Available online at www.sciencedirect.com
SCIENCE DIRECT
Tetrahedron Letters
Tetrahedron Letters 46 (2005) 8953–8957

Synthesis of oligodeoxyribonucleotides containing hydroxymethylphosphonate bonds in the phosphoramidite method and their hybridization properties

Akihiro Ohkubo,[a,c] Katsufumi Aoki,[a] Yusuke Ezawa,[a] Yuichi Sato,[a] Haruhiko Taguchi,[a,c] Kohji Seio[b,c] and Mitsuo Sekine[a,c,*]

[a]*Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226-8501, Japan*
[b]*Division of Collaborative Research for Bioscience and Biotechnology, Frontier Collaborative Research Center, Nagatsuta, Midoriku, Yokohama 226-8501, Japan*
[c]*CREST, JST (Japan Science and Technology Agency), Nagatsuta, Midoriku, Yokohama 226-8501, Japan*

Received 8 August 2005; revised 11 October 2005; accepted 14 October 2005
Available online 7 November 2005

Abstract—A set of diastereomeric TpT phosphoramidite building blocks, **1-f** and **1-s**, containing an alkaline-labile hydroxymethylphosphonate (HMP) linkage were synthesized. Dodecadeoxynucleotides incorporating the HMP bond were also synthesized by use of each stereochemically pure dimer block under mild conditions in the *N*-unprotected phosphoramidite method. Moreover, it turned out that HMP-oligonucleotides derived from a fast-eluted dimer block exhibit higher affinity for DNA single and double strands than those containing a slow-eluted one.

A variety of modified oligonucleotides have been synthesized as inhibitors of gene expression in antisense,[1] decoy,[2] and RNAi[3] strategies. Among them, backbone modified DNA oligomers such as phosphorothioate,[4] phosphoramidate,[5] and methylphosphonate[6] DNA have proved to have high nuclease resistance to endonucleases and exonucleases. Therefore, much attention has been paid to their potential usefulness as antisense drugs. In our previous study,[7] we reported the chemical synthesis of oligothymidylates having hydroxymethylphosphonate (HMP)[8] linkages instead of phosphate diester linkages, as shown in Figure 1. However, three unavoidable problems were reported as described below because HMP oligonucleotides were synthesized by the *H*-phosphonate method.[9] (1) It is very hard to isolate a single isomer of an HMP-modified oligonucleotide because a set of diastereomers having the HMP bond was generated at the modified phosphate linkage. (2) The detailed analysis of HMP-modified duplexes is very difficult because of the presence of this diastereomeric mixture. (3) The isolated yields of modified oligonucleotides are very low, especially, in the synthesis of longer than 10-mer oligonucleotides because of cleavage of the *H*-phosphonate diester bonds due to intermolecular cyclization in the *H*-phosphonate method.[10]

In this letter, we report the synthesis of phosphoramidite dimer building blocks, **1-f** and **1-s** (where **f** and **s** refer to a fast-eluted product and a slow-eluted one, respectively, in silica gel chromatography), containing a HMP bond (Scheme 1) and modified DNA 12mers having HMP bonds to overcome the problems described above by use of **1-f** and **1-s**. Moreover, for the first time, we revealed detailed properties of HMP-DNA such as the ability of duplex formation and base recognition.

In order to obtain the dimer phosphoramidite units **1a**,**b**, the thymidine dimer **3** having an *H*-phosphonate internucleotidic linkage was synthesized by condensation of 5′-*O*-(4,4′-dimethoxytrityl)thymidine *H*-phosphonate (**2**) with 3′-*O*-(*tert*-butyldimethylsilyl)thymidine in the presence of *N*,*N*-bis(2-oxo-3-oxazolidinyl)phosphoric chloride (BOP-Cl) as a condensing agent for 30 min. After the extraction without further purification, the resulting material was converted into the corresponding silyl phosphite intermediate by treatment with *N*,*O*-bis(trimethylsilyl)acetamide (BSA). The reaction of

