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COMMUNICATION

Synthesis of nucleoside oligophosphates by electrophilic activation of phosphorothioate

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We herein report a new synthetic method of nucleoside oligophosphates based on an electrophilic activation of 5'-phosphorothioate nucleotides. Treatment of the phosphorothioate with 2,4-dinitrochlorobenzene (DNCB) efficiently afforded the key activated species, electrophilic thioester nucleotides (EPT-N), which was coupled with various phosphate reagents to afford the target nucleoside oligophosphates, including an mRNA cap analog.

There are many nucleoside oligophosphates, such as NDPs, NTPs, and their conjugates in living organisms. [1–4] The development of practical synthetic methods for these nucleoside oligophosphates and their derivatives is essential for elucidating biological functions and drug discovery. [5–9] For example, in developing antiviral and anticancer nucleoside analogs, the preparation of modified nucleotide triphosphates (NTPs) is necessary to evaluate their recognition and incorporation by the target DNA/RNA polymerases. [5–7] The dinucleotide consisting of 7-methylated guanosine (m^7G) and a nucleoside connected by a 5'-5' triphosphate bond, a so-called cap analog, is a raw material for in vitro enzymatic synthesis of messenger RNA with 5'-cap structures whose function is essential for the translational activity and in vivo stability thereof. [8,9]

Synthetic methods for these nucleoside oligophosphates [9–14] can be classified into two types. The first one is a one-pot method to synthesize the target phosphorylated compounds, such as nucleoside mono-, di-, and triphosphates, from the corresponding nucleosides (Fig. 1A left). [15–19] The Yoshikawa method is the most utilized for synthesizing nucleoside monophosphates, using phosphoryl chloride as a source of phosphate groups. [15] The phosphorylation reaction is 5'-OH selective by using trimethyl phosphate as the solvent and does not require the protection of other hydroxy groups. [16] In this method, nucleoside di- or triphosphates can be synthesized by

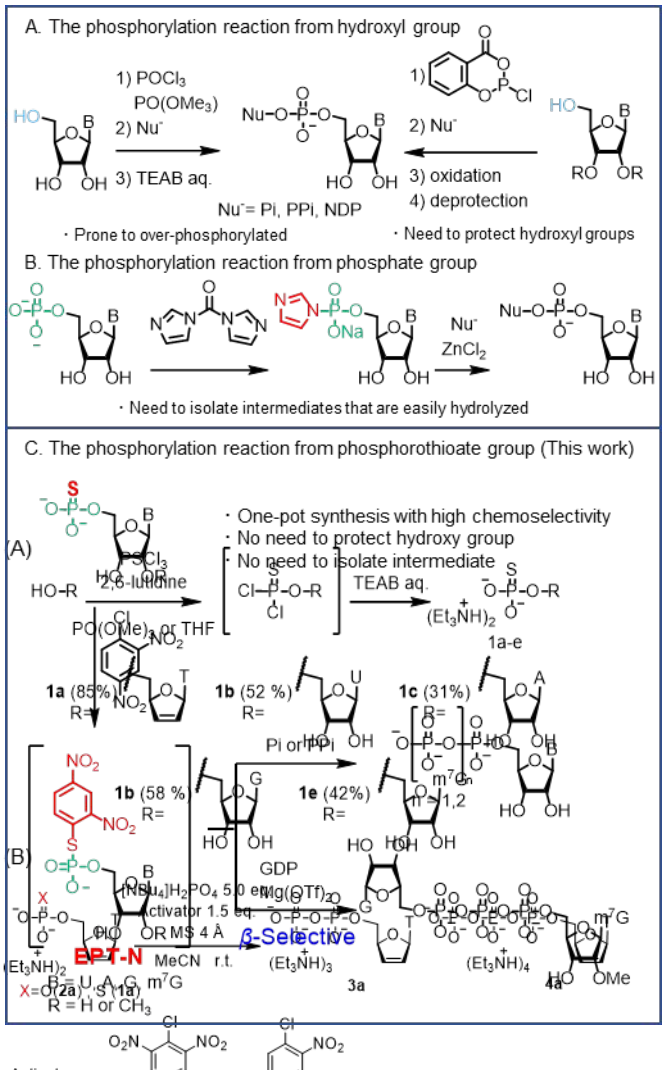

Fig. 1 : Comparison of conventional phosphorylation reactions and this study

entry	X	Activator	Time	Yield[a] (%) 2a	3a	4a
1	O	TNCB	3 h	83	16	1.2
2	S	TNCB	3 h	9.0	70	6.6
3	S	DNCB	7 h	0.3	83	6.7

