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A new approach for pyrophosphate bond formation starting from phosphoramidite derivatives by use of 6-trifluoromethyl-1-hydroxybenzotriazole-mediated O–N phosphoryl migration

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Abstract—A new method for pyrophosphate bond formation in the solid phase was developed by use of phosphoramidite derivatives, which were found to be readily converted by reaction with 6-trifluoromethyl-1-hydroxybenztriazole via an O–N phosphoryl rearrangement into pentavalent phosphotriester intermediates. These intermediates proved to react smoothly with not only phosphomonoesters but also phosphodiesters to give protected pyrophosphate derivatives which, in turn, could be easily deprotected to give the desired pyrophosphate derivatives.

A variety of pyrophosphate compounds such as nucleotide coenzymes and sugar nucleotides[1] play very important roles in biological reactions. The synthesis of such compounds and their analogues has been extensively reported.[1] From the chemical point of view, however, there are three disadvantages in the previous methods[2] for pyrophosphate bond formation: (1) Most of the reactions require much-prolonged periods of time such as 10–24 h. (2) The starting materials such as phosphorimidazolidates[3] and phosphoromorpholidates[4] exhibit inherent high polarity and instability so that the purification and preservation of them is rather difficult. (3) Solvents except highly polar solvents such as water and DMF are not applicable to this reaction because of poor solubility of the starting materials. As a result, the isolated yield of target compounds is usually low.

In connection with our continuous studies on the development of new methods for the chemical synthesis of oligodeoxyribonucleotides, we have recently reported the facile oxidative conversion of several tervalent phosphite benzotriazol-1-yl esters into the corresponding pentavalent benzotriazol-1-yl phosphate derivatives.[5] A preliminary mechanistic study revealed that the conversion was not a simple oxidation, but proceeded via the rearrangement of the phosphite triesters, as exemplified in Scheme 1.[5] Because the resulting benzotriazol-1-yl phosphate derivatives exhibit high reactivity toward various nucleophilic functions, they can be used as new active intermediates for the synthesis of pyrophosphate compounds by the reaction with phosphate nucleophiles. In this paper, we report a new strategy for pyrophosphate bond formation using benzotriazol-1-yl phosphate as a key intermediate. Because the intermediates can be prepared in situ from the chemically stable and lipophilic phosphoramidite compounds, our approach must be promising as a new strategy to overcome the above-mentioned drawbacks of conventional pyrophosphate synthesis methodology. The usefulness of our new strategy was demonstrated by the synthesis of $T^{3'}pp^{5'}T$, $dA^{3'}ppT$, $dC^{3'}pp^{5'}T$ and $dC^{3'}pp^{5'}T$ on polymer supports.

In order to analyze in more detail the O–N phosphoryl rearrangement mediated by HOBt, dimethyl *N*,*N*-diisopropylphosphoramidite **1** was chosen as a model compound. When the phosphoramidite derivative **1** was activated by use of 5 equiv of HOBt, the starting material immediately changed to the corresponding phosphite intermediate **2a** (^{31}P NMR: 146.6 ppm), which, in

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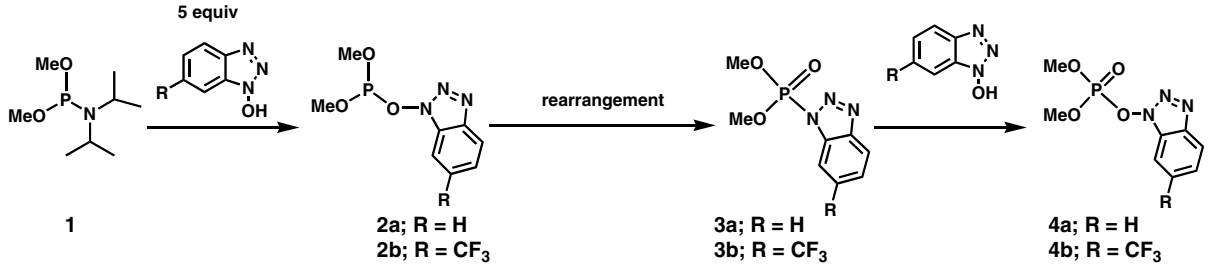


Scheme 1.

turn, was rapidly converted to the phosphotriester intermediate **4a** (^{31}P NMR: 0.8 ppm), as shown in Scheme 1. In the ^{31}P NMR spectrum of the mixture obtained after 5 min, a minor peak of the hydrolyzed product having a P–H bond was observed at 11.8 ppm. Because the phosphite intermediate **2a** could be observed, the rearrangement was thought to be the rate-determining step of the overall reaction. It is likely that the initial rearrangement product **3a** reacts with another HOBt molecule so promptly that we could not observe **3a** in the ^{31}P NMR spectrum of the mixture **4a**. This rearrangement of the phosphite intermediate **2a** proceeded to a degree of 90% after 5 min, as shown in Figure 1A. Completion of this reaction required 15 min. In the case of 6-trifluoromethyl-HOBt (tfHOBt), the rate of the rearrangement increased and the phosphoramidite unit was completely converted into compound **4b** within 5 min, as shown in Figure 1B.

