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

Ryuuya Tawarada, Kohji Seio, and Mitsuo Sekine

Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226-8501, Japan
E-mail: msekine@bio.titech.ac.jp

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

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, ^{31}P NMR analysis, mechanism

Introduction

For internucleotidic bond formation in oligonucleotide synthesis,[1] the *H*-phosphonate method[2] has been used along with the more popular phosphoramidite method.[3] 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.[2b] 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_3) 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.[4] In an attempt to develop a new condensing agent for the *H*-phosphonate method, we found that PPh_3–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}P NMR analysis.

Results and Discussion

When compound **1** was allowed to react with compound **2** in the presence of 5.0 equiv of 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}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}P NMR spectroscopy (Figure 2).

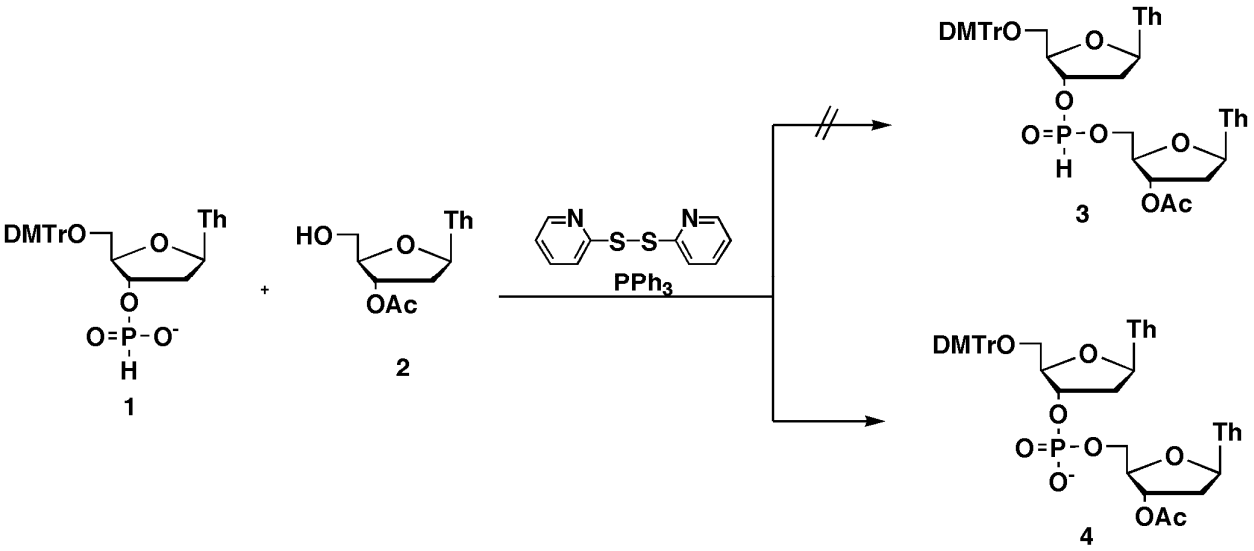

Figure 1. ^{31}P NMR spectra of coupling reaction between **1** and **2** in the presence of PPh_3 and PySSPy.

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.

