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Selective and Facile 5'-De(thio)phosphorylation of Oligodeoxynucleotides Having a 5'-Terminal Phosphorothioate Group by Simple Thermolysis

Hiroyuki Tsuruoka, Koh-ichiroh Shohda, Takeshi Wada and Mitsuo Sekine*

Department of Life Science, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226-8501, Japan

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Abstract: A convenient method for de(thio)phosphorylation of oligodeoxynucleotides containing a 5'-terminal (thio)phosphate group by thermolysis under nearly neutral conditions (pH 6.2-7.0) was developed. At 90 °C about a half of $(pT)_{10}$ was selectively dephosphorylated within 24 h. On the other hand. $psT(pT)_9$ having a 5'-terminal thiophosphate was easily eliminated to give $T(pT)_9$ after 4 h. Thermolysis of $d[psA(pA)_9]$ and d(psCCATTTTCAGAATTGGGTGT) gave the 5'-dethiophosphorylated products. © 1999 Elsevier Science Ltd. All rights reserved.
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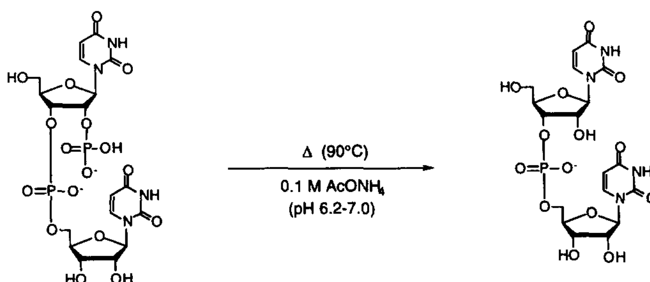
Studies of the chemical properties of various phosphoesters in nucleic acids are of great importance. To date, several research groups have reported the kinetic and mechanistic studies of hydrolysis or migration of the phosphate esters of DNA and RNA [1,2]. In these studies, it has also been disclosed that the properties of three types of phosphoesters, mono-, di- and tri-esters, are quite different as far as the mechanism of hydrolysis is concerned. It has also been pointed out that there is a great difference in the hydrolytic rate between monoesteric and diesteric phosphate groups [2]. In fact, we have recently reported that 2'-*O*-



phosphorylated diribonucleotides bearing proximal 2'-phosphomonoester and 3'-5' phosphodiester linkages underwent site-specific dephosphorylation only at the 2'-phosphomonoester site under neutral conditions [3].

On the other hand, the detailed chemical properties of nucleoside phosphorothioate monoesters, which have been synthesized and utilized in biological applications such as

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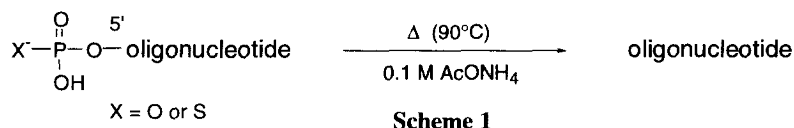
inhibitors and in mechanistic studies on enzyme reactions [4], have not been studied until quite recently, especially as far as the basic kinetics of their hydrolytic behavior are concerned. However, it has long been recognized that phosphorothioate monoesters are more unstable than the corresponding phosphates [5]. Therefore, the 5'-terminal phosphorothioate monoester linkage of oligodeoxyribonucleotides was expected to be hydrolyzed more rapidly and selectively under our thermal conditions.



Scheme 1

We first attempted non-enzymatically the 5'-selective dephosphorylation of decadeoxynucleotide $(pT)_{10}$ **1** and $d(pA)_{10}$ **2** by thermolysis [6]. It was found that $(pT)_{10}$ **1** was selectively dephosphorylated by treatment at 90 °C for 24 h to give $T(pT)_9$ in 50% yield without appreciable hydrolysis of the 3'-5' phosphodiester linkages as shown in Figure 1A.

Since the present dephosphorylation obeyed first-order kinetics, the rate constant was determined to be $3.3 \pm 0.1 \times 10^{-6}$ sec^{-1}. This simple thermal dephosphorylation is of interest because additives such as phosphatases and metal ions are unnecessary. However, application of this non-enzymatic dephosphorylation to $d(pA)_{10}$ **2** as a purine-oligonucleotide and

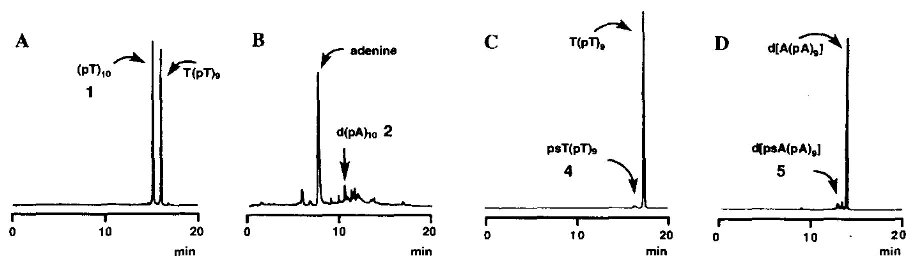


Figure 1. Reversed phase HPLC profiles of 5'-de(thio)phosphorylation of oligonucleotides: (A) **1**, 48 h; (B) **2**, 24 h; (C) **3**, 4 h; (D) **5** , 4 h.

d(pCGCGAATTCGCG) **3**, in which the four common nucleobases are contained, resulted in competitive depurination with the 5'-dephosphorylation to give complex mixtures as shown in Figures 1B and 2A, respectively. Therefore, in the case of oligodeoxynucleotides phosphorylated at the 5'-end, this simple technique should be limited to pyrimidine-rich oligodeoxynucleotide sequences.

