Shibata M.A, Khan I.A, Iinuma M, Shirai T	Natural products for medicine	J Biomed Biotechnol	147120	1	2012年
Kurose H, Shibata M.A, Iinuma M, Otsuki Y	Alterations in cell cycle and induction of apoptotic cell death in breast cancer cells treated with alpha-mangostin extracted from mangosteen pericarp	J Biomed Biotechnol	672428	1-9	2012年
Shibata M.A, Ambati J, Shibata E, Yoshidome K, Harada-Shiba M	Mammary cancer gene therapy targeting lymphangiogenesis:VEGF-C siRNA and soluble VEGF receptor-s, a splicing varian t	Med Mol Morphlo	45	179-184	2012年
柴田雅朗, Jayakrishia Ambati, 柴田映子, Romulo JC Albuquerque, 森本純司, 斯波真理子, 藤岡重和, 伊藤裕子, 大槻勝紀	スプライシング・バリアントである可溶性VEGF受容体2型のマウス乳癌リンパ節移転に対する抑制効果	リンパ学	35	23-28	2012年
柴田雅朗	転移抑制を念頭に置いた乳癌遺伝子治療の実験的研究	乳癌（第2版）－基礎と臨床の最新研究動向－	70	710-713	2012年

ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 11020–11022

www.rsc.org/chemcomm

COMMUNICATION

Synthesis of selenomethylene-locked nucleic acid (SeLNA)-modified oligonucleotides by polymerases†

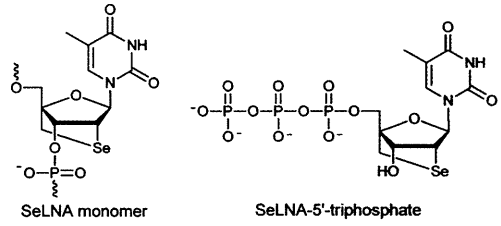
Megan Wheeler,[a] Antoine Chardon,[a] Astrid Goubet,[a] Kunihiko Morihiro,[b] Sze Yee Tsan,[a] Stacey L. Edwards,[a] Tetsuya Kodama,[bc] Satoshi Obika[b] and Rakesh N. Veedu*[a]

Received 5th September 2012, Accepted 24th September 2012
DOI: 10.1039/c2cc36464f

Enzymatic recognition of SeLNA nucleotides was investigated. KOD XL DNA polymerase was found to be an efficient enzyme in primer extension reactions. Polymerase chain reaction (PCR) amplification of SeLNA-modified DNA templates was also efficiently achieved by Phusion and KOD XL DNA polymerases.

Modified nucleotides have become a significant component in oligonucleotide-based therapeutic development as naturally occurring nucleic acids are especially vulnerable to nuclease attack which makes them incompatible as a therapeutic agent. Short single stranded DNA or RNA oligonucleotides called aptamers[1] are functional nucleic acids with significant potential in therapeutic, diagnostic and biosensor development. Aptamers display very high binding affinity and specificity to their targets, ranging from small molecules to complex proteins and even whole cells. Systematic Evolution of Ligands by EXponential enrichment (SELEX)[2] is a technique generally used to develop aptamers. Considering the recent developments in targeted nanotherapy and imaging technologies, the scope of aptamers with high bio-stability has increased tremendously. Aptamers composed of natural nucleotides are rapidly degraded by nucleases. Chemically-modified nucleotides are introduced into aptamers in order to overcome this limitation. However, as SELEX involves several enzymatic protocols, the applicability of the majority of the modified nucleotides is limited in the evolution of aptamers. A locked/bridged nucleic acid (LNA/BNA) nucleotide is one of the most prominent nucleotide analogues developed in recent years with remarkable properties.[3]

Since the first report of LNA/BNA,[4] a series of other derivatives have been reported by modifying the 2′-oxygen atom of the methylene bridge with nitrogen, sulphur or carbon to improve the binding affinity and nuclease resistance.[5] Selenium (Se) is an important atom introduced in the nucleic acids to facilitate the crystal growth and structure determination by X-ray crystallography, especially when it is placed at the 2′-position.[6] Using this strategy, several structures of DNAs, RNAs, and protein–nucleic acid complexes have been solved.[6a] We have recently prepared a new LNA/BNA analogue with a 'Se' atom at the 2′-position called SeLNA (Scheme 1).[7] We envision the development of SeLNA-modified aptamers with the scope to resolve the structure of an aptamer–target complex by X-ray crystallography and also to improve the nuclease resistance. Along this line, the enzymatic recognition of 2′-SeMe NTPs by RNA polymerases was recently reported.[8] Herein, we report the enzymatic recognition of SeLNA nucleotides by polymerases towards generating SeLNA-modified DNA aptamers.



Scheme 1 Structure of SeLNA nucleotide monomer and triphosphate.

To investigate the enzymatic recognition capabilities, a template containing six consecutive SeLNA-T nucleotides (T1, Fig. 1a) after the 19 nucleotide (nt) primer binding region was synthesized first to incorporate natural nucleotides opposite to SeLNA-T nucleotides. Five different polymerases,

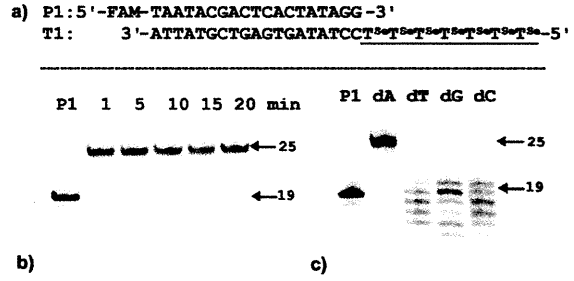


Fig. 1 Primer extension reactions using an SeLNA-template. (a) Primer and template sequences. (b) Reading of SeLNA-T template T1 to incorporate dA by KOD XL DNA polymerase. (c) Fidelity of reading SeLNA-T using all four natural nucleotides. SeLNA-T nucleotides are underlined and denoted by superscript 'Se'.

[a] *School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Brisbane 4072, Australia. E-mail: rakesh@uq.edu.au; Fax: +61 7 33654699; Tel: +61 7 33654611*
[b] *Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita 565-0871, Japan*
[c] *Graduate School of Pharmaceutical Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan*

† Electronic supplementary information (ESI) available: Experimental protocols for the primer extension and PCR reactions and additional gel images. See DOI: 10.1039/c2cc36464f

KOD XL, Phusion High Fidelity (HF), Therminator, *Taq* and Klenow DNA polymerases, were tested by primer extension experiments. The extension product was analysed by 13% denaturing polyacrylamide gel electrophoresis for which the primer DNA was 5′-FAM-labeled. The experiment revealed that KOD XL DNA polymerase was the most efficient enzyme to read SeLNA-T nucleotides of the template strand and extend the primer to full-length (Fig. 1b). Other thermophilic B-family polymerases (Therminator and Phusion HF) tested also yielded the full-length product with slightly reduced yield (Fig. S1a and b, ESI†). Product degradation was observed in the case of Phusion HF DNA polymerase after five minutes of incubation. Out of the two family-A polymerases tested, Klenow DNA polymerase successfully extended the primer to full-length although the yield only improved upon prolonged incubation from one minute to fifteen minutes (Fig. S1c, ESI†). *Taq* DNA polymerase could only extend the primer DNA by three consecutive dA nucleotides failing to afford the full-length extension product even after fifteen minutes of incubation (Fig. S1d, ESI†).

To assess the polymerase fidelity in reading SeLNA-T and to incorporate the correct nucleotide (dA) by following strict Watson–Crick base pairing rules, extension experiments were conducted with KOD XL DNA polymerase using dTTP, dGTP and dCTP. While yielding the expected full-length product with dATP, the polymerase failed to extend the primer with the other three incorrect nucleotides after ten minutes of incubation (Fig. 1c). Prolonged incubation to twenty minutes only resulted in further degradation of the products (data not shown). These results suggest that the polymerase followed the necessary fidelity in reading SeLNA-T nucleotides. Experiments were also conducted by removing Mn^{2+} to investigate its importance in the reaction as Mn^{2+} is known to reduce the 2′-sugar discrimination.[9] The result showed that KOD DNA polymerase was able to yield the full-length extension product with good yield without $MnCl_2$ supplementation in the reaction (Fig. S2, ESI†).

Based on the initial results, we then performed extension experiments using a DNA template (T2, Fig. 2a) containing two SeLNA-T nucleotides along with other nucleotides as a

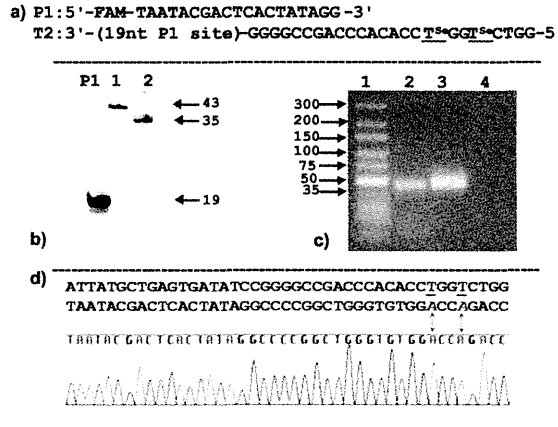


Fig. 2 Primer extension reactions using an SeLNA-template. (a) Primer and template sequences. (b) Extension by KOD XL DNA polymerase, lane P1: primer, lane 1: using all four dNTPs, lane 2: negative control without the 'T' nucleotide. (c) PCR amplification using the purified ssDNA. (d) Alignment of the sequencing chromatogram with the actual product sequence. SeLNA-T nucleotides are underlined and denoted by superscript 'Se'.

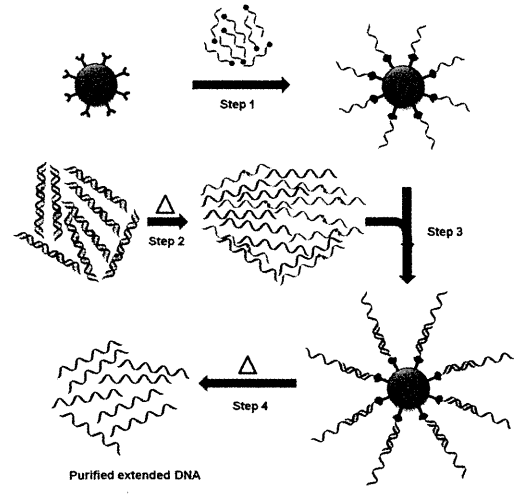


Fig. 3 Purification of the primer extended ssDNA. Step 1: biotinylated short probe DNA is immobilized on streptavidin coated magnetic beads; Step 2: phenol–chloroform extracted extension products were denatured; Step 3: the primer extended top strand is captured by the probe DNA; Step 4: after removing the undesired DNA, the complex is heated to 60 °C and the desired primer extended DNA strand is isolated.

first step towards the application of SeLNA-T nucleotides in aptamer selection by SELEX. KOD XL (Fig. 2b) successfully extended the primer to full-length. The accuracy of the extension product was further verified by sequencing. For sequencing the primer extension product, we isolated and purified the full-length primer extended single-stranded DNA strand (illustrated in Fig. 3) and PCR amplified (Fig. 2c), cloned and sequenced. The sequencing chromatogram clearly matched the actual sequences (Fig. 2d).

The first enzymatic step involved in aptamer selection by the conventional SELEX approach is to amplify the target bound aptamer candidate by PCR. Along this line, we performed PCR amplification using SeLNA-T-modified templates. Templates T2 with two SeLNA-T nucleotides (Fig. 4a) and T3, a 68 nt template with three SeLNA-T nucleotides (Fig. 4b), were used in this experiment. The result revealed that both the

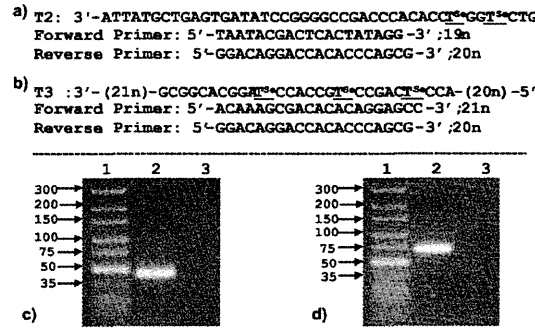


Fig. 4 PCR amplification using SeLNA-T modified DNA templates. (a) Primer sequences specific for template T2; (b) primer sequences specific for template T3; (c) agarose gel electrophoresis image of PCR using template T2, lane 1: DNA size markers in base-pairs, lane 2: PCR using T2, lane 3: PCR without using a template (negative control); (d) agarose gel electrophoresis image of PCR using template T3, lane 1: DNA size markers in base-pairs, lane 2: PCR using template T3, lane 3: PCR without using a template.

templates were successfully amplified using Phusion DNA polymerase (Fig. 4c and d). It is worth mentioning that PCR amplification using SeLNA-T triphosphate in place of dTTP in the reaction mixture with normal DNA templates or SeLNA-T-modified DNA templates was not successful.

Aptamer selection using modified nucleotide containing libraries requires converting the double-stranded DNA copies of the target binders after PCR to the corresponding ssDNA containing modified nucleotides. This is normally achieved by primer extension reaction using modified nucleotide triphosphate. To investigate the capability of polymerases to construct a DNA with SeLNA-T nucleotides, we synthesised the SeLNA-T nucleotide 5′-triphosphate (Scheme 1) using the protocol applied for the synthesis of LNA triphosphates[10] (see ESI† for full characterization). First, we performed the extension experiment using a template (T6, Fig. S5a, ESI†) designed to incorporate seven consecutive SeLNA-T nucleotides in the 3′-end of the primer. KOD XL, Therminator, Phusion, Klenow and *Taq* DNA polymerases were again tested for their capabilities to incorporate SeLNA-T nucleotides. The results showed that KOD XL and Therminator DNA polymerases were able to successively incorporate three and two SeLNA-T nucleotides, respectively (Fig. S3b and c, ESI†). Phusion and Klenow DNA polymerases were only able to incorporate one SeLNA-T nucleotide (Fig. S3d and e, ESI†) whereas *Taq* failed to accept the SeLNA-T nucleotide as a substrate. Later, an experiment was initiated to compare the polymerase recognition capabilities of SeLNA-T and normal LNA-T nucleotides. As expected, the results showed that KOD XL polymerase was able to incorporate three consecutive LNA nucleotides in very good yield and also to afford the full-length product in low yield whereas in the case of SeLNA nucleotides, the enzyme only extended the primer up to three nucleotides (Fig. S4, ESI†).

We further evaluated the efficacy of polymerase to incorporate SeLNA-T nucleotides along with other nucleotides using a longer DNA template. The designed 43 nt template (T4, Fig. 5a) has three sites of incorporation. Positive control (all four natural nucleotide triphosphates) and negative control (dATP, dCTP, dGTP) experiments were also performed in parallel to the SeLNA-T incorporation reactions. KOD XL DNA polymerase efficiently incorporated SeLNA-T nucleotides at the desired positions and extended the product to full-length (Fig. 5b). Prolonged incubation to 40 minutes produced the full-length extension product with very high yield although it was visible after 5 minutes. The extended primer strand with SeLNA-T nucleotide incorporation was isolated and purified as discussed earlier (Fig. 3) and amplified by PCR followed by cloning and sequencing. The sequencing chromatogram clearly matched the expected 'T' nucleotides at the desired positions (Fig. S5, ESI†). Another experiment was performed to investigate the multiple successive incorporation of SeLNA-T nucleotides along with other three nucleotide triphosphates. The designed template (T5, Fig. 5a) has eight consecutive sites of incorporation placed seven nucleotides away from the primer binding region. Remarkably, the results showed that KOD XL polymerase successfully incorporated eight consecutive SeLNA-T nucleotides and extended the primer to full-length (Fig. 5c). However, the yield was poor even after 45 minutes of incubation. Based on our extension experiments performed in the presence and absence of reducing agent DTT (no change observed, data not shown), it is worth mentioning that SeLNA nucleotides do not get oxidized during polymerase reactions.