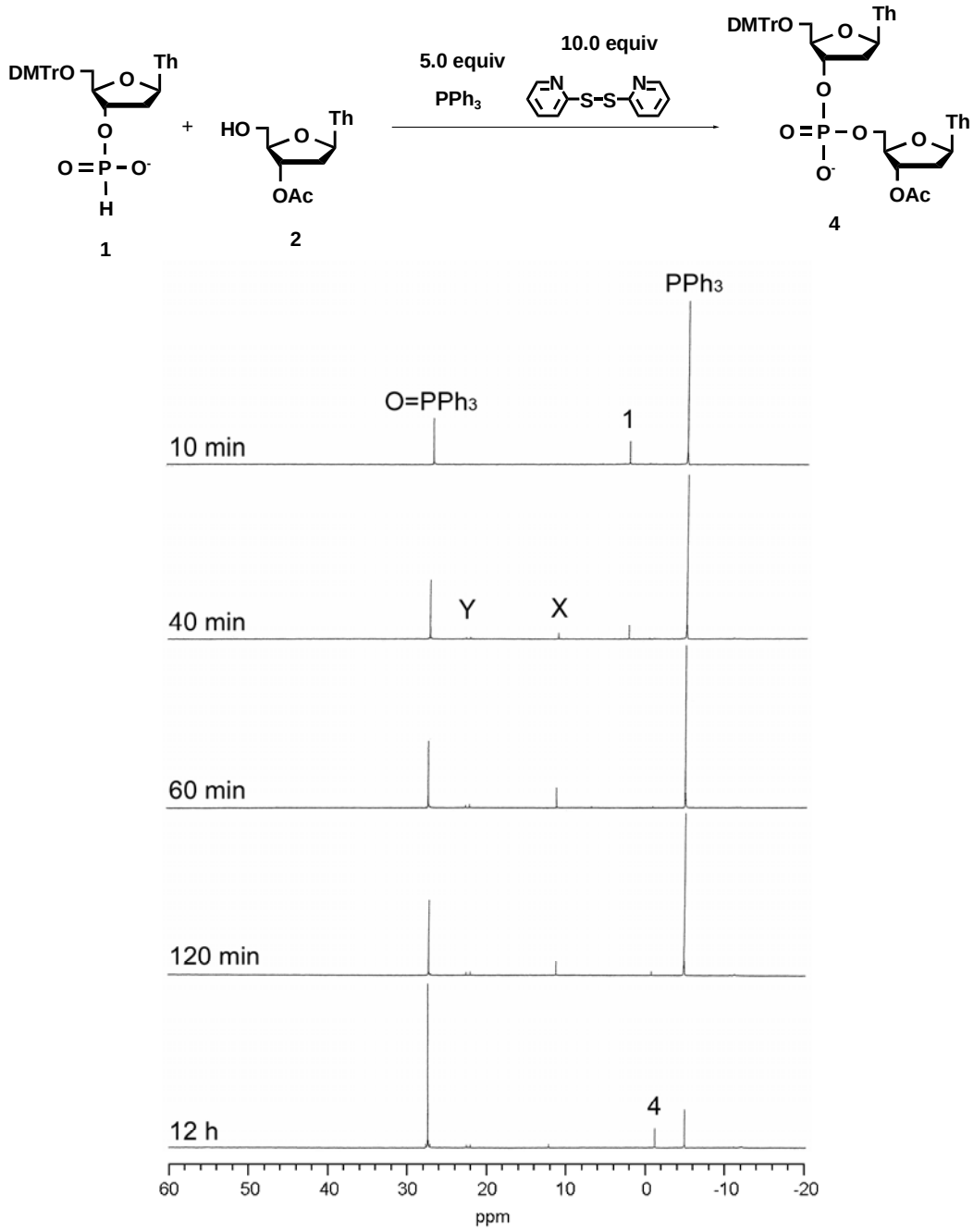

Figure 2. ^{31}P NMR spectrum of the reaction of **1** with **2** in the presence of PPh_3 and PySSPy.

It is known that the ^{31}P NMR peaks of *H*-phosphonate diester and phosphorothioate derivatives are observed at around 10 ppm.[5] 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**),[6] *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_3CN. This reaction gave a diastereomeric mixture of dithymidine *H*-phosphonate derivative **3**.[6] After stirring for 30 min, the mixture was extracted with CH_2Cl_2/triethylammonium carbonate buffer, and the organic layer was concentrated and dissolved in dry pyridine-d_5. The ^{31}P NMR spectrum of the extract showed a set of peaks at 10.30 and 8.71 ppm corresponding to diasteromers **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.

