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Development of an efficient method for phosphorodiamidate bond formation by using inorganic salts

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ABSTRACT

Phosphorodiamidate morpholino oligonucleotides (PMOs) have been extensively applied in antisense strategies for gene regulation because of their high stability in serum and low toxicity. However, chain elongation of PMOs requires long reaction time because few efficient methods have been developed for the formation of phosphorodiamidate bonds. In this Letter, we examined the effect of various additives to improve the reaction efficiency for formation of the phosphorodiamidate bond in the synthesis of PMOs. The addition of certain inorganic salts to the reaction media was found to be more effective. Particularly, lithium bromide was the most effective reagent and led to considerable acceleration (ca. 10-fold improvement).

Chemical modification plays an important role in improving the properties of natural DNA and RNA molecules, including hybridization affinity, base recognition, and nuclease resistance.[1–4] Modification of the internucleotidic phosphate group can significantly increase the nuclease resistance of artificial oligonucleotides.[5–7] In particular, phosphorodiamidate morpholino oligonucleotides (PMOs),[8] which have a unique skeleton containing morpholine rings and phosphorodiamidate moieties shown in Figure 1, have been extensively applied in antisense strategies[9] for gene regulation because of their high stability in serum and low toxicity.[10] Recently, several research groups found that antisense PMOs could efficiently induce functional dystrophin protein expression via exon skipping by restoring in-frame transcripts in Duchenne muscular dystrophy.[11–13]

Summerton et al. previously reported the formation of phosphorodiamidate bonds using 5′-phosphorochloridate morpholino units (Fig. 1b and c) on polymer supports.[14] The PMO units could be readily isolated by silica gel chromatography because of their high chemical stability despite the presence of a P–Cl bond. However, chain elongation of PMOs requires a long reaction time because of their low reactivity. Because few efficient methods have been developed for the formation of phosphorodiamidate bonds, there is a need for further optimization of existing procedures. In this study, we improved the condensation for both the solution- and solid-phase syntheses of PMOs by varying the reaction solvent, base, and additives.

We first synthesized the morpholino-T, morpholino-C, morpholino-A, morpholino-G units (**1**, Fig. 1b, **2** Fig. 1c, **3**, **Fig. 1**d, **4** Fig. 1e, respectively) using Summerton's method.[14] To estimate the efficiency of the condensation using the morpholino-T unit, the solution-phase synthesis of morpholino TT-dimer **6** was performed under various conditions, as shown in Scheme 1 and Table 1. When dimethylimidazolidinone (DMI) was used as the solvent (entry 1), the condensation of morpholino-T derivative **5** with 1.1 equiv of T unit **1** in the presence of 2.2 equiv of *N*-ethylmorpholine (NEM) was completed within 40 min without any side reactions. However, the isolated yield was very low because it was difficult to separate target compound **6** and DMI because of high boiling point

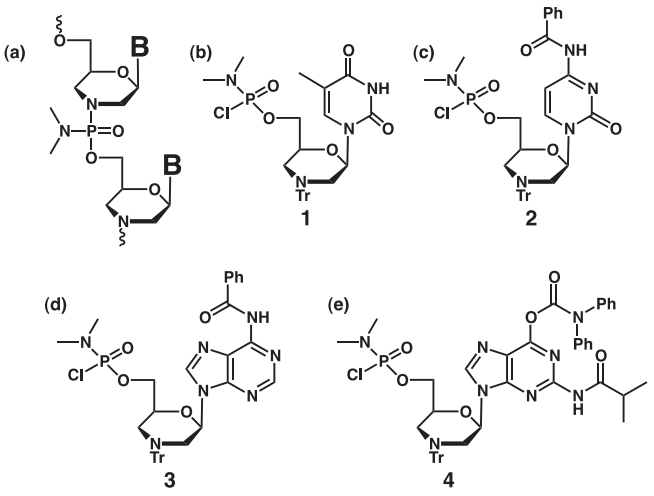

Figure 1. Structure of (a) PMO, (b) morpholino-T unit, (c) morpholino-C unit, (d) morpholino-A unit and (e) morpholino-G unit, Tr: triphenyl methyl group.

