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O-Selectivity and Utility of Phosphorylation Mediated by Phosphite Triester Intermediates in the N-Unprotected Phosphoramidite Method

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Abstract: Previously, O-selective phosphorylation on polymer supports in the N-unprotected phosphora-midite method could not be carried out because the amino groups of dA and dC have high reactivity toward tervalent phosphorus(III)-type phosphitylating reagents. In this paper, we developed a new coupling strategy named the "activated phosphite method" in which the phosphitylation is mediated by phosphite triester intermediates **1**. Application of 1-hydroxybenzotriazole as the promoter to the solid-phase synthesis resulted in excellent O-selectivity of more than 99.7%. This O-selectivity was explained by the frontier molecular orbital interactions between the reactive intermediates and the nucleophiles such as the amino or hydroxyl groups of nucleosides. Furthermore, longer oligonucleotides were synthesized not only by a manual operation but also by a DNA synthesizer. The utility of our new method was demonstrated by the successful synthesis of a base-labile modified oligodeoxyribonucleotide having 4-*N*-acetyldeoxycytidine residues. Finally, DNA 20-mers containing dA or dC could be synthesized in good yields by use of a combined reagent of 6-trifluoromethyl-1-hydroxybenzotriazole and benzimidazolium triflate.

Introduction

In the oligodeoxyribonucleotide synthesis reported to date,[1] the rapid alcoholysis of the P—N bonds has been a key reaction to achieve facile and high-yield formation of the internucleotidic linkage. In the current phosphoramidite approach,[2] P—N bond containing species which are generated by the activation of phosphoramidite units with various tetrazole,[2,3] triazole,[4] and imidazole derivatives[5] act as reactive intermediates. On the other hand, although the alcoholysis to give an internucleotidic bond by the use of reactive intermediates having P—O bonds has also been demonstrated by several authors in development of the

phosphotriester[6] and *H*-phosphonate methods,[1,7] much less interest has been focused on such P—O bond containing intermediates, especially in the phosphoramidite method.[8]

In this paper, we report the intriguing properties of the phosphite intermediates **1a**—**e** which have been discovered by us in our continuing studies to develop new methods for the synthesis of oligodeoxyribonucleotides in the N-unprotected approach.[9–13] These intermediates have a P—O bond activated by substituted benzotriazol-1-yl or 2,4-dinitrophenyl groups and

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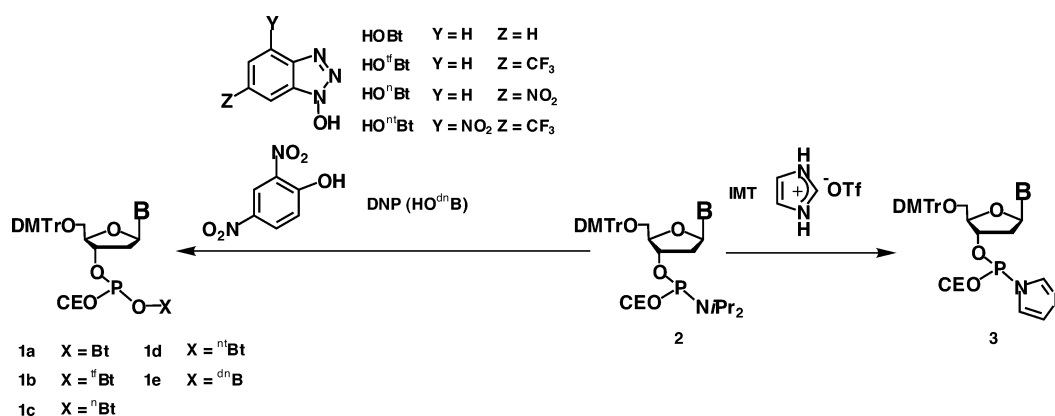
Scheme 1

Table 1. Selectivity of Condensation in the Activated Phosphite and Previous Methods

compd	polymer	final product	% ratio of the desired product						
			IMT	NBT	HOBt	HO^{tf}Bt	HO^{n}Bt	HO^{nt}Bt	DNP
9a	T-HCP	ApT	77.0%	99.2%	99.7%	99.3%	98.8%	96.1%	97.1%
9c	T-HCP	CpT	82.9%	99.0%	99.9%	98.9%	99.8%	92.4%	99.5%
9g	T-HCP	GpT	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%
4	d[ApT]-HCP	TpApT	90.5%	>99.9%	>99.9%	99.1%	97.5%	96.7%	99.6%
4	d[CpT]-HCP	TpCpT	9.7%	99.8%	>99.9%	98.7%	97.2%	64.7%	99.4%
4	d[GpT]-HCP	TpGpT	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%	>99.9%

can be readily generated by activation of the conventional phosphoramidite **2** with 1-hydroxybenzotriazole (HOBt) derivatives or phenol derivatives. Interestingly, the tervalent phosphite intermediates had highly chemoselective reactivity toward the 5′-hydroxyl group of deoxyribonucleosides over the amino group of unprotected nucleobases and allowed chain elongation without using any protecting groups for adenine, guanine, cytosine, and thymine. Moreover, it should be noted that, when HOBt was used as a promoter, the chemoselectivity was much higher than those of the previously reported N-unprotected DNA synthesis.[14]

Here, we report the interesting properties of the phosphite intermediates **1a–e** in detail in terms of reactivity, selectivity, and applicability to the automated synthesis of natural DNA. These unique and excellent results can be explained by ab initio molecular orbital calculations of the phosphite intermediates. Finally, the potential utility of our "activated phosphite method" was demonstrated by the successful synthesis of a modified DNA having base-labile substituents.
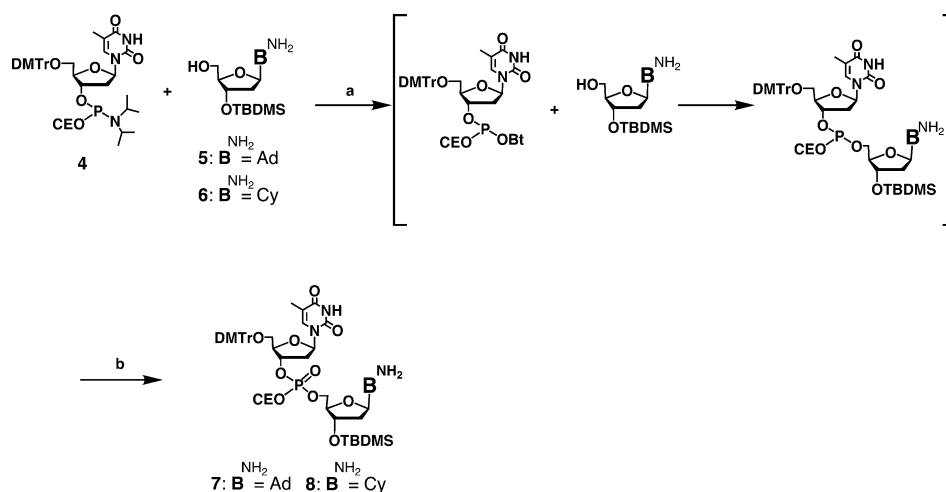
Results and Discussion

Selection of Activators. The activators tested in this study are summarized in Table 1. To find suitable activators for the

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synthesis of TpT on polymer supports, we screened 25 alcohol-type reagents (see Supporting Information). Among these phenol and HOBt derivatives, only five compounds [HOBt,[8a,b] 6-tri-fluoromethyl-HOBt (HO^{tf}Bt),[6c] 6-nitro-HOBt (HO^{n}Bt),[6b,c] 4-ni-tro-6-trifluoromethyl-HOBt (HO^{nt}Bt),[6a] and 2,4-dinitrophenol (DNP)[8c]] showed coupling yields of more than 95%. Although studies on the internucleotidic bond formation by use of HOBt and DNP have been previously reported in the conventional N-protected approach, their properties in the N-unprotected DNA synthesis have not been demonstrated. In Table 1, the results obtained by use of imidazolium triflate (IMT)[12] and 4-nitrobenzimidazolium triflate (NBT)[13] are also shown as references that exhibited relatively high O-selectivity via the formation of reactive intermediates having a P–N bond.
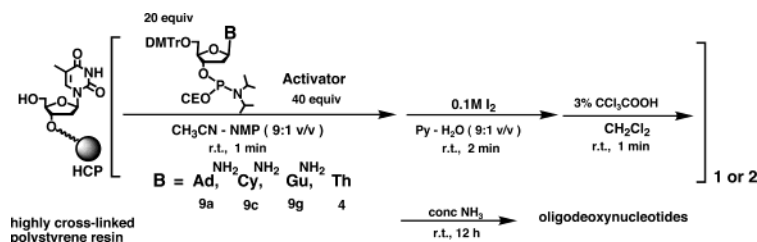
O-Selective Phosphorylation Promoted by HOBt in Solution-Phase Synthesis. The efficiency of HOBt as the promoter for the O-selective phosphorylation was demonstrated by the condensation of 1.2 equiv of the thymidine 3′-phosphoramidite unit **4** with 3′-O-(tert-butyldimethylsilyl)deoxyadenosine [3′-O-tBDMS-deoxyadenosine (**5**)] and 3′-O-tBDMS-deoxycytidine (**6**) in the presence of 2.2 equiv of HOBt in acetonitrile for 5 min at room temperature (RT), as shown in Scheme 2. The amino groups of the adenine and cytosine moieties were not blocked by any protecting groups. After the condensation, oxidation was carried out by use of I$_2$ in pyridine–water for 2 min at room temperature to give the desired d(TpA) and d(TpC) derivatives **7** and **8**. The N-phosphorylated byproducts could not be detected at all by ^{31}P NMR in these reactions. Compounds

Scheme 2 [a]

[a] Reagents and conditions: (a) HOBt, CH₃CN, RT, 5 min; (b) I₂, pyridine−H₂O (9:1, v/v), RT, 5 min.

