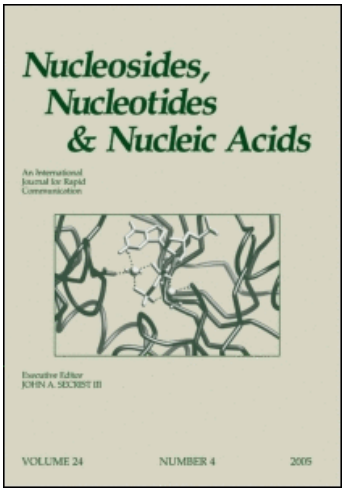

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Stereocontrolled Transesterification for the Synthesis of Chiral Phosphite Triester Backbone

Masatsugu Mizuguchi[ab]; Keisuke Makino[ab]
[a] Department of Polymer Science and Engineering, Kyoto Institute of Technology Matsugasaki, Kyoto, Japan [b] Rational Drug Design Laboratories, Fukushima, Japan

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STEREOCONTROLLED TRANSESTERIFICATION FOR THE SYNTHESIS OF CHIRAL PHOSPHITE TRIESTER BACKBONE[a]

Masatsugu Mizuguchi[†¶] and Keisuke Makino*[†]
Department of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan, and Rational Drug Design Laboratories, Matsukawa-machi, Fukushima 960-12, Japan

ABSTRACT: We have developed stereocontrolled transesterification for the synthesis of chiral phosphite triester backbone under basic conditions. Substrate phosphite triesters with phenoxy derivative substituents as leaving groups were separated into individual stereoisomers and reacted with hydroxyl-containing compounds to form desired backbone.

INTRODUCTION

Phosphorothioate is recognized as the most promising backbone of antisense molecules and applied in many clinical trials[1,2]. This backbone has chiral phosphorus atoms at each phosphorothioate linkage. In spite of the intensive efforts of some groups[3,4], the chemical and biological properties of the isomers have not been fully characterized. In order to elucidate it, synthesis of stereoregulated phosphorothioate oligonucleotides is essential. Stec *et al.* have developed such a method utilizing a pentavalent phosphorous compound, oxathiaphospholane[5]. On the other hand, methods utilizing trivalent phosphorous compounds, which are regarded as more reactive than pentavalent ones[6,7], have not been developed. The chiral phosphite triesters can easily be converted into phosphorothioates by sulfurization with elemental sulfur or other reagents as shown in FIG. 1. The sulfurization reactions[8,9,10,11,12] and subsequent removal of the phosphate protecting groups such as 2-cyanoethyl[13,14] or methyl group[11] can be performed in an absolutely stereoretentive manner (see ref.). For this reason, synthesizing such chiral phosphite triesters is worthwhile. We report herein a stereospecific transesterification reaction of phosphite triesters under basic conditions

[a] Dedicated to Dr. Yoshihisa Mizuno on the occasion of his 75th birthday.
[†] Kyoto Institute of Technology.
[¶] Rational Drug Design Laboratories.

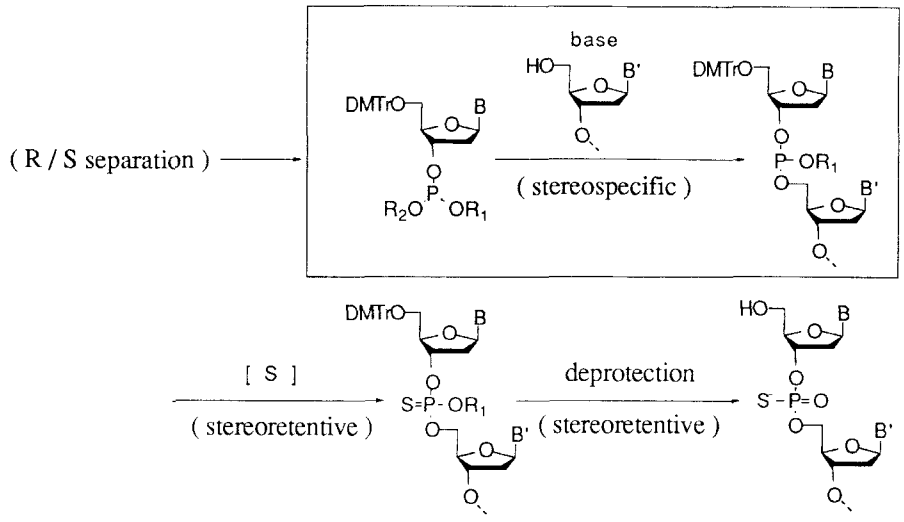


FIG. 1. Proposed scheme for stereocontrolled phosphorothioate synthesis.

