

## Mechanistic studies on oxidative condensation of a thymidine 3'-*H*-phosphonate derivative with 3'-*O*-acetylthymidine

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This paper is dedicated to Prof. Harri Lönnberg on the occasion of his 60th birthday

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### Abstract

Oxidation-reduction condensation using triphenylphosphine and 2,2'-dipyridildisulfide was carried out for forming a phosphodiester bond between a mono-anionic thymidine 3'-*H*-phosphonate derivative and 3'-*O*-acetylthymidine. This reaction proceeded with simultaneous coupling and oxidation. The detailed NMR analysis of this reaction shows that an *S*-(2-pyridyl) phosphorothioate diester derivative was formed as an initial intermediate.

**Keywords:** Oxidation-reduction condensation, oxidative condensation, H-phosphonate chemistry, <sup>31</sup>P NMR analysis, mechanism

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### Introduction

For internucleotidic bond formation in oligonucleotide synthesis,<sup>1</sup> the *H*-phosphonate method<sup>2</sup> has been used along with the more popular phosphoramidite method.<sup>3</sup> The former requires reagents for dehydration between the *H*-phosphonate and alcoholic components. For this purpose, a number of condensing agents such as pivaloyl chloride, 1-adamantanecarbonyl chloride, diphenyl phosphorochloridate, Bop-Cl, PyBOP, and BOMP have been developed.<sup>2b</sup> The initial products of *H*-phosphonate diester synthesis are oxidized in the last stage to give the phosphodiester derivatives.

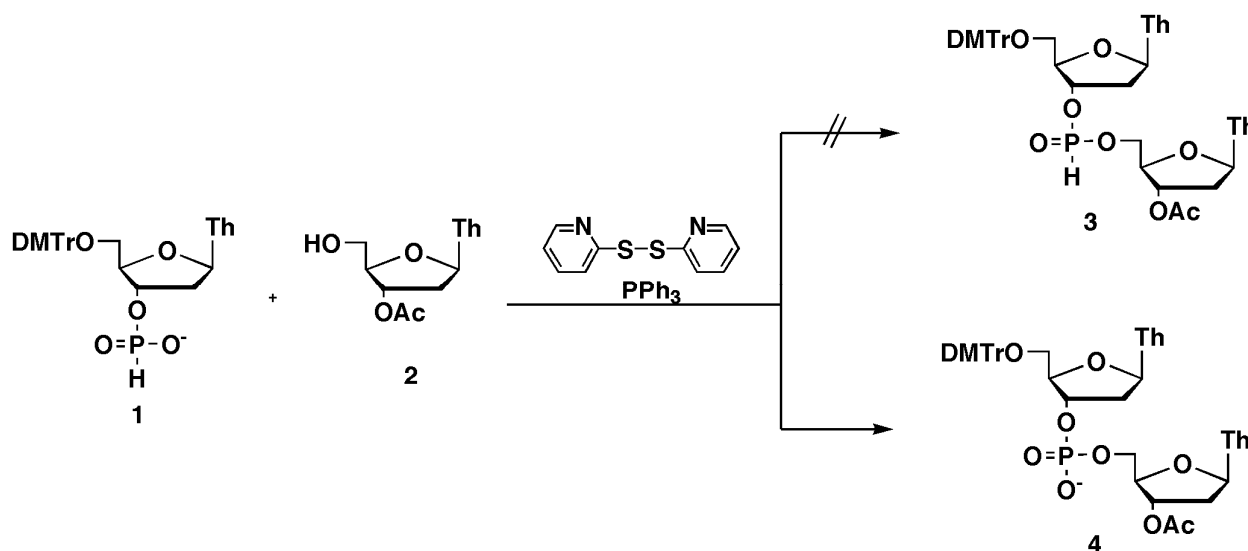
Oxidation-reduction condensation, using triphenylphosphine (PPh<sub>3</sub>) with 2,2'-dipyridyl disulfide (PySSPy) as the condensing agent, was developed in the early years of DNA chemical synthesis by Mukaiyama *et al.* for peptide and oligonucleotide syntheses.<sup>4</sup> In an attempt to develop a new condensing agent for the *H*-phosphonate method, we found that PPh<sub>3</sub>-PySSPy gave phosphodiester derivatives directly when used as the condensing agent for coupling

between 5'-*O*-dimethoxytritylthymidine 3'-*H*-phosphonate (**1**) and 3'-*O*-acetylthymidine (**2**). This result was mechanistically interesting, since no reports have appeared about such a condensation with a simultaneous oxidation reaction. In this paper, we report mechanistic studies of this procedure using  $^{31}\text{P}$  NMR analysis.

