Pergamon

SCIENCE DIRECT®

Tetrahedron Letters 44 (2003) 1703–1707

TETRAHEDRON LETTERS

Synthesis of TMG-capped RNA–DNA chimeric oligonucleotides

Mitsuo Sekine,* Masatoshi Ushioda, Takeshi Wada and Kohji Seio

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

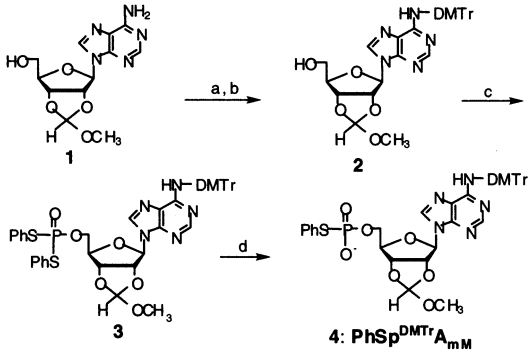
Received 7 August 2002; revised 8 December 2002; accepted 13 December 2002

Abstract—This paper deals with the synthesis of $m_3^{2,2,7}G^{5'}$pppAmpUmpApd(CpTpTpApCpCpT), a 5′-TMG-capped RNA–DNA chimeric oligonucleotide, which is expected to be conveyed to the nucleus by snurportin 1, a nuclear-transport protein. This 5′-TMG-capped RNA–DNA chimeric molecule was synthesized by the enzymatic condensation of $m_3^{2,2,7}G^{5'}$pppAmpUmpA with $A^{5'}$ppd(CpTpTpApCpCpT) in the presence of RNA ligase. An inherent serious side reaction was disclosed in the 5′-adenylation of d(CpTpTpApCpCpT) on the solid support, but the use of an active ester intermediate as the pA donor gave an improved result.

The mechanism of nuclear transport of U1snRNA[1] having a unique 2,2,7-trimethylguanosine (TMG)-cap structure at the 5′-end was clarified by cooperative studies of Lührmann and us in 1998.[2] The mechanism disclosed involves participation of snurportin 1, a specific protein that can bind to the TMG-cap structure of U1snRNA. This TMG-binding protein can also bind to importin β which is well known to carry nucleoproteins from the cytoplasm to the nucleus by triparticle formation of nucleoprotein–importin α–improtin β.[3] Thus, it turned out that the nuclear transport of U1snRNA occurs by formation of a triparticle of U1snRNA–snurportin 1–importin β in a manner very similar to that of nuclear proteins.[4] Namely, snurportin 1 plays an important role in transporting U1snRNA as the substitute of importin β. Based on this finding, we came up with a new idea that, if antigene oligonucleotides can be capped by the TMG-cap structure at the 5′-terminal site, they can be conveyed to the nucleus with the help of snurportin 1 and importin β so that they can bind selectively to DNA duplexes. This process creates a new promising tool in antigene strategy.[5]

In this paper, we report the synthesis of a TMG-capped RNA–DNA chimeric oligonucleotide, $m_3^{2,2,7}G^{5'}$pppAmpUmpApd(CpTpTpApCpCpT) (**26**), as a possible molecule having the above-designed function in antigene strategy.

To synthesize the TMG-capped RNA–DNA chimeric oligonucleotide, we used the RNA ligase-mediated condensation of a 5′-adenylated heptadeoxynucleotide, $A^{5'}$ppd(CpTpTpApCpCpT) (**23**), with $m_3^{2,2,7}G^{5'}$pppAmpUmpA (**24**), i.e. a 5′-terminal fragment of U1snRNA, since we recently established a facile solid-phase synthesis of the latter TMG-capped RNA trimer block without extensive workup.[6] There are few papers regarding the synthesis of RNA–DNA chimeric oligonucleotides by use of RNA ligase.[7] McLaughlin[8] reported the details of RNA ligation of RNA oligomers with 5′-adenylated deoxynucleoside 3′,5′-bisphosphate. Without 5′-adenylation, in general RNA ligase cannot recognize sufficiently the substrate so that the coupling yield is extremely low.[8] Therefore, we studied a more straightforward method for the synthesis of 5′-adenyl-



Scheme 1. *Reagents and conditions*: (a) TMSCl (2 equiv.), pyridine, rt, 20 min; (b) DMTrCl (1.5 equiv.), DMAP (cat.), pyridine, rt, 4 h, 62%; (c) cyclohexylammonium *S*,*S*-diphenylphosphoroditioate (1.2 equiv.), 1*H*-tetrazole (4 equiv.), isodurenedisulfonyl dichloride (2 equiv.), pyridine, rt, 30 min (68%); (d) 5 M pyridinium phosphinate in pyridine–tiethylamine (2:1, v/v), rt, 1 h (93%).