(FIG. 1). Substrate phosphite triesters, which have phenoxy derivative substituents as leaving groups, were separated into individual stereoisomers and used for transesterification reactions with hydroxyl-containing compounds to form desired chiral phosphite triester backbones. Trialkylamines were the most effective bases to maintain high stereospecificity and reactivity. This reaction will be applicable for stereocontrolled synthesis of phosphorothioate oligonucleotides.

RESULTS AND DISCUSSION

Preparation of Substrate Phosphite Triesters and Separation of Their R/S Isomers

Various types of 5'-*O*-dimethoxytritylthymidine 3'-phosphite were prepared as model compounds of the substrates for transesterification. They were readily prepared by the reaction of the corresponding phosphoramidites with the phenol derivatives and 1*H*-tetrazole. After removal of the resultant precipitate by filtration and solvent evaporation, the reaction mixture was applied to silica gel open-column chromatography employing EtOAc/hexane or EtOAc/THF as eluents.

With **1e** or **1f** containing 4-nitrophenyl or pentafluorophenyl groups as R_2, separation of the isomers was unsuccessful because they were unstable in the silica gel column. **1d** (R_2 = 3-nitrophenyl) was successfully separated by adding a small amount of AcOH to

the eluent, but readily underwent racemization after separation. The compounds **1a**, **1b**, and **1c** (R_2 = 4-phenylphenyl, 3-chlorophenyl, and 4-chloronaphthyl, respectively) were separated into individual stereoisomers without any difficulties. The stability of these compounds may be explained by relatively weak electron-withdrawing feature of $-OR_2$. When a methyl group was used as a protecting group R_1 (**2a**), the separation was less efficient than **1a**. The chromatographic properties of the phosphite triesters are summarized in TABLE 1.

Stability of the Substrate Phosphite Triesters

Transesterification reactions described herein proceed under basic conditions. However, in some cases, racemization of substrate phosphite triesters occurred due to nucleophilicity of amines under the present reaction conditions.

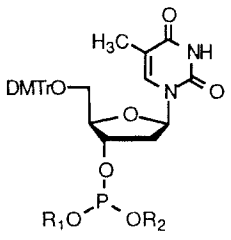
TABLE 1 shows the stability of the phosphite triesters in the presence of amines. Trialkylamines such as triethylamine are suitable for the reaction because of their high basicity and relatively low nucleophilicity. DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) with relatively low nucleophilicity also causes rapid racemization in this reaction system. The possibility of DBU-catalyzed isomerization of *H*-phosphonate is mentioned by Fujii *et al.*[9]. This suggests that addition of DBU to phosphorous atom might also occur in the present system.

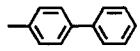
A tentative mechanism for the P-epimerization induced by amines is shown in FIG. 2. In the first step, addition of the amine to the phosphorous atom occurs and subsequently the phenoxide anion is eliminated (**a** to **b**). When the phenoxide anion attacks the intermediate again, the phosphite with original configuration is regenerated (**b** to **a**). On the contrary, addition of the amine to phosphorous followed by elimination of original one causes epimerization of the intermediate (**b** to **c**). Upon subsequent attack by the phenoxide, the intermediate is converted into the epimerized phosphite (**c** to **d**).

