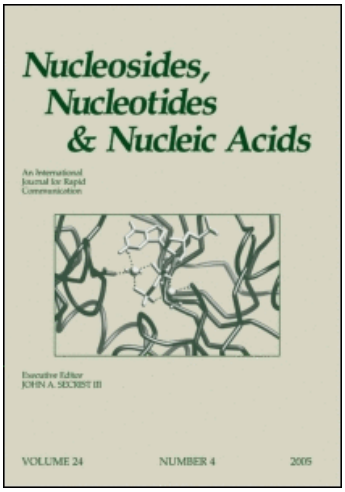

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BIS(2,4,6-TRIBROMOPHENYL) PHOSPHOROCHLORIDATE: A NEW TYPE OF CONDENSING REAGENT IN OLIGONUCLEOTIDE SYNTHESIS

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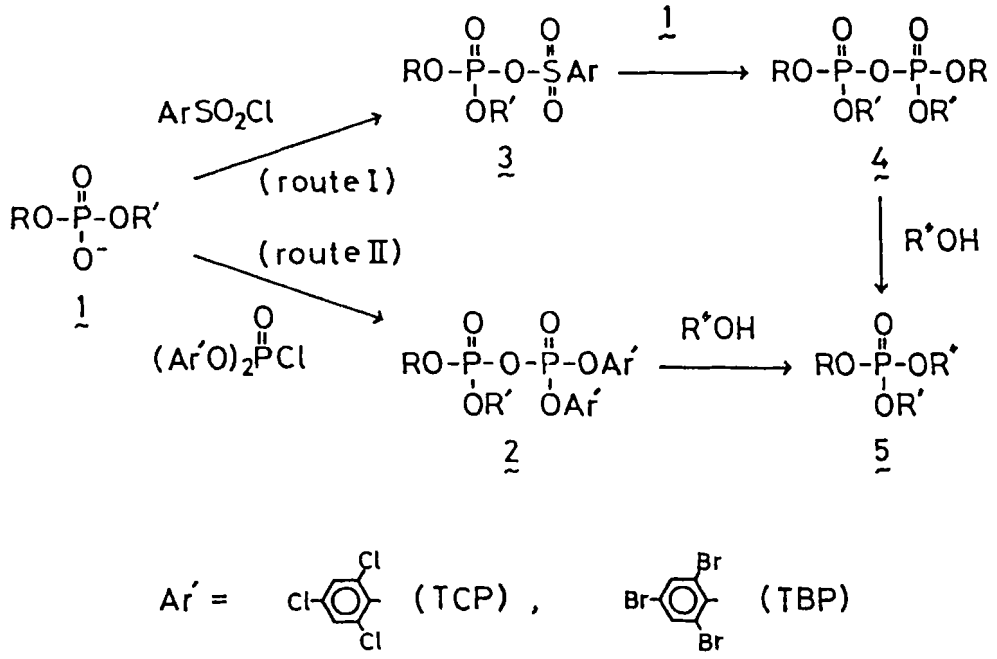
Abstract

The detailed study of internucleotidic bond formation by the use of bis(2,4,6-tribromophenyl) phosphorochloridate (TBP) is described. The ^{31}P NMR analysis suggested that the TBP-mediated condensation proceeded via a reactive unsymmetrical pyrophosphate intermediate, which was readily activated in the presence of 3-nitro-1,2,4-triazole to form internucleotidic bond.

INTRODUCTION

Introduction of the phosphoramidite method to the chemical synthesis of oligodeoxyribonucleotides has resulted in substantial changes in the style of DNA synthesis.[1,2] Rapidness and high reproducibility of this approach enabled us to use practically automated DNA synthesizers to obtain pure and longer DNA fragments easier than before. However, this method still has a disadvantage, i.e., the instability of phosphoramidite reagents. Although white powdered phosphoramidite reagents are commercially available in stable form from several companies, preparation of these pure reagents requires elaborate techniques and extensive know-how in laboratories.

