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Proton-Block Strategy for the Synthesis of Oligodeoxynucleotides without Base Protection, Capping Reaction, and P−N Bond Cleavage Reaction

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Received February 17, 2003

A new *N*-unprotected phosphoramidite method called the "proton-block" approach was developed for the chemical synthesis of oligodeoxynucleotides based on the hitherto simplest and rational principle of acid−base reactions. This concept involves protection of the nucleobases of deoxycytidine and deoxyadenosine with "protons" to convert them to unreactive protonated bases during condensation by use of promoters having pK_a values lower than 2.8. This strategy was applied to the synthesis of d[CpT] and d[ApT] to check the side reactions associated with the base residues. In this "proton-block" method, 5-nitrobenzimidazolium triflate (NBT) was found to be the best promoter, and THF was superior to CH_3CN as the solvent so that the concomitant detritylation due to the inherent acidity of the promoter could be greatly suppressed. Application of this strategy to the solid-phase synthesis gave d[CpT], d[ApT], d[ApA], d[CpC], and d[GpT] as almost single peaks in HPLC analysis. Similarly, d[ApApApT] and d[CpCpCpT] were successfully synthesized without significant side reactions. Finally, d[CpCpCpCpCpCpT] and d[ApApApApApApT] were obtained as the main products. In the case of a longer oligomer, d[CpApGpTpCpApGpTpCpApGpT], a mixed solvent of CH_3CN−*N*-methylpyrrolidone (1:1, v/v) was superior to THF so that the desired oligodeoxynucleotide could be isolated in a satisfactory yield. These results suggest that DNA synthesis can be carried out simply by using the protonated bases at the oligomer level not only without base protection but also without the capping reaction and the posttreatment of branched chains with MeOH−benzimidazolium triflate that previously was requisite. It is concluded that most of the reactions and solvent effects involved in this strategy can be explained in terms of simple acid−base reactions. Some problems associated with the previous posttreatment are also discussed with our own results.

Introduction

Forty-seven years have already passed since Michelson and Todd succeeded in synthesizing a TpT dimer by a chemical approach.[1] After that, Khorana developed the phosphodiester method by use of DCC and 2,4,6-triisopropylbenzenesulfonyl chloride (TPS).[2] In 1967, Letsinger reported the phosphotriester approach[3] and later the phosphite approach.[4] Ultimately, Beaucage and Caruthers devised and developed extensively a practical method called the phosphoramidite approach.[5] Garreg also proposed the *H*-phosphonate approach.[6] Matteucci established this approach using pivaloyl chloride as the condensing reagent.[7] These basic studies have enabled us to synthesize sufficiently pure oligodeoxynucleotides so that custom-made supplies are now available for laboratory use of DNA fragments. However, the principle of these approaches has been based on the use of appropriate protecting groups on the base residues having amino groups.

Adamiak reported the synthesis of an RNA heptamer, CpCpCp^{t6}ApA, of an initiator tRNA anticodon loop in a synthetic strategy without using the protecting group of the adenine residue in the phosphotriester method.[8] Letsinger showed at an early stage of development of the

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10.1021/jo034204k CCC: $25.00
Published on Web 06/13/2003

phosphite approach that *N*-unprotected deoxyguanosine and deoxyadenosine building blocks could be used without protection of their amino groups.[9] Several papers have also reported the synthesis of short (2mer−4mer) RNA oligomers by use of unprotected adenosine derivatives.[10] Actually, later, Gryaznov and Letsinger first demonstrated the DNA synthesis without using base-protecting groups and showed insight into the generality of this possibility.[11] After this breakthrough study, Jones reported the synthesis of a DNA oligomer by the *H*-phosphonate method without using base-protecting groups.[12] Later, in 1997 we also showed a successful synthesis of a DNA 12mer as the main product without base protection by use of the *H*-phosphonate approach in combination with a phosphonium-type of condensing reagent.[13] In this *N*-unprotected *H*-phosphonate approach, we made strenuous efforts to realize selective internucleotidic bond formation to avoid competitive phosphitylation on the base residue, especially from a mechanistic point of view based on HOMO-LUMO interaction between the activated phosphorus intermediates and alcohols. Consequently, it was found that the use of 2-(benzotriazol-1-yloxy)-1,1-dimethyl-2-pyrrolidin-1-yl-1,3,2-diazaphospholidinium hexafluorophosphate (BOMP) resulted in rapid and selective internucleotide bond formation.[13a] However, we also disclosed that the base-unprotected *H*-phosphonate method was accompanied by a side reaction due to intramolecular attack of the 5′-hydroxyl group of a 5′-terminal deoxynucleoside on the 3′-phosphate group to produce one base-lacking oligodeoxynucleotide at every step of the condensation.[13d] Our further efforts to improve this approach failed to eliminate this serious side reaction. A year later, Hayakawa reported a practical procedure of DNA fragments based on the phosphoramidite method without base protection by use of an effective promoter, imidazolium triflate (IMT), for activation of deoxynucleoside-3′-phosphoramidite building blocks on Tenta gel supports.[14a] Hayakawa's method seemed to reach a certain goal in the synthesis of DNA oligomers without base protection[14] but still required extensive workup of the simultaneously formed branched chains by treatment with a more powerful promoter, benzimidazolium triflate (BIT),[15] in methanol at the end of each elongation cycle.[14a]

In our own studies, however, application of their method to the solid-phase synthesis of DNA fragments failed when more widely accessible highly cross-linked polystyrene[16] (HCP) or CPG gel[17] was employed as the polymer support by use of both manual procedures and automated synthesizers, as discussed in the text.

In this paper, we wish to report a new and simple principle designed for the DNA synthesis based on an original approach called the "proton-block" approach that allowed elimination of not only base-protecting groups but also the capping reaction with an acylating reagent and posttreatment with MeOH throughout the protocol. To our best knowledge, this simple "proton-block" method has not been realized to date.[18] The aim of our present study is to synthesize directly DNA fragments without pre- and posttreatment of the base amino function, namely, to find 100% selective internucleotidic bond formation from the beginning. We believe such basic studies without resort to stereotypical concepts would be needed for further development of new methods for the synthesis of artificial DNA molecules having functional groups involving base-labile groups. In this paper, we report the details of our basic studies on the proton-block method directed toward this end.

Results and Discussion

Side Reaction during the Posttreatment with MeOH−BIT. Since the method described by Hayakawa and Kataoka[15a] seemed to be the best in the phosphoramidite approach without base protection, we extensively studied it to clarify the reason a similar solid-phase synthesis by us using the above-mentioned MeOH−BIT system and HCP resin failed. From these studies, it was finally concluded that our unexpected result was attributed to the ester exchange of internucleotidic phosphite intermediates during the posttreatment with MeOH−BIT at every step.[15a]

This side reaction was disclosed as follows: When a trivalent species of DMTrTp$^{(III)}$(OCE)T synthesized on an HCP resin was treated with a 0.5 M solution of BIT in MeOH at room temperature for 2 min,[15a] surprisingly, the oxidation of the resulting mixture with I_2 followed by the successive treatments with 3% trichloroacetic acid and ammonia resulted in a mixture of T and TpT in a 1:2 peak ratio in HPLC, as shown in Figure 2B. This result suggested that the internucleotidic bond was cleaved to a degree of ca. 50%. Contrary to this result, the usual workup of the same resin without using the posttreatment gave an almost single peak of TpT in HPLC, as shown in Figure 2A.

These results implied that serious internucleotidic bond cleavage apparently occurred during the posttreatment, as evidenced by considerable appearance of thymidine at 3 min. It turned out that the phosphite intermediate underwent easy transesterification with

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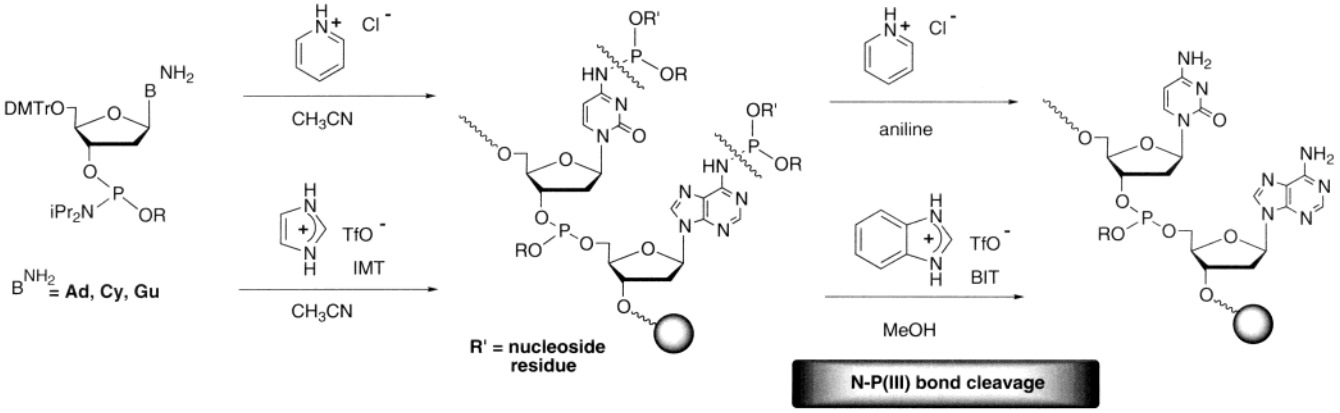


FIGURE 1. Previous *N*-unprotected phosphoramidite approaches reported by Letsinger[11] and Hayakawa.[15a] These methods require posttreatment of branched DNAs on the resin with pyridinium hydrochloride and benzimidazolium triflate in the presence of nucleophiles.

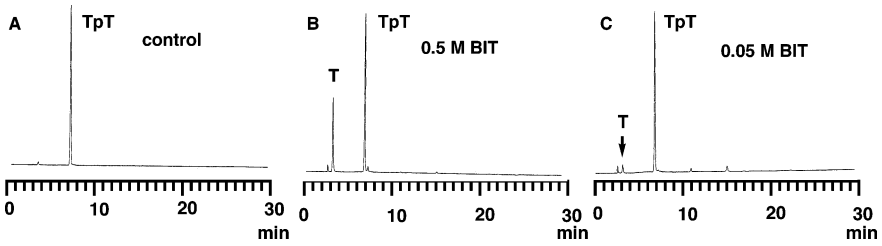
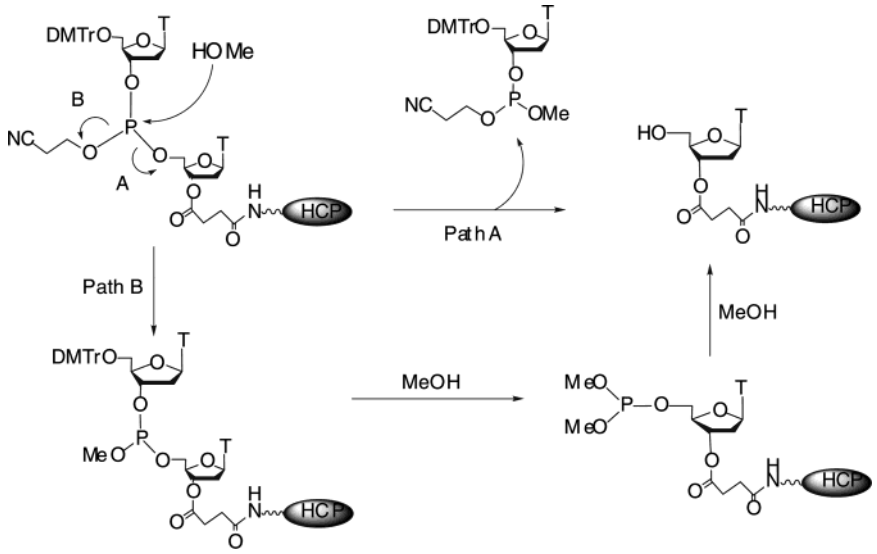


FIGURE 2. Ion-exchange HPLC profiles of the mixtures obtained by condensation of a T-loaded HCP resin with the thymidine phosphoramidite unit **1** in the presence of NBT. Panel A: The mixture obtained when the posttreatment was not carried out after the condensation. Panel B: The mixture obtained when the posttreatment with a 0.5 M solution of BIT in MeOH at room temperature for 2 min was carried out after the condensation. Panel C: The mixture obtained when the posttreatment with a 0.05 M solution of BIT in MeOH at room temperature for 2 min was carried out after the condensation. Solvent system C was used for HPLC.