Scheme 3

7 and **8** were isolated in 91 and 92% yields, respectively, by silica gel column chromatography. These results indicate that the condensation by using HOBt has both sufficient coupling efficiency and high O-selectivity at least under the solution-phase conditions.

O-Selective Phosphorylation Promoted by HOBt in Solid-Phase Synthesis. Next, we examined the O-selective inter-nucleotidic bond formation under solid-phase conditions according to a manual operation shown in Scheme 3. Although CPG resins[15] have been generally used in the synthesis of oligodeoxynucleosides by use of DNA synthesizers, we selected commercially available highly cross-linked polystyrene (HCP) resins[16] since the coupling on the HCP resin was very high and most suitable for the manual operation in our previous studies. In Scheme 3, the final deprotection and release of oligomers from the HCP resin were performed by treatment with concentrated NH₃ aq for 12 h because it took more prolonged time for removal of the cyanoethyl groups from the phosphoramidate residues formed on the nucleobases than those present in the internucleotidic phosphate (O−P(V)) bonds.

Initially, d[ApT] was synthesized on the T-loaded HCP resin by use of IMT as an activator. In this case, selectively O-phosphorylated derivatives (d[ApT] and d[CpT]) were formed with selectivity of only 77.0 and 82.9%, respectively, as shown in Figure 1a,b.

In contrast, the use of HOBt in place of IMT improved the O-selectivity remarkably, and the desired dimers d[ApT] and d[CpT] were formed with selectivity of 99.7 and 99.9%, respectively, even in the presence of a large excess amount of

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the phosphoramidite unit **9a** or **9c** (Figure 1e,f). These results clearly showed that the phosphite intermediate **1a** generated from the phosphoramidite building blocks has excellent chemo-selectivity toward the 5′-hydroxyl group on the resin under these solid-phase conditions.

Since the T-loaded resin was used in these reactions, no free nucleophilic amino groups were present on the HCP resin. The phosphoramidite units **9a** and **9c** have free amino groups, so it is somewhat difficult to estimate the contribution of the N-phosphorylation to the overall side reactions. Therefore, to clarify the reactivity of the phosphite intemediate **1a** generated by HOBt toward the unprotected amino groups of the DNA chain on the resin, we carried out the synthesis of d[TpApT] and d[TpCpT] by the condensation of d[Ap(ce)T]-HCP and d[Cp(ce)T]-HCP, which were synthesized in advance on the HCP resin by use of HOBt as the activator, with a large excess amount of thymidine 3′-phosphoramidite **4** in the presence of HOBt or IMT. These results are shown in Figure 1c,d,g,h. When IMT was used as the promoter, considerable side reactions were detected, as shown in Figure 1c,d. Particularly, the reaction of d[Cp(ce)T]-HCP with **4** gave extremely poor O-selectivity of 9.7% and the N-phosphorylated product (TpC^NHpTpT) was obtained as the major product, as shown in Figure 1d. On the other hand, when HOBt was used as the promoter, the amounts of such N-phosphorylated species became closer to the baseline level, as shown in HPLC profiles of Figure 1g,h, so that the O-selectivity in these cases was calculated to be more than 99.9%. Thus, d[TpApT] and d[TpCpT] were isolated in 89 and 83% yields, respectively.

Similarly, the O-selectivity of the HOBt-promoted phospho-rylation was studied in the d[GpT] and d[TpGpT] synthesis. As the result, N-phosphorylation of the guanine residue could
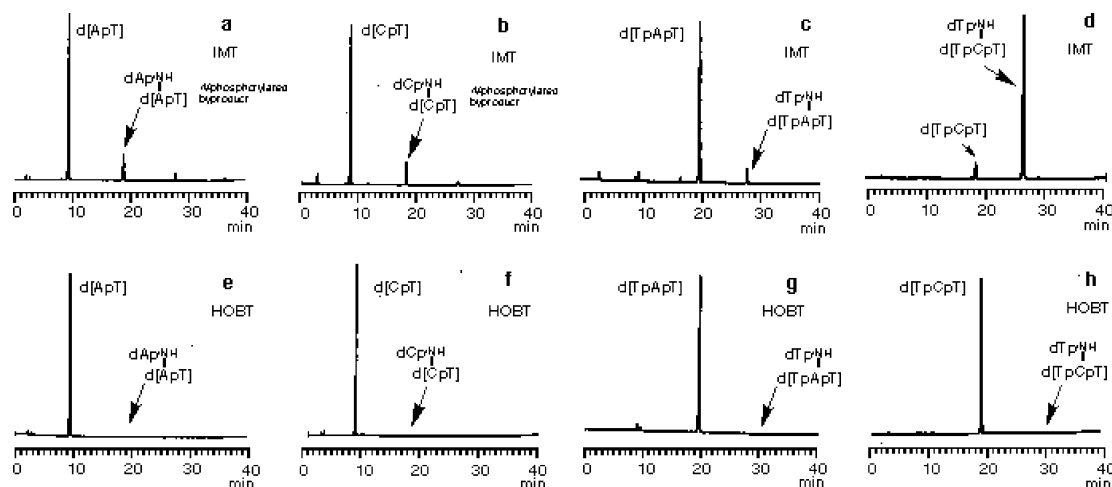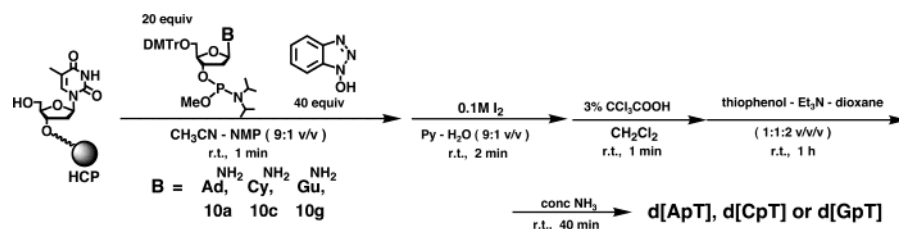
Figure 1. Anion-exchange HPLC profiles of the crude mixture from synthesis with unprotected nucleobases. Solvent system A was used for HPLC. (a) d[ApT], IMT; (b) d[CpT], IMT; (c) d[TpApT], IMT; (d) d[TpCpT], IMT; (e) d[ApT], HOBt; (f) d[CpT], HOBt; (g) d[TpApT], HOBt; (h) d[TpCpT], HOBt.

Scheme 4

not be essentially detected. This observation is in agreement with the previous results of low reactivity of free guanine residues.[10,12,13]

O-Selectivity of the Phosphorylation Promoted by Other Activators. Similar dimer and trimer syntheses were examined by use of other promoters such as NBT, HO^{tf}Bt, HO^{n}BT, HO^{nt}Bt, and DNP, and the results are listed in Table 1 together with the results of IMT and HOBt described above. The results are summarized as follows.

In the synthesis of d[ApT] or d[CpT], it was found that the activated phosphite method using alcohol-type activators dramatically increased the O-selectivity of the phosphorylation compared with that of IMT. (See the first and second rows of Table 1.) Particularly, in the synthesis of d[ApT] and d[CpT], HOBt suppressed N-phosphorylation about 77 and 170 times as much as IMT. It was also shown that HOBt was superior to NBT, used in the "proton-block" method previously reported by us,[13] in terms of O-selectivity. With an increase of the acidity of the promoter, the O-selectivity gradually decreased in the condensation mediated by HOBt derivatives in the order HOBt > HO^{tf}Bt > HO^{n}Bt > HO^{nt}BT. A similar trend was also observed for the synthesis of d[TpApT] and d[TpCpT]. Especially when highly acidic HO^{nt}Bt was used as the promoter in the synthesis of TpCpT, the O-selectivity was very poor among the promoters used in this study. However, the value is still ca. 6.6 times higher than that of IMT. The condensations mediated by DNP constantly showed higher O-selectivity than IMT and HO^{nt}Bt, while the rates are somewhat low compared with those of HOBt in all cases.

In the synthesis of d[GpT] (the third row in Table 1) by use of any promoter, there were also essentially no N-phosphorylated byproducts. On the basis of the data in Table 1, it was concluded that HOBt was the best in terms of the O-selective phospho-

rylation among the HOBt derivatives tested in this study followed by HO^{tf}Bt. It should be also noted that the "proton-block strategy" utilizing NBT also gave satisfactory results comparable to HO^{tf}Bt. The isolated yields in the synthesis of d[ApT], d[CpT], d[GpT], d[TpApT], d[TpCpT], and d[TpGpT] by use of the best activator HOBt were 92, 94, 90, 89, 83, and 84%, respectively.

Effects of Phosphate Protecting Groups on the O-Selectivity and Coupling Efficiency. The steric hindrance around the phosphorus atom generally affects the coupling efficiency in the phosphoramidite method.[17] The smaller the phosphate protecting group is, the higher the coupling efficiency is.[17a] Therefore, we examined whether the steric hindrance around the phosphorus atom affected not only the reactivity but also the O-selectivity in the activated phosphite method. Thus, we examined the O-selectivity of phosphorylation mediated by HOBt and the phosphoramidite unit **10** having a sterically compact methyl group instead of the more bulky 2-cyanoethyl group. The protocol is shown in Scheme 4. For the deprotection of the phosphate group, we used the conditions of thiophenol–triethylamine for 1 h at room temperature.