* Corresponding author. Tel.: +81 45 924 5706; fax: +81 45 924 5772; e-mail: msekine@bio.titech.ac.jp

0040-4039/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2005.10.082

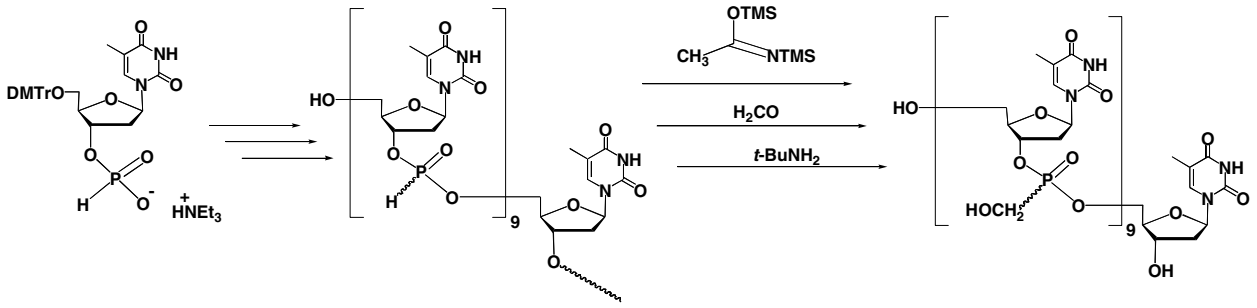
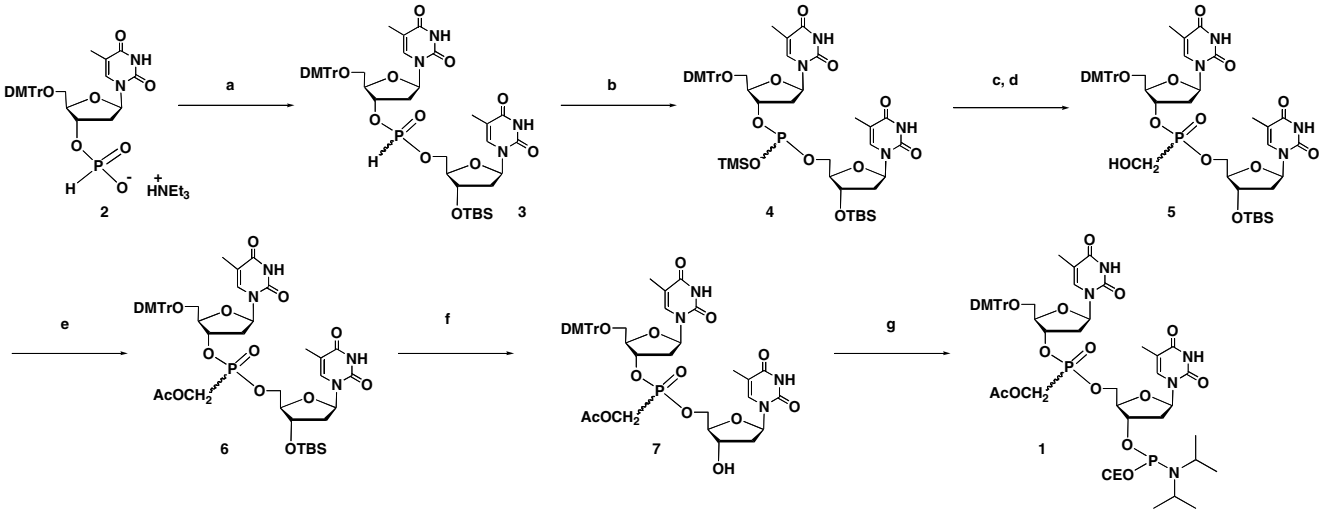


Figure 1. Previous method for the synthesis of HMP-DNA oligomers in the *H*-phosphonate method.

