ORGANIC LETTERS

2012
Vol. 14, No. 1
10–13

Synthesis of 5′-Terminal Capped Oligonucleotides Using O−N Phosphoryl Migration of Phosphoramidite Derivatives

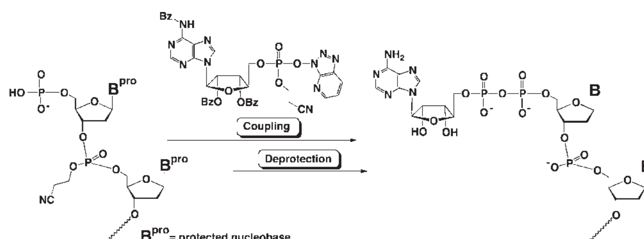
Akihiro Ohkubo, Nobuhiro Tago, Akira Yokouchi, Yudai Nishino, Ken Yamada, Hirosuke Tsunoda, Kohji Seio, and Mitsuo Sekine*

Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226-8501, Japan

msekine@bio.titech.ac.jp

Received September 27, 2011

ABSTRACT



Trivalent phosphoramidite derivatives could be readily converted by reacting with 1-hydroxy-7-azabenzotriazole to phosphotriester intermediates; these intermediates reacted smoothly with phosphorylated compounds to give pyrophosphate derivatives. This new phosphorylation approach enabled a facile and rapid synthesis of 5′-adenylated DNA oligomers ($A^{5'}$ppDNA) on resins using a silyl-type linker. Our new approach could be applied to the synthesis of a 2′-OMe-RNA oligomer containing the 5′-terminal 2,2,7-trimethylguanosine cap structure.

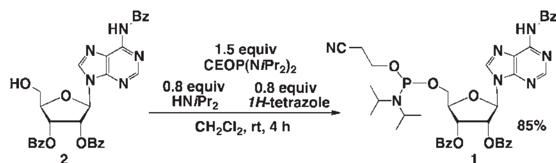
Various nucleotides containing pyrophosphate or triphosphate linkages play a very important role in biological reactions.[1] Among them, 5′-terminal capped oligonucleotides have unique biological properties. For example, 5′-adenylated DNAs ($A^{5'}$ppDNAs) are naturally generated as reaction intermediates catalyzed by DNA[2] and RNA[3] ligase and can serve as substrates in ligations catalyzed by ribozymes and 2′-deoxyribozymes.[4] The well-known 5′-terminal 7-methylguanosine cap structure ($m^7G^{5'}$pppN−) of mRNAs regulates their translation and degradation.[5] In addition, it has also been found that the 5′-terminal 2,2,7-trimethylguanosine cap structure (${m_3}^{2,2,7}G^{5'}$pppN−) of U1snRNA, which serves as a component of RNP particles formed in splicing, is produced in the cytoplasm by hypermethylation of the precursor of U1snRNA having the $m^7G^{5'}$pppN− structure and can bind to snurportin1 (a transport protein) that exclusively carries the hypermethylated molecule into the nucleus.[6] The properties of these 5′-capped oligonucleotides would provide a new insight into gene therapy and gene analysis if a new approach for their efficient chemical synthesis could be developed.

Chiuman and Li previously reported an enzymatic preparation of $A^{5'}$ppDNA using T4 DNA ligase.[7] Piccirilli et al. recently developed a new method for the chemical synthesis of $A^{5'}$ppDNA by using 5′-phosphorimidazolidate

