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O-Selective Condensation Using P−N Bond Cleavage in RNA Synthesis without Base Protection

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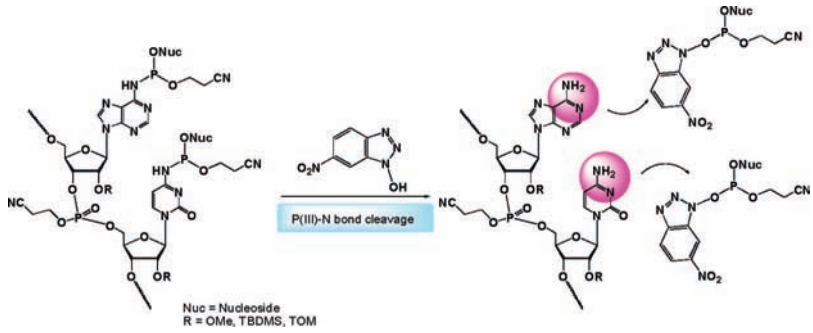
ABSTRACT



In RNA synthesis without base protection, a new method for O-selective condensation with more than 99% selectivity was developed by 6-nitro-HOBt-mediated cleavage of undesired P(III)−N bonds on nucleobase moieties. Moreover, we for the first time succeeded in synthesizing oligoRNAs without base protection.

Development of effective methods for the synthesis of oligodeoxynucleotides having base-labile functional groups, such as oxidatively damaged,[1] N-acylated,[2] and backbone-modified[3] DNA oligomers or aminoacylated RNA molecules,[4] is highly desirable today because the diversification of studies on nucleic acids has progressed considerably. Previously, we reported a so-called "activated phosphite method" for O-selective condensation using phosphite triester intermediates in DNA synthesis.[5] Its O-selectivity reached more than 99% when HOBt or 6-nitroHOBt (HO^{n}Bt) was used as an activator of N-unprotected deoxynucleoside 3′-phosphoramidite building blocks. This high selectivity results from the low reactivity of the phosphite triester intermediates toward the exocyclic amino groups of the adenine and cytosine base moieties compared with hydroxyl groups. Moreover, we could also synthesize DNA oligomers containing base-labile deoxynucleoside derivatives such as N^4-acetyl-2′-deoxycytidine[5a] or N^6-acetyl-8-aza-7-deaza-2′-deoxyadenosine[6] in our activated phosphite method using a silyl-type linker[5b,7] that can be cleaved under neutral conditions.

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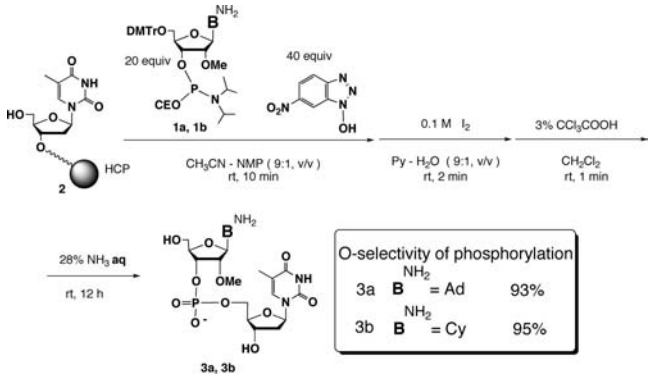
If the O-selectivity of the condensation is very high in this strategy using N-unprotected ribonucleoside 3′-phosphoramidite units, we can similarly synthesize RNA oligomers containing base-labile functional groups. For example, it becomes possible to synthesize non-natural aminoacylated tRNAs[4] by solid-phase synthesis or 2′-*O*-acetoxymethyl RNAs[8] as prodrugs of siRNAs.To examine the selectivity of condensation using N-unprotected RNA phosphoramidite units in our activated phosphite method, we performed the synthesis of 2′-OMe-ApT and 2′-OMe-CpT dimers, as shown in Scheme 1. The *N*-unprotected 2′-OMe RNA monomer