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

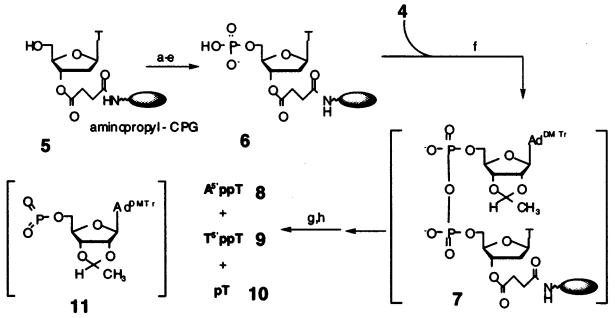
0040-4039/03/$ - see front matter © 2003 Published by Elsevier Science Ltd.
PII: S0040-4039(02)02828-9

ated deoxyoligonucleotides as effective donor molecules for the ligation. Horn and Urdea reported the 5′-phosphorylation of oligodeoxynucleotides synthesized on polymer supports by using a 5′-terminal phosphorylating reagent having a DMTr group,[9] by which the coupling yield of the 5′-phosphorylation can be estimated by UV colorimetric assay of the DMTr cation. In the case of the 5′-adenylation, it is convenient to see if the coupling is successfully carried out by using such an expeditous method. Therefore, we synthesized a DMTr-containing 5′-adenylating reagent, $PhSp^{DMTr}A_{mM}$ (**4**: triethylammonium salt), from 2′,3′-*O*-methoxymethyleneadenosine (**1**), as shown in Scheme 1. Reaction of **1** with DMTrCl gave the *N*-tritylated product **2** in 62% yield. Reaction of **2** with cyclohexylammonium *S*,*S*′-diphenyl phosphorodithioate[10] (PSS) in the presence of isodurenedisulfonyl dichloride[10] (DDS) gave the 5′-*O*-phosphorylated species in 68% yield. The selective dephenylthiolation of **3** with the phosphinate reagent[10,11] gave the *O*,*S*-phosphodiester derivative in quantitative yield. Before its application to the oligomer-level synthesis, we checked the reaction of **4** with a 5′-phosphorylated species **6** derived from a T-loaded aminopropyl-CPG[12] resin.

The reaction of **4** with this resin in the presence of I_2 in pyridine at room temperature for 5 min followed by the successive workup with 1% TFA in CH_2Cl_2 for 1 min and NH_3–EtOH (4:1, v/v) at room temperature for 1 h gave the desired product (**8**: $A^{5'}$ppT) in 48% yield (HPLC). To our surprise, a symmetric pyrophosphate derivative (**9**: $T^{5'}$ppT) was also obtained in 12% yield. A considerable amount (40%) of the unreacted species (**10**: pT) was recovered, as shown in Scheme 2.

The formation of $T^{5'}$ppT (**9**) is unique since we have never observed such symmetric pyrophosphate derivatives in the liquid phase synthesis in our long experience. This result implies that the once-formed $^{DMTr}A^{5'}_{mM}$ppT-polymer (**7**: **X** in Fig. 1) reacted with an activated methaphosphate species (**11**) of **4** to give a triphosphate derivative (**Y**) which *in turn* allowed attack of a neighbouring pT residue (**Z**) to decompose to give $(pT)_2$-polymer (**W**) and $(p^{DMTr}A_{mM})_2$. This might be because some of the pT residues were brought into closer proximity.

To elucidate this hypothesis, an independent reaction of N^4,3′-*O*-dibenzoyl-5′-deoxycytidylic acid (**12**: $pd^{Bz}C_{Bz}$) with 2 equiv. of $PhS\text{-}p^{DMT}A_{mM}$ **4** was carried out in the presence of 6 equiv. of I_2 in pyridine–pyridine-d_5 (3:1, v/v).[13] Actually, the ^{31}P NMR spectrum obtained after 10 min showed that the desired product **13** at −7.5 ppm was formed as a minor product, as shown in Figure 2. Instead, more significant new broad signals **X** and **Y** at around −9 and −18 ppm were observed in the ratio of 2:1. However, it was found that addition of water to the mixture resulted in convergence of the signals to the region of typical pyrophosphate derivatives at −7.5 ppm.[14] From these results, it is likely that the initial resonance signals observed at around −18 ppm are due to the tri-substituted triphosphates[14] **14** and **15** as depicted by asterisks in Figure 2, which were formed by