SCHEME 1. Internucleotidic Bond Cleavage during Posttreatment with MeOH−BIT



MeOH under the above conditions. When a 0.05 M solution of BIT in MeOH was used, the formation of T was considerably suppressed but an appreciable amount of thymidine was still formed, as shown in Figure 2C. The internucleotidic bond cleavage occurred to a degree of ca. 10% in this case. Therefore, it seems to be difficult to reproduce the previous results,[15a] at least when the HCP resin is used.

A plausible mechanism for the internucleotidic bond cleavage giving rise to thymidine is shown in Scheme 1. There are two possible routes to thymidine, as shown in Paths A and B. It is likely that BIT serves as a catalyst for activation of the phosphite triester intermediate. On the basis of these unexpected results, we searched for a better method in the phosphoramidite approach without base protection.

Proton-Block Method. It is well-known that amino groups are more reactive than hydroxyl groups toward electrophilic reagents, but once protonated, their reactivity is decreased.[19] Therefore, we took into account this simple principle in organic chemistry for regulation of the reactivity of the nucleobase amino functions by protonation in the DNA synthesis. The pK_a values of the protonated forms of the four common deoxynucleosides of T, dC, dA, and dG were reported to be −0.5, 4.3, 3.8, and 2.5,[20] as shown in Figure 3 (see Supporting Information). Therefore, the basicity of the bases is ranked in the following order: dC, dA, dG, and T. Thymidine and deoxyguanosine exist in the neutral form under the usual conditions prescribed for the coupling reaction and show no reactivity in the phosphoramidite approach.[5a,11,15a] Compared with these facts, it has been recognized that the base residues of deoxycytidine and deoxyadenosine exhibit considerable reactivity toward reactive species of phosphoramidite reagents activated by promoters in the phosphoramidite approach.[11,15,21]

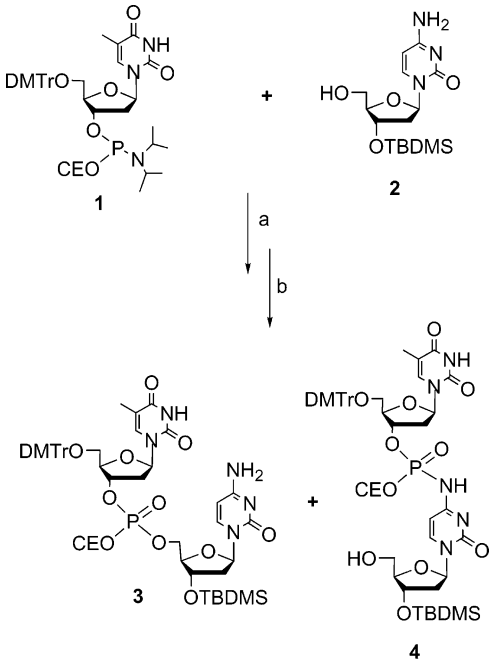
It is expected that deoxycytidine being more basic than deoxyadenosine is more effectively accessible to protonation so that the nucleophilicity of this protonated amino group would substantially decrease when a sufficiently acidic activator can be used in the *N*-unprotected phosphoramidite approach. Therefore, it was expected that less basic (ΔpK_a = 0.5) deoxyadenosine involved more risk of side reactions than deoxycytidine since the possibility of existence of the dissociated (free) adenine residue is higher than that of the dissociated cytosine residue in our approach.

The promoters used in the previous methods, such as 5-(*p*-nitrophenyl)-1*H*-tetrazole (pK_a = 3.7),[22] 5-ethylthio-1*H*-tetrazole (pK_a = 4.28),[23] benzimidazolium triflate (BIT) (pK_a = 4.5),[15a,16] 1*H*-tetrazole (pK_a = 4.8),[5a] pyridinium hydrochloride (pK_a = 5.1),[11,24] *N*,*N*-dimethylanilinium hydrochloride (pK_a = 5.15),[5a] 4,5-dicyanoimidazole (pK_a = 5.2),[25] and imidazolium triflate (IMT) (pK_a = 6.9),[15a] cannot protonate the base residues of dC and dA. The use of these promoters has long been rationalized in terms of avoidance of detritylation of the 5′-*O*-DMTr group.[26,27] This stereotype has been strongly held in this field so that promoters having pK_a values lower than 3.6 have not been examined to date.

To test the possibility of the proton-block method, we extensively searched for more than 70 kinds of promoters having pK_a values lower than 3.6. In our approach, these new promoters have been designed to protonate incoming *N*-unprotected deoxyribonucleoside phosphoramidites without deprotonating the nucleobases of the growing, solid-phase-linked, DNA oligonucleotide. It is expected that the unprotected nucleobases of the oligonucleotide are protonated through TCA treatment at every detrityolation step, and that the activators will not cause deprotonation like those activators exhibiting pK_a as greater than 4.3. It was ultimately found that, among all the activators tested, the three reagents of 5-nitrobenzimidazolium triflate (NBT, pK_a = 2.76), triazolium triflate (TRT, pK_a = 2.85), and 1-hydroxy-4-nitro-6-(trifluoromethyl)benzotriazole (NT-HOBt, pK_a = 2.70)[28] gave satisfactory results in this new strategy. The former two are new compounds that were synthesized as nonhygroscopic fine crystals in this study. When these promoters are used in the unprotected phosphoramidite method, the thymine base remains unprotonated, the guanine base may be partially protonated, and the adenine and cytosine bases can be selectively protonated, i.e., protected by the simplest proton, as depicted in the boxes of Figure 3, which shows the original overall concept we propose in this paper.

To evaluate the efficiency of these promoters, we used the condensation of 1.2 equiv of the thymidine phosphoramidite unit (**1**) with 3′-*O*-tBDMS-deoxycytidine (**2**)[29] in acetonitrile or THF in the presence of 2.2 equiv of an activator for 5 min at room temperature, as shown in Scheme 2. After the coupling, oxidation was carried out by using I_2 in pyridine−water[30] to give the coupling product **3**.

SCHEME 2[a]



[a] Reagents and conditions: (a) activator, THF or CH_3CN, rt, 5 min; (b) I_2, pyridine−H_2O (9:1, v/v), rt, 5 min.

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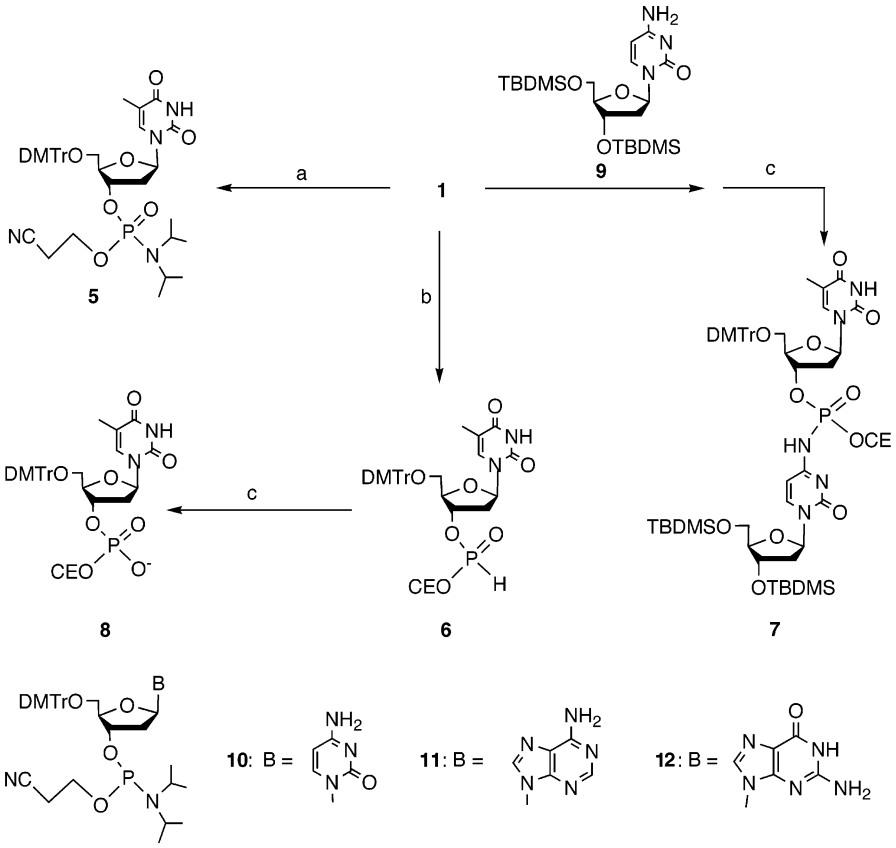
SCHEME 3[a]



[a] Reagents and conditions: (a) *t*-BuOOH in CH_3CN, (b) H_2O-CH_3CN (1:9, v/v), rt, 1 h, (c) I_2 in pyridine$-H_2O$ (9:1, v/v), rt, 5 min, (d) 1*H*-tetrazole in CH_3CN, rt, 1 h.

To analyze these reactions by ^{31}P NMR, we synthesized in advance reference materials **5**−**8** which were expected to be formed as byproducts, as shown in Scheme 3. The phosphoramidate derivative **5**[31] and the hydrolyzed product **6**[26] were obtained quantitatively by oxidation and hydrolysis, respectively, of **1**. The *N*-phosphorylated species **7** was synthesized in 80% yield by reaction of **1** with 3′,5′-*O*-bis(*tert*-butyldimethylsilyl)deoxycytosine **9**[29] in the presence of 1*H*-tetrazole followed by I_2 oxidation. The phosphodiester derivative **8** was also obtained.

Figure 4-A1 shows that, when 5-(ethylthio)-1*H*-tetrazole (EtSTet)[17b,23] was employed as a representative promoter in the reaction of **1** with **2**, the desired product **3** appeared at −1.8 ppm as the main peak in the ^{31}P NMR spectrum of the mixture. Significant amounts of the oxidized product **5** and the phosphodiester product **8** were simultaneously formed. These results reflect the incompleteness of the coupling reaction in the case of EtSTet. Neither the hydrolyzed product **6** nor the *N*-phosphorylated product **4** was detected. It is likely that the once-hydrolyzed product **6** was completely converted to the phosphodiester derivative **8** during the I_2 treatment. Benzimidazolium triflate (BIT, $pK_a = 4.5$)[15a,16] has been reported as the hitherto most powerful activator and is used as the reagent for the posttreatment of trivalent phosphoramidite species attached to the nucleobases in the previous *N*-unprotected phosphoramidite approach, as shown in Figure 1.[15a] As expected, the use of BIT resulted in complete condensation showing two diastereomeric resonance signals at ca. −2 ppm, as shown in Figure 4-B1, and the dimer **3** was isolated in 92% yield. When a new promoter, NBT, was used, the ^{31}P NMR spectrum (Figure 4-C1) of the mixture showed a set of diastereomers of **3** along with a peak at −2.1 ppm which seemed to be the phosphodiester **8**. After purification of the dimer **3**, it was obtained in 94% yield. In the case of NT-HOBt, the ^{31}P NMR spectrum (Figure 4-D1) of the mixture showed the diastereomeric resonance peaks of **3** with two minor peaks between −1.3 and −1.8 ppm. When TRT was used, the ^{31}P NMR spectrum (Figure 4-E1) of the crude mixture showed a main peak at −2 ppm and a minor peak at −2.2 ppm. The former seemed to be an unresolved signal of the diastereomers of **3**, and the latter seemed to be compound **8**. After purification, the dimer was isolated in 95% yield. As shown in these examples, resolution and chemical shifts of the peaks of **3** and **8** tended to change in each case, suggesting that the residues of the promoters delicately affect the whole resonance system. In all cases, however, the isolated product clearly exhibited only the diastereomeric resonance signals of **3**, as shown in Figure 4 (A2−E2). These reactions were done in THF except for the use of TRT since TRT was found to be sparingly soluble in THF.

