Synthesis and Properties of *S,S*-Diaryl Thymidine Phosphorodithioates[1)]

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Appropriately protected or unprotected *S,S*-diphenyl thymidine 3′- or 5′-phosphorodithioates and *S,S*-bis(4-methoxyphenyl) thymidine 3′- or 5′-phosphorodithioates were successfully prepared by the reaction of the thymidine derivatives with cyclohexylammonium *S,S*-diaryl phosphorodithioates in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPS). Stabilities of the thymidylic compounds under acidic or alkaline conditions were described in detail. Several methods for the deprotection of one or both arylthio groups under neutral conditions were also described in connection with the synthesis of oligothymidylates. By the use of the bis(4-methoxyphenylthio)-phosphoryl group, di- and tri-thymidylates were synthesized in high yields.

With the recent remarkable developments of the so-called "phosphotriester method" in oligonucleotide synthesis, the conventional methods for phosphorylation have been reconsidered by introducing new synthetic procedures.[2)] Recently, more considerable attention has been turned to phosphorylating agents which made the phosphotriester products separable by column chromatography on silica gel.[2)]

A few years ago, we described methods for the synthesis of nucleoside *S*-phenyl phosphorothioates by the reaction of nucleotide with a combined reagent of diphenyl disulfide and tributylphosphine[3)] and alternatively by the reaction of nucleoside phosphites with diphenyl disulfide in the presence of a silylating agent *via* highly reactive "nucleoside silyl phosphite" intermediates.[4)] The phenylthio group has been demonstrated as a useful phosphate protecting group in oligonucleotide synthesis.[5)]

In this paper, we wish to report a general method for the synthesis of oligonucleotides by use of *S,S*-diaryl phosphorodithioates as phosphorylating agents for introduction of a 5′-terminal phosphate and promising features of the arylthio groups as "activatable" protecting groups.[6)]

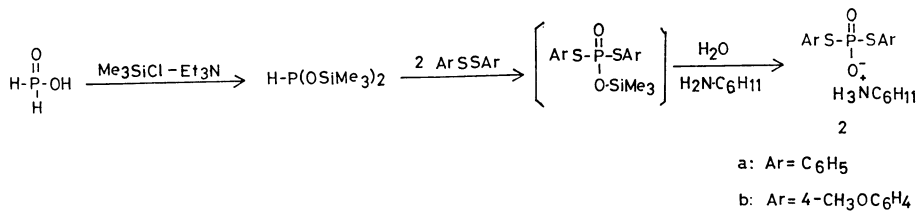
Results and Discussion

In an early work, we showed that *S*-phenyl phosphorodithioate (**1**) could be obtained quantitatively by the reaction of phosphonic acid with diphenyl disulfide in the presence of a trimethylsilylating agent.[4)] However, **1** was too unstable to use as a phosphorylating agent since **1** decomposed rapidly to release benzenethiol in dry pyridine even at room temperature. On the other hand, phosphorothioates of diester-type (*O,S*-phosphorothioates) are known to be rather stable compared with those of monoester-type.[7)] The fact led us to prepare, a new type of compound, *S,S*-diphenyl phosphorodithioate (**2a**) not only to enhance the stability as the phosphorylating agent but also to facilitate the separation of the phosphorylated products by chromatography on silica gel.

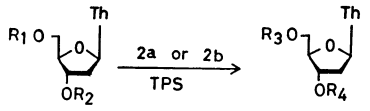
By extending the preparative method of **1**, a convenient synthesis of **2a** was performed: When phosphinic acid with further lower oxidation state than phosphonic acid was allowed to react with 2.1 equiv. of diphenyl disulfide in the presence of each 2.1 equiv. of triethylamine and trimethylsilyl chloride in dry tetrahydrofuran (THF) for 20 h at room temperature, **2a** was obtained as the cyclohexylammonium salt in 83% yield. Cyclohexylammonium *S,S*-bis(4-methoxyphenyl) phosphorodithioate (**2b**) was similarly prepared in 85% yield. Both **2a** and **2b** were found to be quite stable compared with **1** under acidic or alkaline conditions such as 80% acetic acid or 0.1 M (1 M=1 mol dm^{-3}) NaOH at room temperature for several weeks.

An improved method for the preparation of **2a** and **2b** in relatively large scale was established in this laboratory.[8)]

The new phosphorylating agents, **2a** and **2b**, were successfully applied to the synthesis of *S,S*-diaryl nucleoside phosphorodithioates (**3**) by condensing **2a** or **2b** with the corresponding nucleosides in the presence of 2,4,6-triisopropylbenzenesulfonyl chloride (TPS). These results are summarized in Table 1. In these reactions, it is noted that all reactions proceeded cleanly without brownish coloration, which was observed often in the case of coupling reactions using TPS in dry pyridine, to give the phosphorylated products in high yields and that the crystalline cyclohexylammonium salt of **2a** could be used without exchange to the pyridinium salt for the phosphorylation by employing two hold TPS. When **2a** was pre-activated by TPS in order to avoid sulfonylation of 5′-hydroxyl groups of nucleosides by TPS, the 2—5% formation of *S,S*-diphenyl phosphorodithiocychlohexylamidate was always accompanied. However, the by-product could be easily separated from *S,S*-diphenyl nucleoside phosphorodithioates since it was



Scheme 1.



3: Ar = C_6H_5
17: Ar = 4-$CH_3OC_6H_4$

	R_3	R_4
a:	$(ArS)_2P(O)$	Ac
b:	Tr	$(ArS)_2P(O)$
c:	MMTr	$(ArS)_2P(O)$
d:	$(ArS)_2P(O)$	$(ArS)_2P(O)$
e:	$(ArS)_2P(O)$	H
f:	H	$(ArS)_2P(O)$

Tr=trityl
MMTr=monomethoxytrityl

Scheme 2.

eluted with only dichloromethane by silica-gel column chromatography.

The phenylthio group of **3** was found to be quite stable either in dry or aqueous pyridine and also in 80% acetic acid at room temperature for several days. In addition, when **3a** was refluxed in methanol, ethanol, or 2-propanol for 1 h, **3a** was recovered quantitatively and no transesterification between the dithioester and alcohols was observed. The conditions for the selective removal of one and two phenylthio groups and the relationship with other protecting groups are illustrated in Scheme 3.

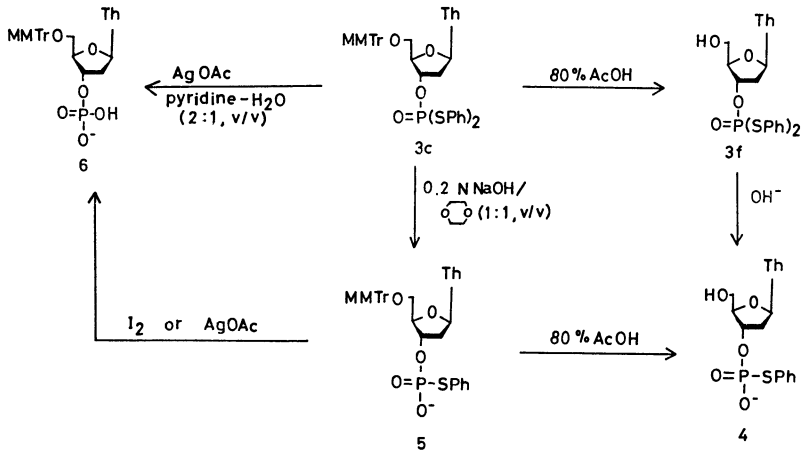
When **3c** was treated with 80% acetic acid at room temperature for 6 h, **3f** was isolated in 94% yield. Under these conditions the loss of the phenylthio group was essentially negligible. Whenever **3c** was heated in 80% acetic acid at 100 °C for 1 h, **3f** could be also obtained in 91% yield. In this case, 3% of *S*-phenyl thymidine 3′-phosphorothioate (**4**) was formed by losing one of two phenylthio groups from **3f**. Treatment of **3c** with 0.2 M NaOH–dioxane (1 : 1, v/v) at room temperature for 15 min gave 5′-*O*-monomethoxytrityl-thymidine *S*-phenyl 3′-phosphorothioate (**5**) quantitatively.

Van Boom[9] and Pfleiderer[10] have recently reported that, when dinucleotide derivatives bearing an aryloxy group in internucleotidic phosphate and an unprotected hydroxyl at the 3′- or 5′-position were treated with NaOH to remove one of two phenoxy groups, the 3′–5′ isomerization of phosphoryl group was accompanied to some extent depending upon the conditions. Therefore, in the case of alkaline treatment of **3e** or **3f** which has an unprotected hydroxyl at the 3′-or 5′-position, the above-mentioned problem should be taken into account.