On the other hand, in the well-known phosphotriester method,[3] more stable phosphodiester components can be chosen as starting material. In this approach, the most



Scheme 1

active compound is a condensing reagent and can be prepared in large quantities from simple organic chemicals. However, the only crucial drawback of this method is the relatively slow rate of condensation.[4] A number of investigators have explored catalysts such as tetrazole,[5] 3-nitro-1,2,4,-triazole,[6] and N-methylimidazole[7-9] in order to accelerate the reaction. However, little attention has been paid for improvement of condensing reagents.[10,11]

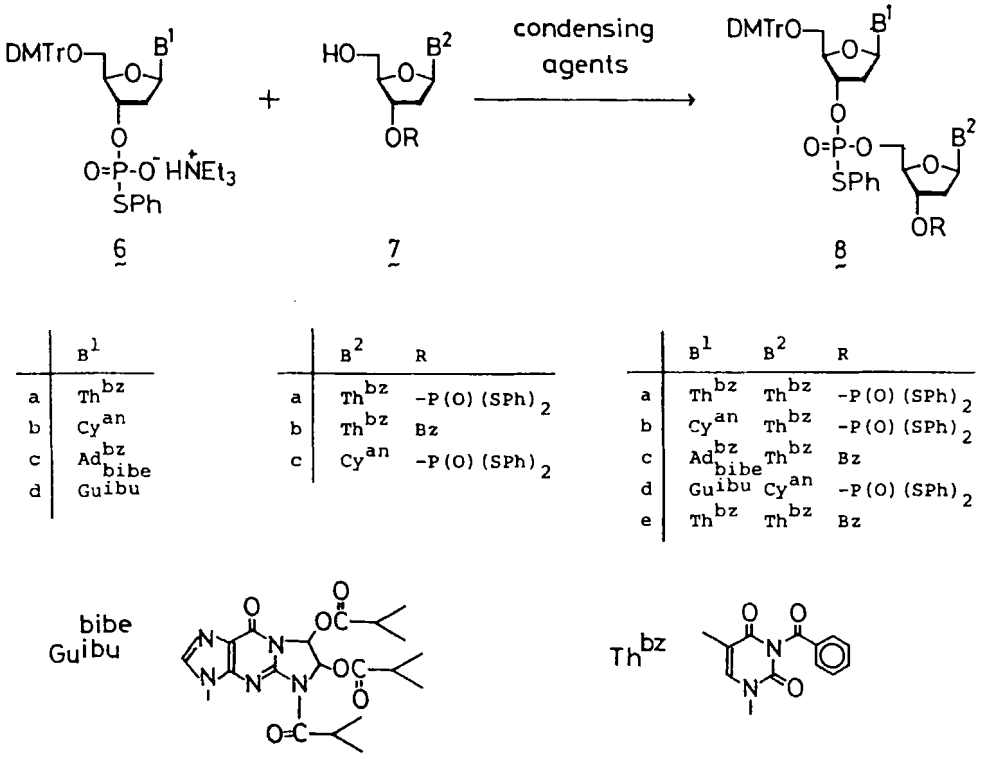
The mechanism of condensation using arenesulfonyl chlorides and azolides has been studied in detail by several research groups.[12-14] The reaction mechanism, which has generally been accepted up to date, is shown in Scheme 1 (route I). The rate-determining step is the alcoholysis at the last stage and a symmetrically tetrasubstituted pyrophosphate (4) exists as a relatively stable intermediate. Nucleophilic catalysts such as pyridine and azoles are required to activate the intermediates (4) for

acceleration of the reaction. Considering the above mechanism, we proposed sterically hindered diaryl phosphorochloridates as condensing reagents (route II). When they are allowed to react with phosphodiester (1), unsymmetrical pyrophosphate (2) can be expected to be formed as more stable species than mixed acid anhydride intermediates (3). Such unsymmetrical pyrophosphates should be more reactive than the symmetrical pyrophosphate (4) to allow facile alcoholysis giving rise to triesters (5). Therefore, we designed two reagents, bis(2,4,6-trichlorophenyl) phosphorochloridate (TCP) and bis(2,4,6-tribromophenyl) phosphorochloridate (TBP).[15] The effectiveness of these reagents has been examined in the synthesis of dithymidylate. This paper describes the detailed study on the condensation promoted by TBP.

Results and Discussion

First, in order to examine the condensing ability of TCP we prepared TCP according to the literature procedure[16] which involved the reaction of phosphorus pentachloride with 2,4,6-trichlorophenol. This known method gave indeed the same compound as described there as far as the melting point was concerned. Therefore, in our preliminary communication, we described this compound as TCP. Later, however, the careful structural analysis based on its elemental analysis and ^{31}P NMR spectrum did not support the structure of such a phosphoromonochloridate. Instead, the compound obtained was determined to be bis(2,4,6-trichlorophenyl) trichlorophosphorane (CTCP). All attempts to convert CTCP into TCP with acetic acid, water, or hexamethyldisiloxane have failed. TCP obtained has always been contaminated with significant amounts of CTCP and/or bis(2,4,6-trichlorophenyl) phosphate.