These results suggested that, in the liquid-phase synthesis of **3** using a small excess amount of **1**, the base modification giving rise to **4** essentially did not occur. It

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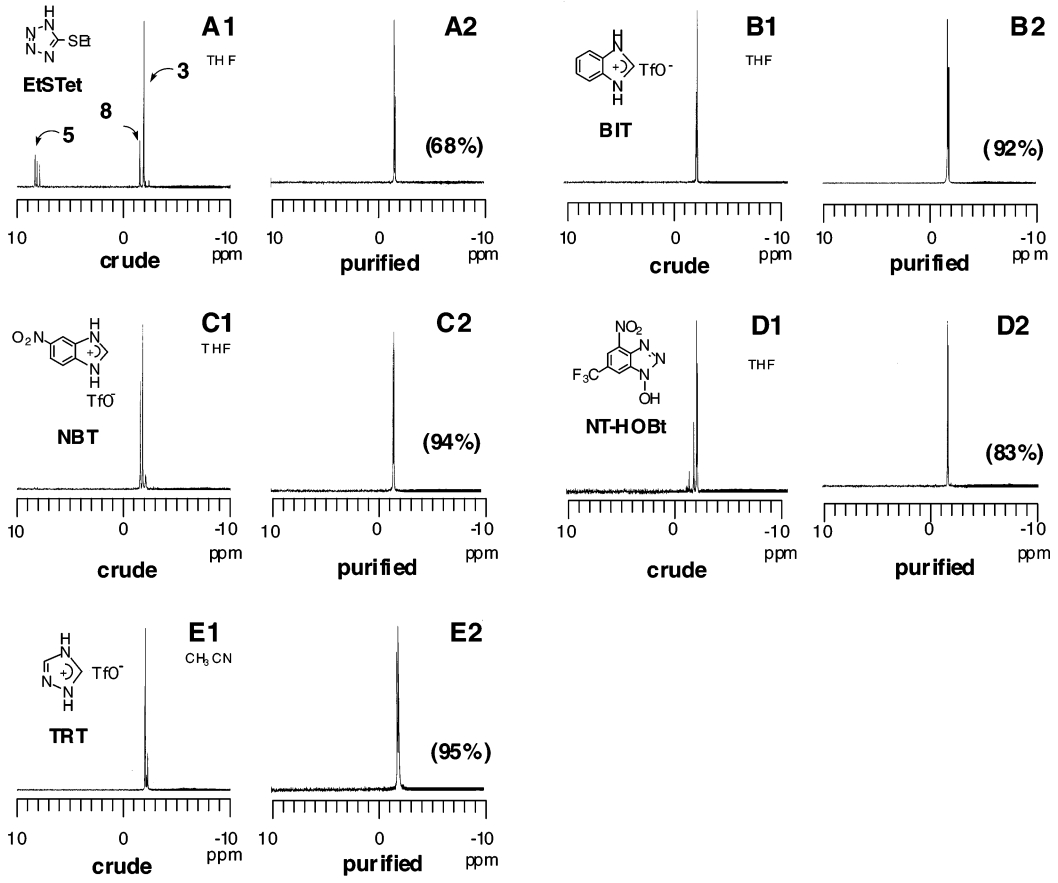


FIGURE 4. ^{31}P NMR spectra of the crude mixture obtained after the reaction of **1** and **2** in the presence of various promoters and the purified dimer **3**: Panel A, 5-(ethylthio)-1*H*-tetrazole (EtSTet); Panel B, benzimidazolium triflate (BIT); Panel C, 4-nitrobenzimidazolium triflate (NBT), Panel D, 4-nitro-6-(trifluoromethyl)benzotriazole-1-ol (NT-HOBt). Numbers 1 and 2 after bold alphabets refer to the spectra of the crude mixture and the purified dimer **3**, respectively. The figure in parentheses is the isolated yield.

TABLE 1. Protocol for the Synthesis of DNA Oligomers on Polymer Supports

entry	manipulation	reagent	time, min
1	washing	CH_2Cl_2	
2	detritylation	3% TCA/CH_2Cl_2	0.5
3	washing	CH_2Cl_2·THF	
4	condensation	phosphoramidite (20 equiv) promoter (40 equiv for dC or dA) (20 equiv for dG or T)	1
5	washing	CH_3CN	
6	oxidation	I_2/pyridine−H_2O (9:1, v/v)	2
7	washing	CH_3CN	

is also noted that under the conditions used for the coupling reaction the elimination of the DMTr group was not detected on TLC.

Among the promoters tested, several reagents such as ethyl nicotinate hydrochloride (pK_a = 3.2) and 4-bromobenzoic acid (pK_a = 3.6) showed nonhygroscopic property and sufficient solubility in organic solvents. However, no reaction occurred when these reagents were used as promoters in THF. These results strongly suggested that the nucleophilic property should be involved in the promoter after the P−N bond activation by an acidic substance, and some intermediates such as a phosphorus benzimidazolide species must be formed to enable the smooth esterification. As the promoter, NBT was found to be the best reagent. Other salts such as the corresponding chloride, hexafluorophosphate (PF_6^-), and nitrate were less effective.

Synthesis of Dideoxynucleotides on Polymer Supports. Compared with the solution-phase synthesis, the solid-phase method requires excess deoxynucleoside-3′-phosphoramidite reagents for condensation. Therefore, a greater risk of side reactions on the base residues of dC and dA was expected. Actually, Hayakawa reported in his phosphoramidite approach without base protection that the use of 25 equiv of monomer units in the presence of imidazolium triflate (IBT) resulted in the occurrence of the base modification of dC and dA to a degree of 8% and 4%, respectively.[15a] It was also expected that the loss of the DMTr group during the condensation becomes more visible because of the excess use of the phosphoramidites. Therefore, to examine the side reaction on the base parts and the detritylation in our proton-block method, we chose the sequence of d[ApT], d[CpT], d[ApA], d[CpC], and d[GpT] to synthesize these dimers on a highly cross-linked polystyrene resin.[17] The T-, dA-, and dC-loaded resins were prepared according to the method described in our previous paper.[13b] For the synthesis of these dimers we used the typical protocol involving the conditions as described in Table 1. This protocol is very simple so that the total time required for the three reactions (detritylation, condensation, and oxidation) is

3.5 min. A 20-equiv sample of a promoter was used in the condensation with 20 equiv of the dG or T phosphoramidite unit, while 40 equiv of a promoter was used in the case of the dC or dA phosphoramidite unit (20 equiv). This is because it is not necessary to protonate the base residues of dG and T and the basic base residues of dC and dA should be simultaneously protonated during the condensation when the P−N bond is activated by the promoter.

In our proton-block method, it is expected that the reaction of an adenine base is a little more serious than that of the cytosine base as far as the *N*-phosphitylation is concerned since the former is weaker by a 0.5 pK_a unit than the latter. Therefore, we checked condensation of a T-loaded resin with the deoxyadenosine phosphoramidite unit **11** by the action of various promoters.

The crude mixtures obtained after successive treatments with trichloroacetic acid (TCA) and ammonia were analyzed by reverse-phase HPLC. 1*H*-Tetrazole is a popular promoter in the phosphoramidite approach. Therefore, we examined how significantly the side reactions occurred when this azole was used. Indeed, this promoter gave byproducts X and X′ at 27 and 29.5 min in HPLC as shown in Figure 5A. Since the DMTr group was not eliminated in this case at all, these byproducts were ascribed to the phosphorylation on the adenine base. This tendency was also observed in the case of BIT, as shown in Figure 5B. Considerable amounts of such byproducts were formed. Even in the case of a mild activator, IMT, which was used for the *N*-unprotected phosphoramidite approach reported by Hayakawa,[15a] a considerable amount of the same byproduct X was formed, as shown in Figure 5C. Actually, the *O*-selectivity of the phosphorylation was estimated to be 77% from the area ratio of the peaks. To determine the structure of the byproduct X, this peak was analyzed by MALDI TOF mass spectroscopy. As a result, this analysis suggested that X has the molecular weight (M + H, calcd 869.21, found 869.21) corresponding to a branched trinucleotide d(^{ApNH}ApT). A minor peak of X′ at 34 min in Panel C was also observed at the region of tetranucleotides in the anion-exchange HPLC. This peak might be a branched tetranucleotide d[^{ApNH}ApApT] or d[Ap^{ApNH}ApT] although this product was not characterized.

Contrary to these facts, the use of NT-HOBt having the pK_a value of 2.70 gave a somewhat improved HPLC profile, as shown in Figure 5D. The formation of a branched trinucleotide was greatly suppressed. TRT also exhibited quite selective formation of d[ApT] in acetonitrile with generation of a lesser amount of the branched trinucleotide (1.4% estimated from the HPLC peaks), but a minor peak (3.6%) at 23.5 min was observed, as shown in Figure 5E. It was confirmed by comparison with the authentic sample that this minor peak was d[ApApT], which was formed owing to the partial detritylation during the condensation followed by further condensation of a newly generated 5′-free hydroxyl group with the excess phosphoramidite unit. In acetonitrile, NBT showed the same byproduct (3.9%) in Figure 5F. More interestingly, it was found that this reagent in THF underwent smooth reaction to give the best result essentially without formation of the base-modified product and the detritylated species, as shown in Figure 5G. These results clearly indicated that the NBT reagent was superior to the other

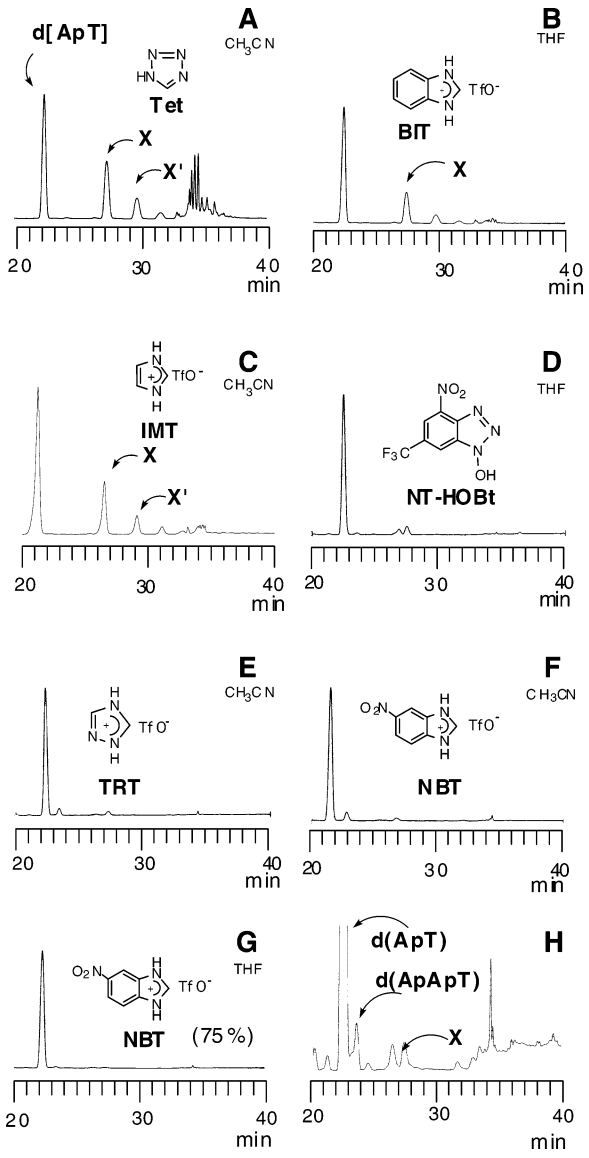


FIGURE 5. The reverse-phase HPLC profiles of crude d[ApT] obtained after the condensation of a T-loaded polystyrene resin with deoxyadenosine 3′-*O*-phosphoramidite building block **10** in various promoters: In each panel, the activator and the solvent used for the condensation are depicted. System B was used for HPLC. In the case of Panel G, the isolated yield of d[ApT] is shown in parentheses. Panel H is an expanded figure of Panel G.

two. The *N*-phosphorylated product having 0.6% area and d[ApApT] having 0.7% area were detected, as shown in Figure 5H. The dimer of d[ApT] was isolated in 75% and was well characterized by ESI mass and enzyme analysis (Experimental Section).