Scheme 2. *Reagents and conditions*: (a) $DMTrO(CH_2)_2\text{-}SO_2(CH_2)_2O\text{-}P(NiPr_2)(OCH_2CH_2CN)$ (20 equiv.) 1*H*-tetrazole (80 equiv.), CH_3CN, rt, 5 min; (b) 0.12 MDMAP, pyridine–Ac_2O (9:1, v/v, rt, 2 min); (c) I_2, pyridine–THF–H_2O (10:10:1, v/v/v), rt, 15 s×3; (d) 2.5% dichloroacetic acid, CH_2Cl_2, rt, 5 s×3; (e) 10% DBU, pyridine–bis(trimethylsilyl)acetamide (1:1, v/v), rt, 10 min; (f) 0.1 M of **4** (20 equiv.) in pyridine, I_2 (60 equiv.), rt, 5 min; (g) trifluoroacetic acid, CH_2Cl_2; (h) NH_3–EtOH (4:1, v/v), rt, 1 h.

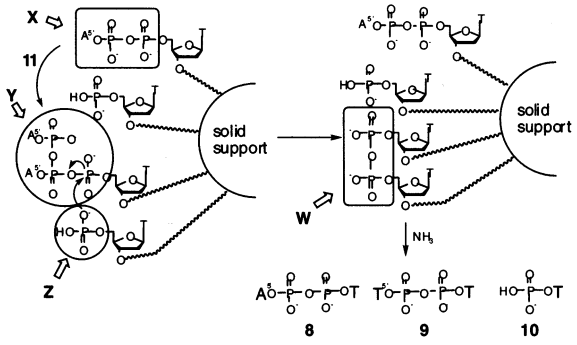


Figure 1. The mechanism of the side reaction observed in the solid-phase synthesis of $A^{5'}$ppT (**8**).

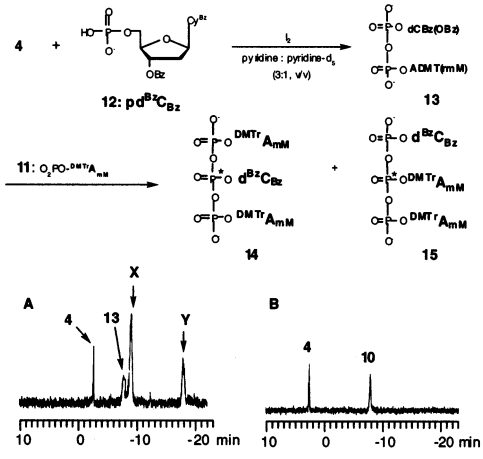


Figure 2. The ^{13}P NMR spectra of the reaction of **4** with **12** in the presence of I_2. Panel A: the spectrum after a mixture of **2** and **12** were treated with I_2 for 10 min. Panel B: the spectrum obtained after the mixture was quenched with water for 30 min.

the reaction of the once-formed initial product **13** with the methaphosphate derivative **11**.

Now, it was concluded that this over-activation of $^{DMTr}A^{5'}_{mM}$ppT-polymer might be due to the extreme reactivity of the generated methaphosphate ester derivative **11** of pA^{DMT}_{mM}, as shown in Figure 2. Therefore, to control the strong reactivity of the methaphosphate intermediate **11**, the phosphorothioate derivative $PhSp^{DMTr}A_{mM}$ **4** was converted to a 6-(trifluoromethyl)-4-nitrobenzotriazolyl-1-oxy ester derivative **17b** by treatment with 3 equiv. of 6-(trifluoromethyl)-1-hydroxyl-4-nitrobenzotriazole **16b** in the presence of 3 equiv. of I_2 in pyridine–pyridine-d_5 (3:1, v/v).

The conversion was completed within 10 min, as evidenced by ^{31}P NMR (Fig. 3) which showed that the starting material at 2.8 ppm disappeared and a new signal at 1.2 ppm was observed as a single peak. Addition of this active ester **17b** to the triethylammonium salt of **12** resulted in slow formation of the desired pyrophosphate derivative **13** which appeared at –8.5 ppm. The pyrophosphate derivative was quantitatively formed after 10 min, as shown in Figure 4(B1). Addition of water after the reaction gave a simple mixture of the desired product **13** and the hydrolysed product $pd(^{DMTr}A_{mM})$ (**18**) of the active ester, as shown in Figure 4(B2).

