ORGANIC LETTERS

2009
Vol. 11, No. 4
967−970

Stereocontrolled Synthesis of Oligoribonucleoside Phosphorothioates by an Oxazaphospholidine Approach

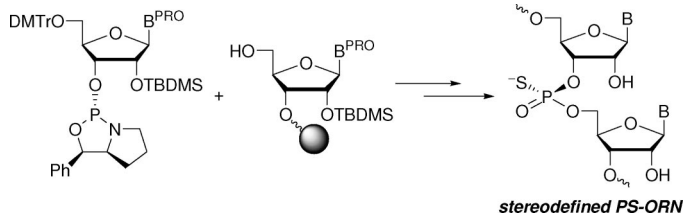
Natsuhisa Oka, Tomoaki Kondo, Satoshi Fujiwara, Yukihiro Maizuru, and Takeshi Wada*

Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Bioscience Building 702, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

wada@k.u-tokyo.ac.jp

Received December 17, 2008

ABSTRACT



Oligoribonucleoside phosphorothioates (PS-ORNs) stereodefined at the phosphorus atoms were synthesized on solid support. Thermal denaturating experiments of the resultant PS-ORNs showed that a backbone consisting of (*Sp*)-PS-linkages as well as stereorandom PS-linkages had an unexpectedly large destabilizing effect on a PS-ORN−ORN duplex, whereas a backbone consisting of (*Rp*)-PS-linkages slightly stabilized a duplex.

RNA-mediated gene silencing systems, particularly RNA interference,[1] have been widely used as powerful tools for gene knockdown because efficient gene silencing can be performed with comparative ease by using relatively short synthetic oligoribonucleotides (ORNs),[2] and their therapeutic applications, as well as the elucidation of the silencing mechanism, are current topics of great interest.[3] Chemically synthesized ORNs with appropriate modifications are requisite for these purposes as probes for the mechanistic elucidation or as drug candidates with enhanced stability to nucleases.[3] Oligoribonucleoside phosphorothioates (PS-ORNs) are one of the most frequently used analogue probes of the functions of internucleotidic linkages. Such molecules can also potentially serve as drug candidates with improved stability to nucleases and cell membrane permeability.[3,4]

One of the most important features of PS-ORNs is the chirality of their phosphorus atoms. PS-ORNs work as probes or drugs through interactions with chiral biomolecules (e.g., nucleic acids, proteins). Therefore, their properties and functions as probes or drugs are theoretically dependent on the configurations of the phosphorus atoms, and elucidation of the dependence is crucial for their applications. However, only a limited number of studies on this subject have been conducted due to the unavailability of stereodefined PS-ORNs.[5−8] Attempts to develop a method to synthesize stereodefined PS-ORNs to date have only succeeded in the synthesis of diastereopure 2−3mers[6a,b] and all-(*Rp*)-PS-ORN 4−12mers with moderate stereoselectivity.[6c,d] Chromatographic separations of ORNs containing internucleotidic

(1) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391*, 806–811.

(2) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494–498.

(3) (a) Tomari, Y.; Zamore, P. D. *Genes Dev.* **2005**, *19*, 517–529. (b) Bumcrot, D.; Manoharan, M.; Koteliansky, V.; Sah, D. W. Y. *Nat. Chem. Biol.* **2006**, *2*, 711–719.

(4) (a) Parrish, S.; Fleenor, J.; Xu, S.; Mello, C.; Fire, A. *Mol. Cell* **2000**, *6*, 1077–1087. (b) Mescalchin, A.; Detzer, A.; Wecke, M.; Overhoff, M.; Wuensche, W.; Sczakiel, G. *Expert Opin. Biol. Ther.* **2007**, *7*, 1531–1538.

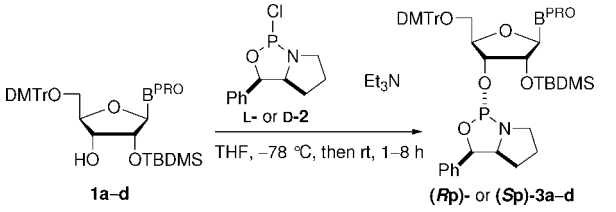
10.1021/ol802910k CCC: $40.75
Published on Web 01/21/2009

phosphorothioate diester linkages (PS-linkages)[7] or dimer building blocks to synthesize ORNs with stereodefined PS-linkages[8] have also been reported, though ORNs containing consecutive stereodefined PS-linkages or fully modified stereodefined PS-ORNs are unavailable.