In the proton-block method there is a possibility that undesired depurination and detritylation might occur during the condensation. To check this possibility, we examined the stability of d[ApT] on the polymer support. After d[ApT] without base protection was synthesized on HCP by use of NBT as a promoter, the resin was treated with a 0.2 M solution of NBT in THF for a prolonged time of 2 h at room temperature. Subsequently, the d[ApT] mixture was released by treatment with ammonia and the mixture obtained was analyzed by RP-HPLC (Figure

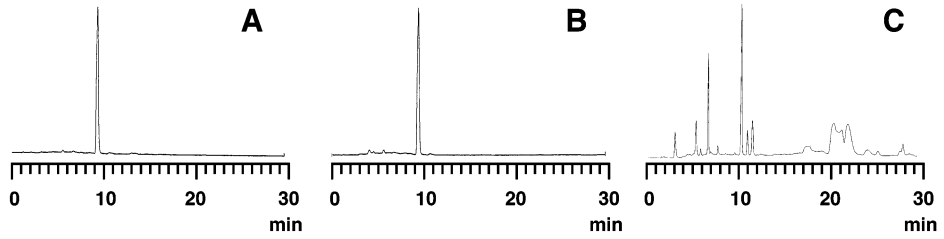
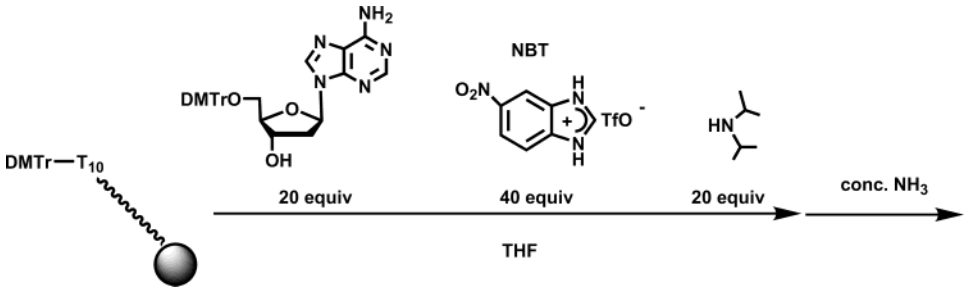


FIGURE 6. Reverse-phase HPLC profiles of the mixtures obtained by treatment of a d[ApT]- or d[A^{Bz}T]-loaded HCP resin withNBT or TCA. Panel A: The mixture obtained when the d[ApT]-loaded HCP resin having dA without the base protecting group was treated with a 0.2 M solution of NBT in THF for 2 h at room temperature. Panel B: The mixture obtained when the d[ApT]-loaded HCP resin having dA without the base protecting group was treated with 3% TCA in CH_2Cl_2 for 2 h at room temperature. Panel C: The mixture obtained when the d[A^{Bz}pT]-loaded HCP resin having *N*-benzoyl dA was treated with 3% TCA in CH_2Cl_2 for 2 h at room temperature. Solvent system A was used for HPLC.

SCHEME 4



6A). The area ratio of depurinated byproducts under these conditions was less than 1%. This fact suggested that depurination rarely occurred in the proton-block strategy.

This result was also confirmed by an independent experiment in the solution phase, using deoxyadenosine which was treated with a 0.2 M solution of NBT in THF at room temperature. It was found that dA was essentially stable in 10 h without decomposition.

To demonstrate the higher stability of unprotected dA than that of *N*-benzoylated dA on the polymer support, the stability of d[ApT]-HCP and d[A^{Bz}pT]-HCP in a 3% TCA solution in CH_2Cl_2 was checked. Consequently, it turned out serious decomposition occurred to give complex HPLC peaks when d[A^{Bz}pT]-HCP was treated with 3% TCA for 2 h at room temperature, as shown in Figure 6C. On the other hand, it was confirmed that d[ApT]-HCP slightly (1.6%) decomposed under the same conditions, as shown in Figure 6B. Apparently, the superiority of the proton-block strategy over the *N*-protected method is confirmed from these results, as far as the suppression of the depurination is concerned.

Since there is a risk of detritylation during the condensation with NBT, we studied in detail the stability of the 5′-terminal DMTr group in a growing chain on CPG and HCP resin in a simpler system which is similar to that of the proton-block method (Scheme 4). Panels A and C in Figure 7 show DMTr-T_{10}, which was synthesized on CPG and HCP resins, respectively, and released by treatment with ammonia. The detritylated byproduct T_{10} was observed at 13 min in reverse-phase HPLC. When DMTr-T_{10}−CPG was treated with a 1:2:1 mixture of 5′-*O*-DMTr deoxyadenosine, NBT, and diisopropylamine in THF for 10 min at room temperature, the loss of the DMTr group to give T_{10} was estimated to be 10%, as shown in Figure 7B. Under similar conditions, DMTr-T_{10}−HCP was found to be more stable, showing the loss

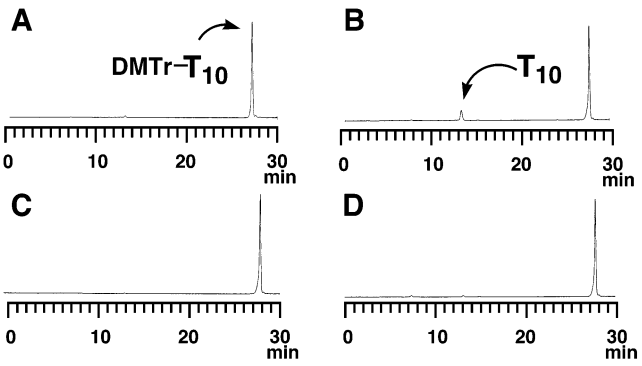


FIGURE 7. Reverse-phase HPLC profiles of the mixtures obtained after keeping a DMTr-T_{10}-loaded CPG or HCP resin under conditions similar to those used in the proton-block method. Panel A: The mixture obtained when DMTr-T_{10} was synthesized on a CPG resin. Panel B: The mixture obtained when the DMTr-T_{10}-loaded CPG resin was treated with NBT (40 equiv), diisopropylamine (20 equiv), and 5′-*O*-DMTr-deoxyadenosine (20 equiv) in THF for 10 min at room temperature. Panel C: The mixture obtained when DMTr-T_{10} was synthesized on a HCP resin. Panel D: The mixture obtained when the DMTr-T_{10}-loaded HCP resin was treated with NBT (40 equiv), diisopropylamine (20 equiv), and 5′-*O*-DMTr-deoxyadenosine (20 equiv) in THF for 10 min at room tempeature. Solvent system A was used for HPLC.

of the DMTr group in less than 1%, as shown in Figure 7D. These results suggested that the HCP resin was more suitable than the CPG resin in the proton-block method.

Next, the dimer d[CpT] was similarly synthesized by condensation of a T-loaded resin with deoxycytidine-3′-phosphoramidite unit **10** in the presence of IMT, NBT, NT-HOBt, or TRT in THF or CH_3CN. When IMT was used as the activator, d[CpT] appeared as the main peak at 8 min in the reverse-HPLC chart but a considerable

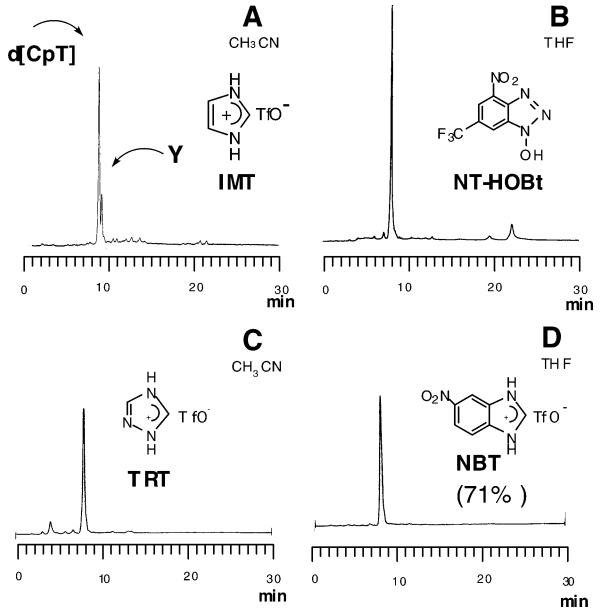


FIGURE 8. The reverse-phase HPLC profiles of crude d[CpT] obtained after condensation of a T-loaded polystyrene resin with the deoxycytidine 3′-*O*-phosphoramidite building block **10** in THF or CH_3CN. In each panel, the activator and the solvent used are depicted. The isolated yield of the dimer after purification by HPLC is shown in parentheses in the case of D. System A was used for HPLC.

amount of byproduct Y was formed, as shown in Figure 8A

According to the analysis with anion-exchange HPLC, the *O*-selectivity of the phosphorylation was calculated to be 83% (data not shown). This peak Y was also analyzed by ESI mass spectroscopy. Consequently, it was determined to be an *N*-phosphorylated byproduct, d[$^{[(dCp)NH}$CpT]. Among the activators tested, NBT gave the most promising result so that an almost single peak was observed in the HPLC chart, as shown in Panels B−D in Figure 8.

In this case, the *O*-selectivity of the phosphorylation was 99.0% and detritylation was not essentially observed, as shown in Figure 8D, since the cytosine base is more basic than the adenine base. This dimer d[CpT] was isolated in 71% yield and was well characterized by ESI mass and enzyme analysis. Under the conditions used for the condensation, no byproduct such as d[CpCpT], which would be derived from the partial detritylation during the condensation and should appear at 9 min in reverse-phase HPLC, were detected.

Next, we examined the detailed side reaction under the conditions where a more straightforward chemoselectivity of the base amino group vs the 5′-hydroxyl group, which are involved in an oligonucleotide growing on the HCP resin, can be estimated. Thus, d[ApT] on an HCP resin was synthesized by the use of NBT in the proton-block method. The thymidine 3′-phosporamidite unit was condensed with the d[ApT]-HCP resin for the synthesis of d[TpApT] by use of two promoters, i.e., IMT in the previous method or NBT in the proton-block method.

Consequently, 9% of the expected undesired byproduct d[Tp^{TpNH}ApT] was detected in the previous method, as shown in Figure 9A, so that the *O*-selectivity was 91%.

