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A Strategy for the Stereoselective Preparation of Thymidine Phosphorothioates with the (*R*) or the (*S*) Configuration at the Stereogenic Phosphorus Atom and Their Application to the Synthesis of Oligodeoxyribonucleotides with Stereochemically Pure Phosphate/Phosphorothioate Chimeric Backbones

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This paper describes a new method for the highly stereoselective preparation of dithymidine phosphorothioates (TpsT) with the (*R*) or the (*S*) configuration at the stereogenic phosphorus atom [(*R*p)- or (*S*p)-TpsT, respectively], together with the synthesis of oligodeoxyribonucleotides with stereochemically pure phosphate/phosphorothioate mixed backbones through the use of (*R*p)- or (*S*p)-TpsT as building blocks. Stereochemically pure (*R*p)- or (*S*p)-TpsT was produced through a five-step process: 1) 1*H*-tetrazole-promoted thermodynamic equilibration of a diastereomeric mixture of allyl (*R*p)- and (*S*p)-thymidine 3′,5′-cyclic phosphate to give the (*S*p) isomer as the major component, 2) stereospecific sulfurization of the allyl (*S*p)-thymidine 3′,5′-cyclic phosphate to afford an allyl (*R*p)-thymidine 3′,5′-cyclic phosphorothioate, 3) regioselective and stereoselective methanolysis of the allyl (*R*p)-thymidine 3′,5′-cyclic phosphorothioate to provide an allyl methyl (*R*p)-thymidine 3′-phosphorothioate as the main product, 4) stereospecific removal of the methyl group from the phosphorothioate moiety to give an allyl (*R*p)-5′-*O*-dimethoxytritylthymidine 3′-phosphorothioate diester, or stereospecific removal of the allyl group from the phosphate moiety to form a methyl (*S*p)-5′-*O*-dimethoxytritylthymidine 3′-phosphorothioate diester, and 5) stereospecific condensation of these (*R*p)- and (*S*p)-diesters with a 5′-*O*-free thymidine in a Mitsunobu reaction to produce the allyl (3′–5′)-linked (*S*p)-dithymidine phosphorothioate and the methyl (3′–5′)-linked (*R*p)-dithymidine phosphorothioate, respectively. The resulting (*R*p)- and (*S*p)-dithymidine phosphorothioates were converted into their 3′-phosphoramidites. Oligodeoxyribonucleotides with stereochemically pure phosphate/phosphorothioate-mixed backbones were then synthesized by use of these phosphoramidites as building blocks.

Introduction

Oligodeoxyribonucleotides in which the backbones are fully modified into phosphorothioate moieties (all-PS oligonucleotides) are important molecules in antisense therapy.[1] Some of them are in fact used as curatives for certain viral diseases, but all-PS oligonucleotides have some drawbacks in therapeutic use: they appear, for example, to bind non-sequence-specifically to cellular proteins[2] and at high concentrations they furthermore competitively inhibit a variety of nucleases and polymerases.[3] The high-concentration use of an all-PS oligonucleotide also produced undesired phenomena in laboratory work and in animal experiments performed to assess the efficacy of the artificial oligonucleotide as a drug. These phenomena included interaction with – and, potentially, the abrogation of – the activity of heparin-binding growth factors,[4] induction of immune stimulatory effects in rodents,[5] complement activation and hypotension in monkeys,[6] and induction of clotting abnormalities in monkeys as a result of direct interactions with thrombin.[6a,6b]

In contrast, analogues with mixed phosphate/phosphorothioate backbones (PO/PS-chimeric oligonucleotides) do not have these drawbacks even at high concentrations.[7] Furthermore, PO/PS-chimeric oligonucleotides are expected to have greater binding affinity to the complementary nucleotides than the all-PS analogues and to show activity similar to that of the all-PS derivatives.[8] Such PO/PS-chimeric oligonucleotides are therefore attractive compounds, and the development of efficient means to synthesize them is an important research subject.

The phosphorothioate internucleotide linkage generates asymmetry at the phosphorus atom, and the chirality of this atom may affect the biological properties of PO/PS-chimeric oligonucleotides, including transport through cell membranes and/or interaction with intracellular biopoly-

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mers such as proteins or nucleic acids, so it is important to develop means to synthesize derivatives of these oligonucleotides with stereochemically pure phosphorothioate linkages. PO/PS-chimeric oligonucleotides have been prepared by several methods, most of which have allowed the synthesis essentially to give mixtures of diastereomers in which the stereogenic phosphorus atoms of phosphorothioate functions have (*R*) and (*S*) configurations [(*R*p)- and (*S*p)-phosphorothioates, respectively].[9] There has been only one example of a stereocontrolled synthesis of PO/PS-chimeric oligonucleotides: a study by Stec et al. employed the oxathiaphosphorane method.[10] Here we present a novel, facile method to synthesize stereochemically pure PO/PS-chimeric oligodeoxyribonucleotides.

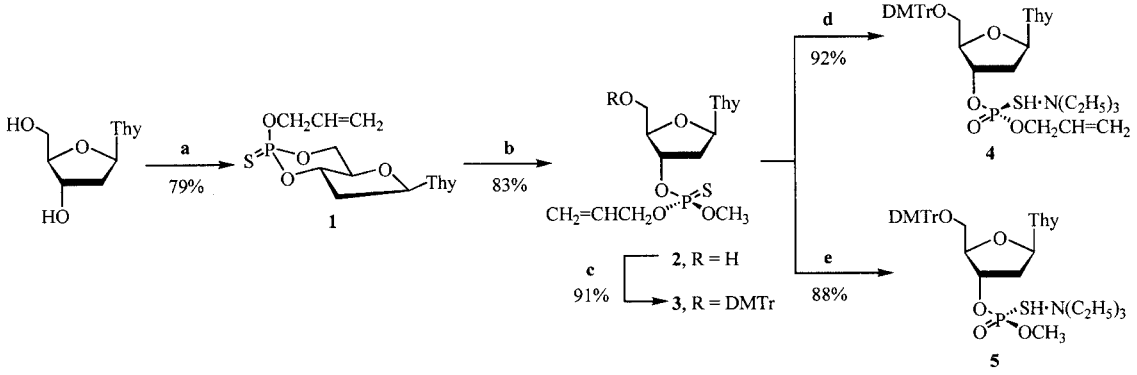
Results and Discussion

The (*R*p)- and (*S*p)-thymidine-3′-phosphorothioic acid diesters **4** and **5** were first prepared stereoselectively by the route shown in Scheme 1. Thymidine (1.0 equiv.) was treated with (allyloxy)[bis(diisopropylamino)]phosphane (1.2 equiv.) with the assistance of 1*H*-tetrazole (3.0 equiv.) in the presence of molecular sieves (4-Å mol. sieves)[11] in acetonitrile at 25 °C for 4 h. The reaction mixture[12] was heated at 55 °C for 36 h[13] and the resulting product was treated with bis[3-(triethoxysilyl)propyl] tetrasulfide[14] (TEST, 1.2 equiv., 25 °C, 2 h) to give the allyl (*R*p)-thymidine 3′,5′-cyclic phosphorothioate **1**, contaminated with a small amount of the (*S*p) isomer.[15] The combined isolated yield of the two isomers was 79% and the ratio of (*R*p) and (*S*p) isomers in this product, estimated by ^{31}P NMR, was 98:2.

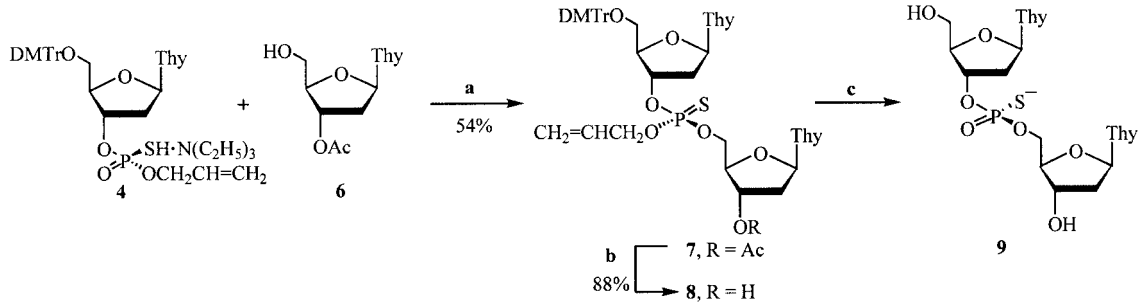
Treatment of **1** (1.0 equiv.) with sodium methoxide (3.0 equiv.) in methanol (25 °C, 24 h) opened the cyclic phosphorothioate ring in a highly stereoselective and regioselective manner to give the allyl methyl (*R*p)-thymidine 3′-phosphorothioate **2** as a major product.[16] In this methanolysis, small amounts of the corresponding (*S*p) isomer (<3%) and allyl methyl thymidine 5′-phosphorothioate were obtained as by-products. The ^{31}P NMR analysis indicated that the product ratio of the nucleoside 3′-phosphorothioate to the nucleoside 5′-phosphorothioate was 90:10 and the isolated yield of **2** was 83%. The structure of this product was supported by the ^{1}H NMR spectrum, showing a signal due to C(3′)-H of the sugar part at δ = 4.16 ppm, and by the ^{31}P NMR spectrum, showing a signal at δ = 66.2 ppm.