a : The yield calculated by peak area of ion-exchange HPLC

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adding monophosphate or pyrophosphate to the phosphorodichloridate intermediates. This technique may produce over-phosphorylated byproducts due to the reaction with highly reactive phosphorodichloridate intermediates, therefore, chromatographic purification of the desired phosphorylated products is sometimes difficult. [17] One-pot nucleotide synthesis from nucleosides has also been reported by using trivalent phosphorylating reagents (P(III)) (Ludwig and Eckstein Method, Fig. 1A right) [18] or condensing reagents such as dicyclohexylcarbodiimide (DCC).[19] However, because reagents are highly reactive, protection of the hydroxy groups is necessary to prevent the phosphorylation at undesired positions.

Second, the synthesis of nucleoside polyphosphates by activating nucleoside monophosphates has also been reported (Fig. 1B). [20–22] The most popular method is to activate phosphoric acid as phosphorimidazolide using carbodiimidazole or imidazole/triphenylphosphine/2,2'-dithiodipyridine. [20,21,23] In this case, the isolation of the phosphorimidazolide intermediate is necessary to inhibit undesired activation, and hydrolysis of the intermediate is an issue to be improved. A reaction to activate the phosphate group with 2,4,6-trinitrochlorobenzene (TNCB) has been reported, but as with the P(III) reagent, this required protection of the hydroxyl group. [22]

In addition to these methods and the milestone work for triphosphate synthesis by Ludwig,[24] new synthetic methods for nucleotide derivatives have been reported as more practical approaches in recent years. [25-30]

As described above, polyphosphorylation reactions to date have issues that need to be improved, such as the need for protective groups on substrates due to the high reactivity of the reagents and the low stability of the reaction intermediates, which makes manipulation difficult. We decided to develop a new simple method for synthesizing polyphosphorylated compounds without protecting the substrates. In addition, the new phosphorylation method is expected to be applied to synthesize cap analogs for in vitro transcriptional synthesis of mRNAs.[8,31]

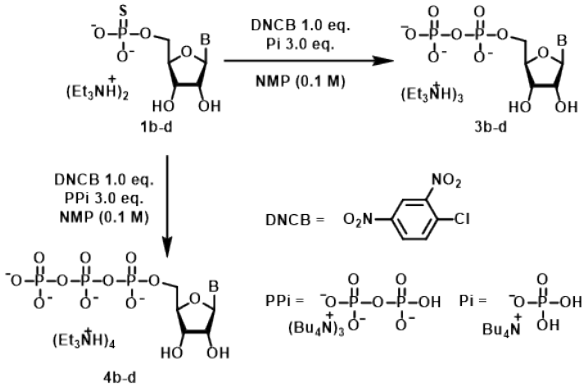
This paper reports a new phosphorylation reaction based on activating phosphorothioate groups (Fig. 1C). The phosphorothioate group, in which one of the oxygen atoms of the phosphate group is replaced by a sulfur atom, can be activated with high chemoselectivity by using the appropriate electrophilic reagents.[32, 33] We previously established a chemical ligation reaction for oligonucleotides based on electrophilic activation of terminal phosphorothioate groups with 2,4-dinitrofluorobenzene (DNFB).[34, 35] We expected that it would be possible to selectively synthesize the target compound even in the presence of multiple phosphate compounds in the system. 2,4,6-Trinitrochlorobenzene (TNCB) or 2,4-dinitrochlorobenzene (DNCB) was reacted with sulfur atoms through aromatic nucleophilic substitution reactions chemoselectively to afford the active intermediates.

Nucleoside thiophosphates (NMPα S), the starting materials for oligophosphates synthesis, were easily synthesized by the Yoshikawa method.[31,36] Namely, the 5' hydroxyl group of nucleosides was selectively reacted with thiophosphoryl chloride in trimethyl phosphate as the solvent, affording the NMPαS in 31-85% yield. (Fig. 2A).