## Results and Discussion

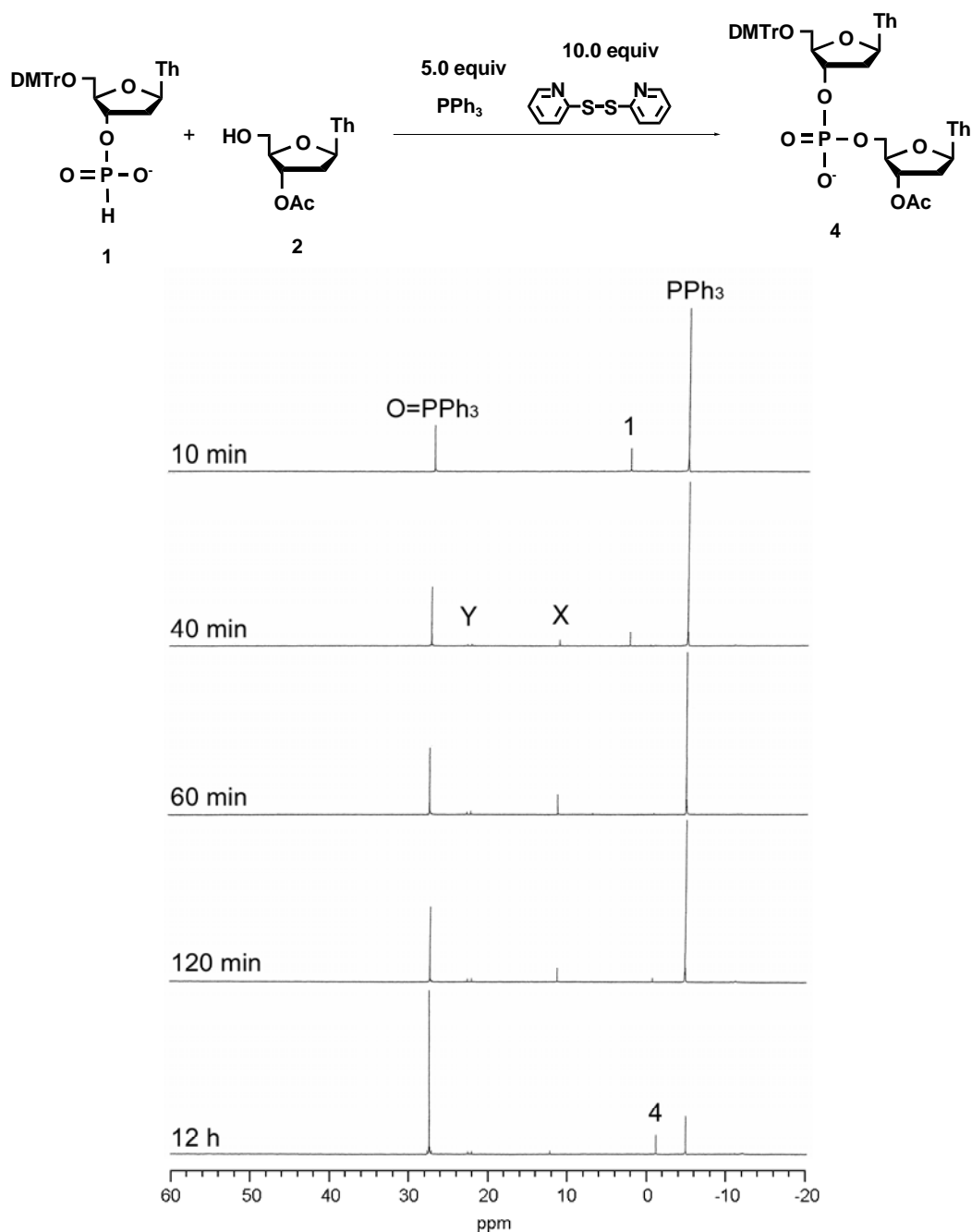
When compound **1** was allowed to react with compound **2** in the presence of 5.0 equiv of  $\text{PPh}_3$  and 10.0 equiv of PySSPy in dry pyridine at room temperature for 12 h, DMTrTpToAc **4** was obtained in 88% yield. The structure of this product was characterized by comparison with an authentic sample synthesized from the phosphoramidite approach, as well as by  $^1\text{H}$  NMR and high-resolution mass analyses.