Transesterification Reaction

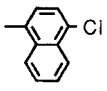
Reaction Mechanism. In order to clarify the mechanism of the transesterification reaction, we followed the time course of the transesterification reaction using a model system (FIG. 3). A purified isomer of 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine 3'-*O*-[2-cyanoethyl(4-phenylphenyl)]phosphite ('**slow**' isomer, diastereomeric purity = 97.1%), triethylamine, and 2-methyl-1-propanol were employed as components of model reaction to form 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine 3'-*O*-[2-cyanoethyl(2-methyl-1-propyl)]phosphite. Conversion and diastereomeric purity of the substrate phosphite triester were determined every hour by normal phase HPLC analysis (FIG. 3). Since two isomers of the final product were not separable by HPLC under the present conditions, their ratio was determined by ^{31}P NMR (see below). As shown in FIG. 3, P-epimerization of the substrate phosphite triester proceeded much slowly relative to the

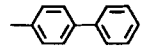
TABLE 1. Properties of substrate phosphite triesters

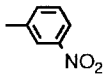


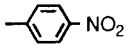
1a: R_1 = 2-cyanoethy, R_2 = 

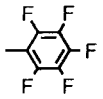
1b: R_1 = 2-cyanoethyl, R_2 = 

1c: R_1 = 2-cyanoethyl, R_2 = 

2a: R_1 = methyl, R_2 = 

1d: R_1 = 2-cyanoethyl, R_2 = 

1e: R_1 = 2-cyanoethyl, R_2 = 

1f: R_1 = 2-cyanoethyl, R_2 = 

compound	isomer separation	eluent	R_f 'fast', 'slow'[b]	resistance against racemization			
				pyridine	NEt_3	MeIm[c]	DBU
1a	possible	EtOAc/hexane=2/1	0.48, 0.44	rather stable	rather stable	unstable	unstable
1b	possible	EtOAc/hexane=2/1	0.43, 0.35	rather stable	rather stable	unstable	unstable
1c	possible	THF/hexane=1/1	0.41, 0.36	unstable	unstable	unstable	unstable
1d	possible	EtOAc/hexane=2/1[a]	0.53, 0.45	unstable	unstable	unstable	unstable
1e	impossible	-	-	-	-	-	-
1f	impossible	-	-	-	-	-	-
2a	difficult	-	-	-	-	-	-

[a] Small amount of AcOH was added.

[b] The R/S isomers of substrate phosphite are designated as '**fast**' and '**slow**' according to the elution volume in open-column chromatography. R_f was determined by thin layer chromatography (Kieselgel 60 F_{254}, Merck).

[c] Abbreviation: MeIm, 1-Methylimidazole.

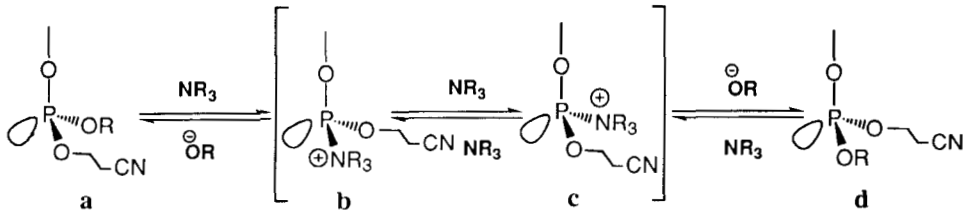


FIG. 2. P-epimerization mechanism.

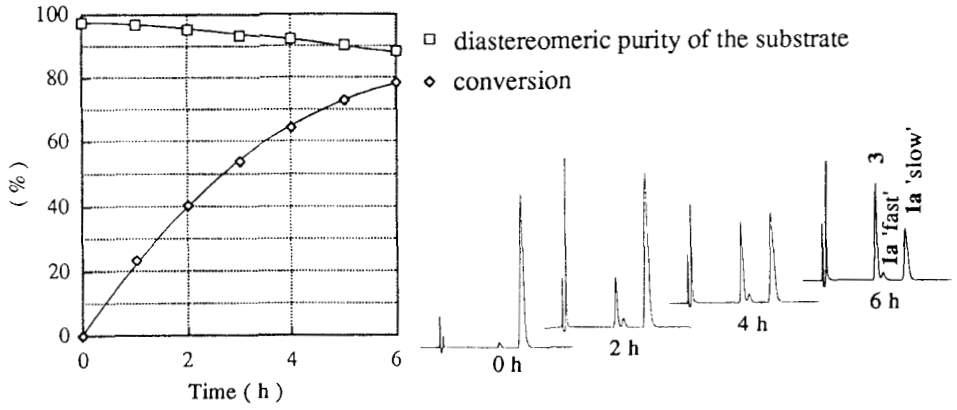


FIG. 3. Time course of the transesterification reaction analyzed by normal phase HPLC. Column: ULTRON VX-SIL 150L X 4.6 5μm (Shinwa Chemical Industries Co., Ltd.); flow rate: 1.0 mL/min; eluent: EtOAc/hexane=1/1; compound **3**: the product (5'-*O*-(4,4'-dimethoyxtrityl)-2'-deoxythymidine-3'-*O*-[2-cyanoethyl(2-methyl-1-propyl)] phosphite, the mixture of both isomers)