The O-selectivity in the synthesis of d[ApT], d[CpT], and d[GpT] showed 99.2, 98.9, and >99.9%, respectively, as calculated by the HPLC profiles shown in Figure 2. These results indicate that the O-selectivity of the activated phosphite method promoted by HOBt decreases slightly due to the steric hindrance of the phosphate protecting group and is as high as that obtained by HO^{tf}Bt, the second best activator in this study. Thus, the activated phosphite method allows the general use of N-

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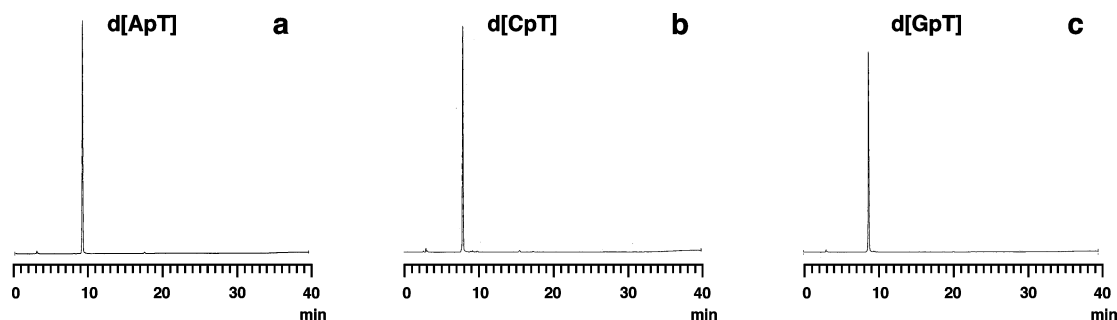
Figure 2. Anion-exchange HPLC profiles of the crude mixtures from synthesis by use of HOBt and N-unprotected phosphoramidite units **14a,c,g**. Solvent system A was used for HPLC. (a) d(ApT); (b) d(CpT); (c) d(GpT).
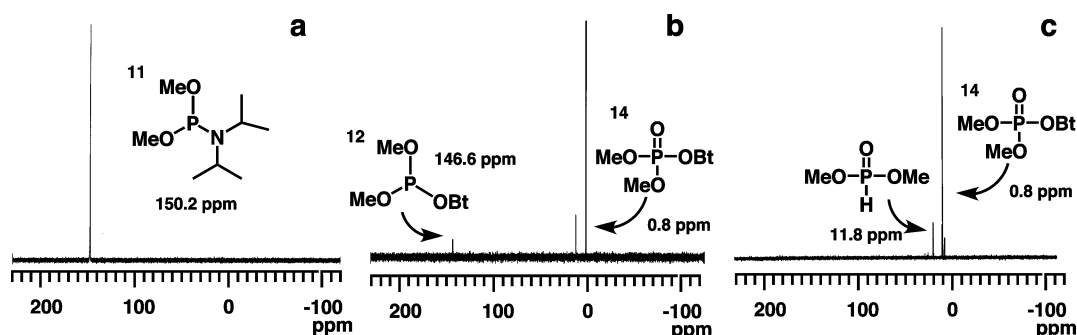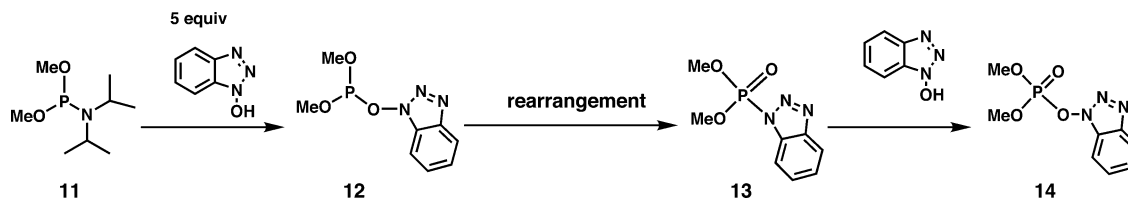

Figure 3. ^{31}P NMR spectra of (MeO)$_2$PN^{i}Pr$_2$ **15** (a) and crude mixture obtained after (MeO)$_2$PN^{i}Pr$_2$ **15** was activated by 0.25 M solution of HOBt in CD$_3$CN−NMP (9:1, v/v) at room temperature for 5 min (b) and for 30 min (c).

Scheme 5

unprotected phosphoramidite units with higher coupling efficiency.

Involvement of Pentavalent Intermediates in the HOBt-Mediated Phosphorylation. In the previous study, we found the conversion of the tervalent phosphite benzotriazol-1-yl ester into the corresponding pentavalent dialkyl phosphorobenzotriazolidate derivative.[11a,18] Actually, the phosphoramidite unit (MeO)$_2$PN^{i}Pr$_2$ **11** (^{31}P NMR: 150.2 ppm) was immediately converted to the corresponding phosphite compound (MeO)$_2$POBt **12** (^{31}P NMR: 146.6 ppm) by treatment with 5 equiv of HOBt as a promoter. The resulting phosphite (MeO)$_2$POBt **12** rapidly changed to the phosphotriester compound (MeO)$_2$P(O)OBt **14** (^{31}P NMR: 0.8 ppm) via the phosphoramidate compound (MeO)$_2$P(O)Bt **12**, as shown in Scheme 5 and Figure 3.

Since there was a possibility of the phosphorylation via the pentavalent phosphorus intermediates in the activated phosphite method, we tried to quantify the contribution of the pentavalent phosphorus intermediate to the overall condensation. For this purpose, we planned the synthesis of the Tp(s)T phosphorothioate dimer **15**, as shown in Scheme 6. When the phosphite intermediate reacts with the 5′-hydroxyl group of thymidine on HCP, the desired product **15** having a phosphorothioate internucleotidic bond (P−S) should be generated by Beaucage's

reagent.[19] On the other hand, when the pentavalent product **18** generated via the rearrangement reacts with the 5′-hydroxyl group, the undesired product **16** having a phosphate backbone (P−O) should be generated (Figure 4). Consequently, the ratio of the P−S compound **12** to the P−O compound **16** reflects the contribution of the pentavalent rearrangement products to the coupling reaction.

For example, the condensation by use of benzimidazolium triflate (BIT), which generates only the tervalent azolide intermediate, followed by the sulfurization resulted in the formation (98.6%) of the desired P−S compound **15** along with 1.4% of the P−O product, as determined from the integrated areas of these HPLC peaks (Figure 5). On the other hand, the use of HOBt as the promoter increased the P−O product to 4.9%. Although this result suggests that the intermediates using the HOBt system involve not only the phosphite benzotriazol-1-yl ester **17a** but also the pentavalent phosphorus compound **18a**, more than 95% of the hydroxyl groups on the HCP resin reacted with the trivalent phosphite intermediate in the HOBt-promoted phosphitylation.

Interestingly, the ratio in the condensation by use of HO^{tf}Bt remarkably increased up to 10%. This increase can be explained in terms of two mechanisms. One might be based on the

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(19) Iyer, R. P.; Egan, W.; Reagan, J. B.; Beaucage, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 1253−1254.
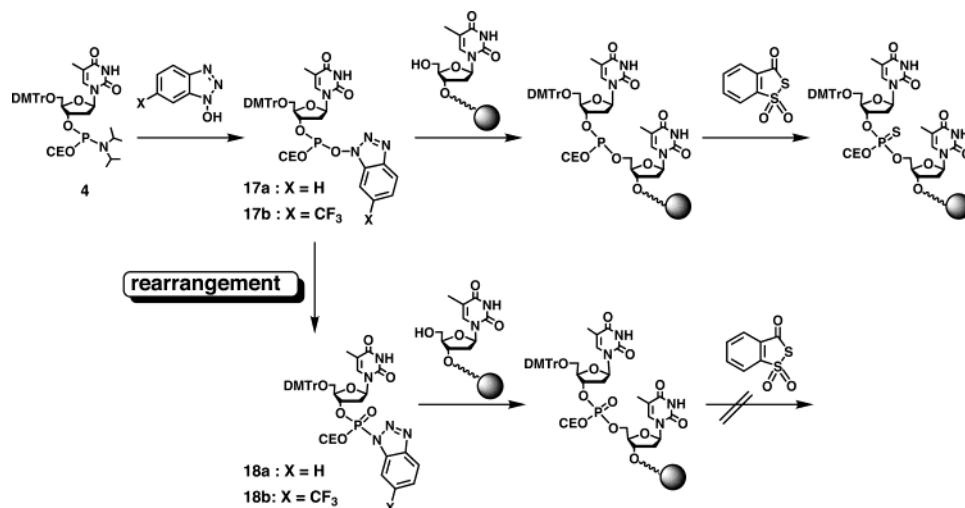
Figure 4. Reaction mechanism of the phosphorylation in the activated phosphite method.
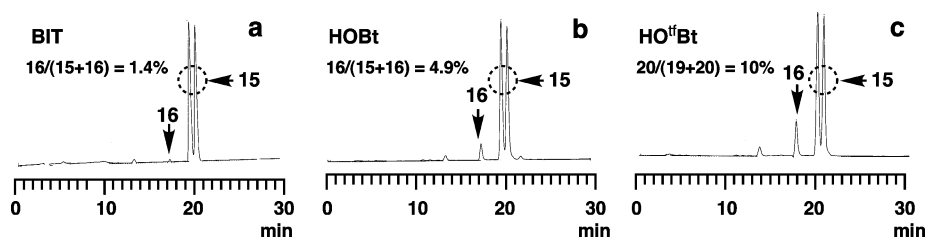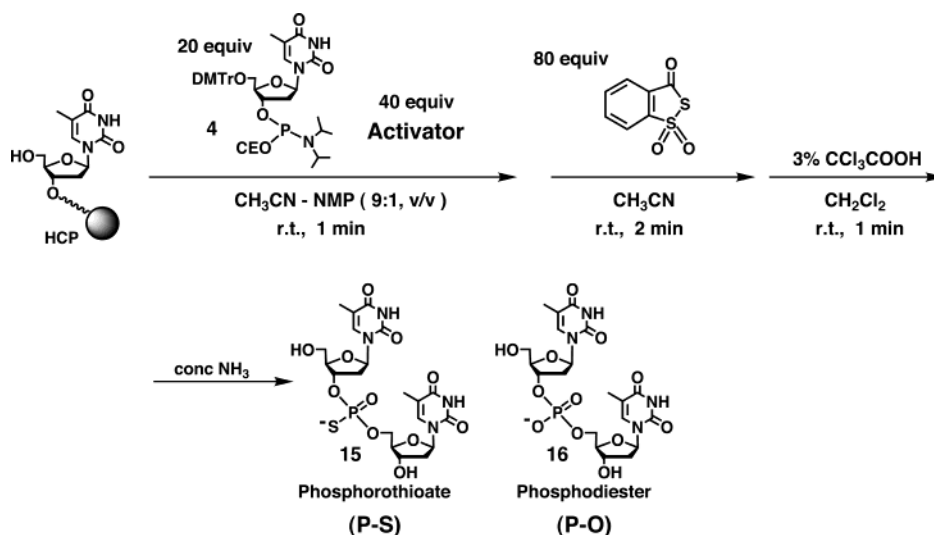