To clarify the oxidative condensation reaction mechanism, we studied the time-course analysis of the products by  $^{31}\text{P}$  NMR spectroscopy (Figure 2).



**Figure 1.**  $^{31}\text{P}$  NMR spectra of coupling reaction between **1** and **2** in the presence of  $\text{PPh}_3$  and PySSPy.

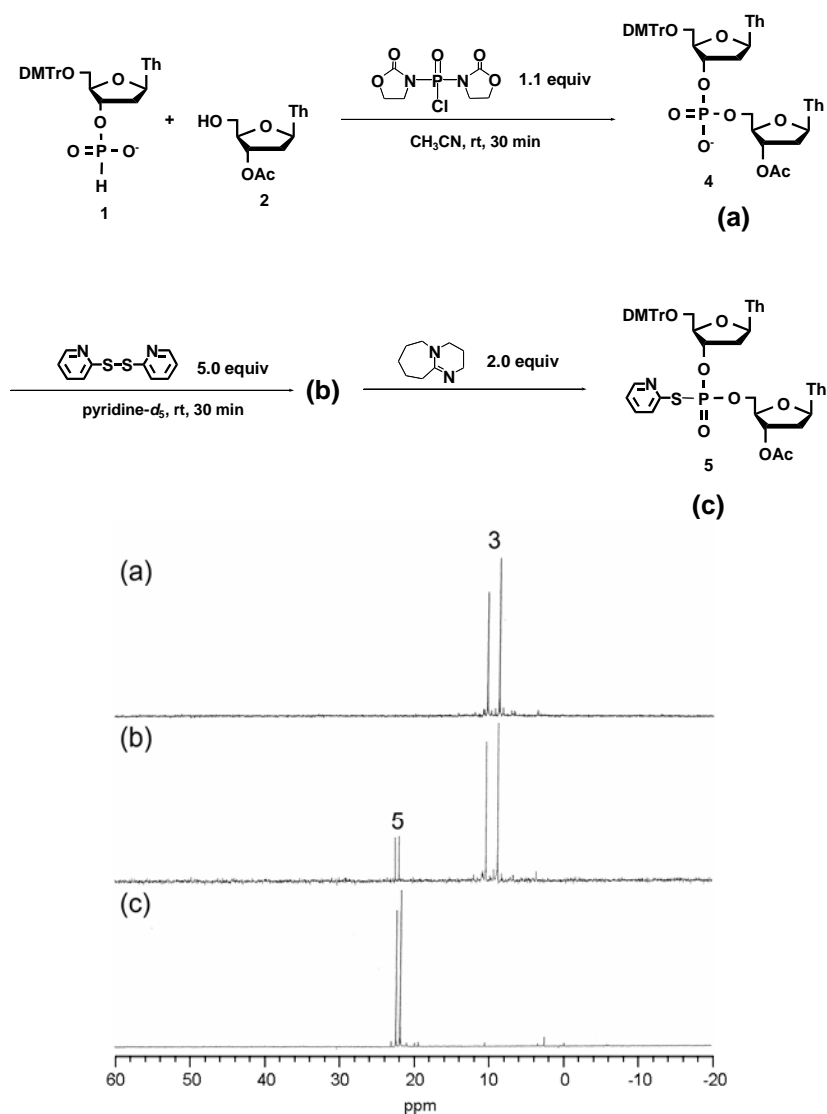
The peak of *H*-phosphonate **1** was observed at 2.44 ppm in pyridine- $d_5$ . After 60 min, this peak disappeared and a new main peak **X** was observed at 11.20 ppm. Moreover, a minor peak **Y** appeared at around 21–22 ppm. The peak **X** slowly decreased, and a peak corresponding to phosphate diester **4** appeared at  $-0.75$  ppm. After 12 h, peak **X** had disappeared.