To overcome this limitation, we developed a method to synthesize stereodefined PS-ORNs by using ribonucleoside 3′-*O*-oxazaphospholidine derivatives as monomer units. As a result, we found that the hybridization affinity of PS-ORNs to the complementary ORNs was highly dependent on the configurations of their phosphorus atoms. The results of the study are described in this paper.

We have reported that a proline-derived bicyclic oxazaphospholidine ring affords the corresponding *trans*-deoxyribonucleoside 3′-*O*-oxazaphospholidines with diastereomer ratio (dr) of >99:1. The resultant oxazaphospholidines were successfully applied in the synthesis of stereodefined oligodeoxyribonucleoside phosphorothioates (PS-ODNs) with dr of >99:1 for each PS-linkage.[9] Similarly, diastereopure *trans*-ribonucleoside 3′-*O*-oxazaphospholidine monomers [**(*Rp*)-** and **(*Sp*)-3a−d**] for PS-ORNs were stereoselectively synthesized from 2′-*O*-TBDMS-protected ribonucleosides **1a−d** and 2-chloro-1,3,2-oxazaphospholidines **L-** and **D-2**, which were derived from L- and D-proline, respectively,[9] in modest to good yields (Table 1). ***trans*-(*Rp*)-** and **(*Sp*)-3a−d** were configurationally assigned based on the $^2J_{PC}$ values of their oxazaphospholidine rings.[9,10]

Table 1. Synthesis of Ribonucleoside 3′-*O*-Oxazaphospholidine Monomers **3a−d**

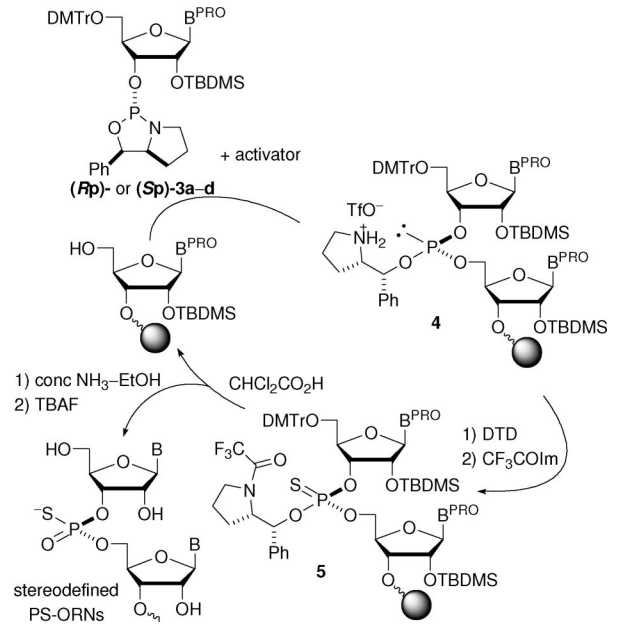


entry	B$^{PRO\alpha}$	**(*Rp*)-** or **(*Sp*)-3**	yield (%)	*trans*/*cis*
1	Ur	**(*Rp*)-3a**	51	>99:1
2	Cy^{ac}	**(*Rp*)-3b**	47	>99:1
3	Ad^{ac}	**(*Rp*)-3c**	51	>99:1
4	Gu^{ce,pac}	**(*Rp*)-3d**	41	>99:1
5	Ur	**(*Sp*)-3a**	75	>99:1
6	Cy^{ac}	**(*Sp*)-3b**	70	>99:1
7	Ad^{ac}	**(*Sp*)-3c**	72	>99:1
8	Gu^{ce,pac}	**(*Sp*)-3d**	46	>99:1

[a] B^{PRO} = protected nucleobase; Ur = uracil-1-yl; Cy^{ac} = N^4-acetylcytosin-1-yl; Ad^{ac} = N^6-acetyladenin-9-yl; Gu^{ce,pac} = O^6-cyanoethyl-N^2-phenoxyacetylguanin-9-yl.