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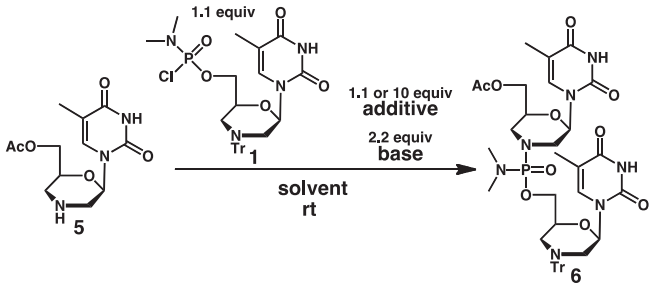


Scheme 1. Solution-phase synthesis of morpholino TT-dimer **6**.

Table 1
Efficiency of condensation of the morpholino-T unit with morpholino T (**5**)

Entry	Solvent	Base (2.2 equiv)	Additive (1.1 equiv or 10 equiv)	Time (min)	Isolated yield (%)
1	DMI	NEM	None	40	40
2	THF	NEM	None	120	88
3	DMF	NEM	None	2	84
4	CH_3CN	NEM	None	40	96
5	CH_3CN	Lutidine	None	10	82
6	CH_3CN	DBU	None	40	17
7	CH_3CN	Diisopropylethylamine	None	30	95
8	CH_3CN	NEM	DMAP (1.1 equiv)	>720	54[a]
9	CH_3CN	NEM	HOBt (1.1 equiv)	>720	80[a]
10	CH_3CN	NEM	4-Methoxypyridine *N*-oxide (1.1 equiv)	720	89
11	CH_3CN	NEM	1*H*-Tetrazole (1.1 equiv)	15	82
12	CH_3CN	NEM	LiI (1.1 equiv)	10	88
13	CH_3CN	NEM	TBAI (1.1 equiv)	60	85
14	CH_3CN	NEM	LiCl (1.1 equiv)	30	98
15	CH_3CN	NEM	LiBr (1.1 equiv)	15	Quant.
16	CH_3CN	NEM	LiBr (10 equiv)	3	Quant.

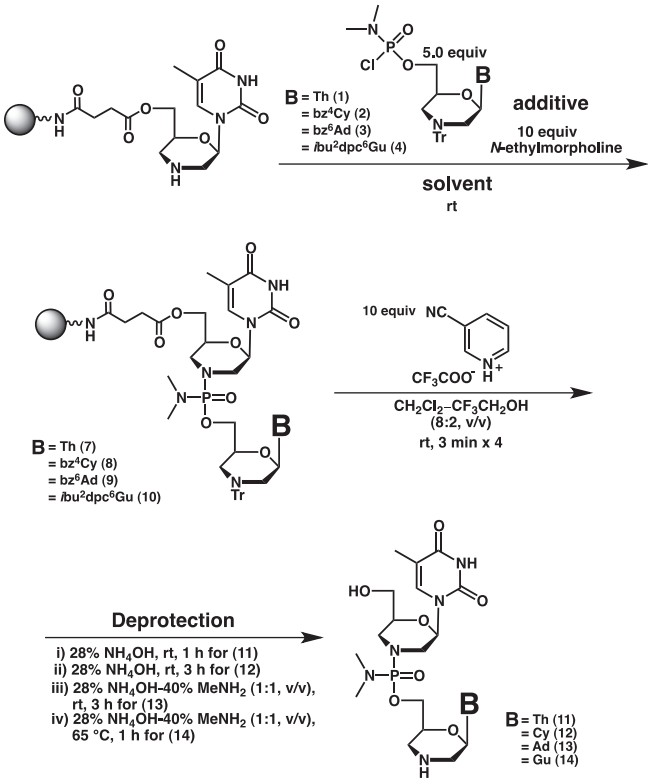
[a] The reaction mixture was worked up after 720 min.

