(AB-d, 2 H, $COOCH_2$), 4.5–4.7 (m, 2 H, OCH_2), 5.2–5.6 (m, 2 H, vinyl H), 5.9–6.3 (m, 1 H, vinyl H), 6.9–8.3 (m, 16 H, ArH).

Anal. Calcd for $C_{34}H_{32}O_5$: C, 78.44; H, 6.20. Found: 78.62; H, 6.13.

(*S*)-4-[(2-Methyl-1-butoxy)carbonyl]phenyl 4-[4-(4-Pentenyloxy)phenyl]benzoate (6). 4-[4-(4-Pentenyloxy)phenyl]-benzoic acid, 3 g (11 mmol), and (*S*)-2-methyl-1-butyl 4-hydroxybenzoate, 2.1 g (11 mmol), were used in the procedure described above. The product was recrystallized from ethanol to give 3.8 g (75%): mp 105 °C to smectic, 198 °C to isotropic; $[\alpha]^{25}_D$ +2.6° (*c* 0.3, chloroform); IR (KBr) 1725 cm^{-1}; NMR ($CDCl_3$) δ 0.9–1.1 (m, 6 H, CH_3), 1.1–2.4 (m, 7 H, CH, CH_2), 4.03 (t, 2 H, OCH_2), 4.17 (AB-d, 2 H, $COOCH_2$), 4.9–5.2 (m, 2 H, vinyl H), 5.6–6.1 (m, 1 H, vinyl H), 6.9–8.3 (m, 12 H, Ar H).

Anal. Calcd for $C_{30}H_{32}O_5$: C, 76.25; H, 6.83. Found: C, 76.54; H, 6.94.

(*S*)-4′-[(2-Methyl-1-butoxy)carbonyl]biphenyl-4-yl 4-[4-(4-Pentenyloxy)phenyl]benzoate (7). 4-[4-(4-Pentenyloxy)-phenyl]benzoic acid, 3 g (11 mmol), and (*S*)-2-methyl-1-butyl 4-(4-hydroxyphenyl)benzoate, 3 g (11 mmol) were used in the procedure described above. The product was recrystallized from ethanol to give 3.3 g: 57%; mp 135 °C to smectic, 295 °C to cholesteric, 315 °C to isotropic; $[\alpha]^{25}_D$ +2.7° (*c* 0.5, chloroform); IR (KBr) 1710, 1730 cm^{-1}; NMR ($CDCl_3$) δ 0.9–1.1 (m, 6 H, CH_3), 1.1–2.4 (m, 7 H, CH, CH_2), 4.04 (t, 2 H, OCH_2), 4.21 (AB-d, 2 H, $COOCH_2$), 4.9–5.2 (m, 2 H, vinyl H), 5.7–6.1 (m, 1 H, vinyl H), 6.9–8.3 (m, 16 H, Ar H).

Anal. Calcd for $C_{36}H_{36}O_5$: C, 78.81; H, 6.61. Found: C, 78.70; H, 6.55.

General Procedure for the Preparation of Mesogenic Polymers. Poly[oxy(methylsilylene)] (Petrarch, 2250 ave molecular weight), 50 mg (0.83 mmol of SiH), and 0.83 mmol of olefin were combined and 2 mL of toluene was added. Chloroplatinic acid, 0.2 mL (0.1 M), in 2-propanol was added, and the solution was heated at 80 °C for 16 h. The polymers were obtained by precipitation with an equal volume of methanol. The products were purified by dissolving them in 1 mL of methylene chloride and precipitating with 2 mL of methanol. This process was repeated 2 times. The product was dried at 80 °C under vacuum for 24 h. The melting characteristics of the polymers are shown in Table I. A polymer was not obtained with monomer **5** because of its limited solubility in any common solvent. A copolymer containing equal amounts of **1** and **2** substituents (PMPS-1,2) was prepared as above from equal amounts of alkenes **1** and **2** and the appropriate amount of poly[oxy(methylsilylene)]. PMPS-1,2 had the following phase transitions: 130 °C glass to smectic, 219 °C smetic to nematic, and 235 °C nematic to isotropic.

Procedure for Testing Stationary-Phase PMPS-1,2. Untreated fused silica columns, 18 m by 0.3 mm i.d. (Hewlett-Packard, Avondale, PA), were statically coated with a 0.25 μm film of SE-54 (a 5% phenyl, 94% methyl, 1% vinyl silicone) or with PMPS-1,2 as was previously described.[16] The SE-54 phase was cross-linked with azo-*tert*-butane.[17] Both columns were conditioned overnight at 280 °C under a nitrogen flow. A Hewlett-Packard Model 5880 gas chromatograph equipped with a flame ionization detector and a flame photometric detector was used. Hydrogen gas at 100 cm s^{-1} was used as the carrier gas. The solute standards were obtained commercially or were synthesized. Figure 3 shows the chromatograms for one specific separation.

Acknowledgment. This work was supported by National Science Foundation Grant No. CHE-8314769.

Registry No. 1, 93001-02-6; 2, 93001-03-7; 3, 93001-04-8; 4, 93039-02-2; 5, 93001-05-9; 6, 93001-06-0; 7, 93001-07-1; 8, 93001-08-2; 9, 58574-03-1; 10, 93001-09-3; 11, 91577-91-2; allyl bromide, 106-95-6; 4-[4-(4-pentenyloxy)phenyl]benzoic acid, 93001-10-6; 5-bromo-1-pentene, 1119-51-3; (*S*)-2-methyl-1-butanol, 1565-80-6; 4-hydroxybenzoic acid, 99-96-7; 4-methoxyphenol, 150-76-5; 4-(4-methoxyphenyl)phenol, 16881-71-3; (*S*)-2-methyl-1-butyl 4-(4-hydroxyphenyl)benzoate, 91577-91-2; 3-methyldibenzothiophene, 16587-52-3; 1-methyldibenzothiophene, 31317-07-4; 2-methyldibenzothiophene, 20928-02-3; 8-methylnaphtho[1,2-*b*]thiophene, 93001-11-7.

(16) Kuei, J. C.; Shelton, J. I.; Castle, L. W.; Kong, R. C.; Richter, B. E.; Bradshaw, J. S.; Lee, M. L. *HRC CC, J. High Resolut. Chromatogr. Chromatogr. Commun.* **1984**, *7*, 13.

(17) Richter, B. E.; Kuei, J. C.; Park, N. J.; Crowley, J. J.; Bradshaw, J. S.; Lee, M. L. *HRC CC, J. High Resolut. Chromatogr. Chromatogr. Commun.* **1983**, *6*, 371.