Figure 5. Reversed-phase HPLC profiles of the crude mixtures from synthesis of TpT having a P−S bond by use of BIT, HOBt, or HO^tfBt. Solvent system was used for HPLC. (a) BIT; (b) HOBt; (c) HO^tfBt.

Scheme 6

assumption that the rearrangement of the tervalent phosphite intermediate **17b** proceeded much faster than that of **17a**. The other might be explained by the assumption that the reactivity of the pentavalent phosphorus compound **18b** is much higher than that of **18a**. Our early study suggested that the rearrangement of a tervalent phosphite generated by HO^tfBt is about 6 times as fast as that by use of HOBt.[18] This result strongly supports the former mechanism.

Theoretical Studies of the Mechanism of the O-Selective Phosphorylation

Previously, we proposed the reaction mechanism for the O-selective condensation based on frontier molecular orbital

(FMO) interaction in our previous N-unprotected *H*-phosphonate method.[11a] Similarly, a plausible reaction mechanism for the O-selective phosphorylation via the phosphite intermediate can be considered as follows. In the first step of the phosphitylation, the lone pair of the phosphorus atom interacts with the hydrogen atom of the hydroxyl group. Since this interaction is expected to increase both the nucleophilicity of the hydroxyl oxygen and the electrophilicity of the phosphorus center, the successive nucleophilic attack of the hydroxyl group toward the phosphorus atom proceeded more readily. In contrast to the hydroxyl group, such an acid−base interaction must be less prominent for the amino group because of the lower acidity of the N−H proton. As a result, the amino groups become less reactive to the
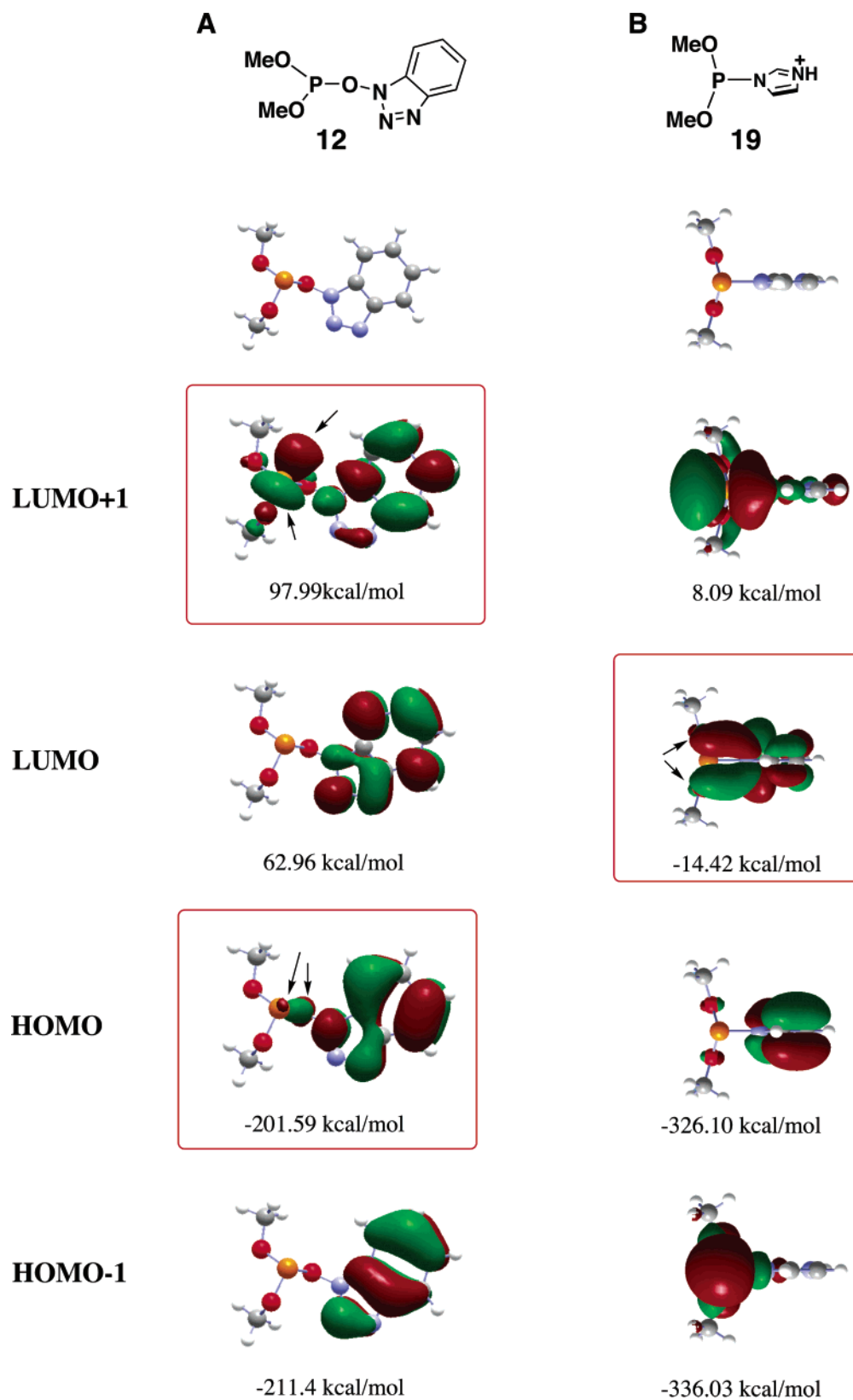
A

MeO
    \P—O—N
MeO

12

B

MeO
    \P—N⁺
MeO   NH

19

LUMO+1

97.99kcal/mol   8.09 kcal/mol

LUMO

62.96 kcal/mol   -14.42 kcal/mol

HOMO

-201.59 kcal/mol   -326.10 kcal/mol

HOMO-1

-211.4 kcal/mol   -336.03 kcal/mol

Figure 6. Optimized geometries and MOs of the reaction intermediates at the HF/6-31G* level: (A) phosphite intermediate **12**; (B) protonated imidazolide intermediate **19**. The MOs are visualized on the threshold of 0.030.

phosphite intermediates. (See the Supporting Information regarding the molecular orbitals (MOs) of the nucleobases and MeOH.)

To evaluate this hypothesis, the calculation of the MO of the phosphite intermediate model **12** at the HF/6-31G* level was

carried out. It was found that there was a large unoccupied orbital on the phosphorus center in the LUMO+1 of the phosphite intermediate **12**, as shown in Figure 6A. Considering the high energy of this LUMO+1 (97.99 kcal/mol), compound **12** seems less reactive toward the nucleophilic hydroxyl and
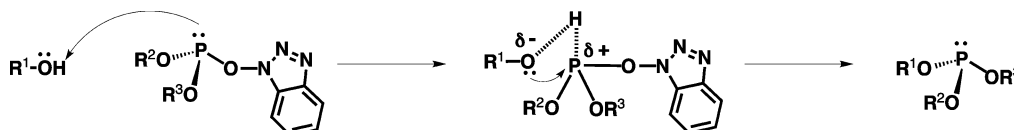
Figure 7. Reaction mechanism of phosphitylation mediated by the phosphite intermediate.

amino groups of deoxyribonucleoside derivatives. However, the intermediate **12** also has an occupied orbital on the phosphorus center which is included in the HOMO that can interact at the hydrogen atom with the LUMO of alcoholic nucleophiles, as shown in Figure 6. It is likely that such two simultaneous FMO interactions between the tervalent phosphorus intermediate and the alcoholic OH group account for the high O-selectivity in our method.

Similarly, it was suggested that the protonated imidazolide intermediate **19** has a very large unoccupied orbital on the phosphorus atom, as shown in Figure 6B. Considering the energy level of the LUMO of the imidazolide (−14.42 kcal/mol), the compound seems to be reactive enough to interact with the HOMO or HOMO−1 on the oxygen atoms of the hydroxyl groups and the nitrogen atoms of the amino groups. Furthermore, the protonation on the phosphorus atom of imidazolide intermediate **19** by the hydroxyl group as shown in Figure 7 seems less prominent because of the low energy level (−336.03 kcal/mol) of the HOMO−1 of the intermediate **19** which corresponds to the lone pair on the phosphorus center. Therefore, the use of such an acid−azole complex salt as the promoter must result in poor O-selectivity in the phosphorylation.

