dryness to afford (S_p)-**11** (50.4 mg, 47 μmol) as a colorless foam. ^{1}H and ^{31}P NMR spectra were identical to those of the authentic sample synthesized by the conventional *H*-phosphonate method.[25] The ^{1}H and ^{31}P NMR spectra of (S_p)-**11** are given in the Supporting Information.

(R_p)-1,8-Diazabicyclo[5.4.0]undec-7-enium 5′-*O*-(*tert*-Butyldiphenylsilyl)thymidin-3′-yl 3′-*O*-(*tert*-Butyldimethylsilyl)thymidin-5′-yl Phosphorothioate [(R_p)-11]. (R_p)-**11** (52.1 mg, 49 μmol) was obtained as a colorless foam, using (S_p)-**5a** (39.6 mg, 60 μmol) as a starting material. The ^{1}H and ^{31}P NMR spectra were identical to those of the authentic sample synthesized by the conventional *H*-phosphonate

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method.[25] The ^{1}H and ^{31}P NMR spectra of (R_p)-**11** are given in the Supporting Information.

1,8-Diazabicyclo[5.4.0]undec-7-enium 5′-*O*-(*tert*-Butyldiphenylsilyl)thymidin-3′-yl 3′-*O*-(*tert*-Butyldimethylsilyl)thymidin-5′-yl Phosphorothioate (11). 11 was synthesized according to the conventional *H*-phosphonate method[25] as a mixture of diastereomers [(R_p)-**11**:(S_p)-**11** = 46:54]. The ^{1}H and ^{31}P NMR spectra of **11** are given in the Supporting Information.

(S_p)-Ammonium Thymidin-3′-yl Thymidin-5′-yl Phosphorothioate [(S_p)-12a]. A Typical Procedure for the Synthesis of (R_p)-12a and (S_p)-12a. (S_p)-**11** (50.4 mg, 47 μmol) was dried by repeated coevaporations with dry pyridine and dry toluene, and then dissolved in triethylamine trihydrofluoride (500 μL). The mixture was stirred for 15 h at rt. A 0.1 M ammonium acetate buffer (3 mL) was then added to the mixture, and the mixture was washed with Et_2O (3 × 3 mL). The combined organic layers were back-extracted with 0.1 M ammonium acetate buffer (3 mL). The combined aqueous layers were then concentrated to dryness under reduced pressure, and the residue was purified by reversed-phase column chromatography [a linear gradient of acetonitrile (0−10%) in 0.1 M ammonium acetate buffer (pH 7.0)] to afford (S_p)-**12a** (20.5 mg, 35.4 μmol) as a colorless foam. R_p:S_p = 0.5:>99.5 [determined by reversed-phase HPLC performed with a linear gradient of 0−10% (30 min) and then 10% (10 min) acetonitrile in 0.1 M ammonium acetate buffer (pH 7.0) at 50 °C at a rate of 1.0 mL/min]. ^{1}H and ^{31}P NMR spectra and the RP-HPLC profile were identical to those of the authentic sample synthesized by the conventional *H*-phosphonate method.[25] The ^{1}H and ^{31}P NMR spectra and the RP-HPLC profile of (S_p)-**12a** are given in the Supporting Information.

(R_p)-Ammonium Thymidin-3′-yl Thymidin-5′-yl Phosphorothioate [(R_p)-12a]. (R_p)-**12a** (20.6 mg, 36 μmol) was obtained as a colorless foam, using (R_p)-**11** (52.1 mg, 49 μmol) as a starting material. R_p:S_p = >99.5:0.5 [determined by reversed-phase HPLC performed with a linear gradient of 0−10% (30 min) and then 10% (10 min) acetonitrile in 0.1 M ammonium acetate buffer (pH 7.0) at 50 °C at a rate of 1.0 mL/min]. ^{1}H and ^{31}P NMR spectra and the RP-HPLC profile were identical to those of the authentic sample synthesized by the conventional *H*-phosphonate method.[25] The ^{1}H and ^{31}P NMR spectra and the RP-HPLC profile of (R_p)-**12a** are given in the Supporting Information.