**Figure 2.**  $^{31}\text{P}$  NMR spectrum of the reaction of **1** with **2** in the presence of  $\text{PPh}_3$  and  $\text{PySSPy}$ .

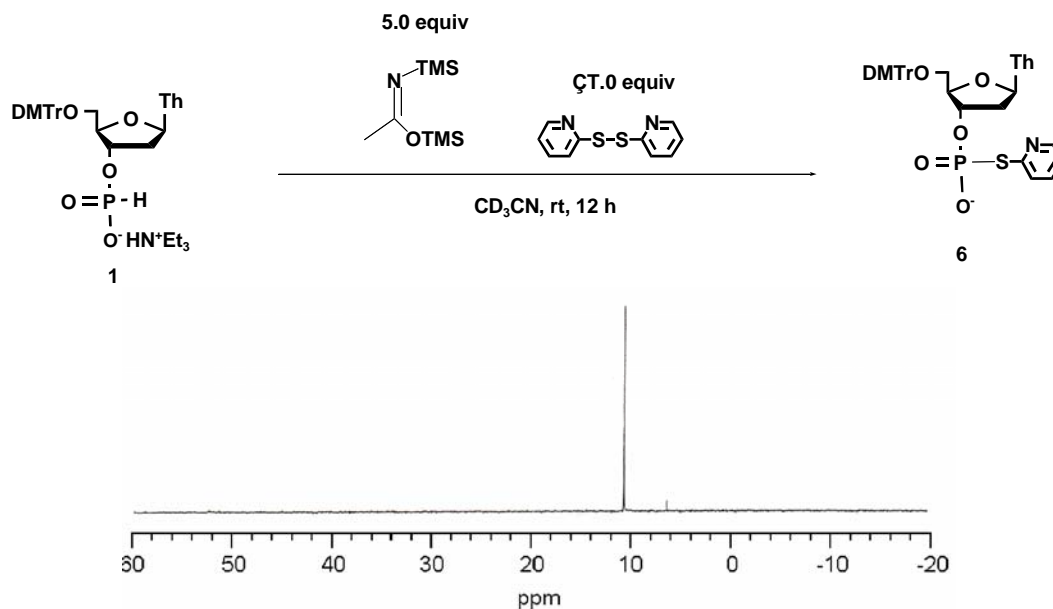
It is known that the  $^{31}\text{P}$  NMR peaks of *H*-phosphonate diester and phosphorothioate derivatives are observed at around 10 ppm.<sup>5</sup> To determine the structures responsible for peaks **X** and **Y**, we synthesized three compounds that are expected to be formed as intermediates in the present reaction: 5'-*O*-dimethoxytritylthymidine(3'-5')3'-*O*-acetylthymidine *H*-phosphonate (**3**),<sup>6</sup> *S*-(2-pyridyl) 5'-*O*-dimethoxytritylthymidine(3'-5')3'-*O*-acetylthymidine phosphorothioate (**5**), and *S*-(2-pyridyl) 5'-*O*-dimethoxytritylthymidine 3'-phosphorothioate (**6**).

*H*-phosphonate **1** was coupled with compound **2** in the presence of Bop-Cl in CH<sub>3</sub>CN. This reaction gave a diastereomeric mixture of dithymidine *H*-phosphonate derivative **3**.<sup>6</sup> After stirring for 30 min, the mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub>/triethylammonium carbonate buffer, and the organic layer was concentrated and dissolved in dry pyridine-*d*<sub>5</sub>. The <sup>31</sup>P NMR spectrum of the extract showed a set of peaks at 10.30 and 8.71 ppm corresponding to diastereomers **3**. When PySSPy was added to the solution, two diastereomeric peaks of the resulting *S*-pyridyl phosphorothiate derivative **5** appeared after 30 min at 22.47 and 21.93 ppm. Furthermore, the *H*-phosphonate diester peak of **3** had completely converged spontaneously with the peak of **5** after addition of DBU.