Compound **2** was subsequently converted into the (*R*p)-5′-*O*-*p*,*p*′-dimethoxytrityl (DMTr) derivative **3** in a 91% yield by treatment with *p*,*p*′-dimethoxytrityl chloride (1.4 equiv.) in a 1:5 (*v*/*v*) mixture of pyridine and DMF (25 °C, 5 h). The phosphorothioate triester **3** was then transformed into the allyl (*R*p)-phosphorothioate diester **4** and the methyl (*S*p)-phosphorothioate diester **5** by chemoselective and stereospecific removal of methyl and allyl groups, respectively. Thus, treatment of **3** with *tert*-butylamine[17] at 35 °C (24 h) removed the methyl group selectively in a stereoretentive manner to give the allyl (*R*p)-phosphorothioate diester **4** as the sole product. The structure of this product was confirmed by the spectroscopic data: no signal due to a methyl group attached to the phosphate function was observed in the ^{1}H NMR spectrum, whilst the ESI mass spectrum indicated a molecular peak at *m*/*z* 679.2713 [calcd. for $C_{34}H_{36}N_2O_9PS$ $[M-H]^-$ 679.1885] and also supported the demethylated structure. Furthermore, the stereochemical genuineness of the product was confirmed by ^{31}P NMR analysis, which indicated one singlet at δ = 52.8 ppm. The yield of **4** was 92%.

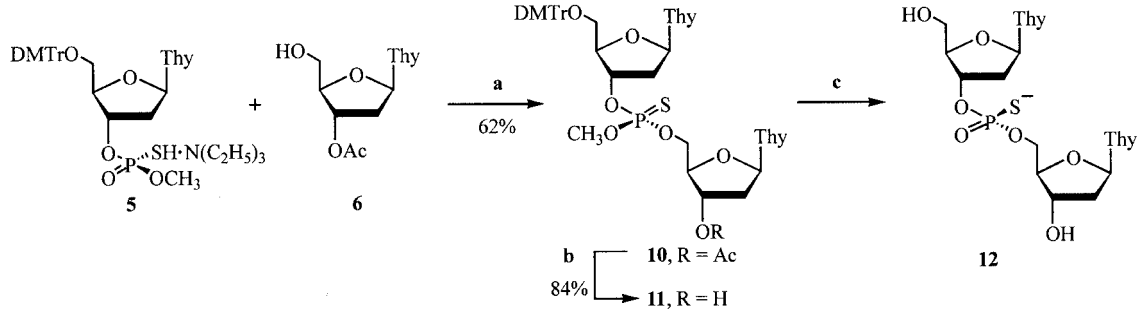
The stereospecific and chemoselective deallylation of **3** (1.0 equiv.), on the other hand, was carried out by treatment with a mixture of chloroform–tris(dibenzylideneacetone)dipalladium(0) complex $[Pd_2(dba)_3{\cdot}CHCl_3]$ (0.05 equiv.) and triphenylphosphane (0.25 equiv.) in THF (25 °C, 2 h)[18] to give **5**, which showed a single signal at δ = 54.3 ppm in its ^{31}P NMR spectrum. This observation confirmed that this product was stereochemically pure, and the ^{1}H NMR spectrum also indicated the stereochemical singleness of the product. The molecular peak at *m*/*z* 653.2584 in the ESI mass spectrum was consistent with **5** [calcd. for $C_{32}H_{34}N_2O_9PS$ $[M-H]^-$ 653.1728].



Scheme 1. Reagents and conditions: a) 1) 1*H*-tetrazole, $CH_2CH{=}CH_2OP[N(i\text{-}C_3H_7)_2]_2$, CH_3CN, mol. sieves (4 Å), 25 °C, 2) 55 °C, 36 h, 3) TEST, 25 °C, 1 h. b) CH_3ONa, CH_3OH, 25 °C, 24 h. c) DMTrCl, DMF/pyridine 5:1 (*v*/*v*), 25 °C, 7 h. d) 1) *t*-$C_4H_9NH_2$, 35 °C, 24 h, 2) $(C_2H_5)_3NH^+HCO_3^-/H_2O$. e) 1) $Pd_2(dba)_3{\cdot}CHCl_3$, $(C_6H_5)_3P$, *n*-$C_4H_9NH_2$, HCOOH, THF, 25 °C, 2 h, 2) $(C_2H_5)_3NH^+HCO_3^-/H_2O$.

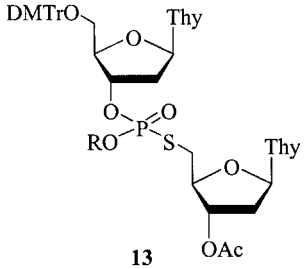


Scheme 2. Reagents and conditions: a) DEAD, $(C_6H_5)_3P$, THF, 55 °C, 3 h. b) CH_3ONa, CH_3OH, 25 °C, 4 h. c) 1) $Pd_2(dba)_3{\cdot}CHCl_3$, $(C_6H_5)_3P$, n-$C_4H_9NH_2$, HCOOH, THF, 25 °C, 3 h, 2) CH_3COOH, 60 °C, 4 h.



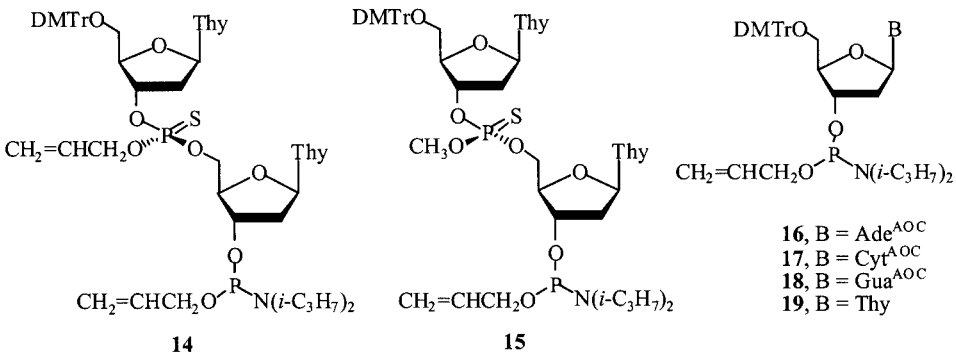
Scheme 3. Reagents and conditions: a) DEAD, $(C_6H_5)_3P$, THF, 55 °C, 3 h. b) CH_3ONa, CH_3OH, 25 °C, 4 h. c) 1) t-$C_4H_9NH_2$, 35 °C, 12 h, 2) CH_3COOH, 60 °C, 4 h.

The nucleoside phosphorothioate diesters **4** and **5** were converted into the corresponding (*S*p)- and (*R*p)-dithymidine phosphorothioates **7** and **10**, respectively, in a stereospecific manner through Mitsunobu reactions[19] with the 5′-*O*-free thymidine **6** (Scheme 2 and Scheme 3). Treatment of **4** or **5** (1.0 equiv.) with **6** (2.0 equiv.) in the presence of diethyl azodicarboxylate (DEAD) and triphenylphosphane (1.3 equiv.) in THF at 55 °C for 3 h gave stereochemically pure **7** or **10**, respectively.[20] Each reaction also afforded a considerable amount of the dithymidine thiaphosphate **13** as a by-product.



The stereochemistry of **7** and **10** was confirmed as follows. The 3′-*O*-acetyl protectors of **7** and **10** were first removed with sodium methoxide in methanol (25 °C, 4 h) to give **8** and **11**, respectively. Subsequently, **8** was converted into **9** by stereospecific, stereoretentive removal of the allyl group on the phosphorothioate moiety in a reaction catalyzed by a chloroform–tris(dibenzylideneacetone)dipalladium(0) complex in the presence of butylammonium formate (25 °C, 3 h) and subsequent removal of the 5′-*O*-DMTr protector by treatment with acetic acid (60 °C, 4 h). Compound **11**, on the other hand, was converted into **12** by successive treatment with *tert*-butylamine (35 °C, 12 h) to remove the methyl protector on the internucleotide linkage and acetic acid (60 °C, 4 h) to eliminate the 5′-*O*-DMTr group. The resulting phosphorothioate diesters **9** and **12** were subjected to enzyme reactions with nuclease P1 in the presence of a Zn^{2+} salt in a Tris·HCl buffer solution[8b,21] and with snake venom phosphodiesterase in the presence of a Mg^{2+} salt in a Tris·HCl buffer solution.[8b,22] The reaction with nuclease P1 digested **9** but did not hydrolyze **12** at all, whilst in contrast the reaction with snake venom phosphodiesterase degraded **12** but left **9** intact. These results indicated that **9** and **12** are phosphorothioates with (*S*) and (*R*) configurations, respectively.

Subsequently, we carried out solid-phase syntheses of four kinds of PO/PS-chimeric oligodeoxyribonucleotides – (*R*p,*R*p)-T[TpsT]T$_7$[TpsT]T$_9$ (**20**), (*R*p)-G[TpsT]CAT (**21**), (*S*p,*S*p)-T[TpsT]T$_7$[TpsT]T$_9$ (**22**), and (*S*p)-G[TpsT]CAT (**23**) – by the phosphoramidite method, using the dithymidine phosphorothioates **8** and **11** as building blocks for the construction of the phosphorothioate components. First, the phosphoramidites **14** and **15**, which are requisite building blocks for the synthesis, were prepared from **8** and **11**, respectively. Compound **8** was converted into **14** in an 85% yield by treatment with (allyloxy)[bis(diisopropylamino)]-phosphane in the presence of diisopropylammonium tetrazolide in acetonitrile containing mol. sieves (3 Å, 25 °C, 3 h), whilst **15** was prepared in a similar way from **11** in a



78% yield. The solid-phase synthesis was carried out on an Applied Biosystems Model 392 DNA/RNA synthesizer with the nucleoside allyl phosphoramidites **16**–**19** and the dinucleoside phosphoramidites **14** or **15** as building units by the standard procedure with a slight modification, in which the phosphoramidite coupling (0.2 min) and subsequent waiting (5.0 min) were carried out once in the cases of **16**, **17**, or **19** as the phosphoramidite whilst this treatment was repeated twice in the cases of **14**, **15**, or **18** as the phosphoramidite (Table 1). Here, the synthesis of oligonucleotides with only (*S*) configurations at the phosphorothioate components was performed with **14** and **16**–**19** as building units, whilst the synthesis of derivatives incorporating phosphorothioate moieties with only (*R*) configurations, on the other hand, employed **15** and **16**–**19** as phosphoramidites. After chain elongation was complete, products were deprotected and detached in all cases by successive treatment with the chloroform–tris(dibenzylideneacetone)dipalladium(0) complex in the presence of butylammonium formate (50 °C, 14 h) to remove the allyl protectors, followed by treatment with concentrated aqueous ammonia to detach the target compound from the solid support (25 °C, 2 h) and to remove the methyl protectors (55 °C, 6 h) in the synthesis of **20** and **21**. The yields of products estimated by conventional trityl assay were 85% for **20**, 87% for **21**, 82% for **22**, and 86% for **23**.