Stereodefined PS-ORNs were manually synthesized on solid supports using the oxazaphospholidine derivatives as monomer units. The synthetic cycle is shown in Scheme 1. The oxazaphospholidine monomers **(*Rp*)-** or **(*Sp*)-3a−d** were allowed to react with the 5′-OH of a nucleoside anchored to a highly cross-linked polystyrene (HCP)[11] or a controlled pore glass (CPG) via a usual succinate linker to afford phosphite intermediates **4**. The phosphites were then sulfurized with *N*,*N*′-dimethylthiuram disulfide (DTD),[12] and unreacted 5′-OH and the secondary amino group derived from the chiral auxiliary were acylated with trifluoroacetylimidazole (CF_3COIm).[9] It was confirmed that CF_3COIm capped the remaining 5′-OH quantitatively without causing any observable desulfurization.[13] Then the 5′-*O*-DMTr group was removed by treatment with 3% dichloroacetic acid/CH_2Cl_2. The cycle was repeated to synthesize the protected PS-ORNs, which were then deprotected and cleaved from the support by treatment with concd NH_3−EtOH (3:1, v/v) at rt. The resultant 2′-*O*-TBDMS-protected PS-ORNs were desilylated with TBAF.

Scheme 1. Synthesis Cycle for Stereodefined PS-ORNs



First, (*Sp*)- and (*Rp*)-dinucleoside phosphorothioates [**(*Sp*)-** and **(*Rp*)-7a−d**][6] were synthesized by using the cycle.

(5) (a) Burgers, P. M. J.; Eckstein, F. *Biochemistry* **1979**, *18*, 592–596. (b) Almer, H.; Stawinski, J.; Strömberg, R.; Thelin, M. *J. Org. Chem.* **1992**, *57*, 6163–6169. (c) Oivanen, M.; Ora, M.; Almer, H.; Strömberg, R.; Lönnberg, H. *J. Org. Chem.* **1995**, *60*, 5620–5627.

(6) (a) Lesnikowski, Z. J. *Nucleosides Nucleotides* **1992**, *11*, 1621–1638. (b) Sierzchala, A.; Okruszek, A.; Stec, W. J. *J. Org. Chem.* **1996**, *61*, 6713–6716. (c) Almer, H.; Stawinski, J.; Strömberg, R. *J. Chem. Soc., Chem. Commun.* **1994**, 1459–1460. (d) Almer, H.; Stawinski, J.; Strömberg, R. *Nucleic Acids Res.* **1996**, *24*, 3811–3820.

(7) Slim, G.; Gait, M. J. *Nucleic Acids Res.* **1991**, *19*, 1183–1188.

(8) Yano, J.; Ohgi, T.; Ueda, T. WO2006/043521-A1, CAN: 144: 450874.

(9) Oka, N.; Yamamoto, M.; Sato, T.; Wada, T. *J. Am. Chem. Soc.* **2008**, *130*, 16031–16037.

(10) Hyodo, M.; Ando, H.; Nishitani, H.; Hattori, A.; Hayakawa, H.; Kataoka, M.; Hayakawa, Y. *Eur. J. Org. Chem.* **2005**, 5216–5223.

(11) McCollum, C.; Andrus, A. *Tetrahedron Lett.* **1991**, *32*, 4069–4072.

(12) Song, Q.; Wang, Z.; Sanghvi, Y. S. *Nucleosides Nucleotides Nucleic Acids* **2003**, *22*, 629–633.

(13) (a) Helínski, J.; Skrzypczýnski, Z.; Wasiak, J.; Michalski, J. *Tetrahedron Lett.* **1990**, *31*, 4081–4084. (b) Bruzik, K. S.; Stec, W. J. *J. Org. Chem.* **1990**, *55*, 6131–6135.

N-(Cyanomethyl)pyrrolidinium triflate (CMPT, **6**),[14] an acidic activator with extremely low nucleophilicity, was used to activate the monomers. Reversed-phase HPLC (RP-HPLC) analysis of the resultant 2mers showed that the yields (96−98%) and diastereoselectivity (>99:1) were sufficient for the synthesis of oligomers (Table 2).[15]