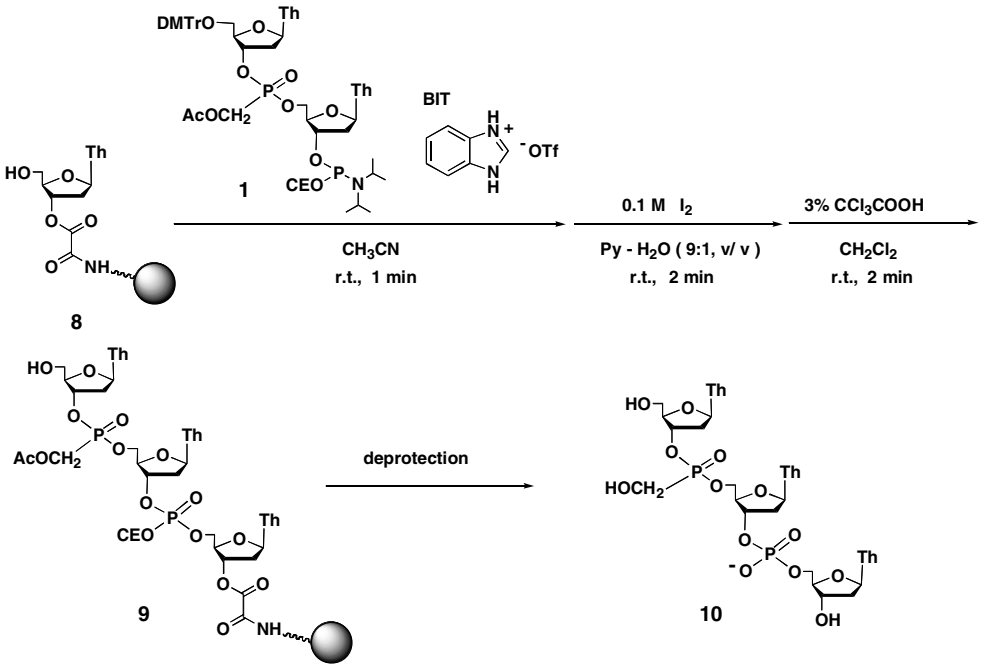


Scheme 1. Regents and conditions: (a) 3′-*O*-(*tert*-butyldimethylsilyl)thymidine (1.05 equiv), BOP-Cl (1.1 equiv), pyridine, rt, 0.5 h; (b) BSA (10 equiv), CH_3CN, rt, 0.5 h; (c) H_2CO (gas), rt, 2 min; (d) *tert*-butylamine–MeOH (1:9, v/v), rt, 15 min; (e) Ac_2O (1.5 equiv), DMAP (0.04 equiv), rt, 2 h; (f) TBAF (2 equiv), AcOH (2 equiv), rt, 4 h; (g) ClP(OCE)N*i*Pr$_2$ (1.5 equiv), EtN*i*Pr$_2$ (1.5 equiv), THF, rt, 15 min.

the resulting silyl phosphite derivative with H_2CO gas followed by treatment with 10% *t*BuNH$_2$/MeOH for 15 min gave the diastereoisomers **5-f** and **5-s**, which could be separated by silica gel column chromatography and isolated in 34% and 32% yields, respectively. Treatment of each **5-f** and **5-s** with Ac_2O gave **6-f** and **6-s**, which in turn were converted to **7-f** and **7-s** by treatment with tetrabutylammonium fluoride (TBAF)–AcOH in overall yields of 61% and 62%, respectively. In these TBAF-mediated reactions, decomposition of the hydroxymethylphophonate linkage was observed. A similar decomposition was also observed when Et_3N-3HF was used. Finally, the desired phosphoramidite dimer units **1a** and **1b** were synthesized in 90% and 79% yields, respectively, by the usual phosphitylation of **7-f** and **7-s** with ClP(OCE)N*i*Pr$_2$.