On the other hand, TBP was readily prepared by a modification of Kozlov's method[17] which involved partial acetolysis of bis(2,4,6-tribromophenyl) trichlorophosphorane (BTCP) with acetic acid. BTCP was obtained in 24% yield by



Scheme 2

Table 1. The conditions and results of the condensation of 6 with 7 by the use of TBP.

dimer	6/7	TBP/6	NT/6	time (min)	yield of 8 (%)
8a	1.2	1.08	0.83	15	88
8b	1.2	1.25	1.67	15	88
8b	1.2	1.08	0.83	10	99
8c	1.2	1.08	1.08	10	83
8d	1.2	1.25	1.67	30	96

the reaction of phosphorus pentachloride with 2 equiv of 2,4,6-tribromophenol.

The optimized yield of TBP from BTCP was 80%. Nonetheless, this reagent can be prepared in relatively large quantities because the starting materials are commercially available at inexpensive prices. TBP thus obtained had a purity of more than 85% which was estimated by its ^{31}P NMR spectrum.

It was reported briefly in our previous paper[15] that TBP could serve as a powerful condensing agent for the synthesis of TpTp, when used in combination with 3-nitro-1,2,4-triazole (NT). In order to examine the generality of TBP as a condensing agent, we applied this reagent to activate the common four kinds of S-phenyl 5'-dimethoxytrityl deoxyribonucleoside 3'-phosphorothioate (6a-d) in the presence of a hydroxyl component to give fully protected dinucleotides (8a-e) (Scheme 2).

As shown in Table 1, the use of slightly excess TBP gave good yields of 8a-d. It is interesting that these reactions required relatively small amounts of condensing agent, whereas at least 3 equiv of arenesulfonylchloride relative to a hydroxyl component (7) has been used in the usual condensation in the presence of NT. In the case of thymidine and deoxyguanosine, the base moieties were fully protected to prevent any possible side reactions. In this study, we used the benzoyl (bz) group at the N^3-position of thymidine[15,18] and the bis(isobutyryloxy)ethylene (bibe) group at the N^1 and N^2 positions of guanosine[19].

To compare the coupling efficiency of TBP with those of more popular condensing reagents of arenesulfonyl chlorides, the following experiments were undertaken. Condensation was performed at room temperature by use of 1.3 equiv each of 6a, a condensing reagent, and an azole relative to 7b. The reaction was stopped regardless of its completion or incompletion after 15 min. The isolated yields of 8e are summarized in Table 2. The TBP-NT system gave the product in 95% yield while the MS-NT system or MSNT resulted in

Table 2. The isolated yield of 8e obtained after 15 min by condensation of 6a with 7b in the presence of several kinds of condensing agents.

condensing agent	azole	time (min)	yield of 8e (%)
TBP	NT	15	95
TBP	Tet	15	84[a)]
MS	NT	15	82[a)]
MS	Tet	15	73[a)]
MSNT	---	15	87[a)]

a) These reactions were not completed in 15 min.

lower yields. TLC showed that the latter reactions were not completed within 15 min. Interestingly, TBP led to the incomplete reaction in the presence of 1H-tetrazole (Tet). Furthermore, the condensation of 6a with 7b in the presence of TBP-NT or MS-NT was estimated by isolation of 8e formed at appropriate times. These results are shown in Fig. 1. It shows that the reaction using TBP was completed in 5 min while the reaction using MS still proceeded even after 30 min.

Next, we examined more detailed studies of the combined use of TBP and NT as a condensing agent. Table 3 revealed that the reaction was accelerated with an increase of NT. When only TBP was increased without an increase of NT, phosphorylation of the 5'-hydroxyl group of 7b with TBP occurred to lead to a lower yield of 8e (data not shown). Consequently, the combination use of a small excess amount of TBP with a large excess amount of NT was most effective to make shorten the condensing reaction maintaining a high yield synthesis of 8e.