On the other hand, under similar conditions, the 5'-terminal thiophosphate group $psT(pT)_9$ **4**, which is known to be resistant to any phosphatase [7], could be easily eliminated to give $T(pT)_9$ in 98% yield. Without appreciable hydrolysis of the 3'-5' phosphodiester and glycosyl bonds, the reaction was completely finished after 4 h, as shown in Figure lC. The 5'-dethiophosphorylation of purine-oligodeoxynucleotide $d[psA(pA)_9]$ **5** also proceeded rapidly to give a main peak (84%) of the dethiophosphorylated product $d[A(pA)_9]$, as shown in Figure 1D. The depurination was observed to a degree of less than ca. 10%, as shown in Figure 1D. When d(psCCATTTTCAGAATTGGGTGT) **6** having the *Tat* splice acceptor site of HIV-l [8] was used as a substrate, the 5'-dethiophosphorylated product was almost the only

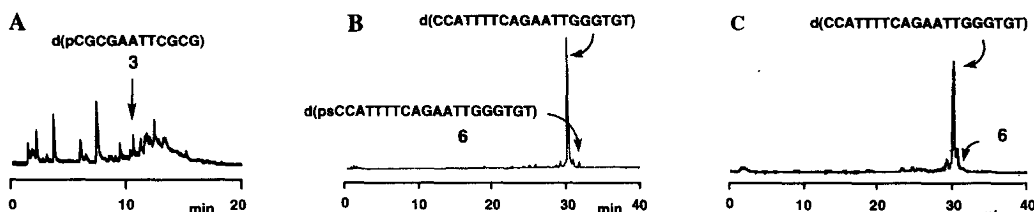


Figure 2. (A) Reversed phase HPLC profile of the mixture obtained by dephosphorylation of **3** in 0.1 M NH_4OAc after 24 h; (B) Ion exchange HPLC profile of the mixture obtained by dethiophosphorylation of **6** in 0.1 M NH_4OAc after 4 h; (C) Ion exchange HPLC profile of the mixture obtained by dethiophosphorylation of **6** in 10 mM phosphate after 8 h. The arrows indicate the position of **3** and **6**.

Table 1. De(thio)phosphorylation of the 5'-Terminal (thio)phosphate Group from Oligodeoxy-nucleotides Having a 5'-Terminal (Thio)phosphate Group by Thermolysis

oligodeoxynucleotide	buffer	yield of de(thio)phosphorylated product (HPLC)	first-order rate constants	
			^{a}k / 10^{-6} sec^{-1}	$t_{1/2}$ (h)
pTTTTTTTTTT (1)	0.1 M NH_4OAc	45% (48 h)	3.3 ± 0.1	58.5
d(pAAAAAAAAAA) (2)	0.1 M NH_4OAc	-	b	
d(pCGCGAATTCGCG) (3)	0.1 M NH_4OAc	8% (6 h)	b	
psTTTTTTTTTT (4)	0.1 M NH_4OAc	98% (4 h)	297 ± 5	0.65
d(psAAAAAAAAAA) (5)	0.1 M NH_4OAc	84% (4 h)	370 ± 5	0.52
d(psCCATTTTCAGAATTGGGTGT) (6)	0.1 M NH_4OAc	79% (4 h)	261 ± 4	0.74
	10 mM Phosphate	69% (8 h)	123 ± 7	1.57
d(pCCATTTTCAGAATTGGGTGT) (7)	10 mM Phosphate	16% (15 h)	b	

[a] The initial concentration of the substrate was 1.5 x 10^{-4} mol dm^{-3}, and the reaction temperature was maintained at 90 °C. The reaction was monitored by HPLC and the first-order rate constant was determined. The method of least-squares was applied to fit experimental data using the equation of ln (c_0 / c) = kt. The c_0 value is the initial concentration of the starting material and c is the concentration of the starting material which remained at time t.

[b] The reaction was complicated with the concomitant hydrolysis of the glycosidic bond so that the kinetic rate could not be determined.

one after 4 h and it was obtained in 79% yield. It turned out that the internucleotidic phosphodiesters were patient enough to hydrolyze during thermolysis.