Table 1. Reaction sequence of the solid-phase synthesis.

Step	Operation	Reagent(s)	Time (min)	
1	washing	CH_3CN	0.4	
2	detritylation	3% Cl_3CCOOH	1.3	
3	washing	CH_3CN	0.5	
4	coupling	0.1 M amidite/CH_3CN + 1*H*-tetrazole/CH_3CN	0.2	x n[a]
5	waiting	CH_3CN	5.0	
6	washing	CH_3CN	0.3	
7	capping	Ac_2O/THF +*N*-methylimidazole/THF	0.2	
8	oxidation	0.1 M *t*-C_4H_9OOH/toluene	10.0	
9	washing	CH_3CN	0.6	

[a] In the cases of **16**, **17**, or **19** as the amidite component this treatment is conducted once (n = 1). In the cases of **14**, **15**, or **18** as the amidite component this treatment is conducted twice (n = 2).

The product structures were confirmed by the enzymatic treatment. The cases of **20** and **22**, with (*R*p) and (*S*p) configurations at all phosphorothioate functions, respectively, are representatively described. Treatment of **20** with nuclease P1 in Tris·HCl (37 °C, 24 h) did not cleave two of the phosphorothioate parts and gave pTpsT (see part A in Figure 1). In contrast, in the digestion of **20** with snake venom phosphodiesterase in Tris·HCl (37 °C, 24 h), the phosphorothioate parts were hydrolyzed and no formation of pTpsT was observed (Figure 1, B). These results suggested that two phosphorothioate functions in **20** have (*R*) configurations. In the case of **22**, on the other hand, its two phosphorothioate bonds were completely hydrolyzed by nuclease P1 in Tris·HCl (37 °C, 24 h) (Figure 1, C), but were not decomposed by snake venom phosphodiesterase in Tris·HCl (37 °C, 24 h), thus giving pTpsT (Figure 1, D). These results confirmed that the absolute configurations of the two phosphorothioate moieties in **22** are both (*S*). In a similar manner, the stereochemistry of the phosphorothioate in **21** and **23** was confirmed.

Conclusions

We have demonstrated a new methodology for the stereoregulated synthesis of PO/PS-chimeric oligodeoxyribonucleotides, the keystone of which is a novel method for preparing the stereochemically pure (*R*p)- and (*S*p)-dithymidine phosphorothioates **7** and **10**, achieved by a combination of stereocontrolled reactions. These involve: 1) 1*H*-tetrazole-promoted condensation of thymidine and (allyloxy)[bis(diisopropylamino)]phosphane followed by thermal equilibration of the resulting product, affording an (*S*p)-thymidine 3′,5′-cyclic phosphate[23] as the major component, 2) stereospecific sulfurization of this major product, giving an allyl (*R*p)-thymidine 3′,5′-cyclic phosphorothioate, 3) regioselective and stereoselective methanolysis of the allyl (*R*p)-thymidine 3′,5′-cyclic phosphorothioate, producing an allyl methyl (*R*p)-thymidine 3′-phosphorothioate as the

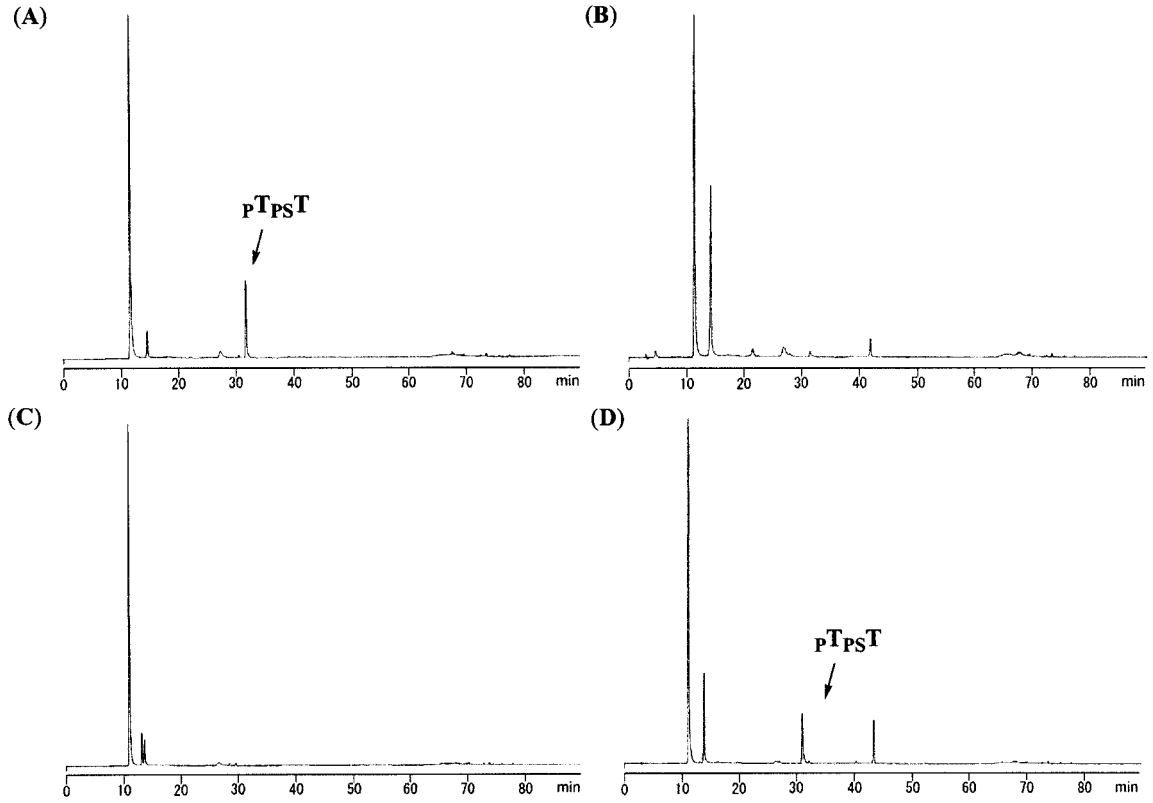


Figure 1. Reversed-phase HPLC profiles: A) treatment of **20** with nuclease P1, B) treatment of **20** with svPDE, C) treatment of **22** with nuclease P1, D) treatment of **22** with svPDE. Conditions: COSMOSIL 5C18-AR-II column (4.6×250 mm); buffer A, 0.1 M triethylammonium acetate in H_2O; buffer B, CH_3CN/H_2O, 4:1; gradient, linear 0–20% B in A for 60 min; detection; 254 nm; flow rate, 1.0 mL·min^{-1}; temperature, 40 °C.

major product, 4) after dimethoxytritylation of the free 5′-hydroxy group in the allyl methyl (*R*p)-thymidine 3′-phosphorothioate, stereospecific removal of the methyl group from the phosphorothioate triester moiety by treatment with *tert*-butylamine, giving an allyl (*R*p)-5′-*O*-dimethoxytritylthymidine 3′-phosphorothioate diester, or stereospecific removal of the allyl group from the phosphate moiety in an organopalladium-catalyzed reaction, producing a methyl (*S*p)-5′-*O*-dimethoxytritylthymidine 3′-phosphorothioate, and 5) stereospecific condensation of the allyl (*R*p)-thymidine 3′-phosphorothioate or the methyl (*S*p)-thymidine 3′-phosphorothioate with a 5′-*O*-free thymidine derivative in Mitsunobu reactions, which results in retention of the stereochemistry of the phosphorothioate function to provide an allyl (3′–5′)-linked (*S*p)-dithymidine phosphorothioate or a methyl (3′–5′)-linked (*R*p)-dithymidine phosphorothioate, respectively. This preparation of **7** and **10** has remarkable advantages not found in existing methods for the stereodefined preparation of related dinucleoside phosphorothioates, including those developed by Stec,[8b,10,24] Beaucage,[25] Agrawal,[26] Just,[27] Wada,[28] and Stawinski.[29] *The most remarkable advantage of this method is that both the (Rp)- and the (Sp)-dithymidine phosphorothioates can be synthesized from a common intermediate, the corresponding (Rp)-thymidine 3′,5′-cyclic phosphate. In other words, this method requires only one synthetic precursor for either kind of dithymidine phosphorothioate with (R) and (S) configurations at the stereogenic phosphorus atom.* In contrast, all the existing methods for the stereodefined synthesis of these two dithymidine phosphorothioates require the preparation, as precursors, of two kinds of suitable thymidine 3′-phosphorus compound, one with the (*R*p) configuration and the other with the (*S*p) configuration, in pure forms. Moreover, the acquisition of these monomer units is troublesome: the methods of Stec, Beaucage, and Stawinski, for example, require the separation of nearly 1:1 mixtures of (*R*p)- and (*S*p)-thymidine 3′-phosphorus compounds such as thymidine 3′-*O*-(2-thio-spiro-4,4-pentamethylene-1,3,2-oxathiaphosphorane) (Stec), a thymidine 3′-(cyclic phosphoramidite) based on *N*-fluoroacetyl-2-aminoethanol (Beaucage), and a thymidine 3′-[1-oxido-2-picolyl-(9-fluorenemethyl) phosphorothioate] (Stawinski). Meanwhile, the methods of Agrawal, Just, and Wada require the preparation of enantiomerically or diastereomerically pure chiral amino alcohols: namely (*R*)- and (*S*)-2-(hydroxymethyl)pyrrolidines (Agrawal), 1,2-di-*O*-cyclopentylidene-5-isopropylamino-D-xylofuranose and its L isomer (Just), (*R*)- and (*S*)-2-(indolyl)ethanol derivatives (Just), or (*R*)- and (*S*)-2-methylamino-1-phenylethanols (Wada), all of these serving as components of their nucleoside 3′-phosphoramidite units. In these methods, the stereochemically pure chiral amino alcohols have to be synthesized individually by two pathways starting from two kinds of suitable optically pure substances. In contrast, our