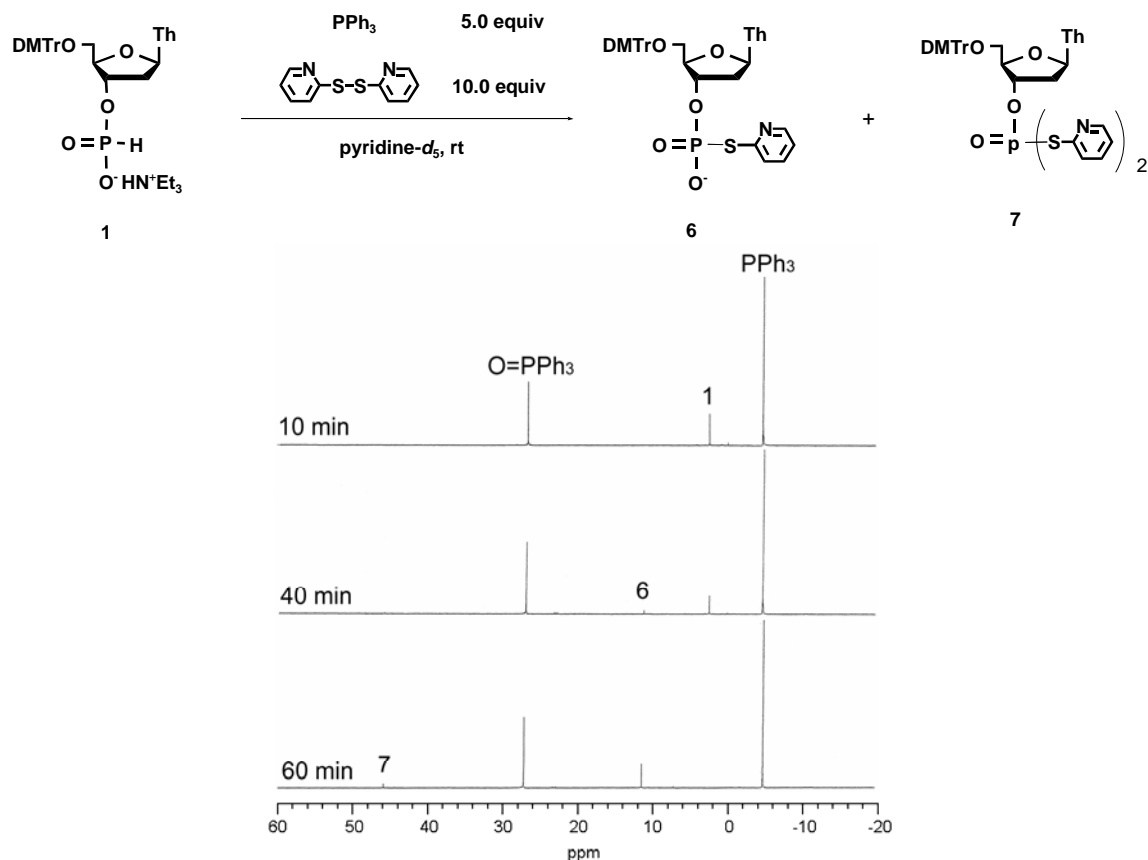


**Figure 3.** <sup>31</sup>P NMR spectrum of the reaction of **1** with **2** in the presence of Bop-Cl.

These results strongly suggested that the minor peak **Y** was due to compound **5**. Moreover, the  $^1\text{H}$ - $^{31}\text{P}$  coupling of peak **X** was not observed on the proton-coupled  $^{31}\text{P}$  NMR measurement (data not shown). This result suggested that compound **X** does not have an H-P(O) bond, which should have a large H-P coupling constant.<sup>7</sup> To synthesize compound **6** according to the well-established procedure,<sup>8</sup> compound **1** was allowed to react with PySSPy in the presence of bis(trimethylsilyl)acetamide (BSA) at room temperature in  $\text{CD}_3\text{CN}$  for 12 h. The  $^{31}\text{P}$  NMR spectrum of the mixture thus obtained showed a single peak at 10.47 ppm. Based on these results, we concluded that peak **X** must have been due to compound **6**.