The synthesis of modified trithymidylate derivatives **10-f** having a HMP bond was carried out to examine the chemical stability of the HMP bond under the conditions prescribed for the DNA synthesis involving detritylation, coupling, and oxidation (Scheme 2). Condensation was performed by use of 20 equiv of the phosphoramidite unit **1-f** with a T-loaded highly cross-linked polystyrene resin[11] having an oxalyl linker[12] in the presence of 40 equiv of benzimidazolium triflate (BIT)[13] as a promoter for 1 min at room temperature, as shown in Scheme 2. After the coupling, oxidation was carried out by using I_2 in pyridine–water. Subsequently, the DMTr group of the terminal 5′-hydroxyl group was removed by the acid treatment using CCl_3COOH. The resulting oligomer **9** was deprotected and released from the resin by treatment with concd NH_3 for 30 min, and analyzed by HPLC. However, complicated decomposition of the target material **10** was observed, as shown in Figure 2a. To avoid the cleavage of the HMP bond, milder conditions for deprotection, that is, 5% *n*PrNH$_2$ for 30 min, were used instead of concd NH_3 for 30 min. Consequently, the side reactions could be significantly suppressed, as shown in Figure 2b. The oligomer **10-f** was isolated in satisfactory yield (63%) and characterized by ESI mass spectrometry.

Moreover, we synthesized three 12mer oligonucleotides containing one or three HMP bonds under similar mild conditions. However, the protecting groups of the nucleobases such as benzoyl and acetyl could not be completely removed by treatment with 5% *n*PrNH$_2$/MeOH for 30 min. Therefore, we tried to apply the activated phosphite method[14] without base protection to the synthesis of oligonucleotides using an ABI 392 synthesizer. These oligomers were also isolated in satisfactory yields



Scheme 2.

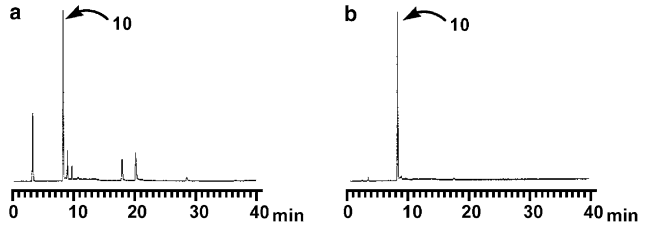


Figure 2. The anion-exchange HPLC of the crude mixture obtained in the synthesis of **10** having a hydroxymethylphosphonate linkage. (a) Deprotection by treatment with concd NH_3 for 30 min; (b) 5% nPrNH$_2$/MeOH for 30 min.

(44%, 37% and 29%) and characterized by MALDI-TOF mass spectrometry.

The measurements of T_m values were carried out to evaluate the thermal stability of duplexes formed HMP-DNAs and their complementary DNA, as shown in Table 1. As a result, it turned out that thermal stability of the duplex containing a dimer block derived from **1-f** increased compared with that of the unmodified duplex ($\Delta T_m = +0.6$ °C). This increase might result from the reduction of an anion charge of the backbone. To the contrary, that of the duplex containing a HMP (slow) linkage greatly decreased ($\Delta T_m = -3.9$ °C). It is well known that modified DNA duplexes having an *R*p isomer of methylphosphonate is thermodynamically more stable than those of the duplexes having an *S*p isomer because of steric hindrance between the methyl group of the *S*p isomer and H-3′.[15] Therefore, these results strongly suggested that the HMP (fast) is an *R*p isomer and the HMP (slow) is an *S*p isomer. In addition, the duplex having three fast HMP linkages was thermodynamically more stable than the natural duplex or the duplex having a HMP (fast) linkage ($\Delta T_m = +1.7$ °C). Analysis of the CD spectra of these modified DNA duplexes suggested that they have a typical B-form (data is not shown).