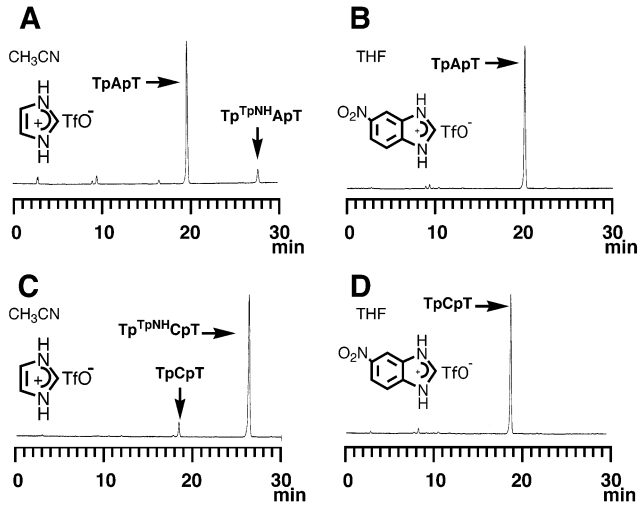


FIGURE 9. The anion-exchange HPLC profiles of crude d[TpApT] or d[TpCpT] obtained after condensation of a d[ApT]- or d[CpT]-loaded polystyrene resin with the thymidine 3′-*O*-phosphoramidite building block **1** for 1 min in THF or CH_3CN. Panels A and B show the mixtures obtained in the synthesis of d[TpApT] by use of IMT and NBT, respectively. Panels C and D show the mixtures obtained in the synthesis of d[TpCpT] by use of IMT and NBT, respectively. In each panel, the activator and the solvent used are depicted. System C was used for HPLC.

Interestingly, the use of NBT resulted in more than 99.9% of *O*-selectivity, as shown in Figure 9B.

Next, the *O*-selectivity was similarly examined in the case of d[TCpT]−HCP. The *O*-selectivity was surprisingly poor (less than 10%), as shown in Figure 9C when IMT was used, while with the use of NBT it was excellent (99.8%), as shown in Figure 9D. These results strongly suggested the usefulness of our new method.

These successful results encouraged us to study other sequences using NBT. Therefore, we focused on this reagent for the synthesis of d[ApA] and d[CpC] having two basic deoxynucleosides. These results are shown in Figure 10A,B. In each case, an almost single peak was observed. When this system was applied to the synthesis of d[GpT], a similar result was obtained, as shown in Figure 10C. Thus, d[ApA], d[CpC], and d[GpT] could be isolated in 70%, 78%, and 82% yields, respectively.

Since at the dimer and trimer levels we succeeded in synthesizing satisfactorily several di- and tri-deoxynucleotides, further studies were done by using the sequences of d[ApApApT] and d[CpCpCpT]. These results are depicted in Figure 11A,B. In each case, the main peak was found to be the desired tetradeoxynucleotide. These oligomers were isolated in satisfactory yields and characterized by MALDI-TOF mass and enzymatic degradation.

Synthesis of Longer Oligodeoxynucleotides in the Proton-Block Approach. Since with an increase of the length of the oligodeoxynucleotide chain the possibility of occurrence of side reactions should be amplified, we evaluated our proton-block approach at the 7mer level. For this purpose, the sequences of d[CpCpCpCpCpCpT] and d[ApApApApApApT] as the target oligomers were chosen. In the synthesis of d[CpCpCpCpCpCpT], the desired product was obtained as the main peak as shown in Figure 12A. However, many minor peaks in the region

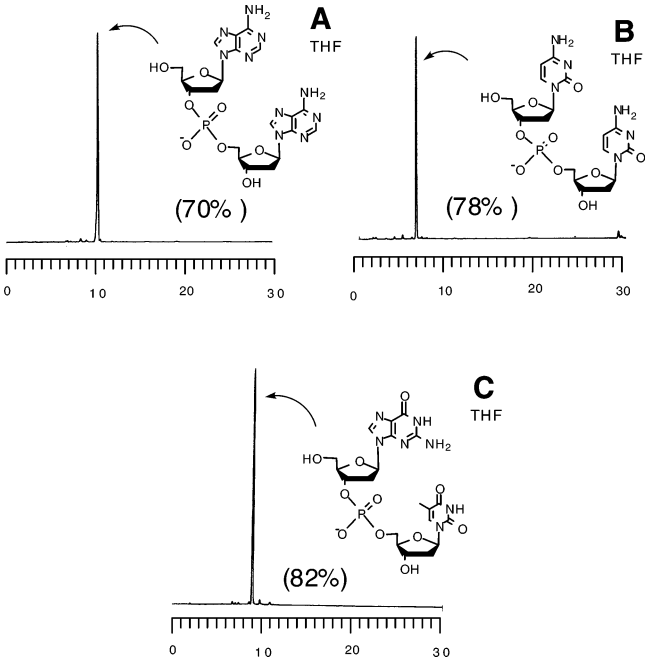


FIGURE 10. The reverse-phase HPLC profiles of the mixtures obtained after the condensation of dC-, dA-, and T-loaded polystyrene resins with the deoxyadenosine 3′-*O*-phosphoramidite building blocks **10**, **11**, and **13** respectively, in various promoters: Panel A, d[ApA], NBT, THF; Panel B, d[CpC], NBT, THF; and Panel C, d[GpT], NBT, THF. The isolated yield of each dimer after purification by HPLC is shown in parentheses. System A was used for HPLC.

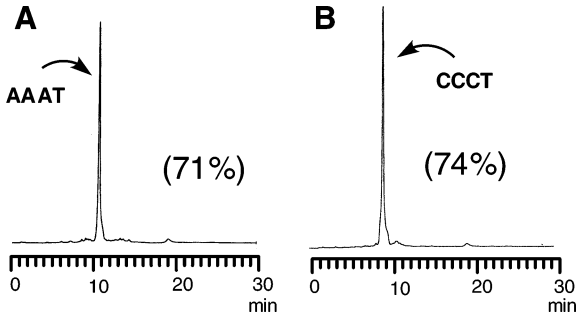


FIGURE 11. The reverse-phase HPLC profiles of the crude mixture obtained by use of NBT in THF. The isolated yield of each tetramer after purification by HPLC is shown in parentheses. System B was used for HPLC.

of retention time of more than 20 min were observed. These complex peaks might be ascribed to the base modification that might occur at any place with the consecutive sequence of dC. The isolated yield of this 7mer was 21%. In the synthesis of d[ApApApApApApT], we were able to obtain a little better yield of 25%, as shown in Figure 12B. These oligomers were characterized by enzyme digestion and MALDI-TOF mass spectroscopy. Since we eliminated both capping and post-P−N bond cleavage reactions from the current DNA protocol, such side reactions arose on a visible scale at the level of longer DNA fragments. Nevertheless, it should be emphasized that it is possible to obtain these oligomers as main products without any posttreatment.

Synthesis of d[CAGTCAGTCAGT] in the Proton-Block Approach and the Choice of a Mixed Solvent System for Better Condensation in Oligomer Synthesis. Finally, we applied our proton-block approach to the synthesis of a 12mer, d(CpApGpTpCpApGpTpCpApGpT). During this synthesis, we encountered a problem in using the solvent, THF. In this solvent, we could not obtain satisfactory yields, particularly in the synthesis of more than 9mer. Therefore, we further searched for better solvent systems. Consequently, at the oligomer level, we found that a mixture of CH_3CN and *N*-methylpyrrolidone (NMP) is much superior to THF. NMP has a pK_a value of −0.71, which is somewhat stronger in basicity than THF (pK_a ca. −2), as shown in Figure 3. The pK_a value of CH_3CN is −10, as mentioned before. Therefore, we can prepare appropriate solvent systems having suitable basicity within the range from pK_a −0.71 to −10 by changing the ratio of the two solvents. It was ultimately found that a 1:1 mixture of CH_3CN and NMP was effective in this study. Consequently, we were able to obtain d(CpApGpTpCpApGpTpCpApGpT) in 18% isolated yield. The crude HPLC chart is shown in Figure 13, which shows a main peak corresponding to the desired 12mer.

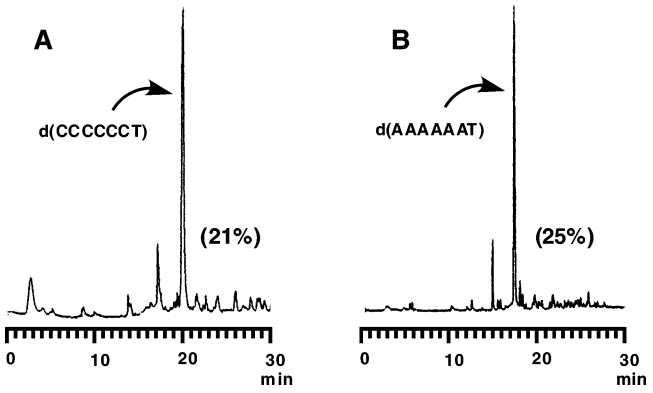


FIGURE 12. The anion-exchange HPLC profiles of the mixtures obtained by use of NBT: Panel A, d[CpCpCpCpCpCpT]; and Panel B, d[ApApApApApApT]. System D was used for HPLC.

It should be emphasized that without using base protection, capping reaction, and posttreatment of the cleavage of concomitant byproducts having the P−N bond, we were able to obtain the 12mer as the highest peak in HPLC. To demonstrate the significance of this result, we also did an independent experiment to synthesize the same 12mer. As mentioned before, Hayakawa reported his successful DNA 60mer synthesis.[15a] In this method, the condensation was carried out by use of imidazolium triflate (IMT). Therefore, we examined the synthesis of d[CpApGpTpCpApGpTpCpApGpT] by using the hitherto best activator, IMT,[15a] as far as the DNA synthesis without base protection is concerned. We checked what happened when the posttreatment with MeOH−BIT was eliminated in his procedure. Consequently, it turned out that, when the posttreatment was eliminated, a complex mixture was obtained, as shown in Figure 14. As seen in Figure 14, a cluster of peaks such as a mountain was observed.

This result implied that multicomplex branched DNA chains were apparently formed, as evidenced by the presence of many peaks around retention times of 27−50 min. This is also supported by an early experiment

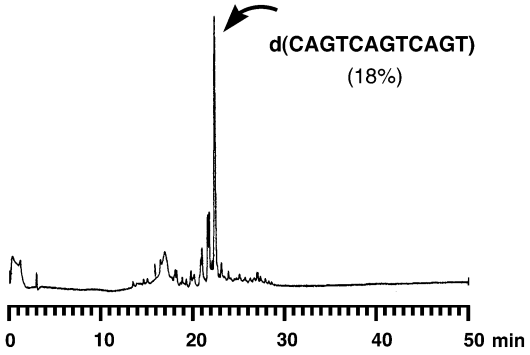


FIGURE 13. The anion-exchange HPLC profiles of the crude d[CAGTCAGTCAGT] obtained by use of NBT in $CH_3CN-NMP$ (1:1, v/v). System D was used for HPLC.

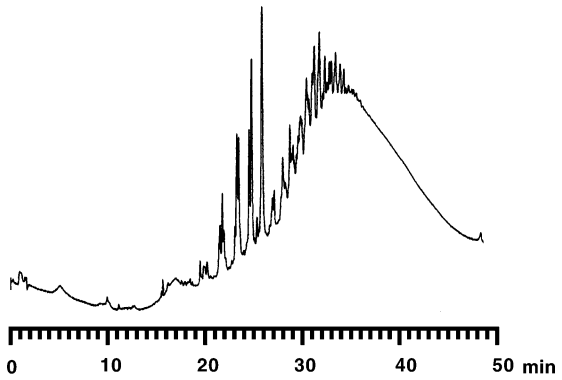


FIGURE 14. The anion-exchange HPLC profiles of the mixture obtained by use of IMT as the activator in CH_3CN in an attempt to obtain d[CAGTCAGTCAGT]. Solvent D was used for HPLC.