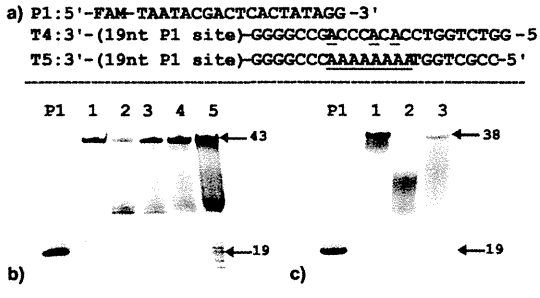


Fig. 5 Enzymatic incorporation using SeLNA-T-5′-triphosphate. (a) Primer and template sequences. (b) Extension by KOD XL DNA polymerase, lane P1: primer, lane 1: using all four dNTPs for 5 minutes, lanes 2–5: incorporation of SeLNA-T nucleotides (dATP, dGTP, dCTP and SeLNA–TTP in the mixture) at 5, 10, 20 and 45 minutes respectively. (c) Successive incorporation of SeLNA-T by KOD XL DNA polymerase, lane P1: primer, lane 1: using all four dNTPs for 5 minutes, lane 2: negative control without the 'T' nucleotide in the reaction mixture, lane 3: incorporation of SeLNA-T nucleotides (dATP, dGTP, dCTP and SeLNA–TTP in the mixture) after 45 minutes.

In summary, we have demonstrated that SeLNA-T nucleotides can be successfully accepted by polymerases. KOD XL DNA polymerase was found to be an efficient enzyme to construct oligonucleotide strands by reading SeLNA-T nucleotides of the template strand and also by incorporating SeLNA-T nucleotides using DNA templates. The findings reported here suggest that the evolution of SeLNA-T nucleotide-modified aptamers can be developed towards the elucidation of the aptamer structure by X-ray crystallography.

This research was supported by The University of Queensland Fellowship schemes awarded to RNV.

Notes and references

1 M. Famulok, J. S. Hartig and G. Mayer, *Chem. Rev.*, 2007, **107**, 3715.
2 R. Stoltenburg, C. Reinemann and B. Strehlitz, *Biomol. Eng.*, 2007, **24**, 381.
3 (*a*) R. N. Veedu and J. Wengel, *RNA Biol.*, 2009, **6**, 321–323; (*b*) R. N. Veedu and J. Wengel, *Chem. Biodiversity*, 2010, **7**, 536.
4 (*a*) S. Obika, D. Nanbu, Y. Hari, K. Morio, Y. In, T. Ishida and T. Imanishi, *Tetrahedron Lett.*, 1997, **38**, 8735; (*b*) S. K. Singh, P. Nielsen, A. A. Koshkin and J. Wengel, *Chem. Commun*, 1998, 455.
5 (*a*) R. Kumer, S. K. Singh, A. A. Koshkin, V. K. Rajwanshi, M. Meldgaad and J. Wengel, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 2219; (*b*) S. K. Singh, R. Kumer and J. Wengel, *J. Org. Chem.*, 1998, **63**, 6078; (*c*) S. K. Singh, R. Kumer and J. Wengel, *J. Org. Chem.*, 1998, **63**, 10035; (*d*) J. Xu, Y. Liu, C. Dupouy and J. Chattopadhyaya, *J. Org. Chem.*, 2009, **74**, 6534.
6 (*a*) L. Lin, J. Sheng and Z. Huang, *Chem. Soc. Rev.*, 2011, **40**, 4591; (*b*) J. Sheng and Z. Huang, *Int. J. Mol. Sci.*, 2008, **9**, 258; (*c*) P. S. Pallan and M. Egli, *Nat. Protocols*, 2007, **2**, 647.
7 K. Morihiro, T. Kodama, K. Tefu, Y. Moai, R. N. Veedu and S. Obika, *Angew. Chem., Int. Ed.*, 2012, submitted.
8 (*a*) T. Santner, V. Siegmund, A. Marx and R. Micura, *Bioorg. Med. Chem.*, 2012, **20**, 2416; (*b*) V. Siegmund, T. Santner, R. Micura and A. Marx, *Chem. Sci.*, 2011, **2**, 2224.
9 S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U. S. A.*, 1989, **86**, 4076.
10 R. N. Veedu, H. V. Burri, P. Kumar, P. K. Sharma, P. J. Hrdlicka, B. Vester and J. Wengel, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 6565.

Organic & Biomolecular Chemistry

RSC Publishing

PAPER

Cite this: Org. Biomol. Chem., 2012, 10, 9639

Synthesis and duplex-forming ability of oligonucleotides containing 4′-carboxythymidine analogs†

Yoshiyuki Hari, Takashi Osawa and Satoshi Obika*

Received 30th August 2012,
Accepted 23rd October 2012

DOI: 10.1039/c2ob26712h

www.rsc.org/obc

Oligonucleotides containing 4′-carboxy-, 4′-methoxycarbonyl-, 4′-carbamoyl-, and 4′-methylcarbamoyl-thymidines, and their 2′-methoxy, 2′-amino or 2′-acetamido analogs were prepared. Their duplex-forming ability with DNA and RNA complements was evaluated by UV melting experiments. Interestingly, 4′-carboxythymidine existing in the *S*-type sugar conformation was found to lead to an increase in the stability of the duplex formed with RNA complements compared to natural thymidine.

Introduction

Modified oligonucleotides with high binding affinity to complementary single-stranded nucleic acids are promising materials applicable to various nucleic acid-based technologies. In particular, those with high affinity for RNA complements are essential for applications in RNA-targeted therapies like antisense methodology and siRNA therapy.[1,2] To date, many artificial oligonucleotides have been developed towards practical use and to acquire increased RNA-binding affinity by introduction of substituents at the 2′-position like 2′-alkoxy groups and bridged chains like 2′,4′-BNA/LNA.[1] This increased binding affinity is due to preorganization of the sugar conformation to that in RNA duplexes, namely the *N*-type sugar conformation. However, in some cases, oligonucleotides, including several modified nucleotides, likely existing in *S*-type conformations, were also observed to increase the stability of duplexes formed with RNA complements. For instance, 4′-methoxymethyl- or 4′-aminomethyl-thymidine units could increase the melting temperature (T_m) value by up to 0.6 °C or 0.5 °C per modification, respectively, depending on the number and position of the introduction.[3] In contrast, 4′-hydroxymethylthymidine[4] and 4′-methylthymidine,[5] an analog without any functional group on the 4′-methyl group, seem to lead to a decrease in the stability. Given this background, we were interested in the effect of thymidines with highly functionalized methyl units at the 4′-position on the thermal stability of the formed duplexes. Here, the synthesis of oligonucleotides bearing 4′-carboxy-, 4′-methoxycarbonyl-, 4′-carbamoyl- and 4′-methylcarbamoyl-thymidines and their 2′-substituted analogs as shown in Fig. 1 was performed and their duplex formation with DNA and RNA complements was studied.

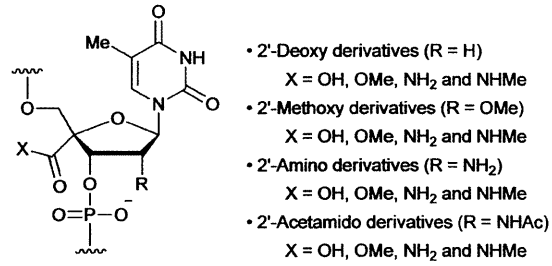


Fig. 1 Structures of 4′-carboxythymidine analogs used in this study.

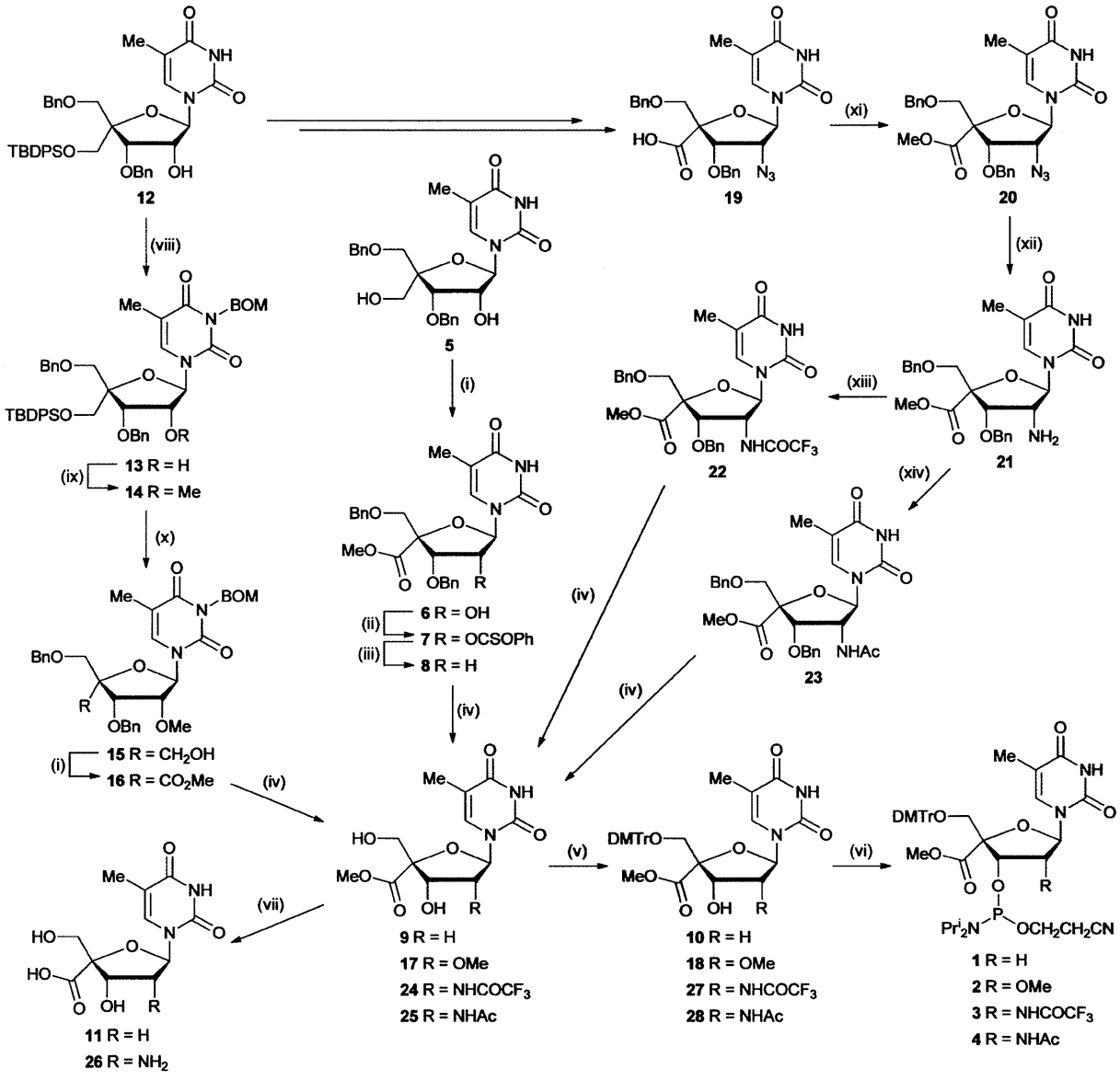
Results and discussion

Synthesis

We planned to convert 4′-methoxycarbonylthymidines into analogs with the desired 4′-carboxylic acid equivalents by base treatment after oligonucleotide synthesis. Thus, the phosphoramidites **1–4** of 4′-methoxycarbonylthymidines were synthesized as shown in Scheme 1. The diol **5**[6] previously reported by Wengel's group was oxidized with a catalytic amount of TEMPO and $PhI(OAc)_2$, and the resulting carboxylic acid was reacted with $TMSCHN_2$ in MeOH to give the methyl ester **6** in 72% over 2 steps. Deoxygenation of **6** afforded the 2′-deoxy compound **8** *via* thiocarbonate **7**. By treatment of **8** with cyclohexene in the presence of $Pd(OH)_2$-C, 4′-methoxycarbonylthymidine **9**[7] was obtained in 45% yield.

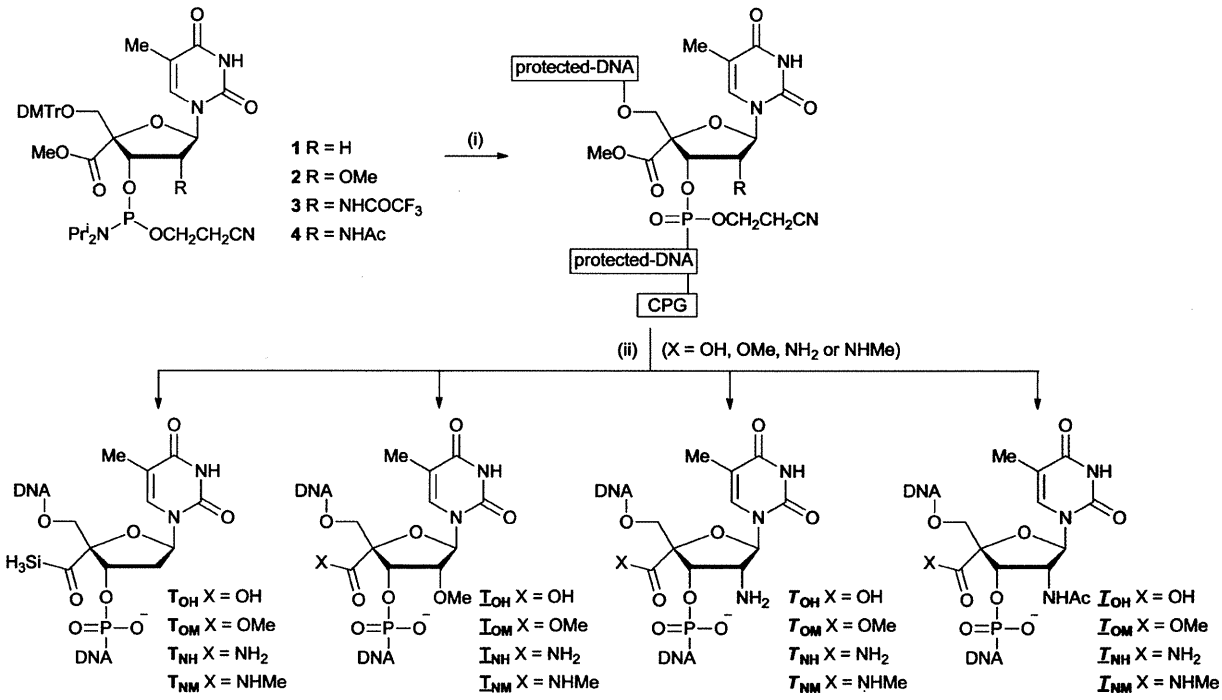
Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita 565-0871, Japan.
E-mail: obika@phs.osaka-u.ac.jp; Fax: +81 6 6879 8204; Tel: +81 6 6879 8200

† Electronic supplementary information (ESI) available: Additional experimental procedures, NMR spectra of all new compounds, and HPLC and MALDI-TOF mass spectra of the synthesized oligonucleotides. See DOI: 10.1039/c2ob26712h



Scheme 1 *Reagents and conditions*: (i) TEMPO, $PhI(OAc)_2$, $MeCN–H_2O$ (1 : 1), rt, 12 h, then $TMSCHN_2$, MeOH, rt, 0.5 h, 52–72% for 2 steps; (ii) PhOCSCl, DMAP, CH_2Cl_2, rt, 0.5 h, 79%; (iii) AIBN, *n*-Bu_3SnH, toluene, reflux, 2 h, 55%; (iv) 20% $Pd(OH)_2$-C, cyclohexene, MeOH, reflux, 10–12 h, 45%-quant.; (v) DMTrCl, pyridine, rt, 1–4 h, 83%-quant.; (vi) $Pr^i_2NP(Cl)OCH_2CH_2CN$, DIPEA, CH_2Cl_2, rt, 2–4 h, 56–95%; (vii) BOMCl, DBU, THF, rt, 0.5 h, 86%; (viii) NaH, MeI, DMF, rt, 1 h, 58%; (ix) TBAF, THF, rt, 11 h, quant.; (x) $TMSCHN_2$, MeOH, rt, 0.5 h, 89%; (xi) $NaBH_4$, $NiCl_2$, MeOH–THF (1 : 1), rt, 0.5 h, 89%; (xii) CF_3CO_2Et, Et_3N, MeOH, rt, 1 h, 92%; (xiii) Ac_2O, pyridine, rt, 1.5 h, quant.