(1) Hindsgaul, O.; Fukuda, M. *Molecular Glycobiology*; Oxford University Press: New York, 1994.
(2) (a) Weiss, B.; Jaquemin-Sablon, A.; Live, T. R.; Fareed, G. C.; Richardson, C. C. *J. Biol. Chem.* **1968**, *243*, 4543–4555. (b) Harvey, C. L.; Gabriel, T. F.; Wilt, E. M.; Richardson, C. C. *J. Biol. Chem.* **1971**, *246*, 4523–4530. (c) Lehman, I. R. *Science* **1974**, *186*, 790–797.
(3) (a) Ohtsuka, E.; Nishikawa, S.; Sugiura, M.; Ikehara, M. *Nucleic Acids Res.* **1976**, *3*, 1613–1623. (b) Uhlebeck, O. C.; Gumport, R. I. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1982; pp 31−58.
(4) (a) Li, Y.; Liu, Y.; Braker, R. R. *Biochemistry* **2000**, *39*, 3106–3114. (b) Sreedhara, A.; Li, Y.; Breaker, R. R. *J. Am. Chem. Soc.* **2004**, *126*, 3454–3460.
(5) (a) Jackson, D. A.; Pombo, A.; Iborra, F. *FASEB J.* **2000**, *14*, 242–254. (b) Parker, R.; Sheth, U. *Mol. Cell* **2007**, *25*, 635–646. (c) Wickens, M.; Goldstrohm, A. *Science* **2003**, *300*, 753–755.
(6) (a) Fischer, U.; Sumpter, V.; Sekine, M.; Satoh, T.; Lührmann, R. *EMBO J.* **1993**, *12*, 573–583. (b) Sekine, M.; Kadokura, M.; Satoh, T.; Seio, K.; Wada, T.; Fischer, U.; Sumpter, V.; Lührmann, R. *J. Org. Chem.* **1996**, *61*, 4412–4422. (c) Ohkubo, A.; Sasaki, K.; Noma, Y.; Tsunoda, H.; Seio, K.; Sekine, M. *Bioorg. Med. Chem.* **2009**, *17*, 4819–24.
(7) Chiuman, W.; Li, Y. *Bioorg. Chem.* **2002**, *39*, 332–349.

10.1021/ol2026075 © 2011 American Chemical Society
Published on Web 12/14/2011

Scheme 1. Synthesis of Adenosine 5′-Phosphoramidite Unit **1**



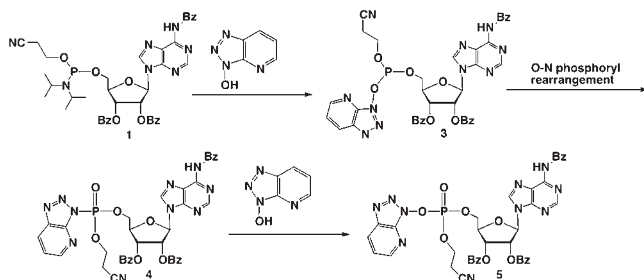


Figure 1. O–N phosphoryl rearrangement of phosphoramidite compound **1** mediated by HOAt.

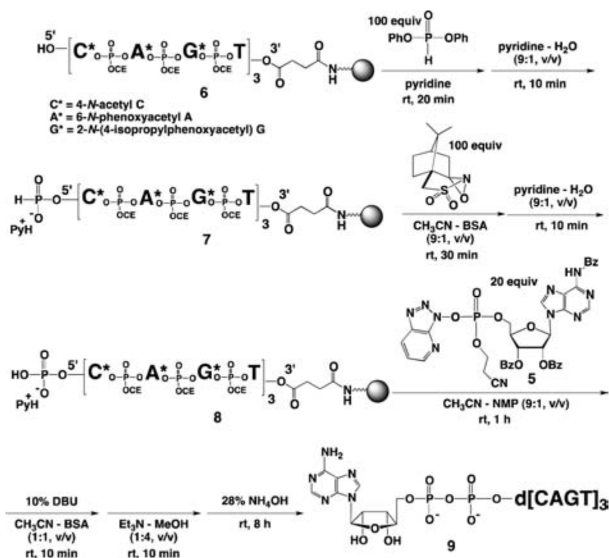
in a solid phase.[8] However, the 5′-terminal adenylation of polythymidylate having more than 10 nucleobases required a considerably longer time, that is, 16–48 h.

We found that trivalent phosphoramidite derivatives could be converted to phosphotriester intermediates by reacting with 1-hydroxybenzotriazole (HOBt) via an O–N phosphoryl rearrangement.[9] These intermediates reacted smoothly with not only phosphomonoesters but also phosphodiesters to give the corresponding pyrophosphate derivatives. Therefore, we were able to demonstrate the efficient synthesis of $A^{5'}$ppDNA and $m_3^{2,2,7}G^{5'}$pppRNA by this new phosphorylation using phosphoramidite derivatives.

We first synthesized adenosine 5′-phosphoramidite unit **1** by phosphitylation of compound **2**, as shown in Scheme 1. ^{31}P NMR analysis showed that, within 10 min, phosphoramidite unit **1** (150 ppm) immediately changed to the corresponding phosphotriester compound **5** (0 ppm) via the O–N phosphoryl rearrangement of phosphite intermediate **3** when the starting material **1** was treated with 5 equiv of HOAt (Figure 1).