The T_m values of the DNA duplexes having a mismatched base pair are also summarized in Table 2. The base recognition ability of the DNA duplex having

Table 1. T_m values[a,b] for DNA 12mer duplexes containing HMP bonds

HMP-DNA 12mer	**5'-d(TpTxTpCpTyTpCpCpTxTpCpT)-3'**
Target DNA 12mer	**3'-d(ApApApGpApApGpGpApApGpA)-3'**

HMP modification	x	y	T_m (°C)	ΔT_m (°C)
None	Phosphodiester	Phosphodiester	34.6	—
1	Phosphodiester	HMP (fast)	35.2	+0.6
1	Phosphodiester	HMP (slow)	30.7	−3.9
3	HMP (fast)	HMP (fast)	36.3	+1.7

[a] T_m values were measured under the following conditions; 10 mM sodium phosphate (pH 7), 100 mM NaCl, 0.1 mM EDTA, 2 μM duplex.
[b] ΔT_m is the difference in T_m between the duplex having HMP linkages and that having natural phosphodiester.

Table 2. T_m values[a,b] for DNA 12mer duplexes containing HMP bonds to evaluate their base recognition ability

HMP-DNA 12mer 5'-d(TpTpTpCpTzTpCpCpTpTpCpT)-3'

Target DNA 12mer 3'-d(ApApApGpXpYpGpGpApApGpA)-3'

z	T_m, °C (ΔT_m, °C)						
	Match	Mismatch					
	-ApA-	-GpA-	-CpA-	-TpA-	-ApG-	-ApC-	-ApT-
Phosphodiester	34.6	28.7	21.0	23.5	24.1	20.1	24.2
HMP (fast)	35.2 (+0.6)	28.4 (−0.3)	20.7 (−0.3)	22.8 (−0.7)	24.1 (0)	18.9 (−1.2)	22.3 (−1.9)
HMP (slow)	30.7 (−3.9)	22.0 (−6.7)	14.7 (−6.3)	19.2 (−4.3)	21.3 (−2.8)	14.1 (−6.0)	17.5 (−4.8)

[a] T_m values were measured under the following conditions; 10 mM sodium phosphate (pH 7), 100 mM NaCl, 0.1 mM EDTA, 2 μM duplex.
[b] ΔT_m is the difference in T_m between the duplex having HMP linkages and that having natural phosphodiester.

Table 3. *T*m values[a,b] for DNA 12mer duplexes containing HMP bonds

HMP-DNA 12mer 5'-d(TpTxTpCpTyTpCpCpTxTpCpT)-3'

Target dsDNA 34mer 5'-d(GpCpApAp ApGpApApGpGpApApGpApC T T
3'-d(CpGpTpTp TpCpTpTpCpCpTpTpCpTpG T T

HMP modification	x	y	T_m, °C (ΔT_m, °C)		
			pH 7.0	pH 6.2	pH 5.4
None	Phosphodiester	Phosphodiester	7.1	20.1	28.8
1	Phosphodiester	HMP (fast)	8.3 (+1.1)	20.9 (+0.8)	29.5 (+0.7)
1	Phosphodiester	HMP (slow)	5.7 (−1.4)	14.0 (−6.1)	22.2 (−6.6)
3	HMP (fast)	HMP (fast)	9.5 (+2.4)	23.9 (+3.8)	34.1 (+5.3)

[a] T_m values were measured under the following conditions; 10 mM sodium cacodylate (pH 7.0, 6.2 and 5.4), 100 mM NaCl, 0.1 mM EDTA, 2 μM triplex.
[b] ΔT_m is the difference in T_m between the triplex having HMP linkages and that having natural phosphodiester.

a fast HMP was higher than that of the natural DNA duplex in all cases. The difference in the T_m value between HMP-DNA duplexes having a matched base pair and those having a mismatched base pair increased in the 0.6–2.5 °C region. In measurement of base recognition ability of the DNA duplex having a slow HMP, it turned out that the base recognition ability was maintained similarly to the case of a fast HMP though these T_m values greatly decreased.

Furthermore, we examined the thermal stability of the triplex containing HMP-DNA, as shown in Table 3. The triplex-formation of HMP-DNA 12mer toward the hairpin DNA 34mer was carried out in sodium cacodylate buffers (pH 7.0, 6.2 and 5.4) containing 100 mM NaCl. Consequently, the thermal stability of the triplex containing HMP-DNA was almost similar to those of duplexes containing HMP-DNA. The thermal stability of the triplex containing a HMP (fast) linkage was higher than that of the triplex containing a HMP (slow) linkage and the triplex containing three HMP (fast) linkages was the most stable among them.