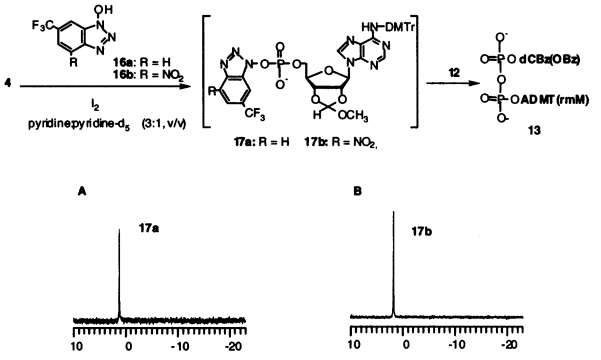


Figure 3. The ^{13}P NMR spectra of the active esters **17a**,**b** obtained by the reaction of **4** with **16a**,**b**.

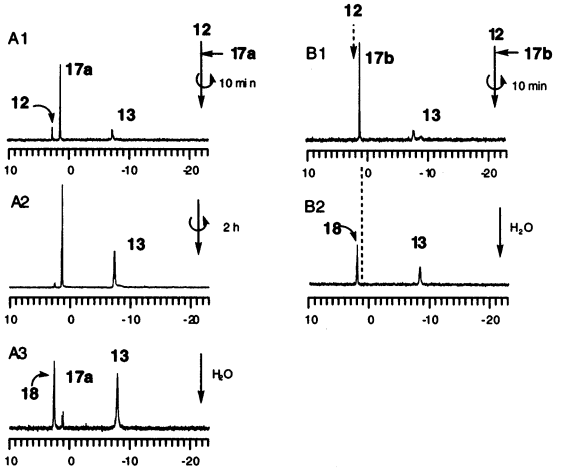
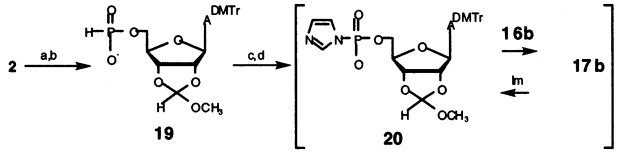


Figure 4. The ^{13}P NMR profiles of the reaction of **12** with **17a** or **17b**.

When the 6-(trifluoromethyl)-1-hydroxybenzotriazole was replaced with 6-(trifluoromethyl)-4-nitrobenzotriazole, the coupling required longer periods of time as shown in Figure 4(A2). Kadokura et al. recently reported a pyrophosphate bond formation by use of such active esters derived from phosphorimidazolidate derivatives.[15] Actually, they found that the use of the active ester from phosphorimidazolidate derivatives gave better results than that from *S*-phenyl phosphorothioate derivatives, although the reason is still unclear. Thus, we also synthesized the active ester **17b** by the same route.

Treatment of **2** with 7 equiv. of diphenyl phosphonate in pyridine at room temperature for 2 h followed by addition of triethylamine–H_2O (1:1, v/v) gave the 5′-phosphonate derivative **19** in 68% yield, as shown in Scheme 3. The reaction of **19** with 1.2 equiv. of trimethylsilylimidazole in the presence of 4 equiv. of triethylamine in CH_3CN–CCl_4 at room temperature for 20 min followed by addition of methanol gave the desired phosphorimidazolidate derivative **20** in quantitative yield, as evidenced by Figure 5(A).

Addition of 6-(trifluoromethyl)-1-hydroxy-4-nitro-benzotriazole to this product gave an equilibrated mixture of the phosphorimidazolidate (–8.5 ppm) and the active ester (0.8 ppm), as shown in Figure 5(B). This mixture was allowed to react with pT-polymer at room temperature for 2 h. Consequently, $A^{5'}$ppT was formed in 64% yield, while the formation of $T^{5'}$ppT was greatly suppressed to only 1% and unreacted pT was recovered in 33% yield. Further attempts to improve the coupling yield failed. The difficulty in completing the reaction is an inherent serious problem. It should be noted that the direct use of **20** without addition of 6-(trifluoromethyl)-