To demonstrate the efficiency of compound **5** as a phosphorylating reagent for pyrophosphate bond formation, the synthesis of $A^{5'}$ppd[CAGT]$_3$ **9** was carried out on highly cross-linked polystyrene (HCP) resins containing a succinyl linker, as shown in Scheme 2. After chain elongation by using a DNA synthesizer, the treatment of resin **6** with 100 equiv of diphenyl phosphonate in pyridine,

Scheme 2. Synthesis of $A^{5'}$ppd[CAGT]$_3$ **9** on Polymer Supports Containing a Succinyl Linker



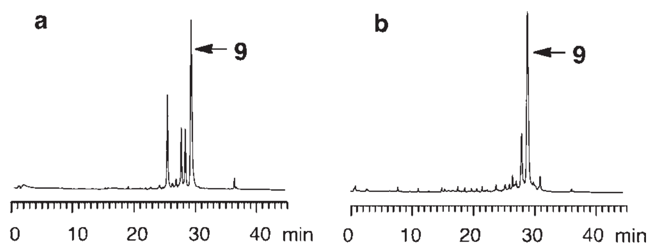
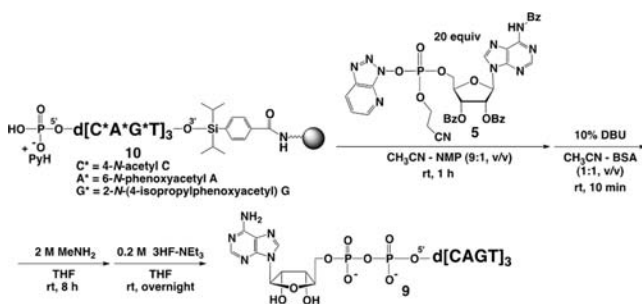


Figure 2. Anion-exchange HPLC profiles of crude mixtures obtained in the synthesis of $A^{5'}$ppd[CAGT]$_3$ **9** by treatment with (a) NH_4OH and (b) Et_3N-3HF.

followed by hydrolysis, afforded *H*-phosphonate compound **7**.[10] Compound **7** was subsequently oxidized using (2*R*, 8*S*)-(+)-(camphorylsulfonyl)oxaziridine in the presence of *N*,*O*-bis(trimethylsilyl)acetamide (BSA). In these steps for 5′-phosphorylation, 2-cyanoethyl groups of internucleotidic phosphate residues were preserved to avoid side reactions in adenylation because, as mentioned earlier, similar phosphorylating reagents reacted easily with not only phosphomonoesters but also phosphodiesters. The condensation of the in situ generated reactive phosphotriester **5** with resin **8** was carried out at room temperature for 1 h. After the removal of the 2-cyanoethyl and trimethylsilyl groups, the target oligomer **9** was released from the resin by treatment with 28% NH_4OH for 8 h. Figure 2a shows the HPLC profiles of the crude mixture obtained in the synthesis of $A^{5'}$ppd[CAGT]$_3$

(8) Dai, Q.; Saikia, M.; Li, N.; Pan, T.; Piccirilli, J. A. *Org. Lett.* **2009**, *11*, 1067–1070.
(9) Ohkubo, A.; Aoki, K.; Seio, K.; Sekine, M. *Tetrahedron Lett.* **2004**, *45*, 979–982.
(10) (a) Ohkubo, A.; Seio, K.; Sekine, M. *J. Am. Chem. Soc.* **2004**, *126*, 10884–10896. (b) Ohkubo, A.; Kasuya, R.; Aoki, K.; Kobori, A.; Taguchi, H.; Seio, K.; Sekine, M. *Bioorg. Med. Chem.* **2008**, *16*, 5345–5351.

Scheme 3. Synthesis of A$^{5'}$ppd[CAGT]$_3$ **9** on Polymer Supports Containing a Silyl-type Linker



9 by treatment with NH_4OH. The decomposition of the target oligonucleotide was unexpectedly observed at 25–27 min. Similar decomposition was also observed in the synthesis of A$^{5'}$ppODN containing other sequences (data not shown).