^{31}P NMR Study of the Mechanism of Activation and Coupling Reactions in the Synthesis of Oligodeoxyribonucleotides by the Phosphotriester Method

Srinivasan Chandrasegaran, Akira Murakami, and Lou-sing Kan*

Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205

Received June 7, 1984

The phosphotriester method provides a rapid and convenient procedure for synthesizing oligonucleotides. The mechanism has been revealed and intermediates have been identified by ^{31}P NMR methodology. It was found that reaction of a 5′-protected nucleoside 3′-(*p*-chlorophenyl phosphate) with mesitylenesulfonyl chloride (MSCl) or 1-(mesitylyl-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) in anhydrous pyridine yields only two products within 5 min, the sulfonic acid–phosphate mixed anhydride **2** and the (3′-3′) symmetrical pyrophosphate tetraester **3** which can be isolated as a mixture. Reaction of **2** and **3** with 3′-*O*-acetylthymidine yields the phosphotriester dimer $[(MeO)_2Tr]NpTOAc$. The reaction rate and yield of dimers are closely dependent on the presence of catalysts. The reaction finished within minutes when tetrazole or 3-nitro-1,2,4-triazole was used. On the contrary, the reaction completed in hours when imidazole or 1,2,4-triazole was used as catalysts. The possible mechanisms are explored and discussed in detail.

The phosphotriester method provides a rapid and convenient procedure for the synthesis of oligonucleotides.[1-4] An important step in the synthesis of oligonucleotides by the phosphotriester approach involves activation of a phosphodiester group with a suitable condensing agent and subsequent condensation with a nucleoside 5′-hydroxyl group to form a new internucleotide linkage. Arenesulfonyl

(1) Itakura, K.; Hirose, T.; Crea, R.; Riggs, A.; Heyneker, H. L.; Bolivar, F.; Boyer, H. W. *Science (Washington, D.C.)* **1977**, *198*, 1056–1063.

(2) Crea, R.; Kraszewski, A.; Hirose, T.; Itakura, K. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5765–5769.

(3) Edge, M. D.; Greene, A. R.; Heathcliffe, G. R.; Meacock, P. A.; Schuch, W.; Scalon, D. B.; Atkinson, T. C.; Newton, C. R.; Markham, A. F. *Nature (London)* **1981**, *292*, 756–762.

(4) Efimov, V. A.; Reverdatto, S. V.; Chakhmakhcheva, O. G. *Nucleic Acids Res.* **1982**, *10*, 6675–6694.

0022-3263/84/1949-4951$01.50/0

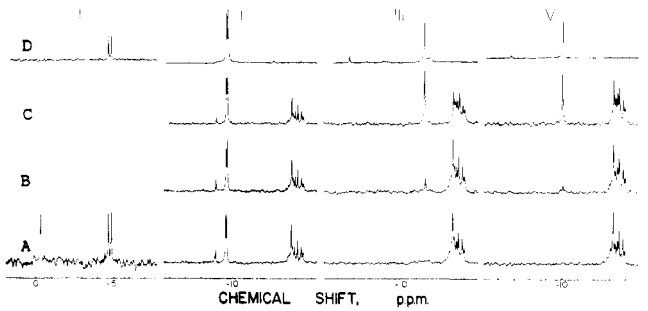


Figure 1. ^{31}P NMR spectra of **2**, **3**, and phosphorazolides. The resonance of ^{31}P of **1** (B = T) is marked as 0 ppm. The system of **2** and **3** plus imidazole (**4**) (column I), 1,2,4-triazole (**5**) (column II), tetrazole (**6**) (column III), and 3-nitro-1,2,4-triazole (**7**) (column IV). IA is the spectrum after 2 min. IIA, IIIA, and IVA are spectra after 10 min. IIB, IIIB, IVB are spectra after 70 min. IIC, IIIC, and IVC are spectra after 6, 20, and 22 h, respectively. Row D are the phosphorazolide spectra corresponding to **4**, **5**, **6**, and **7** from a different route as indicated in Scheme II.

chlorides, though very effective coupling reagents for synthesis by the phosphodiester approach,[5,6] have been shown to be less suitable for the phosphotriester approach.[7] The most suitable condensing agents with regard to short reaction times are arenesulfonyl tetrazoles introduced by Narang and co-workers[8] and arenesulfonyl 3-nitro-1,2,4-triazoles introduced later by Reese and co-workers.[9]

A clear understanding of the mechanism of the coupling reaction is essential for further development of improved condensing reagents. The efficiencies of several condensing reagents and problems of sulfonation were described by Seth and Jay.[10] They used TLC and UV spectroscopy to study the kinetics of phosphotriester formation and the rate of sulfonylation reactions. Based on these studies, they concluded the coupling reaction proceeds via formation of a very reactive phosphorazolide intermediate.

We have studied the intermediates formed during these coupling reactions using noise-decoupled ^{31}P NMR. These studies indicate reactions of a 5′-protected nucleoside 3′-(*p*-chlorophenyl phosphate) (**1**) with mesitylenesulfonyl chloride (MSCl) or 1-(mesitylyl-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) in anhydrous pyridine yield two stable products, the mesitylenesulfonic acid–phosphate mixed anhydride **2** and the (3′-3′) symmetrical pyrophosphate tetraester **3** (the ^{31}P NMR spectrum is provided as Figure 1 in the supplementary material). Recently, Ivanova et al.[11] and Zarytova and Knorre[12] reported that **3** is an intermediate in a similar experiment. Our studies suggest azoles such as imidazole (**4**) and 1,2,4-triazole (**5**) react with **2** and **3** readily to yield the phosphorazolide intermediates instantly (Figure 1). On the other hand, the phosphorazolides were formed at a very slow rate by treating **2** and **3** with azoles such as tetrazole (**6**) and 3-nitro-1,2,4-triazole (**7**) (Figure 1). Namely, less than 5% of this intermediate is formed in an hour, which is usually the time required for a condensation reaction to proceed to completion. However, the rate and yield of the formation of dimer (the target product) is just the reverse in proportion to how fast and how high the yield was of the formation of phosphorazolide. Namely, the dimers formed fast with high yield when **2** (and **3**) and 3′-*O*-acetyl nucleotide were treated with **6** or **7** and no phosphorazolide was observed initially. These results suggest that the phosphorazolides are relatively stable and reluctant to proceed to form dimers. On the other hand, compounds **2** and **3** are the intermediates and play an important role in oligodeoxyribonucleotide synthesis. All these reactions can be monitored closely to ^{31}P NMR spectroscopy.

Results and Discussion

I. ^{31}P NMR Study of the Intermediates Formed in the Phosphotriester Reaction. (a) Formation of Mesitylenesulfonic Acid–Nucleoside 3′-Phosphate Mixed Anhydride 2 and (3′-3′) Symmetrical Pyrophosphate Tetraester 3 Intermediates. Reaction of 5′-*O*-(dimethoxytrityl)thymidine 3′-(*p*-chlorophenyl phosphate) (**1**, B = T) with 1-(mesitylyl-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) in anhydrous pyridine results in the rapid disappearance of the starting diester ^{31}P signal (assigned as 0 ppm) and the subsequent appearance of six new ^{31}P resonances (Figure 1b, supplementary material). These new resonances are observed approximately 30 s after mixing, which is the time required to record the NMR spectrum. Two of the resonances (−12.96 and −13.31 ppm) are assigned to the mesitylenesulfonic acid–nucleoside 3′-phosphate mixed anhydride **2**, while the remaining four resonances (−13.12, −13.25, −13.57, −13.70 ppm) are assigned to the (3′-3′) symmetrical pyrophosphate tetraester **3**. Similar results were observed when **1** (B = T) is reacted with mesitylenesulfonyl chloride (MSCl) and 1-(mesitylyl-2-sulfonyl)tetrazole (MSTe).