To demonstrate the usefulness of such a final product of the phosphotriester type as an efficient phosphorylating reagent for the pyrophosphate bond formation, the synthesis of T$^{3'}$pp$^{5'}$T was carried out by reaction of a thymidine 3′-phosphotriester intermediate **6**, which was prepared in situ from the thymidine 3′-phosphoramidite derivative **5** by the tfHOBt-mediated reaction, with two different phosphate species, that is, the phosphomonoester **10** and the phosphodiester **16**, as described in Methods A and B, respectively, of Scheme 2.

In Method A, the 5′-phosphitylation of a T-loaded highly cross-linked polystyrene (HCP) resin **7**[6] containing a succinate linker[7] was carried out by use of DMTrO(CH$_2$)$_2$- SO$_2$(CH$_2$)$_2$OP(N*i*Pr$_2$)(OCH$_2$CH$_2$CN) **8**[8] that is, a 5′-phosphorylating in the presence of benzimidazolium triflate[9] (BIT). The successive treatment with 0.1 M I$_2$ in pyridine–H$_2$O gave the 5′-phosphorylated

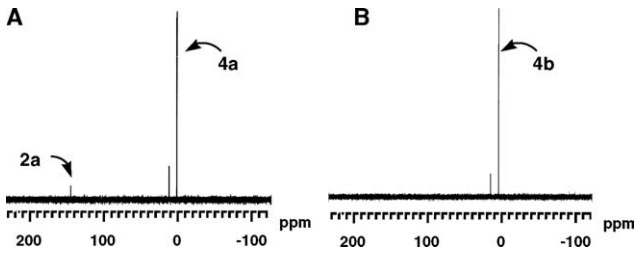
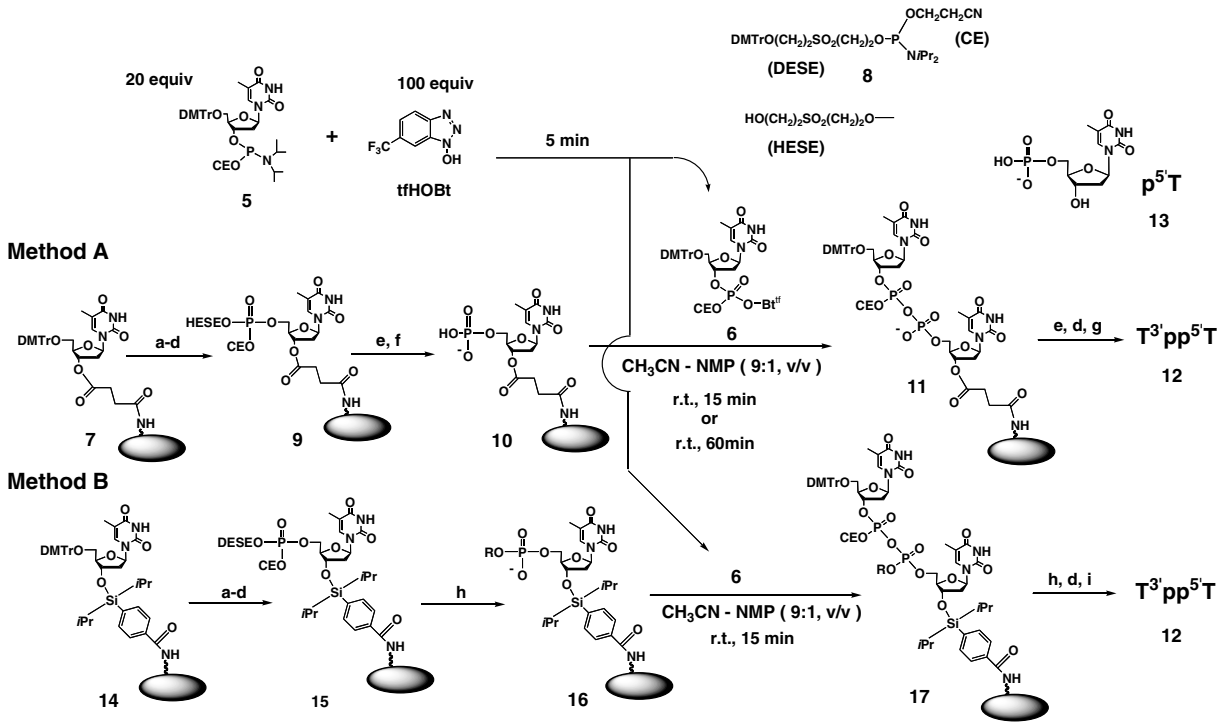