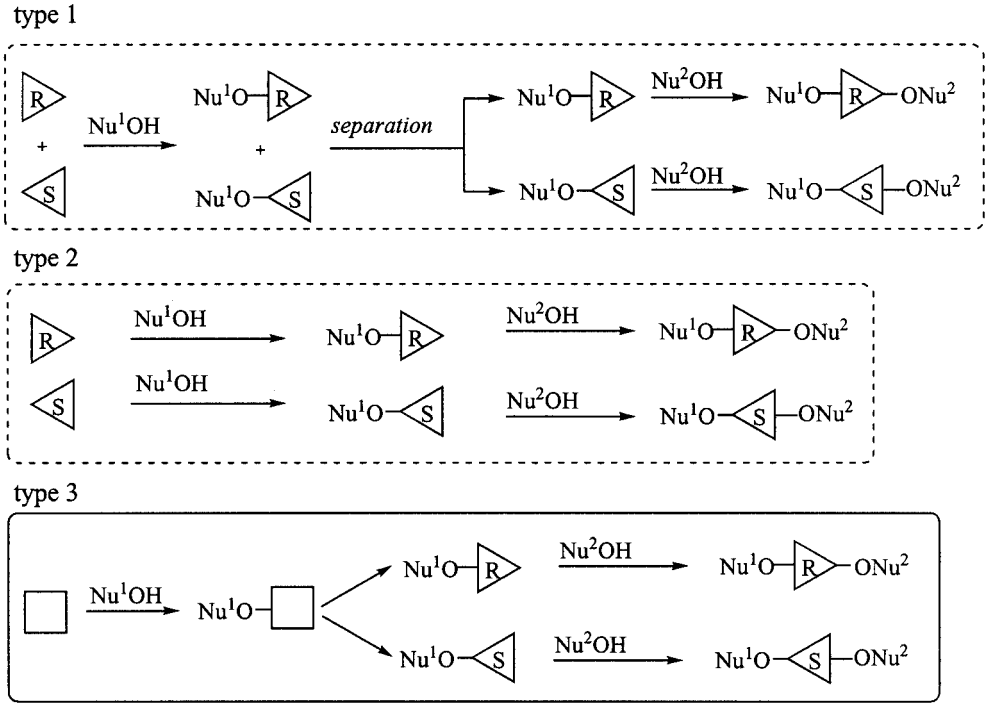


Figure 2. Three typical modes of synthetic strategies.

method does not require any chiral compounds other than a nucleoside for the preparation of the key building unit **1** with the pure (*R*p) configuration, which is another remarkable advantage of our method. To illustrate these matters, three types of strategies for the stereodefined synthesis of (*R*p)- and (*S*p)-dinucleoside phosphorothioates, as outlined in Figure 2, have so far been developed. The Stec and Beaucage syntheses were achieved by the first type of strategy, whilst the syntheses carried out by Agrawal, Just, and Wada used the second type. Meanwhile, the current synthesis is the only use of the third type to have been reported. Among these three strategies, the third is undoubtedly superior in operational simplicity, which is essential in organic synthesis. Accordingly, this approach may serve as a highly useful tool for the synthesis of stereodefined PO/PS-chimeric oligonucleotides.

Experimental Section

General Methods: UV spectra were measured on a JASCO V-500 spectrometer. NMR spectra were taken in $CDCl_3$, unless otherwise noted, on a JEOL JNM-α400 or a ECA-500 instrument. The ^1H, ^{13}C, and ^{31}P NMR chemical shifts are given as δ values in ppm relative to $(CH_3)_4Si$ for ^1H and ^{13}C NMR spectra, 85% H_3PO_4 was used as reference for ^{31}P NMR spectra. ESI-TOF high-resolution mass (HRMS) spectra were obtained with an Applied Biosystems Mariner or a QStar spectrometer. HPLC analysis was carried out with a COSMOSIL 5C18-MS column (Nacalai Tesque, ODS-5 mm, 4.6×250 mm) on a Waters 2695 Separations Module chromatograph with a Waters 2996 Photodiode Array detector. Column chromatography was performed on Nacalai Tesque silica gel 60 (neutral, 75 μm). Unless otherwise noted, synthetic reactions were carried out at ambient temperature. Reactions requiring anhydrous conditions were carried out under argon in flasks dried by heating at 400 °C under 133–400 Pa, or by washing with a 5% solution of dichlorodimethylsilane in dichloromethane followed by anhydrous dichloromethane, and then heating at 100 °C.

Materials and Solvents: Compounds **6**[30] and **16**–**19**,[31] CH_2=$CHCH_2OP[N(i\text{-}C_3H_7)_2]_2$,[18b] $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$,[18b] and a 1.0 M *tert*-butyl hydroperoxide/toluene solution[32] were prepared by the reported methods. Bis[3′-(triethoxysilyl)propyl] tetrasulfide (TEST)[14] was purchased from Shin-etsu (Japan). 1*H*-Tetrazole was used after sublimation of material purchased from Dozindo (Japan). Triphenylphosphane was recrystallized from hexane before use. THF was used after drying by reflux over sodium/benzophenone ketyl. Acetone, acetonitrile, and dichloromethane were distilled from calcium hydride. Deoxyribonucleosides (Yamasa), sodium methoxide (Nakalai Tesque), *p*,*p*′-dimethoxytrityl chloride (Tokyo Kasei), dichloroacetic acid (Tokyo Kasei), triethylamine (Kishida), *tert*-butylamine (Nakalai Tesque), a 40% diethyl azodicarboxylate/toluene solution (Tokyo Kasei), snake venom phosphodiesterase (Funakoshi), Sma I (Takara Bio), nuclease P1 (Yamasa), and a 1 M Tris·HCl buffer solution (Nakalai Tesque) were used as commercially supplied without any purification. Solid and amorphous organic substances were used after drying at 50–60 °C for 8–12 h under 133–400 Pa. Powdery mol. sieves (3 Å) were employed after the commercially supplied (Nakalai Tesque) product had been dried at 200 °C for 12 h under 133–400 Pa.

Synthesis of Allyl (*R*p)-Thymidine 3′,5′-Cyclic Phosphorothioate (1): A mixture of thymidine (4.84 g, 20.0 mmol) and 1*H*-tetrazole (4.20 g, 60.0 mmol) in acetonitrile (250 mL) containing powdered mol. sieves (4 Å) was stirred at 25 °C for 30 min. To this mixture was slowly added, at 0 °C, a solution of CH_2=$CHCH_2OP[N(i\text{-}C_3H_7)_2]_2$ (7.67 mL, 7.29 g, 24.0 mmol) in dichloromethane (25.0 mL). The resulting mixture was stirred at 0 °C for 30 min, at 25 °C for 4 h, and at 55 °C for 24 h. After the mixture had cooled to 25 °C, TEST (11.8 mL, 13.0 g, 24.0 mmol) was added to it, and

the resulting mixture was stirred at the same temperature for 2 h. The reaction mixture was passed through a Celite 545 pad to remove the mol. sieves and the filtrate was concentrated. The residue was dissolved in ethyl acetate (200 mL), and the resulting organic solution was washed with an aqueous sodium hydrogen carbonate solution (200 mL). The aqueous layer was extracted with ethyl acetate (200 mL, 2×), and the combined organic solutions were washed with brine (300 mL), dried with Na_2SO_4 (ca. 150 g), and concentrated to give a viscous oil. This crude material was subjected to silica gel column chromatography with a 1:2 mixture of ethyl acetate and hexane as eluent to afford the desired product **1** (5.68 g, 79%) in a pure form: TLC: R_f = 0.41 (ethyl acetate/hexane, 1:2). ^{1}H NMR: δ = 1.96 (s, 3 H), 2.53–2.65 (m, 2 H), 3.94 (dt, J = 4.4 and 10.4 Hz, 1 H), 4.45 (dt, J = 2.0 and 9.6 Hz, 1 H), 4.53–4.72 (m, 3 H), 4.80 (dd, J = 8.4 and 17.6 Hz 1 H), 5.34 (dd, J = 0.8 and 10.4 Hz, 1 H), 5.45 (dd, J = 1.6 and 17.6 Hz, 1 H), 5.97–6.10 (m, 2 H), 6.98 (d, J = 1.2 Hz, 1 H), 9.05 (br., 1 H) ppm. ^{13}C NMR: δ = 12.5, 35.2 (d, J = 8.2 Hz), 69.2 (d, J = 4.2 Hz), 70.0 (d, J = 11.5 Hz), 73.9 (d, J = 7.4 Hz), 77.2 (d, J = 8.2 Hz), 86.1, 112.0, 119.4, 132.0 (d, J = 7.4 Hz), 136.2, 149.8, 163.4 ppm. ^{31}P NMR: δ = 61.0 ppm. HRMS (ESI$^+$): m/z calcd. for $C_{13}H_{17}N_2O_6PSNa$ $[M+Na]^+$: 383.0437; found 383.0663.