Dimethoxytritylation of the 5′-hydroxyl group in **9** followed by phosphitylation of **10**[7] gave the desired phosphoramidite **1**, a building block for oligonucleotide synthesis. The synthesis of the 2′-methoxy analog **2** was achieved from **11**[8] prepared according to our previous report. After BOM-protection of the nitrogen at the 3-position of **11**, the resulting **12** was methylated to afford **13** by alkoxide formation, followed by treatment with MeI. Desilylation of **13** with TBAF produced **14**, which was converted into methyl ester **15** as before. Removal of benzyl and BOM groups by hydrogenolysis of **15** led to 2′-methoxy monomer **16** quantitatively. Analogous to **1**, the phosphoramidite **2** was prepared from **16** *via* compound **17**. 2′-Amino and 2′-acetamido analogs were synthesized from **18**[9] prepared from **11** according to our previous report. Methyl esterification of **18** using $TMSCHN_2$ yielded **19**, which was reduced by a combination of $NiCl_2$ and $NaBH_4$ to produce **20** in 89% yield. Trifluoroacetylation and acetylation of **20** gave **21** and **22** in high yields, respectively. These compounds **21** and **22** were hydrogenolyzed to afford monomers **23**, protected as the 2′-amino analog, and **24** as the 2′-acetamido one. Dimethoxytritylated **25** and **26**, and subsequently phosphoramidites **3** and **4**, were prepared as before. As expected, it was



Scheme 2 *Reagents and conditions*: (i) oligonucleotide synthesis on an automated DNA synthesizer; (ii) 50 mM NaOH aq., rt, 1.5 h then 55 °C, 12 h (for X = OH), 50 mM K_2CO_3 in MeOH, rt, 2 h (for X = OMe), 28% NH_3 aq., rt, 1.5 h then 55 °C, 12 h (for X = NH_2) or 40% MeNH aq., rt, 1.5 h then 55 °C, 12 h (for X = NHMe).

found from large $J_{1'2'}$ values (6.5–9.0 Hz) obtained by ^{1}H NMR measurements that the monomers **9**, **16**, **23** and **24** existed in *S*-type conformations.[10]

Next, introduction of the phosphoramidites **1–4** into oligonucleotides was performed on an automated DNA synthesizer using standard phosphoramidite chemistry (Scheme 2). For the synthesis of 4′-methoxycarbonyl-modified oligonucleotides, commercially available ultra-mild phosphoramidites‡ as other phosphoramidites were used. After completion of the synthesis on the DNA synthesizer, four base treatments (50 mM NaOH aq., 50 mM K_2CO_3 in MeOH, 28% NH_3 aq. and 40% $MeNH_2$ aq.) afforded cleavage from the resin, deprotection of nucleobases and phosphates, and conversion of 4′-methoxycarbonyl moieties to give the corresponding oligonucleotides containing 4′-carboxy, 4′-methoxycarbonyl, 4′-carbamoyl and 4′-methylcarbamoyl derivatives, respectively. The removal of the standard isobutyryl protecting group of the 2-amino moiety in G was completed by treatment with 50 mM NaOH aq. at 55 °C for 12 h. The list of the synthesized oligonucleotides is shown in Table 1. In all cases, they were obtained in moderate to good yields with high purities and their molecular weights were confirmed by MALDI-TOF-MS.

Table 1 Sequence of the synthesized oligonucleotides[a]

Oligonucleotide	Sequence
ON1a	5′-GCGTTTT$_{OH}$TTGCT-3′
ON1b	5′-GCGTTTT$_{OM}$TTGCT-3′
ON1c	5′-GCGTTTT$_{NH}$TTGCT-3′
ON1d	5′-GCGTTTT$_{NM}$TTGCT-3′
ON2a	5′-GCGTTTT$_{OH}$TTGCT-3′
ON2b	5′-GCGTTTT$_{OM}$TTGCT-3′
ON2c	5′-GCGTTTT$_{NH}$TTGCT-3′
ON2d	5′-GCGTTTT$_{NM}$TTGCT-3′
ON3a	5′-GCGTTTT$_{OH}$TTGCT-3′
ON3b	5′-GCGTTTT$_{OM}$TTGCT-3′
ON3c	5′-GCGTTTT$_{NH}$TTGCT-3′
ON3d	5′-GCGTTTT$_{NM}$TTGCT-3′
ON4a	5′-GCGTTTT$_{OH}$TTGCT-3′
ON4b	5′-GCGTTTT$_{OM}$TTGCT-3′
ON4c	5′-GCGTTTT$_{NH}$TTGCT-3′
ON4d	5′-GCGTTTT$_{NM}$TTGCT-3′
ON5a	5′-GCGTTT$_{OH}$T$_{OH}$T$_{OH}$TGCT-3′
ON6a	5′-GCGTT$_{OH}$TT$_{OH}$TT$_{OH}$GCT-3′

[a] Bold letters: Modified thymidines (see Scheme 2).

Evaluation of duplex-forming ability

UV melting experiments of the synthesized **ON1–4** with single modification and DNA or RNA complement, 5′-d(AGCAAAAAACGC)-3′ or 5′-r(AGCAAAAAACGC)-3′, were carried out and the results are summarized in Table 2. In general, the binding affinity of singly modified **ON1–4** against complementary DNA and RNA was greatly affected by the 2′-substituent and almost no effect by the 4′-modification was observed. The order of stabilization was 2′-deoxy (**ON1a–d**), 2′-methoxy (**ON2a–d**), 2′-amino (**ON3a–d**) and 2′-acetamido (**ON4a–d**) groups. 2′-Amino and 2′-acetamido modifications led to an especially large decrease in the duplex stability compared to that of the unmodified DNA/DNA duplex (T_m = 51 °C). It is

‡Purchased from Glen Research.

Table 2 UV melting experiments with DNA and RNA complements[a,b]

Oligonucleotide	DNA complement T_m (°C)	DNA complement ΔT_m/mod.[c,d] (°C)	RNA complement T_m (°C)	RNA complement ΔT_m/mod.[c,d] (°C)
ON1a	51	0	47	+1
ON1b	51	0	46	0
ON1c	51	0	46	0
ON1d	51	0	46	0
ON2a	48	−3	44	−2
ON2b	48	−3	43	−3
ON2c	48	−3	43	−3
ON2d	48	−3	43	−3
ON3a	38	−13 (−2)	41	−5 (+3)
ON3b	37	−14 (−3)	37	−9 (−1)
ON3c	38	−13 (−3)	38	−8 (0)
ON3d	38	−13 (−3)	37	−9 (−1)
ON4a	35	−16	35	−11
ON4b	35	−16	35	−11
ON4c	35	−16	35	−11
ON4d	35	−16	34	−12

[a] Conditions: 10 mM sodium phosphate buffer (pH 7.2) and 100 mM NaCl. The final concentration of each oligonucleotide used was 4 μM. The sequences of DNA and RNA complements were 5′-d(AGCTTTTTTCGC)-3′ and 5′-r(AGCTTTTTTCGC)-3′, respectively. [b] T_m values of unmodified DNA/DNA and DNA/RNA duplexes were 51 °C and 46 °C, respectively. [c] The changes in T_m per modification compared with the unmodified duplexes are shown. [d] In parentheses, the changes in T_m per modification compared with the duplexes by 5′-GCGTT*T*TTTGCT-3′ (*T* = 2′-aminothymidine) are shown. The T_m values of the duplexes with DNA and RNA complements were 40 °C and 38 °C, respectively.

known that 2′-amino modification drastically destabilizes duplexes[11] and a UV melting experiment of 2′-aminothymidine-modified oligonucleotide, 5′-GCGTT*T*TTTGCT-3′ (*T* = 2′-aminothymidine), under the same conditions showed decreased T_m values of 40 °C and 38 °C by 11 °C and 8 °C compared to those of unmodified DNA/DNA and DNA/RNA duplexes, respectively (see ESI† for the preparation of 2′-aminothymidine-modified oligonucleotide). From these points, the decreased binding affinity of 2′-amino-modified **ON3a–d** was considered to be reasonable. In the case of 2′-acetamido modification (**ON4a–d**), the destabilization of the formed duplex was likely caused by the steric bulkiness of the 2′-acetamido group.

It was very interesting that 4′-carboxy modifications of thymidine and 2′-aminothymidine (**ON1a** and **ON3a**) had some apparent effect on the duplex stability with RNA complements. The T_m values of **ON1a** and **ON3a** were increased by 1 °C and 3 °C compared to those of the 4′-unmodified oligonucleotides, 46 °C for a natural DNA/RNA duplex and 37 °C for the duplex between 2′-amino-modified oligonucleotide and RNA complement, respectively, though the T_m value of **ON3a** was much lower than that of **ON1a** due to destabilization by the 2′-amino modification.[11] Very recently, Leumann's group reported that the existence of a carboxy group at the C6′-position of tricyclo-DNA did not perturb the pairing affinity of tricyclo-DNA with an RNA complement and in their case, carboxy and carbamoyl modifications apparently increased stability of the duplexes

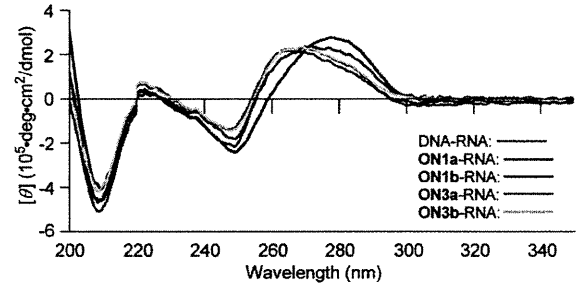


Fig. 2 CD spectra of duplexes with RNA complements by DNA, **ON1a**, **ON1b**, **ON3a** and **ON3b**.

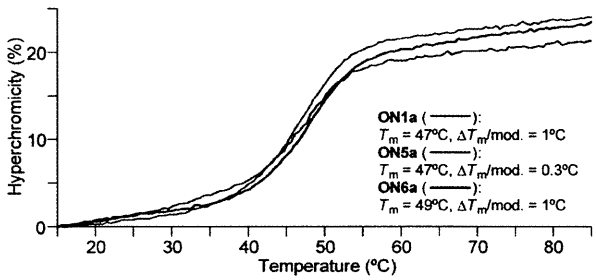


Fig. 3 UV melting profiles of duplexes with RNA complements by **ON1a**, **ON5a** and **ON6a**.

formed with RNA.[12] However, in our case, only thymidine and 2′-aminothymidine with modifications by 4′-carboxy groups stabilized the duplex with RNA compared to their 4′-unmodified ones. To study global conformational changes in duplexes of **ON1a**, **ON1b**, **ON3a** and **ON3b** with RNA complements, circular dichroism (CD) spectroscopy was measured (Fig. 2). As a result, no major change of the global conformations was observed although in the case of **ON1b** containing 4′-methoxycarbonylthymidine the positive band at around 260–280 nm was red-shifted.

Duplex-forming ability of oligonucleotides **ON5a** and **ON6a** including three 4′-carboxythymidine units consecutively and alternately led to the highest stabilization of the duplex with an RNA complement. Under the same conditions shown in Table 2, the ability was compared to singly modified **ON1a** and the UV melting profiles are displayed in Fig. 3. The T_m value of **ON5a** with consecutive modifications was comparable to that of **ON1a** with a single modification. This indicated that consecutive modifications led to a significant decrease in ΔT_m/mod. (+1 °C → +0.3 °C), perhaps due to slight electrostatic repulsion by contiguous carboxylate ions. On the other hand, the T_m value of **ON6a** with alternate modifications was 49 °C. Its ΔT_m/mod. was 1 °C, almost the same as that of **ON1a**, and it formed a stable duplex with an RNA complement. The ^{1}H NMR spectrum of 4′-carboxythymidine[13] exhibited a $J_{1'2'}$ value of 6.7 Hz, meaning that it preferentially adopted the *S*-type conformation.[10] These results could indicate that 4′-carboxythymidine significantly stabilized the duplex with an RNA complement compared to 4′-methoxymethylthymidine (ΔT_m/

mod. = up to 0.6 °C) or 4′-aminomethylthymidine (ΔT_m/mod. = up to 0.5 °C), which also adopted *S*-type conformations.

Experimental

General

All moisture-sensitive reactions were carried out in well-dried glassware under a N_2 atmosphere. ^1H, ^{13}C and ^{31}P spectra were recorded on JEOL JNM-EX300 and JEOL JNM-EX400 spectrometers. Chemical shifts are reported in parts per million referenced to tetramethylsilane (δ = 0.00 ppm) for ^1H NMR spectra, $CDCl_3$ (δ = 77.0 ppm) and CD_3OD (δ = 49.0 ppm) for ^{13}C NMR spectra, and phosphoric acid (δ = 0.00 ppm) for ^{31}P NMR spectra. IR spectra were recorded on a JASCO FT/IR-4200 spectrometer. Optical rotations were recorded on a JASCO P-2200 polarimeter. FAB mass spectra were measured on JEOL JMS-600 or JEOL JMS-700 mass spectrometers. MALDI-TOF mass spectra were recorded on a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer. Fuji Silysia silica gel PSQ-60B (0.060 mm) and FL-60D (0.060 mm) were used for flash column chromatography. For HPLC, SHIMADZU LC-10AT$_{VP}$, SHIMADZU SPD-10A$_{VP}$ and SHIMADZU CTO-10$_{VP}$ instruments were used.

3′,5′-Di-*O*-benzyl-4′-methoxycarbonyl-5-methyluridine (6). PhI(OAc)$_2$ (3.86 g, 12.0 mmol) and TEMPO (170 mg, 1.09 mmol) were added to a solution of compound 5[6] (2.55 g, 5.45 mmol) in MeCN–H_2O (1 : 1, 30 mL) at room temperature. The reaction mixture was stirred for 12 h at room temperature. The resulting mixture was concentrated *in vacuo*, and the residue (5.98 g) was co-evaporated with anhydrous MeCN three times, and dissolved in anhydrous MeOH (30 mL). $TMSCHN_2$ (2 M in *n*-hexane, 3.0 mL, 6.0 mmol) was added to the solution at room temperature. The reaction mixture was stirred for 0.5 h at room temperature. The resulting mixture was concentrated *in vacuo* and the residue (6.11 g) was purified by column chromatography (silica gel, 150 g, *n*-hexane–EtOAc = 2 : 3) to give compound **6** (1.94 g, 72% for 2 steps from 5) as a white foam.

Mp: 62–64 °C. $[\alpha]_D^{23}$ −35.4 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3449, 3177, 3065, 3033, 2952, 2870, 1713, 1472, 1455, 1373, 1279, 1240 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.59 (d, J = 1.0 Hz, 3H), 3.72 (s, 3H), 3.76–3.82 (m, 2H), 4.05 (d, J = 10.0 Hz, 1H), 4.55–4.74 (m, 5H), 6.21 (d, J = 6.5 Hz, 1H), 7.25–7.41 (m, 11H), 9.05 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.09, 52.74, 71.83, 73.86, 74.36, 75.28, 80.24, 89.08, 89.20, 111.51, 127.61, 128.02, 128.25, 128.26, 128.53, 128.69, 135.59, 136.59, 136.82, 150.76, 163.54, 169.58. MS (FAB): m/z = 497 $[M + H]^+$. HRMS (FAB): calcd for $C_{26}H_{29}N_2O_8$ $[M + H]^+$, 497.1924, found, 497.1923.

