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A new strategy for the synthesis of oligodeoxynucleotides directed towards perfect *O*-selective internucleotidic bond formation without base protection

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Abstract—Deoxyadenosine and deoxycytidine have nucleophilic amino groups so that the undesired *N*-phosphitylation of these amino groups occurred in the previous phosphoramidite methods without base protection. We report that the *N*-phosphitylation could be considerably suppressed in our new HOBt-mediated coupling strategy via phosphite intermediates as reactive species. Thus, 99.7–99.9% *O*-selective internucleotidic bond formation was achieved.

The chemical synthesis of oligodeoxynucleotides has contributed to biological and structural studies of nucleic acids.[1] Recently, much attention has been paid to the development of more straightforward methods for the synthesis of DNA fragments in the post-genome days. Particularly, our interest has been focused on a synthetic strategy without base protection, since the procedure prescribed for protection and deprotection of the *N*-protecting groups can be eliminated.[2–5]

Letsinger first proposed the phosphoramidite method[6] without nucleobase protection by use of pyridinium chloride as a promoter in 1991.[3] Later, Hayakawa changed this hygroscopic reagent to nonhygroscopic imidazolinium triflate (IMT) and succeeded in synthesizing oligodeoxyribonucleotides.[5] However, these precedents require an additional post-treatment with pyridinium hydrochloride/aniline[3] or benzimidazolium triflate (BIT)/MeOH[5] for decomposition of undesired *N*-phosphitylated side chains containing P(III)–N bonds on the base residues at every coupling step. In addition, we have quite recently reported that the latter method was not generally applicable.[7a,8]

In an attempt to eliminate this P–N bond cleavage step, we have recently developed a new phosphoramidite approach called 'proton-block method' based on the hitherto simplest principle of acid–base reactions.[7] However, the yield of the product was low in the case of longer oligodeoxynucleotides because byproducts exponentially increased. As a matter of fact, completely *O*-selective phosphitylation has not yet been achieved in the phosphoramidite method to date.

On the other hand, we previously reported *O*-selective internucleotidic bond formation by the *H*-phosphonate method.[9] In this method, we observed that phosphite intermediates generated from *H*-phosphonate building blocks reacted scarcely with the exocyclic amino groups of the base residues. This high *O*-selectivity was explained in terms of two kinds of HOMO–LUMO zinteractions between the activated phosphite species and the 5′-hydroxyl function. Based on these previous results, we considered that, if phosphite-type intermediates could be generated from deoxynucleoside phosphoramidite derivatives by an alcohol-type activator, they would not react with the amino groups.

Keywords: Chemical synthesis of DNA; HOBt; *O*-Selective phosphorylation; Activator; Phosphoramidite approach without base protection.
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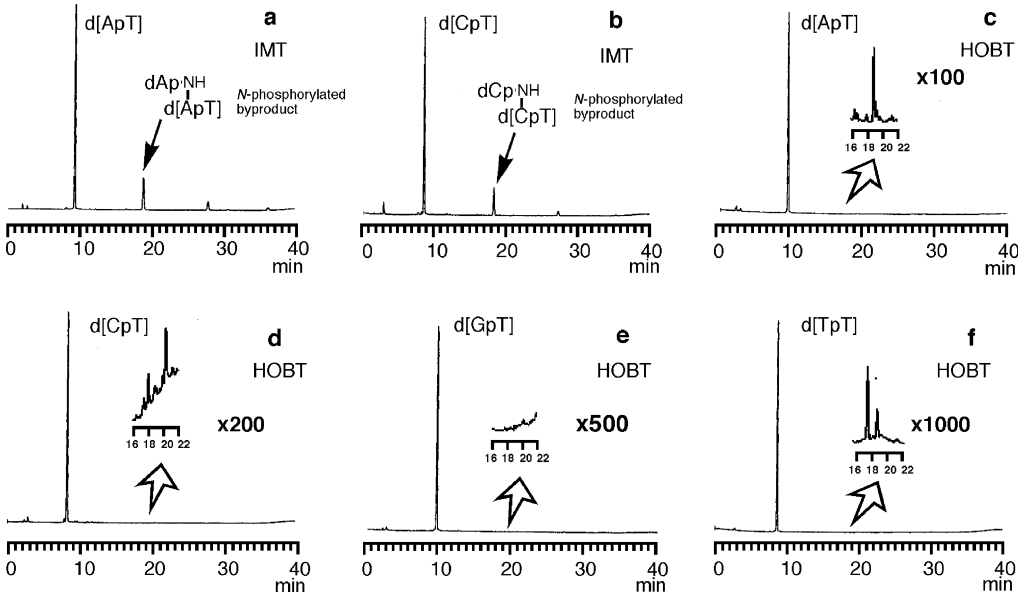

Figure 1. The anion-exchange HPLC profiles of the crude mixtures from synthesis with unprotected nucleobases. (a) d[ApT], IMT; (b) d[CpT], IMT; (c) d[ApT], HOBt; (d) d[CpT], HOBt; (e) d[GpT], HOBt; (f) d[TpT], HOBt.