When **3e** was treated with 0.2 M NaOH–dioxane (1 : 1, v/v) at room temperature for 15 min, two new

TABLE 1. YIELDS AND ELEMENTAL ANALYSIS OF *S,S*-DIARYL THYMIDINE PHOSPHORODITHIOATE DERIVATIVES (**3** AND **17**)

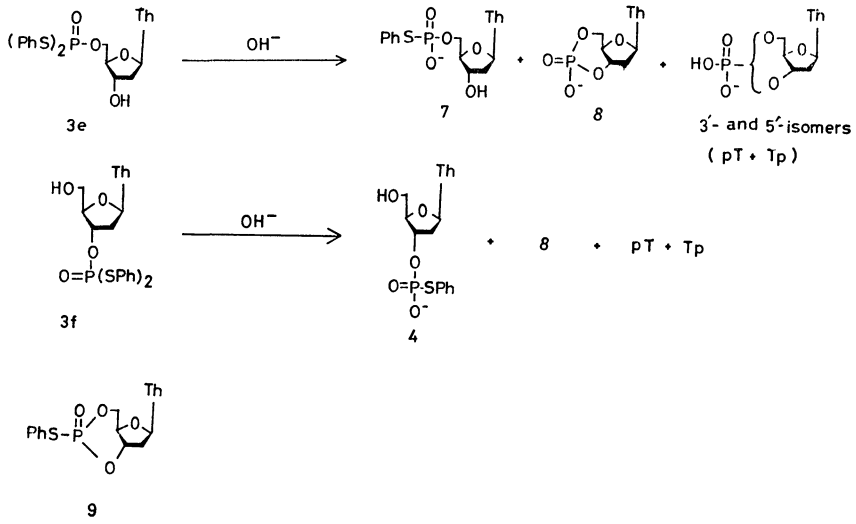
Compound	Yield/%	Formula	Calcd (%)			Found (%)		
			C	H	N	C	H	N
3a	92	$C_{24}H_{25}O_7N_2PS_2$	52.55	4.59	5.11	52.34	4.61	4.93
3b	96	$C_{41}H_{37}O_6N_2PS_2$	65.76	4.98	3.74	66.08	5.45	3.42
3c	95	$C_{42}H_{39}O_7N_2PS_2$	64.77	5.05	3.60	64.81	5.07	3.51
3d	88	$C_{34}H_{32}O_7N_2P_2S_4$	52.98	4.18	3.63	53.34	4.32	3.56
3e	66	$C_{22}H_{23}O_6N_2PS_2$	52.17	4.58	5.53	51.68	4.77	5.20
3f	—	$C_{22}H_{23}O_6N_2PS_2$	52.17	4.58	5.53	52.41	4.50	5.43
17a	95	$C_{26}H_{29}O_9N_2PS_2$	51.31	4.80	4.60	51.89	4.54	4.47
17c	93	$C_{44}H_{43}O_9N_2PS_2$	62.99	5.17	3.34	63.26	5.49	3.15
17e	71	$C_{24}H_{27}O_8N_2PS_2$	50.88	4.80	4.94	50.20	4.85	4.93
17f	—	$C_{24}H_{27}O_8N_2PS_2$	50.88	4.80	4.94	50.77	4.97	4.78



Scheme 3.

Table 2. ^{1}H-NMR spectra of phosphorylated products **3** and **17**

Compound	C_1'H	C_2'Ha C_2'Hb	C_3'H	C_4'H	C_5'Ha C_5'Hb	CH_3	C=CH	Others
3a	6.37(dd) $J_{1'-2'a}$=9.0 $J_{1'-2'b}$=6.0	2.03—2.63(m)	5.21(dd) $J_{2'-3'}$=6.9 $J_{3'-4'}$=2.1	4.24(m)	4.51(m)	1.90(s)	7.28(s)	2.16 (3H, s, OC(O)CH_3), 7.14—7.74 (10H, m, ArH), 9.41 (1H, br. s, NH)
3b	6.40(t) J=5.5	2.35 (m)	5.39(m)	4.04(m)	3.36(m)	1.40(s)	—	7.00—7.73 (26H, m, C=CH and ArH), 9.47 (1H, br. s, NH)
3c	6.42(t) J=7.2	2.32(t) J=3.7	5.33(m)	4.05(m)	3.38(m)	1.39(s)	—	6.85 (2H, d, J=8.4, ArH), 7.09—7.79 (23H, m, C=CH and ArH), 9.49 (1H,br. s, NH)
3d	6.32(dd) $J_{1'-2'a}$=6.7 $J_{1'-2'b}$=5.4	2.03—2.63(m)	4.15(m)	4.24 ———	4.55(m)	1.87(s)	—	7.23—7.73 (11H, m, C=CH and ArH), 9.32 (1H, br. s, NH)
3e	6.53(t) J=6.0	1.90—2.53(m)	4.23— 4.77(m)	4.17(m)	4.23—4.77(m)	1.83(s)	7.26(s)	7.08—7.67 (10H, m, ArH), 9.33 (1H, br. s, NH)
3f	6.22(t) J=7.0	2.31(m)	5.35(m)	4.04(m)	3.73(m)	1.88	7.31(s)	2.16 (1H, s, OH), 7.18—7.75 (10H, m, ArH), 9.48 (1H, br. s, NH)
17a	6.41(dd) $J_{1'-2'a}$=9.0 $J_{1'-2'b}$=6.0	2.03—2.50(m)	5.23(dd) $J_{3'-4'}$=2.0 $J_{2'-3'}$=6.0	4.24(dd) $J_{3'-4'}$=2.0 $J_{4'-5'}$=2.8	4.50(dd) $J_{4'-5'}$=2.0 J_{P-H}=7.0	1.90(s)	7.37(s)	2.14 (3H, s, OC(O)CH_3), 6.87 (2H, d, J=8.4, ArH), 7.55 (2H, dd, J=8.4, J=2.0, ArH), 9.46 (1H, br. s, NH)
17c	6.38(t) J=7.0	2.37(m)	5.36(m)	4.10(m)	3.38(m)	1.40(s)	—	3.77 (6H, s, OCH_3), 6.85 (4H, d, J=8.8, ArH), 6.93 (2H, d, J=8.8, ArH),7.10—7.64 (17H, m, C=CH and ArH)
17e	6.38(t) J=6.5	2.07—2.50(m)	4.31— 4.63(m)	4.18(m)	4.31—4.63(m)	1.84	7.30(s)	3.84 (6H, s, OCH_3), 6.89 (2H, d, J=8.4, ArH), 7.45 (4H, dd, J=8.4, J_{P-H}=2.1, PS-C=CH), 9.39 (1H, br. s, NH)
17f	6.21(t) J=7.0	2.35(m)	5.32(m)	4.08(m)	4.08(m)	1.90	7.35(s)	3.83 (6H, s, OCH_3), 6.96 (4H, d, J=9.0, O-C=CH), 7.56 (4H, dd, J=9.0, J_{P-H}=2.0, S-C=CH), 9.49 (1H, br. s, NH)

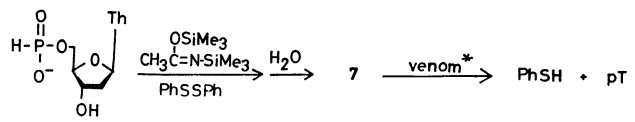


Scheme 4.

spots of R_f 0.53 and 0.20 (Solvent I) were observed other than the major spot which showed the R_f value corresponding to *S*-phenyl thymidine 5'-phosphorothioate (**7**) on TLC. The nucleotidic substance of R_f 0.53 was determined as thymidine 3',5'-cyclic phosphate (**8**) by comparison with the authentic sample. The yield of **8** was estimated to be 12% spectroscopically. The other minor spot was identified with thymidine 3'-or 5'-phosphate formed in 1% yield.

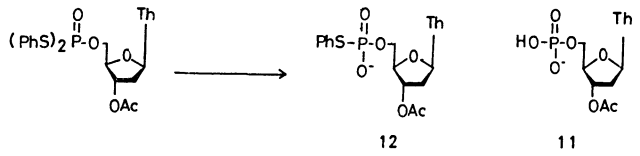
Since it is known that the 3'–5' isomerization of phosphoryl group occurs through a cyclic triester intermediate,[9] if the 3'–5' isomerization occurs during the alkaline treatment of **3e**, thymidine 3',5'-cyclic phosphate derived from thymidine *S*-phenyl 3',5'-cyclic phosphorothioate (**9**) by further alkaline hydrolysis of **9** should be expected as a by-product. Our result indicated that during this alkaline treatment the cyclization reaction along with elimination of one of two phenylthio groups took place at least to the extent of 12%. It is possible that, *S*-phenyl thymidine 3'-phosphorothioate (**4**) might be produced from **9**, if the bond of P–O of **9** at the 5'-position was competitively hydrolyzed with cleavage of the ester bond of P–O at the 3'-position or with cleavage of P–S bond of **9**. Therefore, the nucleotidic material corresponding to **7**, which was estimated to be formed in 87% yield, was further confirmed whether it contained TpSPh (**4**), although it was not separated from PhSpT (**7**) by paper chromatography and electrophoresis.

In a previous paper,[4] we reported that pure PhSpT (**7**) was obtained from thymidine 5'-phosphite by the silylation in the presence of 1.1 equiv. of diphenyl disulfide in quantitative yield. It was found that this pure **7** was completely degraded by snake venom phosphodiesterase in Tris buffer (pH 8). On the other



* *Venom phosphodiesterase*

Scheme 5.



Scheme 6.

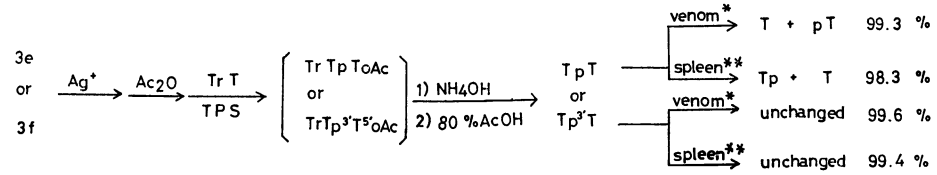
hand, **4** was found to be resistant to the same enzyme. The results are of interest in connection with the report by Nussbaum and Cook that *S*-ethyl thymidine 5'-phosphorothiate (**10**) served as a substrate of snake venom phosphodiesterase.[7]

On the basis of this finding, the nucleotidic substance obtained by alkaline treatment from **3e** was incubated at pH 8 with snake venom phosphodiesterase for 1 h. The nucleotidic substance was degraded in more than 99.3% to give thymidine 5'-phosphate as the sole degraded product. This result indicates that the P–S bond predominantly cleaved on hydrolysis of **9**. Therefore, it is concluded that the 3'–5' isomerization of hydroxy(phenylthio)phosphoryl group is essentially negligible in alkaline treatment of **3e**.