Synthesis of Allyl Methyl (*R*p)-Thymidin-3′-yl Phosphorothioate (2): A solution of sodium methoxide in methanol (1.0 M, 15.0 mL) was slowly added at 0 °C to a stirred solution of **1** (1.80 g 5.00 mmol) in methanol (50.0 mL). The reaction mixture was stirred at 25 °C for 24 h, and ethyl acetate (500 mL) was added. The organic solution was washed with an ammonium chloride-saturated aqueous solution (100 mL), the aqueous layer was extracted with ethyl acetate (200 mL, 2×), and the combined organic layers were washed with brine (300 mL), dried with Na_2SO_4 (ca. 150 g), and concentrated. The resulting crude product was subjected to silica gel column chromatography with elution with a 1:2 mixture of ethyl acetate and hexane to afford **2** (1.63 g, 83% yield): TLC R_f = 0.37 (ethyl acetate/hexane, 1:2). ^{1}H NMR: δ = 1.93 (s, 3 H), 2.49 (dd, J = 4.4 and 7.2 Hz, 1 H), 2.58 (br. s, 1 H), 3.78 (d, J = 13.6 Hz, 3 H), 3.89–3.99 (m, 2 H), 4.24 (dd, J = 2.4 and 5.2 Hz, 1 H), 4.58 (ddt, J = 1.2, 2.4, and 6.8 Hz, 2 H), 5.18–5.23 (m, 1 H), 5.29 (dd, J = 1.2 and 10.5 Hz, 1 H), 5.39 (ddd, J = 1.5, 2.9, and 17.3 Hz, 1 H), 5.90–5.99 (m, 1 H), 6.20 (t, J = 6.8 Hz, 1 H), 7.45 (d, J = 1.2 Hz, 1 H), 8.61 (br. s, 1 H) ppm. ^{13}C NMR: δ = 12.5, 38.2 (d, J = 5.7 Hz), 54.7 (d, J = 5.7 Hz), 62.1, 69.1 (d, J = 4.9 Hz), 78.2 (d, J = 4.9 Hz), 85.5 (d, J = 5.7 Hz), 86.5, 111.3, 118.8, 132.1 (d, J = 7.4 Hz), 136.6, 150.4, 163.7 ppm. ^{31}P NMR: δ = 66.2 ppm. HRMS (ESI$^+$): m/z calcd. for $C_{14}H_{21}N_2O_7PSNa$ $[M+Na]^+$: 415.0699; found 415.0911.

Dimethoxytritylation of Allyl Methyl (*R*p)-Thymidin-3′-yl Phosphorothioate (2) to Provide Allyl Methyl (*R*p)-5′-*O*-(*p,p*′-Dimethoxytrityl)thymidin-3′-yl Phosphorothioate (3): A solution of **2** (834 mg, 2.12 mmol) in a 5:1 mixture of *N*,*N*-dimethylformamide (20.0 mL) and pyridine (4.00 mL) was chilled to 0 °C, and small portions of *p,p*′-dimethoxytrityl chloride (1.01 g, 2.97 mmol) were added. The resulting homogeneous mixture was stirred at 0 °C for 30 min and then at 25 °C for 7 h. Ethyl acetate (100 mL) was added to the reaction mixture, and the resulting organic solution was washed with a sodium hydrogen carbonate-saturated aqueous solution (50 mL). The aqueous layer was extracted with ethyl acetate (100 mL, 2×), and the organic layer was washed with brine (50 mL), dried with Na_2SO_4 (ca. 30 g), and concentrated to give an oily residue. This crude product was purified by silica gel column chromatography with a 1:1 mixture of ethyl acetate and hexane as eluent to afford **3** (1.34 g, 91% yield): TLC R_f = 0.43 (ethyl acetate/hexane, 1:1). ^{1}H NMR: δ = 1.45 (s, 3 H), 2.36–2.43 (m, 1 H), 2.59–2.64 (m, 1 H), 3.45–3.46 (m, 2 H), 3.69 (d, J = 13.7 Hz, 3 H), 3.80 (s, 6 H), 4.27 (d, J = 1.9 Hz, 1 H), 4.55 (dd, J = 5.6 and 10.2 Hz, 2 H), 5.27–5.40 (m, 3 H), 5.88–5.98 (m, 1 H), 6.44 (dd, J = 5.4 and 8.5 Hz, 1 H), 6.84–6.86 (m, 4 H), 7.23–7.41 (m, 9 H), 7.56 (s, 1 H), 8.25 (br. s, 1 H) ppm. ^{13}C NMR: δ = 11.7, 39.2 (d, J = 5.7 Hz), 54.6 (d, J = 5.8 Hz), 55.3, 63.3, 69.0 (d, J = 5.0 Hz), 78.8 (d, J = 4.2 Hz), 150.1, 158.8, 163.3 ppm. ^{31}P NMR: δ = 68.5 ppm. HRMS (ESI$^+$): m/z calcd. for $C_{35}H_{39}N_2O_9PSNa$ $[M+Na]^+$: 717.2006; found 717.2423.

Synthesis of Allyl (*R*p)-5′-*O*-(*p,p*′-Dimethoxytrityl)thymidin-3′-yl Phosphorothioate Triethylammonium Salt (4): A solution of the phosphorothioate triester **3** (1.00 g, 1.44 mmol) in *tert*-butylamine (25.0 mL) was stirred at 35 °C for 24 h. Evaporation of the reaction mixture gave a yellow oil, which was dissolved in dichloromethane (20 mL). The solution was washed with an aqueous triethylammonium hydrogen carbonate solution (0.1 M, 10 mL, 2×), the organic layer was dried with magnesium sulfate (ca. 20 g) and concentrated, and the resulting residue was subjected to silica gel column chromatography with a 1:9 methanol/dichloromethane mixture. Fractions including the desired compound were combined and concentrated to give a residual material, which was again dissolved in dichloromethane (5.0 mL). The solution was shaken with an aqueous solution of triethylammonium hydrogen carbonate (0.1 M, 10 mL, 2×) to convert the phosphorothioic acid completely into its triethylammonium salt. The resulting organic solution was dried with magnesium sulfate (ca. 20 g) and concentrated, the residue was dissolved in dichloromethane (2.0 mL), and the solution was poured into petroleum ether (200 mL). The precipitates were collected by filtration and dried to give the phosphorothioate diester **4** (1.04 g, 92% yield): TLC R_f = 0.37 (methanol/dichloromethane, 1:9). ^{1}H NMR ([D$_6$]DMSO): δ = 1.17 (t, J = 7.2 Hz, 9 H), 1.34 (s, 3 H), 2.24–2.40 (m, 2 H), 3.05–3.15 (m, 7 H), 3.28–3.34 (m, 1 H), 3.74 (s, 6 H), 4.16–4.24 (m, 3 H), 5.03 (dd, J = 1.7 and 10.5 Hz, 2 H), 5.19 (ddd, J = 2.0, 3.9, and 17.3 Hz, 1 H), 5.82–5.91 (m, 1 H), 6.20 (dd, J = 5.6 and 8.5 Hz, 1 H), 6.88–6.90 (m, 4 H), 7.22–7.40 (m, 9 H), 7.49 (s, 1 H), 9.23 (br. s, 1 H), 11.32 (s, 1 H) ppm. ^{13}C NMR: δ = 8.56, 11.5, 39.5 (d, J = 3.3 Hz), 45.6, 55.2, 64.0, 67.0 (d, J = 5.8 Hz), 76.7 (d, J = 4.9 Hz), 84.7, 85.6 (d, J = 5.8 Hz), 87.0, 111.1, 113.3, 116.2, 127.0, 128.2, 130.2, 134.6 (d, J = 8.2 Hz), 135.4, 135.6, 135.9, 144.4, 150.4, 158.7, 163.7 ppm. ^{31}P NMR ([D$_6$]-DMSO): δ = 52.8 ppm. HRMS (ESI$^-$): m/z calcd. for $C_{34}H_{36}N_2O_9PS$ $[M-H]^-$: 679.1885; found 679.2713.