Ammonium Thymidin-3′-yl Thymidin-5′-yl Phosphorothioate (12a). 12a was synthesized according to the conventional *H*-phosphonate method[25] as a mixture of diastereomers [(R_p)-**12a**:(S_p)-**12a** = 41:59]. ^{1}H and ^{31}P NMR spectra and the RP-HPLC profile of **12a** are given in the Supporting Information.

Typical Procedure for Manual Solid-Phase Synthesis. Each cycle of the chain elongation consisted of detritylation (3% TCA in CH_2Cl_2; 3 × 5 s), washing (CH_2Cl_2 followed by CH_3CN), coupling (0.2 M monomer **13** and 1.0 M **1f** in CH_3CN; 90 s), washing (CH_3CN), capping [Ac_2O−*N*-methylimidazole−THF (1:2:7, v/v/v); 30 s], washing (CH_3CN), sulfurizing (0.5 M Beaucage reagent in CH_3CN; 60 s), and washing (CH_3CN). The average yield per cycle was estimated to be 97−98% by $DMTr^+$ assay. After the chain elongation, the DMTr group was removed by treatment with 3% TCA in CH_2Cl_2 (3 × 5 s), and washed with CH_2Cl_2. The oligomer on the HCP resin was then treated with 25% NH_3(aq)−pyridine (9:1, v/v) for 30 min (in the cases of **15**, **16**, and **18**) or 15 h (in the case of **17**) at 55 °C to remove the chiral auxiliaries and the protecting groups of the nucleobases and also to release the oligomer from the HCP resin. The HCP resin was removed by filtration and washed with H_2O. The filtrate was concentrated to dryness. The residue was dissolved in H_2O (1 mL) and washed with Et_2O (3 × 1 mL), and the combined washings were back-extracted with H_2O (1 mL). The combined aqueous layers were concentrated to dryness. The resulting crude product was analyzed and/or purified by reversed-phase HPLC with a linear gradient of 0−20% acetonitrile in 0.1 M ammonium acetate buffer (pH 7.0) for 60 min at 50 °C at a rate of 0.5 mL/min.

***all*-(R_p)-[T_{PS}]$_3$T (15).** According to the typical procedure described above, 5′-*O*-(DMTr)thymidine 3′-*O*-succinate bound to HCP (18.5 mg, 0.5 μmol) gave **15** as a crude product. The average yield per cycle was estimated to be 98% by $DMTr^+$ assay. The generation of [T_{PS}]$_3$T was confirmed by MALDI-TOF-MS analysis [*m*/*z* 1201 $(M - H^+)^-$]. RP-HPLC analysis showed that the retention time of the main product was identical to that of the fastest eluted diastereomer of *random*-[T_{PS}]$_3$T, which indicated that the main product was **15**.[8,26] The *P*-configurations of **15** were confirmed by svPDE digestion; the main product was completely digested with svPDE (the experimental procedure is described below).[8] The yield of **15** determined by RP-HPLC was 80%. The average diastereoselectivity was estimated on the basis of the RP-HPLC profiles to be >97%.

***all*-(S_p)-[T_{PS}]$_3$T (16).** According to the typical procedure described above, 5′-*O*-(DMTr)thymidine 3′-*O*-succinate bound to HCP (18.5 mg, 0.5 μmol) gave **16** as a crude product. The average yield per cycle was estimated to be 98% by $DMTr^+$ assay. The generation of [T_{PS}]$_3$T was confirmed by MALDI-TOF-MS analysis [*m*/*z* 1201 $(M - H^+)^-$]. RP-HPLC analysis showed that the retention time of the main product was identical to that of the slowest eluted diastereomer of *random*-[T_{PS}]$_3$T, which indicated that the main product was **16**.[8,26] The *P*-configurations of **16** were confirmed by nuclease P1 digestion; the main product was completely digested with nuclease P1 (the experimental procedure is described below).[8] The yield of **16** determined by RP-HPLC was 78%. The average diastereoselectivity was estimated on the basis of the RP-HPLC profiles to be >95%.