Similarly, treatment of **3f** with 0.2 M NaOH–dioxane (1 : 1, v/v) for 15 min gave a main product of R_f 0.71, **8**, and thymidine 3'- or 5'-phosphate in 76, 21, and 3% yields, respectively. When the product of R_f 0.71 was incubated with snake venom phosphodiesterase, the nucleotidic material remained unchanged and recovered in more than 97%.

This result shows that the 3'–5' isomerization was not observed in the case of **3f**.

Next, complete removal of both phenylthio groups by one-step from **3a** was examined. In spite of facile transformation of S-phenyl nucleoside phosphorothioates to nucleotides by the action of aqueous iodine,[3,4] **3a** was found to be extremely stable toward oxidizing agents such as iodine, sodium periodate, iodosobenzene, hydrogen peroxide, *N*-chlorosuccinimide, and *N*-bromosuccinimide. Removal of both phenylthio groups by use of transition metal salts was examined, since it was expected that sulfur atom has a strong affinity for



*Venom phosphodiesterase **Spleen phosphodiesterase

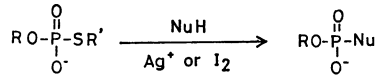
Scheme 7.

the transition metals such as copper, mercury, and silver. Copper(I) chloride, copper(II) chloride, copper-(II) acetylacetonate, copper(II) acetate, mercury(II) acetate, mercury(II) chloride, silver acetate, and silver nitrate were examined. The former three copper salts were not effective. For example, the use of 16 molar equiv. of copper(II) acetylacetonate in pyridine–water (2 : 1, v/v) at room temperature for 24 h gave only 3'-*O*-acetylthymidine *S*-phenyl 5'-phosphorothioate (**12**) in a poor yield. Among the mercury salts, mercury(II) acetate was not effective, but treatment of **3a** with 16 equiv. of mercury(II) chloride at room temperature for 16 h gave **11** quantitatively. On the other hand, two silver salts showed almost the same results as mercury-(II) chloride. From the three effective metal salts, silver acetate was finally chosen as an effective and mild agent for removal of both phenylthio groups. The details of one-step removal of both phenylthio groups from **3a** are summarized in Table 3. This deblocking reaction was carried out at room temperature for 16 h using 16 molar equiv. of silver acetate in pyridine–water (2 : 1, v/v). After the deblocking reaction was completed, the excess of silver acetate, disilver salt of nucleotide, and AgSPh could be converted to the mixture of acetic acid, pyridinium salt of nucleotide, and benzenethiol, respectively, by bubbling hydrogen sulfide with the formation of insoluble silver sulfide which could be easily separated off by centrifugation or filtration. Thus, from **3a**, **3b**, **3c**, and **3d**, the corresponding deblocked nucleotides (**11a**, **11b**, **11c**, and **11d**) were obtained by paper chromatography in 99, 97, 96, and 95 yields, respectively. The use of cation exchange resin (DIAION SK IB, pyridinium form) was also useful for removal of silver ions. In this case, AgSPh was not converted sufficiently to benzenethiol. However, it could be filtered off with the resin.

Table 3. Deprotection of arylthio groups from phosphorylated compounds (**3**)

Phosphorylated compound	Conditions		Product (%)	
	AgOAc (equiv.)	Time h	pT	ArSpT
$(PhS)_2pT$	4	3	46	9
	6	3	55	20
	8	3	55	46
	8	18	97	0
	16	3	80	15
	16	18	100	0
$(PhS)_2pTOAc$	16	18	99 (pTOAc)	0 (PhSpTOAc)
$(4\text{-}CH_3OC_6H_4S)_2pT$	16	18	87	10
	20	18	98	0
$MMTrTp(SPh)_2$	16	18	96 (MMTrTp)	0 (MMTr-TpSPh)

Furthermore, in these silver ion-catalyzed hydrolysis reactions, the 3'–5' isomerization of phosphoryl group was also examined by taking **3e** and **3f**. Each mononucleotidic substance corresponding to pT or Tp obtained by treatment of **3e** or **3f** with silver acetate in aqueous pyridine as described above was acetylated with acetic anhydride in pyridine followed by condensation with 5'-*O*-tritylthymidine in the presence of TPS. Consequently, trityl containing dinucleotide corresponding to TrTpTOAc or $TrTp^{3'}T^{5'}OAc$ was obtained. The dinucleotide derivatives were deprotected by successive treatment with concentrated ammonium hydroxide and with 80% acetic acid to afford unprotected dinucleotides corresponding to TpT and $Tp^{3'}T$. The dinucleotidic substance (90.1 OD) originally derived from **3e** was degraded by snake venom phosphodiesterase in more than 99.3% to give T (45.4 OD) and pT (47.4 OD) in the ratio of 1.00 : 0.96 and by spleen phosphodiesterase in more than 98.3% to give Tp (42.8 OD) and T (46.2 OD) in the ratio of 1.00 : 1.08. On the other hand, the dinucleotidic substance derived from **3f** was recovered in more than 99.6 and 99.4% yields, respectively, when it was incubated with snake venom phosphodiesterase and with spleen phosphodiesterase under the same conditions. The results indicate clearly that the 3'–5' isomerization of phosphoryl group does not take place during the silver ion-catalyzed hydrolysis in the case of **3e** and **3f**. No isomerization was also suggested from the fact that thymidine 3',5'-cyclic phosphate (**8**) could not be detected.



Scheme 8.

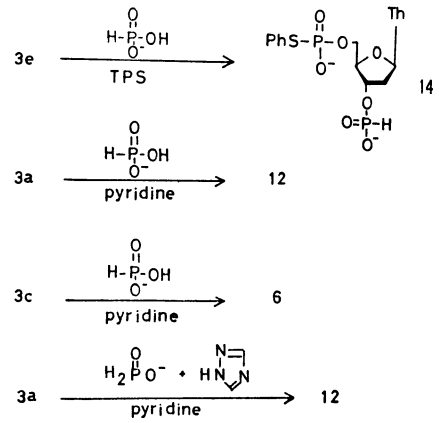
It has been well known that a diester type of phosphorothioates represented as RS–P(O)(OR')O$^-$ can be activated oxidatively on sulfur atom by oxidizing agents such as iodine and sodium metaperiodate to produce a metaphosphate derivative, R'OP(O)=O, which in turn reacts with various nucleophiles to afford the corresponding phosphorylated products. This reaction has been utilized for the synthesis of nucleoside di- and tri-phosphates by Nussbaum.[7] Furthermore, it has recently been reported that unsymmetrical α,γ-dinucleosides triphosphates could be obtained in good

yields by activating the phenylthio group of P¹-*S*-phenyl P²-ribonucleoside 5′-pyrophosphorothioates in the presence of silver salts.[6]

Therefore, conversion of phosphorodithioates to phosphoromonothioates under milder conditions should be required.

In connection with our previous results in oligonucleotide synthesis *via* the phosphotriester method where the phenylthio group was used as an internucleotidic phosphate protecting group,[5] we have found that one of two phenylthio groups can be removed selectively from bis(phenylthio)phosphoryl group under very mild conditions.

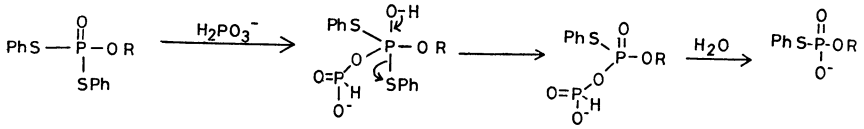
In order to introduce a phosphite group (p_3) into 3′-hydroxyl of **3e** for the preparation of $(PhS)_2pTp_3$ (**13**), **3e** was treated with 4 equiv. of phosphonic acid in the presence of TPS in dry pyridine for 24 h according to the procedure reported early. However, **13** could not be obtained but a product (**14**: $PhSpTp_3$) leaving one of two phenylthio groups was obtained as the main product. The formation of **14** might be explained as a result of elimination of one phenylthio group from the expected compound (**13**) by the catalytic action of phosphonic acid. Consequently, the independent reaction of **3a** with 4 equiv. of phosphonic acid under the same conditions was tested. When **3a** was treated with 4 equiv. of phosphonic acid in dry pyridine at room temperature for 24 h, *S*-phenyl 3′-*O*-acetylthymidine 5′-phosphorothioate (**12**) was obtained quantitatively. The reaction was keenly specific because acetyl group could not be removed at all under the same conditions. It was also found that under the conditions monomethoxytrityl group and trityl group were stable as indicated in the following experiment. When **3c** was treated with 6 equiv. of phosphonic acid in dry pyridine for 20 h, TLC showed a negligible loss of monomethoxytrityl group, and *S*-phenyl 5′-*O*-monomethoxytritylthymidine 3′-phosphorothioate (**5**) was obtained in 86% yield. In comparison with phosphonic acid, phosphates, such as 2,2,2-trichloroethyl phosphate, made only negligible effect on such deblocking reaction.



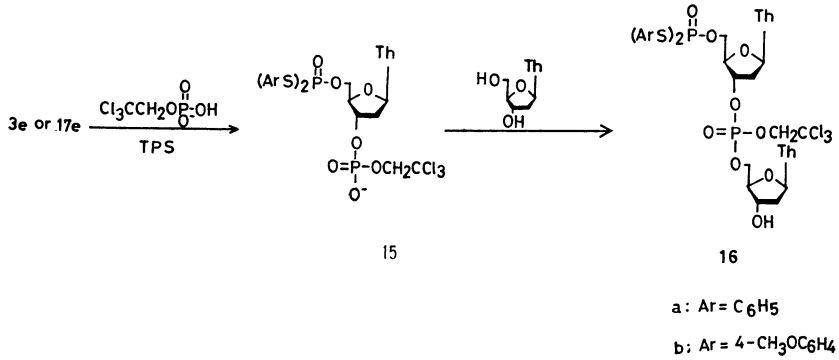
Scheme 9.