Synthesis of Methyl (*S*p)-5′-*O*-(*p,p*′-Dimethoxytrityl)thymidin-3′-yl Phosphorothioate Triethylammonium Salt (5): Triphenylphosphane (6.56 mg, 25.0 μmol) and $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$ (5.18 mg, 5.00 μmol) were added to a solution of the phosphorothioate triester **3** (65.9 mg, 100 μmol) in THF (1.00 mL), and the resulting mixture was stirred at 25 °C for 30 min. To this mixture were added, at 0 °C, butylamine (19.0 μL, 14.1 mg, 200 μmol) and formic acid (7.50 μL, 9.18 mg, 200 μmol). The reaction mixture was stirred at 25 °C for 2 h and was then concentrated to afford a yellow oil. This material was dissolved in dichloromethane (5.0 mL) and washed with a solution of triethylammonium hydrogen carbonate in water (0.1 M, 5.0 mL, 2×). The organic layer was dried with magnesium sulfate (ca. 10 g) and concentrated, the residue was subjected to silica gel column chromatography with a 1:9 methanol/dichloromethane mixture, collected fractions containing the desired product were concentrated, and the resulting residue was dissolved in dichloromethane (5.0 mL). In order to convert the phosphorothioic acid completely into the triethylammonium salt, the solution was again treated with an aqueous triethylammonium hydrogen carbonate solution (0.1 M, 5.0 mL, 2×). The organic layer was dried with magnesium sulfate (ca. 10 g) and concentrated, the

residue was dissolved in dichloromethane (1.0 mL) and poured into petroleum ether (100 mL), and the precipitates were collected by filtration and dried to give the target compound **5** (66.5 mg, 88% yield): TLC R_f = 0.21 (methanol/dichloromethane, 1:9). ^{1}H NMR ([D$_6$]DMSO): δ = 1.17 (t, J = 7.2 Hz, 9 H), 1.37 (s, 3 H), 2.25–2.38 (m, 2 H), 3.07 (q, J = 7.3 Hz, 6 H), 3.15 (dd, J = 2.7 and 10.2 Hz, 1 H), 3.27–3.29 (m, 1 H), 3.74 (s, 6 H), 4.11 (d, J = 2.4 Hz, 1 H), 5.00–5.03 (m, 1 H), 6.18 (dd, J = 5.9 and 8.3 Hz, 1 H), 6.88–6.90 (m, 4 H), 7.22–7.41 (m, 9 H), 7.48 (s, 1 H), 9.49 (br. s, 1 H), 11.32 (s, 1 H) ppm. ^{13}C NMR: δ = 8.62, 11.6, 39.8 (d, J = 4.1 Hz), 45.7, 52.9 (d, J = 6.5 Hz), 55.2, 63.9, 76.5 (d, J = 4.9 Hz), 84.7, 85.1 (d, J = 5.8 Hz), 87.0, 111.1, 113.3, 127.0, 128.2, 130.1, 135.4, 135.6, 135.8, 144.4, 150.4, 158.7, 163.8 ppm. ^{31}P NMR ([D$_6$]DMSO): δ = 54.3 ppm. HRMS (ESI$^-$): *m*/*z* calcd. for $C_{32}H_{34}N_2O_9PS$ [M – H]$^-$: 653.1728; found 653.2584.

Synthesis of 3′-*O*-(Acetyl)thymidin-5′-yl Allyl (*S*p)-5′-*O*-(*p*,*p*′-Dimethoxytrityl)thymidin-3′-yl Phosphorothioate (7): A solution of DEAD in toluene (40%, 0.35 mL, 0.77 mmol) was added at 0 °C to a solution of triphenylphosphane (202 mg, 0.77 mmol) in THF (20 mL). After the mixture had been stirred at 0 °C for 30 min, **4** (460 mg, 0.59 mmol) and the nucleoside **6** (335 mg, 1.18 mmol) were added at the same temperature and the reaction mixture was stirred at 60 °C for 3 h. According to ^{31}P NMR analysis, this reaction mixture included not only the desired compound **13** but also a considerable amount (ca. 42%) of the by-product **13** (R^1 = CH_2=$CHCH_2$), showing a ^{31}P NMR signal at δ = 26.5 ppm. The reaction mixture was diluted with ethyl acetate (50 mL) and washed with an ammonium chloride-saturated aqueous solution (20 mL). The aqueous layer was extracted with ethyl acetate (50 mL, 2×), and the collected organic solutions were washed with brine (20 mL), dried with Na_2SO_4 (ca. 50 g), and concentrated. The resulting residue was subjected to column chromatography on silica gel with a 1:9 mixture of methanol and dichloromethane, followed by a 1:30:10 mixture of methanol, ethyl acetate, and hexane as eluents to afford **7** (301 mg, 54% yield): TLC R_f = 0.57 (methanol/dichloromethane, 1:9), R_f = 0.43 (methanol/ethyl acetate/hexane, 1:30:10). ^{1}H NMR: δ = 1.47 (s, 3 H), 1.94 (s, 3 H), 2.10–2.19 (m, 4 H), 2.35–2.44 (m, 2 H), 2.60 (dd, J = 5.6 and 12.8 Hz, 1 H), 3.46 (d, J = 2.4 Hz, 2 H), 3.80 (s, 6 H), 4.13–4.35 (m, 4 H), 4.60 (dd, J = 5.6 and 10.8 Hz, 2 H), 5.21–5.41 (m, 4 H), 5.88–5.97 (m, 1 H), 6.36 (dd, J = 5.6 and 9.2 Hz, 1 H), 6.41 (dd, J = 5.2 and 8.4 Hz, 1 H), 6.85 (d, J = 8.8 Hz, 4 H), 7.22–7.40 (m, 10 H), 7.58 (s, 1 H), 8.81 (s, 1 H), 8.85 (s, 1 H) ppm. ^{13}C NMR: δ = 11.8, 12.6, 21.0, 37.1, 39.1 (d, J = 5.8 Hz), 55.3, 63.3, 67.5 (d, J = 5.8 Hz), 69.4 (d, J = 4.9 Hz), 74.4, 79.6 (d, J = 4.1 Hz), 82.6 (d, J = 9.1 Hz), 84.4, 84.6, 84.7 (d, J = 5.8 Hz), 87.3, 111.6, 111.9, 113.4, 119.5, 127.2, 128.1, 130.1, 131.7 (d, J = 7.4 Hz), 134.8, 135.1 (2 C), 135.2, 144.1, 150.4, 150.5, 158.8, 163.6, 163.7, 170.4 ppm. ^{31}P NMR: δ = 67.0 ppm. HRMS (ESI$^+$): *m*/*z* calcd. for $C_{46}H_{51}N_4O_{14}PSNa$ [M + Na]$^+$: 969.2752; found 969.3589.

Synthesis of 3′-*O*-(Acetyl)thymidin-5′-yl (*R*p)-5′-*O*-(*p*,*p*′-Dimethoxytrityl)thymidin-3′-yl Methyl Phosphorothioate (10): A DEAD/toluene solution (40%, 0.40 mL, 0.88 mmol) was added at 0 °C with stirring to a solution of triphenylphosphane (231 mg, 0.88 mmol) in THF (23 mL). Stirring at this temperature was continued for 30 min. To this mixture, at the same temperature, were added the phosphorothioate **5** (510 mg, 0.68 mmol) and the nucleoside **6** (387 mg, 1.36 mmol). The reaction mixture was stirred at 60 °C for 3 h. ^{31}P NMR analysis showed that this reaction mixture included the desired compound **10** and a considerable amount (ca. 33%) of the by-product **13** (R^1 = CH_3), showing a ^{31}P NMR signal at δ = 27.1 ppm. Ethyl acetate (100 mL) was added to this reaction mixture, and the mixture was washed with an ammonium chloride-saturated aqueous solution (50 mL). The aqueous layer was extracted with ethyl acetate (100 mL, 2×), and the organic layers were collected and washed with brine (50 mL). After drying, concentration of the organic solution afforded a residual material, which was subjected to silica gel column chromatography with elution with a 1:9 mixture of methanol and dichloromethane followed by a 1:30:10 mixture of methanol, ethyl acetate, and hexane to give **10** (389 mg, 62% yield): TLC R_f = 0.60 (methanol/dichloromethane, 1:9), R_f = 0.38 (methanol/ethyl acetate/hexane, 1:30:10). ^{1}H NMR: δ = 1.46 (s, 3 H), 1.95 (s, 3 H), 2.11 (s, 3 H), 2.16–2.25 (m, 1 H), 2.37–2.50 (m, 2 H), 2.58 (dd, J = 5.6 and 14.0 Hz, 1 H), 3.45 (br., 2 H), 3.69 (d, J = 14.0 Hz, 3 H), 3.80 (s, 6 H), 4.18–4.23 (m, 2 H), 4.28–4.38 (m, 2 H), 5.28–5.30 (m, 1 H), 5.35–5.39 (m, 1 H), 6.34–6.41 (m, 2 H), 6.85 (d, J = 8.8 Hz, 4 H), 7.23–7.70 (m, 11 H), 8.65 (s, 1 H), 8.73 (s, 1 H) ppm. ^{13}C NMR: δ = 11.8, 12.5, 20.9, 37.2, 39.0 (d, J = 5.8 Hz), 54.8 (d, J = 4.9 Hz), 55.3, 63.3, 67.7 (d, J = 5.8 Hz), 74.4, 79.7 (d, J = 3.3 Hz), 82.7 (d, J = 9.1 Hz), 84.4, 84.6 (d, J = 6.6 Hz), 84.8, 87.3, 111.7 (2 C), 113.4, 127.2, 128.1, 130.1, 135.0 (2 C), 135.1, 135.2, 144.1, 150.5, 150.6, 158.8, 163.8 (2 C), 170.6 ppm. ^{31}P NMR: δ = 68.0 ppm. HRMS (ESI$^+$): *m*/*z* calcd. for $C_{44}H_{49}N_4O_{14}PSNa$ [M + Na]$^+$: 943.2596; found 943.3270.