and similar polarity. As shown in entry 2, the condensation in THF was completed in 120 min. On the other hand, when DMF was used as the solvent, some byproducts were observed (entry 3), although the reaction time was very short (within 2 min). As shown in entry 4, the condensation in CH_3CN was completed after 40 min without any side reactions, and target dimer **6** was isolated in high yield (96%). These results indicate that CH_3CN is more suitable than other solvents for the solution-phase synthesis of PMOs considering the reaction time and isolated yield.

To decrease the reaction time, we varied the base from NEM to lutidine, DBU, and diisopropylethylamine. Although the reaction using lutidine was faster than that using NEM (10 min, entry 5), the isolated yield was lower. In the condensation using DBU, the isolated yield surprisingly decreased to 17% owing to accompanying complicated side reactions (entry 6). As shown in entry 7, the use of diisopropylethylamine gave a result similar to that using NEM. Therefore, we chose NEM as a suitable base reagent for the synthesis of PMOs.

Next, we examined the effects of various additives on the condensation for the synthesis of PMOs. The addition of typical nucleophilic catalysts, such as 4-dimethylaminopyridine (DMAP), 1-hydroxybenzotriazole (HOBt), and 4-methoxypyridine N-oxide, markedly retarded the reaction, all of which required more than 720 min for completion (entries 8–10). On the other hand, the condensation using 1*H*-tetrazole was completed in only 15 min (entry 11); however, the isolated yield was lower than that for the reaction without an additive. Therefore, we examined the effects of another series of additives on the reaction rate. In these examples, a series of inorganic lithium salts (LiX, X = I, Br, and Cl) and a quaternary ammonium salt (Bu_4NI) were employed.

As shown in entry 12, in the presence of LiI, the reaction time was decreased to 10 min, although the isolated yield of the desired product decreased owing to the formation of some byproducts. Compared with this result, Bu_4NI was less effective, as shown in entry 13. However, in the presence of LiBr, the condensation was complete in 15 min without any side reactions, and target compound **6** was quantitatively isolated (entry 15). In sharp contrast, the addition of LiCl had little effect on the condensation (entry 14). These results indicate that the addition of inorganic salts (LiBr and LiI) could induce the halogen-exchange reaction of compound **1**, leading to a more reactive species.[15] Moreover, it was found that the addition of excess LiBr (10 equiv) resulted in considerable acceleration (reaction time of only 3 min, ca. 10-fold improvement), as shown in entry 16.



Scheme 2. Synthesis of PMO dimers **11**–**14** on polymer supports.

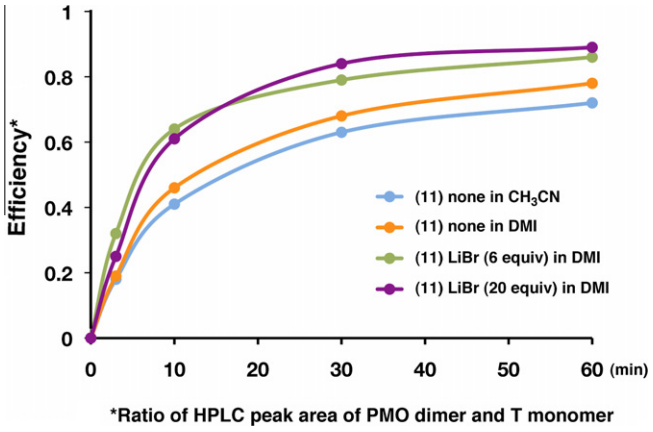


Figure 2. Reaction efficiency for the synthesis of morpholino-T dimers **11** on polymer supports.