transesterification reaction. After 6 hours, diastereomeric purity and the conversion of the substrate were 88.3% and 78.3%, respectively. Then the solvent was evaporated and diastereomeric purity of the final product was determined as 94% by ^{31}P NMR analysis (FIG. 4). Comparing diastereomeric purity of the starting substrate and the final product, we estimated the overall stereoselectivity of this reaction to be 97%.

FIG. 5 shows the possible routes of this reaction system. They include P-epimerization ($\mathbf{r_1}$), stereoretentive transesterification ($\mathbf{r_2}$), and stereoinvertive transesterification ($\mathbf{r_2'}$). High purity of the product can be well explained if it is assumed that the transesterification reaction proceeds exclusively in a single stereospecific pathway (i.e., either $\mathbf{r_2}$ or $\mathbf{r_2'}$) and stereoselectivity of the reaction is affected only by

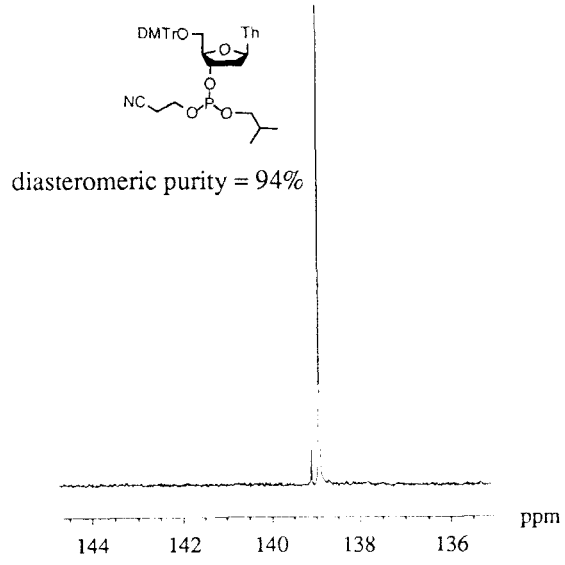


FIG. 4. ^{31}P NMR spectrum of the product obtained by the transesterification reaction.

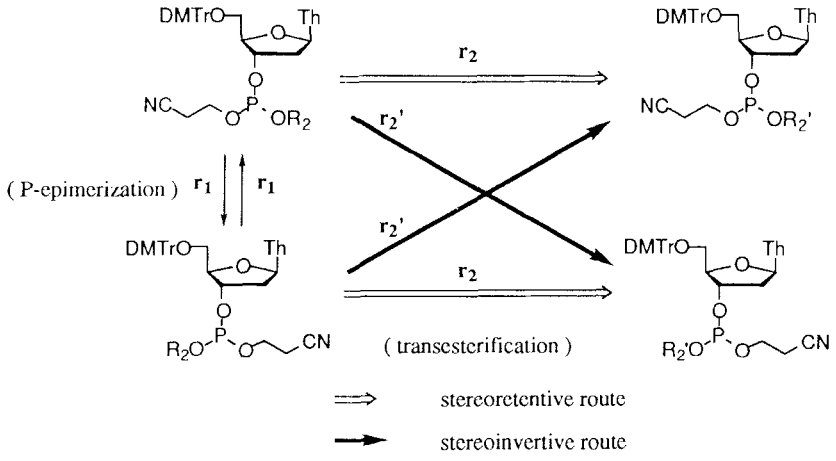


FIG. 5. Possible reaction pathways.

epimerization of the substrate at phosphorous atom. In this mechanism, diastereomeric purity of the final product is determined by the isomer ratio of the starting phosphite triester.