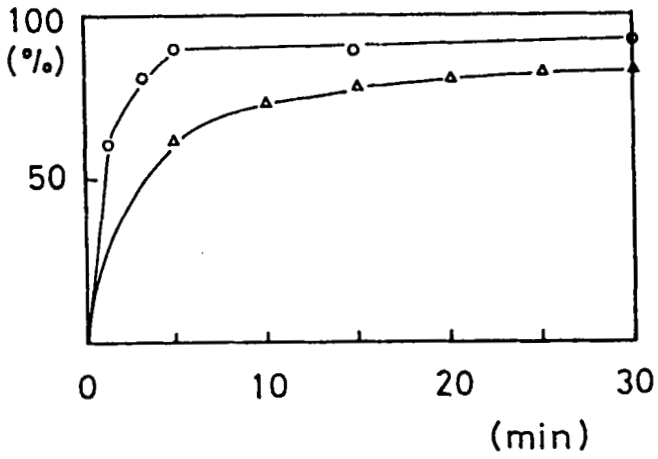
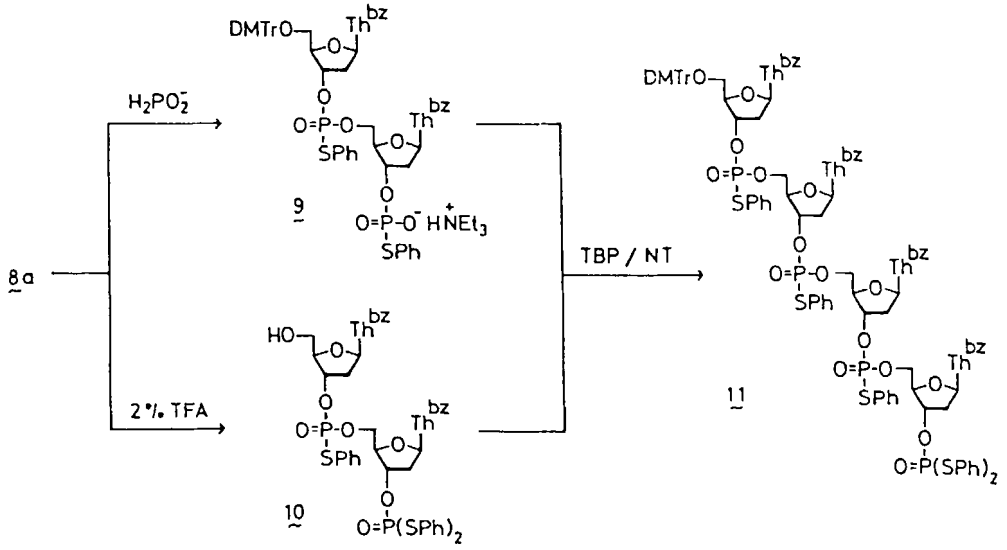


Figure 1. Rate of the formation of 8e by using 1.3 equiv each of TBP and NT (O) or 1.3 equiv each of MS and NT (Δ) in pyridine (0.1 M based on 7b).

Table 3. The effect of NT on condensation of 6a with 7b by use of TBP.

TBP/6a	NT/6a	time (min)	yield of 8e (%)
1.08	3.25	15	95
1.08	3.25	5	93
1.08	3.75	3	96

To examine the utility of TBP in block condensation, the fully protected tetrathymidylate (11) was synthesized by a strategy of dimer plus dimer (Scheme 3). The dimer (8a) was converted to the phosphodiester component (9) and the hydroxyl component (10) in 90% and 99% yields by treatment with pyridinium phosphinate (PHP)/pyridine and 2% trifluoroacetic acid (TFA)/dichloromethane, respectively, as reported in our previous paper.[20,21] Condensation of 9 with 10 was carried out by use of 1.5 equiv of TBP and 2.0 equiv of NT



Scheme 3.

at room temperature for 15 min. The tetramer (11) was obtained in an isolated yield of 63%.

To confirm the structure of the product, its deprotection was performed as follows:

1) 0.1 M NaOH for removal of the phenylthio group of triester.
2) conc. NH_3 for removal of the benzoyl group on the thymine residure
3) I_2 for removal of the remaining phenylthio group of diester.
4) 80% acetic acid for removal of the 5'-DMTr group.