When 3 equiv of MSNT are used in the reaction, the ratio of **2** to **3** is 2:1. When 0.5 equiv of MSNT are used, the ratio of **2** to **3** changes to 1:1. This result suggests nucleoside phosphate **1** initially reacts with MSNT to form **2**. Subsequent reaction of **2** with another molecule of **1** results in formation of **3**. The proposed reaction pathway is shown in Scheme I.

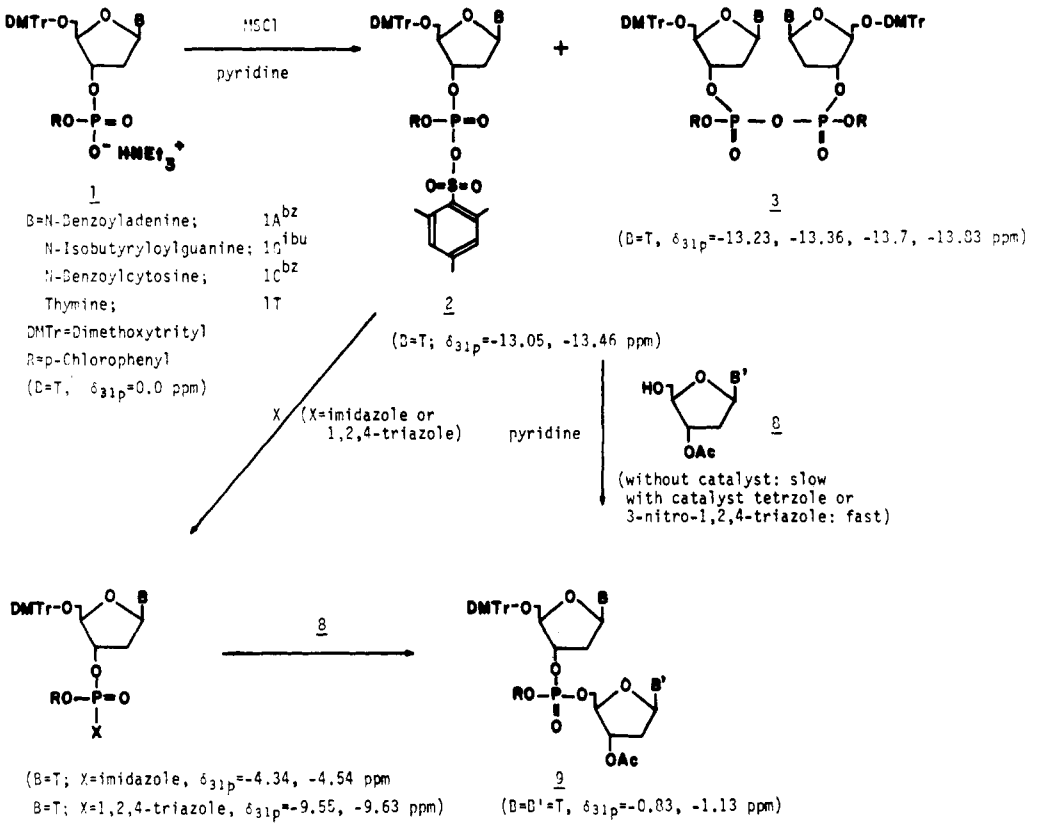
The identity of **3** was confirmed by preparation of **3** by an independent route. As shown in Scheme II, reaction of a 5′-*O*-dimethoxytrityl nucleoside with *p*-chlorophenyl phosphorodichloridate (δ of ^{31}P: 8.48 ppm) yields the 5′-*O*-dimethoxytrityl nucleoside 3′-(*p*-chlorophenyl phosphoromonochloridate), $[(MeO)_2Tr]NpCl$. Two phosphorus resonances are observed at 4.39 and 4.01 ppm which correspond to the two diastereoisomers of $[(MeO)_2Tr]NpCl$. Reaction of $[(MeO)_2Tr]NpCl$ with **1** yields **3**. The ^{31}P NMR spectrum of this compound consists of four lines (−13.23, −13.46, −13.70, and −13.83 ppm) whose chemical shift values correspond to those of intermediate **3** obtained by reaction of **1** with MSCl (Figure 2, supplementary material). Addition of mesitylenesulfonic acid dihydrate to **3** results in the instantaneous formation of **1**. This is seen by the disappearance of the four ^{31}P signals of **3**, with concurrent appearance of a new peak at 0 ppm which corresponds to the phosphorus resonance of **1** (Scheme II).

It is interesting to note that reaction of **1** with 1-(mesitylyl-2-sulfonyl)imidazole (MSI) or 1-(mesitylyl-2-sulfonyl)-1,2,4-triazole (MST) do not form **2** and **3** even after 24 h, according to the ^{31}P NMR. Only one signal at 0 ppm which corresponds to the phosphorus resonance of **1** was seen all the time.

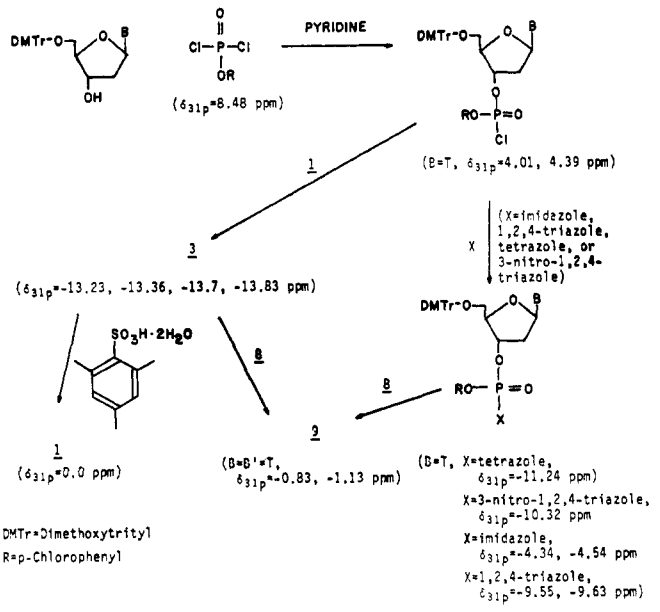
(b) Formation of 5′-*O*-Dimethoxytrityl Nucleoside 3′-(*p*-Chlorophenyl phosphorazolide) Intermediates. Reaction of **1** with MSNT (or MSTe) in pyridine led to