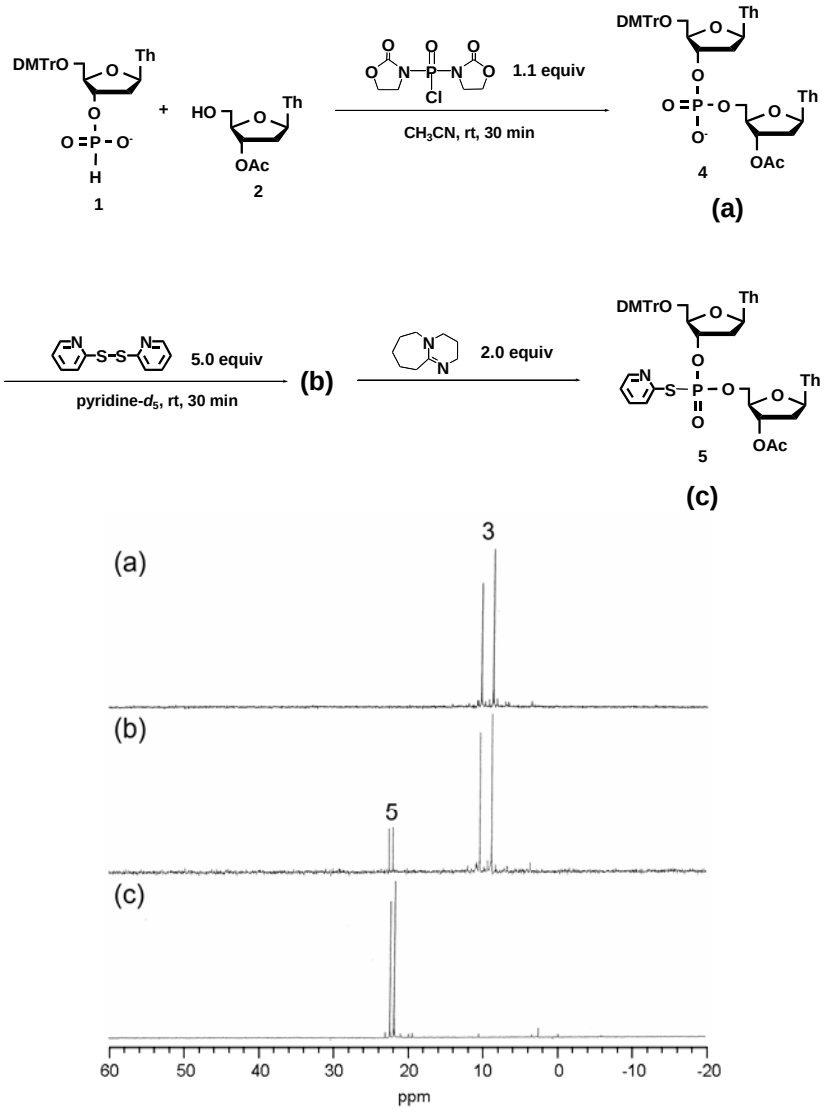

Figure 3. ^{31}P NMR spectrum of the reaction of **1** with **2** in the presence of Bop-Cl.

These results strongly suggested that the minor peak **Y** was due to compound **5**. Moreover, the ^{1}H–^{31}P coupling of peak **X** was not observed on the proton-coupled ^{31}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.[7] To synthesize compound **6** according to the well-established procedure,[8] compound **1** was allowed to react with PySSPy in the presence of bis(trimethylsilyl)acetamide (BSA) at room temperature in CD_3CN for 12 h. The ^{31}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**.