***all*-(R_p)-d[$C_{PS}A_{PS}G_{PS}$]T (17).** According to the typical procedure described above, 5′-*O*-(DMTr)thymidine 3′-*O*-succinate bound to HCP (18.5 mg, 0.5 μmol) gave **17** as a crude product. The average yield per cycle was estimated to be 98% by $DMTr^+$ assay. The generation of d[$C_{PS}A_{PS}G_{PS}$]T was confirmed by MALDI-TOF-MS analysis [*m*/*z* 1220 $(M - H^+)^-$]. RP-HPLC analysis showed that the retention time of the main product was identical to that of the fastest eluted diastereomer of *random*-d[$C_{PS}A_{PS}G_{PS}$]T, which indicated that the main product was **17**.[8,26] The *P*-configurations of **17** were confirmed by svPDE digestion; the main product was completely digested with svPDE (the experimental procedure is described below).[8] The yield of **17** determined by RP-HPLC was 75%. The average diastereoselectivity was estimated on the basis of the RP-HPLC profiles to be >94%.

***all*-(R_p)-[T_{PS}]$_9$T (18).** According to the typical procedure described above, 5′-*O*-(DMTr)thymidine 3′-*O*-succinate bound to HCP (18.5 mg, 0.5 μmol) gave **18** [1.52 A_{260} units, 17.7 nmol (35%) based on the assumption of 7% hypochoromicity: UV (H_2O) λ_{max} 267 nm, λ_{min} 236 nm] after purification of 1/10 of the crude product by RP-HPLC with a linear gradient of 0−20% acetonitrile in 0.1 M ammonium acetate buffer (pH 7.0) for 60 min at 50 °C at a rate of 0.5 mL/min. MALDI-TOF-MS: *m*/*z* 3121 $(M - H^+)^-$. **18** was completely digested by incubation with svPDE for 1 h at 37 °C; <4% of **18** was digested by incubation with nuclease P1 for 1 h at 37 °C (the experimental procedure is described below).[8]

Digestion with svPDE. A 10 μL sample of the crude **15**, **17** dissolved in 500 μL of H_2O (<10 nmol), or purified **18** (5 nmol) was diluted with 50 mM Tris−HCl buffer (pH 8.0, 100 μL). Snake venom phosphodiesterase (20 units) was added, and the mixture was incubated for 1 h at 37 °C. The enzyme was inactivated upon heating (100 °C, 1 min), and then the mixture was analyzed by RP-HPLC at 260 nm.

Digestion with Nuclease P1. A 10 μL sample of the crude **16** dissolved in 500 μL of H_2O (<10 nmol) or purified **18** (5 nmol) was diluted with 50 mM sodium acetate buffer (pH 5.3, 100 μL). Nuclease P1 (20 units) was added, and the mixture was incubated for 1 h at 37 °C. The enzyme was inactivated upon heating (100 °C, 1 min), and then the mixture was analyzed by RP-HPLC at 260 nm.

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Supporting Information Available: ^{1}H and ^{31}P NMR spectra of crude (5*R*)-**3a** and distilled (5*R*)-**3a**, ^{1}H spectrum of crude **3d**, experimental details for the configurational assignment of **5**, experimental details and characterization data for **1a**−**e**,**g**−**n**, **2a**−**f**, **5a**−**f**, **6b**,**d**, and **13a**−**d**, ^{1}H and ^{31}P NMR spectra of (R_p)-**11**, (S_p)-**11**, and **11** (mixture of diastereomers), ^{1}H and ^{31}P NMR spectra and the RP-HPLC profile of (R_p)-**12a**, (S_p)-**12a**, and **12a** (mixture of diastereomers), RP-HPLC profiles of the mixtures obtained by the digestion of crude **15** with svPDE, crude **17** with svPDE, **18** with svPDE, crude **16** with nuclease P1, and **18** with nuclease P1, and RP-HPLC profiles of *random*-[T_{PS}]$_3$T and *random*-d[$C_{PS}A_{PS}G_{PS}$]T (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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