(5) Jacob, T. M.; Khorana, H. G. *J. Am. Chem. Soc.* **1964**, *86*, 1630–1635.
(6) Lohrmann, R.; Khorana, H. G. *J. Am. Chem. Soc.* **1966**, *88*, 829–833.
(7) Katagiri, N.; Itakura, K.; Narang, S. A. *J. Am. Chem. Soc.* **1975**, *97*, 7332–7337.
(8) Stawinski, J.; Hozumi, T.; Narang, S. A. *Can. J. Chem.* **1976**, *54*, 670–672.
(9) Reese, C. B.; Titmas, R. C.; Yau, L. *Tetrahedron Lett.* **1978**, 2727–2730.
(10) Seth, A. K.; Jay, E. *Nucleic Acids Res.* **1980**, *8*, 5445–5459.
(11) Ivanova, E. H.; Khalimskaya, L. M.; Romanenko, V. P.; Zarytova, V. F. *Tetrahedron Lett.* **1982**, *23*, 5447–5450.
(12) Zarytova, V. F.; Knorre, D. G. *Nucleic Acids Res.* **1984**, *12*, 2091–2110.

Scheme I



Scheme II



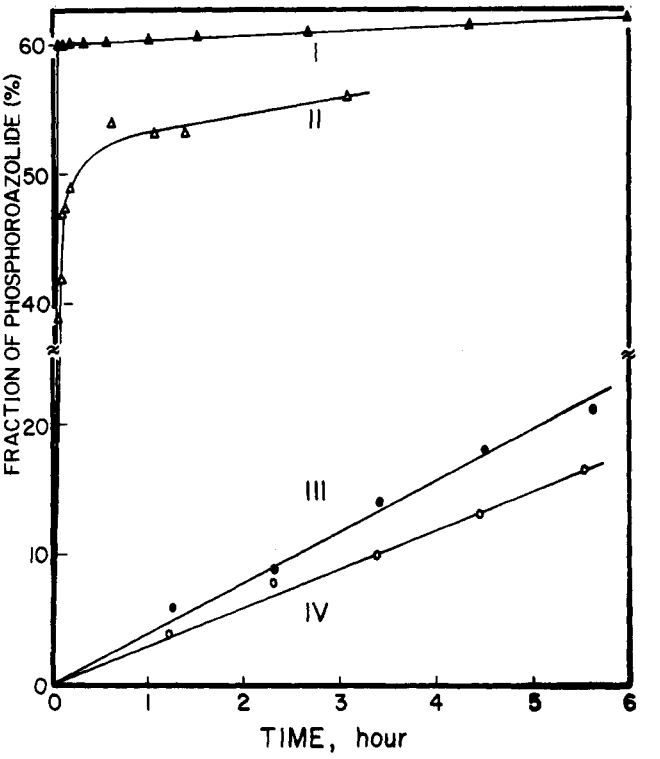


Figure 2. Rate of formation of the phosphorazolide by the reaction of **2** and **3** with azoles. Reaction of **2** and **3** with imidazole (**4**), 1,2,4-triazole (**5**), tetrazole (**6**), and 3-nitro-1,2,4-triazole (**7**) are shown by I, II, III, and IV, respectively.

the formation of intermediates **2** and **3**. No distinct nucleoside 3′-phosphorazolide adducts were found under the detection of ^{31}P NMR within 1 h. However, the intermediates **2** and **3** (prepared by reacting **1** with MSCl) can subsequently react with **4**, **5**, **6**, or **7** to form phosphorazolides. ^{31}P NMR was used to monitor the formation of nucleoside 3′-phosphorazolides as a function of time (Figure 2). The attack of **4** on **2** and **3** occurs very rapidly (within 2 min which is the time required to record the spectrum) to form nucleoside 3′-phosphorimidazolide. This is seen by the disappearance of the six resonances due to **2** and **3** and the appearance of two new signals (−4.34 and −4.54 ppm) in the ^{31}P NMR spectrum (Figure 1, IA; Figure 2, I). The identity of nucleoside 3′-phosphorimidazolide was confirmed by preparation through an independent route: reaction of 5′-protected nucleoside 3′-(*p*-chlorophenyl phosphoromonochloridate) with **4** or the reaction of 5′-protected nucleoside with *p*-chlorophenyl phosphorodiimidazolide yields the nucleoside 3′-phosphorimidazolide whose ^{31}P resonances occur at −4.33

and −4.54 ppm (Figure 1, ID), which have an identical chemical shift data as previously studied. The reaction of **5** with **2** and **3** produces nucleoside 3′-phosphoro-1,2,4-triazolide as evidenced by the appearance of two new signals (−9.55 and −9.63 ppm) in the ^{31}P NMR spectrum (Figure 1, IIA,B,C). This reaction occurs at a slower rate than that of **4** and can be monitored as a function of time (Figure 2, II). Reaction of 5′-protected nucleoside 3′-(*p*-chlorophenyl phosphoromonochloridate) with **5** or 5′-protected nucleoside with *p*-chlorophenyl phosphoroditriazolide yielded nucleoside 3′-phosphorotriazolide, whose ^{31}P NMR signals (−9.55 and −9.63 ppm) (Figure 2, IID) were exactly identical with those in Figure 1, II-A,B,C, thereby confirming the identity of nucleoside 3′-phosphoro-1,2,4-triazolide.

Unlike **4** or **5**, the attack of **6** and **7** on **2** and **3** in pyridine is extremely slow (Figure 2, III,IV). Even after an hour, less than 5% of nucleoside 3′-phosphorotetrazolide or nucleoside 3′-phosphoro-3-nitro-1,2,4-triazolide are formed. New resonances of these phosphorazolides appear at −11.24 ppm and −10.32 ppm, respectively, in each of their ^{31}P NMR spectrum (Figure 1 IIIA,B,C; IVA,B,C). Only single resonance signals were observed for both the nucleoside 3′-phosphorotetrazolide and nucleoside 3′-phosphoro-3-nitro-1,2,4-triazolide. The identity of these compounds was also confirmed by preparation of these by an independent route (Figure 1, IIID, IVD). Namely, azole adducts are formed when 5′-protected nucleoside 3′-(*p*-chlorophenyl phosphoromonochloridate) react with **6** and **7** or by reaction of 3′-protected nucleoside with *p*-chlorophenyl phosphorodiazolides. The ^{31}P resonances of these phosphorazolides occur at −11.24 and −10.32 ppm, respectively, thereby confirming the identity of nucleoside 3′-phosphorotetrazolide and nucleoside 3′-phosphoro-3-nitro-1,2,4-triazolide (Figure 1, IIID, IVD).

Accidental chemical shift equivalence of the diastereoisomers of these phosphorazolides could account for observing a single resonance in their ^{31}P NMR spectra. It is interesting to point out the fact that the chemical shift differences of the two diastereoisomers decreased in the following order: phosphorimidazolides ($\delta\Delta$ = 0.20 ppm) > phosphorotriazolides (0.08 ppm) > phosphoro-3-nitro-1,2,4-triazolide ≈ phosphorotetrazolides ($\delta\Delta \simeq$ 0.00 ppm).