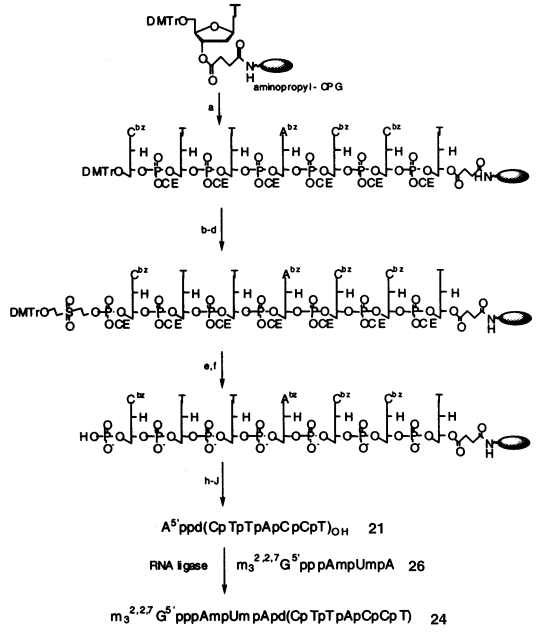
Scheme 3. *Reagents and conditions*: (a) diphenylphosphonate (7 equiv.), pyridine, rt, 2 h; (b) triethylamine–H_2O (1:1, v/v), rt, 30 min, 68% from **2**; (c) trimethylsilylimidazole (1.2 equiv.), triethylamine (4 equiv.), CH_3CN–CCl_4 (1:1, v/v), rt, 20 min; (d) MeOH, rt, 5 min, quant.

Figure 5. The ^{13}P NMR spectra of the mixture of **20** and **17b** obtained by the reaction of **20** with **16b** in pyridine at room temperature for 15 min. Panel A: before addition of **16b**. Panel B: after addition of **16b**.

1-hydroxy-4-nitro-benzotriazole gave lower yields of the product.

Application of this method to the synthesis of $A^{5'}$ppd(CpTpTpApCpCpT) (**21**) was carried out by use of 6-*N*,2′,3′-*O*-tribenzoyladenosine 5′-phosphorimidazolidate (**22**) which was similarly synthesized from the corresponding 5′-phosphonate derivative (**23**) and activated by addition of 6-(trifluoromethyl)-1-hydroxy-4-nitrobenzotriazole, as shown in Scheme 4. Thus, the desired 5′-adenylated product **21** was isolated in 16% yield. Since, usually, the isolated yields of unmodified oligodeoxynucleotides are ca. 30%, this isolated yield is reasonable in consideration of the efficiency of ca. 65% in the pyrophosphate bond formation.

Finally, we used this 5′-adenylated oligodeoxynucleotide derivative to obtain $m_3^{2,2,7}G^{5'}$pppAmpUmpApd-(CpTpTpApCpCpT) (**24**). To demonstrate the efficiency of the adenylation on ligase reaction, we studied both reactions of $A^{5'}$ppd(CpTpTpApCpCpT) (**21**) and pd(CpTpTpApCpCpT) (**25**) with $m_3^{2,2,7}G^{5'}$pppAmpUmpA (**26**)[6] in the presence of RNA ligase under the conditions described in Figure 6. It was clearly shown that the adenylated species gave better results than the unmodified oligodeoxynucleotide. After 48 h, the product was formed in 58% yield (HPLC) while the product was formed in only 10% in the case of the unmodified oligodeoxynucleotide. Separation of the desired product by HPLC followed by workup gave $m_3^{2,2,7}G^{5'}$pppAmpUmpApdd(CpTpTpApCpCpT) (**24**) in an isolated yield of 19%.



Scheme 4. *Reagents and conditions*: (a) chain elongation; (b) $DMTrO(CH_2)_2SO_2(CH_2)_2O$-P(N*i*Pr$_2$)($OCH_2$-$CH_2CN$) (20 equiv.), 1*H*-tetrazole (80 equiv.), CH_3CN, rt, 5 min; (c) 0.12 M DMAP, pyridine–Ac_2O (1:9, v/v), rt, 2 min; (d) I_2, pyridine–THF–H_2O (10:10:1, v/v/v), rt, 15 s×3; (e) 2.5% dichloroacetic acid, CH_2Cl_2, rt, 5 s×3; (f) 10% DBU, pyridine–bis(trimethylsilyl)acetamide (1:1, v/v), rt, 10 min; (h) an equilibrilized (40 equiv., 0.1 M) of **22** and the active ester obtained by pre-activation of **22** by addition of 6-(trifluoromethyl)-1-hydroxy-4-nitrobenzotriazole (120 equiv.) at rt for 10 min, pyridine, rt, 2 h; (i) NH_3–EtOH (4:1, v/v), 55°C, 4 h.