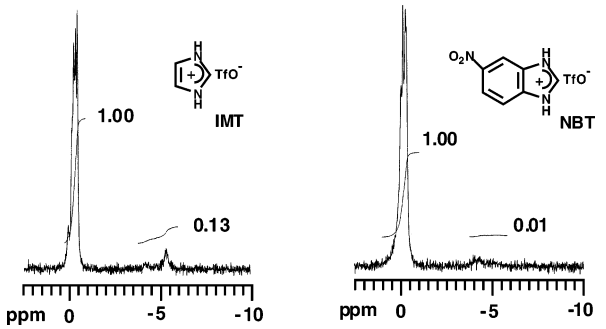


FIGURE 15. ^{31}P NMR spectrum of crude $d[CAGT]_3$. Panel A: The profile of $d[CAGT]_3$ by use of IMT; Panel B: The profile of $d[CAGT]_3$ by use of NBT.

at the dimer synthesis, as shown in Figure 5C. Therefore, as shown in Figure 13, our new reagent, NBT, has proved to work very well as an effective activator in the proton-block method.

The *O*-selectivity of Figure 13 or Figure 14 was evaluated by ^{31}P NMR. Figure 15A shows the NMR profile of the crude mixture involving $d(CAGT)_3$, which was obtained by use of IMT, and Figure 15B shows that obtained by use of NBT. There were broad signals of *N*-phosphorylated byproducts at −4 to −6 ppm in both cases, but the *O*-selectivity in the proton-block method was 13-fold over that calculated in the previous method. At a glance, it seems that this difference is not significant when the dramatic change between Figures 13 and 14 is compared. However, an increase of branched chains on the 12mer increases simultaneously the number of phosphodiester linkages on the branched chains so that the 13-fold difference sufficiently explains the outlooks of Figures 13 and 14, as evidenced by our own theoretical simulation (data not shown). These results indicated that our new strategy can suppress considerably *N*-phosphorylation compared with the previous methods in the synthesis of oligodeoxynucluotides with mixed sequences.

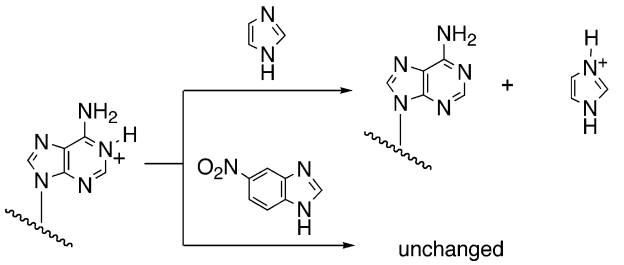
Side Reactions Associated with the Proton-Block Approach. It was a matter of concern that the use of more acidic activators would cause depurination of deoxyadenosine and deoxyguanosine.[26a] However, we could not observe this kind of problem during this study. It is known that the depurination rate of *N*-unprotected deoxyadenosine is slower than that of *N*-benzoyldeoxyadenosine.[32,33] Therefore, it is likely that, since depurination occurs via a diprotonated species, it is more difficult to protonate the position 7 of the protonated deoxyadenosine form than that of *N*-benzoyldeoxyadenosine by the action of NBT.

Since our new activators ranked between deoxyguanosine ($pK_a = 2.5$) and deoxyadenosine ($pK_a = 3.8$) in the pK_a list, as shown in Figure 3, deoxyguanosine is partially protonated theoretically by NBT, while deoxyadenosine is mostly protonated by these activators and deoxycytidine is completely protected. However, the growing chain of oligodeoxynucleotides on the polymer was treated by the action of TCA for removal of the DMTr group. The TCA-treated resin was washed with dichloromethane. Therefore, all three basic deoxynucleosides of dG, dA, and dC formed salts with TCA ($pK_a = 0.52$) after the TCA treatment. As a matter of fact, this protonated resin was allowed to react with phosphoramidite reagents in the presence of NBT. This manipulation resulted in a complex mixture in the reaction system. It is expected that the TCA salts of dG, dA, and dC come to acquire the ability as activators of the P−N bond of the phosphoramidites but the efficiency of the activation of such TCA salts has yet to be evaluated in this study. This situation becomes more complex with an increase of the length of oligodeoxynucleotides since the relative ratio of the TCA salts to NBT would increase. When a DNA 10mer $d[(Xp)_9T]$ containing dA, and/or dC as X is synthesized in the proton-block method, it is expected that, at the last coupling, nine TCA salts are generated on the polymer. Usually, we used 40 equiv of an activator and 20 equiv of a phosphoramidite building unit. Probably, this side effect is one reason the HPLC pattern becomes complicated at the oligomer level.

In our approach, the nucleobases are initially protonated by treatment with TCA and these protonated bases are preserved during the condensation, since the diisopropylamine generated by condensation is effectively neutralized by triflic acid and, simultaneously, the free, less basic activator (5-nitrobenzimidazole $pK_a = 2.76$) that cannot deprotonate the nucleobases is generated. On the other hand, the previous method[15a] with IMT produces imidazole ($pK_a = 6.9$), which is more basic than

(32) York J. L. *J. Org. Chem.* **1981**, *46*, 2171−2173.
(33) Schaller, H.; Khorana, H. G. *J. Am. Chem. Soc.* **1963**, *85*, 3828−3835. Moon, M. W.; Nishimura, S.; Khorana, H. G. *Biochemistry* **1966**, *5*, 937−945.

SCHEME 5



the adenine and cytosine bases having pK_a values of 3.8 and 4.2, respectively, so that the side reactions on the base moieties cannot be avoided. As a matter of course, the use of IMT resulted in considerable amounts of *N*-phosphorylated byproducts, as evidenced by Figures 5C, 8A, 9A, and 9C as well as Figure 14.

In the whole reaction system of our proton-block method, the nitrogen of the phosphoramidite seems to be the most basic site. However, this basicity is not known, because the phosphoramidite building unit is too unstable to allow examination of this fundamental physicochemical property. Since the phosphoramidite bond can be activated by IMT (pK_a = 6.9), the pK_a value would be at least more than 7.

Superiority of THF or CH_3CN−NMP as the Solvent in Condensation. In our study we found that THF is superior to CH_3CN as the solvent as far as avoidance of the loss of the DMTr group during condensation is concerned. Previous activators, such as 1*H*-tetrazole, BIT, and 5-(ethylthio)-1*H*-tetrazole, usually work well in CH_3CN. We also observed that these reagents having pK_a values higher than 3.7 become less effective in THF than in CH_3CN as far as the reaction rate is concerned. The reason there were significant differences both in detritylation during the condensation and in the reaction rate of condensation between CH_3CN and THF is apparently explained in terms of the basicity of the solvents used. CH_3CN and THF have pK_a values[34] of ca. −10 and ca. −2, respectively, showing there is a difference of 10^8 in the dissociation constant between the protonated forms of CH_3CN and THF. The following data[35] are available for discussion about the evaluation of the basicity of the 5′-oxygen: $CH_3C(O)OH$, pK_a = 4.79; *p*-MeO-C_6H_4-$CH_2C(O)OH$, pK_a = 4.45; and C_6H_5-$CH_2C(O)OH$, pK_a = 4.30. From these data, the substituents of phenyl and *p*-methoxyphenyl are generally recognized as electron-withdrawing groups. In addition, it is also expected that the sugar residue serves as an electron-withdrawing group since there are β- and γ-oxygens at the 4′- and 3′-positions that can abstract electrons. However, once this ethereal 5′-oxigen is protonated, the ether bond cleavage more easily occurs than that of the protonated THF since a much more stable trityl cation is generated. Therefore, a more basic solvent, THF, would serve as a buffer site more accessible to protonation than the 5′-ether site so that the risk of direct protonation of the DMTr ether bond causing the detritylation would decrease. Thus, the choice of THF enabled us to use more acidic activators such as NBT for activation of phosphoramidite building units.

The difference in pK_a between NBT and the 5′-trityl ether is expected to be ca. 5 units. This difference allows the concomitant detritylation in CH_3CN as exemplified by Figure 5F, but selective internucleotidic bond formation can be done in a more basic solvent of THF, as shown in Figure 5G (THF).

In addition, we checked the stability of the DMTr group on a DMTrT-HCP resin (0.25 μmol) in a 0.1 M solution of NBT (50 μmol) in CH_3CN, THF, and CH_3CN−NMP (1:1, v/v) at 25 °C to evaluate the solvent effect. Consequently, it was found that the amounts of the DMTr group released from the resins after 5 min were 40%, 26%, and 2% for CH_3CN, THF, and CH_3CN−NMP (1:1, v/v), respectively. These results clearly suggested that the detritylation can be suppressed more effectively by use of a more basic solvent system. It should also be noted that under the actual conditions used for the proton-block method, the actual loss of the DMTr group can be expected to be far less than those obtained in the above experiments since essentially basic phosphoramidite units were not added.

Therefore, it might be ideal if we can use a little more basic solvent than THF as far as the avoidance of the detritylation is concerned. In this manner, the 1:1 mixture of CH_3CN−NMP used for the synthesis of the DNA 12mer is the better choice of the solvent system.

Conclusion

The present method evolved from an idea that most of the important items (the P−N bond activation, solvent effects, detritylation, and related side reactions) involved in the DNA synthesis can all be recognized as very simple "acid−base reactions". This new concept would stimulate a new possibility of DNA synthesis without base protection. Although visible side reactions were observed at the level of longer DNA fragments, this proton-block method would provide new insight into the synthesis of artificially modified DNA oligomers and extremely base-sensitive functional groups. When the proton-block method is employed for the synthesis of such fragile modified DNA oligomers, it is necessary to use a linker that can be removed under neutral conditions in place of the succinate linker. Quite recently, we also developed a useful silyl-type linker capable of removal by treatment with Bu_4NF under neutral conditions.[36] Therefore, the combined use of the present proton-block method and this linker would provide a new tool for the synthesis of base-sensitive DNA oligomers.

We also reported previously the preparation of HCP resin-linked 3′-terminal deoxynucleotide derivatives, which must be required for the general synthesis of oligodeoxynucleotides having dA, dG, or dC at the 3′-terminal site. These polymer-linked deoxynucleoside derivatives have two DMTr groups at the 5′- and *N*-positions. Therefore, it will be possible to synthesize any sequences without basic conditions such as ammonia by use of the proton-block method, the silyl-type linker, and these polymers.

However, to improve the proton-block approach, extensive work to find more effective activators and solvents

(34) The list of the basicity of common solvents is now available from http://mail.chor.unipd.it/wisor/lecture_notes/acidbase.pdf (Dr. A. Bagno's lab, Centro CNR, Padova, Italy).

(35) The pK_a values were calculated by use of Advanced Chemistry Development (ACD) Software Solaris V4.76 (copyright 1994−2003 ACD) in SciFinder 2002.

(36) Kobori, A.; Miyata, K.; Ushioda, M.; Seio, K.; Sekine, M. *Chem. Lett.* **2002**, 16−17.

has yet to be done. Further studies are now underway in this direction.