Utility of the Activated Phosphite Method for the Automated Synthesis of Longer and Modified Oligodeoxyribonucleotides. To demonstrate the superiority of our activated phosphite method, we previously reported the synthesis of d[AAAAAAT], d[CCCCCCT], d[GGTGGTGGT], and d[CAGTCAGTCAGT] by a manual operation, and the N-phosphorylation side reactions were checked by anion-exchange HPLC (see Supporting Information). Two consecutive couplings at each cycle were employed to increase the coupling efficiency of the condensation to as high as possible. In the synthesis of d[AAAAAAT], the desired product was obtained as the main peak. Likewise, there was no detectable peak arising from N-phosphorylation in the synthesis of d[CCCCCCT]. These results suggested that the N-phosphorylations were almost completely suppressed even in the successive sequence of dA or dC. In these cases, the coupling efficiency was more than 99% (trityl cation assay). d[AAAAAAT] and d[CCCCCCT] were satisfactorily isolated in 48 and 67% yields, respectively.

The amounts of the (n − 1)-mers lacking a nucleotide increased to some extent in the synthesis of G-rich sequence d[GGTGGTGGT] because of the somewhat lower average coupling yield, 98.1%. However, no N-phosphorylated byproduct was observed as in the cases of d[GpT] and d[TpGpT]. The excellent O-selective phosphorylation was also observed in the synthesis of d[CAGTCAGTCAGT]. Although (n − 1)-mer side products were observed because of the lower coupling efficiency at the dG positions, they could be separated by HPLC. The 12-mer was isolated in 36% yield. These oligomers were well characterized by enzyme digestion and MALDI-TOF mass spectroscopy.

Next, we tried to apply the activated phosphite method to the manipulation using a DNA synthesizer in order to elucidate

the practical usefulness of the method. Unexpectedly, it was found that the coupling efficiency by using HOBt as the promoter was not high enough when it was applied to an ABI 392 DNA synthesizer. The coupling efficiency was 97%, although two consecutive couplings at each cycle were employed. In our previous study, it was found that the use of more acidic promoters in the phosphoramidite chemistry could increase the coupling efficiency.[20] Therefore, we chose a more acidic activator, HO^{tf}BT. Consequently, it was found that this system gave the best result of high O-selectivity comparable with HOBt in the manual operation, as shown in Table 1. We used a 0.2 M solution of HO^{tf}Bt in CH_3CN−N-methyl-2-pyrrolidinone (NMP) (15:1, v/v) instead of a 0.45 M CH_3CN solution of 1*H*-tetrazole used in the protocol of the current DNA synthesis. The synthetic protocol was the same as the manual operation described above.

We reexamined which resin was the most suitable for the coupling by use of HO^{tf}Bt in the automated DNA synthesis. We synthesized TpT on the synthesizer in a single-coupling mode on HCP (ABI), primer support, TentaGel, ArgoPore, and CPG by use of a 0.2 M solution of HO^{tf}Bt in CH_3CN−NMP (15:1, v/v). As a result, the coupling yields were found to be 99.0, 96.4, 98.7, 88.0, and 97.8%, respectively. These results showed HCP was the choice of reagent in the automated activated phosphite method.

On the basis of these results, the automated synthesis of d[CAGTCAGTCAGT] on HCP was carried out by use of HO^{tf}Bt. The HPLC profile of the mixture obtained was similar to that of the 12-mer in the manual operation, as shown in Figure 8a. It was also confirmed that the N-phosphorylated byproducts are rarely observed. The isolated yield was 32%. This result indicates that the activated phosphite method with the use of HO^{tf}Bt can be applied to the DNA synthesizer system.

In the same protocol we also synthesized an α-oligodeoxyribonucleotide 12-mer d[TC*TTC*C*TTC*TTT] having 5-methylcytosine base (C*), which was frequently used in antisense or antigene method because the nucleobase has a stacking effect and is more basic than a cytosine base.[21] Since the 3-nitrogen atom of this nucleobase is protonated more easily than that of cytosine, a Hoogsteen base pair of this nucleobase with a guanine base is easily formed in DNA triple helix. It is also well-known that α-oligodeoxyribonucleotides have high resistance to nucleases. [22]

In the synthesis of the α-DNA 12-mer, the desired product was obtained as the main peak, as shown in Figure 8b. The N-phosphorylations were almost completely suppressed even in the presence of 5-methyl-dC. In these cases, the coupling efficiency was more than 99% (trityl cation assay). The α-DNA

(20) Moriguchi, T.; Yanagi, T.; Kunimori, M.; Wada, T.; Sekine, M. *J. Org. Chem.* **2000**, *65*, 8229−8338.
(21) (a) Kemal, O.; Brown, T.; Burgess, S.; Bishop, J.-D.; Leigh-Brown, A. J. *Nucleosides Nucleotides* **1991**, *10*, 555−561. (b) Morvan, F.; Rayner, B.; Imbach, J.-L. *Anti-Cancer Drug Des.* **1991**, *6*, 521−529.
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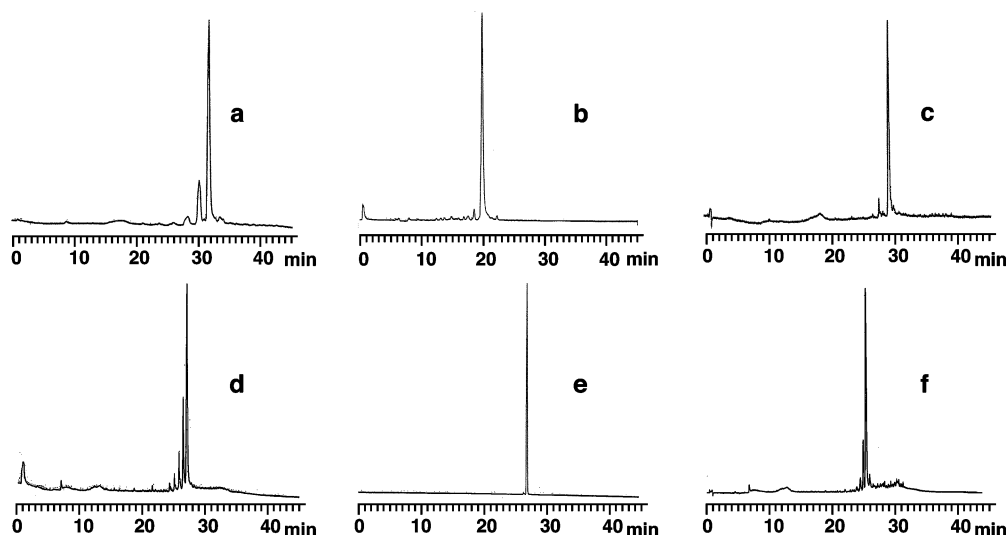
Figure 8. Anion-exchange HPLC profiles of the crude mixtures obtained by using a 0.2 M HO^{tf}Bt solution (method A) or a 0.2 M HO^{tf}Bt + BIT solution (method B) under the condition used for ABI 392 DNA synthesizer. Solvent system C or D was used for HPLC. (a) d(CAGTCAGTCAGT), method A, solvent system C; (b) α-d(TC*TTC*C*TTC*TTT), method A, solvent system D; (c) d(GA^{ac}ATCAGC^{ac}C^{ac}TCAT, method A, solvent system C; (d) d(CCCCCTTTTTCTCTCTCTCT), method A, solvent system D; (e) d(CCCCCTTTTTCTCTCTCTCT), method B, solvent system D; (f) d(TTAAAAAT-TATTAAATTATT), method B, solvent system D.

αDNA12mer (1)   3'-d(T p T p T p C*p T p T p C*p C*p T p T p C*p T)-5'   C* = 5-methylcytidine

βDNA12mer (2)   5'-d(T p T p T p C p T p T p C p C p T p T p C p T)-3'

target β ssDNA12mer   3'-d(A p A p A p G p A p A p G p G p A p A p G p A)-5'

target β dsDNA34mer
5'-d(Gp Cp Ap Ap Ap Gp Ap Ap Gp Gp Ap Ap Gp Ap C ^{T} T
3'-d(Cp Gp Tp Tp Tp Cp Tp Tp Cp Cp Tp Tp Cp Tp G $_T$ T

	target β ssDNA12mer [a]	T_m C° target β dsDNA34mer [b]		
		pH7.0	pH6.2	pH5.4
(1)	51.1	16.3	26.3	33.6
(2)	34.6	7.1	20.1	28.8

Figure 9. Oligonucleotides and T_m values of their duplexes or triplexes. (a) Experiments were carried out at 2 μM duplex concentration in a buffer containing 10 mM sodium phosphate, pH 7, 100 mM sodium chloride in the presence of 0.1 mM EDTA. (b) Experiments were carried out at 2 μM triplex concentration in a buffer containing 10 mM sodium cacodylate, 100 mM sodium chloride.

12-mer α-d[TC*TTC*C*TTC*TTT] was satisfactorily isolated in 61% yield. This oligomer was characterized by MALDI-TOF mass spectroscopy. This α-DNA 12-mer had a high ability of duplex formation (parallel strand duplex) or triplex formation toward ssDNA or dsDNA having the compensate sequence compared with the *β*-DNA 12-mer d[TTTCTTCCTTCT], as shown in Figure 9.