Since we observed considerable depurination during dephosphorylation of **2** or **3**, the reaction media employed for the present reaction was carefully examined. As the result, it turn out that the pH of the mixture changed from pH 7.0 up to pH 6.2 because of the inherent volatile property of ammonium acetate probably releasing ammonia even in a sealed tube. This change occurred rapidly during the first 60 min to reach pH 6.3, and thereafter the solution was rather constantly kept at pH 6.2-6.3. This pH change would contribute to acceleration of the dephosphorylation at the elevated temperature of 90 °C. In order to know the genuine de(thio)phosphorylation under actually neutral conditions at 90 °C, we also examined the use of 10 mM phosphate buffer (pH 7.0) [9]. This buffer has proved to be essentially unaffected by the temperatures ranging from 25 °C to 90°C at which pH was measured to be 6.99 after 24 h. Treatment of **6** under these conditions gave the dethiophosphorylated product in 69% yield after 8 h. The reaction velocity was retarded two times (k = 123 ± 7 x 10^{-6} sec^{-1}) compared with the use of 0.1 M ammonium acetate (k = 261 ± 4 x 10^{-6} sec^{-1}). This difference in kinetic rate is basically due to the above mentioned change of pH in the latter buffer. Treatment of **7** in 10 mM phosphate buffer at 90 °C for 15

h gave the dephosphorylated product (less than 15%) but prolonged reaction resulted in considerable decomposition with depurination as observed in the dephosphorylation of **2** and **3**. It is likely that depurination occurs even at neutral conditions at such a higher temperature. Similar observations have already been reported in the hydrolysis of DNA under neutral conditions at high temperatures [10].

These results strongly suggested that a 5'-terminal thiophosphoryl group can be rather selectively removed from a medium-sized oligonucleotide having four common bases. Since oligonucleotides thiophosphorylated at their 5'-terminal site can be prepared by both enzymatic and organochemical methods by use of [γ-S]ATP [11] and thiophosphorylating reagents [12], respectively, the present simple method for the selective 5'-terminal dethiophosphorylation would provide a variety of new techniques utilizing the transient protection strategy of the 5'-terminus of oligodeoxynucleotides, especially in molecular biology. Simultaneously, it should be noted that oligodeoxynucleotides having a 5'-terminal thiophosphate group must be carefully handled because of the above mentioned inherent instability at higher temperatures.

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References and Notes

[1] (a) Oivanen M, Lönnberg H. Trends Org. Chem. 1991;2:183-198. (b) Oivanen M, Lönnberg H. J. Org. Chem. 1989;54:2556-2560. (c) Kuusela S, Lönnberg H. J. Phys. Org. Chem. 1993;6:347-356. (d) Järvinen P, Oivanen M. Lönnberg H. J. Org. Chem. 1991;56:5396-5401. (e) Kosonen M. Lönnberg H. J. Chem. Soc. Perkin Trans. 2 1995;1203-1209.

[2] (a) Cox JR, Ramsay OB. Chem. Rev. 1964;64:317-352. (b) Westheimer FH. Chem. Rev. 1981;81:313-325.

[3] (a) Tsuruoka H, Shohda K, Wada T, Sekine M. J. Org. Chem. 1997;62:2813-2822.

[4] (a) Eckstein F. Angew. Chem. 1983;22:423-434. (b) Herschlag D, Piccirilli JA, Cech TR. Biochemistry 1991;30:4844-4854. (c) Koziolkiewicz M, Stec W. J. Biochemistry 1992;31:9460-9466.

[5] (a) Eckstein F. J. Am. Chem. Soc. 1966;88:4292-4294. (b) Eckstein F. J. Am. Chem. Soc. 1970;92:4718-4723. (b) Ora M, Oivanen M, Lönnberg, H. J. Chem. Perkin Trans. 2 1996;771-774.

[6] The reaction was carried out in 100 μL of 0.1 M NH_4OAc or 10 mM phosphate buffer at 90 °C by use of a 1.5 ml eppendorf tube tightly closed with a screw cap and an O-ring seal in a thermos bottle, the lid of which was closed to keep the same temperature in both the solution and the whole tube.

[7] Goody RS, Eckstein F. J. Am. Chem. Soc. 1971;93:6252-6257.

[8] Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalski JA, Whitenhorn EA, Baumeister K, Ivanoff L, Petteway Jr. SR, Pearson ML, Lautenberger JA, Papas TS, Ghrayeb J, Chang NT, Gallo RC, Wong-Staal F. Nature 1985;313:277-284.

[9] For precise measurement of pH at such a high temperature with the accuracy of ± 0.1 pH, an ISFET pH meter (IQ Sci. Instr. Inc., USA) was used with a 3.5 mm micro tube probe.

[10] Greer S, Zamenhof S. J. Mol. Biol. 1962;4:123-141; Shapiro R, Kang S. Biochemistry 1969;8:1806-1810.

[11] Beltz WR, O'Brien K. J. Fed. Proc. Fed. Am. Soc. Exp. Biol. 1981;40:1849.

[12] Chu BCF, Orgel LE. Postsynthesis Functionalization of Oligonucleotides. In: Agrawal S, editor; Protocols for Oligonucleotide Conjugates, Totowa, NJ: Humana Press, 1994: 145-165.