The resulting material was applied to a DEAE-Sephadex A25 column. Elution with a linear gradient (0.05-2.0 M) of triethylammonium bicarbonate gave 88 OD units at A_{260} (28%) of the perfectly deprotected tetramer from 10 μmol of 11. The product was characterized by reversed phase HPLC compared with an authentic tetramer and by enzymatic digestion with spleen phosphodiesterase.

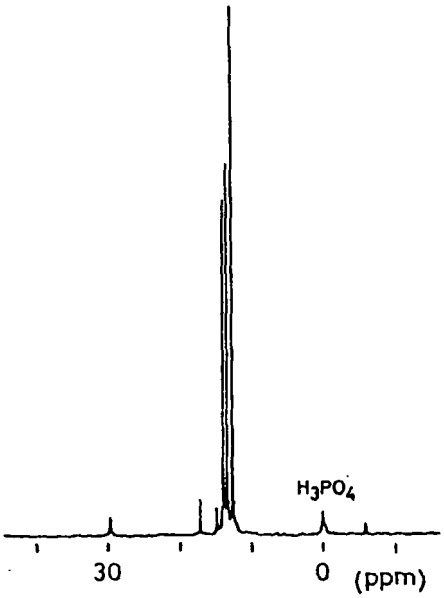
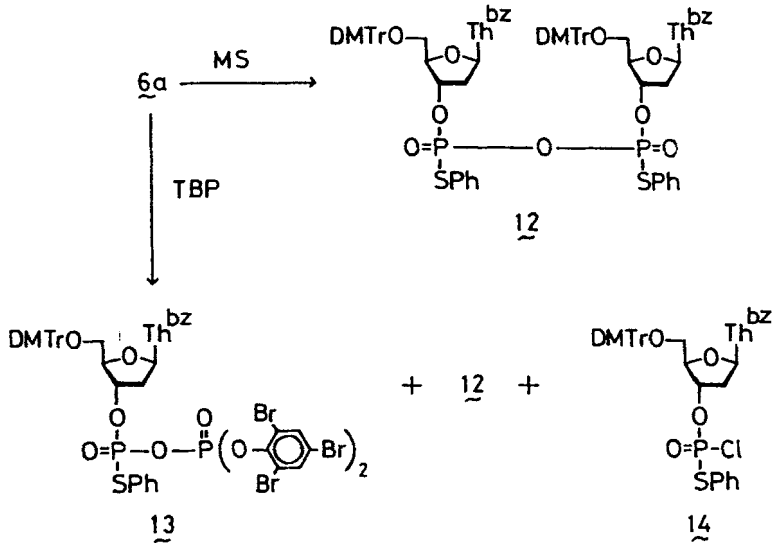


Figure 2. ^{31}P NMR spectra of symmetrical dinucleoside pyrophosphate 12. 6a was treated with 1.1 equiv of MS in pyridine. 85% H_3PO_4 was used as an external standard.

^{31}P NMR STUDIES

It is interesting to analyze how the reaction using TBP proceeds very fast. To clarify the difference in mechanism between the reactions using TBP and arenesulfonyl chlorides such as MS, we studied the detailed ^{31}P NMR analysis of the TBP-promoted condensation. When the phosphodiester (6a) was mixed with 1.1 equiv of MS in pyridine, the ^{31}P NMR spectrum of the mixture gave triple signals at 13.81, 13.23, 12.50 ppm as shown in Fig. 2. These resonance peaks were assigned to an symmetrical pyrophosphate (12) derived from two molecules of 6a (Scheme 4). The signal of 6a at 11.24 ppm completely disappeared.

Fig. 3 shows the ^{31}P NMR spectrum of the mixture obtained when 6a was treated with 1.1 equiv of TBP in pyridine. TBP and its hydrolyzed phosphate (TBPOH) gave resonance signals at -9.35 and -13.18 ppm, respectively, in