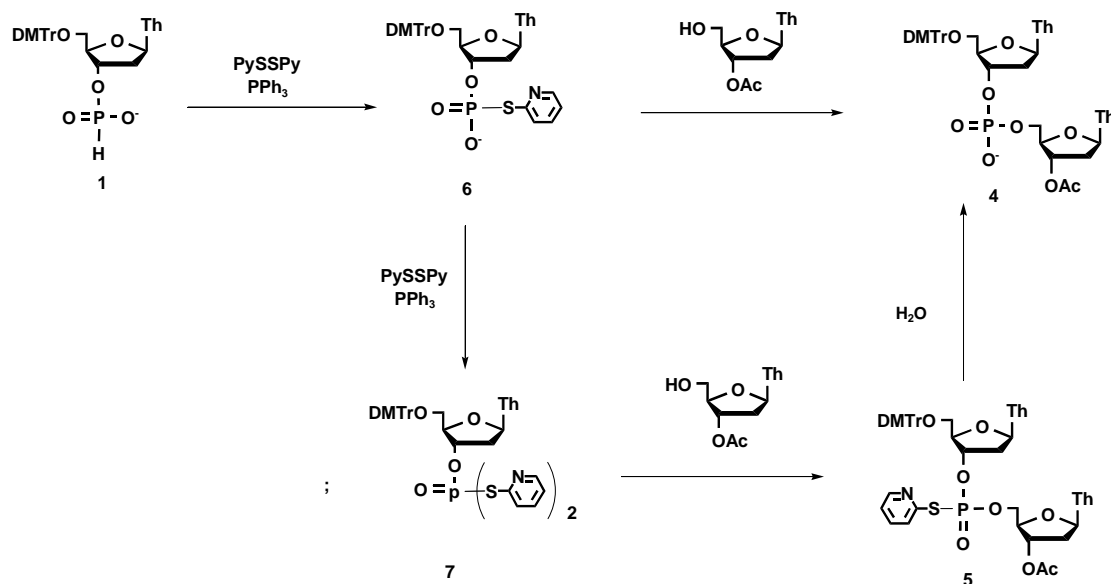
**Figure 4.**  $^{31}\text{P}$  NMR spectrum of the reaction of compound **1** with PySSPy in the presence of bis(trimethylsilyl)acetamide.



**Figure 5.**  $^{31}\text{P}$  NMR spectra of the reaction of **1** with  $\text{PPh}_3$  and PySSPy.

If this assumption is correct, compound **6** could be obtained under the conditions without adding alcohol **2**. To confirm this, compound **1** was dissolved in  $\text{pyridine-}d_5$ , and  $\text{PPh}_3$  and PySSPy were added. After 60 min, the peak of compound **6** was observed at 11.15 ppm, as expected. Interestingly, another peak at 45.70 ppm appeared: It is likely that this peak, from its chemical shift, was due to phosphorodithioate derivative **7**.<sup>9</sup> However, it was difficult to synthesize compound **7** using a different procedure, because it was very unstable and highly reactive. In independent experiments, it was found that compound **4** remained intact when treated with excess  $\text{PPh}_3$  and PySSPy in the presence or absence of 2-mercaptopyridine (data not shown).

Compound **6** was previously used as an activated ester to obtain phosphoramidate derivatives.<sup>10</sup> However, the reaction of this activated ester with nucleosides proceeded slowly. From these results, it was plausible that compound **5** was derived from the reaction of phosphorodithioate **7** with **2** (Figure 6).



**Figure 6.**  $^{31}\text{P}$  NMR spectrum of the reaction of compound **1** with PySSPy in the presence of bis(trimethylsilyl)acetamide.

