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First Synthesis of *H*-Phosphonate Oligonucleotides Bearing *N*-Unmodified Bases

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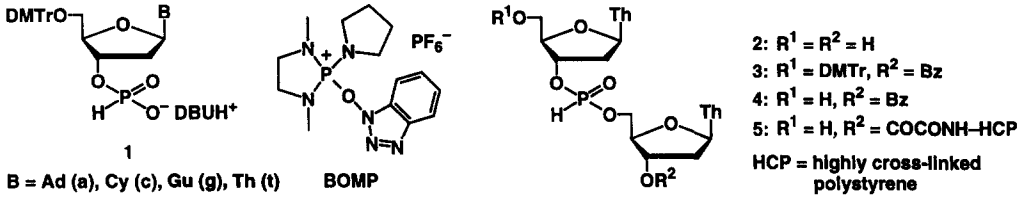
Abstract: Oligodeoxyribonucleotides bearing *H*-phosphonate internucleotidic linkages and unmodified nucleobases were synthesized for the first time by the new *H*-phosphonate method using *N*-unprotected monomers. The 6-hydroxyhexyl phosphonates at both the 5' and 3' ends were found to be highly effective for stabilization of the *H*-phosphonate oligonucleotides. Solid-phase synthesis of *H*-phosphonate oligodeoxyribonucleotides containing dA, dC, dG, and T was achieved.

Keywords: nucleic acids; solid-phase synthesis

Although *N*-protected *H*-phosphonate oligodeoxyribonucleotides have frequently been used as versatile intermediates for the synthesis of DNA and its analogs [1], *N*-unmodified *H*-phosphonate oligonucleotides have not been synthesized. This is due to their inherent instability under the basic conditions prescribed for removal of the base protecting groups. At the dimer level, however, Ogilvie and Hata have first reported the synthesis and nuclease-resistant properties of dinucleotide analogs Up(H)U [2] and Tp(H)T [3] which did not require the base protecting group. Quite recently, we have reported a new *H*-phosphonate approach using the *N*-unprotected monomers (**1a**, **1c**, **1g**, and **1t**), a new phosphonium condensing reagent BOMP (Scheme 1), and *N*-sulfonyloxaziridines as oxidizing reagents [4,5].

Scheme 1



The new method enabled us to eliminate undesirable side reactions, which have been reported for the conventional *H*-phosphonate method, such as base modifications, acylation of the 5'-hydroxyl group, acylation of the internucleotidic *H*-phosphonate linkages, formation of the trivalent phosphorus species, and hydrolysis of the *H*-phosphonate backbone during the oxidation step [1]. The new strategy is apparently suitable for the synthesis of base-sensitive oligonucleotides. We report here the first successful synthesis of *H*-phosphonate

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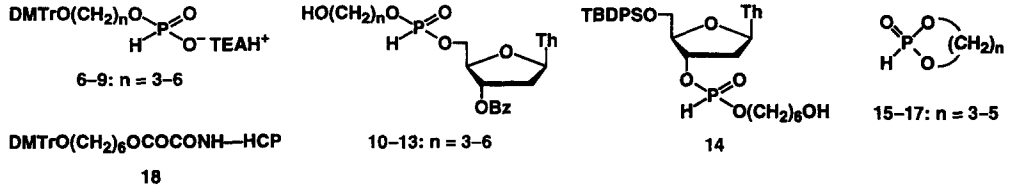
oligodeoxyribonucleotides bearing the unmodified bases by use of the new *H*-phosphonate strategy.