In summary, Figure 2 shows the rate of the formation of phosphorazolides from **2** and **3** as a function of time with four different catalysts. This plot clearly shows that formation of phosphorazolide from **2** and **3** using **6** and **7** is extremely slow but rapid using **4** and **5**. The amount of phosphorotetrazolide linearly increases to 28% after 20 h, while that of phosphoro-3-nitro-1,2,4-triazolide to 22% after 22 h. This linear increase of phosphorotetrazolide and phosphoro-3-nitro-1,2,4-triazole with time suggest that the nucleophilic attack of **6** and **7** on **2** and **3** in pyridine is very slow and they are not close to completion after almost a day of reaction.

Thus, the rate of formation of phosphorazolide is directly related to the nucleophilicities of the catalysts. Both **4** and **5**, especially **4**, can serve as strong nucleophilic attack reagents in pyridine.[12] On the other hand, **6** and **7** are relatively poor nucleophilic reagents.[13,14] Thus, the formation of phosphorazolide with these reagents was slow.

(c) Formation of Dimer [(MeO)$_2$Tr]TpTOAc. Both the mixed anhydride **2** and the pyrophosphate **3** react with 3′-*O*-acetylthymidine (**8**, B′ = T) to yield the dinucleoside phosphotriester [(MeO)$_2$Tr]NpTOAc. Dimer formation

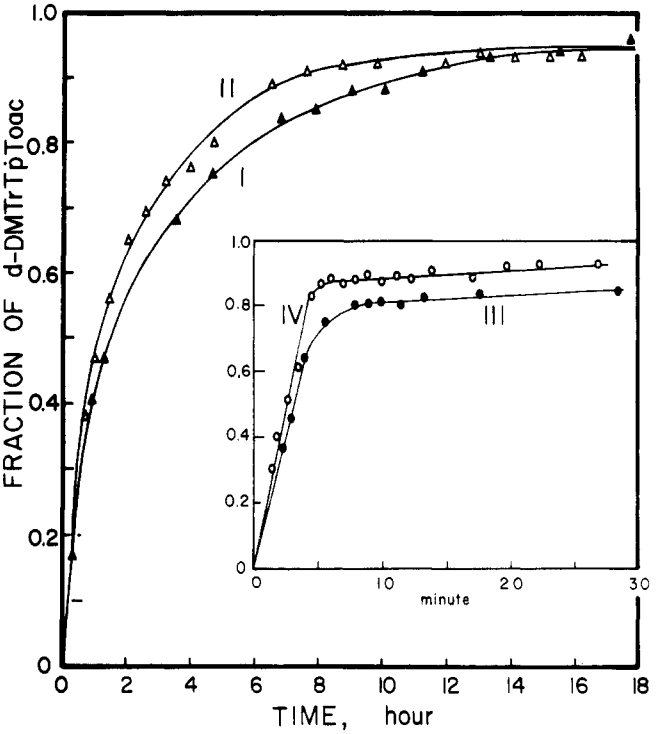


Figure 3. Rate of formation of the phosphotriester product [(MeO)$_2$Tr]TpTOAc by treating **2** and **3** with **8**. Curves I, II, III, and IV correspond to imidazole (**4**), 1,2,4-triazole (**5**), tetrazole (**6**), or 3-nitro-1,2,4-triazole (**7**), which were used as catalyst, respectively.

is very slow in the absence of any catalyst and is only 30% complete after 16 h. However, this reaction proceeds rapidly in the presence of either **6** or **7** and is complete within 10 min at 22 °C (Figure 3). But the reaction proceeds slowly in the presence of **4** and **5** and is about 80% complete after 6 h (Figure 3). Formation of dimer leads to appearance of two new resonances (−0.83, −1.13 ppm) in the ^{31}P NMR spectrum (Figure 1c, supplementary material). The rate of formation of dimer can be monitored by the increase in the intensity of these two signals and the decrease in the intensity of the signals of the phosphorazolides and **2** and **3**. Figure 3 clearly shows the results of percent dimer formed as a function of time. The rate of dimer formation follows the order: 3-nitro-1,2,4-triazole (**7**) > tetrazole (**6**) >>> 1,2,4-triazole (**5**) > imidazole (**4**).

Thus, these results state five facts: First, the initial intermediates in condensation reaction are **2** and **3**. Second, the phosphorazolides can be formed from **2** or **3** in general. However, the reaction rate and yield of the product are closely dependent on the reagent used. Phosphorimidazolide and phosphoro-1,2,4-triazolide were formed very rapidly with high yields, and on the contrary, the phosphorotetrazolide and phosphoro-3-nitro-1,2,4-triazolide were detected in small amounts after 1 h by treating **2** and **3** by **4**, **5**, **6**, and **7**, respectively. Third, the reaction of **2** and **3** with **8** is a nucleophilic reaction. Fourth, this nucleophilic reaction is facilitated by acid catalysts. A protonation may most likely occur on the P=O oxygen to generate a partial "plus" charge on the phosphorus atom and the 5′-OH function group attacks the phosphorus to form a dimer. Fifth, phosphorimidazolide and phosphoro-1,2,4-triazolide form dimer very slowly by reacting with **8**. In this case of using **4** or **5** as catalysts, stable phosphorazolide already formed from **2** and **3** through nucleophilic reaction. Thus, the 5′-OH group of **8** has to compete with either **4** or **5**. This may

(13) Katritsky, A. R.; Ed. "Physical Methods in Heterocyclic Chemistry"; Academic Press: New York, 1963; Vol. 1.
(14) Brown, E. J. *Aust. J. Chem.* **1980**, *22*, 2251.

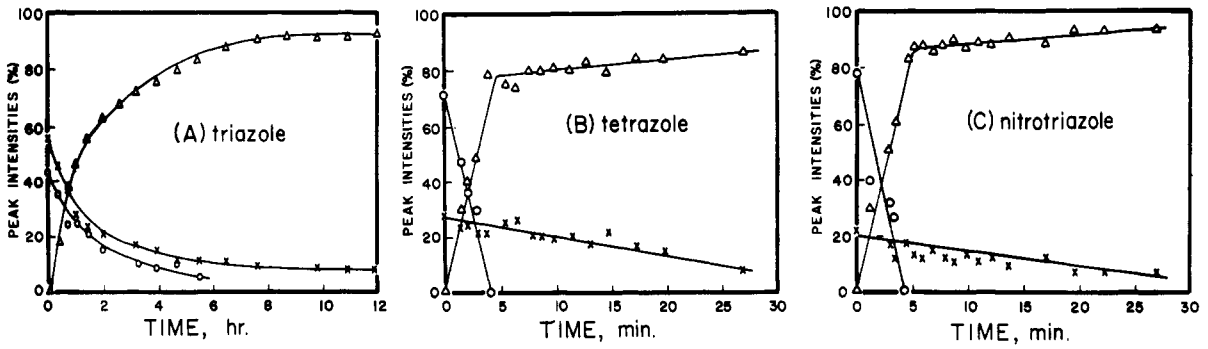


Figure 4. Rates of disappearance of the mixture of **2** and **3** (O) and the phosphorazolide (X) and the subsequent formation of [$(MeO)_2$Tr]TpTOAc (Δ) are shown by treating with azoles: (A) 1,2,4-triazole (**5**), (B) tetrazole (**6**), and (C) 3-nitro-1,2,4-triazole (**7**).

well explain the mechanism of dimer condensation reaction (Schemes I and II).