3′,5′-Di-*O*-benzyl-4′-methoxycarbonyl-2′-*O*-phenoxythiocarbonyl-5-methyluridine (7). Under a nitrogen atmosphere, DMAP (493 mg, 4.04 mmol) and PhOCSCl (0.33 mL, 2.42 mmol) were added to a solution of compound **6** (1.00 g, 2.02 mmol) in anhydrous CH_2Cl_2 (20 mL) at 0 °C. The reaction mixture was stirred at room temperature for 0.5 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (1.52 g) was purified by column chromatography (silica gel, 30 g, *n*-hexane–EtOAc = 1 : 1) to give compound 7 (1.01 g, 79%) as a white foam.

Mp: 58–61 °C. $[\alpha]_D^{23}$ −47.6 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3182, 3033, 2952, 2868, 2614, 2484, 1954, 1867, 1715, 1591, 1489, 1470, 1361, 1277 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.57 (d, J = 1.0 Hz, 3H), 3.70 (s, 3H), 3.86 (d, J = 10.0 Hz, 1H), 4.10 (d, J = 10.0 Hz, 1H), 4.59 (d, J = 11.5 Hz, 1H), 4.61 (d, J = 10.5 Hz, 1H), 4.68 (d, J = 10.5 Hz, 1H), 4.68 (d, J = 11.0 Hz, 1H), 4.80 (d, J = 6.0 Hz, 1H), 5.79 (dd, J = 6.0, 7.5 Hz, 1H), 6.66 (d, J = 7.5 Hz, 1H), 6.96–7.40 (m, 16H), 8.80 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.06, 52.69, 71.83, 73.91, 75.47, 78.70, 81.50, 86.38, 89.67, 111.84, 115.28, 121.55, 126.79, 127.71, 127.85, 128.05, 128.25, 128.38, 128.66, 129.57, 135.75, 136.74, 136.85, 150.26, 153.28, 163.47, 168.82, 194.42. MS (FAB): m/z = 633 $[M + H]^+$. HRMS (FAB): calcd for $C_{33}H_{33}N_2O_9S$ $[M + H]^+$, 633.1907, found, 633.1920.

3′,5′-Di-*O*-benzyl-4′-methoxycarbonylthymidine (8). Under a nitrogen atmosphere, *n*-Bu_3SnH (0.81 mL, 3.00 mmol) and AIBN (33.1 mg, 0.202 mmol) were added to a solution of compound 7 (1.00 g, 1.58 mmol) in anhydrous toluene (20 mL) at room temperature. The reaction mixture was refluxed for 2 h. The resulting mixture was concentrated *in vacuo* and the residue (2.00 g) was purified by column chromatography (silica gel, 60 g, *n*-hexane–EtOAc = 1 : 1) to give compound **8** (424 mg, 55%) as a white foam.

Mp: 72–74 °C. $[\alpha]_D^{23}$ +24.6 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3183, 3065, 3034, 2952, 2920, 2871, 1695, 1496, 1454, 1434, 1399, 1365, 1281, 1207 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.56 (d, J = 1.0 Hz, 3H), 2.13–2.22 (m, 1H), 2.53–2.61 (m, 1H), 3.75 (s, 3H), 3.88 (d, J = 10.0 Hz, 1H), 4.08 (d, J = 10.0 Hz, 1H), 4.48–4.61 (m, 5H), 6.58 (t, J = 6.5 Hz, 1H), 6.96–7.40 (m, 10H), 7.52 (d, J = 1.0 Hz, 1H), 9.27 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.03, 37.71, 52.42, 70.98, 72.42, 73.74, 79.59, 85.70, 89.90, 111.08, 127.41, 127.48, 127.62, 127.87, 128.11, 128.39, 128.48, 128.58, 135.81, 136.98, 137.06, 150.24, 163.84, 169.80. MS (EI): m/z = 480 (M^+, 14.3), 355 (11.8), 261 (13.6), 248 (14.2), 217 (85.5), 181 (29.5), 157 (13.8), 127 (16.3), 91 (100). HRMS (EI): calcd for $C_{26}H_{28}N_2O_7$ $[M^+]$, 480.1897, found, 480.1900.

4′-Methoxycarbonylthymidine (9).[7] Under a nitrogen atmosphere, a solution of compound **8** (420 mg, 0.874 mmol) in MeOH (15 mL) and cyclohexene (8.8 mL, 87 mmol) were added to a suspension of 20% $Pd(OH)_2$ on carbon (307 mg, 0.437 mmol) in MeOH (5.0 mL) at room temperature. The reaction mixture was refluxed for 12 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue (360 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 20 : 1 to 7 : 1) to give compound **9** (118 mg, 45%) as a white foam.

^1H NMR (400 MHz, CD_3OD) δ: 1.87 (d, J = 1.0 Hz, 3H), 2.32–2.36 (m, 2H), 3.75 (s, 3H), 3.92 (d, J = 12.0 Hz, 1H), 3.96 (d, J = 12.0 Hz, 1H), 4.57 (dd, J = 5.5, 6.5 Hz, 1H), 6.48 (t, J = 6.5 Hz, 1H), 7.74 (d, J = 1.0 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD)

δ: 12.47, 40.56, 52.55, 64.25, 73.33, 87.12, 93.37, 111.70, 138.43, 152.26, 166.40, 172.39.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-methoxycarbonylthymidine (10).[7] Under a nitrogen atmosphere, DMTrCl (120 mg, 0.355 mmol) was added to a solution of compound **9** (71.0 mg, 0.236 mmol) in anhydrous pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (210 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 15 : 1) to give compound **10** (118 mg, 83%) as a white foam.

^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.38 (d, *J* = 1.0 Hz, 3H), 2.37–2.56 (m, 2H), 3.58 (d, *J* = 10.0 Hz, 1H), 3.70 (d, *J* = 10.0 Hz, 1H), 3.73 (s, 3H), 3.78 (s, 6H), 4.77 (dd, *J* = 3.0, 6.5 Hz, 1H), 6.65 (dd, *J* = 6.0, 8.0 Hz, 1H), 6.81–7.40 (m, 13H), 7.55 (d, *J* = 1.0 Hz, 1H), 9.51 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 11.60, 39.99, 52.59, 55.17, 65.03, 73.62, 85.66, 87.17, 91.55, 111.36, 113.19, 127.15, 127.95, 128.06, 130.08, 134.77, 134.89, 136.29, 143.91, 150.34, 158.61, 158.63, 163.93, 170.71.

3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonylthymidine (1). Under a nitrogen atmosphere, DIPEA (0.17 mL, 0.97 mmol) and i-Pr_2NP(Cl)-OCH_2CH_2CN (52 μL, 0.23 mmol) were added to a solution of compound **10** (117 mg, 0.194 mmol) in anhydrous CH_2Cl_2 (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (166 mg) was chromatographed (silica gel, 10 g, *n*-hexane–EtOAc = 2 : 3) to give **1** with a small amount of impurity (99.8 mg), which was reprecipitated from *n*-hexane–$CHCl_3$ to give compound **1** (86.7 mg, 56%) as a white powder.

Mp: 88–90 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.05–1.19 (m, 12H), 1.32 (d, *J* = 1.5 Hz, 1.5H), 1.33 (d, *J* = 1.5 Hz, 1.5H), 2.41–2.65 (m, 4H), 3.50–3.80 (m, 15H), 4.84–4.91 (m, 0.5H), 4.98–5.06 (m, 0.5H), 6.61 (t, *J* = 8.0 Hz, 0.5H), 6.65 (t, *J* = 8.0 Hz, 0.5H), 6.82–7.41 (m, 13H), 7.59 (d, *J* = 1.5 Hz, 0.5H), 7.62 (d, *J* = 1.5 Hz, 0.5H), 9.26 (brs, 1H). ^{31}P NMR (161 MHz, $CDCl_3$) δ: 149.89, 150.02. MS (FAB): *m*/*z* = 803 $[M + H]^+$. HRMS (FAB): calcd for $C_{42}H_{52}N_4O_{10}P$ $[M + H]^+$, 803.3421, found, 803.3430.

3-*N*-Benzyloxymethyl-3′,5′-di-*O*-benzyl-4′-*tert*-butyldiphenylsiloxymethyl-5-methyluridine (12). Under a nitrogen atmosphere, DBU (0.71 mL, 4.8 mmol) and BOMCl (0.61 mL, 4.4 mmol) were added to a solution of compound **11**[8] (2.60 g, 3.67 mmol) in anhydrous THF (15 mL) at 0 °C. The reaction mixture was stirred at room temperature for 0.5 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (3.40 g) was purified by column chromatography (silica gel, 60 g, *n*-hexane–EtOAc = 3 : 1) to give compound **12** (2.62 g, 86%) as a white foam.

Mp: 40–42 °C. $[\alpha]_D^{24}$ −6.8 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3409, 3065, 3031, 2930, 2858, 1711, 1668, 1496, 1455, 1428, 1362, 1265, 1209 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.06 (s, 9H), 1.62 (d, *J* = 1.0 Hz, 3H), 3.76–3.82 (m, 4H), 4.64 (d, *J* = 11.0 Hz, 1H), 4.68 (s, 2H), 4.76 (d, *J* = 11.0 Hz, 1H), 5.45 (d, *J* = 9.5 Hz, 1H), 5.49 (d, *J* = 9.5 Hz, 1H), 6.00 (d, *J* = 2.0 Hz, 1H), 7.19–7.68 (m, 26H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.76, 19.02, 26.75, 64.11, 70.47, 71.83, 72.10, 73.66, 73.86, 74.50, 78.06, 88.20, 92.17, 110.90, 127.53, 127.60, 127.67, 127.80, 127.87, 127.96, 128.03, 128.09, 128.22, 128.52, 128.55, 129.92, 129.95, 132.06, 132.09, 135.24, 135.62, 137.18, 137.25, 137.97, 151.18, 163.46. MS (FAB): *m*/*z* = 827 $[M + H]^+$. HRMS (FAB): calcd for $C_{49}H_{55}N_2O_8Si$ $[M + H]^+$, 827.3728, found, 827.3726.

3-*N*-Benzyloxymethyl-3′,5′-di-*O*-benzyl-4′-*tert*-butyldiphenylsiloxymethyl-2′-*O*-methyl-5-methyluridine (13). Under a nitrogen atmosphere, NaH (127 mg, 3.17 mmol) was added to a solution of compound **12** (2.50 g, 3.02 mmol) in anhydrous DMF (15 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. MeI (0.20 mL, 3.2 mmol) was added to the above mixture at 0 °C, and the mixture was further stirred at room temperature for 1 h. After the addition of H_2O at 0 °C, the mixture was extracted with Et_2O. The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (3.21 g) was purified by column chromatography (silica gel, 50 g, *n*-hexane–EtOAc = 3 : 1) to give compound **13** (1.48 g, 58%) as a colorless oil.

$[\alpha]_D^{24}$ +15.4 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3068, 3031, 2955, 2930, 2858, 1707, 1668, 1463, 1428, 1389, 1362, 1298, 1254, 1211 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.05 (s, 9H), 1.45 (d, *J* = 1.0 Hz, 3H), 3.36 (s, 3H), 3.68 (d, *J* = 11.0 Hz, 1H), 3.71 (dd, *J* = 3.5, 5.5 Hz, 1H), 3.92 (d, *J* = 11.0 Hz, 1H), 4.02 (d, *J* = 11.0 Hz, 1H), 4.11 (d, *J* = 11.0 Hz, 1H), 4.27 (d, *J* = 5.5 Hz, 1H), 4.45 (d, *J* = 12.0 Hz, 1H), 4.50 (d, *J* = 12.0 Hz, 1H), 4.58 (d, *J* = 12.0 Hz, 1H), 4.66 (d, *J* = 12.0 Hz, 1H), 4.69 (s, 2H), 5.46 (d, *J* = 9.5 Hz, 1H), 5.50 (d, *J* = 9.5 Hz, 1H), 5.82 (d, *J* = 3.5 Hz, 1H), 7.20–7.71 (m, 26H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.52, 19.24, 26.88, 58.61, 64.47, 70.36, 70.67, 72.06, 73.97, 73.53, 75.55, 83.23, 87.75, 87.88, 109.66, 127.53, 127.59, 127.61, 127.64, 127.70, 127.87, 127.98, 128.20, 128.34, 128.47, 128.53, 129.56, 129.69, 132.94, 133.40, 134.73, 135.56, 135.80, 137.40, 137.51, 138.00, 150.72, 163.46. MS (FAB): *m*/*z* = 841 $[M + H]^+$. HRMS (FAB): calcd for $C_{50}H_{57}N_2O_8Si$ $[M + H]^+$, 841.3884, found, 841.3882.

3-*N*-Benzyloxymethyl-3′,5′-di-*O*-benzyl-4′-hydroxymethyl-2′-*O*-methyl-5-methyluridine (14). TBAF (1 M in THF, 1.8 mL, 1.8 mmol) were added to a solution of compound **13** (1.40 g, 1.66 mmol) in THF (20 mL) at room temperature. The reaction mixture was stirred at room temperature for 11 h. The reaction was concentrated *in vacuo* and the residue (2.54 g) was purified by column chromatography (silica gel, 50 g, *n*-hexane–EtOAc = 3 : 1 to 1 : 1) to give compound **14** (1.07 g, quant.) as a colorless oil.

$[\alpha]_D^{19}$ +78.3 (*c* 1.10, $CHCl_3$). IR: ν_{max} (KBr): 3499, 3064, 3030, 2941, 2867, 1708, 1668, 1496, 1455, 1362, 1274, 1209 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.48 (d, *J* = 1.0 Hz, 3H), 2.75 (t, *J* = 7.0 Hz, 1H), 3.59 (s, 3H), 3.68 (d, *J* = 10.5 Hz, 1H), 3.75–3.87 (m, 4H), 4.40 (d, *J* = 6.0 Hz, 1H), 4.47 (d, *J* = 11.0 Hz, 1H), 4.48

(d, J = 11.5 Hz, 1H), 4.53 (d, J = 11.5 Hz, 1H), 4.68 (s, 2H), 4.75 (d, J = 11.0 Hz, 1H), 5.44 (d, J = 10.0 Hz, 1H), 5.47 (d, J = 10.0 Hz, 1H), 6.08 (d, J = 2.5 Hz, 1H), 7.19–7.38 (m, 15H), 7.63 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.48, 59.14, 63.59, 70.30, 70.33, 72.04, 73.00, 73.48, 75.86, 83.28, 87.21, 89.28, 109.82, 127.50, 127.56, 127.60, 127.66, 127.99, 128.15, 128.48, 128.51, 134.52, 137.08, 137.14, 137.86, 150.59, 163.30. MS (EI): m/z = 602 (M^+, 14.0), 511 (16.3), 496 (96.4), 249 (10.2), 235 (20.5), 181 (34.0), 140 (23.5), 111 (24.1), 99 (37.4), 91 (100). HRMS (EI): calcd for $C_{34}H_{38}N_2O_8$ [M^+], 602.2628, found, 602.2623.

3-*N*-Benzyloxymethyl-3′,5′-di-*O*-benzyl-4′-methoxycarbonyl-2′-*O*-methyl-5-methyluridine (15). $PhI(OAc)_2$ (1.23 g, 3.80 mmol) and TEMPO (53.9 mg, 0.345 mmol) were added to a solution of compound **14** (1.04 g, 1.73 mmol) in MeCN–H_2O (1 : 1, 20 mL) at room temperature. The reaction mixture was stirred for 12 h at room temperature. The resulting mixture was concentrated *in vacuo*, and the residue (1.88 g) was co-evaporated with anhydrous MeCN three times, and dissolved in anhydrous MeOH (10 mL). $TMSCHN_2$ (2M in *n*-hexane, 0.95 mL, 1.9 mmol) was added to this solution at room temperature. The reaction mixture was stirred for 0.5 h at room temperature. The resulting mixture was concentrated *in vacuo* and the residue (1.26 g) was purified by column chromatography (silica gel, 30 g, *n*-hexane–EtOAc = 3 : 1 to 2 : 1) to give compound **15** (566 mg, 52% for 2 steps from **14**) as a colorless oil.