Experimental Section

General Remarks. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 270, 68, and 109 MHz, respectively. The chemical shifts were measured from tetramethylsilane or DSS for ^{1}H NMR spectra, $CDCl_3$ (77 ppm) or DSS (0 ppm) for ^{13}C NMR spectra, and 85% phosphoric acid (0 ppm) for ^{31}P NMR spectra. UV spectra were recorded on a U-2000 spectrometer. CD spectra were recorded on a J-500 C spectrometer with a 0.5-cm cell. TLC was performed by the use of Kieselgel 60-F-254 (0.25 mm). Column chromatography was performed with silica gel C-200 purchased from Wako Co. Ltd., and a minipump for a goldfish bowl was conveniently used to attain sufficient pressure for rapid chromatographic separation. Reversed-phase column chromatography was performed by the use of μBondapak C-18 silica gel (prep S-500, Waters). Reversed-phase HPLC was performed with the following systems. System A:, A Waters Aliance system was used with an Waters 3D UV detector and a μBondasphere 5 μm C18 100 Å column (3.9 × 150 mm) at 50 °C with a linear gradient (0−10%) of CH_3CN in 0.1 M NH_4OAc, pH 7.0, at a flow rate of 1.0 mL/min for 30 min. System B: A linear gradient (0−30%) of CH_3CN in 0.1 M NH_4OAc was used. The other conditions are the same as those of System A. System C: An anion-exchange HPLC was done on a Waters Aliance system with a Waters 3D UV detector and a Waters Spherisorb S5 SAX analytical column (4.6 × 250 mm). A linear gradient (0−30%) of solvent I (20% CH_3CN in 0.5 M potassium phosphate) in solvent II (20% CH_3CN in 0.005M potassium phosphate) was used at 50 °C at a rate of 1.0 mL/min for 30 min. System D: An anion-exchange HPLC was done on a Shimadzu LC-10 AD VP with a Shimadzu 3D UV detector and a Gen-PakTM FAX column (Waters, 4.6 × 100 mm). A linear gradient (0−60%) of Solvent III (1 M NaCl in 25 mM phosphate buffer (pH 6.0)) in solvent IV (25 mM phosphate buffer (pH 6.0)) was used at 50 °C at a flow rate of 1.0 mL/min for 45 min. If the HPLC analysis after 45 min is necessary, a linear gradient (60−100%) of Solvent III in Solvent IV was further used at 50 °C at a flow rate of 1.0 mL/min for an additional 7 min. Gel permeation was performed by use of Sephadex G-10 or G-15 after swelling with sterilized water, and elution was done with sterilized water. Pyridine was distilled two times from *p*-toluenesulfonyl chloride and from calcium hydride and then stored over 4A molecular sieves. MALDI-TOF mass was performed by use of Voyager RP (PerSeptive Biosystems Inc.). Highly cross-linked polystyrene was purchased from Perkin-Elmer, ABI. NT-HOBt was synthesized according to the literature method.[28] The thymidine-3′-*O*-phosphoramidite was prepared according to the standard method.[37] The deoxycytidine-, dexyadenosine-, and deoxyguanosine-*O*-3′-phosphoramidite derivatives were synthesized by the method reported by Gryaznov and Letsinger.[11] Elemental analyses were performed by the Microanalytical Laboratory, Tokyo Institute of Technology at Nagatsuta.

The Stability of Internucleotidic Phosphite Triester Linkage on HCP During Posttreatment with MeOH−BIT. After condensation of a T-loaded HCP resin (1 μmol) with thymidine phosphoramidite unit **1** in the general procedure, the resin was treated with a 0.5 or 0.05 M solution of BIT in MeOH (400 μL) for 2 min. After the subsequent oxidation with I_2 in pyridine−H_2O (9:1, v/v, 500 μL) for 2 min, the dimer was deprotected and released from the polymer support by treatment with concentrated aq NH_3 (500 μL) for 40 min. The polymer support was removed by filtration and washed with H_2O (3 × 1 mL). The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in water and analyzed by anion-exchange HPLC.

5-Nitrobenzimidazolium Triflate (NBT). To a solution of 5-Nitrobenzimidazole (7.1 g, 44 mmol) in MeOH−ether (1:1, v/v, 50 mL) was added dropwise triflic acid (3.9 mL, 44 mmol) with vigorous stirring at 0 °C. After being stirred for 10 min, the mixture was poured into ether (200 mL). The resulting precipitates were collected by filtration and washed with ether (300 mL) to give the triflate (13.2 g, 96%): mp 138−140 °C (ethyl acetate); ^{1}H NMR (DMSO) δ 7.97 (d, 1H, J = 8.9 Hz), 8.30−8.34 (m, 1H), 8.63 (d, 1H, J = 2.2 Hz), 9.51 (s, 1H); ^{13}C NMR (DMSO) δ 111.3, 113.4, 115.4, 118.2, 120.7, 122.9, 127.6, 130.9, 135.0, 144.7, 145.0. Anal. Calcd for $C_8H_6F_3N_3O_5S$: C, 30.68; H, 1.93; N, 13.42; F, 18.20. Found: C, 30.60; H, 1.91; N, 13.44; F, 18.28.

Triazolium Triflate (TRT). This compound was synthesized by use of 1,2,4-triazole (3.0 g 44 mmol) in the same method as described above. The yield was 9.4 g (98%): mp 162−164 °C (ethyl acetate-acetonitrile); ^{1}H NMR (DMSO) δ 9.31 (s, 1H), 9.33 (s, 1H); ^{13}C NMR (DMSO) δ 113.4, 118.1, 122.8, 127.6, 142.3. Anal. Calcd for $C_3H_4F_3N_3O_3S$: C, 16.4; H, 1.84; N, 19.17; F, 26.01; S, 14.63. Found: C, 16.33; H, 1.75; N, 19.35; F, 25.82; S, 14.30.

Ethyl Nicotinate Hydrochloride. To a solution of ethyl nicotinate (27.8 g, 183 mmol) in ether (100 mL) was added dropwise hydrochloride (4.0 M solution in dioxane, 45.8 mL, 183 mmol) with vigorous stirring at 0 °C. After being stirred for 10 min, the mixture was poured into ether (500 mL). The resulting precipitates were collected by filtration and washed with ether (800 mL) to give the triflate (30 g, 87%): mp 122−124 °C (ethyl acetate); ^{1}H NMR (DMSO) δ 1.34 (t, 3H, J = 7.0 Hz), 4.37 (dd, 2H, J = 4.9 Hz, J = 14.0 Hz), 7.86 (dd, 1H, J = 5.3 Hz, J = 8.0 Hz), 8.62 (dd, 1H, J = 3.4 Hz, J = 8.0 Hz), 8.97 (dd, 1H, J = 1.6 Hz, J = 5.4 Hz), 9.17 (d, 1H, J = 1.6 Hz), 11.94 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.1, 62.3, 127.3 128.5, 143.1, 144.3, 146.2, 162.2. Anal. Calcd for $C_8H_6F_3N_3O_5S$: C, 51.21; H, 5.37; N, 7.47. Found: C, 51.13; H, 5.37; N, 7.43.

Synthesis of the *N*-Unprotected d[TpC] Derivative 3. A mixture of the thymidine 3′-*O*-phosphoramidite derivative[29] **1** (300 mg, 0.403 mmol) and 3′-*O*-(*tert*-butyldimethylsilyl)-deoxycytidine **2** (116 mg, 0.336 mmol) was rendered anhydrous by repeated coevaporation successively with dry pyridine (×3), dry toluene (×3), and dry CH_2Cl_2 (×3), and finally dissolved in dry THF (3 mL). To the mixture was added an appropriate activator (0.672 mmol). After the mixture was stirred at room temperature for 1 h, a 1 M solution of I_2 (pyridine−water, 9:1, v/v, 3 mL) was added to the mixture. After being stirred at room temperature for 2 min, the mixture was partitioned between $CHCl_3$ (50 mL) and aqueous 5% $Na_2S_2O_3$ (30 mL). The organic phase was collected, washed twice with aqueous 5% $Na_2S_2O_3$ (30 mL), filtered, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (10 g) with 1% pyridine containing hexane−$CHCl_3$ (50:50−0:100, v/v) and then 1% pyridine containing $CHCl_3$−MeOH (100:0−97:3, v/v) to give the fractions containing **3**. The fractions were collected, evaporated under reduced pressure, and finally evaporated by repeated coevaporation three times each with toluene and CH_2Cl_2 to remove the last traces of pyridine to give the title compound **3** (NBI, 315 mg, 94%; NT-HOBt, 280 mg, 83%; TRT, 319 mg, 95%; BIT, 310 mg, 92%; 5-(ethylthio)-1*H*-tetrazole, 228 mg, 68%): ^{1}H NMR ($CDCl_3$) δ 0.06 (s, 6H), 0.86 (s, 9H), 1.39 (s, 3H), 2.10−2.81 (m, 6H), 3.32−3.58 (m, 2H), 3.78 (s, 6H), 3.82−4.02 (m, 1H), 4.10−4.38 (m, 5H), 5.10−5.22 (m, 2H), 6.15−6.34 (m 3H), 6.76−6.82 (m, 4H), 7.23−7.33 (m, 9H), 7.46 (s, 1H), 7.71 (d, 1H, J = 6.8 Hz), 9.10−9.55 (br, s, 2H); ^{31}P NMR ($CDCl_3$) δ −1.58, −1.66; ^{13}C NMR ($CDCl_3$) δ −4.8, −4.6, 11.8, 17.9, 19.6, 19.7, 19.8, 25.7, 39.0, 41.2, 55.3, 62.5, 62.6, 63.3, 70.4, 77.2, 79.9, 84.2, 94.9, 111.8, 113.2, 116.2, 116.4, 123.6, 127.1, 127.9, 128.0, 128.9, 129.9, 134.7, 134.8, 135.8, 140.3, 143.8, 149.6, 150.6, 150.7, 155.3, 158.6, 163.7, 163.8,

(37) Sinha, N. D.; Biernat, J.; McManus, J.; Köster, H. *Nucleic Acids Res.* **1984**, *12*, 4539−4557. For the synthesis of **1**, the phosphitylating reagent of chloro(2-cyanoethoxy)(diisopropylamino)phosphine was prepared by: Tanimura, H.; Maeda, M.; Fukazawa, T.; Sekine, M.; Hata, T. *Nucleic Acids Res.* **1989**, *17*, 8135−8147.

165.5; MS *m*/*z* calcd for M + H 1001.3883, found 1001.3887. Anal. Calcd for $C_{40}H_{49}N_4O_9P\cdot 2H_2O$: C, 56.74; H, 6.31; N, 8.10. Found: C, 56.95; H, 6.04; N, 7.90.

Cyanoethyl 5′-*O*-(4,4′-Dimethoxytrityl)thymidine-3′-yl *N*-Diisopropylphosphoramidate (5). To a solution of the thymidine phosphoramidite unit **1** (470 mg, 0.63 mmol) in CH_3CN (5 mL) was added *t*-BuOOH (5 M solution in decane, 0.5 mL, 2.5 mmol). After being stirred at room temperature for 10 min, the mixture was diluted with $CHCl_3$. The $CHCl_3$ layer was washed three times with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (10 g) with hexane−$CHCl_3$ (50:50−0:100, v/v) containing 1% pyridine and then with MeOH−$CHCl_3$ (0:100−3:97, v/v) containing 1% pyridine to give the title compound (468 mg, 80%): ^{1}H NMR ($CDCl_3$) δ 1.09−1.35 (m, 15H), 2.33−2.72 (m, 4H), 3.34−3.50 (m, 4H), 3.77 (s, 6H), 4.00−4.29 (m, 3H), 5.03 (br s, 1H), 6.43 (dd, 1H, J = 5.4 Hz, J = 8.9 Hz), 6.81 (d, 4H, J = 8.9 Hz), 7.13−7.59 (m, 10H), 8.66 (d, 1H, J = 8.6 Hz); ^{31}P NMR ($CDCl_3$) δ 8.03, 8.33; ^{13}C NMR ($CDCl_3$) δ 11.7, 19.6, 39.4, 46.3, 46.4, 63.4, 77.3, 84.3, 84.4, 87.0, 87.1, 111.5, 111.6, 113.1, 113.2, 126.9, 127.1, 127.6, 127.9, 128.1, 128.9, 129.0, 130.0, 134.8, 134.9, 135.0, 135.3, 139.3, 143.8, 143.9, 150.2, 158.4, 158.6, 163.4; MS *m*/*z* calcd for M + H 761.3505, found 761.3317. Anal. Calcd for $C_{40}H_{49}N_4O_9P\cdot{}^1/_2H_2O$: C, 62.41; H, 6.55; N, 7.27. Found: C, 62.66; H, 6.62; N, 7.14.