Furthermore, we demonstrated the synthesis of a modified DNA 13-mer d[GC^{ac}ATCAGC^{ac}C^{ac}TCAT] having three 4-*N*-acetyldeoxycytidine residues[23] (dC^{ac}). We previously reported that acetylation of the 4-*N*-amino group of deoxycytidine stabilizes DNA duplexes, and the base recognition ability of this modified base is very similar to that of deoxycytidine. However, this *N*-acetyl group is sensitive to basic conditions that are required for removal of N-protecting group in the conventional DNA synthesis. Because the deacylation of the acetyl group occurs in aqueous ammonia, we used our new original T-loaded HCP resin having a silyl-type linker[24] which could be cleaved by treatment with tetrabutylammonium fluoride

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(24) Kobori, A.; Miyata, K.; Ushioda, M.; Seio, K.; Sekine, M. *Chem. Lett.* **2002**, 16−17.
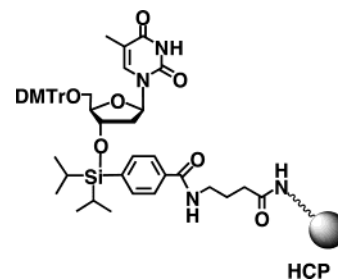
Figure 10. New original T-loaded HCP resin having a silyl-type linker.

(TBAF) without using aqueous ammonia, as shown in Figure 10. (Detailed information on this linker will be reported in another paper.) The protocol of the synthesis was the same as that of the synthesis on the HCP resin containing a succinate linker described above. After the final condensation, the cyanoethyl groups were removed by treatment with a 10% solution of 1,8-diazabicyclo[5,4,0]-7-undecene (DBU) in CH$_3$-CN at room temperature for 1 min. The crude oligodeoxyribonucleotide was released from the resin by treatment with a 1 M TBAF−AcOH solution in THF, and analyzed by HPLC (Figure 8c). The panel 8c shows both excellent O-selectivity and high coupling efficiency. The modified 13-mer was isolated in 33%

DNA13mer	5'-d(G p X p ApT p C p Ap G p X p Xp T pC p Ap T)-3'	
target DNA13mer	3'-d(C p Gp TpA p Gp T pC pG p Gp ApG p Tp A)-5'	

X	Tm℃	ΔTm℃
C	56.2	—
C^{ac}	59.9	+ 3.7

Figure 11. Oligonucleotides and T_m values of their duplexes. Experiments were carried out at 2 μM duplex concentration in a buffer containing 10 mM sodium phosphate, pH 7, 100 mM sodium chloride in the presence of 0.1 mM EDTA.
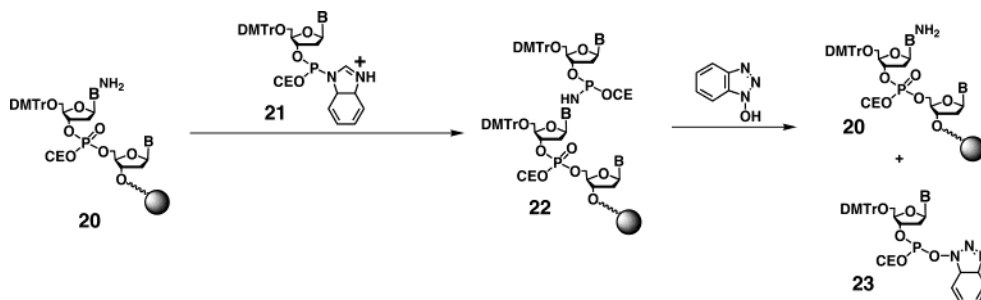

Figure 12. Possiblility of cleavage of N−P(III) bonds in activated phosphite.

yield. This oligomer was characterized by MALDI-TOF mass spectroscopy.

This 13-mer proved to have strong affinity for ssDNA having the complementary sequence compared with DNA 13-mer d[GCATCAGCCTCAT] without an *N*-acetyl group, as shown in Figure 11.

The above-mentioned successful synthesis of the base-labile modified deoxyoligoribonucleotide strongly demonstrates the usefulness of the "activated phosphite" method for the synthesis of base-labile functional DNA molecules.

Further Improvement of Activated Phosphite Approach. Furthermore, a protocol for the automated DNA synthesis using a 0.2 M solution of HO^{tf}Bt was applied to the synthesis of a DNA 20-mer d[CCCCCTTTTTCTCTCTCTCT], as shown in Figure 8d. The amounts of the $(n − 1)$-mers lacking a nucleotide increased to some extent in the HCP profile because the coupling efficiency was not complete.

We have also made strenuous efforts to increase the coupling efficiency in our activated phosphite approach. As a result, it turned out that the coupling efficiency significantly increased when the condensation was carried out in the presence of benzimidazolium triflate (BIT) in addition to HO^{tf}Bt. The desired oligomer was obtained as a main peak with very high coupling efficiency and O-selectivity, as shown in Figure 8e. Actually, the 20-mer could be isolated in 81% yield. This result strongly suggests that the activated phosphite method could make the synthesis of antigene molecules having only pyrimidine sequences very facile and immediate. Under similar conditions using a combined reagent of HO^{tf}Bt + BIT, a DNA 20-mer d[TTAAAAATTATTAAATTATT] was also synthesized (Figure 8f). Although both the coupling efficiency and the O-selectivity were somewhat lower, we could synthesize the desired compound as a major product without P−N bond cleavage and capping treatment. This oligomer was isolated in 24% yield and characterized by enzyme digestion and MALDI-TOF mass spectroscopy.

Possibility of Cleavage of N−P(III) Bonds in Activated Phosphite Approach. Although the condensation in the presence of benzimidazolium triflate (BIT) in addition to HO^{tf}Bt

was the best in our strategy as described above, there was a possibility that, even if the branched chain species **22** is formed by reaction of a dimer **20** with an activated phosphoramidite derivative **21**, it might be converted to the parent material **20** and the activated phosphite triester **23** due to the reaction of N−P(III) bond cleavage by HOBt, as shown in Figure 12.

Therefore, we carried out the experiment as shown in Scheme 7 to examine whether cleavage of N−P(III) bonds occurred in the coupling reaction. Three consecutive condensations of a Cp(OCE)T-loaded HCP resin **24** with the thymidine phosphoramidite unit **4** in the presence of IMT as a promoter were carried out to generate an N−P(III) bond on the cytosine base. A mixture **24** and **25** of the resin having the N−P(III) bond was divided into three portions. The first portion was oxidized and deprotected (method A). The second was treated with HOBt before oxidation (method B). The last was treated with the phosphoramidite unit **4** in the present of HOBt before oxidation (Scheme 7).

Method A gave a 2:3 mixture of C^{NH_2}pT **27** and C^{NHpT}pT **26**, as shown in Figure 13a. However, it turned out that posttreatment with 20 equiv of HOBt in CH₃CN for 1 min resulted in cleavage of the N−P(III) bond of **25** on polymer supports, as shown in Figure 13b. The efficiency of the cleavage was determined to be 85% from the integrated areas of HPLC peaks of C^{NH_2}pT **27** and C^{NHpT}pT **26** compared with Figure 13a. On the other hand, posttreatment of a mixture of **25** and **24** with the T-phosphoramidite unit and HOBt gave a result similar to that of method B, as shown in Figure 13c. These results showed that HOBt in the activated phosphite approach could promote not only O-selective phosphitylation but also cleavage of the N−P(III) bond.

In conclusion, even if the N−P(III) bond is formed on the cytosine residue, it can be removed by the action of HOBt regardless of the presence or absence of phosphoramidite units to regenerate the parent N-unprotected oligonucleotides with the release of an activated phosphite triester like **23**. This is the essential reason the condensation of the present approach by use of BIT + HO^{tf}Bt as promoters also retained high O-selectivity in the internucleotidic bond formation.
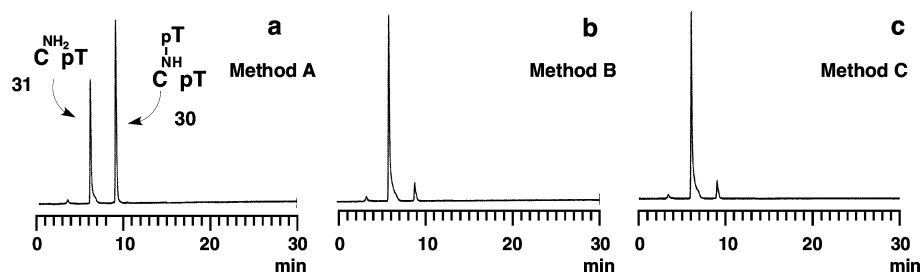
Figure 13. Anion-exchange HPLC profiles of the crude mixtures obtained by three condensations of a CpT derivative loaded HCP resin with the thymidine phosphoramidite unit in the presence of IMT(40 equiv). (a) The mixture obtained when the posttreatment was not carried out after the condensation. (b) The mixture obtained when the posttreatment with HOBt (20 equiv) in CH$_3$CN at room temperature for 1 min was carried out after the condensation. (c) The mixture obtained when the posttreatment with HOBt (40 equiv) and T-phosphoramidite (20 equiv) in CH$_3$CN at room temperature for 1 min was carried out after the condensation.