$[\alpha]_D^{21}$ +9.7 (c 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3064, 3031, 2952, 2927, 2867, 1762, 1713, 1673, 1497, 1454, 1363, 1278, 1239, 1210 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.62 (d, J = 1.0 Hz, 3H), 3.40 (s, 3H), 3.69 (s, 3H), 3.80 (d, J = 10.5 Hz, 1H), 3.96 (dd, J = 5.5, 6.0 Hz, 1H), 4.05 (d, J = 10.5 Hz, 1H), 4.35 (d, J = 5.5 Hz, 1H), 4.57 (d, J = 11.5 Hz, 1H), 4.60 (d, J = 11.5 Hz, 1H), 4.62 (d, J = 11.5 Hz, 1H), 4.68 (s, 2H), 4.79 (d, J = 11.5 Hz, 1H), 5.46 (d, J = 9.5 Hz, 1H), 5.49 (d, J = 9.5 Hz, 1H), 6.40 (d, J = 6.0 Hz, 1H), 7.23–7.38 (m, 15H), 7.42 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.78, 52.52, 58.87, 70.47, 71.48, 72.03, 73.77, 74.22, 78.38, 82.81, 87.96, 88.78, 110.63, 127.50, 127.53, 127.56, 127.65, 127.67, 127.82, 128.18, 128.26, 128.54, 128.62, 134.44, 136.87, 137.30, 137.83, 151.01, 163.24, 169.33. MS (EI): m/z = 630 (M^+, 3.6), 539 (10.8), 524 (52.8), 247 (8.3), 91 (100). HRMS (EI): calcd for $C_{35}H_{38}N_2O_9$ [M^+], 630.2577, found, 630.2559.

4′-Methoxycarbonyl-2′-*O*-methyl-5-methyluridine (16). Under a nitrogen atmosphere, a solution of compound **15** (550 mg, 0.872 mmol) in MeOH (8.0 mL) and cyclohexene (4.4 mL, 44 mmol) were added to a suspension of 20% $Pd(OH)_2$ on carbon (306 mg, 0.436 mmol) in MeOH (2.0 mL) at room temperature. The reaction mixture was refluxed for 12 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue (350 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 15 : 1 to 7 : 1) to give compound **16** (295 mg, quant.) as a white foam.

Mp: 112–114 °C. $[\alpha]_D^{21}$ −16.4 (c 1.00, CH_3OH). IR: ν_{max} (KBr): 3506, 3285, 3050, 2955, 2833, 1702, 1471, 1378, 1277, 1218 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD) δ: 1.88 (d, J = 1.0 Hz, 3H), 3.39 (s, 3H), 3.75 (s, 3H), 3.81 (d, J = 12.0 Hz, 1H), 4.00 (d, J = 12.0 Hz, 1H), 4.05 (dd, J = 5.0, 7.5 Hz, 1H), 4.45 (d, J = 5.0 Hz, 1H), 6.20 (d, J = 7.5 Hz, 1H), 7.75 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ: 12.45, 52.72, 58.50, 65.14, 72.05, 83.28, 88.44, 92.48, 112.13, 138.22, 152.41, 166.17, 171.68. MS (FAB): m/z = 331 [M + H]$^+$. HRMS (FAB): calcd for $C_{13}H_{19}N_2O_8$ [M + H]$^+$, 331.1141, found, 331.1149.

5′-*O*-(4,4′-Dimethoxytrityl)-4′-methoxycarbonyl-2′-*O*-methyl-5-methyluridine (17). Under a nitrogen atmosphere, DMTrCl (380 mg, 1.12 mmol) was added to a solution of compound **16** (285 mg, 0.863 mmol) in anhydrous pyridine (5.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (710 mg) was purified by column chromatography (silica gel, 15 g, $CHCl_3$–MeOH = 30 : 1) to give compound **17** (464 mg, 85%) as a white foam.

Mp: 81–91 °C. $[\alpha]_D^{22}$ +4.8 (c 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3469, 3224, 3065, 2953, 2936, 2837, 1697, 1607, 1580, 1509, 1464, 1387, 1252 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.40 (d, J = 1.0 Hz, 3H), 3.10 (d, J = 5.0 Hz, 1H), 3.54 (s, 3H), 3.56 (d, J = 12.0 Hz, 1H), 3.73 (d, J = 12.0 Hz, 1H), 3.74 (s, 3H), 3.78 (s, 6H), 4.05 (t, J = 5.0 Hz, 1H), 4.60 (t, J = 5.0 Hz, 1H), 6.26 (d, J = 5.0 Hz, 1H), 6.83–7.40 (m, 13H), 7.48 (d, J = 1.0 Hz, 1H), 9.96 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.61, 52.52, 55.11, 58.67, 64.76, 71.39, 82.33, 87.11, 87.20, 88.47, 111.44, 113.20, 123.67, 127.12, 127.92, 128.00, 130.02, 134.62, 134.83, 135.19, 136.07, 143.85, 149.41, 150.37, 158.64, 163.96, 169.91. MS (FAB): m/z = 655 [M + Na]$^+$. HRMS (FAB): calcd for $C_{34}H_{36}N_2NaO_{10}$ [M + Na]$^+$, 655.2268, found, 655.2286.

3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonyl-2′-*O*-methyl-5-methyluridine (2). Under a nitrogen atmosphere, DIPEA (0.63 mL, 3.5 mmol) and i-$Pr_2NP(Cl)OCH_2CH_2CN$ (0.18 mL, 0.84 mmol) were added to a solution of compound **17** (444 mg, 0.702 mmol) in anhydrous CH_2Cl_2 (5.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (639 mg) was chromatographed (silica gel, 10 g, *n*-hexane–EtOAc = 1 : 1) to give **2** with a small amount of impurity (511 mg), which was reprecipitated from *n*-hexane–$CHCl_3$ to give compound **2** (450 mg, 77%) as a white powder.

Mp: 86–88 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.04–1.19 (m, 12H), 1.37 (d, J = 1.0 Hz, 2.1H), 1.38 (d, J = 1.0 Hz, 0.9H), 2.42–2.47 (m, 1.4H) 2.61–2.65 (m, 0.6H), 3.51–3.80 (m, 18H), 4.04–4.13 (m, 1H), 4.65–4.72 (m, 1H), 6.29 (d, J = 5.5 Hz, 0.7H), 6.32 (d, J = 6.0 Hz, 0.3H), 6.81–7.41 (m, 13H), 7.45 (d, J = 1.0 Hz, 0.3H), 7.51 (d, J = 1.0 Hz, 0.7H), 8.15–8.21 (m, 1H). ^{31}P NMR (161 MHz, $CDCl_3$) δ: 151.02, 151.56. MS (FAB): m/z = 833 [M + H]$^+$. HRMS (FAB): calcd for $C_{43}H_{54}N_4O_{11}P$ [M + H]$^+$, 833.3527, found, 833.3551.

(2′*R*)-2′-Azido-3′,5′-di-*O*-benzyl-4′-methoxycarbonylthymidine (19). Under a nitrogen atmosphere, $TMSCHN_2$ (2.0 M in *n*-hexane, 0.13 mL, 0.26 mmol) was added to a solution of compound

18[9] (107 mg, 0.211 mmol) in anhydrous THF–MeOH (1 : 1, 2.0 mL) at room temperature. The reaction mixture was stirred at ambient temperature for 0.5 h. The resulting mixture was concentrated *in vacuo* and the residue (113 mg) was purified by column chromatography (silica gel, 5 g, *n*-hexane–EtOAc = 2 : 1) to give compound **19** (97.5 mg, 89%) as a white foam.

Mp: 159–161 °C. $[\alpha]_D^{31}$ −50.9 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3179, 3066, 3031, 2954, 2927, 2878, 2110, 1755, 1691, 1664, 1455, 1376, 1274 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.67 (d, J = 1.0 Hz, 3H), 3.67 (s, 3H), 3.77 (dd, J = 6.0, 8.5 Hz, 1H), 3.79 (d, J = 10.0 Hz, 1H), 4.06 (d, J = 10.0 Hz, 1H), 4.41 (d, J = 6.0 Hz, 1H), 4.57 (d, J = 11.5 Hz, 1H), 4.62 (d, J = 11.5 Hz, 1H), 4.62 (d, J = 11.0 Hz, 1H), 4.81 (d, J = 11.0 Hz, 1H), 6.49 (d, J = 8.5 Hz, 1H), 7.28–7.39 (m, 11H), 8.31 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.29, 52.78, 63.82, 71.81, 74.04, 75.40, 81.50, 86.14, 89.81, 111.96, 127.75, 127.76, 128.22, 128.24, 128.27, 128.46, 128.48, 128.82, 128.84, 134.93, 136.45, 136.69, 150.39, 163.59, 168.95. MS (FAB): m/z = 522 $[M + H]^+$. HRMS (FAB): calcd for $C_{26}H_{28}N_5O_7$ $[M + H]^+$, 522.1998, found, 522.1973.

(2′*R*)-2′-Amino-3′,5′-di-*O*-benzyl-4′-methoxycarbonylthymidine (20). Under a nitrogen atmosphere, $NiCl_2$ (9.2 mg, 0.0709 mmol) and $NaBH_4$ (80.5 mg, 2.13 mmol) were added to a solution of compound **19** (370 mg, 0.709 mmol) in anhydrous THF–MeOH (1 : 1, 2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 0.5 h. The resulting mixture was filtrated and the filtrate was concentrated *in vacuo*. The residue (550 mg) was purified by column chromatography (silica gel, 20 g, $CHCl_3$–MeOH = 40 : 1) to give compound **20** (313 mg, 89%) as a white powder.

Mp: 75–78 °C. $[\alpha]_D^{28}$ −27.0 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3372, 3178, 3066, 3031, 2948, 2923, 2867, 1759, 1703, 1496, 1472, 1454, 1394, 1364, 1278 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ: 1.60 (d, J = 1.0 Hz, 3H), 3.58 (dd, J = 6.0, 8.5 Hz, 1H), 3.69 (s, 3H), 3.80 (d, J = 10.0 Hz, 1H), 4.09 (d, J = 10.0 Hz, 1H), 4.21 (d, J = 6.0 Hz, 1H), 4.58 (d, J = 11.5 Hz, 1H), 4.63 (d, J = 11.0 Hz, 1H), 4.66 (d, J = 11.5 Hz, 1H), 4.68 (d, J = 11.0 Hz, 1H), 6.14 (d, J = 8.5 Hz, 1H), 7.26–7.41 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.21, 52.63, 58.75, 72.46, 73.96, 75.56, 82.39, 89.12, 89.83, 111.31, 127.76, 128.08, 128.23, 128.27, 128.53, 128.59, 128.81, 130.79, 135.58, 137.01, 137.03, 151.00, 163.80, 169.85. MS (FAB): m/z = 496 $[M + H]^+$. HRMS (FAB): calcd for $C_{26}H_{30}N_3O_7$ $[M + H]^+$, 496.2086, found, 496.2070.

(2′*R*)-3′,5′-Di-*O*-benzyl-4′-methoxycarbonyl-2′-trifluoroacetamidothymidine (21). Under a nitrogen atmosphere, Et_3N (12 μL, 0.085 mmol) and CF_3CO_2Et (24 μL, 0.19 mmol) were added to a solution of compound **20** (84.4 mg, 0.170 mmol) in anhydrous MeOH (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 h. The resulting mixture was concentrated *in vacuo* and the residue (134 mg) was purified by column chromatography (silica gel, 5 g, *n*-hexane–EtOAc = 1 : 1) to give compound **21** (92.3 mg, 92%) as a white foam.

Mp: 75–77 °C. $[\alpha]_D^{22}$ −19.8 (*c* 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3235, 3070, 2928, 2863, 1709, 1556, 1469, 1386, 1276 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.89 (d, J = 0.5 Hz, 3H), 3.71 (s, 3H), 3.83 (d, J = 10.0 Hz, 1H), 4.03 (d, J = 10.0 Hz, 1H), 4.48 (d, J = 6.5 Hz, 1H), 4.51 (d, J = 11.0 Hz, 1H), 4.57 (d, J = 11.0 Hz, 1H), 4.59 (d, J = 11.5 Hz, 1H), 4.64 (d, J = 11.5 Hz, 1H), 4.71 (dt, J = 6.5, 8.0 Hz, 1H), 6.43 (d, J = 8.0 Hz, 1H), 7.25–7.41 (m, 11H), 7.68 (d, J = 8.0 Hz, 1H), 9.44 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.16, 52.87, 55.66, 71.21, 73.96, 75.66, 79.59, 87.18, 90.06, 112.26, 115.50 (q, J = 286 Hz), 127.82, 127.86, 128.35, 128.40, 128.58, 128.69, 128.84, 128.95, 134.99, 136.19, 136.75, 150.84, 157.75 (q, J = 38 Hz), 163.63, 169.38. MS (FAB): m/z = 592 $[M + H]^+$. HRMS (FAB): calcd for $C_{28}H_{29}F_3N_3O_8$ $[M + H]^+$, 592.1908, found, 592.1886.

(2′*R*)-4′-Methoxycarbonyl-2′-trifluoroacetamidothymidine (23). Under a nitrogen atmosphere, a solution of compound **21** (95.5 mg, 0.162 mmol) in MeOH (4.0 mL) and cyclohexene (0.80 mL, 8.0 mmol) were added to a suspension of 20% Pd-$(OH)_2$ on carbon (57.0 mg, 0.0810 mmol) in MeOH (1.0 mL) at room temperature. The reaction mixture was refluxed for 10 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue (55.6 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 15 : 1) to give compound **23** (40.8 mg, 62%) as a white foam.

Mp: 108–110 °C. $[\alpha]_D^{22}$ −5.7 (*c* 1.00, MeOH). IR: ν_{max} (KBr): 3481, 3261, 3068, 2957, 2841, 1710, 1552, 1471, 1388, 1276 cm^{-1}. ^1H NMR (400 MHz, CD_3OD) δ: 1.90 (d, J = 1.0 Hz, 3H), 3.77 (s, 3H), 3.84 (d, J = 12.0 Hz, 1H), 4.08 (d, J = 12.0 Hz, 1H), 4.45 (d, J = 6.0 Hz, 1H), 4.75 (dd, J = 6.0, 8.5 Hz, 1H), 6.32 (d, J = 8.5 Hz, 1H), 7.81 (d, J = 1.0 Hz, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ: 12.48, 52.78, 56.83, 65.09, 72.89, 87.99, 93.60, 112.52, 117.20 (q, J = 285 Hz), 137.77, 152.68, 159.21 (q, J = 38 Hz), 166.17, 171.46. MS (FAB): m/z = 412 $[M + H]^+$. HRMS (FAB): calcd for $C_{14}H_{17}F_3N_3O_8$ $[M + H]^+$, 412.0969, found, 412.0966.

(2′*R*)-5′-*O*-(4,4′-Dimethoxytrityl)-4′-methoxycarbonyl-2′-trifluoroacetamidothymidine (25). Under a nitrogen atmosphere, DMTrCl (80.0 mg, 0.238 mmol) was added to a solution of compound **23** (61.1 mg, 0.149 mmol) in anhydrous pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (152 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 50 : 1) to give compound **25** (95.7 mg, 90%) as a white foam.

Mp: 127–130 °C. $[\alpha]_D^{22}$ −3.4 (*c* 0.66, $CHCl_3$). IR: ν_{max} (KBr): 3414, 3271, 3066, 2955, 2839, 1703, 1608, 1509, 1466, 1386, 1293, 1255 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 1.40 (d, J = 1.0 Hz, 1H), 3.08 (d, J = 4.0 Hz, 1H), 3.58 (d, J = 10.0 Hz, 1H), 3.69, (d, J = 10.0 Hz, 1H), 3.78 (s, 3H), 3.80 (s, 6H), 4.64 (dd, J = 4.0, 5.0 Hz, 1H), 4.39 (dt, J = 5.0, 8.5 Hz, 1H), 6.39 (d, J = 8.5 Hz, 1H), 6.86 (d, J = 8.5 Hz, 1H), 6.85–7.38 (m, 13H), 7.53 (d, J = 1.0 Hz, 1H), 8.06 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.49, 52.97, 55.30, 55.72, 65.43, 72.78, 85.91, 88.01, 91.04, 112.44, 113.49, 115.57 (q, J = 286 Hz), 127.50, 128.27, 130.28, 134.49, 134.69, 135.63, 135.65, 143.58, 151.36, 157.38, 157.95 (q, J = 38 Hz), 158.94, 158.97, 164.39, 169.85. MS (FAB): m/z = 714 $[M + H]^+$, HRMS (FAB): calcd for $C_{35}H_{35}F_3N_3O_{10}$ $[M + H]^+$, 714.2276, found, 714.2247.