Compared with the usual condensing agents such as pivaloyl chloride, diphenyl phosphorochloridate, and BOMP<sup>2b</sup> used for the *H*-phosphonate approach, the condensing agent  $\text{PPh}_3$ -PySSPy used for the oxidation-reduction condensation showed its inherent, and entirely different, properties. If this reagent could work as a simple dehydrating reagent between *H*-phosphonate **1** and alcohol **2**, the reaction was expected to proceed initially like the usual *H*-phosphonate method. Our results did not suggest this course of reaction, and thereby, at the initial stage, the *H*-phosphonate function was promptly oxidized to the less reactive five-valent species, so that slow condensation with oxidation was observed.

In addition, we could not detect the formation of symmetrical pyrophosphate derivatives, which were exclusively observed when *S*-phenyl nucleoside phosphorothioate derivatives were activated by condensing agents such as triisopropylbenzenesulfonyl chloride (TPS).<sup>11,12</sup>

## Conclusions

In summary, it is clear that the coupling reaction between *H*-phosphonate monoester **1** and alcohol **2** using  $\text{PPh}_3$  and PySSPy occurred with simultaneous oxidation to give phosphodiester **4** as the main product. In this reaction, *H*-phosphonate monoester **1** is initially oxidized by  $\text{PPh}_3$  and PySSPy to give *S*-(2-pyridinyl) phosphorothioate **6**. This intermediate compound **6** slowly reacts with alcohol **2** to give phosphodiester **4**. Since phosphodiester **4** did not react with  $\text{PPh}_3$  and PySSPy, the main route to **5** might be the reaction of **7** with **2**. This unique condensing reaction yielding phosphodiester products from alkyl phosphonates and alcohols via a one-step

reaction could be used when both components are easily available, and when the final products can be purified without difficulty.

## Experimental Section

**General Procedures.** Solvents were obtained from commercial sources. Pyridine was distilled after being refluxed over *p*-toluenesulfonyl chloride for several hours, redistilled from CaH<sub>2</sub>, and stored over 4 Å molecular sieves. Other dry solvents were stored over 4 Å molecular sieves. <sup>1</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR spectra were obtained at 500, 126, and 203 MHz, respectively. The chemical shifts were measured from tetramethylsilane (0 ppm) for <sup>1</sup>H NMR, CDCl<sub>3</sub> (77.0 ppm) for <sup>13</sup>C NMR, and 85% H<sub>3</sub>PO<sub>4</sub> as an external standard for <sup>31</sup>P NMR.

**DMTrTpToAc 4.**<sup>13</sup> A solution of 5'-*O*-dimethoxytritylthymidine 3'-*H*-phosphonate **1** (213 mg, 0.3 mmol) and 3'-*O*-acetylthymidine **2** (170 mg, 0.6 mmol) in dry pyridine (3.0 ml) under argon was treated with triphenylphosphine (393 mg, 1.5 mmol) and PySSPy (660 mg, 3.0 mmol). After stirring for 12 h at room temperature, the reaction mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (20 ml) and 0.1 M triethylammonium carbonate buffer (20 ml × 2). The combined organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated under reduced pressure. The residue was purified by silica gel chromatography (C-200, 5g, hexane/CHCl<sub>3</sub>, 80% and CHCl<sub>3</sub>/MeOH, 0 to 10%). The product was evaporated and diluted with CH<sub>2</sub>Cl<sub>2</sub>. The solution was extracted with CH<sub>2</sub>Cl<sub>2</sub> (20 ml) and 0.1 M triethylammonium carbonate buffer (20 ml × 2). The combined organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated under reduced pressure to give the triethylammonium salt of DMTrTpTAc **4** as foam (260 mg, 88%). <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 500 MHz) δ 1.25 (9H, t, *J* = 7.08 Hz), 1.32 (3H, s), 1.92 (3H, s), 2.06 (3H, s), 2.25–2.27 (2H, m), 2.33–2.39 (1H, m), 2.62–2.65 (1H, m), 2.94–2.95 (6H, m), 3.36–3.78 (1H, m), 3.49–3.51 (1H, m), 3.78 (6H, m), 4.02–4.06 (1H, m), 4.09–4.12 (2H, m), 4.32 (1H, br), 5.01 (1H, br), 5.27 (1H, br), 6.40 (1H, t, *J* = 7.08 Hz), 6.45 (1H, dd, *J* = 5.37, 9.03 Hz), 6.79–6.80 (4H, m), 7.20–7.38 (10H, m), 7.62 (1H, s), 7.78 (1H, s); <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 126 MHz) δ 9.3 (s), 11.8 (s), 12.7 (s), 21.2 (s), 37.4 (s), 39.8 (s), 45.9 (s), 53.7 (s), 55.4 (s), 64.2 (s), 65.8 (s), 75.7 (s), 76.8 (s), 84.0 (s), 84.6 (s), 84.8 (s), 85.5 (s), 87.2 (s), 111.5 (s), 111.8 (s), 113.4 (s), 127.3 (s), 128.1 (s), 128.5 (s), 130.4 (s), 135.5 (s), 135.6 (s), 135.9 (s), 136.2 (s), 144.5 (s), 151.1 (s), 151.2 (s), 158.9 (s), 164.2 (s), 170.6 (s); <sup>31</sup>P-NMR (CDCl<sub>3</sub>, 203 MHz), δ -1.11.