Scheme 1. Synthesis of 2′-OMe-ApT (**3a**) and 2′-OMe-CpT (**3b**) Dimers in the Activated Phosphite Method Using HO^{n}Bt



building units **1a**,**b** were synthesized by treatment of commercially available *N*-acylated 2′-OMe RNA phosphoramidite units with methylamine in THF.[9] These units reacted with the hydroxyl group of thymidine on highly cross-linked polystyrene (HCP) resins[10] **2** in the presence of 6-nitro-HOBt (HO^{n}Bt) for 10 min at room temperature. The resulting dimers were released from the resins by treatment with concd ammonia. The reaction selectivity was estimated by HPLC analysis of the mixture thus obtained. Unfortunately, these condensations resulted in very low selectivities of 93% and 95% for 2′-OMe-ApT (**3a**) and 2′-OMe-CpT (**3b**), respectively. These results suggested that a new strategy superior to the activated phosphite method was required for RNA synthesis without base protection.Therefore, to increase O-selectivity, we focused on the pioneering study reported by Gryaznov and Letsinger on the N-unprotected synthesis of DNA oligomers using a two-step reaction of condensation and successive P−N bond cleavage.[11] In their strategy using tetrazole or pyridinium chloride as an activator, N-branched oligonucleotides containing trivalent P−N bonds were generated as byproducts. They found that these P−N bonds could be efficiently cleaved by treatment with pyridinium hydrochloride as an acidic promoter in the presence of aniline before the oxidation step. However, this reaction was slow and in equilibrium with the reverse reaction, as shown in Figure 1.

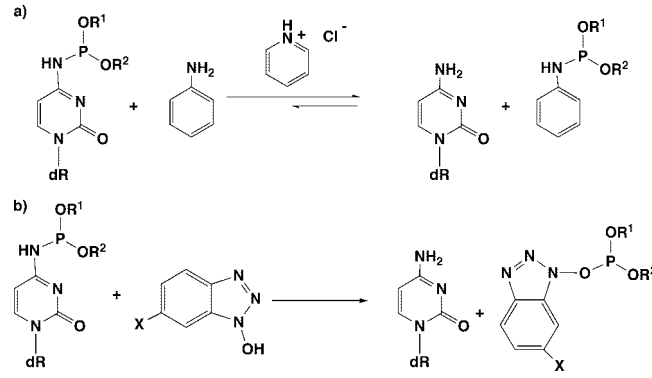


Figure 1. Trivalent P−N bond cleavage of a nucleobase using (a) pyridinium chloride and aniline or (b) HOBt derivatives.

We considered that if HOBt derivatives could be used for the P−N bond cleavage in place of pyridinium hydrochloride and aniline, the reverse reaction might be considerably suppressed. Therefore, we investigated the efficiency of P−N bond cleavage using HOBt derivatives.

First, we examined this efficiency in the DNA dimer synthesis of d[ApT] (**5a**) and d[CpT] (**5b**). After the coupling of N-unprotected deoxynucleoside 3′-phosphoramidite derivatives **4a**,**b**[5] with dT-loaded HCP **2** in the presence of benzimidazolium triflate (BIT),[12] P−N bond cleavage was performed by treatment with pyridinum hydrochloride or HOBt derivatives for 1 min. Subsequently, oxidation of the resulting phosphite intermediate and release of the desired dimers from resin were performed by treatment with concd ammonia. The O-selectivity of each two-step coupling reaction was evaluated by anion-exchange HPLC analysis of the reaction mixture obtained, as shown in Table 1.

When the post-treatment was eliminated, the *O*-selectivities of condensation in the synthesis of d[ApT] and d[CpT] were 56% and 90%, respectively. The selectivity of the synthesis of d[ApT] with P−N bond cleavage using pyridinium hydrogen chloride significantly increased to 96%, but it was still not enough to synthesize oligonucleotides. The selectivities in the synthesis of d[ApT] and d[CpT] using HOBt were similar to that using pyridinium hydrochloride.