Cyanoethyl 5′-*O*-(4,4′-Dimethoxytrityl)thymidine-3′-yl Phosphonate (6). To a solution of the thymidine phosphoramidite unit **1** (470 mg, 0.63 mmol) in CH_3CN-H_2O (9:1, v/v, 3 mL) was added 1-*H*-tetrazole (88 mg, 1.26 mmol). After being stirred at room temperature for 10 min, the mixture was diluted with $CHCl_3$. The $CHCl_3$ layer was washed three times with brine, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel to give the title compound (354 mg, 85%): ^{1}H NMR ($CDCl_3$) δ 1.34 (s, 3H), 2.38−2.70 (m, 4H), 3.30−3.48 (m, 2H), 3.69 (s, 6H), 4.10−4.21 (m, 3H), 5.17 (s, 1H), 6.36 (dd, 1H, J = 5.4 Hz, J = 8.1 Hz), 6.76 (d, 4H, J = 8.6 Hz), 6.82 (d, 1H, J = 725.0 Hz), 7.06−7.45 (m, 10H), 9.77 (d, 1H, J = 11.3 Hz); ^{31}P NMR ($CDCl_3$) δ 7.57, 7.60; ^{13}C NMR ($CDCl_3$) δ 11.9, 19.8, 19.9, 20.1, 39.2, 55.4, 60.5, 60.6, 60.7, 63.1, 77.6, 84.3, 87.3, 111.7, 113.3, 116.5, 123.9, 125.3, 127.2, 128.0, 130.1, 135.0, 135.2, 149.2, 150.7, 150.8, 158.7, 164.1; MS *m*/*z* calcd for M + Na 684.2086, found 684.2135. Anal. Calcd for $C_{34}H_{36}N_3O_9P$: C, 61.72; H, 5.72; N, 6.29. Found: C, 61.73; H, 5.78; N, 6.35.

4-*N*-[5′-*O*-(4,4′-Dimethoxytrityl)thymidine-3′-yl](2-cyanoethyl)phosphoryl-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)-deoxycytidine (7). A mixture of the thymidine 3′-*O*-phosphoramidite unit **1** (500 mg, 0.67 mmol) and 3′,5′-*O*-bis(*tert*-butyldimethylsilyl)deoxycytidine **9** (204 mg, 0.449 mmol) was rendered anhydrous by repeated coevaporation successively three times each with dry pyridine, dry toluene, and dry CH_2Cl_2 and finally dissolved in dry CH_3CN (5 mL). To the solution was added 1*H*-tetrazole (70 mg, 0.674 mmol). After the mixture was stirred at room temperature for 1 h, a 1 M solution of I_2 in pyridine−H_2O (9:1, v/v, 3 mL) was added to the mixture. After being stirred at room temperature for 2 min, the mixture was diluted with $CHCl_3$. The $CHCl_3$ layer was washed three times with aqueous 5% $Na_2S_2O_3$, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (10 g) with hexane−$CHCl_3$ (50:50−0:100, v/v) containing 1% pyridine and then with MeOH−$CHCl_3$ (0:100−3:97, v/v) containing 1% pyridine to give the title compound (400 mg, 80%): ^{1}H NMR ($CDCl_3$) δ 0.07 (s, 6H), 0.10 (s, 6H), 0.79 (s, 9H), 0.82 (s, 9H), 1.39 (s, 3H), 2.10−2.81(m, 6H), 3.32−3.58 (m, 2H), 3.78 (s, 6H), 3.82−4.02 (m, 1H), 4.10−4.38 (m, 5H), 5.10−5.22 (m, 2H), 6.15−6.34 (m, 3H), 6.76−6.82 (m, 4H), 7.23−7.33 (m, 9H), 7.46 (s, 1H), 7.71 (m, 1H); ^{31}P NMR ($CDCl_3$) δ 5.28, 5.16; ^{13}C NMR ($CDCl_3$) δ −5.5, −5.4, −4.9, −4.6, 11.6, 17.9, 18.3, 19.5, 19.6, 25.6, 25.8, 39.1, 41.8, 55.2, 60.9, 61.0, 62.1, 63.2, 70.8, 76.9, 84.3, 84.5, 84.6, 85.4, 86.9, 87.7, 111.2, 113.1, 116.5, 126.9, 127.7, 129.8, 134.9, 135.0, 135.2, 140.2, 143.8, 143.9, 150.2, 150.3, 158.4, 159.9, 16.37; MS *m*/*z* calcd for M + H 1115.4747, found 1115.4748. Anal. Calcd for $C_{55}H_{75}N_6O_{13}PSi_2$: C, 59.33; H, 6.78; N, 7.53. Found: C, 59.80; H, 7.00; N, 7.30.

2-Cyanoethyl 5′-*O*-(4,4′-Dimethoxytrityl)thymidine-3′-yl Phosphate (8). To a solution of compound **6** (260 mg, 0.39 mmol) in pyridine−H_2O (9:1, v/v, 3 mL) was added a 0.1 M solution of I_2 in pyridine−H_2O (9:1, v/v, 20 mL). After being stirred at room temperature for 2 min, the mixture was diluted with $CHCl_3$. The $CHCl_3$ layer was washed three times with aqueous 5% $Na_2S_2O_3$, dried over Na_2SO_4, filtered, and evaporated under reduced pressure to give **8** (300 mg 99%): ^{1}H NMR ($CDCl_3$) δ 1.28−1.45 (m, 12H), 2.17−2.67 (m, 4H), 3.04 (dd, 1H, J = 7.29 Hz, J = 14.6 Hz), 3.38 (d, 1H, J = 8.91 Hz), 3.49 (d, 1H, J = 7.83 Hz), 3.78 (s, 6H), 3.98 (s, 2H), 4.29 (s, 1H), 4.97 (s, 1H), 6.42 (s, 1H), 6.83 (d, 4H, J = 8.91 Hz), 7.23−7.63 (m, 10H), 8.52 (br s, 1H), 12.23 (br s, 1H); ^{31}P NMR ($CDCl_3$) δ −1.17; ^{13}C NMR ($CDCl_3$) δ 8.6, 11.6, 19.8, 19.9, 45.6, 55.1, 60.1, 77.3, 84.4, 86.8, 110.9, 112.8, 113.0, 17.7, 125.0, 126.9, 127.7, 128.0, 128.8, 129.9, 135.1, 135.2, 135.3, 135.4, 144.0, 158.4, 163.9. Anal. Calcd for $C_{40}H_{51}N_4O_{10}P\cdot 1H_2O$: C, 60.29; H, 6.71; N, 7.01. Found: C, 60.39; H, 6.97; N, 6.52.

Typical Procedure for Solid-Phase Synthesis. Each cycle of chain elongation consisted of detritylation (3% trichloroacetic acid in CH_2Cl_2, 2 mL, 1 min), washing [CH_2Cl_2 (1 mL × 3), THF (1 mL × 3)], coupling [an appropriate phosphoramidite unit (20 μmol) in THF (200 μL), NBT (12.5 mg, 40 μmol) in THF (200 μL), 1 min], and washing (THF (1 mL × 3)), oxidation (0.1 M I_2, pyridine−H_2O (9/1, v/v), 2 min), and washing [pyridine (1 mL × 3), CH_3CN (1 mL × 3), CH_2Cl_2 (1 mL × 3)]. Generally, the average yield per cycle was estimated to be 97−99% by the DMTr cation assay. After chain elongation, the DMTr group was removed by treatment with 3% trichloroacetic acid in CH_2Cl_2 (2 mL) for 1 min, and the resin was washed with CH_2Cl_2 (1 mL × 3) and CH_3CN (1 mL × 3). The oligomer was deprotected and released from the polymer support by treatment with concentrated aq NH_3 (500 μL) for 40 min. The polymer support was removed by filtration and washed with CH_3CN (3 × 1 mL). The filtrate was evaporated and purified by reversed-phase HPLC or anion-exchange HPLC.

The Stability of the DMTr Group on Polymer Supports under Acidic Conditions Similar to Those Used for the Condensation of the Proton-Block Method. DMTr-ON T_{10} (1 μmol) on a HCP resin or CPG resin was synthesized by use of an ABI 392 DNA/RNA synthesizer. The resin was treated with a THF solution (400 μL) of NBT (12.4 mg, 40 μmol), diisopropylamine (2.8 μL, 20 μmol), and 5′-O-DMTr-deoxyadenosine (11.0 mg, 20 μmol) for 10 min. The oligomer was deprotected and released from the polymer support by treatment with concentrated aq NH_3 (500 μL) for 40 min. The polymer support was removed by filtration and washed with H_2O (1 mL × 3). The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in water and analyzed by reversed-phase HPLC.

The Stability of d[ApT] on Polymer Supports under Acidic Conditions Similar to Those Used for Detritylation in the Proton-Block Method. d[ApT] (1 μmol) without base protection on HCP were synthesized by use of the proton-block method. The resin was treated with a 0.2 M solution of NBT in THF (200 μL) or 3% trichloroacetic acid (400 μL). The dimer was deprotected and released from the polymer support by treatment with concentrated aq NH_3 (500 μL) for 40 min. The polymer support was removed by filtration and washed with H_2O (3 × 1 mL). The filtrate was evaporated and purified by anion-exchange HPLC.

Next [ApT] (1 μmol) with an *N*-benzoyl group on HCP were synthesized by use of the general procedure. Then the resin was treated with 3% trichloroacetic acid (400 μL). the dimer was deprotected and released from the polymer supports by treatment with concentrated aq NH_3 (500 μL) for 40 min. The

polymer support was removed by filtration and washed with H_2O (3 × 1 mL). The filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in water and analyzed by anion-exchange HPLC.

Polymer Support Synthesis of Oligodeoxynucleotides. d(ApT), mass (M + H) calcd 556.1558, found 556.1532; d(CpT) mass, (M + H) calcd.532.1445, found 532.1443; d(GpT), mass (M + H) calcd 572.1507, found 572.1475; d(ApA) calcd 565.1673, found 565.1618; d(CpC), mass (M + H) calcd 517.1449, found 517.1404; d(ApApApT), mass (M + H) calcd 1182.2710, found 1182.2849; d(CpCpCpT), mass (M + H) calcd 1110.2374, found 1110.2199; d(ApApApApApApT), mass (M − H) calcd 2119.43, found 2119.71; d(CpCpCpCpCpCpT), mass (M − H) calcd 1975.36, found 1975.50.

Enzyme Assay of Modified Oligonucleotides. The enzymatic digestion was performed by using an appropriate oligodeoxynucleotide (0.5 OD) snake venom phosphodiesterase (4 μL) and calf intestine alkaline phosphatase (2 μL) in 50 μL of alkaline phosphatase buffer (pH 9.0) at 37 °C for 40 min. After the enzymes were deactivated by heating at 100 °C for 1 min, the solution was diluted and filtered by a 0.45-μm filter (Millex-HV, MILLIPORE). The mixture was analyzed by reversed-phase HPLC. d(CpCpCpT): dC:T 3.00:1.12. d(ApApApT): dA:T 3.00:1.09. d(CpCpCpCpCpCpT): dC:T 6.0 0:0.97. d(ApApApApApApT): dA:T 6.00:1.11. d(CpApGpTpCpApGpTpCpApGpT): dA:dG:dC:T 1.01:1.21:1.00:0.95.

Acknowledgment. This work was supported by a Grant from "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS-RFTF97I00301) and by CREST of JST (Japan Science and Technology Corporation) for the Synthesis of DNA Oligomers on Polymer Supports.

Supporting Information Available: Basicity of nucleobases and promoters for activation of phosphoramidites in the proton-block approach (Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

JO034204K