Scheme 4

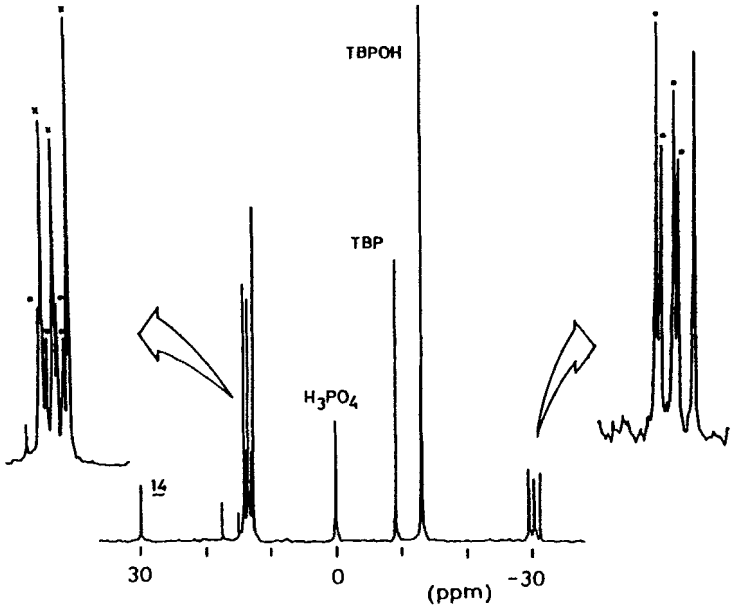


Figure 3. ^{31}P NMR spectra of TBP-promoted reaction intermediates. 6a was treated with 1.1 equiv of TBP in pyridine. 85% H_3PO_4 was used as an external standard. Symmetrical pyrophosphate 12 (x), unsymmetrical pyrophosphate 13 (Q) and phosphorochloridate 14 were observed.

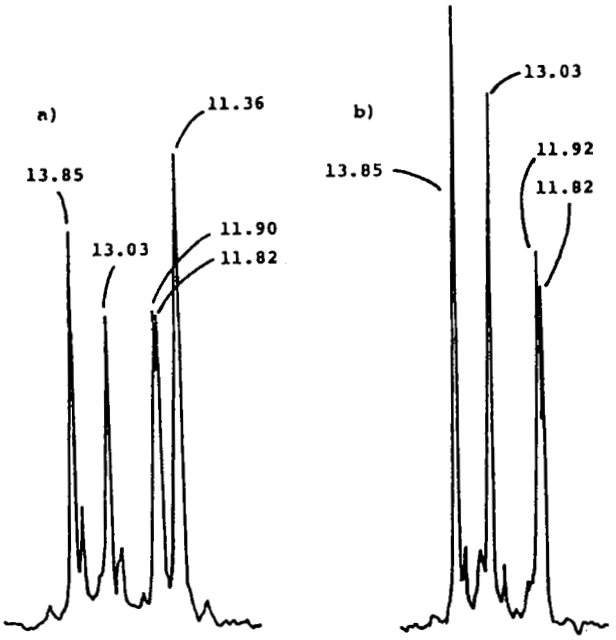


Figure 4. ^{31}P NMR spectra of nucleotide intermediates caused by TBP in dioxane. a) 6a was treated with 1.2 equiv each of TBP and lutidine in dioxane. b) 6a was treated with 1.2 equiv each of TBP and pyridine in dioxane.

pyridine. In this case, triple signals, which was assignable to 12 by the result of Fig. 2, was also observed. The other four peaks around these peaks of 12 were assigned as a double doublet due to the phosphorothioate of an unsymmetrical pyrophosphate (13). The resonance signals due to another phosphorus of 13 was observed at -29.98, -30.20, -30.89, and -31.11 ppm with equal intensity in the high magnetic field. These signals indicated that the unsymmetrical pyrophosphate (13) exists in the reaction mixture as one of important active intermediates for internucleotidic bond formation. The ratio of 12 to 13 was 3.7 : 2.9. The ratio would be changed in the actual condensation whereupon NT was present. A peak at -31.92 ppm was a resonance signal of tetrakis(2,4,6-tribromophenyl)

pyrophosphate formed from two molecules of TBP. Two peaks at 30.00 and 29.85 ppm were attributed to a doublet of a phosphorochloridate intermediate (14) as reported in our previous paper.[23] The reason of the appearance of the signal of 14 was not clear but phosphodiesters with such an electron withdrawing group as the phenylthio group seemed to be readily converted to phosphorochloridates by the reaction of 12 and/or 13 with the chloride ion generated during pyrophosphate formation. In fact, the signal of 14 was observed in the case of MS in Fig. 2. A similar observation has recently been reported by Garreg and Stawinski.[24]