To increase the efficiency of P−N bond activation of the undesired byproducts, we employed two methods using HOBt derivatives. One involves addition of BIT to the reaction mixture, based on our previous report that the coupling efficiency of phosphoramidite units using HOBt

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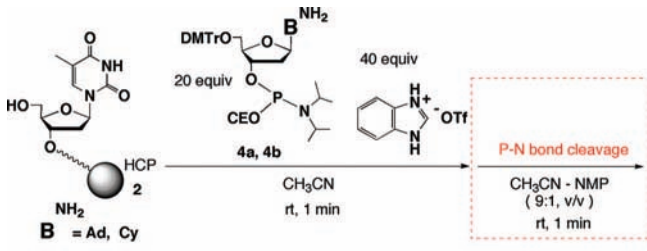
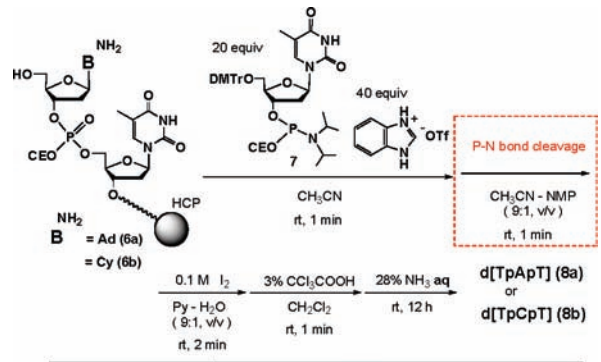
Table 1. *O*-Selectivity[a] of Phosphorylation with P−N Bond Cleavage in d[ApT] (**5a**) and d[CpT] (**5b**) Synthesis



final product	reagents for P-N bond cleavage					
	—	Py-HCl aniline	HOBt	BIT HOBt	HO^{tf}Bt	HO^{n}Bt
d[ApT] (5a)	56%	96%	95%	89%	98%	>99%
d[CpT] (5b)	90%	>99%	>99%	>99%	>99%	>99%

[a] Estimated by anion-exchange HPLC analysis.

Table 2. *O*-Selectivity[a] of Phosphorylation with P−N Bond Cleavage in d[TpApT] (**8a**) and d[TpCpT] (**8b**) Synthesis



final product	reagents for P-N bond cleavage				
	—	HOBt	BIT HOBt	HO^{tf}Bt	HO^{n}Bt
d[TpApT] (8a)	91%	94%	96%	>99%	>99%
d[TpCpT] (8b)	90%	>99%	>99%	>99%	>99%

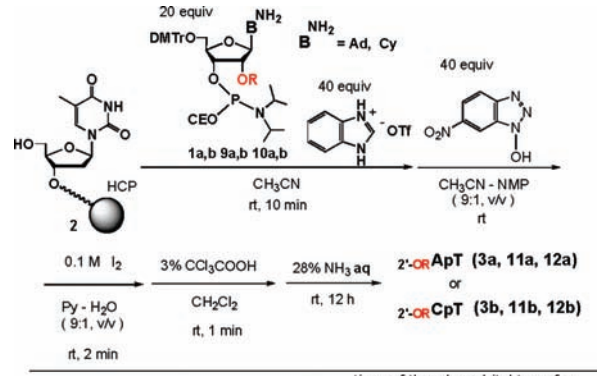
[a] Estimated by anion-exchange HPLC analysis.

derivatives and BIT was higher than that using only HOBt derivatives.[5b] However, O-selectivity decreased to 89% in the d[ApT] synthesis, as shown in Table 1. The other method was devised to increase the acidity of HOBt derivatives by addition of electron-withdrawing groups, such as trifluoro or nitro groups. As a result, the efficiency of P−N bond cleavage increased when using 6-trifluoro-HOBt (HO^{tf}Bt) or HO^{n}Bt in place of HOBt. In particular, the selectivity using HO^{n}Bt was more than 99% in each synthesis. These results demonstrated that the efficiency of P−N cleavage using HO^{n}Bt was much higher than that reported in the previous method using pyridinum hydrochloride and aniline.