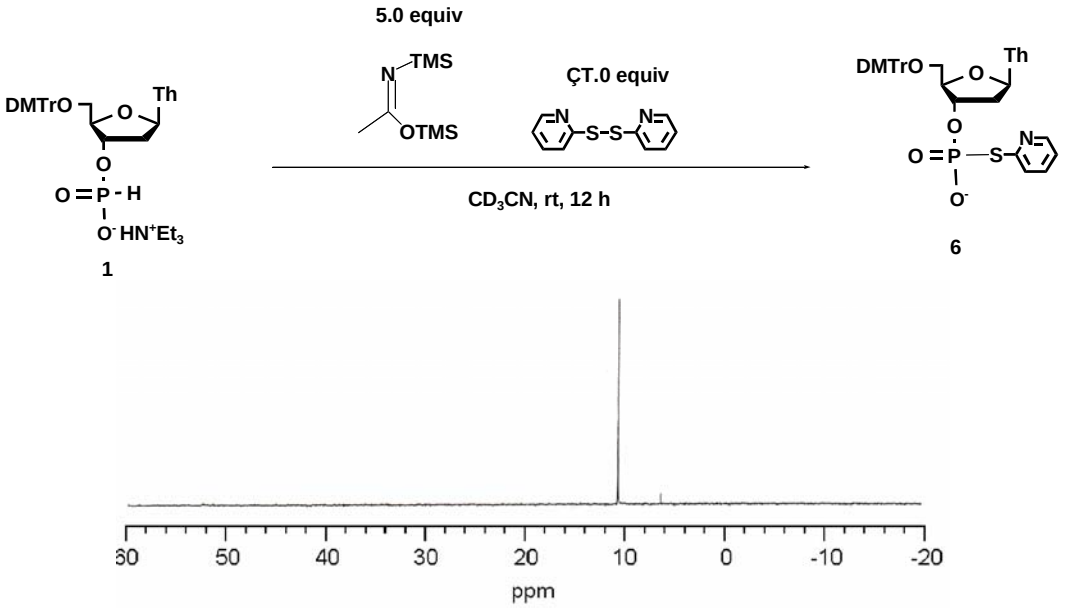

Figure 4. ^{31}P NMR spectrum of the reaction of compound **1** with PySSPy in the presence of bis(trimethylsilyl)acetamide.

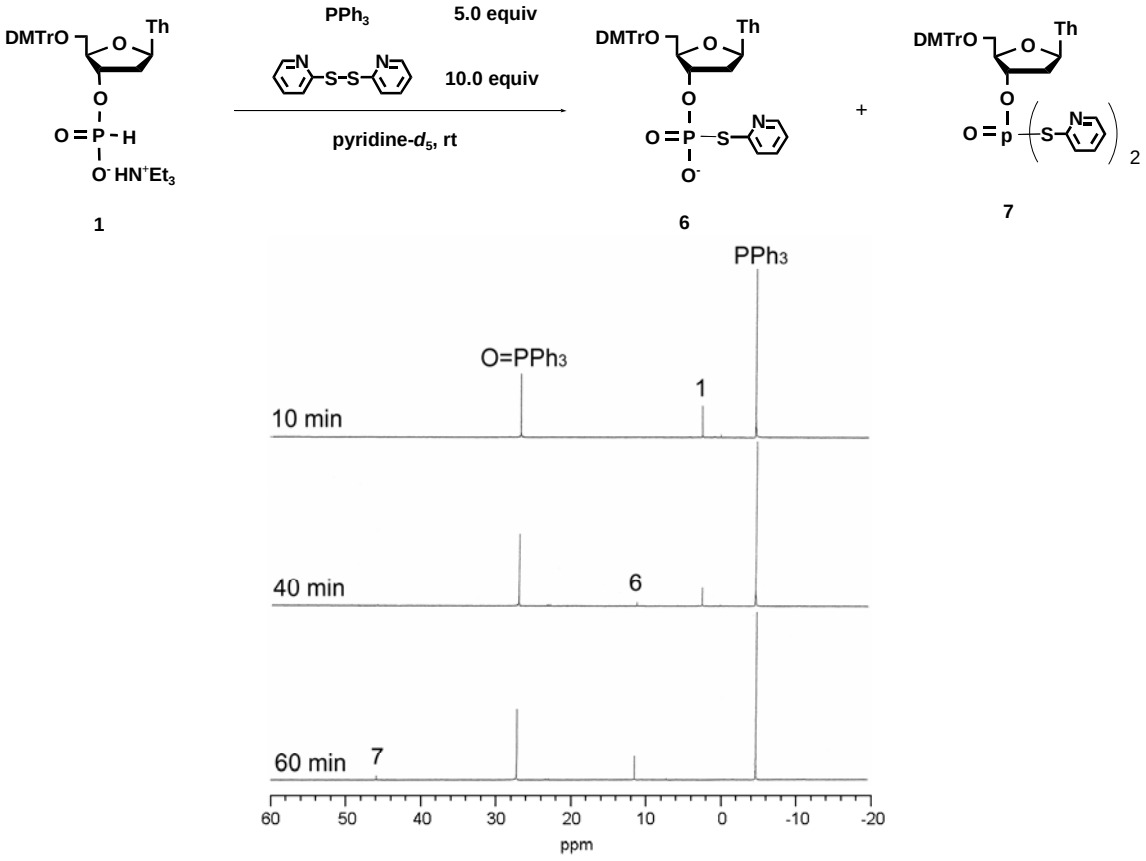

Figure 5. ^{31}P NMR spectra of the reaction of **1** with PPh_3 and PySSPy.