(2′*R*)-3′-*O*-[2-Cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonyl-2′-trifluoroacetamidothymidine (3). Under a nitrogen atmosphere, DIPEA (0.11 mL, 0.62 mmol) and i-$Pr_2NP(Cl)OCH_2CH_2CN$ (30 μL, 0.14 mmol) were added to a solution of compound **25** (88.7 mg, 0.124 mmol) in anhydrous CH_2Cl_2 (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (122 mg) was chromatographed (silica gel, 10 g, $CHCl_3$–MeOH = 50 : 1) to give **6** with a small amount of impurity (119 mg), which was reprecipitated from *n*-hexane–$CHCl_3$ to give compound **3** (102 mg, 95%) as a white powder.

Mp: 85–88 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.07–1.16 (m, 12H), 1.40 (d, J = 1.0 Hz, 1.5H), 1.43 (d, J = 1.0 Hz, 1.5H), 2.36–2.80 (m, 2H), 3.45–3.80 (m, 15H), 4.86–5.08 (m, 2H), 6.35 (d, J = 6.0 Hz, 0.5 H), 6.40 (d, J = 6.0 Hz, 0.5H), 6.86–7.52 (m, 15H), 7.95–7.99 (m, 1H). ^{31}P NMR (160 MHz, $CDCl_3$) δ: 151.96, 153.04. MS (FAB): m/z = 914 $[M + H]^+$. HRMS (FAB): calcd for $C_{44}H_{52}F_3N_5O_{11}P$ $[M + H]^+$, 914.3355, found, 914.3348.

(2′*R*)-2′-Acetamido-3′,5′-di-*O*-benzyl-4′-methoxycarbonylthymidine (22). Under a nitrogen atmosphere, Ac_2O (80 μL, 0.85 mmol) was added to a solution of compound **20** (352 mg, 0.710 mmol) in anhydrous pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1.5 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (438 mg) was purified by column chromatography (silica gel, 15 g, $CHCl_3$–MeOH = 30 : 1) to give compound **22** (408 mg, quant.) as a white foam.

Mp: 85–88 °C. $[\alpha]_D^{24}$ −31.4 (c 1.10, $CHCl_3$). IR: ν_{max} (KBr): 3317, 3204, 3067, 3033, 2952, 2927, 2867, 1762, 1683, 1543, 1455, 1374, 1283 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.56 (d, J = 1.0 Hz, 3H), 1.84 (s, 3H), 3.67 (s, 3H), 3.81 (d, J = 10.0 Hz, 1H), 4.03 (d, J = 10.0 Hz, 1H), 4.39 (d, J = 6.0 Hz, 1H), 4.53 (d, J = 11.5 Hz, 1H), 4.57 (d, J = 11.5 Hz, 1H), 4.60 (d, J = 12.0 Hz, 1H), 4.68 (d, J = 12.0 Hz, 1H), 4.77 (dt, J = 6.0, 8.5 Hz), 6.41 (d, J = 8.5 Hz, 1H), 6.98 (d, J = 8.5 Hz), 7.26–7.39 (m, 10H), 7.51 (d, J = 1.0 Hz, 1H), 9.84 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.16, 22.76, 52.62, 55.45, 71.72, 73.85, 75.48, 80.68, 86.61, 90.07, 111.83, 127.75, 128.11, 128.14, 128.18, 128.21, 128.45, 128.50, 128.75, 135.46, 136.89, 137.00, 151.15, 163.83, 169.42, 171.02. MS (FAB): m/z = 538 $[M + H]^+$. HRMS (FAB): calcd for $C_{28}H_{32}N_3O_8$ $[M + H]^+$, 538.2191, found, 538.2194.

(2′*R*)-2′-Acetamido-4′-methoxycarbonylthymidine (24). Under a nitrogen atmosphere, a solution of compound **22** (380 mg, 0.707 mmol) in THF–MeOH (1 : 1, 8.0 mL) and cyclohexene (3.6 mL, 35 mmol) were added to a suspension of 20% Pd-$(OH)_2$ on carbon (199 mg, 0.283 mmol) in THF–MeOH (1 : 1, 2.0 mL) at room temperature. The reaction mixture was refluxed for 10 h. The resulting mixture was filtered and the filtrate was concentrated *in vacuo*. The residue (330 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 7 : 1 → 4 : 1) to give compound **24** (181 mg, 72%) as a white powder.

Mp: 162–163 °C. $[\alpha]_D^{27}$ −15.2 (c 0.85, MeOH). IR: ν_{max} (KBr): 3483, 3288, 3066, 2961, 2833, 1682, 1530, 1471, 1439, 1377, 1291 cm^{-1}. ^{1}H NMR (300 MHz, CD_3OD) δ: 1.89 (s, 3H), 1.94 (s, 3H), 3.75 (s, 3H), 3.81 (d, J = 11.5 Hz, 1H), 4.05 (d, J = 11.5 Hz, 1H), 4.34 (d, J = 6.0 Hz, 1H), 4.70 (dd, J = 6.0, 9.0 Hz, 1H), 6.20 (d, J = 9.0 Hz, 1H), 7.81 (s, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ: 12.48, 22.37, 52.68, 56.35, 65.30, 73.23, 88.12, 93.46, 112.11, 138.04, 152.82, 166.22, 171.67, 173.82. MS (FAB): m/z = 358 $[M + H]^+$. HRMS (FAB): calcd for $C_{14}H_{20}N_3O_8$ $[M + H]^+$, 358.1252, found, 358.1252.

(2′*R*)-2′-Acetamido-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonylthymidine (26). Under a nitrogen atmosphere, DMTrCl (186 mg, 0.550 mmol) was added to a solution of compound **24** (65.5 mg, 0.183 mmol) in anhydrous pyridine (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with EtOAc. The combined organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (315 mg) was purified by column chromatography (silica gel, 10 g, $CHCl_3$–MeOH = 20 : 1) to give compound **26** (123 mg, quant.) as a white foam.

Mp: 142–144 °C. $[\alpha]_D^{27}$ −19.5 (c 1.00, $CHCl_3$). IR: ν_{max} (KBr): 3489, 3323, 3188, 3063, 3017, 2953, 2835, 1694, 1607, 1579, 1509, 1465, 1378, 1291 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.34 (d, J = 1.0 Hz, 3H), 1.99 (s, 3H), 3.51 (d, J = 10.0 Hz, 1H), 3.69 (d, J = 10.0 Hz, 1H), 3.64 (d, J = 4.5 Hz, 1H), 3.73 (s, 3H), 3.78 (s, 6H), 4.57 (dd, J = 4.5, 5.5 Hz, 1H), 5.02 (dt, J = 5.5, 8.5 Hz, 1H), 6.36 (d, J = 8.5 Hz, 1H), 6.64 (d, J = 8.5 Hz, 1H), 6.84–7.42 (m, 13H), 7.55 (d, J = 1.0 Hz, 1H), 9.16 (brs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.56, 23.00, 52.84, 54.95, 55.26, 65.56, 72.71, 85.99, 87.74, 90.75, 112.05, 113.40, 127.33, 128.14, 128.25, 130.23, 130.25, 134.55, 134.77, 135.29, 143.63, 151.26, 158.85, 163.63, 170.21, 171.11. MS (FAB): m/z = 660 $[M + H]^+$. HRMS (FAB): calcd for $C_{35}H_{38}N_3O_{10}$ $[M + H]^+$, 660.2559, found, 660.2538.

(2′*R*)-2′-Acetamido-3′-*O*-[2-cyanoethoxy(diisopropylamino)phosphino]-5′-*O*-(4,4′-dimethoxytrityl)-4′-methoxycarbonylthymidine (4). Under a nitrogen atmosphere, DIPEA (0.15 mL, 0.86 mmol) and i-$Pr_2NP(Cl)OCH_2CH_2CN$ (45 μL, 0.21 mmol) were added to a solution of compound **26** (113 mg, 0.127 mmol) in anhydrous CH_2Cl_2 (2.0 mL) at 0 °C. The reaction mixture was stirred at room temperature for 4 h. After addition of sat. $NaHCO_3$ at 0 °C, the mixture was extracted with CH_2Cl_2. The combined organic layer was washed with sat. $NaHCO_3$, water and brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue (210 mg) was chromatographed (silica gel, 10 g, $CHCl_3$–MeOH = 20 : 1) to give **4** with a small amount of impurity (113 mg), which was reprecipitated from *n*-hexane–$CHCl_3$ to give compound **4** (103 mg, 68%) as a white powder.

Mp: 92–95 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.08–1.18 (m, 12H), 1.35 (s, 1.8H), 1.37 (s, 1.2H), 2.00 (s, 1.8H), 2.03 (s, 1.2H), 2.40–2.64 (m, 2H), 3.51–3.80 (m, 15H), 4.68–4.76 (m, 1H), 4.99–5.07 (m, 1H), 6.23–6.41 (m, 2H), 6.83–7.43 (m, 13H), 7.53 (m, 1H), 8.15–8.21 (m, 1H). ^{31}P NMR (160 MHz,

$CDCl_3$) δ: 152.39, 152.99. MS (FAB): m/z = 860 $[M + H]^+$. HRMS (FAB): calcd for $C_{44}H_{55}N_5O_{11}P$ $[M + H]^+$, 860.3637, found, 860.3608.

Synthesis of oligonucleotides

Phosphoramidites **1–4** were used and the 0.2 μmol scale synthesis of oligonucleotides was performed on an automated DNA synthesizer (Applied Biosystems Expedite™ 8909) using a standard phosphoramidite protocol (DMTr-ON mode). **ON1–6** were prepared by cleavage from the CPG supports, deprotection of nucleobase and phosphate moieties, and conversion of 4′-carboxyl units by treatment with the corresponding base [50 mM NaOH aq., rt, 1.5 h then 55 °C, 12 h (for **ON1a–6a**); 50 mM K_2CO_3 in MeOH, rt, 2 h (for **ON1b–4b**); 28% NH_3 aq., rt, 1.5 h then 55 °C, 12 h (for **ON1c–4c**); or 40% MeNH aq., rt, 1.5 h then 55 °C, 12 h (for **ON1d–4d**)]. After neutralization with 1% HCl aq. for **ON1a–6a** and **ON1b–4b**, the solvent was concentrated *in vacuo*. For **ON1a–6a** and **ON1b–4b**, removal of ammonia or methylamine was carried out *in vacuo*. The crude **ON1–6** were purified with Sep-Pak® Plus C18 cartridges (Waters) followed by reversed-phase HPLC (Waters XBridge® MS C_{18} 2.5 μm, 10 × 50 mm). The composition of the **ON1–6** was confirmed by MALDI-TOF mass analysis. Yields and MALDI-TOF-MS data ($[M - H]^-$) for **ON1–6**; **ON1a**, 43% yield, found 3676.23 (calcd 3676.37); **ON1b**, 30% yield, found 3690.24 (calcd 3690.40); **ON1c**, 30% yield, found 3674.55 (calcd 3675.39); **ON1d**, 28% yield, found 3689.01 (calcd 3689.51); **ON2a**, 38% yield, found 3706.23 (calcd 3706.40); **ON2b**, 33% yield, found 3720.98 (calcd 3720.42); **ON2c**, 32% yield, found 3705.91 (calcd 3705.41); **ON2d**, 29% yield, found 3719.61 (calcd 3719.44); **ON3a**, 43% yield, found 3690.69 (calcd 3691.39); **ON3b**, 51% yield, found 3706.06 (calcd 3705.41); **ON3c**, 40% yield, found 3689.73 (calcd 3690.40); **ON3d**, 61% yield, found 3704.78 (calcd 3704.43); **ON4a**, 36% yield, found 3732.43 (calcd 3733.42); **ON4b**, 50% yield, found 3746.94 (calcd 3747.45); **ON4c**, 52% yield, found 3731.93 (calcd 3732.44); **ON4d**, 28% yield, found 3746.03 (calcd 3746.46); **ON5a**, 29% yield, found 3765.21 (calcd 3764.39); **ON6a**, 49% yield, found 3764.75 (calcd 3764.39).

UV melting experiments

UV melting experiments were carried out on SHIMADZU UV-1650 and SHIMADZU UV-1800 spectrophotometers equipped with T_m analysis accessory. **ON1–6** and ssDNA or ssRNA were dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 100 mM NaCl to give a final concentration of each strand of 4 μM. The samples were annealed by heating at 100 °C followed by slow cooling to 15 °C. The melting profiles were recorded at 260 nm from 15 °C to 85 °C at a scan rate of 0.5 °C min^{-1}. The two-point average method was employed to obtain the T_m values and the final ones were determined by averaging three independent measurements which were accurate to within 1 °C.

CD measurements

Under the same conditions as those of UV melting experiments, CD measurements were carried out from 350 nm to 200 nm at 4 °C on a JASCO J-720W spectropolarimeter.

Conclusions

The synthesis of oligonucleotides including 16 kinds of 4′-carboxythymidines was achieved by base treatment after oligonucleotide synthesis. Evaluation of their duplex-forming ability with DNA or RNA complements found that 4′-carboxythymidine led to an increase in stability of the duplex formed with RNA. In the recent report by Leumann's group, tc-DNA with a hexadodecyloxycarbonyl unit enhanced cellular uptake.[12] Therefore, the carboxyl group of 4′-carboxythymidine that we synthesized in this work might be practically useful as a prodrug functional group. Thus, 4′-carboxythymidine was considered to be a potent candidate for targeting RNA.

Acknowledgements

This work was supported in part by the Program to Disseminate Tenure Tracking System of the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT), a Grant-in-Aid for Scientific Research on Innovative Areas (no. 22136006) from MEXT, and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

Notes and references

1 Reviews: T. P. Prakash, *Chem. Biodiversity*, 2011, **8**, 1616; T. Yamamoto, M. Nakatani, K. Narukawa and S. Obika, *Future Med. Chem.*, 2011, **83**, 339.
2 Reviews: J. K. Watts, G. F. Deleavey and M. J. Damha, *Drug Discovery Today*, 2008, **13**, 842; S. Shukla, C. S. Sumaria and P. I. Pradeepkumar, *ChemMedChem*, 2010, **5**, 328.
3 G. Wang and W. E. Seifert, *Tetrahedron Lett.*, 1996, **37**, 6515.
4 H. Thrane, J. Fensholdt, M. Renger and J. Wengel, *Tetrahedron*, 1995, **51**, 10389.
5 R. Liboska, J. Snášel, I. Barvík, M. Buděšínký, R. Pohl, Z. Točík, O. Páv, D. Rejman, P. Novák and I. Rosenberg, *Org. Biomol. Chem.*, 2011, **9**, 8261.
6 S. K. Singh, P. Nielsen, A. A. Koshkin and J. Wengel, *Chem. Commun.*, 1998, 455; A. A. Koshkin, S. K. Singh, P. Nielsen, V. K. Rajwanshi, R. Kumar, M. Meldgaard, C. E. Olsen and J. Wengel, *Tetrahedron*, 1998, **54**, 3607.
7 J. Gaster and A. Marx, *Chem.-Eur. J.*, 2005, **11**, 1861.
8 A. R. Shrestha, Y. Hari, A. Yahara, T. Osawa and S. Obika, *J. Org. Chem.*, 2011, **76**, 9891.