**<sup>31</sup>P NMR analysis of formation of 4.** A solution of 5'-*O*-dimethoxytritylthymidine 3'-*H*-phosphonate **1** (71 mg, 0.1 mmol) and 3'-*O*-acetylthymidine **2** (56 mg, 0.2 mmol) in pyridine-*d*<sub>5</sub> (0.5 ml) was treated with triphenylphosphine (131 mg, 0.5 mmol) and PySSPy (220 mg, 1.0 mmol). After 10, 40, 60, 120 min, and 12 h, <sup>31</sup>P NMR spectrum was measured; the results are shown in Figure 2.



**<sup>31</sup>P NMR analysis of formation of 3 and 5.** A solution of 5'-*O*-dimethoxytritylthymidine 3'-*H*-phosphonate **1** (213 mg, 0.3 mmol) and 3'-*O*-acetylthymidine **2** (127 mg, 0.45 mmol) in dry pyridine (3.0 ml) under argon was treated with Bop-Cl (90 mg, 0.33 mmol). After stirring for 30 min at room temperature, the reaction mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub> (20 ml) and 0.1 M triethylammonium carbonate buffer (20 ml × 2). The combined organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated under reduced pressure. The residue was co-evaporated with toluene and pyridine. The residue was dissolved with pyridine-*d*<sub>5</sub> (1.5 ml) and the <sup>31</sup>P NMR spectra was measured. PySSPy (660 mg, 3.0 mmol) was added to the solution. The mixture was stirred at room temperature for 30 min, and the <sup>31</sup>P NMR spectra was measured. DBU (90 μl, 0.6 mmol) was added to the mixtures. After 5 min, the <sup>31</sup>P NMR spectra was measured; the results are shown in Figure 3.

**<sup>31</sup>P NMR analysis of formation of 6 using BSA and PySSPy.** Compound **1** (71 mg, 0.1 mmol) was allowed to react with PySSPy (110 mg, 0.5 mmol) in the presence of BSA (122 μl, 0.5 mmol) at room temperature in CD<sub>3</sub>CN (0.5 ml). After 12 h, the <sup>31</sup>P NMR spectra was measured; the results are shown in Figure 4.

**<sup>31</sup>P NMR analysis of the reaction of 1, PPh<sub>3</sub>, and PySSPy.** A solution of 5'-*O*-dimethoxytritylthymidine 3'-*H*-phosphonate **1** (71 mg, 0.1 mmol) in pyridine-*d*<sub>5</sub> (0.5 ml) was treated with triphenylphosphine (131 mg, 0.5 mmol) and PySSPy (220 mg, 1.0 mmol). After 10, 40, and 60 min, <sup>31</sup>P NMR spectrum was measured; the results are shown in Figure 5.

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