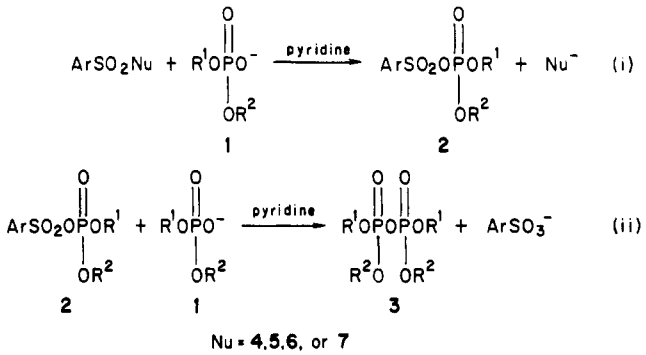
Since the chemical shifts of ^{31}P atom of all constituents in the condensation reaction have distinctly different values (Figure 1), the amount of each component in the mixture can be followed by ^{31}P NMR spectroscopy. Thus, we designed the following experiment to further support our findings. First, **1** reacts with MSCl to generate the intermediates **2** and **3** mixtures. Then this mixture was reacted with **4**, **5**, **6**, and **7** separately in different (but reasonable) time periods in order to generate sufficient amount of phosphorazolides. As shown in Figure 4, 29% phosphorotetrazolide, 22% phosphoro-3-nitro-1,2,4-triazolide, and 56% phosphoro-1,2,4-triazolide were formed after 20, 22, and 6 h by treating **2** and **3** with **6**, **7**, and **5**, respectively. These mixtures then were treated with **8**, and the ^{31}P signal in solution was closely monitored by NMR as a function of time. These results are shown in Figure 4: Plots of the disappearance of **2** and **3**; the disappearance of phosphorazolides; and the formation of dimer by detection of the ^{31}P signal against time. In the case of **5** (Figure 4A) the disappearance of **2** and **3** closely follows that of phosphoro-1,2,4-triazolide, suggesting that **2** and **3** are converted to the relatively stable phosphoro-1,2,4-triazolide before **8** is added then both react with 5′-hydroxy function of **8** at a similar rate to form dimer. However, in the cases of **6** (Figure 4B) and **7** (Figure 4C), **2** and **3** disappear very rapidly (within 5 min in both cases) which is a direct response to the formation of dimers (Figure 4B, 4C). The phosphorazolides on the other hand disappear rather slowly. Thus, in this case, the rate of the formation of dimer is dominated by the disappearance of **2** and **3**.

Without any acidic catalysts, **2** and **3** also convert to dimer at a slow rate as does phosphorazolide (Figure 4A). The reduction of **2** and **3** and phosphoro-1,2,4-triazolide are almost at equal rate and the coupling reaction close to completion is about 12 h (Figure 4A), which is much longer than the same reaction treated with either **6** or **7** (Figure 4B, 4C).

Thus, the previous observations confirmed the **2** and **3** are active with weak acidic catalysts such as **6** and **7**. Phosphorazolides have also formed but they convert to dimer slowly. The increased rate of the coupling reaction may be facilitated via protonation as previously described. This assumption can also be proved by NMR.

We observed that the addition of **6** to a mixture of **2** and **3** in anhydrous pyridine results in no change in the pattern of the ^{31}P resonances. The retention of the same pattern of the ^{31}P NMR spectrum indicates neither **2** nor **3** has reacted with **6**. However, the six resonances are shifted downfield by approximately 1.3 ppm. The downfield shift of the ^{31}P resonances could reflect decreased electron density around the phosphorus atoms of **2** and **3** resulting from hydrogen bonding with **6**. Furthermore, the C-H proton of tetrazole can be studied in pyridine-d_5 by proton NMR. The resonance signal of this proton (9.41 ppm) is shifted slightly downfield to 9.47 ppm when a mixture of **2** and **3** is added to the solution. This downfield shift may also result from hydrogen bond formation between **2** (and **3**) and **6**.

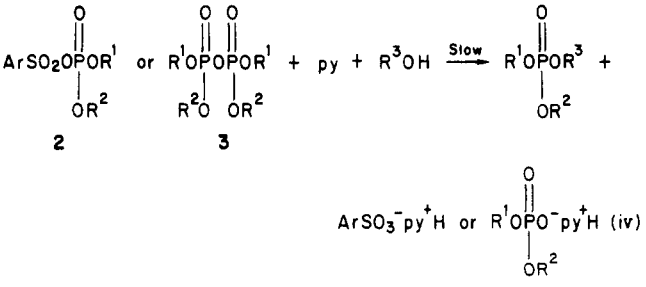
In conclusion, it is clear from our data (Figures 1–4) that phosphotriester condensation in pyridine using arenesulfonyl azolides proceeds via two distinct steps: (1) the intermediate formation (pyrophosphate and mixed anhydride); (2) the phosphotriester product formation. This was also observed by Zarytova and Knorre.[12] The first step of the process can be described as follows:



Azoles liberated in eq i probably participate in an equilibrium (eq iii). Based on our results (Figure 1), we