Next, d[TpApT] (**8a**) and d[TpCpT] (**8b**) trimers were synthesized to examine the *O*-selectivity of condensation using a thymidine phosphoramidite unit **3c** toward the nucleobase (A or C) containing an amino group on polymer supports, as shown in Table 2. d[ApT]- and d[CpT]-loaded HCP resins **6a**,**b** were synthesized in advance by the method depicted in Table 1. The resins **6a**,**b** were treated with an excess thymidine phosphoramidite unit **7** in the presence of BIT with or without HOBt derivatives. When the condensation did not involve a post-treatment of P−N bond cleavage, the selectivities in the synthesis of d[TpApT] (**8a**) and d[TpCpT] (**8b**) were 91% and 90%, respectively. Among the HOBt derivatives tested as P−N bond cleavage reagents, HO^{tf}Bt and HO^{n}Bt proved to give O-selectivities of more than 99%. Interestingly, the efficiency of P−N bond cleavage using HOBt and BIT increased to 96% in the d[TpApT] (**8a**) synthesis unlike the decrease in the efficiency in the d[ApT] (**5a**) synthesis.

Figure S1 (Supporting Information) shows the HPLC profile of the crude mixture obtained in the synthesis of an oligodeoxynucleotide containing 20 bases by condensation using 0.2 M BIT and P−N bond cleavage using 0.1 M HO^{n}Bt. The target 20-mer oligonucleotide was obtained as the main product and satisfactorily isolated in 22% yield. This result was similar to the results of the usual DNA synthesis in the previous activated phosphite method using HO^{n}Bt and BIT as activators.

Table 3. *O*-Selectivity[a] of Phosphorylation with P−N Bond Cleavage in 2′-OR-AT and 2′-OR-CT Synthesis



final product	R	B NH2	time of the phosphityl transfer		
			—	1 min	2 min
2′-OMeApT (**3a**)	Me	Ad	76%	97%	>99%
2′-OMeCpT (**3b**)	Me	Cy	74%	>99%	>99%
2′-OTBDMSApT (**11a**)	TBDMS	Ad	90%	98%	>99%
2′-OTBDMSCpT (**11b**)	TBDMS	Cy	93%	>99%	>99%
2′-OTOMApT (**12a**)	TOM	Ad	76%	96%	>99%
2′-OTOMCpT (**12b**)	TOM	Cy	88%	>99%	>99%

[a] Estimated by anion-exchange HPLC analysis.

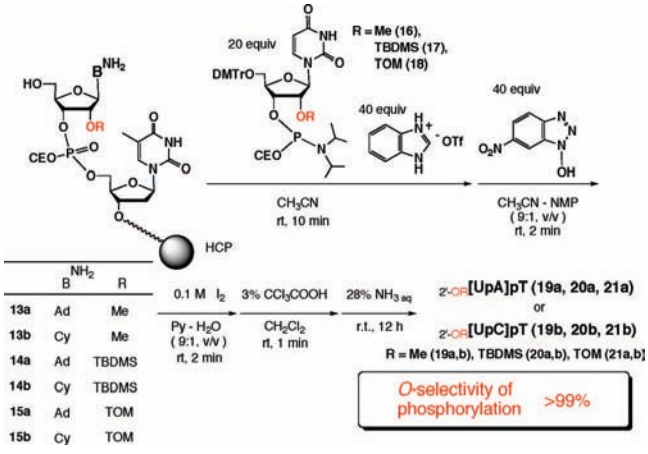
Subsequently, we also examined the efficiency of P−N bond cleavage with HO^{n}Bt in RNA synthesis using *N*-unprotected phosphoramidite units **1a**,**b**, **9a**,**b**, and **10a**,**b** having methyl (Me), *tert*-butyldimethylsilyl (TBDMS), and triisopropylsilyloxymethyl (TOM) groups, respectively, as 2′-protecting groups, as summarized in Table 3. Compounds

9a,**b**, and **10a**,**b** were also synthesized by treatment of the corresponding *N*-acylated phosphoramidite units with methylamine, similar to the synthesis of **1a**,**b**.

As shown in Table 3, the synthesis without P−N bond cleavage resulted in poor O-selectivities of 74−93%, which were similar to those in the DNA synthesis described above. However, the selectivity increased to more than 99% for P−N bond cleavage using HO^{n}Bt for prolonged periods of 2 min regardless of the kind of 2′-protecting group, while the selectivity of synthesis with the P−N bond cleavage for 1 min was not adequate (96−99%).