Therefore, we extensively searched for suitable activators to realize this idea. In this paper, we report that 1-hydroxybenzotriazole [HOBt ($pK_a = 5.3$)][10] was the most effective as an activator in the *N*-unprotected phosphoramidite approach.

To check the *O*/*N*-selectivity of internucleotidic bond formation of the previously reported methods, several experiments were conducted. When IMT, previously reported as the best reagent for the *O*-selective phosphitylation,[5a] was used in the solid-phase synthesis of d[ApT] and d[CpT] on highly cross-linked polystyrene[11] (HCP) resins using a manual operation, the *N*-phosphorylated derivatives of deoxyadenosine and deoxycytidine were considerably formed in 23% and 17% yields, respectively, as shown in Figure 1a and b.

To examine the side reaction of the base parts in our HOBt-mediated internucleotidic bond formation on HCP, d[ApT], d[CpT], d[GpT] and d[TpT] were synthesized according to Figure 2.

The efficiency of the condensation should be increased as much as possible to avoid the capping reaction using acetic anhydride, which would react readily with the amino groups of oligodeoxynucleotides. Thus, two consecutive couplings at each cycle were employed, and this operation was superior to the one-coupling mode.

The result of the synthesis of d[ApT] is shown in Figure 1c and Table 1. There was almost no peak corresponding to the *N*-phosphorylated product at around 18.5 min. Hundred-fold expansion of the HPLC chart exhibited the remarkably high selectivity (the ratio of *O*/*N*-phosphorylation in this condensation) that was estimated to be 99.7:0.3. The condensation in the synthesis of d[CpT] using HOBt was also almost perfectly *O*-selective (99.9%), as shown in Figure 1d. In the case of d[GpT] and d[TpT], similarly, no *N*-phosphorylated byproducts were detected at all, as shown in Figure 1e and f. Thus, these products, d[ApT], d[CpT], d[GpT] and d[TpT], were isolated in 92%, 94%, 90% and 92% yields, respectively.

Furthermore, the condensation of d[Ap(ce)T]-HCP, d[Cp(ce)T]-HCP and d[Gp(ce)T]-HCP, which were prepared by the same method as described in Figure 2,

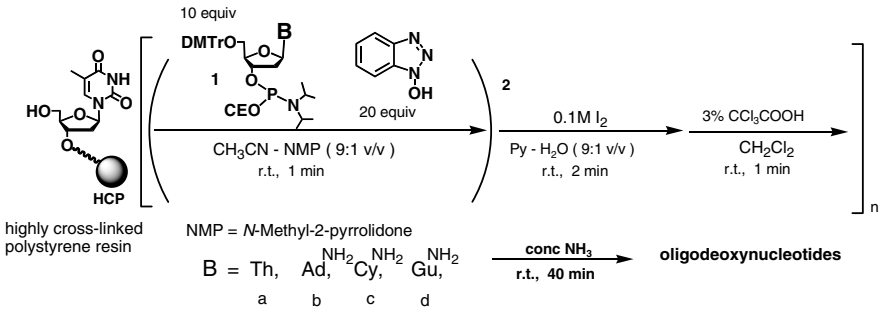

Figure 2. Solid-phase synthesis of oligodeoxynucleotides in the HOBt approach.

Table 1. The *O*-selectivity of condensation in the HOBt approach

Amidite	Polymer	Final product	*O*-Selectivity (%)		
			IMT	HOBt	tf-HOBt
1b	d[T]-HCP	d[ApT]	77.0	99.7	99.3
1c	d[T]-HCP	d[CpT]	82.9	99.9	98.9
1d	d[T]-HCP	d[GpT]	>99.9	>99.9	>99.9
1a	d[Ap(ce)T]-HCP	d[TpApT]	90.5	>99.9	99.1
1a	d[Cp(ce)T]-HCP	d[TpCpT]	9.7	>99.9	98.7
1a	d[Gp(ce)T]-HCP	d[TpGpT]	>99.9	>99.9	>99.9

with **1a** was carried out in the presence of HOBt. These results are summarized in Table 1. The amounts of these *N*-phosphorylated species became closer to the noise level as shown in the expanded HPLC spectra of Figure 3c–e so that the *O*-selectivity in these cases was calculated to be more than 99.9%. Thus, d[TpApT], d[TpCpT] and d[TpGpT] were isolated in 89%, 83% and 84% yields, respectively. When IMT was used as the activator, considerable side reactions were detected, as shown in Figure 3a and b and Table 1. Particularly, the reaction of d[Cp(ce)T]-HCP with **1a** gave extremely poor selectivity of 9.7%. Surprisingly, the *N*-phosphorylated product was formed as the major product, as shown in Figure 3b. On the other hand, the use of a more acidic HOBt derivative, 1-hydroxy-6-(trifluoromethyl)benzotriazole (tf-HOBt, $pK_a = 3.94$), resulted in a slight decrease of the *O*-selectivity compared with HOBt, as shown in Table 1.