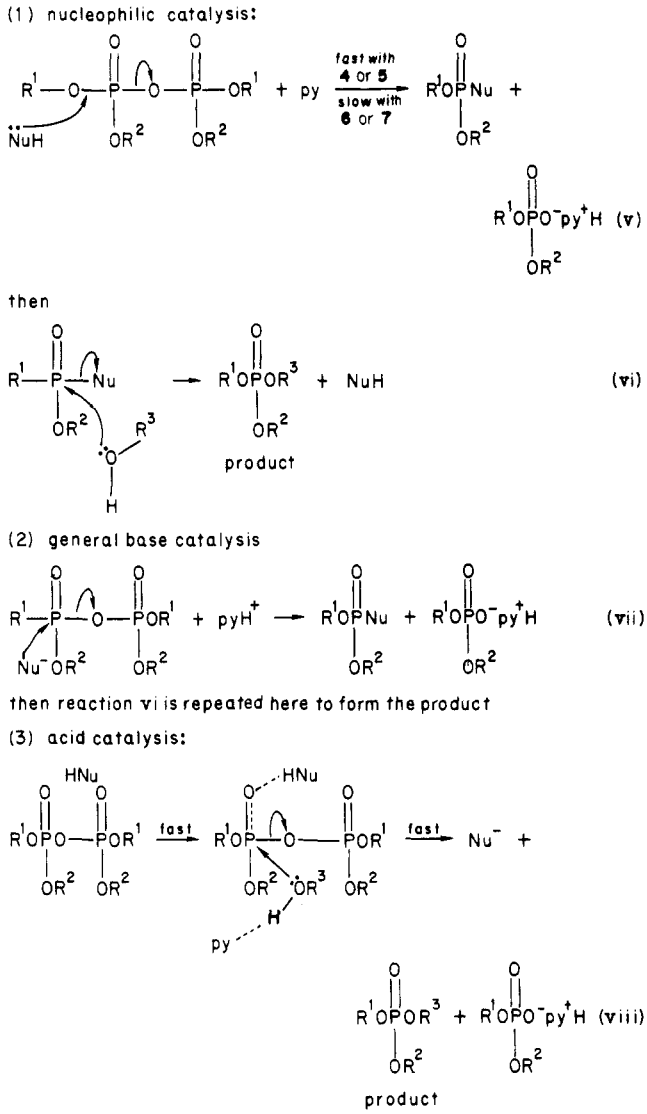
$$Nu^- + Py^+H \rightleftarrows NuH + py \qquad \text{(iii)}$$

propose the following: In the absence of azoles, intermediates **2** and **3** react very slowly with R^3OH to from the dideoxyribonucleoside *p*-chlorophenyl phosphotriester (eq iv). In the presence of azoles, especially **6** or **7**, the



phosphotriester formation is drastically accelerated (Figure 4). This clearly suggests some catalytic role for these azoles. Three different types of catalysis can be considered (Chart I). Identical schemes for the activation of mixed anhydride as that of pyrophosphate is applicable. We could detect phosphorazolides by ^{31}P NMR (Figure 1). With azoles such as **4** or **5**, the phosphorimidazolide and phosphorotriazolide formed (Figure 2). This suggests that

Chart I



with **4** or **5** the phosphotriester formation is dominated by nucleophilic or base-catalyzed reactions. However, reaction vi proceeds very slowly (Figure 3). On the other hand, formation of phosphorotetrazolide and phosphoronitrotriazolide are formed very slowly (<5% after 1 h in pyridine solution (Figures 1 and 2)). Furthermore, triethylamine does not catalyze the phosphotriester formation,[10] thereby suggesting that contribution through general base catalysis is small. It seems that eq viii is the most probable route for phosphotriester when tetrazole and 3-nitrotriazole are used (Figure 3). ^{31}P NMR studies clearly support this hypothesis. Thus, it is clear that the phosphotriester formation using arenesulfonyl azolides in pyridine proceed via different routes depending on the catalyst used.

II. Synthesis of Dideoxyribonucleotides via 2 and 3. (a) Isolation of the Intermediates 2 and 3. A mixture of the mixed anhydride **2** and the pyrophosphate **3** can be isolated as a solid after precipitation from pentane when MSCl is used in the activation reaction. The ^{31}P NMR spectrum of this precipitate is identical with the spectrum of the intermediate observed in situ. A solution of **2** and **3** in anhydrous pyridine is stable for several days. The precipitate of **2** and **3** may be stored for several months under anhydrous pentane. Attempts to isolate **2** and **3** by precipitation when MSNT is used in the activation reaction were not successful since **7** also precipitates from pentane. The presence of this contaminant leads to the decomposition of both **2** and **3**.

Table I. Stereoisomeric Yields of the Triester Product Using MSNT

base	MSNT (equiv)	stereoisomer,[a] % I	II
T	0.5	50	50
	3.0	67	33
C	3.0	68	32
A	3.0	60	40
G	3.0	50	50

[a] Yields were calculated from ^{31}P NMR spectra.

(b) Synthesis of Dideoxyribonucleotides Using 2 and 3. The same phosphate intermediates are obtained when the four protected nucleoside 3′-(*p*-chlorophenyl phosphates) (**1**, B = A^{bz}, C^{bz}, G^{ibu}, and T) react with MSCl. These intermediates react rapidly with **8** (B = T) in the presence of **6** and **7** to yield the corresponding phosphotriester dimer [$(MeO)_2$Tr]NpTOAc (**9**) (N = A^{bz}, C^{bz}, G^{ibu}, and T).

The mixture of **2** and **3** serves as a useful reagent for the syntheses of oligonucleotides. As described above, these compounds may be isolated by precipitation free of contaminating MSCl. Although **2** and **3** are very sensitive to acid and moisture, they may be stored in solid form in dry pentane under dry argon atmosphere. These intermediates have been used in conjunction with **7** to prepare dideoxyribonucleotides on an insoluble polystyrene support. The syntheses include the following steps: (i) removal of the dimethoxytrityl group, (ii) reaction of the liberated 5′-hydroxyl group with a mixture of **2** and **3** in the presence of **7** to yield the phosphotriester product, and (iii) capping of any unreacted hydroxyl groups with acetic anhydride.

These steps are repeated using **2** and **3** with various nucleosides until the desired sequence is assembled. Step iii is not required for the synthesis of dideoxyribonucleotides but is essential for the synthesis of longer oligonucleotides. It is possible to recover unreacted **2** and **3** which can then be converted to 5′-protected nucleoside 3′-(*p*-chlorophenyl phosphate) by reaction with aqueous mesitylenesulfonic acid. The phosphate can be purified by liquid chromatography and recycled to prepare **2** and **3**.

The progress of activated coupling reactions of four different deoxynucleoside 3′-phosphates using MSNT as determined by observing the intensities of the dimer ^{31}P NMR signals vs. time are shown in Figure 3 of the supplementary material. The coupling reactions proceed to completion within 10–15 min. Two distinct ^{31}P signals corresponding to the diastereoisomers of [$(MeO)_2$Tr]-NpTOAc are seen. As shown in Table I, the ratio of the diastereoisomers varies slightly depending upon the base. The amount of MSNT used to activate the 5′-protected nucleoside 3′-phosphate also slightly affects the ratio of stereoisomers formed. However, these different ratios of diastereoisomers have no effect on the final yield of the products. Extra ^{31}P resonances other than those due to products were observed in the case of the purine nucleotides. This suggests purine nucleotides are more susceptible to formation of side products than are pyrimidine nucleotides during the coupling reactions. No attempt was made to identify these side products since they were formed in only small amounts (≤10%).

The yields obtained from coupling reactions performed in the presence and absence of **7** are summarized in Table II. As expected, in the absence of **7** the yields of the dimers are very low. Yields equal to or better than those obtained when MSNT is used as a condensing agent in the

Table II. Comparison of the Yields of Dimers Using the Isolated Intermediates 2 and 3 with and without 3-Nitro-1,2,4-triazole (7)

compd	t_R,[a] min	yields, % (+)-7	(−)-7
d-TpT	10.2	92	40
d-CpT	8.7	98	20
d-ApT	10.8	99	37
d-GpT	9.5	87	24

[a] Retention time of the deprotected dimers on the reversed-phase HPLC (see text for details).

traditional phosphotriester approach are observed when 7 is added to the coupling reaction. Because unreacted MSNT is not present in the coupling reaction, sulfonylation of support-bound nucleoside 5′-OH groups will not occur.[15] In addition, potential adduct formation between guanine and 7[16] should be prevented by using this procedure.