9 A. Yahara, A. R. Shrestha, T. Yamamoto, Y. Hari, T. Osawa, M. Yamaguchi, M. Nishida, T. Kodama and S. Obika, *ChemBioChem*, DOI: 10.1002/cbic.201200506.
10 C. Altona and M. Sundaralingam, *J. Am. Chem. Soc.*, 1973, **95**, 2333.
11 H. Aurup, T. Tuschl, F. Benseler, J. Ludwig and F. Eckstein, *Nucleic Acids Res.*, 1994, **22**, 20.
12 J. Lietard and C. J. Leumann, *J. Org. Chem.*, 2012, **77**, 4566.
13 H. Hřebabecký and A. Holý, *Collect. Czech. Chem. Commun.*, 1997, **62**, 1128.

Journal of Controlled Release 163 (2012) 119–124

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Efficient reduction of serum cholesterol by combining a liver-targeted gene delivery system with chemically modified apolipoprotein B siRNA

Jeong-Hun Kang [a], Yoichi Tachibana [a], Satoshi Obika [b], Mariko Harada-Shiba [c], Tetsuji Yamaoka [a,*]

[a] Department of Biomedical Engineering, Advanced Medical Engineering Center, National Cerebral and Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan
[b] Division of Bioorganic Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Department of Molecular Innovation in Lipidology, National Cerebral and Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan

ARTICLE INFO

Article history:
Received 18 March 2012
Accepted 29 August 2012
Available online 4 September 2012

Keywords:
Apo B
Pullulan
Liver-targeted delivery
Cholesterol

ABSTRACT

Apolipoprotein B (Apo B) is a key amphipathic glycoprotein compound in the metabolism of plasma lipoproteins (mainly very low-density lipoprotein (VLDL) and LDL). Inhibition of Apo B synthesis by short interfering RNA (siRNA) targeting Apo B (Apo B siRNA) is very efficient for serum LDL reduction. In the present study, the chemically modified Apo B siRNA (Apo B–siBNA) with the increased enzymatic stability was selected. We developed a cationic conjugate for efficient delivery of Apo B–siBNA into the liver by introducing pullulan with different molecular weights (MWs) (5900 and 107,000) into polyethylenimine (PEI). Introduction of pullulan into PEI dramatically decreased mortality and lung damage after systemic injection of the conjugate/Apo B–siBNA complexes into mice. The PEI–pullulan carrier prepared with high MW pullulan (107,000) was more stable in the blood stream and showed higher fluorescence levels in the liver for a longer time than the carrier prepared with low MW pullulan (5900). Moreover, efficient reduction of serum LDL and Apo B mRNA in the liver was observed in mice injected with PEI–pullulan (MW, 107,000)/Apo B–siBNA, whereas there was no or little change in serum LDL and Apo B mRNA in livers of mice treated with Apo B–siBNA alone, PEI/Apo B–siBNA, and PEI–pullulan (MW, 5900)/Apo B–siBNA. These results suggest that combining a liver-targeted gene delivery system with chemically modified Apo B siRNA efficiently reduces the level of serum LDL and Apo B mRNA in the liver.

1. Introduction

Liver-targeted gene delivery systems are very useful to treat liver (hepatic) diseases such as hepatocellular carcinoma, hepatitis B and C, and liver-based metabolic diseases. Many viral and non-viral vectors (carriers) for liver-targeted gene delivery have been developed for either *in vivo* or *ex vivo/in vitro* use. Cationic polymers containing liver-specific molecular ligands (e.g., galactose), which are recognized by membrane receptors [1–3], or lipid-based formulations [4,5] have been studied as non-viral vectors for liver-targeted gene delivery. Viral-based vectors for liver-targeted gene delivery are recognized mainly by hepatocyte membrane receptors [6–8]. However, it is necessary to solve the problem of immune response for viral vectors to be effective liver-targeted gene therapy. On the other hand, hydrodynamic transfection is also used to deliver naked genes to the liver by intravenous injection of a large volume of gene-containing solution at a high velocity. This is a simple, efficient, and liver-specific method for *in vivo* gene delivery [9–11].

Recently, our group developed a cationic conjugate for liver-targeted gene delivery by introducing pullulan into polyethylenimine (PEI). Pullulan is a water-soluble polysaccharide and is useful for liver targeting because of its high affinity for the hepatocyte asialoglycoprotein receptor [12,13]. Naked genes were unstable and were rapidly degraded, but PEI–pullulan conjugates formed stable complexes with genes and protected them from enzymatic degradation. Moreover, the introduction of pullulan into PEI showed low toxicity *in vivo* and an increase in liver-targeting efficiency [14].

Apolipoprotein B (Apo B) is a key amphipathic glycoprotein compound in the metabolism of plasma lipoproteins (mainly very low-density lipoprotein (VLDL) and LDL). Apo B present in plasma lipoproteins is divided into 2 types: Apo B-48 and Apo B-100. Apo B-48 is produced by the small intestine, and Apo B-100 is secreted by the liver; however, Apo B-100 is an essential component of plasma lipoproteins (VLDL and LDL) [15–17]. Elevated levels of Apo B and LDL increase the risk of atherosclerotic diseases such as coronary artery disease and heart disease [18,19]. Inhibition of Apo B synthesis by short interfering RNA (siRNA) targeting of the Apo B gene (Apo B siRNA) efficiently reduces serum LDL [20,21].

In the present study, we used chemically modified Apo B siRNA due to its increased enzymatic stability. A PEI–pullulan conjugate

* Corresponding author.
E-mail address: yamtet@ri.ncvc.go.jp (T. Yamaoka).

0168-3659/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jconrel.2012.08.030

was developed for efficient delivery of chemically modified Apo B siRNA into the liver. Two PEI–pullulan conjugates with different molecular weights (MWs; 5900 and 107,000) were synthesized. We investigated whether the MW of pullulan can affect gene transfection efficiency and therapeutic effectiveness. Combining a liver-targeted gene delivery system with chemically modified Apo B siRNA reduced both serum LDL and Apo B mRNA in the liver.

2. Materials and methods

2.1. Synthesis of PEI–pullulan carrier

A mixture of 48.6 mg pullulan with different MWs (5900 and 107,000; 0.3 unit mmol) and 24.3 mg of carbonyldiimidazole (CDI; 0.15 mmol) was stirred in 30 ml of anhydrous dimethylsulfoxide (DMSO) at room temperature. After 4 h, 13.2 mg of linear polyethyleneimine (PEI; MW, 22 kDa; 0.3 mmol) was added to the mixture and further stirred at room temperature in a nitrogen-rich atmosphere for 1 day. The mixture was dialyzed against water for 3 days in a Spectra Pore membrane bag with an MW molecular weight cut-off of 10,000 (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA), followed by lyophilization; this resulted in the production of a PEI–pullulan powder (Fig. S1).

Nitrogen content in the PEI–pullulan was determined by acid–base titration using automatic potentiometric titrator (877 Titrino plus; Metrohm Ltd., Herisau, Switzerland). Exactly 4.8 mg of PEI–pullulan was dissolved in 8 mL of 150 mM NaCl to a final concentration of 0.6 mg/mL and the pH of the PEI–pullulan solution was set to 12 with NaOH. The solution was subsequently titrated with 0.1 M HCl.

2.2. Fluorescein-labeled Apo B siBNA

The chemically modified gene *Apo B* siRNA (hereafter referred to as Apo B–siBNA) used in this study was synthesized by Gene Desing Inc. (Osaka, Japan). The sequence was as follows: 5′-GTcaTcacacugaa TacCaaudTdT-3′ and 3′-dTdTcaguagugugacuuaugguua-5′; uppercase letters (G, C, and T) indicate 2′-O,4′-C-methylene bridged nucleic acid or locked nucleic acid (2′,4′-BNA/LNA) (Fig. S2) [22–24]. Lowercase letters indicate siRNA sequences. Alexa Fluor 750 (Invitrogen, Tokyo, Japan) was used as an amine-reactive dye. The fluorescein-labeled Apo B–siBNA was dialyzed against water containing 0.1% diethylpyrocarbonate (DEPC) for 2 days in a dialysis membrane with an MW cutoff of 3500, followed by lyophilization.

2.3. Size and zeta-potential of complexes

PEI–pullulan and Apo B–siBNA complexes at several nitrogen/phosphorus (N/P) ratios (24, 48, 96, and 192) were prepared by incubating both the Apo B–siBNA and the polymer in ultrapure distilled water (Invitrogen) for 30 min. The final concentration of Apo B–siBNA was adjusted to 1 μg/mL with water (pH 7.3). The diameters of the complexes were determined using a Zetasizer (Malvern Instruments, Malvern, UK) with a helium/neon (He/Ne) laser at a detection angle of 173° at 25 °C.

2.4. Electrophoresis of the carrier/Apo B siBNA complex

For electrophoresis experiments, various concentrations of PEI–pullulan were mixed with the Apo B–siBNA in ultrapure distilled water at room temperature for 30 min and then analyzed by 19% polyacrylamide gel electrophoresis.

2.5. Biodistribution of carrier/Apo B siBNA complexes

All animal studies were performed in accordance with the Guidelines for Animal Experiments established by the Ministry of Health, Labour and Welfare of Japan, and by the National Cerebral and Cardiovascular Center Research Institute. Six-week-old male C57BL/6J mice (Japan SLC Inc., Shizuoka, Japan) weighing approximately 22 g were used in this study. The mice were maintained in a temperature-controlled room (22 °C) with a 12-h light–dark cycle and were provided a standard pellet diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water ad libitum. The mice were sacrificed at 3, 6, 12, or 24 h after systemic injection of complexes. Each tissue type (lung, heart, liver, spleen, and kidney) was excised. Images were obtained with the Maestro In Vivo Imaging System (Cambridge Research & Instrumentation, Woburn, MA, USA). The signal level for each tissue was indicated by total signal/area.

2.6. Serum cholesterol and Apo B mRNA in the liver after systemic injection of complexes

Male 6-week-old C57BL/6J mice were provided a standard pellet diet (control group) or a high-fat diet for 10 days before experiment, and it continued during the experimental period of time. PEI/Apo B–siBNA complexes (N/P = 3) or PEI–pullulan/Apo B–siBNA complexes (N/P = 192) were injected daily via the tail vein for 3 days (on day 11, 12, and 13; 25 μg Apo B–siBNA per mouse per day). Next day (on day 14), the mice were sacrificed, and blood samples were collected from the caudal vena cava. Serum samples were separated by high-performance liquid chromatography (HPLC) and measured cholesterol levels by enzymatic method.

For determining Apo B mRNA concentration in the liver, total RNA from tissue was extracted using the QuickGene RNA tissue kit S 2 (RT-S2; FUJIFILM Co., Tokyo, Japan) according to the manufacturer's recommendations. Complementary DNA (cDNA) was synthesized by reverse-transcription of total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) and was subjected to real-time PCR (StepOnePlus; Applied Biosystems, Foster, CA, USA).

Glutamyl oxaloacetic transaminase (GOP) and glutamyl pyruvic transaminase (GTP) were detected with a transaminase assay kit (Transaminase C II-test; Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's instructions.

3. Results

3.1. Complex diameters and zeta-potentials

The amount of pullulan (MW, 5900 and 107,000) in the polymer was estimated to be 40.8 and 40.1 mol%, respectively. Complexes of carrier and Apo B–siBNA were prepared at several N/P ratios (24, 48, 96, and 192) and were detected using a Zetasizer. Particle sizes decreased with increasing N/P ratios. At N/P ratios of 96 and 192, sizes of PEI–pullulan/Apo B–siBNA complexes were <260 nm and their zeta-potentials were almost neutral (Table S1). On the other hand, sizes of PEI/Apo B–siBNA complexes were <200 nm for N/P ratios of 1.5 to 48 (data not shown) [14].

3.2. Electrophoresis of carrier/Apo B–siBNA complexes

PEI–pullulan carriers were mixed with Apo B–siBNA at several N/P ratios. The complexes were analyzed by electrophoresis. Bands corresponding to free Apo B–siBNA in the PEI/Apo B–siBNA complexes were not observed when the carrier was present at N/P ratios of >3. In contrast, Apo B–siBNA migration was suppressed in PEI–pullulan/Apo B–siBNA complexes with NP ratios of >96 (Fig. 1).

3.3. In vivo toxicity of carrier/Apo B–siBNA complexes

PEI/Apo B–siBNA and PEI–pullulan/Apo B–siBNA complexes were injected into mice. PEI/Apo B–siBNA complexes with an N/P ratio of

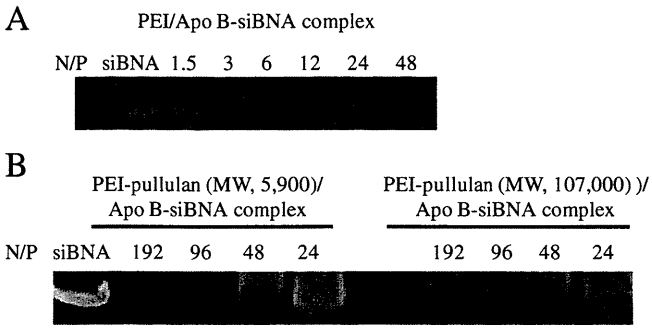


Fig. 1. Gel electrophoresis experiment. Electrophoresis of (A) PEI/Apo B–siBNA and (B) PEI–pullulan/Apo B–siBNA complexes. Various concentrations of the polymer were mixed with Apo B–siBNA and analyzed by 19% polyacrylamide gel electrophoresis.

6.0 caused death after systemic injection. Notably, all mice died when complexes with N/P ratios of ≥12 were injected (data not shown). Dying mice lapsed into dyspnea less than 30 min after injection and showed hemorrhage-like dark red regions in the lung. On the other hand, no mortality was observed in mice injected with PEI–pullulan/Apo B–siBNA complexes with N/P ratios of 96 to 192 (Fig. 2). These results suggested that introduction of pullulan into PEI reduces low mortality.

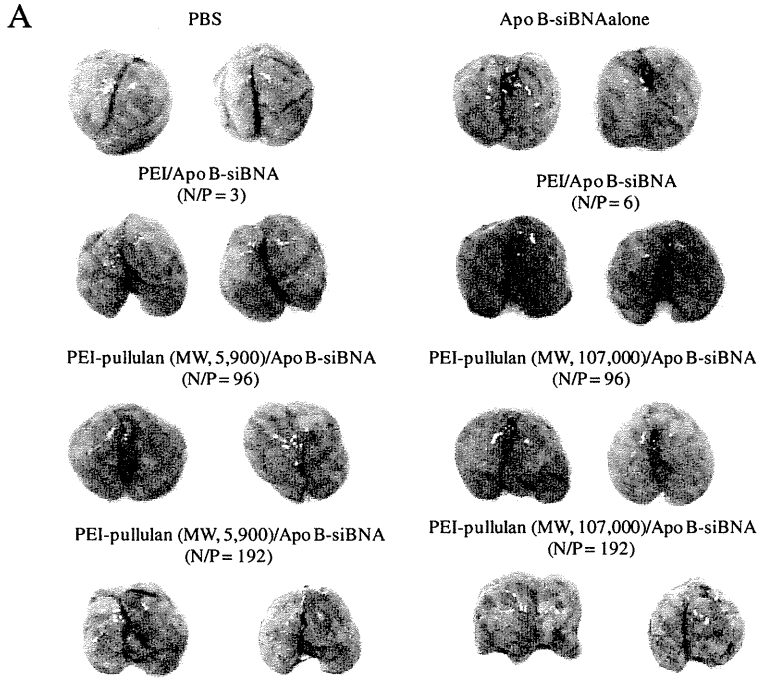
3.4. Biodistribution of carrier/Apo B–siBNA complex

Apo B–siBNA (25 μg/mouse) formed a complex with PEI at an N/P ratio of 3, and with PEI–pullulan at an N/P ratio of 192. Complexes were injected via the tail vein. Fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was measured at 3, 6, 12, or 24 h after injection. At 3 and 6 h after injection of PEI/siBNA, fluorescence was detected mainly in the lung and kidney. PEI–pullulan (MW, 5900)/Apo B–siBNA complex showed high fluorescent signals in the liver and kidney, but PEI–pullulan (MW, 107,000)/Apo B–siBNA complex-injected mice showed high fluorescence levels in the liver for a long time (Fig. 3 and Fig. S3). These data indicate that a PEI–pullulan carrier with high MW pullulan (MW, 107,000) is more stable in the blood stream compared with that containing low MW pullulan (MW, 5900).