In order to extend the catalyst we examined to use phosphinic acid ($pK_a=1.1$)[11] which has a little smaller pK_a value than phosphonic acid ($pK_{a'}=1.3$).[11] It was found that phosphinic acid is much more soluble in pyridine than phosphonic acid and that the velocity of the deprotection was considerably accelerated for the dearylthioation compared with phosphonic acid: When **3a** was treated with 1.2 equiv. of phosphinic acid in dry pyridine, **3a** disappeared completely after 10 h and **12** was formed in 97% yield along with 3′-*O*-acetylthymidine-5′-phosphate and the corresponding pyrophosphate derivative. Furthermore, an effect of addition of 1,2,4-triazole was tried for the deblocking reaction. The compound, **3a**, was treated with 1.2 equiv. each of phosphinic acid and 1,2,4-triazole at room temperature. The deblocking reaction occured only in 61% yield after 3 h. For the complete deprotection, it took about one day. Although the addition of 1,2,4-triazole reduced the reaction velocity, the deblocking reaction proceeded selectively and gave **12** in quantitative yield without any visible by-products.

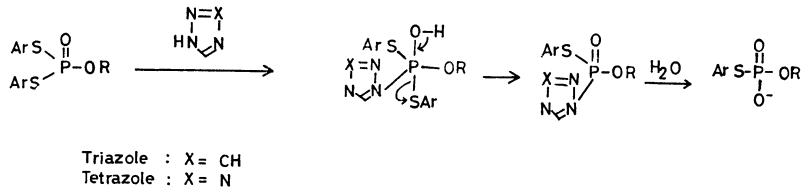
Next, the synthesis of protected dithymidine diphosphate derivatives was examined by using **3e** as the 5′-terminal nucleotide component. Compound **3e** was



Scheme 10.



Scheme 11.



Scheme 12.

phosphorylated with 2,2,2-trichloroethyl phosphate[12] in the presence of TPS and then condensed with thymidine by use of 1-(*p*-nitrobenzenesulfonyl)-1*H*-1,2,4-triazole (NBST).[13] However, the desired dinucleotide (**16a**) was obtained unexpectedly in a lower yield of 44% than expected. In this reaction, a partial dephenylthioation was observed during the second coupling reaction. Such deprotection could be hardly observed during the phosphorylation step using 2,2,2-trichloroethylphosphate and TPS or the condensation step of introducing the bis(phenylthio)phosphoryl group into nucleoside hydroxyls using TPS. The above remarkable difference seemed to be attributed to the kind of condensing agent. The lower yield of **16a** by use of NBST was considered as a result of nucleophilic attack of 1,2,4-triazole to phosphorus of the phosphorodithioate followed by elimination of benzenethiol to give a phosphotriazole intermediate. During the phosphorylation 1,2,4-triazole accumulates up to at least equimolar amount. The phosphotriazole is easily hydrolyzed during work-up of the reaction mixture to form the deblocked product. This catalytic effect of triazole was also confirmed by the following experiment. By treatment of **3a** in the presence of one equiv. of 1,2,4-triazole in dry pyridine at room temperature for 72 h, **12** was formed in 31% yield. In the case of imidazole, **12** was found in 67% yield under the same conditions. On the other hand, tetrazole gave **12** only in 4% yield. Thus, it was concluded that the catalytic effect of these azoles on the undesirable deprotection of the phenylthio group decreases in the following order: imidazole>triazole<tetrazole. The powerful catalytic effect of imidazole might be utilized for the selective removal of the phenylthio group from the bis(phenylthio)phosphoryl group as well as that of phosphonic acid or phosphinic acid described precedingly. In fact, **12** was obtained in 96% yield by treatment with 10 equiv. of imidazole for 28 h. The details of the selective deprotection are summarized in Table 4.

The above results indicate that arenesulfonyl imidazales should not be used condensing agents for phosphorylation of nucleotides having bis(phenylthio)-phosphoryl group. Accordingly, TPS was used for the formation of internucleotidic bond and **16a** was obtained in 92% yield. In this case, the monomethoxytritylation after the condensation was performed so as to remove the by-product of the 3′–3′ isomer.[14]

Since neutral condensing agents such as NBST, 1-(2,4,6-triisopropylbenzenesulfonyl)-1*H*-tetrazole (TPSTe), and 1-(8-quinolinesulfonyl)-1*H*-tetrazole (QSTe) have become apparently more desirable than TPS in several aspects as reported in a number of labolatories,[15–21] our efforts were focused on the exploration of protecting groups more stable towards the above-mentioned azoles. In conclusion, it was found that 4-methoxyphenylthio group having an electron-donating group on benzenethiol was sufficiently stable.

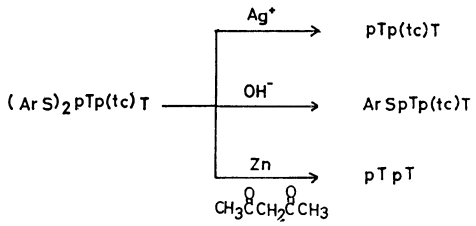
Table 4. Treatment of **3a** and **17a** with imidazole, 1,2,4-triazole, and tetrazole

Azole	$(ArS)_2$pTOAc Ar	Ratio of Azole/$(ArS)_2$pTOAc	Pyridine–H_2O (ml)	(ml)	Time h	Product[a] (%) ArSpTOAc	pTOAc
Imidazole	$(PhS)_2$pTOAc	10	1.0	—	6	63	0
		10	1.0	—	28	96	1
		10	1.0	0.1	6	81	5
		1	0.2	—	72	67	3
1,2,4-Triazole		10	1.0	—	6	4	0
		10	1.0	—	72	21	0
		1	0.2	—	72	31	1
Tetrazole		10	1.0	—	6	<1	0
		10	1.0	—	72	5	0
		1	0.2	—	72	4	0
Imidazole	$(4\text{-}CH_3OC_6H_4S)_2$pTOAc	10	10	—	72	0	0
		10	1.0	—	72	4	0
		1	0.2	—	72	14	0
1,2,4-Triazole		1	0.2	—	72	2	0
Tetrazole		10	1.0	—	72	0	0
		1	0.2	—	72	0	0

a) The yields of PhSpTOAc and 4-$CH_3OC_6H_4$SpToAc were calculated by using ε values of PhSpT [λ_{max} 267 mm (ε 8.6×10^3)] and 4-$CH_3OC_6H_4$SpT [λ_{max} 267 mm (ε 9.2×10^3)].

The introduction of bis(4-methoxyphenylthio)phosphoryl group into hydroxyls of nucleosides could be readily carried out as well as that of the bis(phenylthio)phosphoryl group as shown in Table 1. The promising feature of 4-methoxyphenylthio group is demonstrated in the following experiment: When 3'-*O*-acetylthymidine *S*,*S*-di(4-methoxyphenyl) 5'-phosphorodithioate (**17a**) was treated with one equiv. of 1,2,4-triazole in pyridine at room temperature for 72 h, only 2% of the corresponding deprotected product (**18**) was formed. Therefore, the deprotection reaction can be suppressed within 1% under the usual conditions using NBST in coupling reactions. In fact, when *S*,*S*-di(4-methoxyphenyl) thymidine 5'-phosphorodithioate (**17a**) was phosphorylated with 2,2,2-trichloroethyl phosphate by use of TPS and successively condensed with thymidine by employing NBST followed by the monomethoxytritylation of the mixture, the corresponding dithymidine diphosphate derivative (**16b**) was obtaind in 90% yield. In a similar manner, the corresponding trithymidylate, (4-$CH_3OC_6H_4S$)$_2$pTp(tc)Tp(tc)T, was obtained in 82% yield from **16b**.

Removal of Protecting Groups from the Oligomers. Selective removal of one of two phenylthio or two 4-methoxyphenylthio groups from **16a** or **16b** was also carried out by using 0.2 M NaOH–dioxane (1 : 1, v/v) at room temperature for 20 min or by phosphonic acid in pyridine containing a small amount of water at room temperature for 24 h. The product, PhSpTp(tc)T, or 4-$CH_3OC_6H_4$SpTp(tc)T was easily converted to pTp(tc)T by treatment with iodine in aqueous pyridine. This two-step procedure for removal of both arylthio groups is efficient for obtaining pTp(tc)T in high yields. When zinc/acetylacetone in DMF–pyridine[22] was employed for removal of 2,2,2-trichloroethyl group of **16a**, the phenylthio groups were together removed and pTpT was obtained by one-step treatment in 71% yield. It seems to be plausible that the active $ZnCl^+$ formed along with 1,2-dichloroethylene serves as an activating reagent of the phenylthio group to liberate ZnCl(SPh) or $Zn(SPh)_2$. In order to remove 2,2,2-trichloroethyl group selectively, benzenethiol was added as a scavenger of $ZnCl^+$. Contrary to our expectation, pTpT was obtained in 84% yield from **16a**. Under the same conditions, pTpT was obtained in 35% yield from **16b** and the desired product, (4-$CH_3OC_6H_4S$)$_2$-pTpT, was obtained in 34% yield.



Scheme 13.