In the standard solid-phase synthesis of DNA, oligonucleotides are anchored to solid supports *via* a succinyl linker. However, *H*-phosphonate oligonucleotides readily decomposed upon the aqueous ammonia treatment prescribed for the cleavage of this linker. A more labile oxalyl linker, which can be cleaved by treatment with *n*-$PrNH_2$ under anhydrous conditions, has been described by Letsinger for the synthesis of base-sensitive oligonucleotide derivatives [6]. Fortunately, dialkyl phosphonates are sufficiently stable to primary alkylamines [3,7]. For example, a 3',5'-*O*-protected *H*-phosphonate dimer **3** was stable in *n*-$PrNH_2$-$CDCl_3$ (1:4, v/v) at 25 °C for several hours (^{31}P NMR). Actually, an *H*-phosphonate dimer **5** bound to the highly cross-linked polystyrene (HCP) [8] *via* an oxalyl linker was liberated by treatment with *n*-$PrNH_2$-CH_2Cl_2 (1:4, v/v) at rt for 30 min [6]. However, the desired dimer **2** could not be obtained at all. In a similar manner, an *H*-phosphonate dimer **4** having the 5'-hydroxyl group decomposed in *n*-$PrNH_2$-$CDCl_3$ (1:4, v/v) with a half-lifetime of 30 min [9]. These results indicate that the *H*-phosphonate dimers were considerably destabilized in the presence of the 3' or 5' terminal hydroxyl group which would cause the intramolecular attack on the phosphorous atom under anhydrous basic conditions. In order to confirm the intramolecular reaction of the hydroxyl group to the neighboring *H*-phosphonate diesters, the stability of 3'-*O*-benzoylthymidin-5'-yl hydroxyalkyl phosphonates (**10–13**), which have different methylene chain lengths, was investigated. Phosphonylating reagents **6–9** were prepared by a modification of the method reported by Agrawal [10] in 53-69% yields. These compounds (1.5 equiv) were condensed with 3'-*O*-benzoylthymidine in the presence of 3 equiv of *N,N*-bis(2-oxo-3-oxazolidin-1-yl)phosphonic chloride (BOP-Cl) in pyridine for 5 min followed by detritylation with 1% TFA in CH_2Cl_2 for 10 min to give 3'-*O*-benzoylthymidin-5'-yl hydroxyalkyl phosphonates (**10–13**, Scheme 2) [11].

Scheme 2



First, 3'-*O*-benzoylthymidin-5'-yl 3-hydroxypropyl phosphonate **10** (n = 3) was treated with *n*-$PrNH_2$-$CDCl_3$ (1:4, v/v) at 25 °C and the reaction was monitored by ^{31}P NMR. After 5 min, the signals of **10** (9.12 and 10.33 ppm) completely disappeared and a new signal was observed at 3.22 ppm with a $^1J_{PH}$ value of 676.2 Hz as the sole product. These results clearly suggested that the formation of a six-membered cyclic *H*-phosphonate, 2-oxo-1,3,2-dioxaphosphorinane **15** [12,13]. In addition, TLC analysis of the reaction mixture indicated the formation of 3'-*O*-benzoylthymidine. Similarly, 3'-*O*-benzoylthymidin-5'-yl 4-hydroxybutyl phosphonate **11** (n =

4) gave the seven-membered cyclic *H*-phosphonate, 2-oxo-1,3,2-dioxaphosphepane **16** (11.24 ppm, $^1J_{PH}$ = 706.8 Hz) within 5 min [13]. In the case of 3'-*O*-benzoylthymidin-5'-yl 5-hydroxypentyl phosphonate **12** (n = 5), relatively slow formation of the eight-membered cyclic *H*-phosphonate, 2-oxo-1,3,2-dioxaphosphocane **17** (7.08 ppm, $^1J_{PH}$ = 708.1 Hz) was observed [13]. The half-lifetime of **12** was 150 min. It was found that 3'-*O*-benzoylthymidin-5'-yl 6-hydroxyhexyl phosphonate **13** (n = 6) was almost completely stable in *n*-$PrNH_2$-$CDCl_3$ (1:4, v/v) at 25 °C for several hours. After 3 days, about a half amount of **13** was decomposed to give several products. On the other hand, 5'-*O*-*t*-butyldiphenylsilyl-3'-yl 6-hydroxyhexyl phosphonate **14** [11] was found to have similar stability under the same conditions. Consequently, the 6-hydroxyhexyl phosphonates at both the 5' and 3' ends were found to be highly effective to avoid the intramolecular attack of the terminal hydroxyl groups to the neighboring *H*-phosphonate diesters.