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 pyridine-d_5, and 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**.[9] 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 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.[10] 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).

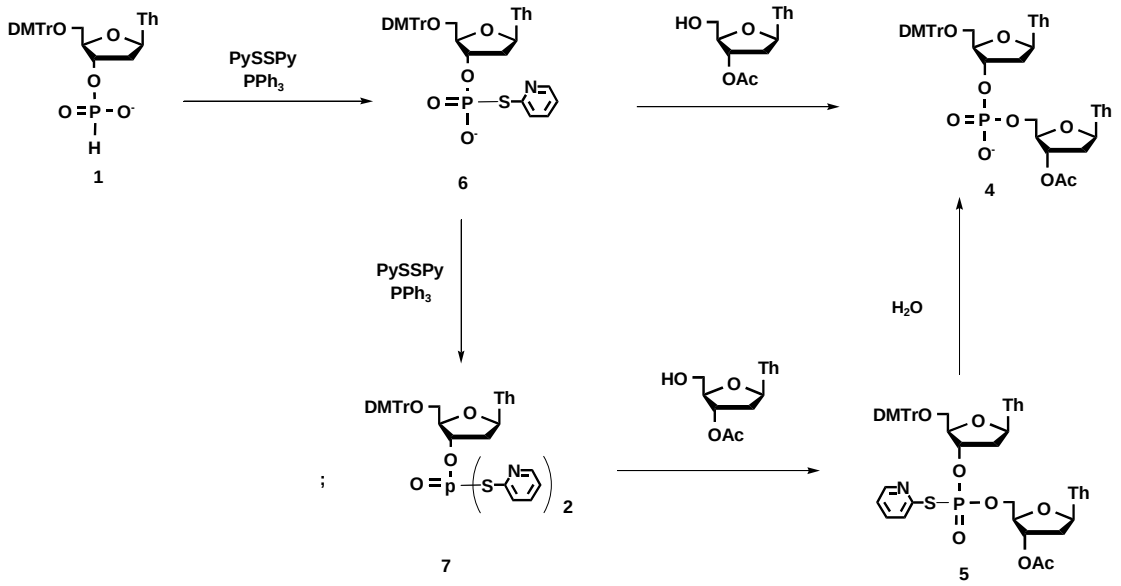

Figure 6. ^{31}P NMR spectrum of the reaction of compound **1** with PySSPy in the presence of bis(trimethylsilyl)acetamide.

Compared with the usual condensing agents such as pivaloyl chloride, diphenyl phosphorochloridate, and BOMP[2b] used for the *H*-phosphonate approach, the condensing agent 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).[11,12]

Conclusions

In summary, it is clear that the coupling reaction between *H*-phosphonate monoester **1** and alcohol **2** using 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 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 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 alchohols 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_2, and stored over 4Å molecular sieves. Other dry solvents were stored over 4 Å molecular sieves. ^{1}H, ^{13}C, and ^{31}P NMR spectra were obtained at 500, 126, and 203 MHz, respectively. The chemical shifts were measured from tetramethylsilane (0 ppm) for ^{1}H NMR, $CDCl_3$ (77.0 ppm) for ^{13}C NMR, and 85% H_3PO_4 as an external standard for ^{31}P NMR.

DMTrTpToAc 4.[13] 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_2Cl_2 (20 ml) and 0.1 M triethylammonium carbonate buffer (20 ml × 2). The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was purified by silica gel chromatography (C-200, 5g, hexane/$CHCl_3$, 80% and $CHCl_3$/MeOH, 0 to 10%). The product was evaporated and diluted with CH_2Cl_2. The solution was extracted with CH_2Cl_2 (20 ml) and 0.1 M triethylammonium carbonate buffer (20 ml × 2). The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give the triethylammonium salt of DMTrTpTAc **4** as foam (260 mg, 88%). ^{1}H-NMR ($CDCl_3$, 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); ^{13}C-NMR ($CDCl_3$, 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); ^{31}P-NMR ($CDCl_3$, 203 MHz), δ −1.11.