Preparation of Allyl (*S*p)-5′-*O*-(*p*,*p*′-Dimethoxytrityl)thymidin-3′-yl Thymidin-5′-yl Phosphorothioate (8) by Deacetylation of (*S*p)-3′-*O*-(Acetyl)thymidin-5′-yl Allyl 5′-*O*-(*p*,*p*′-Dimethoxytrityl)thymidin-3′-yl Phosphorothioate (7): Sodium methoxide in methanol solution (1.0 M, 55.0 μL, 55.0 μmol) was slowly added at 0 °C to a solution of **7** (103 mg, 109 μmol) in methanol (4.00 mL). The reaction mixture was warmed up to 25 °C and stirred at this temperature for 4 h, diluted with ethyl acetate (20 mL), and washed with an ammonium chloride-saturated aqueous solution (5.0 mL). The aqueous layer was extracted with ethyl acetate (20 mL, 2×), and the combined organic layers were washed with brine (10 mL), dried with Na_2SO_4 (ca. 30 g), and concentrated to give an oily product. This crude product was purified by silica gel column chromatography with a 1:9 mixture of methanol and dichloromethane as eluent to afford **8** (86.8 mg, 88% yield): TLC R_f = 0.53 (methanol/dichloromethane, 1:9). ^{1}H NMR: δ = 1.48 (s, 3 H), 1.92 (s, 3 H), 2.10–2.17 (m, 1 H), 2.35–2.45 (m, 2 H), 2.60 (dd, J = 5.6 and 13.2 Hz, 1 H), 3.46 (d, J = 2.4 Hz, 2 H), 3.80 (s, 6 H), 4.08–4.32 (m, 4 H), 4.46 (t, J = 2.8 Hz, 1 H), 4.59 (dd, J = 5.6 and 10.8 Hz, 2 H), 5.26–5.40 (m, 3 H), 5.87–5.97 (m, 1 H), 6.32 (t, J = 6.4 Hz, 1 H), 6.41 (dd, J = 5.2 and 8.8 Hz, 1 H), 6.85 (d, J = 9.2 Hz, 4 H), 7.22–7.40 (m, 10 H), 7.56 (d, J = 1.2 Hz, 1 H), 9.36 (s, 1 H), 9.53 (s, 1 H) ppm. ^{13}C NMR: δ = 11.8, 12.6, 39.0 (d, J = 4.9 Hz), 40.1, 55.3, 63.4, 67.6 (d, J = 6.6 Hz), 69.3 (d, J = 4.1 Hz), 71.3, 79.6 (d, J = 4.9 Hz), 84.5, 84.6 (d, J = 8.2 Hz), 84.7 (d, J = 4.1 Hz), 85.1, 87.3, 111.5, 111.9, 113.4, 119.3, 127.2, 128.1, 130.1, 131.8 (d, J = 6.9 Hz), 135.1, 135.2, 135.6, 144.2, 150.7, 150.9, 158.8, 164.0, 164.2 ppm. ^{31}P NMR: δ = 65.7 ppm. HRMS (ESI$^+$): *m*/*z* calcd. for $C_{44}H_{49}N_4O_{13}PSNa$ [M + Na]$^+$: 927.2647; found 927.3500.

Preparation of (*R*p)-5′-*O*-(*p*,*p*′-Dimethoxytrityl)thymidin-3′-yl Methyl Thymidin-5′-yl Phosphorothioate (11) by Deacetylation of (*R*p)-3′-*O*-(Acetyl)thymidin-5′-yl 5′-*O*-(*p*,*p*′-Dimethoxytrityl)thymidin-3′-yl Methyl Phosphorothioate (10): Deacetylation of **10**, affording **11**, was carried out in a manner similar to that described above for preparation of **8** from **7**. From 19.0 mg (21.0 μmol) of **10**, 15.5 mg of **11** (84% yield) was produced: TLC R_f = 0.54 (methanol/dichloromethane, 1:9). ^{1}H NMR: δ = 1.50 (s, 3 H), 1.92 (s, 3 H), 2.16–2.23 (m, 1 H), 2.31–2.38 (m, 1 H), 2.44 (ddd, J = 3.4, 5.8 and 9.8 Hz, 1 H), 2.68 (dd, J = 4.9 and 13.6 Hz, 1 H), 3.40 (dd, J = 3.4 and 10.7 Hz, 1 H), 3.69 (d, J = 6.8 Hz, 3 H), 3.79 (s, 6 H), 4.13–4.15 (m, 1 H), 4.24–4.35 (m, 3 H), 4.52–4.55 (m, 1 H), 5.26–5.29 (m, 1 H), 6.29–6.33 (m, 2 H), 6.85 (d, J = 8.8 Hz, 4 H), 7.22–7.41

(m, 10 H), 7.58–7.59 (m, 1 H), 9.22 (s, 1 H), 9.83 (s, 1 H) ppm. ^{13}C NMR: δ = 11.9, 12.4, 39.4 (d, J = 3.3 Hz), 40.5, 54.8 (d, J = 5.8 Hz), 55.3, 63.4, 67.4 (d, J = 6.6 Hz), 71.0, 80.2 (d, J = 3.3 Hz), 84.5 (d, J = 8.2 Hz), 84.6 (d, J = 7.4 Hz), 84.8, 85.3, 87.4, 111.1, 112.0, 113.4, 127.3, 128.4, 130.1, 134.9, 135.1 (2 C), 144.1, 150.6, 151.2, 158.8, 163.8, 163.9 ppm. ^{31}P NMR: δ = 68.1 ppm. HRMS (ESI$^+$): m/z calcd. for $C_{42}H_{47}N_4O_{13}PSNa$ $[M+Na]^+$: 901.2490; found 901.2828.

Preparation of the Phosphoramidite 14 from 8: Diisopropylammonium 1*H*-tetrazolide (281 mg, 1.64 mmol) was added to a solution of **14** (738 mg, 0.82 mmol) in acetonitrile (27.0 mL) containing powdery mol. sieves (3 Å), and the resulting solution was stirred at 25 °C for 30 min. CH_2=$CHCH_2OP[N(i\text{-}C_3H_7)_2]_2$ (0.39 mL, 350 mg, 1.23 mmol) was slowly added at the same temperature to this solution, and the mixture was stirred for an additional 3 h. The reaction mixture was filtered to remove the mol. sieves, and the filtrate was concentrated. The residue was dissolved in ethyl acetate (150 mL) and washed with an aqueous sodium hydrogen carbonate solution (100 mL), the aqueous layer was extracted with ethyl acetate (150 mL, 2×), and the combined organic solutions were washed with brine (150 mL), dried with Na_2SO_4 (ca. 100 g), and concentrated to provide a residual product, which was dissolved in dichloromethane (5.0 mL) and poured into petroleum ether (500 mL). The precipitates were collected by filtration and dried in a vacuum to give the phosphoramidite **14** (765 mg, 85% yield) as a mixture of two diastereomers: TLC R_f = 0.39 (ethyl acetate/hexane, 2:1). ^{1}H NMR: δ = (mixture of two diastereomers) 1.16 (d, J = 6.8 Hz, 12 H), 1.43 (s, 3 H), 1.91 (s, 3 H), 2.05–2.14 (m, 1 H), 2.36–2.49 (m, 2 H), 2.56–2.61 (m, 1 H), 3.40–3.47 (m, 2 H), 3.55–3.63 (m, 2 H), 3.79 (s, 6 H), 4.06–4.24 (m, 6 H), 4.45–4.61 (m, 3 H), 5.12 (d, J = 10.4 Hz, 1 H), 5.23–5.39 (m, 4 H), 5.87–5.96 (m, 2 H), 6.31 (t, J = 6.4 Hz, 1 H), 6.41 (t, J = 7.2 Hz, 1 H), 6.84 (d, J = 8.8 Hz, 4 H), 7.22–7.40 (m, 10 H), 7.58 (s, 1 H), 8.92 (br. s, 2 H) ppm. ^{13}C NMR: δ = (mixture of two diastereomers) 11.7, 12.6, 24.5 (3 C), 24.6 (3 C), 24.7, 39.2 (2 C), 39.5 (2 C), 43.1, 43.2, 55.3, 63.4, 64.2 (2 C), 64.4 (2 C), 67.3, 69.3 (2 C), 72.6, 72.8, 73.0, 79.4, 79.5, 83.7, 83.8 (2 C), 83.9, 84.1, 84.2 (2 C), 84.3, 84.4, 84.7, 84.8, 85.0 (2 C), 87.3, 111.4, 111.7, 113.4, 115.8, 116.0, 119.3 (2 C), 127.2, 128.1 (2 C), 130.1, 131.7, 131.8 (2 C), 131.9, 135.1 (2 C), 135.2, 135.3 (4 C), 135.4, 144.1,150.4, 150.5, 158.8, 163.9 ppm. ^{31}P NMR: δ = (mixture of two diastereomers) 67.4, 147.5, 147.9.

Preparation of the Phosphoramidite 15 from 10: According to the procedure used to produce **14**, **15** (343 mg, 78% yield) was prepared as a mixture of two diastereomers on the basis of the reaction of **10** (358 mg, 0.41 mmol) and CH_2=$CHCH_2OP[N(i\text{-}C_3H_7)_2]_2$ (0.20 mL, 179 mg, 0.62 mmol): TLC R_f = 0.54 (ethyl acetate/hexane, 2:1). ^{1}H NMR: δ = (mixture of two diastereomers) 1.17 (d, J = 3.4 Hz, 6 H), 1.19 (d, J = 3.9 Hz, 6 H), 1.44 (s, 3 H), 1.93 (s, 3 H), 2.14–2.23 (m, 1 H), 2.35–2.60 (m, 3 H), 3.44 (s, 2 H), 3.56–3.68 (m, 5 H), 3.79 (s, 6 H), 4.05–4.32 (m, 6 H), 4.50–4.54 (m, 1 H), 5.13–5.16 (m, 1 H), 5.26–5.29 (m, 1 H), 5.34–5.38 (m, 1 H), 5.88–5.99 (m, 1 H), 6.27–6.31 (m, 1 H), 6.39 (dd, J = 5.9 and 8.8 Hz, 1 H), 6.84 (d, J = 8.8 Hz, 4 H), 7.24–7.39 (m, 10 H), 7.55 (s, 1 H), 8.37 (br. s, 2 H) ppm. ^{13}C NMR: δ = (mixture of two diastereomers) 11.7, 12.8, 24.4, 24.5, 24.6, 24.7, 39.0, 39.1, 39.5 (3 C), 39.6, 43.1, 43.2, 54.7, 54.8, 55.3, 63.3, 64.2, 64.4, 67.3, 67.4 (2 C), 67.5, 72.6, 72.8 (2 C), 72.9, 79.4 (2 C), 83.8, 84.2 (2 C), 84.3, 84.6 (2 C), 85.2, 85.3, 87.3, 111.3, 111.7, 113.4, 115.8, 116.0, 127.2, 128.1, 130.1, 135.0, 135.1, 135.2, 135.3 (2 C), 135.4, 135.5, 135.6 ppm. ^{31}P NMR: δ = (mixture of two diastereomers) 68.7, 147.6, 148.0.