Experimental Section

Materials. The protected nucleosides and 5′-protected nucleoside 3′-(*p*-chlorophenyl cyanoethyl phosphates) were obtained from ChemGene Chemical Company. Pyridine-d_5 (Cambridge Isotope Laboratories) was dried over calcium hydride overnight. Dioxane was distilled from calcium hydride and stored over calcium hydride. Mesitylenesulfonyl chloride (MSCl), *p*-chlorophenyl phosphorodichloridate, tetrazole (6), and 3-nitro-1,2,4-triazole (7) were purchased from Aldrich Chemical Company. MSCl was recrystallized from pentane and *p*-chlorophenyl phosphorodichloridate was distilled before use. All chemical manipulations were carried out under an atmosphere of dry argon. MSNT, MST, MSTe, and MSI were synthesized according to procedures reported in the literature.[8,9]

NMR Spectroscopy. Both ^{1}H and noise-decoupled ^{31}P NMR spectra were recorded on a Bruker WM-300 wide bore NMR spectrometer located in the JHU-Baltimore Biomedical NMR Facility Center. The NMR tubes (5 mm) were flushed with dry argon before use. Anhydrous pyridine-d_5 was used as the solvent and its most upfield deuterium resonance was used as a lock signal. The signal of phosphorus in the 5′-protected nucleoside 3′-(*p*-chlorophenyl phosphate) was designated as 0 ppm. The amount of each species was determined by integration of the corresponding phosphorus signal (with an error of ±5%). The reaction starts at the mixing of reagents in the NMR tube. This sample tube then rapidly put in the pretuned magnet and for detecting ^{31}P signals. About 20 s is needed for this procedure. The time of sampling is dependent on the reaction rate: ranging from few accumulations for fast reactions or several hundreds accumulations for slow reaction. All data were stored in a Diablo-31 single platter hard disk for later analysis.

General Procedure for the Preparation of 5′-Protected Nucleoside 3′-(*p*-Chlorophenyl phosphate) Triethylammonium Salts. The 5′-protected nucleoside 3′-(*p*-chlorophenyl cyanoethyl phosphate) [(MeO)$_2$Tr]NpCE (1 mmol), was dissolved in pyridine (17 mL). The solution was treated with triethylamine (6 mL) and water (6 mL) and then stirred overnight at room temperature. The solution was evaporated to dryness by using a vacuum pump. The residue was dissolved in methylene chloride (50 mL) which was then extracted with triethylammonium bicarbonate (3 × 50 mL). The organic layer was dried over anhydrous sodium sulfate and filtered, and the filtrate was concentrated to 5 mL on a water aspirator. This solution was added dropwise to 300 mL of pentane with constant stirring. The resulting precipitate was filtered immediately by using a Buchner funnel. The solid was initially dried under vacuum for 2 h and then dried in the presence of P_2O_5 by using a vacuum pump.

General Procedure for Isolation of Intermediates 2 and 3. The 5′-protected nucleoside 3′-(*p*-chlorophenyl phosphate) (40 μmol) was dissolved in 0.15 mL of THF and the solution was added to MSCl (60 μmol) in the presence of 0.05 mL of collidine in a 1-mL vial fitted with a serum cap. The activation reaction was allowed to proceed for 15 min at room temperature. The precipitated collidine hydrochloride was centrifuged and the supernatant was removed with a 1-mL syringe. The supernatant was added to dry pentane which contained 0.05 mL of collidine. The excess MSCl is soluble in pentane. The pentane was removed and the precipitate was dried under vacuum for several minutes. The precipitate is very sensitive to acid and moisture. The precipitate can be stored for long periods under dry argon in dry pentane without noticeable decomposition. The ^{31}P NMR (40 MHz) spectrum of the precipitate was identical with that reported by Ivanova et al.[11] (data not shown).

Synthesis of Dideoxyribonucleotides on a Polystyrene Support. Polystyrene esterified with 5′-*O*-(dimethoxytrityl)-3′-*O*-succinylthymidine (10 mg, 0.7 μmol) was treated with 1 M $ZnBr_2$ (dissolved in 2-propanol–methylene chloride (15:85 v/v) (3 × 2 mL)) for 5 min and washed with 2-propanol–methylene chloride (15:85 v/v, 6 × 2 mL), 0.1 M triethylammonium acetate in DMF (3 × 2 mL), pyridine (6 × 2 mL), and finally with ether (6 × 2 mL). The support was coevaporated with pyridine in the presence of P_2O_5. The support was treated with a mixture of intermediates 2 and 3 (20 equiv, 14 μmol) in 20 μL of anhydrous pyridine. A solution of the catalyst, 3-nitro-1,2,4-triazole (42 μmol) (7), dissolved in pyridine (20 μL) was then added to the support. Heating is required to make 7 dissolve in pyridine. The reaction was carried out for 2 h at room temperature. The support was then washed with pyridine (6 × 2 mL) and ether (6 × 2 mL).

The cleavage of the dideoxyribonucleotides from the support and the removal of the protecting groups were carried out by sequential treatment of the support with 50% NH_4OH/pyridine at room temperature overnight and then at 65 °C for 1 h followed by treatment with 80% acetic acid for 30 min.[17] The solution was evaporated to dryness, dissolved in 50% ethanol, and analyzed by HPLC on an analytical C-18 reversed phase column. The yields obtained are shown in Table II.

Acknowledgment. We are indebted to Professor Paul O. P. Ts'o, Dr. P. S. Miller, and Dr. M. P. Reddy for their valuable suggestions and discussion and to the JHU–Baltimore Biomedical NMR Facility Center (established by NIH Grant GM 27512) where all NMR spectra were run. This work was supported by NCI Grant CA 27111.

Supplementary Material Available: The spectral data for the formation of compounds 2 and 3 by two different routes (Figures 1 and 2); the rate of formation of dimers in the coupling reaction of four different deoxyribonucleotides using MSNT as monitered by the ^{31}P NMR signal (Figure 3) (3 pages). Ordering information is given on any current masthead page.

(15) Gait, M. J.; Popov, S. G. *Tetrahedron Lett.* **1980**, *21*, 2841–2842.
(16) Reese, C. B.; Ubasawa, A. *Nucleic Acids Res.* **1980**, Symp. Ser. No. 7, 5–21.
(17) Miller, P. S.; Cheng, D. M.; Dreon, H.; Jayaraman, K.; Kan, L.-S.; Leutzinger, E. E.; Pulford, S. M.; Ts'o, P. O. P. *Biochemistry* **1980**, *19*, 4688–4698.