Scheme 7
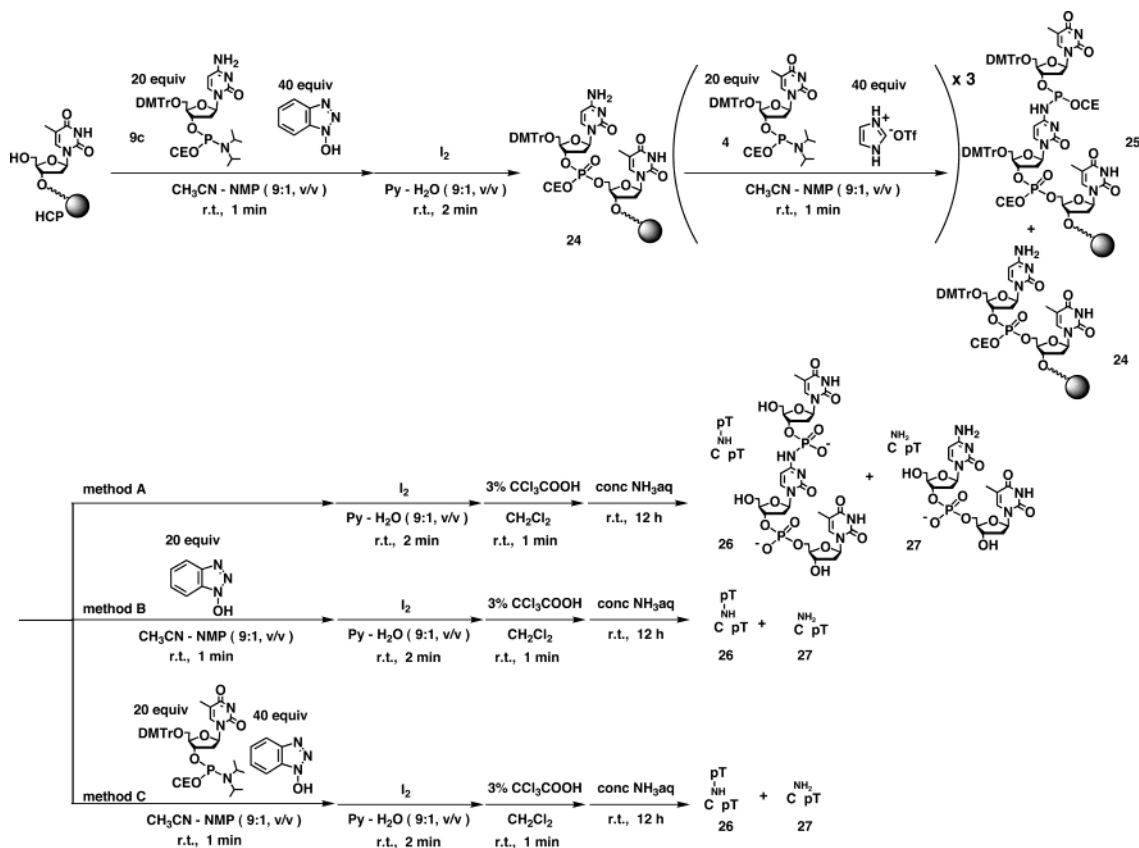
Experimental Section

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 for ^{1}H NMR spectra, CDCl$_3$ (77 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. 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. HPLC was performed using the following systems. System A: Anion-exchange HPLC was done on an 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$_3$CN in 0.5 M potassium phosphate) in solvent II (20% CH$_3$CN in 0.005 M potassium phosphate) was used at 50 °C at a rate of 1.0 mL/min for 30 min. System B: Reversed-phase HPLC was done on a Waters Aliance system with a Shimadzu 3D UV detector and a Gen-PakTM FAX column (Waters, 4.6 × 100 mm) Waters 3D UV detector and a μBondasphere 5μ C18 100 Å column (3.9 × 150 mm). A linear gradient

(0–30%) of solvent III (CH$_3$CN) in solvent IV (0.1 M NH$_4$Osc buffer (pH 7.0)) was used at 50 °C at a flow rate of 1.0 mL/min for 30 min. System C: 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 V (1 M NaCl in 25 mM phosphate buffer (pH 6.0)–10% CH$_3$CN) in solvent VI (25 mM phosphate buffer (pH 6.0)–10% CH$_3$CN) was used at 50 °C at a flow rate of 1.0 mL/min for 45 min. System D: 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 (0–80%) solvent V (1 M NaCl in 25 mM phosphate buffer (pH 6.0)–10% CH$_3$CN) in solvent VI (25 mM phosphate buffer (pH 6.0)–10% CH$_3$CN) was used at 50 °C at a flow rate of 1.0 mL/min for 45 min. ESI mass spectrometry was performed by use of a Mariner (PerSeptive Biosystems Inc.). MALDI-TOF mass was performed by use of a Voyager RP (PerSeptive Biosystems Inc.). Highly cross-linked polystyrene was purchased from Perkin-Elmer, ABI. The thymidine-3′-*O*-phosphoramidite was prepared according to the standard method. The deoxycytidine-, deoxyadenosine-,

Table 2. Reaction Cycle for the Synthesis of Oligodeoxyribonucleotides by Use of ABI 392 DNA Synthesizer

step	operation	reagent(s)	time, min
1	washing	CH_3CN	0.2
2	detritylation	3% Cl_3CCOOH/CH_2Cl_2	1.5
3	washing	CH_3CN	0.4
4	coupling	0.1 M amidite + 0.2 M $HO^{tf}Bt$ in $CH_3CN-NMP$ (15:1, v/v)	1.0
5	coupling	0.1 M amidite + 0.2 M $HO^{tf}Bt$ in $CH_3CN-NMP$ (15:1, v/v)	1.0
6	washing	CH_3CN	0.2
7	oxidation	0.1 M I_2 in $Py-H_2O-THF$ (v/v/v)	0.5
8	washing	CH_3CN	0.4

and deoxyguanosine-*O*-3′-phosphoramidite derivatives were synthesized by the method reported by Gryaznov and Letsinger.[10]

Ab Initio Calculations. All ab initio molecular orbital calculations were carried out using the Gaussian 94 program on a Cray C-916/12256 supercomputer. Geometry optimizations were carried out at the HF/6-31G* level and single-point energy calculations were carried out at the MP2/6-31G* level involving electronic correlation to obtain accurate energies and atomic charges.

Synthesis of the N-Unprotected d[TpA] Derivative. A mixture of the thymidine 3′-*O*-phosphoramidite derivative **4** (267 mg, 0.36 mmol) and 3′-*O*-(*tert*-butyldimethylsilyl)deoxyadenosine **5** (109 mg, 0.3 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 CH_3CN (5 mL). To the mixture was added HOBt (97 mg, 0.72 mmol). After the mixture was stirred at room temperature for 5 min, a 1 M solution of I_2 in 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 **7**. To remove the last traces of pyridine, the fractions were collected, evaporated under reduced pressure, and finally evaporated by repeated coevaporation three times each with toluene and CH_2Cl_2 to give **7** (280 mg, 91%): ¹H NMR ($CDCl_3$) δ 0.11 (s, 6H), 0.90 (s, 6H), 1.40 (s, 3H), 2.30–2.95 (m, 6H), 3.28–3.47 (m, 2H), 3.76 (s, 6H), 4.01–4.30 (m, 6H), 4.73 (d, 1H, *J* = 2.4 Hz), 5.11 (s, 1H), 6.33–6.52 (m, 4H), 6.80 (dd, 4H, *J* = 3.0 Hz, *J* = 9.0 Hz), 7.23–7.33 (m, 9H), 8.02 (d, 1H, *J* = 2.7 Hz), 8.36 (d, 1H, *J* = 4.59 Hz); ³¹P NMR ($CDCl_3$) δ −2.19, −1.84; ¹³C NMR ($CDCl_3$) δ −4.7, −4.5, 11.7, 11.8, 17.9, 19.1, 19.4, 19.5, 19.6, 25.7, 39.8, 39.9, 55.2, 62.0, 62.1, 62.3, 63.1, 71.5, 71.7, 77.2, 79.1, 79.4, 83.8, 83.9, 84.2, 84.4, 85.1, 86.9, 87.0, 111.4, 111.5, 113.2, 115.9, 116.2, 119.4, 119.6, 125.1, 127.0, 127.8, 128.0, 128.1, 128.9, 129.9, 134.9, 139.3, 139.5, 143.8, 143.9, 148.9, 151.1, 151.3, 152.6, 155.7, 158.5, 164.5, 164.7. MS *m/z* calcd for M + H 1025.3994, found 1025.3987.

In a similar manner, the N-unprotected d[TpC] derivative **8** was synthesized: ¹H NMR ($CDCl_3$) δ −0.01 (s, 6H), 0.86 (s, 9H), 1.29 (s, 3H), 2.11–2.71 (m, 6H), 3.33 (d, 1H, *J* = 8.6 Hz), 3.45 (d, 1H, *J* = 9.7 Hz), 3.67 (s, 6H), 3.92 (s, 1H), 4.13–4.23 (m, 5H), 5.10–5.22 (m, 1H), 5.92 (dd, 1H, *J* = 7.1 Hz, *J* = 17.6 Hz), 6.15 (m, 1H), 6.36 (dd, 1H, *J* = 5.1 Hz, *J* = 8.9 Hz), 6.77 (d, 4H, *J* = 8.9 Hz), 7.23–7.33 (m, 9H), 7.47 (s, 1H), 7.61 (dd, 1H, *J* = 4.3 Hz, *J* = 7.3 Hz); ³¹P NMR ($CDCl_3$) δ −1.67, −1.58; ¹³C 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, 70.0, 70.4, 77.2, 79.9, 84.2, 84.4, 86.1, 94.9, 111.8, 113.2, 116.2, 116.4, 123.6, 127.1, 127.9, 128.0, 128.9, 129.9, 134.8, 134.9, 135.8, 140.3, 143.8, 149.6, 150.6, 150.7, 155.3, 158.6, 163.7, 163.8, 165.5. MS *m/z* calcd for M + H 1001.3882, found 1001.3876.