Based on this assumption, diastereomeric purity of the final product can be calculated by the following equations, where diastereomeric purity of the product is represented by the ratio of the integral of the major substrate diastereoisomer reacted (in this case, '**slow**') and that of total substrate reacted.

$$\text{diastereomeric purity (\%)} = \frac{\int_0^t p\,r\,dt}{\int_0^t r\,dt} \times 100$$

$$= \frac{\int_0^C p\,dC}{\int_0^C dC} \times 100 \quad = \frac{\int_0^C p\,dC}{C} \times 100$$

where, p represents substrate diastereomeric purity,

r represents transesterification reaction rate,

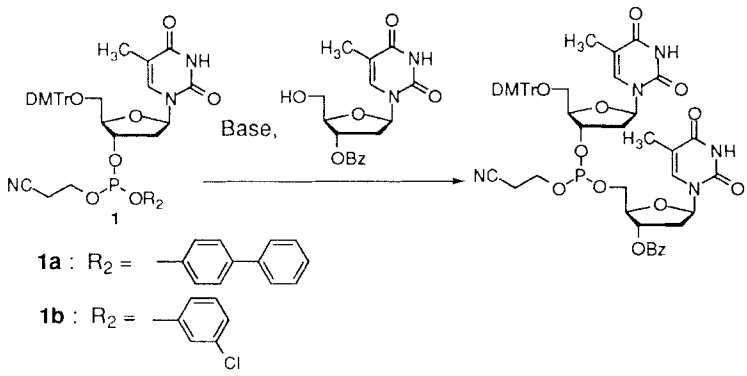
C represents conversion,

and, $\frac{dC}{dt} = \alpha r$ (α: constant)

Using this equation along with C and p values experimentally determined every hour by HPLC, diastereomeric purity of the product is calculated as 94.5 %. The value obtained by ^{31}P NMR (94 %) nicely fits the calculated value, confirming the proposed mechanism. If the assumption is inappropriate and both routes ($\mathbf{r_2}$ and $\mathbf{r_2'}$) proceed simultaneously, the diastereomeric purity of the product must be smaller than the calculated value. Thus, we have concluded that one of the two possible routes (either $\mathbf{r_2}$ or $\mathbf{r_2'}$) proceeds exclusively in the transesterification reaction. However, there is no experimental implication as to which reaction is the exclusive route so far.

Transesterification with Nucleoside. TABLE 2 shows the typical results of the transesterification reactions of **1a** and **1b** with 3'-benzoylthymidine using triethylamine as a base. The bulky 3'-benzoylthymidine has lower transesterification reactivity compared with 1-methyl-2-propanol. Therefore, P-epimerization which reduces diastereomeric purity became more dominant from 3'-benzoylthymidine than 1-methyl-2-propanol. Although transesterification of **1b** occurs much faster than **1a**, diastereomeric purity of the product from **1b** is only slightly higher than from **1a** due to

TABLE 2. Results of transesterification reactions



entry	substrate/isomer/equiv.	base/solvent	reaction time (hr)	yield (%)	stereoselectivity (%)
1	**1b** / 'slow' / 3	NEt_3 / CH_3CN	1.25	77	65
2	**1b** / 'fast' / 19	NEt_3 / py	0.5	55	86
3	**1b** / 'slow' / 34	NEt_3 / py	1.0	85	80
4	**1a** / 'slow' / 19	NEt_3 / py	7.5	37	78

higher P-epimerization reactivity of **1b** (see entries 1 and 4). We obtained better stereoselectivity when large equivalent of the substrate phosphite triester was employed (entries 1 and 2). Prolonged reaction time resulted in the reduction of stereoselectivity, though the conversion increased (entries 2 and 3). That is because large amount of epimerized substrate produced in entry 3 was converted into the product.

According to the proposed mechanism (FIG. 5), overall stereoselectivity of the transesterification reaction is determined by relative rate of transesterification (either $\mathbf{r_2}$ or $\mathbf{r_2'}$) and P-epimerization ($\mathbf{r_1}$). P-epimerization is caused by addition of the amine or the phenoxide anion produced by transesterification reaction. Accordingly, if high concentration of substrate phosphite triester is used, transesterification rate can be accelerated while the P-epimerization rate remains unchanged. Therefore, employing such conditions is an effective strategy for the optimization of the reaction system.