Table 2. Synthesis of Dinucleoside Phosphorothioates **7a**−**d**[a]

entry	monomer	product[b]		yield (%)[c]	*R*p/*S*p[c]
1	**(*R*p)-3a**	(*S*p)-U_SU	**(*S*p)-7a**	98	>1:99
2	**(*R*p)-3b**	(*S*p)-C_SU	**(*S*p)-7b**	97	>1:99
3	**(*R*p)-3c**	(*S*p)-A_SU	**(*S*p)-7c**	98	>1:99
4	**(*R*p)-3d**	(*S*p)-G_SU	**(*S*p)-7d**	97	>1:99
5	**(*S*p)-3a**	(*R*p)-U_SU	**(*R*p)-7a**	98	>99:1
6	**(*S*p)-3b**	(*R*p)-C_SU	**(*R*p)-7b**	98	>99:1
7	**(*S*p)-3c**	(*R*p)-A_SU	**(*R*p)-7c**	96	>99:1
8	**(*S*p)-3d**	(*R*p)-G_SU	**(*R*p)-7d**	98	>99:1

[a] CMPT **6** was used as activator. [b] Subscript "S" = PS-linkage. [c] Determined by RP-HPLC.

Given this result, we investigated the synthesis of stereodefined PS-ORNs. All-(*S*p)- and all-(*R*p)-$[U_S]_3U$ and $A_SG_SC_SU$ 4mers (Table 3, **12**−**15**) were synthesized by using

Table 3. Synthesis of Stereodefined PS-ORNs **12**−**17**[a]

entry	oligonucleotide[b]	activator	coupling yield (%)[c]
1	all-(*S*p)-$[U_S]_3U$ **12**	CMPT **6**	94
2	all-(*R*p)-$[U_S]_3U$ **13**	CMPT **6**	90
3	all-(*R*p)-$[U_S]_3U$ **13**	CMPT **6**	88
4	all-(*S*p)-$A_SG_SC_SU$ **14**	CMPT **6**	90
5	all-(*R*p)-$A_SG_SC_SU$ **15**	CMPT **6**	67
6	all-(*R*p)-$A_SG_SC_SU$ **15**	BTT **10**	84
7	all-(*R*p)-$A_SG_SC_SU$ **15**	DCI **11**	90
8	all-(*R*p)-$A_SG_SC_SU$ **15**	BIT **8**	97
9	all-(*R*p)-$[U_S]_9U$ **16**	BIT **8**	92
10	all-(*R*p)-$[U_S]_9U$ **16**	PhIMT **9**	97
11	all-(*S*p)-$[U_S]_9U$ **17**	PhIMT **9**	99

[a] HCP was used in entries 1 and 2, and CPG was used in entries 3−11. [b] Subscript "S" = PS-linkage. [c] Average coupling yields were determined by RP-HPLC for 4mers **12**−**15**, and by $DMTr^+$ assay for 10mers **16**, **17**.

CMPT **6**. However, an RP-HPLC analysis of the resultant 2′-*O*-TBDMS-protected 4mers showed that the average coupling yields were not sufficient for the synthesis of long oligomers (67−94%, entries 1−5). The coupling efficiency for (*R*p)-PS-linkages was lower than that for (*S*p)-PS-linkages; the difference in coupling efficiency between the (*R*p)- and (*S*p)-monomers could be attributed to the chirality of ribose.[16] The lower coupling yields for the synthesis of PS-ORNs compared to those for PS-ODNs[9] are due to the steric hindrance of the 2′-*O*-TBDMS groups. Use of a CPG in the place of an HCP did not improve the results (entries 2 and 3).

To solve the problem of low coupling efficiency, we turned our attention to the use of nucleophilic azoles, which have been widely used as activators in the phosphoramidite chemistry.[17] We employed 5-benzylthio-1*H*-tetrazole (BTT **10**),[18] 4,5-dicyanoimidazole (DCI, **11**),[19] and benzimidazolium triflate (BIT, **8**)[20] to synthesize all-(*R*p)-$A_SG_SC_SU$ **15** and found that the coupling efficiency was greatly improved in all of these cases (entries 6−8). In particular, BIT **8** gave **15** with sufficient average coupling efficiency for the synthesis of long oligomers (entry 8). The RP-HPLC profiles of the resultant all-(*R*p)-2′-*O*-TBDMS-protected $A_SG_SC_SU$ 4mers showed that the ratios of diastereoisomers obtained by using **8**, **10**, or **11** were as high as that obtained by using CMPT **6**.[15] To examine the diastereoselectivity of the synthesis of each PS-linkage promoted by azole-type activators, (*S*p)- and (*R*p)-U_SU were synthesized by using BIT **8**. An RP-HPLC analysis showed that (*S*p)- and (*R*p)-2′-*O*-TBDMS-U_SU were synthesized with dr of >99:1 (97% yield) and 96:4 (98% yield), respectively.