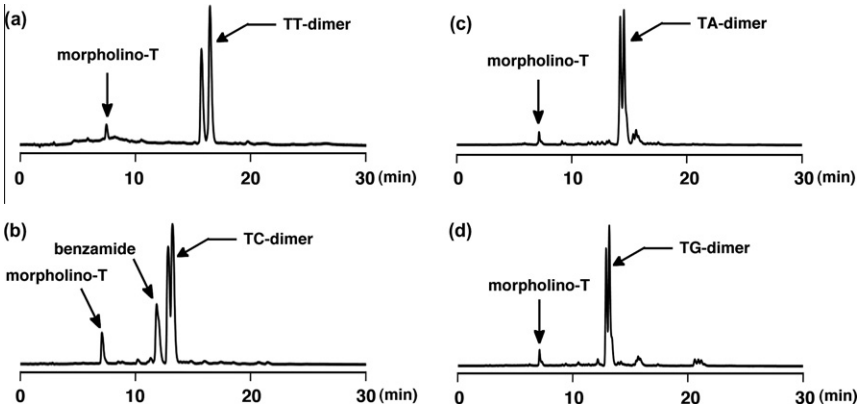


Figure 3. Reversed-phase HPLC profiles of the mixtures containing (a) PMO TT-dimer **11**, (b) PMO TC-dimer **12**, (c) PMO TA-dimer **13** and (d) PMO TG-dimer **14** after the 60-min condensation on polymer supports.

In addition, we attempted to further improve the conditions for the synthesis of PMOs using polymer supports. The condensation of morpholino units **1–4** with morpholino T-loaded polystyrene was performed in the presence of different bases and additives. Following the condensation, which was performed under various conditions, the Tr group was removed under mild acidic conditions,[16] and target PMO dimers **11–14** were released from the resins by treatment with 28% NH_4OH or AMA[17] for 1–12 h, as shown in Scheme 2. The crude mixtures thus obtained were analyzed by reversed-phase HPLC, and the reaction efficiencies were evaluated by the ratio of the peak area of target dimers **11–14** to the combined areas of all peaks of PMO observed by HPLC. The light blue line in Figure 2 shows the time course of the formation of dimer **11**. The efficiency of the condensation in CH_3CN was unexpectedly low owing to aggregation of the resin. To avoid this problem, we changed the solvent to DMI, which was previously reported as a suitable solvent for solid-phase synthesis.[14] As a result, the reaction efficiency improved, as shown by the orange line in Figure 2. In addition, it was found that the reaction efficiency was enhanced by the addition of 6 equiv of LiBr (light green line), consistent with the results of the solution-phase synthesis mentioned above. However, the addition of a large excess of LiBr (20 equiv) did not further accelerate the reaction (purple line in Fig. 2). Figure 3a shows the reversed-phase HPLC profile of the crude mixture obtained from the 60-min condensation using 20 equiv of LiBr. Target dimer **11** was isolated by reversed-phase HPLC in 45% yield and characterized by electrospray ionization mass spectrometry. Furthermore, we performed the synthesis of other PMO dimers **12–14** using 20 equiv of LiBr in DMI. These reaction efficiencies were as high as that in the synthesis of TT-dimer **11**, as shown in Figure 3b and c. The isolated yields of these dimers **12–14** were 30%, 42%, and 46%, respectively.

In summary, we examined the effects of various additives to improve the reactivity of intermediates involved in the condensation. Catalysts that are typically used in oligonucleotide and peptide syntheses and nucleophilic substitutions did not show an enhancement of the reaction rate. Similarly, organic salts like TBAI showed no improvement in the efficiency of the condensation. However, the addition of certain inorganic salts to the reaction media was found to be more effective. Particularly, lithium bromide was the most effective reagent and led to considerable acceleration (ca. 10-fold improvement). The additive effect of LiBr was also observed in the synthesis of PMO dimers **11–14** on polymer supports. Further studies are now in progress.

Acknowledgments

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