The use of strong bases such as triethylamine or DBU may cause decomposition of protecting groups or cleavage of solid-support linkages. However, Suska *et al.* performed the synthesis of phosphorothioate 27-mer by the 'oxathiaphospholane' method

using a high concentration of DBU[4], suggesting that our method can be used for synthesis of phosphorothioate oligonucleotides with reasonable length.

CONCLUSION

Chiral phosphite triesters with modified phenoxy groups as leaving groups, especially 4-phenylphenoxy group and 3-chlorophenoxy group, can serve as good substrates of stereocontrolled phosphite backbone synthesis. Transesterification reactions of the phosphite triesters under basic conditions are fully stereospecific. However, overall stereoselectivity is affected by P-epimerization of the substrate occurring prior to the transesterification reaction. It is essential to control the P-epimerization for reaction optimization.

EXPERIMENTAL SECTION

^1H NMR and ^{31}P NMR spectra were obtained on either QE-300 (General Electric) or ARX-500 (Bruker). 85% H_3PO_4 was used as an external standard in ^{31}P NMR analysis.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine 3'-O-[2-cyanoethyl(4-phenylphenyl)]phosphite **(1a).** 3'-*O*-[2-Cyanoethoxy(*N*,*N*-diisopropylamino)phosphino]-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine (374 mg, 0.50 mmol) and 4-phenylphenol (86.6 mg, 0.51 mmol) were dissolved in 1 mL of acetonitrile. To the solution was added 1*H*-tetrazole (36.2 mg, 0.51 mmol) in 1 mL acetonitrile. After 1.5 h, the resultant precipitate was filtrated off. The solvent was evaporated and the residue was applied to silica gel open-column chromatography employing EtOAc/hexane (1/1) as eluents. The fractions were collected to give two isomers. Yield: '**fast**' isomer 120.4 mg, '**slow**' isomer 98.1 mg, and the mixture of both isomers, 129.1 mg. The total yield was 347.6 mg (0.42 mmol, 85%). Anal. Calcd for $C_{46}H_{44}N_3O_9P$: C, 67.89; H, 5.45; N, 5.16. Found: C, 67.77; H, 5.62; N, 5.13. UV (MeOH) λ_{max} 237 nm (ε 26400), 255 (25400), for '**fast**', ^1H NMR ($CDCl_3$) δ 1.47 (3 H, s, CH_3), 2.39 (1 H, ddd, J = 13.8, 8.9, and 6.0 Hz, 2"-H), 2.52~2.62 (1 H, m, 2'-H), 2.60 (2 H, t, J = 6.7 Hz, CH_2CN), 3.37 (1 H, dd, J = 10.8 and 3.2 Hz, 5'-H), 3.53 (1 H, dd, J = 10.8 and 3.2 Hz, 5"-H), 3.77 (6 H, s, OCH_3), 4.12 (2 H, q, J = 6.7 Hz, $POCH_2$), 4.23 (1 H, d, J = 3.6 Hz, 4'-H), 5.12~5.18 (1 H, m, 3'-H), 6.47 (1 H, dd, J = 8.5 and 6.0 Hz, 1'-H), 6.81~7.61 (23 H, m, Ar-H and 5-H), 8.52 (1 H, s, NH), ^{31}P NMR ($CDCl_3$) δ 132.7, for '**slow**', ^1H NMR ($CDCl_3$) δ 1.48 (3 H, s, CH_3), 2.41 (1 H, ddd, J = 13.8, 8.9, and 6.0 Hz, 2"-H), 2.55~2.67 (1 H, m, 2'-H), 2.66 (2 H, t, J = 6.6 Hz, CH_2CN), 3.37 (1 H, dd, J = 10.8 and 3.2 Hz, 5'-H), 3.50 (1 H, dd, J = 10.8 and 3.2 Hz, 5"-H), 3.76 (6 H, s, OCH_3), 4.08~4.19 (2 H, m, $POCH_2$), 4.23 (1 H, d, J = 3.6 Hz, 4'-H), 5.20~5.25 (1 H, m, 3'-H), 6.49 (1 H, dd, J = 8.5 and 6.0 Hz, 1'-H), 6.80~7.60 (23 H, m, Ar-H and 5-H), 9.50 (1 H, s, NH), ^{31}P. NMR ($CDCl_3$) δ 132.9.

***Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine 3'-O-[2-cyanoethyll(3-chlorophenyl)]phosphite* (1b).** The same procedure was used as the synthesis of **1a**. 3'-*O*-[2-cyanoethoxy(*N*,*N*-diisopropylamino)phosphino]-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine (500 mg, 0.67 mmol), 3-chlorophenol (94.9 mg, 0.74 mmol), and 1*H*-tetrazole (51.8 mg, 0.74 mmol) were reacted to give the products, '**fast**' 142.9 mg, '**slow**' 170.3 mg, and the mixture 57.8 mg. The total yield was 371.0 mg (0.48 mmol, 72%). Anal. Calcd for $C_{40}H_{39}N_3O_9PCl$: C, 62.22; H, 5.09; N, 5.44. Found: C, 62.27; H, 5.13; N, 5.55. UV (MeOH) λ_{max} 268 nm (ε 12900), for '**fast**' ^{1}H NMR ($CDCl_3$) δ 1.48 (3 H, s, CH_3), 2.38 (1 H, ddd, J = 14.1, 8.1, and 6.2 Hz, 2"-H), 2.50~2.60 (1 H, m, 2'-H), 2.58 (2 H, t, J = 6.7 Hz, CH_2CN), 3.36 (1 H, dd, J = 10.8 and 3.3 Hz, 5'-H), 3.53 (1 H, dd, J = 10.8 and 3.3 Hz, 5"-H), 3.79 (6 H, s, OCH_3), 4.00~4.16 (2 H, m, $POCH_2$), 4.21 (1 H, d, J = 2.4 Hz, 4'-H), 5.09~5.14 (1 H, m, 3'-H), 6.45 (1 H, dd, J = 8.1 and 6.2 Hz, 1'-H), 6.81~7.61 (18 H, m, Ar-H and 5-H), 9.08 (1 H, s, NH) , ^{31}P NMR ($CDCl_3$) δ 132.7, for '**slow**', ^{1}H NMR ($CDCl_3$) δ 1.47 (3 H, s, CH_3), 2.39 (1 H, ddd, J = 14.1, 8.1, and 6.0 Hz, 2"-H), 2.53~2.57 (1 H, m, 2'-H), 2.66 (2 H, t, J = 6.7 Hz, CH_2CN), 3.35 (1 H, dd, J = 10.8 and 3.3 Hz, 5'-H), 3.50 (1 H, dd, J = 10.8 and 3.3 Hz, 5"-H), 3.78 (6 H, s, OCH_3), 4.13 (2 H, q, J = 6.7 Hz, $POCH_2$), 4.20 (1 H, d, J = 3.5 Hz, 4'-H), 5.15~5.21 (1 H, m, 3'-H), 6.46 (1 H, dd, J = 8.5 and 6.0 Hz, 1'-H), 6.81~7.61 (18 H, m, Ar-H and 5-H), 9.50 (1 H, s, NH), ^{31}P NMR ($CDCl_3$) δ 133.0.

Analysis of Reaction Mechanism. With the substrate phosphite triester **1a**, the '**slow**' isomer (20 mg, 24.6 μmol) was dissolved in the mixture of 2-methyl-1-propanol (100 μL) and triethylamine (300 μL), then the reaction was allowed to proceed with stirring at room temperature. Sample was taken every one hour and the amount of substrate isomers ('**fast**' and '**slow**') were analyzed by normal phase HPLC. The product, 5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxythymidine 3'-*O*-[2-cyanoethyl(2-methyl-1-propyl)]phosphite, was also analyzed in the same way. After 6 h of reaction, the solvent was evaporated immediately and diastereomeric purity of the product was determined by integrated signal intensity of each diastereoisomers in ^{31}P NMR analysis.

General Procedure of Transesterification Reactions. To the mixture of substrate phosphite triester and 3'-benzoylthymidine were added triethylamine and pyridine (or acetonitrile). After the specific time, the amines (and the solvent) were evaporated immediately. Conversion was determined by normal phase HPLC and stereoselectivity was analyzed by ^{31}P NMR spectroscopy.

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