To test the solvent effect of the TBP-promoted condensation, we used lutidine as non-nucleophilic base in the place of pyridine in dioxane. When the phosphodiester and TBP were mixed without pyridine half amount of 6a (11.36 ppm) remained unchanged and a small amount of 13 was formed as shown in Fig. 4 a). On the other hand, when reagent amount of pyridine was present instead of lutidine, the resonance peak of 6a was completely disappeared as shown in Figure 4 b). It was obviously seen that aromatic amines or azoles with sufficient nucleophilicity like pyridine were necessary to activate TBP. Similar results in the case of arenesulfonyl chlorides were noted by Knorre's group.[13]

Experimental

Melting points were determined on a Mitamura Melt-pointer apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 60 MHz on a Hitachi R24-B spectrometer using tetramethylsilane as an internal standard. ^{31}P NMR spectra were obtained on a JEOL-PS-100 at 40.26MHz using 85% H_3PO_4 as an external standard. UV spectra were measured on a Hitachi 220 A spectrophotometer. Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd. and minipump for a gold fish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. TLC was performed on precoated TLC plates of silica gel 60 F-254 (Merck).

Pyridine was distilled twice from p-toluenesulfonyl chloride and from calcium hydride and then stored over molecular sieves 4A.

Elemental analysis was performed by the Microanalytical Laboratory, Tokyo Institute of Technology, at Nagatsuta.

Bis(2,4,6-tribromophenoxy) trichlorophosphorane (BTCP). 2,4,6-Tribromophenol (66.2 g, 0.2 mol) recrystalized from ethanol (0.5 ml/g) was rendered anhydrous by coevaporation with dry benzene and dissolved in dry benzene (300 ml). Commercially available phosphorus pentachloride (20.8 g, 0.1 mol) was heated at 50-60 °C under reduced presure to remove phosphorus oxychloride. The benzene solution was added and the mixture was refluxed for 90 min. Removal of the solvent in vacuo and recrystallization from dry benzene (550 ml) gave 19.6 g (24%, 94% purity) of BTCP: mp, 110-120 °C; ^{31}P NMR (pyridine) -57.98 ppm.

Anal. Calcd for $C_{12}H_4O_2Br_6Cl_3P$: C, 18.09; H, 0.51; Cl, 13.35; Br, 60.10: Found C, 18.41; H, 0.51; Cl+Br, 72.62.

Bis(2,4,6-tribromophenyl) phosphorochloridate (TBP). Glacial acetic acid (855 µl, 15 mmol) was added dropwise to a suspension of BTCP (12 g, 15 mmol) in dry benzene (40 ml), and the mixture was refluxed for 30 min. After the solvent was removed in vacuo, the residue was coevaporated with dry benzene (20 ml) and recrystallized from acetonitrile (300 ml) to give 8.9 g (80%) of TBP. mp 150 °C; ^{31}P NMR (pyridine) -9.35 ppm.

Anal. Calcd for $C_{12}H_4Br_6ClO_3P$: C, 19.42; H, 0.54; Cl, 4.87; Br, 64.61: Found; C, 19.43; H, 0.57; Cl+Br, 70.95.

General Procedure for Condensation. A mixture of a phosphodiester, a hydroxyl component, and 3-nitro-1,2,4-triazole was rendered anhydrous by repeated coevaporation with dry pyridine (1 ml x 3) and finally dissolved in dry pyridine (1 ml/0.1 mmol of the hydroxyl component). A condensing reagent was added to the solution and the mixture

was stirred at room temperature. The reaction was monitored by silica gel thin layer chromatography. When the hyroxyl component disappeared on TLC or an appropriate time was passed, the mixture was transferred into a separatory funnel with $CHCl_3$ (10 ml) and washed three times with water containing 5% $NaHCO_3$ (10 ml). The washings were combined and back-extracted with $CHCl_3$ (10 ml). The $CHCl_3$ extract was washed with 5% $NaHCO_3$ (10 ml) and mixed with the former organic extract. The combined organic extract was dried over anhydrous Na_2SO_4 and evaporated. After coevaporation with toluene to remove the last traces of pyridine, the desired product was purified by chromatography on a silica-gel (20 g) column with CH_2Cl_2-hexane (8:2 to 10:0, v/v). The amounts of the reagents and the yields of the products were described in the text.