In the synthesis of d[A_6T] and d[C_6T], the desired products were obtained as the main peaks, as shown in Figure 3f and g. These results indicate that *N*-phosphorylation was greatly suppressed even in the consecutive sequence of dA or dC. In these cases, the coupling efficiency was ca. 99% (trityl cation assay). Thus, d[A_6T] and d[C_6T] were isolated in 48% and 67% yields, respectively. As a dG-rich sequence, we chose d[GGT]$_3$ having the same number of dG as the above 7-mers, since sequences such as d[G_6T] having consecutive dGs tend to aggregate giving complex HPLC patterns. In this case, the average coupling yield was 98.1% so that the desired 9-mer was formed as the predominant peak with small amounts of the $n-1$-mers (Fig. 3h). Consequently, d[GGT]$_3$ was isolated in 48% yield. In a similar manner, a DNA 12-mer d[CAGT]$_3$ could be isolated in 36% yield (Fig. 3i). The $n-1$-mers observed in Figure 3i is due mainly to the somewhat lower coupling efficiency at the dG positions. In the ^{31}P NMR spectrum of crude d[CAGT]$_3$, there were no significant resonance signals of *N*-phosphorylated byproducts. These isolated oligomers were well characterized by enzyme digestion and MALDI-TOF mass spectroscopy.

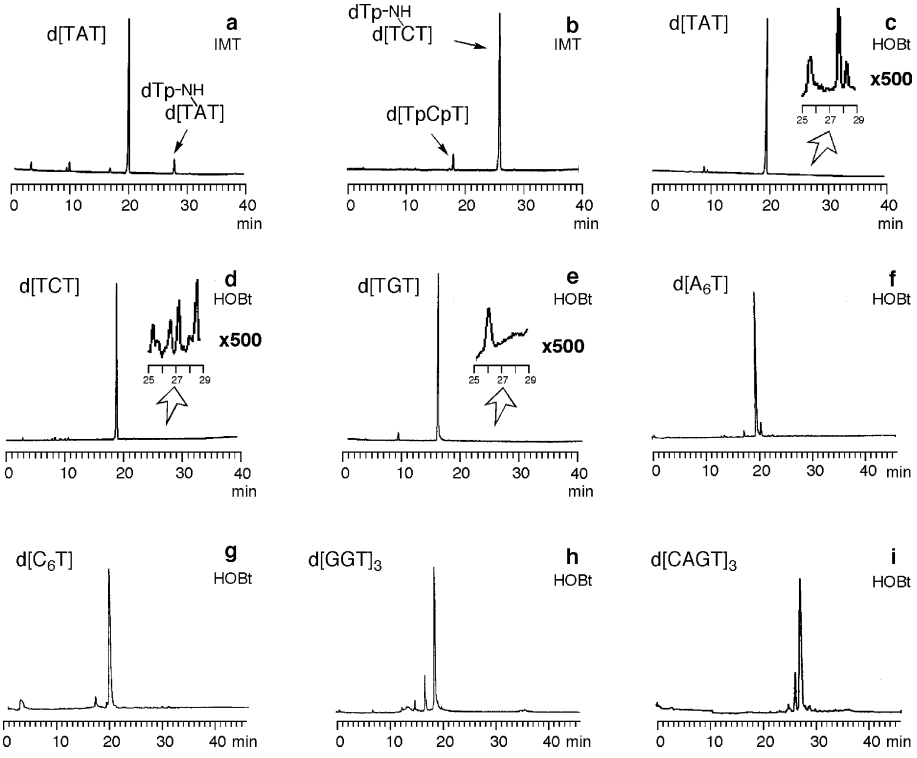

Figure 3. The anion-exchange HPLC profiles of the crude mixtures obtained by the solid-phase synthesis. (a) d[TAT], IMT; (b) d[TCT], IMT; (c) d[TAT], HOBt; (d) d[TCT], HOBt; (e) d[TGT], HOBt; (f) d[A_6T], HOBt; (g) d[C_6T], HOBt; (h) d[GGT]$_3$, HOBt; (i) d[CAGT]$_3$, HOBt.

In conclusion, we have realized the hitherto highest *O*-selective phosphorylation and synthesizing oligodeoxyribonucleotides in satisfactory yields without using base amino protecting groups, capping reaction and post-treatment for the P–N bond cleavage after condensation. This new strategy would provide new insight into the synthesis of base-sensitive modified oligonucleotides and the high-throughput synthesis of DNA fragments on DNA chips. Further studies are now under way.

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