Synthesis of terminally modified oligonucleotides and their hybridization dependence on the size of the target RNAs†

Kohji Seio,* Yusuke Takaku, Kazuya Miyazaki, Sayako Kurohagi, Yoshiaki Masaki, Akihiro Ohkubo and Mitsuo Sekine*

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We have developed new artificial oligonucleotide probes that show selective recognition for short RNA targets over long RNA targets. Our results suggested that modification of the termini of the oligonucleotide probes by bulky substituents such as cyclohexyl and 4-(3,6,9-trioxaundecylenedioxy)phenyl (Bzcr) groups significantly improved the selectivity of the probes toward the short RNA targets. The selectivity was further improved by the addition of a phosphate group on the cyclohexane ring. Although much improved selectivity toward short RNA targets is desirable in a general sense, it is particularly applicable to the selective detection of matured-miRNA over pre-miRNAs.

Introduction

MicroRNAs are a group of noncoding RNAs having 18–25 nucleotide residues,[1] which regulate a wide range of biological functions such as development, differentiation, and metabolism.[2–7] Several methods have been reported for the analysis of miRNAs by use of microarray[8–11] and PCR[12–13] technologies. It is well known that mature miRNAs are excised from longer pre-miRNAs having stem–loop structures.[1] Therefore, miRNA detection needs to be performed in both a sequence- and size-selective manner. In general, mature miRNAs can be detected by the above-mentioned detection methods because of the lower abundance of pre-miRNAs[11,14] (the stem–loop structures of pre-miRNAs that block the hybridization of the oligonucleotide probes), and the inefficiency of the enzymatic labeling of the large pre-miRNAs.[11] However, recent studies have revealed that the processing of pre-miRNA and pri-miRNAs are regulated post-transcriptionally and that the pre-miRNAs have become more abundant than the mature miRNAs in some cases.[15–16] Therefore, methods that can selectively detect mature miRNAs are required for more detailed miRNA analyses. Based on this consideration, we attempted to develop new 2′-*O*-methyl-RNA probes that could bind to short RNA targets more tightly than long RNA targets, simply by adding bulky or anionic substituents to the termini of the oligonucleotide probes (Fig. 1). We methylated the 2′-*O*-positions of the probes considering the superiority of the 2′-*O*-methyl-RNA to DNA and RNA in terms of the strong affinity toward RNA targets.[17] It was expected that terminal modification would induce short-RNA selectivity, either by lowering the stability of the duplexes with the long RNA targets by steric and electrostatic interactions (Fig. 1a), or by stabilizing the duplexes through terminal stacking effects (Fig. 1b).

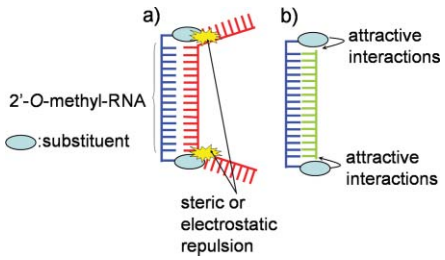

Fig. 1 Schematic representation of the possible factors affecting the stability of duplexes of the terminally substituted oligonucleotide (blue) with long RNA (red) and short RNA (green). a) The duplex with long RNA can be destabilized by steric and electrostatic repulsion. b) The duplex with short RNA can be stabilized by attractive interactions such as stacking or van der Waals interactions at the termini.

As the substituents, we chose the *N*-carbamoylnucleosides having bulky groups (Scheme 1A) and synthesized the corresponding phosphoramidites **1a–1f**, as shown in Schemes 1B, 3, 5 and 6, and the oligonucleotides, as shown in Schemes 4 and 7. Previously, incorporation of *N*-arylcarbamoylnucleosides into DNA duplexes was reported to stabilize DNA duplexes by intercalation into the opposite strands.[18] We hypothesized that the replacement of the intercalative aryl group to non-intercalative groups such as the cyclohexyl and polyether rings might result in the de-stabilization of duplexes containing long RNAs by steric interactions, while the stability of duplexes containing short RNAs (Fig. 1) could be maintained or increased. In addition, we studied the effects of adding a phosphate group (carried out by incorporating dA^{ChcmP}; Scheme 1A) on the hybridization and size discrimination of the target RNAs.