Fig. 3 : Synthesis of nucleoside di- and triphosphates

With the starting materials in hand, the synthesis of nucleoside diphosphates was investigated using stavudine 5'- phosphate or phosphorothioate as a model substrate. After TNCB or DNCB was added to an acetonitrile solution of nucleoside phosphorothioate and tetrabutylammonium phosphate at room temperature. The conversion yield was calculated based on the area data in ion-exchange high-performance liquid chromatography (HPLC) at a specific time (Fig. 2B), and the reaction mixture was analyzed by ion-exchange HPLC (Fig. S1). While the reaction with **2a** and TNCB afforded the target material **3a** in 16% yield with a significant amount of the remaining starting material (entry 1), the reaction with **1a** and TCNB proceeded more efficiently to yield **3a** in 70% after 3 h (entry 2). However, this condition also afforded 9.0% nucleoside monophosphate **2a** and 6.6% nucleoside triphosphate **4a**. This might be due to the high reactivity of the activator, TNCB, which further activated the diphosphate to react with the tetrabutylammonium phosphate. In addition, **2a** was easily formed by hydrolysis of the intermediate due to the low stability of the intermediate. We hence evaluated DNCB, a less

Fig. 2 A) Synthesis of NMPαS, B) Selective activation of phosphorothioate groups compared to phosphate groups.



entry	Substrate	phosphate	product	Yield (%)
1	UMPαS (**1b**)	Pi	UDP (**3b**)	72[a] (69)[b]
2	AMPαS (**1c**)	Pi	ADP (**3c**)	74 (63)
3	GMPαS (**1d**)	Pi	GDP (**3d**)	88 (77)
4	UMPαS (**1b**)	PPi	UTP (**4b**)	76 (62)
5	AMPαS (**1c**)	PPi	ATP (**4c**)	74 (59)
6	GMPαS (**1d**)	PPi	GTP (**4d**)	83 (58)

a : The yield calculated by peak area of ion-exchange HPLC
b : isolated yield

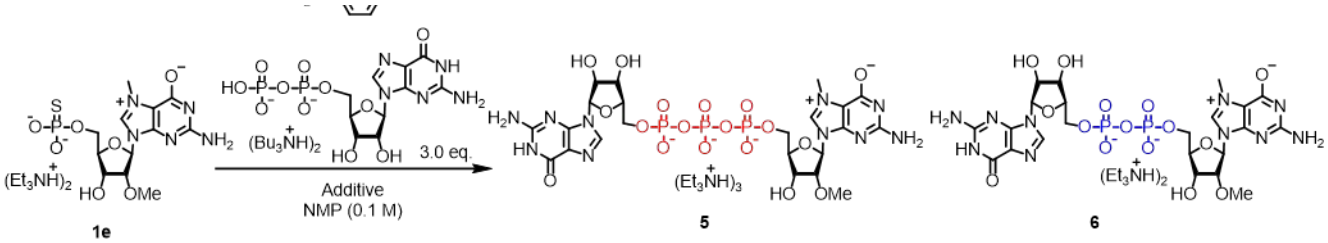
reactive activator. Under this condition, the reaction time was longer due to reduced reactivity, but the yield was improved

because hydrolysis of the intermediates was effectively suppressed. (entry 3)

Next, natural nucleoside diphosphates and triphosphates were synthesized using the corresponding ribonucleoside thiophosphates as starting materials to react with tetrabutylammonium salt of phosphate or pyrophosphate and DCNB (Figs. 3, S2). *N*-methylpyrrolidone (NMP) was selected as the solvent because of the solubility of the substrate. In contrast to the model experiment, little hydrolysis of the activating intermediate occurred without adding molecular sieves. Furthermore, no excess phosphorylation was observed by reducing the equivalent amount of activator (Fig. S2). Purification by anion exchange chromatography allowed the isolation of the target nucleotides in high yields. The side reactions of unprotected 2' or 3'- hydroxy groups at ribose or nucleobases were not observed, which suggested that the nucleoside oligophosphates could be synthesized efficiently without the need for protecting groups.