Removal of both phenylthio or both 4-methoxyphenylthio groups from the fully protected dinucleotide (**16a**) or (**16b**) was carried out as follows: Treatment of **16a** or **16b** with 16 equiv. of silver acetate in pyridine–water (2 : 1, v/v) at room temperature for 16 h or 18 h gave the deprotected dinucleotide pTp(tc)T in 98 or 97% yield. The successive treatment of the deprotected dinucleotide with zinc powder in DMF–pyridine (2 : 1, v/v) in the presence of acetylacetone gave pTpT in quantitative yield. On the other hand, treatment of (4-$CH_3OC_6H_4S$)$_2$pTp(tc)Tp(tc)T with 20 equiv. of silver acetate in aqueous pyridine followed by zinc/acetylacetone in DMF–pyridine gave pTpTpT in 97% yield.

Experimental

Proton magnetic resonance spectra were recorded at 60 Hz on a Varian A-60 spectrometer. Infrared spectra were obtained on a Hitachi 124 spectrophotometer. Melting points were taken on a Fisher-Johns melting point block. Reagent grade pyridine was distilled by addition of *p*-toluenesulfonyl chloride and stored over calcium hydride for several weeks. Paper chromatography was performed using the descending technique on Toyo Roshi No. 51 or Whatman 3 MM paper. The solvent systems used for paper chromatography were: isopropyl alcohol–concentrated ammonium hydroxide–water (7: 1: 2, v/v) (Solvent I); ethyl alcohol–1 M ammonium acetate (pH 7.5) (7: 3, v/v) (Solvent II); butyl alcohol–water (84: 16, v/v) (Solvent III). Paper electrophoresis was carried out on Toyo Roshi No. 51A (15 cm × 60 cm) impregnated with the solvents described by Markham[23] and 1200 V for 1.5 h (buffer I) or at 1500 V for 1.5 h (buffer II) using an apparatus similar to that described by Markham and Smith;[23] buffer I, 0.05 M potassium phosphate (pH 8.0); buffer II, 0.05 M potassium phosphate (pH 6.0). For separation of appropriately protected nucleotide derivatives of triester-type, silica gel (C-200) purchased from Wako Chemical Co. was used. The eluent was monitored by thin layer chromatography using pre-coated plates of silica-gel 60 F-254 purchased from Merck Chemical Co. The yields of water-soluble nucleotidic products were estimated spectrophotometrically after elution of the bands from paper chromatograms. Absorbances were determined with a Hitachi spectrophotometer by the absorbancy for a blank cut from the paper adjacent to the product spot. Snake venom and calf spleen phosphodiesterases were purchased from Boehringer Mannheim Co.

S,S-*Diphenyl Phosphorodithioate (2a).* Sodium Phosphinate (5.30 g, 50 mmol) was converted to pyridinium salt by passing the aqueous solution through DIAION SK 1B (Mitsubishi Kasei Kogyo Co., pyridinium form). Elution was performed with 500 ml of water. The eluent was evaporated and the residue was dried by repeated coevaporations with pyridine. The resulting white solid was mixed with diphenyl disulfide (22.89 g, 105 mmol) and dissolved in 400 ml of dry tetrahydrofuran (THF). Trimethylsilyl chloride (13.1 ml, 105 mmol) and triethylamine (14.8 ml, 105 mmol, were added dropwise to the THF solution at 0 °C. After the addition the mixture was warmed to room temperature and stirred for 20 h. A precipitate of triethylamine hydrochloride was filtered off and the filtrate was evaporated to dryness. The residue was treated with 2 M hydrochloric acid (200 ml) and extracted with dichloromethane (CH_2Cl_2) (4 × 100 ml). The extracts were combined and evaporated to dryness. The residue was dissolved in chloroform (200 ml) and treated with cyclohexylamine (5.73 ml, 50 mmol). The solvent was removed *in vacuo* to give an oily material which solidified after washing with three 50 ml portions of hexane. The solid was recrystallized from water containing a small ammount of THF to afford 15.8 g (83%) of **2a**: mp 177—

179 °C; IR(KBr) 3040, 2950, 1629, 1580, 1523, 1476, 1388, 1220, 1210, 1052 cm^{-1}; NMR (DMSO-d_6–CD_3OD, 1: 1, v/v) δ 1.00—1.57 (5H, m, protons of cyclohexane ring), 1.57—2.11 (5H, m, protons of cyclohexane ring), 2.86 (1H) m, CH–N), 7.29 (3H, m, ArH), 7.56 (2H, m, ArH).

Found: C, 56.71; H, 6.47; N, 3.75; S, 16.52%. Calcd for $C_{18}H_{24}O_2S_2NP$: C, 56.67; H, 6.34; N, 3.67; S, 16.81%.

S,S-*Bis(4-methoxyphenyl)phosphorodithioate.* The title compound (18.8 g, 85%) was prepared according to the same procedure as described above by using bis(4-methoxyphenyl) disulfide (29.2 g, 105 mmol); mp 174—175 °C; IR(KBr) 3050, 2920, 2830, 1588, 1535, 1488, 1450, 1390, 1282, 1238, 1210, 1175, 1050, 1028 cm^{-1}.NMR (DMSO-(DMSO-d_6–CD_3OD, 1: 1, v/v) 1.00—1.64 (m, 5H, protons of cyclohexane ring), 1.64—2.32 (m. 5H, protons of cyclohexane ring), 2.90 (m, 1H, CH–N), 3.82 (s, 6H, OCH_3), 6.89 (d, 4H, J=9.0 Hz, ArH), 7.58 (d, 4H, J=9.0 Hz, ArH).

Found: C, 54.42; H, 6.44; N, 3.12; S, 14.73%. Calcd for $C_{20}H_{28}O_4NS_2P$: C, 54.40; H, 6.39; N, 3.17; S, 14.52%.

Introduction of S,S-*Diaryl Phosphorodithioyl Group into Appropriately Protected Nucleosides. Typical Procedure A) 3'-O-Acethylthymidine* S,S-*Diphenyl 5'-Phosphorodithioate (3a)*: TPS 727 mg (2.4 mmol) was added to **2a** (458 mg, 1.2 mmol) in dry pyridine (2.0 ml) and it was kept at room temperature for 30 min. 3'-O-Acethylthymidine[19] (284 mg, 1 mmol), was added. The mixture was stirred at room temperature for 20 h. The solution was diluted with CH_2Cl_2 (20 ml) and then water (20 ml) was added. The aqueous layer was further treated with CH_2Cl_2 (3×20 ml) and the organic layers were combined and dried over Na_2SO_4. The solution was concentrated *in vacuo* to dryness. The last traces of pyridine was removed by coevaporation with toluene (3× 10 ml). The residue was treated with ethyl acetate (20 ml) whereupon pyridinium salt of 2,4,6,triisopropylbenzenesulfonic acid was separated. The white precipitate was filtered off and washed with cold ethyl acetate (10 ml). The filtrate and washings were combined, dried over Na_2SO_4, and evaporated to a gum. The residue was dissolved in a small amount of benzene and applied to a column on silica gel (20 g). Elution with benzene–ethyl acetate (1: 2, v/v) gave 505 mg (92%) of **3a** as a white foam:

B) S,S-*Diaryl Thymidine 5'-Phosphorodithioates (3d and 17d)*: Compound **2a** (2.1 g, 5.5 mmol) was coevaporated with dry pyridine (3×5 ml) and dissolved in dry pyridine (10 ml). To the solution was added TPS (3.33 g, 11 mmol). The mixture was stirred at room temperature for 1 h and then well pulverized thymidine (1.21 g, 5.0 mmol) was added. After stirring at room temperature for 22 h, the mixture was diluted with chloroform (40 ml) and then water (40 ml) was added. The organic layer was collected and the aqueous layer was further extracted with chloroform (3×10 ml). The organic extracts were combined, dried over Na_2SO_4, evaporated to dryness, and coevaporated with toluene (10 ml). The residue was treated with ethyl acetate (100 ml) and a white precipitate was filtered off. The filtrate was evaporated *in vacuo* and dissolved in dry pyridine after repeated evaporations with dry pyridine (3×5 ml). To the solution was added monomethoxytrityl chloride (577 mg, 2.0 mmol). After stirring at room temperature for 15 h, the mixture was diluted with chloroform (50 ml) and then water (50 ml) was added. The aqueous layer was further extracted with chloroform (2×10 ml) and the organic extracts were combined and dried over Na_2SO_4. The solution was evaporated to dryness *in vacuo*, coevaporated with CH_2Cl_2–toluene (3×5 ml), and the residue chromatographed on silica gel with THF (5: 1, v/v) to afford **3d** (3.34 g, 66%) as a white foam.

Removal of Monomethoxytrityl Group from 3c. S,S-*Diphenyl Thymidine 3'-Phosphorodithioate (3f)*: Compound **3c** (779 mg, 1 mmol) was dissolved in 80% acetic acid (50 ml) and the solution was kept at room temperature for 6 h. The mixture was evaporated to dryness *in vacuo*, and coevaporated with pyridine (5 ml) and then with toluene (3×5 ml). The residue was chromatographed on silica gel with CH_2Cl_2–methanol to give **3e** (477 mg, 94%): UV_{max} (MeOH) 256 nm (ε 14×10³), UV_{min} (MeOH) 245 nm (ε 13.5×10³). The data of its elemental analysis and ^{1}H-NMR spectrum are shown in Tables 1 and 2.