^{31}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_5 (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, ^{31}P NMR spectrum was measured; the results are shown in Figure 2.

^{31}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_2Cl_2 (20 ml) and 0.1 M triethylammonium carbonate buffer (20 ml × 2). The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was co-evaporated with toluene and pyridine. The residue was dissolved with pyridine-d_5 (1.5 ml) and the ^{31}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 ^{31}P NMR spectra was measured. DBU (90 µl, 0.6 mmol) was added to the mixtures. After 5 min, the ^{31}P NMR spectra was measured; the results are shown in Figure 3.

^{31}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_3CN (0.5 ml). After 12 h, the ^{31}P NMR spectra was measured; the results are shown in Figure 4.

^{31}P NMR analysis of the reaction of 1, PPh_3, and PySSPy. A solution of 5′-*O*-dimethoxytritylthymidine 3′-*H*-phosphonate **1** (71 mg, 0.1 mmol) in pyridine-d_5 (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, ^{31}P NMR spectrum was measured; the results are shown in Figure 5.

Acknowledgements

This work was supported by a Grant from CREST of JST (Japan Science and Technology Agency) and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This work was supported in part by a grant of the Genome Network Project from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by the COE21 project.

References

1. For recent reviews on oligonucleotide synthesis see (a) Cobb, A. J. A. *Org. Biomol. Chem.* **2007**, *5*, 3260. (b) Reese, C. B. *Org. Biomol. Chem.* **2005**, *3*, 3851. (c) Pon, R. T. *Artificial DNA* **2003**, 1.
2. (a) Stawinski, J.; Strömberg, R. *Methods Mol. Biol.* **2005**, *288*, 81. (b) Strömberg, R.; Stawinski, J. *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrome, D. E., Glick, G. D., Jones, R. A., Eds.; Wiley: New York, 2000; pp 3.4.1-3.4.11.

3. (a) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859. (b) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223. (c) Köster, H.; Biernat, J.; McManus, J.; Wolter, A.; Stumpe, A.; Narang, Ch. K.; Sinha, N. D. *Tetrahedron* **1984**, *40*, 103. (d) Michalski, J.; Dabkowski, W. *Top. Curr. Chem*. **200**4, *232*, 93.
4. (a) Mukaiyama, T.; Matsheda, R.; Suzuki, M. *Tetrahedron Lett*. **1970**, *11*, 1901. (b) Mukaiyama, T.; Hashimoto, M. *J. Am. Chem. Soc*. **1972**, *94*, 8528.
5. (a) Hotoda, H.; Wada, T.; Sekine, M.; Hata, T. *Tetrahedron Lett*. **1987**, *28*, 1681. (b) Regberg, T.; Stawinski, J.; Strömeberg, R. *Nucleosides Nucleotides* **1988**, *7*, 23.
6. Strömberg, R.; Stawinski, J. *Nucleic Acids Res. Symposium Series* **1987**, *18*, 185.
7. *Phosphorus-31 NMR*, Grenstein, D. G., Ed.; Academic Press Inc. **1984**, pp 38-42.
8. Hata, T.; Sekine, M. *Tetrahedron Lett*. **1974**, *15*, 3943.
9. Sekine, M.; Heikkila, J.; Hata, T. *Tetrahedron Lett*. **1987**, *28*, 5691.
10. Mukaiyama, T.; Hashimoto, M. *Tetrahedron Lett*. **1971**, *12*, 2425.
11. Sekine, M.; Mori, H.; Hata, T. *Tetrahedron Lett*. **1979**, *20*, 1145.
12. Garegg, P. J.; Stawinski, J.; Strömberg, R. *J. Chem. Soc. Perkin Trans. II* **1987**, *9*, 1209.
13. (a) Köster, H.; Cramer, F. *Just. Lieb. Ann. Chem*. **1972**, *766*, 6. (b) Dreef, C. E; Dreef-Tromp, C. M.; van der Marel, g. A.; van Boom, J. H. *Synlett*, **1990**, 481.