To avoid the decomposition resulting from treatment with NH_4OH, we used HCP resins containing a silyl linker,[10] which can be cleaved under neutral conditions, instead of a succinyl linker (Scheme 3). After the phosphorylation and adenylation of an N-acylated oligomer immobilized on the resin were performed in a manner similar to that described above, the resin was treated with 2 M methylamine/THF to cleave the protecting groups used for the amino groups of the nucleobases.[11] The target oligomer **9** was subsequently released from the resin by treatment with 0.2 M Et_3N-3HF for 12 h, as shown in Figure 2b. The decomposition of the 5′-adenylated oligomer was surprisingly decreased by using the neutral conditions, and the target oligomer **9** could be isolated in 54% yield. The oligomer was characterized by MALDI-TOF mass spectroscopy. Note that the reaction time for adenylation was considerably reduced by using our phosphorylating reagent **5** because the previous methods for the adenylation of oligomers required a considerably longer time of more than 16 h.

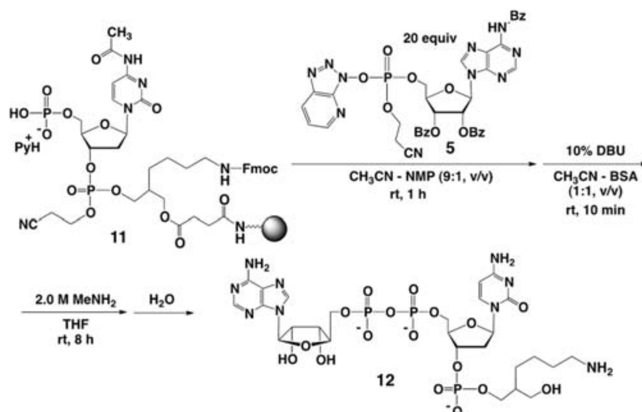
Next, we synthesized 5′-adenylated 5′-deoxycytidylic acid (A$^{5'}$ppdC-NH_2 **12**) with an aminoalkyl group, which can be efficiently linked to the 3′ terminus of RNAs by ligation with T4 RNA ligase.[12] After the adenylation of 5′-phosphorylated compound **11** and the removal of the protecting groups, the resulting resin was treated with 2 M methylamine/THF for 8 h, as shown in Scheme 4. The solution of methylamine was removed by filtration, and the resin was rinsed with anhydrous acetonitrile. Finally, the desired product was eluted from the resin using water according to Dellinger's procedure.[13] Figure 4a shows the reversed phase HPLC profiles of the mixture thus obtained. HPLC purification gave dimer **12** in 40% yield, and the isolated material was characterized by MALDI-TOF mass spectroscopy. These results indicate that A$^{5'}$ppDNA can be efficiently synthesized without

(11) Ohkubo, A.; Sakamoto, K.; Miyata, K.; Taguchi, H.; Seio, K.; Sekine, M. *Org. Lett.* **2005**, *7*, 5389–5392.

(12) Sekine, M.; Kusuoku, H. *Nucleosides Nucleotides* **1992**, *11*, 1713–1730.

(13) Dellinger, D. J.; Timár, Z.; Myerson, J.; Sierzchala, A. B.; Turner, J.; Ferreira, F.; Kupihár, Z.; Dellinger, G.; Hill, K. W.; Powell, J. A.; Sampson, J. R.; Caruthers, M. H. *J. Am. Chem. Soc.* **2011**, *133*, 11540–11556.

Scheme 4. Synthesis of A$^{5'}$ppdC-NH_2 **12**



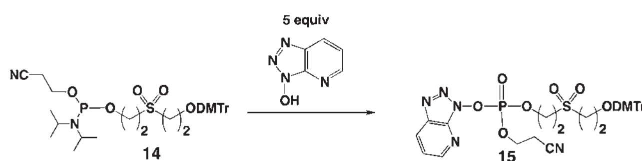
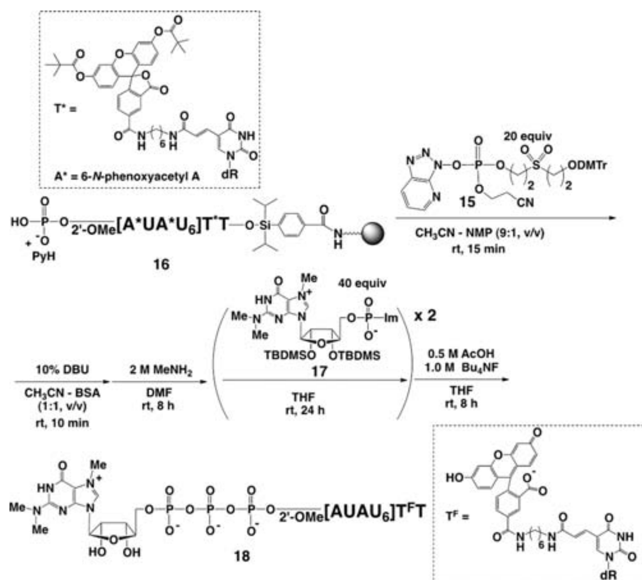