Conversion of 3c to 5: To a solution of **3c** (389.5 mg, 0.5 mmol) in dioxane (12 ml) was added 0.2 M NaOH (11 ml) with stirring. The mixture was kept at room temperature for 15 min and then neutralized through a column (1 cm×27 cm) of DIAION SK 1B (NH_4^+ form). The column was washed with dioxane–water (2: 1, v/v, 60 ml), and the eluent and washings were combined and concentrated to *ca.* 20 ml. The aqueous solution was extracted with hexane (3×20 ml) for removal of benzenethiol and then passed through a column (1 cm×27 cm) of DIAION SK 1B (Na^+ form). The column was eluted with dioxane–water (2: 1, v/v, 200 ml) and the eluent was concentrated to *ca.* 20 ml. The solution was extracted with CH_2Cl_2 (3×20 ml) by addition of a few milliliters of saturated sodium chloride. The organic layer was dried over Na_2SO_4, concentrated to 5 ml, and poured into hexane (100 ml). A white precipitate was collected and dried over P_4O_{10} *in vacuo* to give sodium salt (310 mg, 81%) of **5**: UV_{max} (H_2O) 266 nm (ε 10.4×10³), UV_{min} (H_2O) 255 nm (ε 9.5×10³), UV_{sh} (H_2O), 232 nm (ε 1.9×10³).

Found: C, 56.56; H, 5.10; N, 3.52%. Calcd for $C_{36}H_{34}NO_8PSNa\cdot 3H_2O$: C, 56.69; H, 5.29; N, 3.67%.

Conversion of 5 to 4: The sodium salt of **6** (140.7 mg, 0.2 mmol) obtained in the above experiment was treated with 80% acetic acid (10 ml) at room temperature for 6 h. Then water (20 ml) was added and the aqueous solution was extracted with CH_2Cl_2 (3×20 ml). The aqueous solution was evaporated and lyophilized to give a hygroscopic sodium salt (87 mg, 100%) of **4**: UV_{max} (H_2O) 266 nm (ε 9.2×10³), 245 nm (ε 7.6×10³), UV_{min} (H_2O) 249 nm (7.6×10³), 230 nm (ε 6.2×10³).

Found: C, 40.50; H, 4.41; N, 5.6%. Calcd for $C_{16}H_{18}O_7N_2PSNa\cdot 2H_2O$: C, 40.68; H, 4.70; N, 5.93%.

Alkaline Treatment of 3e and 3f and Enzymatic Assay of the Resulting Diesters: To a solution of **3e** (93 mg, 0.184 mmol) in dioxane (4.4 ml) was added 0.2 M NaOH (4.4 ml) with stirring. After being stirred at room temperature for 15 min, the solution was passed through a column (1 cm×2 cm) of DIAION SK 1B (NH_4^+ form) and the elution was performed with dioxane–water (1: 1, v/v, 60 ml). The eluent was concentrated to *ca.* 10 ml, and extracted with ether (3×10 ml) for removal of benzenethiol. The aqueous solution was concentrated and applied to Whatman 3MM paper developed with Solvent I to afford **7** (R_f 0.72, 87%), **8** (R_f 0.53, 12%), and pT (+Tp) (R_f 0.20, 1%).

A similar treatment of **3f** gave **4** (R_f 0.71, 76%), **8** (R_f 0.47, 21%), and Tp (+pT) (R_f 0.18, 3%).

The ammonium salt (138 OD) of **7**, obtained from **3e** in the above experiment, was incubated with snake venom phosphodiesterase (70 μg) in Tris buffer (pH 8, 1.4 ml) at 37 °C for 1 h. After incubation the mixture was treated with pyridine (1 ml) and chromatographed on Whatman 3MM paper developed with Solvent I to give pT (R_f 0.20, 130 OD) as the sole nucleotidic material.

In a similar manner, the ammonium salt (143 OD) of **4**, obtained by alkaline treatment of **3f**, was incubated under the same conditions. The paper chromatography showed unchanged **4** (138 OD) and Tp (R_f 0.20, 2 OD).

Enzymatic Assay of the Authentic Samples (7 and 4): The ammonium salt (112 OD) of **7**, obtained from thymidine 5'-phosphite, was incubated with snake venom phosphodiesterase (66 μg) in Tris buffer (pH 8.0, 1.32 ml) at 37 °C for 1 h. One tenth of the incubation mixture was treated with pyridine (0.1 ml) and analyzed with paper electrophoresis (pH 8.0), which showed a single band of pT (12.5 OD).

The ammonium salt (76.1 OD) of **4**, obtained by treatment of **5** with 80% acetic acid in the previous experiment, was incubated with snake venom phosphodiesterase (38 μg) in Tris buffer (pH 8, 0.76 ml) at 37 °C. After 1, 6, and 20 h, 0.1 ml aliquots of the incubation mixture were analyzed with paper electrophoresis (pH 8.0), and the bands corresponding to pT were eluted with water, and the yields of pT were spectroscopically calculated to be 1.5% (1 h), 7% (6 h), and 11% (20 h), respectively. These results showed that this enzyme is essentially resistant to TpSPh within 1 h but degrades it gradually to Tp and benzenethiol for prolonged incubation.

One-step Removal of Both Phenylthio Groups from Bis(phenylthio)-phosphoryl Group. Typical Procedure A): To a solution of silver acetate (97 mg, 0.58 mmol) in pyridine–water (2 : 1, v/v, 3 ml) was added **3a** (20.0 mg, 36.5 μmol) and the homogeneous solution was vigorously stirred at room temperature. The solution turned gradually to a suspension. After being stirred for 18 h, the suspension was bubbled with hydrogen sulfide gas until a clear supernatant solution had been obtained. The precipitate was removed by centrifugation (or sometime by filtration) and washed with water (2×5 ml). The supernatant and washings were concentrated to *ca.* 2—3 ml and chromatographed on Whatman 3MM paper developed with solvent II to give a single band of R_f 0.20. Elution of the band with water afforded pTOAc (315 OD, 99%).

B): To a solution of **3c** (77.9 mg. 0.1 mmol) in pyridine (5 ml) was added water (2.5 ml) and then silver acetate (267 mg, 1.6 mmol). The resulting solution was stirred vigorously at room temperature for 18 h and then bubbled with hydrogen sulfide gas at 0 °C until a clear supernatant solution had been obtained. The resulting suspension was evaporated *in vacuo* to remove excess hydrogen sulfide and then diluted with pyridine–water (2 : 1, v/v, 10 ml). The black precipitate was removed by centrifugation, and the supernatant and washings with pyridine–water (2 : 1, v/v, 3×2 ml) were combined, evaporated, and chromatographed on Whatman 3MM paper developed with Solvent I. A band of R_f 0.45 was eluted with water to give MMTrTp (37.1 OD, 96%).

Treatment of 3e and 3f with Silver Acetate in Aqueous Pyridine. In the Case of 3e: To a solution of **3e** (33.9 mg, 0.767 mmol) in pyridine (3.9 ml) was added water (1.9 ml) and then silver acetate (205 mg, 1.23 mmol). The mixture was stirred vigorously at room temperature for 16 h. The resulting suspension was bubbled with hydrogen sulfide gas until a clear supernatant had been obtained. The precipitate of silver sulfide was centrifuged and the supernatant solution was decanted. The precipitate was washed with pyridine–water (2 : 1, v/v, 2×3 ml), and the supernatant and washings were collected and then evaporated to dryness. The residue was dried by repeated evaporations with dry pyridine (4×3 ml) and treated with acetic anhydride (0.22 ml) in dry pyridine (0.77 ml) at room temperature for 12 h. Then ice–water (20 ml) was added and the mixture was extracted with chloroform (3×20 ml). The aqueous layer was evaporated to dryness and a benzene adduct of TrT (35.9 mg, 0.064 mmol) was mixed. The mixture was dried by repeated evaporations with dry pyridine (4×3 ml) and treated with TPS (46.5 mg, 0.15 mmol) in dry pyridine (0.8 ml) at room temperature for 3 h. Then the mixture was quenched with water (20 ml) and extracted with chloroform (3×10 ml). The organic extracts were collected, evaporated to dryness *in vacuo*, and treated with 80% acetic acid (10 ml) at 100 °C for 20 min. After removal of the solvent *in vacuo*, the residue was treated with concd NH_4OH–MeOH (1 : 1, v/v, 20 ml) at room temperature for 10 h. Then the mixture was evaporated to dryness and chromatographed on Whatman 3MM paper developed with Solvent I. A band of R_f 0.43 corresponding to TpT was eluted with water to give a nucleotidic material of 194 OD.

In a similar manner, a nucleotidic material (203 OD) corresponding to Tp$^{3'}$T was obtained from **3f** (40.5 mg, 0.08 mmol) *via* TrTp$^{3'}$T$^{5'}$ OAc.

Enzymatic Assay of the Dithymidine Monophosphate Derivatives Obtained in the Above Experiments. A): TpT (90.1 OD, 4.92 mol) obtained from **3e** was incubated with snake venom phosphodiesterase (50 μg) in 0.1 M Tris buffer (pH 8, 1.0 ml) at 37°C for 12 h. After addition of pyridine (1 ml), the mixture was chromatographed on Whatman 3MM paper developed with Solvent I to give T (45.4 OD, 96.1%) and pT (47.4 OD, 100%).

The same substrate (90.1 OD, 4.92 mol) was incubated with spleen phosphodiesterase (140 μg) in 0.05 M NH_4OAc (1.0 ml) at 37 °C for 8 h. Paper chromatography of the mixture gave T (46.2 OD, 98%) and Tp (42.8 OD, 90%). In the above two enzymatic assays, the band corresponding to the original substrate was eluted with water and its recovery was spectroscopically calculated and estimated to be 0.71% (0.64 OD) and 1.7% (1.5 OD), respectively. B) Tp$^{3'}$T (90.7 OD, 4.96 μmol) obtained from **3f** was incubated with snake venom phosphodiesterase and spleen phosphodiesterase under the same conditions as described in the above enzymatic assays. In both cases, the bands corresponding to the substrate unchanged and pT (or Tp) were cut and eluted with water. The recovery of Tp$^{3'}$T was 100% (91 OD) and 98.6% (87 OD), respectively, after treatment with snake venom and spleen phosphodiesterases. pT and Tp eluted were estimated to be less than 0.3% (0.16 OD) and 0.6% (0.36 OD).