In summary, we have synthesized phosphoramidite dimer building block **1** containing a HMP bond and oligonucleotides 12mer having HMP bonds in the phosphoramidite approach. Moreover, we for the first time revealed detailed properties of HMP-DNA such as the ability of duplex or triplex formation and base recognition. As a result, it was proved that oligonucleotides containing HMP (fast) linkage have not only high ability of duplex or triplex formation but also high ability of base recognition. These results suggest the usefulness of an oligonucleotide containing HMP (fast) as a DNA drug in antisense methodology or a DNA probe of DNA microarray. Further studies are now underway in this direction.

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 also supported by the COE21 project.

References and notes

1. Crooke, S. T. *Antisense Nucleic Acid Drug Dev* **1998**, *8*, vii–viii.
2. Murakami, A.; Yamamoto, Y.; Namba, M.; Iwase, R.; Yamaoka, T. *Bioorg. Chem.* **2001**, *29*, 223–233.
3. Dorsett, Y.; Tuschl, T. *Nature Rev. Drug Discovery* **2004**, *3*, 318–329.
4. (a) Stec, W. J.; Zon, G.; Egan, W.; Stec, B. *J. Am. Chem. Soc.* **1984**, *106*, 6077–6079; (b) Marti, G.; Egan, W.; Noguchi, P.; Zon, G.; Matsukura, M.; Broder, S. *Anti-*

sense Res. Dev. **1992**, *2*, 27; (c) Gao, W. Y.; Han, F. C.; Egan, W.; Cheng, Y. C. *Mol. Pharmacol.* **1992**, *41*, 223.
5. (a) Mag, M.; Engels, J. W. *Nucleic Acids Res.* **1989**, *17*, 5973–5988; (b) Chen, J.; Schultzs, R. S.; Lloyd, D. H.; Gryaznov, A. M. *Nucleic Acids Res.* **1995**, *23*, 2661–2668.
6. (a) Miller, P. S.; Agris, C. H.; Murakami, A.; Reddy, M. P.; Spitz, S. A.; Ts'o, P. O. *Nucleic Acids Res.* **1983**, *11*, 6225–6242; (b) Jager, A.; Engels, J. *Tetrahedron Lett.* **1983**, *24*, 877–880; (c) Stec, W. J.; Zon, G.; Egan, W.; Byrd, R. A.; Phillips, L. R.; Gallo, K. A. *J. Org. Chem.* **1985**, *50*, 3908–3913.
7. Wada, T.; Sekine, M. *Tetrahedron Lett.* **1995**, *36*, 8845–8848.
8. Lesnikowski, Z. J. *Bioorg. Chem.* **1994**, *22*, 128–139.
9. *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A., Eds.; John Wiley & Sons: New York, and references cited therein.
10. Wada, T.; Honda, F.; Sato, Y.; Sekine, M. *Tetrahedron Lett.* **1999**, *40*, 915–918.
11. McCollum, C.; Andrus, A. *Tetrahedron Lett.* **1991**, *32*, 4069–4072.
12. Wada, T.; Mochizuki, A.; Sato, Y.; Sekine, M. *Tetrahedron Lett.* **1998**, *39*, 5593–5596.
13. Hayakawa, Y.; Kataoka, M.; Noyori, R. *J. Org. Chem.* **1996**, *61*, 7996–7997.
14. (a) Ohkubo, A.; Ezawa, Y.; Seio, K.; Sekine, M. *J. Am. Chem. Soc.* **2004**, *126*, 10884–10896; (b) Ohkubo, A.; Seio, K.; Sekine, M. *Tetrahedron Lett.* **2004**, *45*, 363–366.
15. Bower, M.; Summer, M.; Powell, C.; Shinozuka, K.; Reagan, J. B.; Zon, G.; Wilson, W. D. *Nucleic Acids Res.* **1987**, *15*, 4915–4930.