Figure 1. ^{31}P NMR spectra of the crude mixtures obtained after the phosphoramidite **1** was activated by HOBt (panel A) and tfHOBt (panel B) at room temperature for 5 min.

product **9**. The simultaneous removal of the cyanoethyl (CE) and DMTrO(CH$_2$)$_2$SO$_2$(CH$_2$)$_2$ (DESE) groups from **9** was performed as follows: Treatment of the resin **9** with 3% trichloroacetic acid in CH$_2$Cl$_2$ followed by the successive treatments with 10% DBU in BSA–pyridine (1:1, v/v)[10] and Et$_3$N–MeOH (1:1, v/v) gave the phosphomonoester **10** on the HCP resin. The condensation of the in situ-generated reactive phosphotriester **6** with the HCP resin **10** was carried out at room temperature for 15 min. After the cyanoethyl group and the DMTr group were removed from the resulting pyrophosphate derivative **11** via a two-step procedure, the nucleotidic materials released from the resin by the action of concd ammonia were analyzed by HPLC. As shown in Figure 2A, the desired product T$^{3'}$pp$^{5'}$T **12** was obtained in 81% yield (HPLC) as the exclusive product except for a minor peak at 8.6 min of p$^{5'}$T **13**, which resulted from failure of the condensation. Even if the reaction time for the condensation was extended to 60 min, the coupling efficiency was unchanged (81%), as shown in Figure 2B. This result indicates that the condensation for the pyrophosphate bond formation does not require more than 15 min. Because previous methods for pyrophosphate bond formation required much-prolonged periods of time of more than 12 h, it should be noted that the reaction time was considerably reduced by using our method.

Method B involves the following new devices to obtain the phosphodiester intermediate **16**. The 5′-phosphorylation of a T-loaded HCP resin **14** having a silyl-type linker recently reported by us[11] was similarly carried out to give the fully protected 5′-phosphorylated product **15**. In order to selectively remove one of the two 5′-phosphate protecting groups, the resin **15** was treated successively with 10% DBU in CH$_3$CN and 3% TCA in CH$_2$Cl$_2$. Since this operation contained no silylating reagents, one of the two phosphate protecting groups, that is, 2-cyanoethyl and HOCH$_2$CH$_2$SO$_2$CH$_2$CH$_2$ (HESE), could be removed to give the phosphodiester **16** where the latter might remain as the substituent 'R' predominantly over the former. This is because the cyano group serves as a stronger electron-withdrawing group than the HOCH$_2$CH$_2$SO$_2$ group so that the 2-cyanoethyl group might be more rapidly removed than the HOCH$_2$CH$_2$SO$_2$CH$_2$CH$_2$ group. Generally, phosphodiester compounds have been recognized to be inert to conventional phosphorylating reagents such as phosphorimidazolidates and phosphoromorpholidates, particularly in the liquid-phase synthesis. However, it



Scheme 2. (a) 3% CCl_3COOH, CH_2Cl_2, rt, 30 s; (b) **8** (20 equiv) BIT (40 equiv) CH_3CN, rt, 1 min; (c) 0.1 M I_2, pyridine–H_2O (9:1, v/v), rt, 2 min; (d) 3% CCl_3COOH, CH_2Cl_2, rt, 30 s; (e) 10% DBU, pyridine–bis(trimethylsilyl)acetamide (1:1, v/v), rt, 10 min; (f) Et_3N–MeOH (1:4, v/v), rt, 20 s; (g) concd NH_3, rt, 40 min; (h) 10% DBU, CH_3CN, rt, 1 min; (i) 1 M TBAF, THF, rt, 1 h.

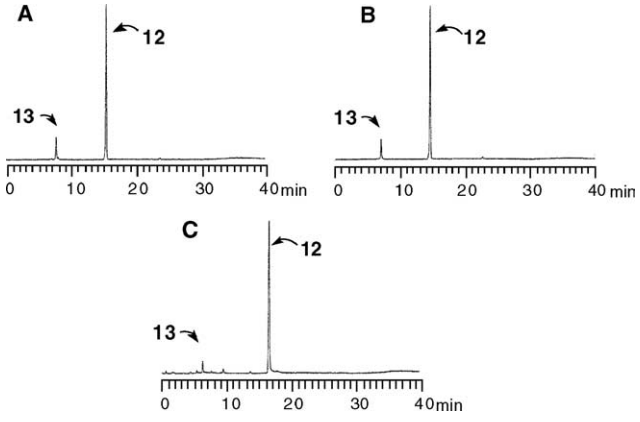


Figure 2. The amino-exchange HPLC profiles of the mixtures released from the resins **11** and **17** obtained after condensation of **6** with **10** and **16**, respectively. **A**: Method A, reaction time 15 min; **B**: Method A, reaction time 60 min; **C**: Method B, reaction time 15 min.