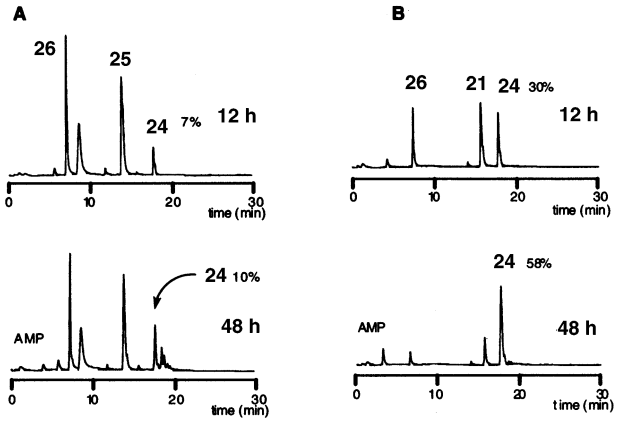


Figure 6. Anion exchange HPLC profile of the crude **24** obtained by ligation of a mixture of **26** with **25** or **21**. Panel A: without 5′-adenylation; 50 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 10 mM DTT, 10 mM ATP. Panel B: with 5′-adenylation; 50 mM Tris–HCl (pH 8), 10 mM $MgCl_2$, 10 mM DTT.

In conclusion, we were able to synthesize the TMG-capped RNA–DNA chimeric molecule. It should be noted that the 5′-adenylation on the polymer support should be improved. Now work is under way in this direction.

Acknowledgements

This work was supported by a Grant-in-Aid (14208074) for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan.

References

1. Massenet, S.; Mougin, A.; Branlant, C. In *Modification and Editing of RNA*; Grosjean, H.; Benne, R., Eds. Posttranscriptional modifications in the U small nuclear RNAs. ASM Press: Washington, DC, 1988; pp. 201–227.
2. Huber, J.; Cronshagen, U.; Kadokura, M.; Marshallsay, C.; Wada, T.; Sekine, M.; Lührmann, R. *EMBO J.* **1998**, *17*, 4114–4126 and references cited therein.
3. Will, C. L.; Lührmann, R. *Curr. Opin. Cell Biol.* **2001**, *13*, 290–301.
4. Izaurralde, E.; Adams, S. *RNA* **1998**, *4*, 351–364.
5. *Pharmaceutical Aspects of Oligonucleotides*; Couvreur, P.; Malvy, C., Eds.; Taylor & Francis: Philadelphia, 2000.
6. (a) Kadokura, M.; Wada, T.; Seio, K.; Moriguchi, T.; Huber, J.; Lührmann, R.; Sekine, M. *Tetrahedron Lett.* **2001**, *42*, 8853–8856; (b) Sekine, M.; Kadokura, M.; Satoh, T.; Seio, K.; Wada, T.; Fischer, U.; Sumper, V.; Lührmann, R. *J. Org. Chem.* **1996**, *61*, 4412–4422.

7. England, T. E.; Bruce, A. G.; Uhlenbeck, O. C. *Methods Enzymol*. **1980**, *65* (Nucleic Acids, Pt. I), 65–74.
8. Hoffmann, P. U.; McLaughlin, L. W. *Nucleic Acids Res*. **1987**, *15*, 5289–5303.
9. Horn, T.; Urdea, M. S. *Tetrahedron Lett*. **1986**, *27*, 4705–4708.
10. Sekine, M.; Matsuzaki, J.; Hata, T. *Tetrahedron Lett*. **1981**, *22*, 3209–3212.
11. (a) Sekine, M.; Hamaoki, K.; Hata, T. *Bull. Chem. Soc. Jpn*. **1981**, *54*, 3815–3816; (b) Sekine, M.; Hata, T. *Curr. Org. Chem*. **1998**, *3*, 25–66.
12. Groeger, G.; Seliger, H. *Nucleosides Nucleotides* **1988**, *7*, 773–778.
13. Usman, N.; Pon, R. T.; Ogilvie, K. K. *Tetrahedron Lett*. **1985**, *26*, 4567–4570.
14. Ludwig, J.; Eckstein, F. *J. Org. Chem*. **1989**, *54*, 631–635.
15. Kadokura, M.; Wada, T.; Urashima, C.; Sekine, M. *Tetrahedron Lett*. **1997**, *38*, 8359–8362.