Deprotection of the Tetramer (11). Fully protected tetramer (24 mg, 10 μmol) was dissolved in pyridine (1 ml) and cooled to 0 °C in an ice bath. To the solution, ice-cold 0.2 M NaOH (1 ml) was added and the mixture was stirred at 0 °C. After 30 min the mixture was subjected on a short column of Dowex 50 W x 2 (pyridinium form, 5 ml) and washed with pyridine-water (1:1, v/v, 20 ml). The washing was collected and evaporated. The resulting gum was dissolved in conc. NH_3 (15 ml) and the solution was stirred at room temperature for 1 h. The solvent and ammonia were removed under reduced pressure carefully. The redisue was dissolved in pyridine (1 ml) and iodine (76 mg, 0.6 mmol) was added to the solution followed by addition of water (0.5 ml) and then stirred at room temperature for 2 h. The mixture was concentrated, dissolved in water (5 ml) again, washed with benzene (20 ml) and ether (10 ml x 2), and coevaporated several times with ethanol. The resulting material was treated with 80% acetic acid (20 ml) at room temperature for 15 min. The solution was evaporated and coevaporated with water repeatedly. The oil containing the deprotected tetramer was dissolved in 0.5 M triethylammonium

bicarbonate (TEAB) (pH 7.0, 10 ml) and washed with ether (20 ml x 3) and evaporated again. Finally purification by column chromatography was performed using Sephadex A 25 (preequilibrated in 0.05 M TEAB, 1.5 x 50 cm) eluted with 0.05 to 2.0 M TEAB. The tetramer (88 A_{260} OD units) was obtained in 28% yield as a white powder after lyophilization. The compound was identified by enzymatic digestion with snake venom phosphodiesterase.

REFFERENCES

1) N. D. Sinha, J. Biernat, J. McManus, and H. Köster, Nucleic Acids Res., 12, 4539 (1984).
2) L. J. McBride, R. Kierzek, S. L. Beaucage, and M. H. Caruthers, J. Am. Chem. Soc., 108, 2040 (1986).
3) R. L. Letsinger, K. K. Ogilvie, ibid., 89, 4801 (1967).
4) A. K. Seth, E. Jay, Nucleic Acids Res., 22, 5445 (1980).
5) J. Stawinski, T. Hozumi, and S. A. Narang, Can. J. Chem., 54, 670 (1976).
6) C. B. Reese, R. C. Titmas, and L. Yau, Tetrahedron Lett., 19, 2727 (1978).
7) V. A. Efimov, S. V. Reverdatto, and O. G. Chakhamakhcheva, ibid., 23, 961 (1982).
8) Idem., Nucleic Acids Res., 10, 6675 (1982).
9) T. Wakabayashi and S. Tachibana, Chem. Pharm. Bull., 30, 3951 (1982).
10) J. Stawinski, T. Hozumi, S. A. Narang, C. P. Bahl, and R. Wu, Nucleic Acid Res., 6, 1371 (1979).
11) C. B. Reese, R. C. Titmas, and L. Yau, Tetrahedron Lett., 19, 2727 (1978).
12) E. M. Ivanova, L. M. Kalimskaya, U. P. Romanenko, V. F. Zarytova, ibid., 23, 5447 (1983).
13) V. F. Zarytova, D. G. Knorre, Nucleic Acids Res., 12, 2091 (1984).
14) S. Chandrasegaran, A. Murakami, L. Kan, J. Org. Chem., 49, 4951 (1984).
15) J. Matsuzaki, H. Hotoda, M. Sekine, and T. Hata, Tetrahedron Lett., 25, 4019 (1984).

16) R. Anschütz, Ann., 454, 106 (1927).
17) E. S. Kozlov, R. P. Kolesink, L. G. Dubenko, M. I. Povolotskii, Zh. Obsch., Khim., 49, 769 (1979).
18) M. Sekine, M. Fujii, H. Nagai, and T. Hata, Synthesis, 1119 (1987).
19) M. Sekine, J. Matsuzaki, and T. Hata, Tetrahedron Lett., 23, 5287 (1982).
20) M. Sekine, K. Hamaoki, T. Hata, Bull. Chem. Soc. Jpn., 54, 3815 (1981).
21) M. Sekine, J. Matsuzaki, T. Hata, Tetrahedron Lett., 22, 3209 (1981).
22) Idem., Tetrahedron, 41, 5279 (1985).
23) J. Matsuzaki, H. Hotoda, M. Sekine, and T. Hata, Tetrahedron Lett., 27, 5645 (1986).
24) P. J. Garegg, T. Regberg, J. Stawinski, and R. Strömberg, ibid., 27, 2665 (1986).

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