was surprisingly found that the in situ-generated phosphate benzotriazol-1-yl ester **6** underwent smooth coupling with the phosphodiester species **16** as shown in Figure 2C, which suggested excellent coupling efficiency (94%). We strongly feel that it is necessary for us to reconsider our stereotype of general pyrophosphate bond formations. In addition to this finding, it should be also emphasized that the existence of the hydroxyl group of the $HOCH_2CH_2SO_2CH_2CH_2$ residue attached to the 5′-phosphate group on the resin **16** did not affect the pyrophosphate bond formation. Actually, no byproducts arising from phosphorylation of the hydroxyl group were detected. The silyl-type linker in place of the succinate linker was used in Method B because it was reported that treatment of succinate linker-containing resins with DBU in the absence of BSA causes serious hydrolysis of the succinate ester linkage via a DBU-mediated intramolecular cyclization of this linker.[10b,12] Thus, by use of Method B, $T^{3'}pp^{5'}T$ could be isolated in 81% yield and characterized by LC-MS.[13]

Furthermore, we synthesized $d[C^{3'}pp^{5'}T]$, $d[A^{3'}pp^{5'}T]$, and $d[G^{3'}pp^{5'}T]$ to demonstrate the generality of our new method. In the synthesis of these dimers, the protocol of Method A was used except for the prolonged ammonia treatment (12 h) for the deprotection and the release from HCP resin since the commercially available *N*-protected phosphoramidite units, 4-*N*-acetyldeoxycytidine-3′-*O*-phosphoramidite, 6-*N*-phenoxyacetyldeoxyadenosine-3′-*O*-phosphoramidite, and 2-*N*-*p*-isopropylphenoxyacetyl-deoxyguanosine-3′-*O*-phosphor-amidite. The efficiency of these condensations for 15 min were ascertain to be as high as that in synthesis of $T^{3'}pp^{5'}T$, as shown in Figure 3. The isolated yields of $d[C^{3'}pp^{5'}T]$, $d[A^{3'}pp^{5'}T]$, and $d[G^{3'}pp^{5'}T]$ were 83, 79, and 84%, respectively, and characterized by LC-MS.[14]

In conclusion, we have proposed a new convenient strategy for the formation of pyrophosphate bonds by the use of readily available phosphoramidite derivatives and demonstrated the usefulness of our new pyrophosphorylation strategy as evidenced by the synthesis of $T^{3'}pp^{5'}T$, $C^{3'}pp^{5'}T$, $A^{3'}pp^{5'}T$, and $G^{3'}pp^{5'}T$. One benefit of our method is that the reaction time can be considerably reduced compared with the previous

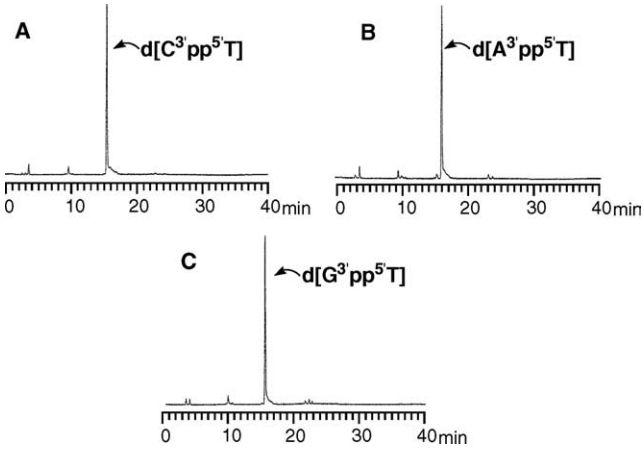


Figure 3. The anion-exchange HPLC profiles of the crude mixtures from the synthesis by use of Method A. **A**: d[C$^{3'}$pp$^{5'}$T]; **B**: d[A$^{3'}$pp$^{5'}$T]; **C**: d[G$^{3'}$pp$^{5'}$T].

methods. Another advantageous feature is that phosphoramidite derivatives for this reaction are more stable than the previous starting materials such as phosphorimidazolidates and phosphoromorpholidates during purification or preservation. More detailed studies of the mechanism of this new pyrophosphorylation and the high-throughput synthesis of sugar nucleotides in the solution phase and 5′-capped oligonucleotides on polymer supports[15] are now under way.

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