The established reaction condition was also applied for the synthesis of 5',5'-dinucleoside triphosphate of m7G and G (5, m7G (5') ppp (5') G)), the cap analog for in vitro transcription for the preparation of capped mRNA.[7] 7-Methylguanosine phosphorothioate and guanosine 5'-diphosphate (GDP) were added to an NMP solution of DNCB (Fig. 4), and the reaction mixture was analyzed by ion-exchange HPLC (Fig. S3). Under the initial conditions (entry 1), the target dinucleotide triphosphate **5** was formed in 53% yield, and the side-product 5',5'-dinucleoside diphosphate **6** in 39% yield. This side-product formation was considered to be due to the degradation of GDP to guanosine 5'-monophosphate (GMP) because of the acidification in the system during the reaction. Even though the DIPEA was added, no improvement was observed (entry 2). Reaction at a lower temperature, 0° C, did not also improve the yield (entry 3). Notably, the hydrolysis of the activated

Fig. 4: Synthesis of 5',5'-dinucleoside triphosphate



entry	DNCB (eq.)	DIPEA (eq.)	Additive (3.0 eq.)	Temp.	Time (h)	Yield[a] (%) 5	6
1[c]	1.1	0	-	r.t.	2	53	39
2[c]	1.1	1.1	-	r.t.	2	53	41
3[c]	1.1	0	-	0℃	2	49	38
4	1.1	0	-	r.t.	2	49	38
5	13	0	$Ca(OTf)_2$	r.t.→40℃	72	60	18
6	13	0	$Mg(OTf)_2$	r.t.→40℃	72	72 (70)[b]	7

a : The yield calculated by peak area of HPLC
b : Isolated yield
c : Adding molecular sieves 4 Å (400 mg/mmol)

intermediate was not observed in the absence of molecular sieves (entry 4), so molecular sieves were not used in