Solid-phase synthesis was started from a 6-[(dimethoxytrityl)oxy]hexyl oxalate bound to the HCP resin (**18**) and was terminated by phosphonylation of the 5'-terminal hydroxyl group with triethylammonium 6-[(dimethoxytrityl)oxy]hexyl phosphonate **9** [14]. Isolation of the *H*-phosphonate oligonucleotides is only successful when the base treatment is performed under strictly anhydrous conditions. From a practical point of view, the base treatment should be carried out in the presence of a neutral silylating reagent, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), to eliminate traces of water. The trimethylsilyl groups in the oligomer were hydrolyzed selectively by treatment with H_2O-CH_3CN (1:1, v/v) under neutral conditions without appreciable degradation of the products. For instance, a tetramer d[Hexp(H)Cp(H)Ap(H)Gp(H)Tp(H)Hex] ("Hex" refers to the 6-hydroxyhexyl group) was synthesized from **18** (0.5 μmol) with the average coupling yield of 99% (DMTr cation assay) by using the *N*-unprotected monomers (**1a**, **1c**, and **1g**) and BOMP as a condensing reagent [4]. The tetramer was released from the solid support by treatment with *n*-$PrNH_2$-BSTFA-CH_3CN (2:1:2, v/v/v) for 30 min. After removal of the reagents and solvents, the tetramer was desilylated by treatment with H_2O-CH_3CN (1:1, v/v) to give crude d[Hexp(H)Cp(H)Ap(H)Gp(H)Tp(H)Hex] in 84% yield (15.5 A_{260}). Figure 1 shows the ^{31}P NMR spectra of the product. The multiple signals of the diastereomeric isomers were observed in the region around 10.97-11.29 ppm (Figure 1A). The proton-coupled spectrum (Figure 1B) indicated that the average value of the $^1J_{PH}$ was estimated to be 728.8 Hz which is characteristic of *H*-phosphonate diesters. The product was successfully characterized by FAB mass spectrometry [15]. Unfortunately, the *H*-phosphonate tetramer was partially decomposed during reversed-phase HPLC [16]. In order to estimate the purity of the product, the *H*-phosphonate tetramer was oxidized to the corresponding phosphodiester derivative by treatment with (1*S*)-(+)-(8,8-dichlorocamphorsulfonyl)oxaziridine (DCSO) in the presence of *N,O*-bis(trimethylsilyl)benzamide (BSB) in CH_3CN [17] and the crude product was analyzed by reversed-phase HPLC (Figure 2A). Purity of the product was estimated to be 89%. The resulting crude d(HexpCpApGpTpHex) was treated with snake venom phosphodiesterase followed by calf intestinal alkaline phosphatase to give dC, dG, dA, and TpHex in the ratio of 1.00:0.92:0.92:1.08 (Figure 2B).

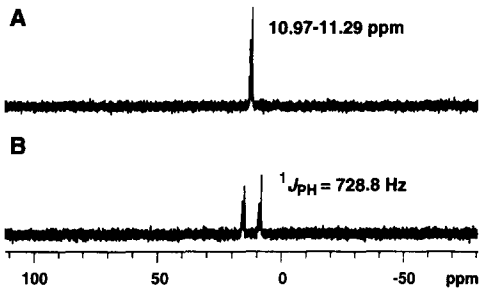


Figure 1. ^{31}P NMR spectra of d[Hexp(H)Cp(H)Ap(H)Gp(H)-Tp(H)Hex] in D_2O-CD_3CN (1:1,v/v): (A) a proton-decoupled spectrum, (B) a proton-coupled spectrum.

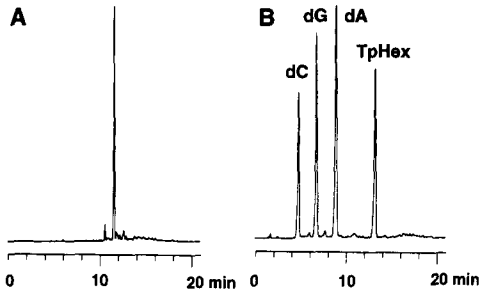


Figure 2. Reversed-phase HPLC profiles: (A) crude Hex-pCpApGpTpHex; (B) after treatment with snake venom phosphodiesterase and calf intestinal alkaline phosphatase.