Reaction of 3e with Phosphonic Acid in the Presence of p-*Toluenesulfonyltriazole.* To a solution of phosphonic acid (0.124 mmol) in pyridine–water (9 : 1, v/v, 1 ml) added **3e** (15.7 mg, 0.02 mmol). The mixture was rendered anhydrous by repeated evaporations with pyridine (5×4 ml) and treated with 1-(*p*-toluenesulfonyl)-1*H*-triazole (27.4 mg, 0.124 mmol) in dry pyridine (0.5 ml) at room temperature for 24 h. Then the mixture was quenched with water (10 ml) and extracted with CH_2Cl_2 (3×10 ml). The organic layer was analyzed by TLC ("Avicel" plate, Funakoshi Co.), which showed no significant spots containing phosphate group indicating that the Hanes-Isherwood test (spray) was negative. TLC of the aqueous layer showed a main product of R_f 0.49 (Solvent I) which was formed almost quantitatively. The spot did not change when concd NH_4OH was added to an aliquot of the aqueous layer. When one third of the aqueous layer was dried by repeated evaporations with dry pyridine (4×1 ml) and treated with 2,2'-dipyridyl disulfide (4.4 mg, 0.02 mmol) in the presence of *N,O*-bis(trimethylsilyl)acetamide (0.1 ml) in dry pyridine (0.2 ml) at room temperature for 2 h, the spot changed to a new one of R_f 0.23 (Solvent I) or 0.39 (Solvent III) corresponding ot PhSpTp. The spot was also converted to a new spot of R_f 0.84 (Solvent I) when one third of the aqueous layer was treated with diphenyl disulfide in place of 2,2'-dipyridyl disulfide. The spot of R_f 0.84 was identical with PhSpTpSPh obtained by treatment of **3d** (29 mg, 0.038 mmol) with 0.2 M NaOH–dioxane (1 : 1, v/v, 8 ml) at room temperature for 15 min. Treatment of PhSpTpSPh

with 20 equiv. of iodine in pyridine–water (2 : 1, v/v) gave pTp of R_f 0.05 (Solvent I). The changes in the above transformations of the product of R_f 0.23 were also followed by paper electrophoresis. The data are shown as follows: PhSpTp$_3$: Rm (pH 8.0, relative to pT) 0.94; PhSpTp: 1.10; PhSpTpSPh: 0.82; pTp: 1.18. These results suggested that the initial product from the reaction of **3e** with phosphonic acid was PhSpTp$_3$.

*Selective Removal of One Phenylthio Group from **3a** by Using Phosphonic acid.* By dissolving phosphonic acid (2.05 g, 25 mmol) in THF using 25 ml measuring flask, 1 M solution of phosphonic acid was prepared. The standard solution of 2.4 ml was taken, evaporated, and dried by repeated evaporations with dry pyridine (3×1 ml). To the pyridinium phosphonate was added **3a** (27.4 mg, 0.05 mmol) in dry pyridine (*ca.* 0.5 ml). The weight of the mixture was 537 mg. Aliquots from the reaction mixture after 0.5, 1, 7, 11, and 21 h were taken out, weighed, and applied to paper electrophoresis (pH 6.0) at 1500 V for 1 h. A band of Rm 0.8 (to pT) was eluted with 0.1 M phosphate buffer (pH 7.0) and the yield was estimated by UV spectrophotometer.

*Conversion of **3c** to **5** by Use of Phosphonic Acid.* The pyridinium salt (2.4 mmol) of phosphonic acid and **3c** (312 mg, 0.4 mmol) were mixed, dried by repeated evaporations using dry pyridine (4×1 ml) and finally dissolved in dry pyridine (4 ml). After being stirred at 30 °C for 15 h, the solution was treated with CH_2Cl_2 (20 ml) and water (20 ml). The aqueous solution was further extracted with CH_2Cl_2 (3×10 ml). The CH_2Cl_2 extracts were combined dried over Na_2SO_4, concentrated to *ca.* 2 ml, and poured into hexane (50 ml). A white precipitate was collected and dried over P_4O_5 *in vacuo* for 12 h to give the free acid of **5** (236 mg, 86%). The product was almost homogeneous on TLC [R_f 0.42 (silica gel plate), Solvent III].

Synthesis of (PhS)$_2$pTp(tc)T. Pyridinium salt (0.6 mmol) of 2,2,2-trichloroethyl phosphate and **3e** (203.1 mg, 0.5 mmol) were mixed, dried by repeated coevaporations with dry pyridine (4×3 ml), and treated with TPS (379 mg, 1.25 mmol) in dry pyridine (2.5 ml) at room temperature for 20 h. Then CH_2Cl_2 (20 ml) and water (20 ml) were added. The aqueous layer was extracted with CH_2Cl_2 (3×10 ml). The CH_2Cl_2 extracts were combined, dried over Na_2SO_4, and evaporated to dryness, and then thymidine (183 mg, 0.75 mmol) was added. The mixture was rendered anhydrous by repeated coevaporations with dry pyridine (4×4 ml), dissolved in dry pyridine (1.0 ml), and finally treated with *p*-nitrobenzenesulfonyl 1,2,4-triazole (153 mg, 0.6 mmol) at room temperature for 24 h. Then CH_2Cl_2 (20 ml) and water (20 ml) were added to the solution. The aqueous layer was further extracted with CH_2Cl_2 (3×10 ml) and the extracts were combined and evaporated to dryness after dryning over Na_2SO_4. The residue was evaporated with toluene (3×4 ml) to remove the last traces of pyridine and chromatographed on silica gel with CH_2Cl_2–methanol to give the title compound (209 mg, 44%): NMR ($CDCl_3$) δ 1.81 (3H, s, C=C–CH_3), 1.90 (3H, C=CH_3), 2.13—2.67 (4H, 2′H), 4.44 (2H, m, 4′H), 4.24—4.70 (5H, m, OH and 5′H), 4.70 (2H, d, J_{P-H}=7 Hz, Cl_3CCH_2–O–P), 5.00—5.47 (2H, m, 3′H), 6.33 (2H, m, 1′H), 7.20—7.73 (12H, m, ArH and)H): UV_{max} (MeOH) 261 nm (ε 21.4×10^3), UV_{min} (MeOH) 241 nm (ε 16.6×10^3).

Found: C, 43.32; H, 3.95; N, 5.87%. Calcd for $C_{34}H_{37}O_{13}N_4S_2P_2Cl_3$: C, 43.35; H, 3.96; N, 5.95%.

Similarly, the use of TPS (303 mg, 1 mmol) in the second coupling reaction gave (PhS)$_2$pTp(tc)T (433 mg, 92%). This compound was further treated with monomethoxytrityl chloride (308 mg, 1 mmol) in dry pyridine (10 ml) for 6 h in order to remove the 3′–3′ isomer. After the usual workup, pure (PhS)$_2$pTp(tc)T (400 mg) was obtained by chromatography. For further transformation or deprotection, this purified material was used.

*Treatment of **3a** or **17a** with Imidazole, 1,2,4-Triazole, and Tetrazole. General Procedure*: To a solution of **3a** or **17a** in dry pyridine was added an appropriate azole. The reaction conditions and the results are summarized in Table 4. The total weight of the reaction mixture was measured and 10—20 mg aliquots of the mixture after the times described in Table 4 were taken to be analyzed by paper electrophoresis.

*Synthesis of (4-$CH_3OC_6H_4S$)$_2$pT(tc)T (**16b**).* Pyridinium salt (513 mg, 1.2 mmol) of 2,2,2-trichloroethyl phosphate was dried by repeated evaporations with dry pyridine (4×5 ml) and finally dissolved in dry pyridine (5 ml). TPS (727 mg, 2.4 mmol) was added to the mixture and the solution was stirred at room temperature for 21 h. Then CH_2Cl_2 (30 ml) and water (30 ml) were added. The organic layer and further extracts with CH_2Cl_2 (3×10 ml) from the aqueous layer were combined, dried over Na_2SO_4, and concentrated to dryness. The residue was rendered anhydrous by repeated evaporations with dry pyridine (5×4 ml) and treated with NBST (509 mg, 2 mmol) in dry pyridine (1 ml) for 1 h. Then well pulverized thymidine (363 mg, 1.5 mmol) was added to the mixture. The mixture was stirred at room temperature for 26 h. Then CH_2Cl_2 (30 ml) and water (30 ml) were added and the organic layer was collected, and then the aqueous solution was further extracted CH_2Cl_2 (3×10 ml). The organic extracts were combined, dried over Na_2SO_4, and concentrated to dryness. The residue was coevaporated with toluene (4×4 ml), and chromatographed on silica gel with CH_2Cl_2–methanol to afford **16b** (902 mg, 90%). The crude product containing the 3′–3′ isomer was further treated with monomethoxytrityl chloride (308 mg, 1 mmol) in pyridine (10 ml) at room temperature for 6 h to remove the 3′–3′ isomer and the purified material (798 mg) was used for further transformation and deprotection: NMR($CDCl_3$) δ 1.82 (6H, m, CH_3), 2.00—2.50 (4H, m, 2′H), 3.75 (6H, OCH_3), 4.00—4.27 (2H, m, 4′H), 4.27—4.57 (5H, m, CHOH and 5′H), 4.60 (2H, d, J_{P-H}=7 Hz, Cl_3CCH_2O–P), 4.90—5.32 (1H, m, CH–O–PS), 6.08—6.56 (2H, m, 1′H), 6.85 (4H, d, J=8.4 Hz, ArH), 7.24 (2H, s, C=CH), 7.58 (4H, dd, J=2 Hz, J=8.4 Hz, ArH); UV_{max} (MeOH) 257 nm (ε 35.7×10^3), UV_{min} (MeOH) 225 nm (ε 18.6×10^3).