Moreover, 2′-OR-[UpA]pT (**19a**, **20a**, **21a**) and 2′-OR-[UpC]pT (**19b**, **20b**, **21b**) trimers, where R is methyl, TBDMS, or TOM, were synthesized to examine the *O*-selectivity of the uridine units **16**, **17**, and **18** toward the nucleobase A or C containing an amino group on polymer supports, as shown in Scheme 2. The selectivity also increased to more than 99% for

Scheme 2. Synthesis of 2′-OR-[UpA]pT and 2′-OR-[UpC]pT Trimers with P−N Bond Cleavage Using HO^{n}Bt[a]



[a]Estimated by anion-exchange HPLC analysis.

P−N bond cleavage using HO^{n}Bt for 2 min.

Furthermore, we synthesized RNA−DNA chimeric oligomers having 5 and 21 bases (an siRNA analogue of Maitogen-protein kinase 14[13]) and two RNA 10-mers using P−N bond cleavage with HO^{n}Bt. In the synthesis of 2′-OMe-[UCAG]T, r[U_4CU_5], and r[U_4AU_5] using *N*-unprotected phosphoramidite units having the 2′-*O*-methyl or TBDMS group (including the N-unprotected 2′-OMeG unit **22**), elongation was performed by condensation using 0.2 M BIT followed by P−N bond cleavage using 0.1 M HO^{n}Bt. Thus, the target oligonucleotides could be obtained as the main products and isolated in 64%, 14%, and 17% yields, respectively (Figure 2a−c). Finally, we tried to synthesize an RNA−DNA chimeric oligonucleotide 21-mer in a similar manner. However, the crude mixture involved large amounts

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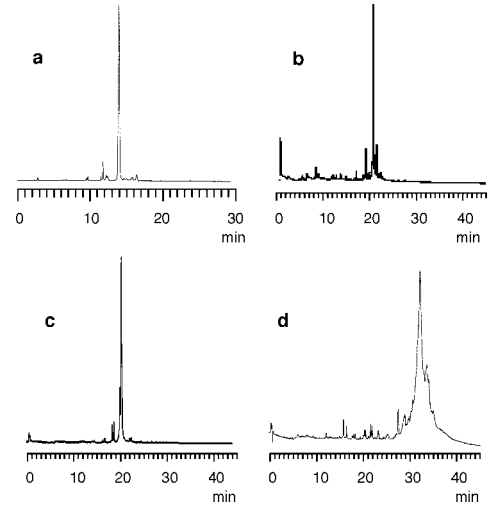

Figure 2. Anion-exchange HPLC profile of the crude mixture obtained in the sysnthesis of RNA−DNA chimeric oligomers and an RNA oligomer: (a) 2′-OMe-[UCAG]T, (b) r[U_4CU_5], (c) r[U_4AU_5], and (d) 2′-OMe-[CCUACAGAGAACUGCGGUU]-TT.

of byproducts containing P−N bonds (data not shown) because the *O*-selectivity of the condensation gradually decreased due to increase in the number of amino groups. Therefore, we added HO^{n}Bt to a solution of the activator, BIT, to suppress the reactivity of the reaction intermediate toward the amino groups. Figure 2d shows the anion-exchange HPLC profile of the crude mixture obtained in the synthesis of the RNA−DNA chimeric oligonucleotide 21-mer. The target oligonucleotide was obtained as a main product although peaks of byproducts were also observed. The oligomer was isolated in 11% yield, as shown in Figure S2, and characterized by MALDI-TOF mass spectroscopy.

In summary, we determined that the O-selectivity of condensation increased to more than 99% by the P−N bond cleavage using HO^{n}Bt not only in DNA synthesis but also in RNA synthesis without base protection. Moreover, we could for the first time synthesize RNA oligomers using our new strategy. These results encourage us to study the synthesis of base-labile RNAs such as an aminoacylated RNA. Further studies are now under way in this direction.

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Supporting Information Available: Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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