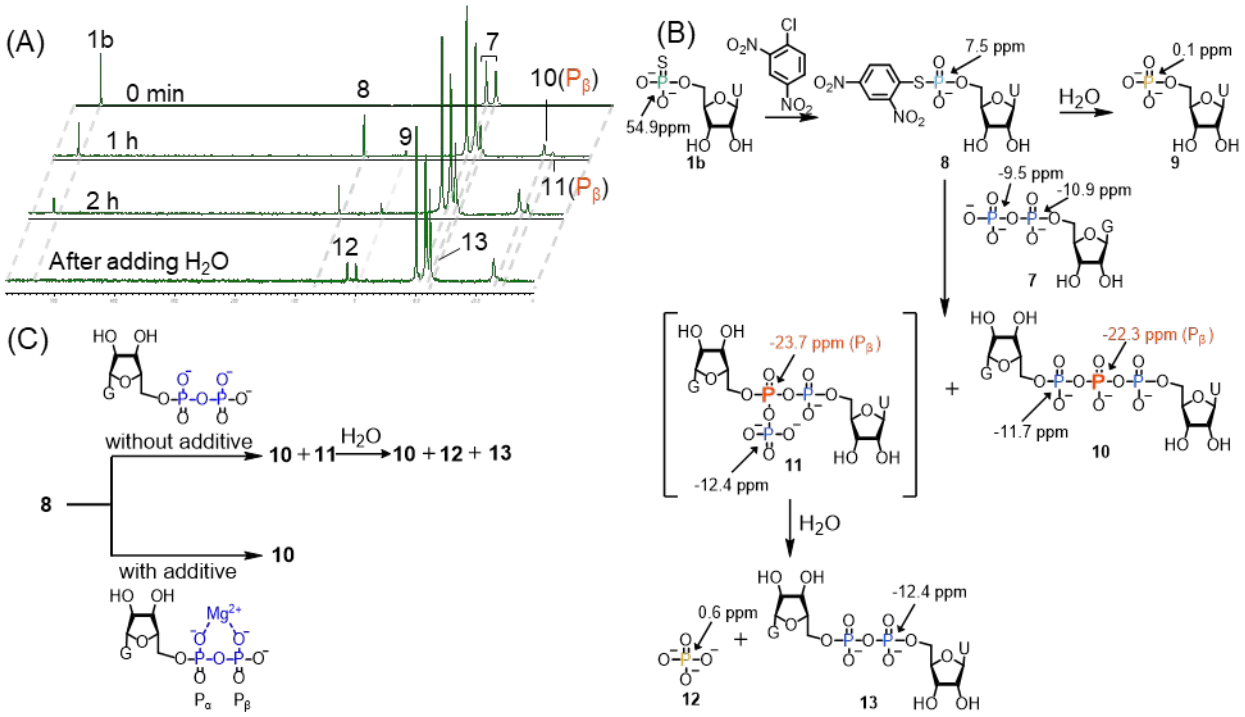


Fig. 5 : ^{31}P NMR analysis of the phosphorylation reaction based on the activation of phosphorothioate, A) Stacked ^{31}P NMR spectra: the first spectrum is of purified starting materials (**1b**, **7**), the second spectrum was obtained after addition of DNCB and reaction for 1 hr in NMR tube, the third spectrum was obtained after reaction for 2 hr in NMR tube, and the fourth spectrum was obtained after addition of water, B) Expected pathways to produce desired triphosphate (**10**) and undesired diphosphate (**13**) based on ^{31}P NMR chemical shifts of the corresponding phosphorous species, C) Control of the side reactions with divalent metal cation.

subsequent studies. To understand how this side product was formed, we performed a time-course ^{31}P NMR analysis (Fig. 5A). Before the addition of the activator, characteristic signals of phosphorothioate groups (54.9 ppm) and GDP (-9.5, -10.9 ppm) were observed (Fig. 5A). As the reaction proceeded (1 and 2 h), a signal of the activating intermediate **8** (7.5 ppm) was newly observed. At the same time, two characteristic signals (-22.3 and -23.7 ppm) were observed around -20 ppm, which would correspond to β-phosphate of the sequential triphosphate structure of **10** and **11**.[37] After quenching the reaction with water, a signal of -23.7 ppm disappeared, and signals at 0.6 ppm and -12.4 ppm were newly observed. These results suggested that the α-phosphate group of GDP reacts with **8** as a nucleophile to give then branched triphosphate **11**, which was eventually hydrolyzed upon water quenching (Fig. 5B)[37] to afford **13**, which was characterized by NMR and MS analysis after the isolation. It was assumed that the reactivity of the phosphate groups at the α- and β-positions of GDP are comparable, resulting in low selectivity and giving byproducts.

Therefore, we expected that the reactivity of the α-phosphate group would be suppressed by coordinating a divalent metal ion to the diphosphate of GDP, and the β-phosphate would selectively react (Fig. 5C). Divalent metal cation salts such as calcium and magnesium triflate were examined as an additive (entries 5, 6) considering the solubility in NMP. Adding these metal salts effectively inhibited the formation of **6**, especially the latter additive, which suppressed the formation of the diphosphate down to 7% (entry 6). However, the addition of metal salts also suppressed the formation of compound **5**, so that the activator had to be added every few hours (up to 13 eq.), and the temperature was raised to 40 ° C to consume the starting material completely. Ion exchange HPLC analysis of the reaction mixture showed 72% conversion to **5**, and purification by ion exchange chromatography allowed the isolation of the target compound in 70% yield. Classically, the synthesis of mRNA cap based on phosphorothioate activation was achieved by Hata *et al*.[38,39] with a phenylthioester intermediate. Although these methods are historically important, the use of I_2 as oxidant during coupling reaction and relatively tedious protocols for the substrate preparation would limit the substrate scope for synthesizing the analogs thereof. The use of a more reactive thioester intermediate in the current method, dinitrophenylthioester rather than phenylthioester, would also be preferable for the synthesis of various analogs synthesis in high yield.

In summary, we developed a new reaction for synthesizing oligophosphates based on the activation of the 5'-phosphorothioate group by an electrophile DNCB. This reaction can selectively activate the phosphorothioate group, even in the presence of potentially competitive multiple phosphate groups in the reaction mixture. In addition to natural nucleoside di-and triphosphates, the established reaction was successfully applicable even to the synthesis of cap analog for mRNA preparation, where the control of the reaction pathway in favor of the target linear triphosphate over the branched one was achieved by the addition of the divalent metal salt. The reaction developed and notion reported herein would be helpful for the

synthesis of many types of biologically relevant nucleoside oligophosphates, and the investigation in such direction is now undergoing in our group.

Conflicts of interest

There are no conflicts to declare.

Acknowledgments

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