The fact that azole-type activators also afforded PS-ORNs with high diastereoselectivity is rather surprising because highly nucleophilic azole-type activators are known to epimerize diastereopure acyclic phosphoramidites as well as monocyclic oxazaphospholidines by repetitive nucleophilic attacks upon the chiral phosphorus atoms, resulting in the formation of phosphite triesters of low diastereopurity,[14,16,21] whereas less-nucleophilic CMPT **6** is considered to activate oxazaphospholidines only by *N*-protonation, without affecting the chiral phosphorus atoms.[14] Although the mechanism of the highly diastereoselective formation of phosphites promoted by azoles is still not clear, it may be attributed to the configurational stability of the proline-derived bicyclic oxazaphospholidine ring structure.[9]

All-(*R*p)-$[U_S]_9U$ **16** was then synthesized by using BIT **8** or *N*-(phenyl)imidazolium triflate (PhIMT) **9**, which has been reported as one of the best activators for the synthesis of unmodified ORNs.[20b] We found that PhIMT **9** afforded **16** with excellent coupling efficiency (entry 10). All-(*S*p)-$[U_S]_9U$ (**17**) was also efficiently synthesized by using PhIMT **9** (entry 11). Although oligomers containing phosphodiester linkages were observed as major byproducts[12] due to incomplete manual sulfurization, the desired all-(*R*p)- and all-(*S*p)-$[U_S]_9U$ were generated in good yields and diastereoselectivity.[15] After desilylation by TBAF, **16** and **17** were isolated by RP-HPLC in 6 and 11% yields, respectively, and identified by MALDI-TOF-MS.[15]

Stability of the resultant all-(*R*p)- and all-(*S*p)-$[U_S]_9U$ (**16**, **17**) to snake venom phosphodiesterase (svPDE)[22] and

(14) Oka, N.; Wada, T.; Saigo, K. *J. Am. Chem. Soc.* **2003**, *125*, 8307–8317.
(15) See the Supporting Information.
(16) Yu, D.; Kandimalla, E. R.; Roskey, A.; Zhao, Q.; Chen, L.; Chen, J.; Agrawal, S. *Bioorg. Med. Chem.* **2000**, *8*, 275–284.
(17) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223–2311.
(18) Welz, R.; Müller, S. *Tetrahedron Lett.* **2002**, *43*, 795–797.
(19) Vargeese, C.; Cater, J.; Yegge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. *Nucleic Acids Res.* **1998**, *26*, 1046–1050.
(20) (a) Hayakawa, Y.; Kataoka, M.; Noyori, R. *J. Org. Chem.* **1996**, *61*, 7996–7997. (b) Hayakawa, Y.; Kawai, R.; Hirata, A.; Sugimoto, J.; Kataoka, M.; Sakakura, A.; Hirose, M.; Noyori, R. *J. Am. Chem. Soc.* **2001**, *34*, 8165–8176.
(21) Stec, W. J.; Zon, G. *Tetrahedron Lett.* **1984**, *25*, 5279–5282.

nuclease P1 (nP1)[23] was investigated. These enzymes have been used for configurational assignment of PS-ODNs, whereas only a limited number of experiments have been reported for stereodefined PS-ORNs.[5−8] According to these reports, svPDE is (*R*p)-specific at least for dinucleoside phosphorothioates[5,6] and ORNs containing PS-linkages,[7] whereas nP1 is not always completely (*S*p)-specific and hydrolyzes (*R*p)-PS-linkages at a much slower rate than that for the (*S*p)-isomers depending on the nucleobases[6b] and lengths[24] of ORNs. All-(*R*p)- and all-(*S*p)-$[U_S]_9U$ (**16**, **17**) were incubated with svPDE or nP1 over 16 h under standard conditions. All-(*R*p)-$[U_S]_9U$ (**16**) and all-(*S*p)-$[U_S]_9U$ (**17**) were completely hydrolyzed by svPDE and nP1, respectively, whereas 53% of **16** and 23% of **17** were hydrolyzed by nP1 and svPDE, respectively.[15] The results indicate that at least 47% of **16** and 77% of **17** were stereochemically homogeneous. However, considering that they were completely hydrolyzed by nP1 and svPDE, respectively, and hydrolysis by nP1 may not be completely (*S*p)-specific, diastereopurity of these PS-ORNs is likely to be higher than these values.