Figure 3. O–N phosphoryl rearrangement of phosphoramidite compound **14** mediated by HOAt.

Scheme 5. Synthesis of m$_3^{2,2,7}$G$^{5'}$ppp[2′-OMe(AUAU$_6$)T^{F}T] **18**



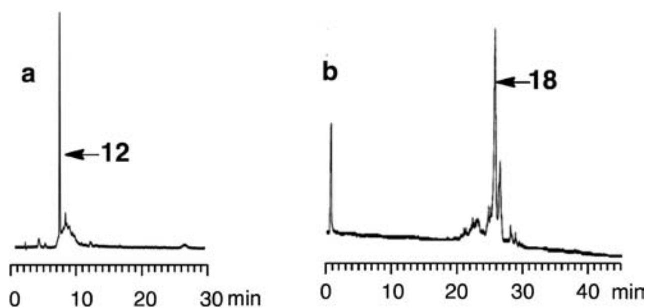


Figure 4. Reversed phase HPLC or anion-exchange profiles of the crude mixtures obtained in the synthesis of 5′-terminal capped oligonucleotides **12** and **18**: (a) $A^{5'}ppdC\text{-}NH_2$ **12** and (b) $m_3^{2,2,7}G^{5'}ppp[2'\text{-}OMe(AUAU_6)T^FT]$ **18**.

serious decomposition by using methylamine, even on the resin containing a succinyl linker.

Moreover, employing our new approach, we also carried out the synthesis of $m_3^{2,2,7}G^{5'}$pppRNA on polymer supports using O−N phosphoryl rearrangement (Scheme 5). Trivalent reagent **14** for phosphorylation could be readily converted to phosphotriester intermediate **15** by treatment with 5 equiv of HOAt within 10 min in a manner similar to that described for the O−N phosphoryl rearrangement of compound **1** (Figure 3). The 5′-terminal pyrophosphate bond was formed by the reaction of the protected oligonucleotide immobilized on the resin with the in situ generated compound **15** for 15 min, as shown in Scheme 5. After the deprotection of the 2-cyanoethyl, sulfonylethyl, and *N*-acyl groups, the 5′-terminal capping reaction was performed by treating the residue with $m_3^{2,2,7}$G phosphorimidazolide unit **17**.[6c] The target oligomer **18** was subsequently deprotected and released from the resin by treatment with 1 M tetrabutylammonium fluoride in the presence of 0.5 M AcOH for 8 h, as shown in the HPLC profiles in Figure 4b. The target oligomer **18** could be isolated in 12% yield and was characterized by MALDI-TOF mass spectroscopy. These results show that our approach is useful for not only the 5′-adenylation of oligomers but also the formation of the 5′-terminal triphosphate bond.

In conclusion, we have established a new convenient approach for the 5′-terminal capping reaction of oligonucleotides on polymer supports by using the phosphoramidite compound. Thus, 5′-adenylated DNA oligomers **9** and **12** and 5′-(2,2,7-trimethyl)guanosine capped 2′-OMe-RNA **18** could be efficiently and rapidly synthesized. One advantage of our approach is that the reaction time can be considerably reduced compared with that of previous methods. Another advantage is that phosphoramidite derivatives used in these reactions are more stable than general reagents, such as phosphorimidazolidates, during purification and preservation. These results indicate that our approach might be very useful for 5′-terminal capping modification of gene therapeutic oligonucleotides. Further studies in this direction are currently in progress.

Acknowledgment. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Izumi Science and Technology Foundation. This study was also partly supported by a grant from the global COE project, Ministry of Education, Culture, Sports, Science and Technology, Japan.

Supporting Information Available. Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.