Typical Procedure for Solid-Phase Synthesis. A. Manual Operation. A thymidine-loaded HCP (1 µmol, 28 µmol/g, succinate linker) was used. Each cycle of chain elongation consisted of the following

steps: (1) detritylation (3% trichloroacetic acid in CH_2Cl_2, 2 mL, 1 min); (2) washing [CH_2Cl_2 (1 mL × 3), CH_3CN (1 mL × 3)]; (3) the first coupling [an appropriate phosphoramidite unit (10 µmol) in $CH_3CN-NMP$ (150 µL, 9:1, v/v), HOBt (6.3 mg, 20 µmol) in $CH_3CN-NMP$ (150 µL, 9:1, v/v), 1 min]; (4) washing [CH_3CN (1 mL × 3)]; (5) the second coupling [an appropriate phosphoramidite unit (10 µmol) in $CH_3CN-NMP$ (150 µL, 9:1, v/v), HOBt (6.3 mg, 20 µmol) in CH_3-$CN-NMP$ (150 µL, 9:1, v/v), 1 min]; (6) washing [CH_3CN (1 mL × 3)]; (7) oxidation [0.1 M I_2, pyridine–H_2O (9/1, v/v), 2 min]; and (8) 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 98–99% by the DMTr cation assay. After chain elongation was finished, 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 NH_3 aq (500 µL) for 40 min or 12 h. The polymer support was removed by filtration and washed with CH_3CN (1 mL × 3). The filtrate was evaporated and purified by reversed-phase HPLC or anion-exchange HPLC.

On the other hand, in synthesis of the dimers d[ApT], d[CpT], and [GpT] by use of the phosphoramidite units **14a,c,g** having a methyl group as the protecting group of the phosphate group, 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) after chain elongation was finished. The methyl group was deprotected by treatment with the solution of thiophenol–triethylamine–dioxane (1:1:2, v/v/v, 400 µL) for 1 h at room temperature, 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 NH_3 aq (500 µL) for 40 min or 12 h. The polymer support was removed by filtration and washed with CH_3CN (1 mL × 3). The filtrate was evaporated and purified by reversed-phase HPLC or anion-exchange HPLC.

B. Synthesis of Phosphorothioate Thymidine Dimer Tp(s)T 15. A thymidine-loaded HCP (1 µmol, 28 µmol/g, succinate linker) was used. Each cycle of chain elongation consisted of the following steps: (1) detritylation (3% trichloroacetic acid in CH_2Cl_2, 2 mL, 1 min); (2) washing [CH_2Cl_2 (1 mL × 3), CH_3CN (1 mL × 3)]; (3) coupling [thymidine phosphoramidite unit (14.8 mg, 20 µmol) in $CH_3CN-NMP$ (150 µL, 9:1, v/v), activator (20 µmol) in $CH_3CN-NMP$ (150 µL, 9:1, v/v), 1 min]; (4) washing [CH_3CN (1 mL × 3)]; (5) sulfurization [0.2 M 3*H*-1,2-benzodithiol-3one-1,1-dioxaide, CH_3CN (400 µL), 2 min]; and (6) washing [pyridine (1 mL × 3), CH_3CN (1 mL × 3), CH_2Cl_2 (1 mL × 3)]. After chain elongation was finished, 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 NH_3 aq (500 mL) for 40 min. The polymer support was removed by filtration and washed with CH_3CN (1 mL × 3). The filtrate was evaporated and purified by reversed-phase HPLC.

C. Automated Operation. The synthesis of oligodeoxyribonucleotides by use of ABI 392 DNA synthesizer was carried out according to the reaction cycle shown in Table 2. In the synthesis of d(CAGT)₃,

the oligomer after chain elongation was deprotected and released from the polymer support by treatment with concentrated NH_3 aq (500 μL) for 40 min. The polymer support was removed by filtration and washed with CH_3CN (1 mL × 3). The filtrate was evaporated and purified by reversed-phase HPLC or anion-exchange HPLC.

On the other hand, in the synthesis of d[GC^{ac}ATCAGC^{ac}C^{ac}TCAT], the oligomer after chain elongation was deprotected by treatment with a 10% DBU solution in CH_3CN (500 μL) for 1 min. Then, the oligodeoxyribonucleotide mixture was released from the resin by treatment with a 1 M TBAF−AcOH (1:1) solution in THF (500 μL) for 1 h. The polymer support was removed by filtration and washed with CH_3CN (1 mL × 3). The filtrate was evaporated and purified by reversed-phase HPLC or anion-exchange HPLC.

In the synthesis of d[CCCCCTTTTTCTCTCTCTCT] and d[T-TAAAAATTATTAAATTATT] a solution of HO^{tf}Bt (0.2 M) + BIT (0.2 M) in CH_3CN−NMP (15:1, v/v) was used as a promoter instead of a 0.2 M solution of HO^{tf}Bt.

The mass spectral data of oligodeoxynucleotides are as follows: d[ApT] mass (M + H) calcd 556.1558, found 556.1523; d[CpT] mass (M + H) calcd 532.1445, found 532.1443; d[GpT] mass (M + H) calcd 572.1507, found 572.1503; d[Tp(s)T] **15** mass (M − H) calcd 561.4794, found 561.5063; d[TpT] **16** mass (M − H) calcd 545.4135, found 545.5045; [TpApT] mass (M + H) calcd 860.2017, found 572.1503; [TpCpT] mass (M + H) calcd 836.1905, found 836.1918; [TpGpT] mass (M + H) calcd 876.1967, found 876.2162; d[AAAAAAT] mass (M − H) calcd 2119.43, found 2119.12; d[CCCCCCT],mass (M − H) calcd 1975.36, found 1975.22; d[CAGTCAGTCAGT] mass (M − H) calcd 3643.65, found 3642.62; d[GC^{ac}ATCAGC^{ac}C^{ac}TCAT] mass (M − H) calcd 4017.72, found 4018.85; d[TC*TTC*C*TTC*TTT] mass (M + H) calcd 3584.41, found 3584.37; d[CCCCCTTTTCTCTCTCTCT] mass (M + H) calcd 5868.23, found 5869.92; d[TTAAAAATTAT-TAAATTATT] mass (M + Na) calcd 6130.31, found 6132.69.

Enzyme Assay of 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 [500 mM Tris·HCl (pH 9.0), 10 mM $MgCl_2$] 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. For d[CCCCCCT], dC:T = 6.00:1.03; for d[AAAAAAT], dA:T = 6.00:0.97; for d[CAGT-CAGTCAGT], dA:dG:dC:T = 1.00:1.20:0.95:0.94.; for d[CCCCC-TTTTCTCTCTCTCT], dC:T = 1.00:0.99; for d[TTAAAAATTAT-TAAATTATT], dA:T =1.00:0.94.

T_m **Measurement. A. Duplex.** An appropriate oligonucleotide (2 μmol) and its complementary ssDNA 12-mer (2 μmol) were dissolved in a buffer consisting of 150 mM NaCl, 10 mM sodium phosphate, and 0.1 mM EDTA adjusted to pH 7.0. The solution was kept at 80 °C for 10 min for complete dissociation of the duplex to single strands, cooled at the rate of −1.0 °C/min, and kept at 15 °C for 10 min. After that, the melting temperatures (T_m) were determined at 260 nm using a UV spectrometer (Pharma Spec UV-1700, Shimadzu) by increasing the temperature at the rate of 1.0 °C/min.

B. Triplex. An appropriate oligonucleotide (2 μmol) and its complementary hairpin DNA 34-mer (2 μmol) were dissolved in three kinds of buffer consisting of 150 mM NaCl, 10 mM sodium phosphate, and 0.1 mM EDTA adjusted to pH 7.0, 6.2, or 5.4. The solution was kept at 80 °C for 10 min for complete dissociation of the triplex to single strands, cooled at the rate of −0.5 °C/min, and kept at 0 °C for 10 min. After that, the melting temperatures (T_m) were determined at 260 nm using a UV spectrometer (Pharma Spec UV-1700, Shimadzu) by increasing the temperature at the rate of 0.5 °C/min.

Conclusion

For the first time, we have showed a new method for phosphorylation giving the hitherto highest O-selectivity and high coupling yield by use of HOBt or 6-trifluoromethyl-HOBt. This new approach enabled us to synthesize medium-sized oligodeoxyribonucleotides without using base protecting groups in both the solution phase and the automated solid phase. We proposed the reaction mechanism of the high O-selectivity by considering the FMO interactions between the reaction intermediates and the nucleophiles on the basis of the quantum chemical calculations. It is expected that more ideal tervalent phosphorus intermediates can be designed on the basis of the theoretical calculations. We also demonstrated the first successful synthesis of an base-labile modified oligodeoxyribonucleotide having three 4-*N*-acetyldeoxycytidines by use of the O-selective phosphorylation in the activated phosphite method without base protection. This method prompts us to study the synthesis of base-labile DNAs such as oxidatively damaged DNAs or, in the near future, functional DNAs and RNAs with various alkali-labile modified structures. Further studies are now underway in this direction.

Supporting Information Available: List of alcohol-type activators tested in this study; ^{1}H, ^{32}P, and ^{13}C NMR data and ESI mass spectra of **7**, **8**, **15**, and **16**; mass spectra of all synthesized oligonucleotide derivatives; enzyme assay of oligonucleotides; T_m data of modified DNA duplexes; molecular orbitals of 9-methyladenine, 9-methylguanine, 1-methylcytosine, and methanol; and program of DNA synthesizer (ABI 392) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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