3.5. Reduction of serum cholesterol and Apo B mRNA in the liver after systemic injection

For 14 days, no difference was observed in the body weight between control (standard pellet diet-fed mice) and high-fat diet-fed mice, but total cholesterol (TC) increased in a time-dependent manner in mice fed a high-fat diet (Fig. S4). Ten days after the provision of diet, each sample was injected into the mice via the tail vein for 3 days (1 injection/day).

No change in body weight was observed following injection of the complexes (Fig. S5). A significant decrease in the TC level was observed in PEI–pullulan (MW, 107,000)/Apo B–siBNA complex-injected mice. PEI–pullulan (MW, 5900)/Apo B–siBNA complex-injected mice showed a slight but insignificant reduction in TC (Fig. 4A). Especially, cholesterol levels in LDL and VLDL fraction dramatically decreased in the PEI–pullulan (MW, 107,000)/Apo B–siBNA complex-injected mice (Fig. 4B). Moreover, no differences in GOP and GTP levels were identified among groups, indicating that the complexes did not damage the liver (Fig. S6).



B

PBS	Apo B-siBNA alone	PEI/Apo B-siBNA		PEI-pullulan (MW, 5,900)/Apo B-siBNA		PEI-pullulan (MW, 107,000)/Apo B-siBNA	
		N/P = 3	N/P = 6	N/P = 96	N/P = 192	N/P = 96	N/P = 192
0/3	0/3	0/4	2/4	0/3	0/4	0/3	0/4

Fig. 2. Lung damage and mortality. Lung damage (A) and mortality after systemic injection (B) of PBS, Apo-B siBNA alone, or polymer/siBNA complexes into mice. Numbers in B indicate the number of dead mice as a function of the total number of mice.

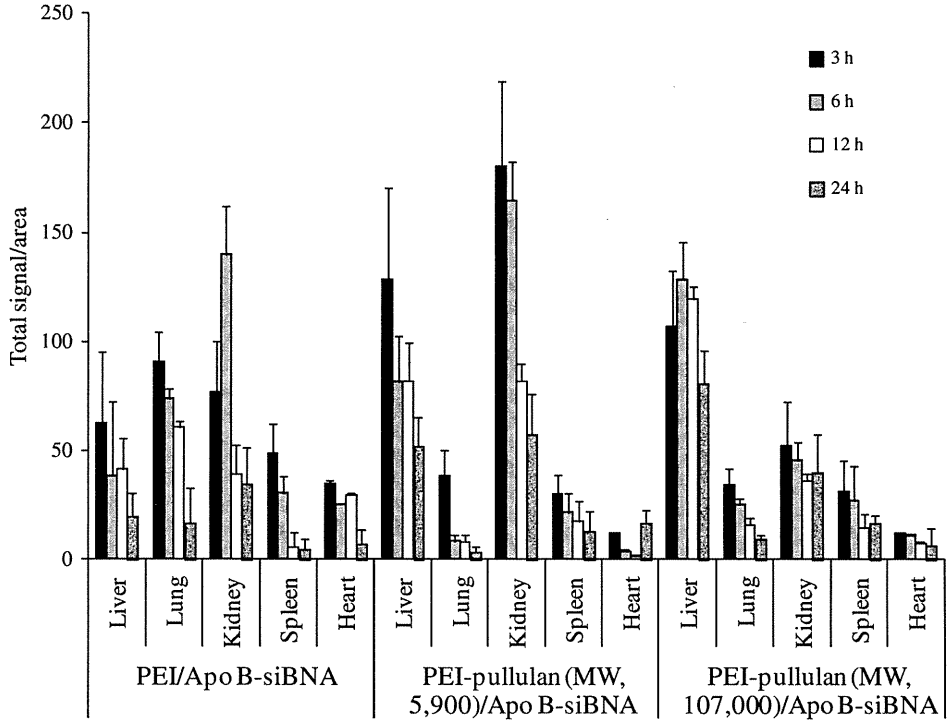


Fig. 3. Biodistribution after injection of each sample. The Apo B–siBNA was bound with PEI at an N/P ratio of 3 and with PEI–pullulan at an N/P ratio of 192. The fluorescence in each tissue (heart, lung, liver, spleen, and kidney) was detected, and the signal level for each tissue was indicated by total signal/area.

Finally, we examined whether the complexes could reduce Apo B mRNA levels in the liver. Significant reduction in Apo B mRNA level was identified in the livers of mice injected with PEI–pullulan (MW, 107,000)/Apo B–siBNA complex. However, the injection of Apo B–siBNA alone did not reduce the level of TC or Apo B mRNA in liver tissue (Fig. 5). In addition, there was no change in serum TC and Apo B mRNA in livers of mice injected with PEI–pullulan carrier alone or separately injected with equal volumes of PEI–pullulan carrier and Apo B–siBNA (Fig. S7).

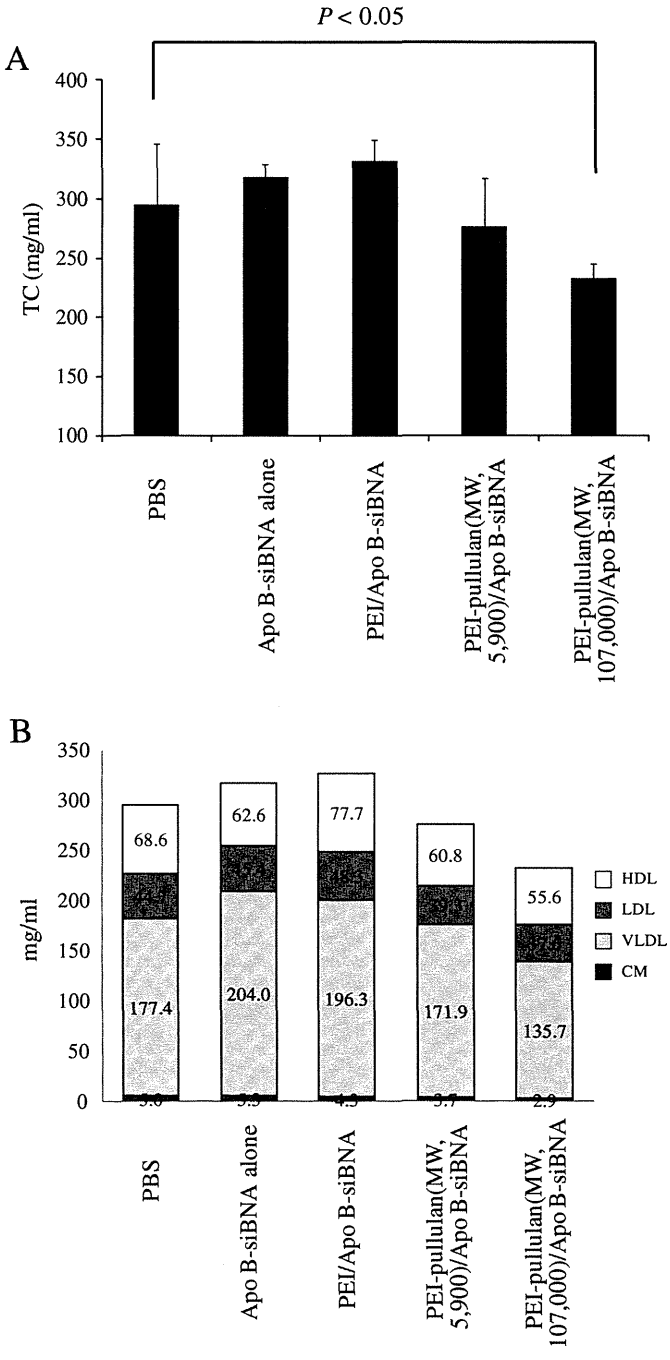
4. Discussion

PEI is one of the most popular synthetic polymer with a high cationic charge density. Its strong buffering capacity over a wide range of pH values plays an important role in the release of genes from the endosome after endocytosis. However, it is necessary to overcome the high *in vivo* toxicity and mortality caused by systemic injection of PEI-based complexes [25,26]. In agreement with previous studies [27,28], we observed a mortality rate of 50% after injection of PEI/Apo B–siBNA complexes with an N/P ratio of 6 into mice (Fig. 2). Interestingly, the amount of free PEI remaining after complex formation with genes correlates with mouse mortality [29]. For reducing *in vivo* toxicity and mortality, the introduction of poly(ethylene glycol) (PEG) [28] and removal of free PEI after complex formation [29] have been widely studied. In the present study, introduction of pullulan into PEI dramatically decreased mortality and lung damage in mice after systemic injection. Moreover, PEI–pullulan has no effect on GOT and GPT levels. These data indicate that PEI–pullulan is a gene carrier with very low toxicity.

We used a linear 22-kDa PEI for complex formation with Apo B–siBNA and for synthesizing the PEI–pullulan carrier. When these PEI/gene complexes were transfected via systemic administration, the main target was the lung. Lower levels of transfection were found in the brain, heart, liver, spleen, and kidney [27]. In the present study, the level of fluorescence was higher in the lungs than in other tissues (heart, liver, and spleen) at all times after intravenous injection of the PEI/Apo B–siBNA complex at an N/P ratio of 3 (Fig. 3). Fluorescence in the kidney may be caused by elimination of biodegraded free fluorescein from the system. However, PEI–pullulan/Apo B–siBNA showed higher liver-targeting ability than PEI/Apo-B–siBNA. A PEI–pullulan carrier with high MW pullulan (107,000) shows higher fluorescence levels in the liver for a longer time than that with low MW pullulan (5900) (Fig. 3). Similarly, although effects on gene expression can be affected by the polymer charge or N/P ratios, high MW pullulan complexes (>20,000) resulted in more efficient gene expression than low MW pullulan complexes (<20,000) [30,31].

High liver-targeting ability of a PEI–pullulan carrier is caused by high affinity of pullulan for the hepatic asialoglycoprotein receptor. Hepatitis viruses (e.g., HBV and HCV) [32,33] and saccharides (e.g., galactose and pullulan) [34] are recognized by the asialoglycoprotein receptor and are thus able to enter hepatocytes. After systemic injection, pullulan-based carriers transfer genes into hepatocytes via endocytosis after binding to the asialoglycoprotein receptor [35,36]. The present study indicates that PEI–pullulan/Apo B–siBNA complexes are more readily transfected into the liver than into other tissues (lung, heart, and spleen) (Fig. 3).

Several excellent targets have been investigated for the reduction of serum LDL levels. For example, proprotein convertase subtilisin/kexin type 9 (PCSK9), which inhibits the recycling of the LDL receptor [37–39]; squalene synthase [38,39]; 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase, HMGCR) [40,41]; and Apo B, which is related to cholesterol synthesis, are all logical targets for gene therapy. The present study focused on Apo B, which is critical for LDL synthesis and metabolism. Apo B siRNA was used for inhibition of Apo B expression, and the gene was chemically modified to increase its stability. However, there was no change in serum LDL and Apo B mRNA levels in the liver in mice treated with Apo B–siBNA alone. A previous study has also reported that administration of naked Apo B siRNA, without formulation or chemical conjugation, at doses higher than 50 mg/kg shows no silencing of Apo B mRNA in the liver and no change of serum LDL levels in mice [20]. However, when a single injection of the cationic liposome formulated with chemically modified Apo B siRNA into monkeys [21] and mice [42] at doses of 1 and 3 mg/kg, respectively, significant reduction of the level of serum LDL and Apo B mRNA in the liver has been



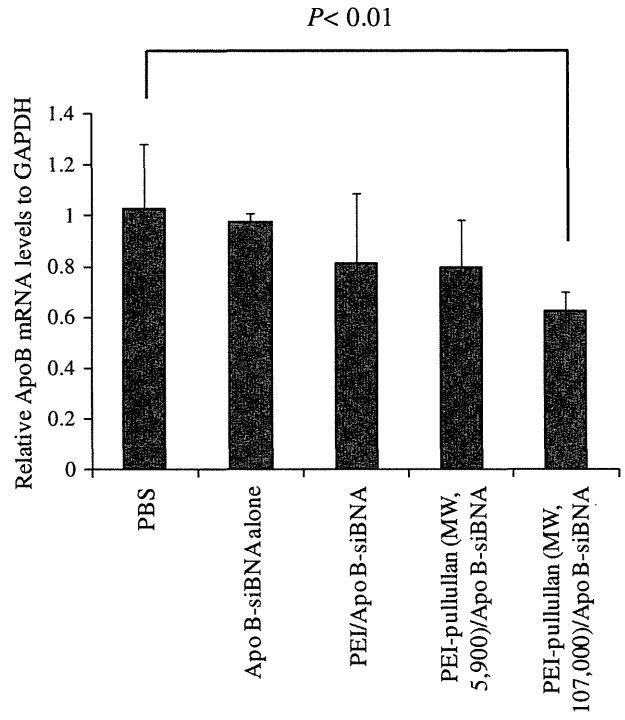


Fig. 5. Apo B mRNA in the liver after systemic injection of each sample. The Apo B–siBNA was bound with PEI at an N/P ratio of 3 and with PEI–pullulan at an N/P ratio of 192.

Fig. 4. Serum TC. The levels of serum TC (A) and lipoprotein classes (B) after systemic injection of each sample. The Apo B–siBNA was bound with PEI at an N/P ratio of 3 and with PEI–pullulan at an N/P ratio of 192.

identified. These results mean that naked Apo B siRNA molecules are unstable in the bloodstream and show low transfer efficacy. Chemical modification of siRNA and use of carriers may be useful for increasing therapeutic efficiency of Apo B siRNA. Although cationic liposomes are very benefic carriers for delivering Apo B siRNA into liver cells, they have no specificity to liver cells. Therefore, liver-targeted delivery system can increase the therapeutic potential of Apo B siRNA.

Several liver-targeted delivery methods for Apo B siRNA have been reported such as direct conjugation of Apo B siRNA to lipophilic molecules (e.g., cholesterol and α-tocopherol) [20,43] or to carriers containing liver-targeted ligands (e.g., *N*-acetylgalactosamine) [44]. In the present study, we used a liver-targeted gene delivery system (PEI–pullulan) to increase the therapeutic efficacy of chemically modified Apo B siRNA (Apo B–siBNA). The PEI–pullulan/Apo B–siBNA complex is almost neutral at an N/P ratio of 192 (Table S1). This indicates that the binding of the complexes to serum proteins containing negative or positive charges may be very low. However, the complexes can be efficiently transferred into hepatocytes by the receptor-mediated endocytosis because the pullulan shows high affinity for the hepatocyte asialoglycoprotein receptor. In fact, reduction in level of serum LDL and Apo B mRNA was observed in the livers of mice injected with PEI–pullulan (MW, 107,000)/Apo B–siBNA (Figs. 4 and 5).

5. Conclusion

Apo B-100 is an essential component of plasma lipoproteins and inhibition of its synthesis by Apo B siRNA efficiently reduces serum LDL. We synthesized a cationic conjugate (PEI–pullulan) for efficient delivery of chemically modified Apo B-siRNA (Apo B–siBNA) into the liver by introducing pullulan with different MWs (5900 and 107,000) into PEI. Introduction of pullulan into PEI dramatically reduced *in vivo* toxicity and mortality after systemic injection of the conjugate/ApoB–siBNA into mice. The PEI–pullulan carrier prepared with high MW pullulan (107,000) was more stable in the blood stream and showed higher fluorescence levels in the liver for a longer time and more efficient reduction of serum LDL and Apo B mRNA than the carrier prepared with low MW pullulan (5900). These results strongly suggest that combining the liver-targeted gene delivery system with Apo B–siBNA significantly reduces the level of serum LDL and Apo B mRNA in the liver.

Acknowledgments

This work was supported by a research grant from the Ministry of Health, Labour and Welfare (MHLW), Japan, and by the Program for