Found: C, 43.52; H, 4.19; N, 5.61%. Calcd for $C_{36}H_{41}O_{15}N_4Cl_3P_2S_2$: C, 43.15; H, 4.12; N, 5.59%.

*Deprotection of the Fully Protected Dithymidine Diphosphates (**16a**) and (**16b**).* The protected dinuclotide, **16a**, (7.9 mg, 8.4 μmol) was treated with silver acetate (22,4 mg, 0.134 mmol) in pyridine–water (2 : 1, v/v, 0.8 ml) at room temperature for 16 h. Then the mixture was bubbled with hydrogen sulfide gas at 0 °C until a clear supernatant had been obtained, and the suspension was sucked by aspirator for a few minutes for removal of excess hydrogen sulfide. The black precipitate was removed off centrifugation by and washed with pyridine–water (2×2 ml). The supernatant and washings were combined and evaporated to dryness *in vacuo*. The residue was dissolved in DMF–pyridine (2: 1, v/v, 0.8 ml). The analysis of the solution with paper electrophoresis (pH 6.0) showed a single spot of Rm 0.50 (relative to pT) corresponding to pTp(tc)T (151 OD, 98%). To the DMF–pyridine solution was added zinc powder (50 mg) and acetylacetone (0.08 ml) and the mixture was stirred vigorously at room temperature for 8 h. Then water (5 ml) and DIAION SK 1B (NH_4^+ form, 5 ml) were added with stirring. The supernatant was passed through a column (1 cm×5 cm) of DIAION SK 1B (NH_4^+ form) and the resin was washed with concd NH_4OH–water (1 : 1, v/v, 30 ml). The eleuent was evaporated and

chromatographed on Whatman 3MM developed with Solvent II. A band of R_f 0.18 was eluted with water to give pTpT (150 OD, 98%). b) In a similar manner, pTpT (250 OD, 99.5%) was obtained from **16b** (11.27 μmol). In this case, 20 equiv. of silver acetate was used and the time for complete removal of 4-methoxyphenylthio group was 18 h.

Conversion of (PhS)$_2$pTp(tc)T to PhSpTp(tc)T. A): (PhS)$_2$pTp(tc)T (7.3 mg, 7.74 μmol) was treated with 0.2 M NaOH–dioxane (1 : 1 (v/v), 0.2 ml) at room temperature for 20 min. Then DIAION SK 1B (NH_4^+ form, 1 ml) was added to the mixture and the supernatant was analyzed by paper electrophoresis and paper chromatography. The electrophoresis showed a single spot of Rm 0.51 (relative to pT, pH 6.0) corresponding to PhSpTp(tc)T [$UV_{max}(H_2O)$ 266 nm, UV_{min} (H_2O) 236 nm, $UV_{sh}(H_2O)$ 250 nm] which appreared at R_f 0.77 (Solvent I) in paper chromatography.

B): (PhS)$_2$pTp(tc)T (7.3 mg, 7.74 μmol) was mixed with pyridinium salt of phosphonic acid (0.046 mmol) and dissolved in pyridine–water (19 : 1, v/v, 0.2 ml). The solution was kept with stirring at 30 °C for 2 d. Paper chromatography and paper electrophoresis of the solution showed the same product as described in the above experiment the yield was nearly quantitative.

Conversion of PhSpTp(tc)T to pTp(tc)T. PhSpTp(tc)T obtained by alkaline treatment of (PhS)$_2$pTp(tc) was dissolved in pyridine–water (2 : 1, v/v, 1 ml) and iodine (392 mg, 1.55 mmol) was added with continuous stirring. The mixture was stirred at room temperature for 30 min and then 1 M sodium sulfite was added until the color of iodine had disappeared. The clear solution was diluted with pyridine (5 ml) and the resulting salt (mainly Na_2SO_4) was filtered off and washed with pyridine–water (5 : 1, v/v, 5 ml). The filtrate and washings were combined, concentrated, and analyzed with paper chromatography. A single spot of pTp(tc)T (R_f 0.18 (Solvent II), 121 OD, 90%) was obtained.

*Conversion of (4-CH$_3$OC$_6$H$_4$S)$_2$pTp(tc)T (**16b**) to 4-CH$_3$OC$_6$H$_4$SpTp(tc)T.* To a solution of **16b** (5.8 mg, 5.8 μmol) in dioxane (0.2 ml) was added 0.2 M NaOH (0.2 ml). After being stirred at room temperature for 20 min, the solution was passed through a column (1 cm × 1 cm) of DIAION SK 1B (pyridinium form) and the resin was washed with pyridine–water (1 : 1, v/v, 10 ml). The eluent was concentrated to *ca.* 4 ml. The weight of the condensed solution was 3.94 g. One gram of the solution was analyzed with paper electrophoresis to afford three spots of Rm 0.57, 0.85, and 1,22 (relative to pT, pH 6.0). The fast running spot was estimated to be pTpT or TpTp (2.2 OD, 8.4%). The middle spot may be cyclic dithymidine diphosphate derivative, $\overline{|\text{pTpT}|}$ (2.2 OD, 8.2%). The slowest moving spot was 4-MeOC$_6$H$_4$SpTp(tc)T (22.0 OD, 83%, $UV_{max}(H_2O)$ 264 nm, 241 nm; UV_{min} (H_2O) 252 nm, 231 nm).

*One-step Conversion of **16a** to pTpT.* To a solution of **16a** (9.4 mg, 0.01 mmol) in DMF–pyridine (2 : 1, v/v, 0.7 ml) were added acetylacetone (0.07 ml) and zinc powder (0.5 mmol). The mixture was vigorously stirred at room temperature for 8 h. After being passed through a column (1 cm × 3 cm) of DIAION SK 1B (NH_4^+ form), the mixture was analyzed by paper electrophoresis. Compounds pTpT and (PhS)$_2$pTpT were formed in 61 and 22% yields, respectively. Some alternative conditions for the deprotection with zinc-acetylacetone are summarized in Table 4.

Synthesis of (4-CH$_3$OC$_6$H$_4$S)$_2$pTp(tc)Tp(tc)T. A mixture of **16b** (501.6 mg, 0.5 mmol) and 2,2,2-trichloroethyl phosphate (126.3 mg, 0.55 mmol) was rendered anhydrous by repeated evaporations with dry pyridine (4 × 3 ml) and finally dissolved in dry pyridine (5 ml). To the solution was added TPS (333.6 mg, 1.1 mmol) and the mixture was stirred at room temperature for 2 d. Then CH_2Cl_2 (20 ml) and water (20 ml) were added. The aqueous solution was extracted with CH_2Cl_2 (3 × 10 ml). The organic extracts were combined and concentrated to dryness. The residue was mixed with well pulverized thymidine (145.3 mg, 0.6 mmol). The mixture was rendered anhydrous by repeated evaporations with dry pyridine (4 × 4 ml) and dissolved in dry pyridine (3.0 ml). It was treated with NBST (280 mg, 1.1 mmol) at room temperature for 2 d. Then CH_2Cl_2 (20 ml) and water (20 ml) were added. The aqueous layer was extracted with CH_2Cl_2 (3 × 10 ml), and the organic extracts were combined and concentrated and dried by repeated coevaporation with dry pyridine (3 × 4 ml). The residue was finally dissolved in dry pyridine (3 ml) and allowed to react with monomethoxytrityl chloride (154 mg, 0.5 mmol) at room temperature for 6 h. Then the mixture was extracted by addition of CH_2Cl_2 (20 ml) and water (20 ml). The aqueous layer was extracted with CH_2Cl_2 (2 × 10 ml) and the organic extracts were combined, dried over Na_2SO_4, and concentrated to dryness. The residue was coevaporated with toluene (3 × 5 ml) and chromatographed on silica gel with CH_2Cl_2–methanol to afford the title compound (593 mg, 82%).

Deprotection of (4-CH$_3$OC$_6$H$_4$S)$_2$pTp(tc)Tp(tc)T. To a solution of (4-CH$_3$OC$_6$H$_4$S)$_2$pTp(tc)Tp(tc)T (28.8 mg, 0.02 mmol) in pyridine–water (2 : 1, (v/v), 2.5 ml) was added silver acetate (66.8 mg, 0.4 mmol). The mixture was stirred vigorously at room temperature for 24 h. The resulting suspension was bubbled with hydrogen sulfide gas until a clear supernatant had been obtained at 0 °C (*ca.* 5 min). The black precipitate was removed off by centrifugation and washed with pyridine–water (2 : 1, v/v, 2 × 2 ml). The supernatant and washing were combined and evaporated to dryness. The residue was dissolved in DMF–pyridine (1 : 1, v/v, 1 ml). To the solution was added acetylacetone (0.4 ml) and then zinc powder (80 mg). The mixture was stirred at room temperature for 3 h. Then water (4 ml) was added and the solution was passed through a column (1 cm × 5 cm) of DIAION SK 1B (NH_4^+ form). The resin was washed with water (30 ml). The eluent and washings were combined, concentrated to *ca.* 2 ml, and chromatographed on Whatman 3MM paper to afford pTpTpT (470 OD, 97%) which appeared at the position of 9.6 cm from the starting point after development by *i*-PrOH–concd NH_4OH–H_2O (6 : 1 : 3, v/v/v) for 3 d.

Enzymatic Assay of pTpTpT. Compound pTpTpT (85 OD) obtained in the above experiment was incubated with snake venom phosphodiesterase (40 μg) in 0.1 M Tris buffer (pH 8, 1 ml) at 37 °C for 18 h. After addition of pyridine (1 ml) the mixture was analyzed by paper chromatography using Whatman 3MM paper to give pT (91 OD) as a single degradation product.

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