In a similar manner, an *H*-phosphonate decathymidylate Hexp(H)[Tp(H)]$_{10}$Hex was synthesized in 82% yield (crude, 30.7 A_{260}). In this case, the average coupling yield was 99% (DMTr cation assay) and the purity of the product, which was estimated as the phosphodiester derivative after oxidation, was 94%. The product was characterized by ^{31}P NMR as well as FAB mass spectrometry [18].

The present method enabled us to synthesize *N*-unmodified *H*-phosphonate oligodeoxyribonucleotides. These oligomers would be useful for the synthesis of a wide variety of base-sensitive DNA analogs. Physicochemical and biological studies of *H*-phosphonate oligodeoxyribonucleotides are now in progress.

Acknowledgments

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References and Notes

[1] Froehler BC. In: Agrawal S. editor. Protocols for oligonucleotide and analogs, synthesis and properties. methods in molecular biology. Totowa: Humana Press, 1993;20:63–80 and references cited therein.
[2] Ogilvie KK, Nemer M. Tetrahedron Lett. 1980;21:4145–4148.
[3] Kume A, Fujii M, Sekine M, Hata T. J. Org. Chem. 1984;49:2139–2143.
[4] Wada T, Sato Y, Honda F, Kawahara S, Sekine M. J. Am. Chem. Soc. 1997;119:12710–12721.
[5] Wada T, Mochizuki A, Sato Y, Sekine M. Tetrahedron Lett. 1998;39:5593–5596.
[6] Alul RH, Singman CN, Zhang G, Letsinger RL. Nucleic Acids Res. 1991;19:1527–1532.
[7] Sobkowski M, Stawinski J, Sobowska A, Kraszewski A. J. Chem. Soc. Perkin I 1994;1803–1808.
[8] McCollum C, Andrus A. Tetrahedron Lett. 1991;32:4069–4072.
[9] In this case, thymidine 3',5'-cyclic *H*-phosphonate could not be detected by ^{31}P NMR. The signals of thymidin-3'-yl phosphonate (3.29 ppm, $^1J_{PH}$ = 615.2 Hz) and 3'-*O*-benzoylthymidin-5'-yl phosphonate (4.38 ppm, $^1J_{PH}$ = 614.0 Hz) were observed. These products must be formed *via* the thymidine 3',5'-cyclic *H*-phosphonate. However, the highly strained 3',5'-cyclic nucleotide may be quickly hydrolyzed by traces of water existing in the solvent to give the *H*-phosphonate monoesters.
[10] Agrawal S. In: Agrawal S. editor. Protocols for oligonucleotide conjugates, synthesis and analytical techniques. methods in molecular biology. Totowa: Humana Press, 1994;26:93–120.
[11] These compounds were partially decomposed during silica gel column chromatography. Therefore, we used these compounds without further purification. Purity of the phosphorus components of the products was higher than 98% in each case which was estimated by ^{31}P NMR.
[12] Osward AA. Can. J. Chem. 1959;37:1498–1504. Nifant'ev ÉE, Nasonovski IS, Borisenko AA. Zh. Obsh. Khim. 1970;40:1248–1251. ibid. idem. 1971;41:2368–2371.
[13] The authentic cyclic *H*-phosphonates were synthesized by the reaction of diphenyl phosphonate with the corresponding diols (1 equiv) in *n*-$PrNH_2$-$CDCl_3$ (1:4, v/v).
[14] Pyridine containing 1% of TFA was used as a solvent to suppress the *N*-phosphonylation of deoxycytidine with **9** and BOMP.
[15] FAB^+ $(M+H)^+$ Calcd for $C_{51}H_{77}N_{15}O_{25}P_5$: 1455.12. Found 1455.09.
[16] A linear gradient of 0-30% MeCN in 0.1 M NH_4OAc (pH 7.0) at 50 °C was used for HPLC. Further efforts should be made to isolate *H*-phosphonate oligonucleotides without decomposition.
[17] Wada T, Mochizuki A, Sato Y, Sekine M. Tetrahedron Lett. 1998;39:7123–7126.
[18] ^{31}P NMR (CD_3CN-H_2O, 1:1, v/v) δ 10.95-11.49, $^1J_{PH}$ = 736.1 Hz; FAB^+ $(M+H)^+$ Calcd for $C_{112}H_{158}N_{20}O_{65}P_{11}$: 3165.29. Found 3165.25.