Next, the effects of the *P*-configurations of PS-ORNs on duplex stability were investigated. Thermal denaturating experiments were conducted for the duplexes of all-(*R*p)-$[U_S]_9U$ (**16**), all-(*S*p)-$[U_S]_9U$ (**17**), stereorandom $[U_S]_9U$ (**18**), and natural $[U_O]_9U$ (**19**) with the complementary $[A_O]_9A$ (**20**) (subscript "O" = phosphodiester linkage). The resultant melting curves showed that the thermal stability of the duplexes was significantly affected by the configurations of their phosphorus atoms (Figure 1). The T_m value of all-(*R*p)-$[U_S]_9U$ **16**−$[A_O]_9A$ **20** was 28.9 °C (red), which was slightly higher than that of the natural counterpart $[U_O]_9U$ **19**−$[A_O]_9A$ **20** (25.7 °C, purple). In sharp contrast, no distinct T_m value was observed above 4 °C for the duplex of all-(*S*p)-$[U_S]_9U$ **17** with **20** (blue). In addition, a duplex of stereorandom $[U_S]_9U$ **18** with **20** had a low T_m value (10.3 °C, green). Thus, the results showed that a backbone consisting of (*R*p)-PS-linkages slightly stabilizes a PS-ORN−ORN duplex, whereas a backbone consisting of (*S*p)-PS-linkages or stereorandom PS-linkages has a great destabilizing effect on the duplex.[25]

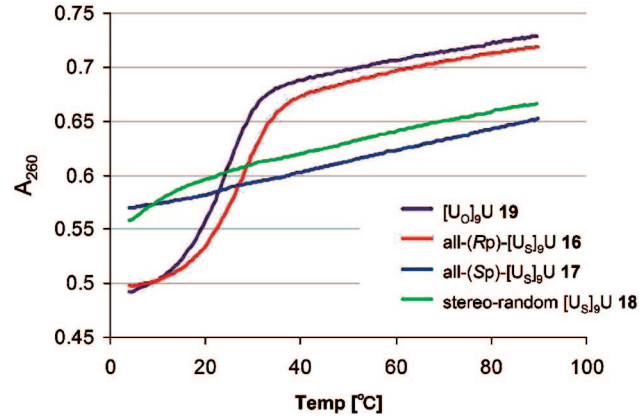


Figure 1. UV melting curves of $[U_S]_9U$−$[A_O]_9A$ duplexes.

In conclusion, we have succeeded in the first synthesis of stereodefined PS-ORNs by the oxazaphospholidine method and demonstrated that the thermal stability of PS-ORN−ORN duplexes is significantly affected by the configurations of the phosphorus atoms of PS-ORNs. In particular, the large destabilizing effect of (*S*p)-PS-linkages including those in a stereorandom PS-backbone on PS-ORN−ORN duplexes is notable because nonstereoregular PS-linkages have been widely used to modify ORNs. In contrast, stereodefined (*R*p)-linkages would be useful to modify ORNs without compromising their binding affinity to the complementary RNA sequences.

Acknowledgment. We thank Professor Kazuhiko Saigo (University of Tokyo) for helpful suggestions. This research was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) Japan and the New Energy and Industrial Technology Development Organization (NEDO) Japan for its Functionalized RNA Project.

Supporting Information Available: Experimental details, NMR spectra, and RP-HPLC profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

OL802910K

(22) (a) Burgers, P. M. J.; Eckstein, F. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4798–4801. (b) Bryant, F. R.; Benkovic, S. J. *Biochemistry* **1979**, *18*, 2825–2829.

(23) Potter, B. V. L.; Connolly, B. A.; Eckstein, F. *Biochemistry* **1983**, *22*, 1369–1377.

(24) Griffiths, A. D.; Potter, B. V. L.; Eperon, I. C. *Nucleic Acids Res.* **1987**, *15*, 4145–4162.

(25) Reference 8 has described that ORNs having an all-(*R*p)- or all-(*S*p)-PO/PS-alternating backbone, which were synthesized by using dimer building blocks, had higher and lower affinity to the complementary ORN, respectively, compared to the unmodified ORN counterpart.