Solid-Phase Synthesis of PO/PS-Chimeric Oligodeoxyribonucleotides: The nucleotide chain was elongated by the reaction cycle shown in Table 1, with the use of suitable phosphoramidites **16**–**19** as building units. Subsequently, the trityl-on or trityl-off product was subjected to deprotection and detachment from the solid support as follows to give the desired oligonucleotide.

(*R*p,*R*p)-T[TpsT]T$_7$[TpsT]T$_9$ (20): After the nucleotide chain elongation was complete, the trityl-on protected oligonucleotide bound on CPG supports was washed with acetonitrile and dried in vacuo. The resulting solid material was mixed with triphenylphosphane (59.0 mg, 0.23 mmol), butylamine (0.60 mL, 444 mg, 6.0 mmol), formic acid (0.23 mL, 281 mg, 6.0 mmol), and $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$ (23.3 mg, 22.5 μmol) in THF (5 mL), and the resulting mixture was vigorously shaken until the THF phase had changed from a dark violet color to yellow/orange. The heterogeneous mixture was then stirred at 50 °C for 14 h. After the supernatant fluid had been decanted, the resulting CPG supports were washed successively with THF (1 mL, 2×) and acetone (1 mL, 2×). Subsequently, the CPG supports were stirred in an aqueous sodium *N*,*N*-diethyldithiocarbamate (ddtc) solution (0.1 M, 1 mL) for 15 min. The mixture was filtered to remove the aqueous solution, and the solid material was then washed with acetone (1 mL, 3×) and water (1 mL, 2×). The resulting CPG supports were again subjected to the treatment with the sodium ddtc solution described above and were then washed with acetone and water. The solid supports were stirred in an aqueous concentrated NH_4OH solution (2 mL) at 25 °C for 2 h, and the supernatant fluid was separated by decantation and then heated at 55 °C for 6 h. The resulting solution was freeze-dried to give a residual material, which was dissolved in a solution of triethylammonium acetate in water (0.1 M, 2 mL). The solution was subjected to HPLC purification on COSMOSIL 5C18-AR-II (4.6×250 mm) under the following conditions (buffer mobile phase: A = 0.1 M triethylammonium acetate in water, B = H_2O/acetonitrile (1:4), linear gradient of 0–100% B in A for 60 min; temperature: 40 °C; flow rate: 1.000 mL·min^{-1}; detection: UV 254 nm) (see Supporting Information). Fractions containing the desired 5′-*O*-dimethoxytritylated oligonucleotide were collected and concentrated. The resulting gummy product was dissolved in an aqueous solution of acetic acid (0.4 M, 2 mL), and the solution was stirred at 60 °C for 6 h. The reaction mixture was concentrated to furnish the target PO/PS-chimeric oligonucleotide **20**, showing a single peak by HPLC analysis [column: COSMOSIL 5C18-AR-II (4.6×250 mm); mobile phase: A = 0.1 M triethylammonium acetate in water, B = H_2O/acetonitrile (1:4), linear gradient of 0–20% B in A for 60 min; temperature: 40 °C; flow rate: 1.000 mL·min^{-1}; detection: UV 254 nm] (see Supporting Information).

(*R*p)-G[TpsT]CAT (21): The trityl-off protected oligonucleotide bound on CPG supports was washed with acetonitrile and dried under reduced pressure. A mixture of the resulting solid material, triphenylphosphane (21.0 mg, 88.0 μmol), butylamine (240 μL, 178 mg 2.4 mmol), formic acid (90 μL, 110 mg, 22.4 mmol), and $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$ (9.1 mg, 8.8 μmol) in THF (2 mL) was vigorously shaken. When the original dark violet color of the THF layer had changed to yellowish orange, the mixture was warmed up to 50 °C and stirred at the same temperature for 14 h. The supernatant fluid was removed by decantation, and the CPG supports were washed with THF (1 mL, 2×) followed by acetone (1 mL, 2×), and were then treated with a sodium ddtc aqueous solution (0.1 M, 1 mL) for 15 min. After the sodium ddtc solution had been removed by filtration, the CPG supports were washed with acetone (1 mL, 3×) and water (1 mL, 2×). Treatment with the sodium ddtc solution and the succeeding washing were repeated. The resulting CPG supports were treated with an aqueous concentrated NH_4OH solution (2 mL) at 25 °C for 2 h, and the supernatant fluid was separated by filtration. The GPG supports were

washed with a small amount of water and the aqueous filtrates were combined and heated at 55 °C for 6 h. Freeze-drying of the resulting solution afforded a gummy material, which was dissolved in a solution of triethylammonium acetate in water (0.1 M, 2 mL). The resulting solution was purified by HPLC as described in the preparation of **20**, and the reaction mixture was concentrated to give **21** (see Supporting Information for details about the purity of this product).

(*Sp*,*Sp*)-T[TpsT]T$_7$[TpsT]T$_9$ (22): The CPG supports binding the trityl-on protected oligonucleotide were washed with acetonitrile and dried in vacuo. The resulting solid material was mixed with triphenylphosphane (65.6 mg, 0.25 mmol), butylamine (0.60 mL, 444 mg, 6.0 mmol), formic acid (0.23 mL, 281 mg, 6.0 mmol), and $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$ (25.9 mg, 25.0 μmol) in THF (5.0 mL), the resulting mixture was vigorously shaken until the solution had changed from a dark violet color to yellow/orange, and the mixture was then heated with stirring at 50 °C for 14 h. After the supernatant fluid had been removed by decantation, the resulting CPG supports were washed successively with THF (1 mL, 2×) and acetone (1 mL, 2×), and were then treated with a solution of sodium ddtc in water (0.1 M, 1 mL) for 15 min. The solid supports were separated by filtration and washed with acetone (1 mL, 3×) followed by water (1 mL, 2×). The sodium ddtc treatment and succeeding washing were repeated once again. The CPG supports were stirred in an aqueous concentrated NH_4OH solution (2 mL) at 25 °C for 2 h, and the aqueous layer was separated by filtration and concentrated by freeze-drying. The resulting residual material was treated with aqueous acetic acid (0.4 M) at 60 °C for 6 h. Concentration of the whole reaction mixture afforded **22** (for details about the purity of this product, see Supporting Information).

(*Sp*)-G[TpsT]CAT (23): The trityl-off product attached to CPG supports was washed with acetonitrile and dried in vacuo. This dried solid was mixed with triphenylphosphane (26.2 mg, 0.10 mmol), butylamine (240 μL, 178 mg, 2.4 mmol), formic acid (90 μL, 110 mg, 2.4 mmol), and $Pd_2[(C_6H_5CH{=}CH)_2CO]_3{\cdot}CHCl_3$ (10.4 mg, 10 μmol) in THF (2 mL), and the mixture was vigorously shaken to change the dark violet color of the THF layer to light orange. The heterogeneous mixture was heated at 50 °C and stirred at this temperature for 14 h, the supernatant fluid was decanted, and the resulting CPG supports were washed successively with THF (1 mL, 2×) and acetone (1 mL, 2×). Subsequently, the solid material was stirred with an aqueous sodium ddtc solution (0.1 M, 1 mL, 15 min). The aqueous phase was removed by filtration, and the solid material was washed with acetone (1 mL, 3×) and water (1 mL, 2×). This sodium ddtc solution treatment and succeeding washings were repeated again. The resulting CPG supports were stirred in a concentrated aqueous NH_4OH solution (2 mL) at 25 °C for 2 h. The solid material was removed by filtration, and the filtrate was freeze-dried to furnish a residual product. Concentration of the whole reaction mixture gave **23** (for details about the purity of this product, see Supporting Information).

Treatment of a PO/PS-Chimeric Oligonucleotide with an Enzyme and HPLC Analysis of the Resulting Product: A mixture of the PO/PS-chimeric oligonucleotide and a suitable enzyme, out of snake venom phosphodiesterase (svPDE), Serratia marcescens (Sma I), and nuclease P1, was incubated at 37 °C for 24 h. The reaction mixture was heated at 100 °C for 3 min to inactivate the enzyme and then subjected to RP-HPLC analysis on COSMOSIL 5C18-AR-II (4.6×250 mm) under the following conditions: buffer mobile phase: A = 0.1 M triethylammonium acetate in water, B = H_2O/acetonitrile (1:4), linear gradient of 0–20% B in A for 60 min; temperature: 40 °C; flow rate: 1.000 mL·min^{-1}; detection: UV 254 nm.

Supporting Information Available (see also the footnote on the first page of this article): ^1H, ^{13}C, and ^{31}P NMR spectra and ESI-TOF high-resolution mass spectra of **1**–**5**, **7**, **8**, **10**, and **11**; ^1H, ^{13}C, and ^{31}P NMR spectra of **14** and **15**; RP-HPLC profiles of the mixtures obtained by the digestion of **9** and **12** with svPDE and with nuclease P1. ESI-TOF high-resolution mass spectra of **20**–**23**; RP-HPLC profiles of crude **20**–**23** and purified **20**–**23**; RP-HPLC profiles of the mixture obtained by the digestion of **20**–**23** with svPDE and with nuclease P1; and the program for the solid-phase synthesis of **20**–**23** on an Applied Biosystems 392 DNA/RNA Synthesizer. This material is available free of charge through the Internet at http://www.eurjoc.org.

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