Results and discussion

Preparation of cytidine 3′-phosphoramidite derivatives 1a–c and oligonucleotides incorporating them

We first designed the deoxycytidine derivatives dC^{Chcm}, dC^{Bzcr}, and their 3′-phosphoramidite derivatives **1a** and **1b** shown in Scheme 1.

Department of Life Science, Tokyo Institute of Technology, 4259 Nagatsuka, Midori-ku, Yokohama, 226-8501, Japan. E-mail: kseio@bio.titech.ac.jp, msekine@bio.titech.ac.jp; Tel: +81 45 924 5706

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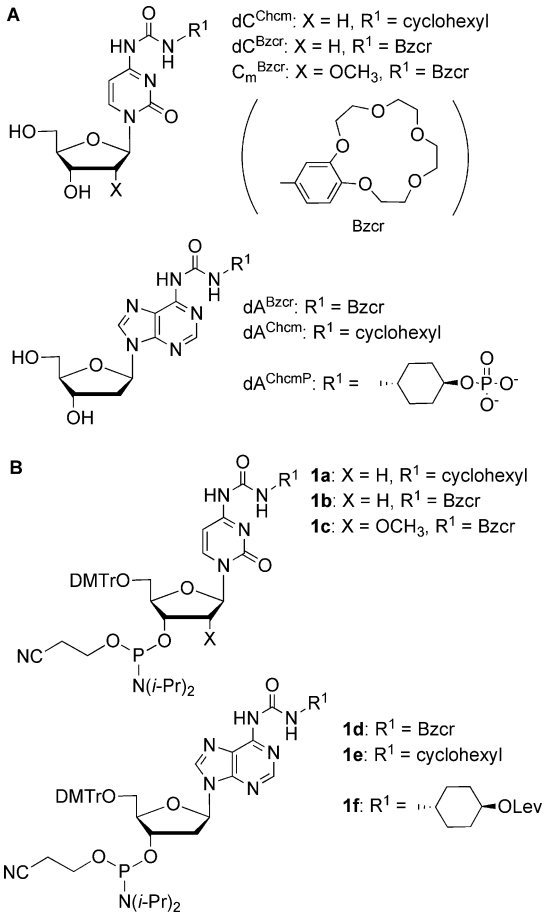
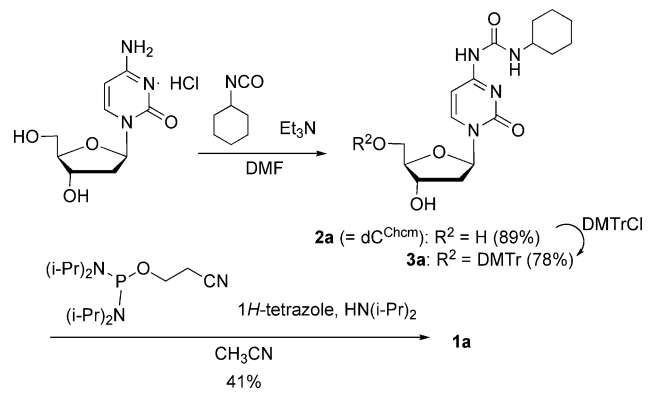


Scheme 1

Compounds **1a** and **1b** have an *N*-cyclohexylcarbamoyl group and a 4-(3,6,9-trioxaundecylenedioxy)phenyl moiety (abbreviated as Chcm and Bzcr (**b**en**zo**-**cr**own ether) in this paper) on the amino group as a bulky substituent, chosen because of the commercial availability of the reagents. We also designed C_m^{Bzcr} and its phosphoramidite compound **1c**, the 2′-*O*-methyl derivative of **1b**, to examine the effects of the sugar structure on the hybridization properties.

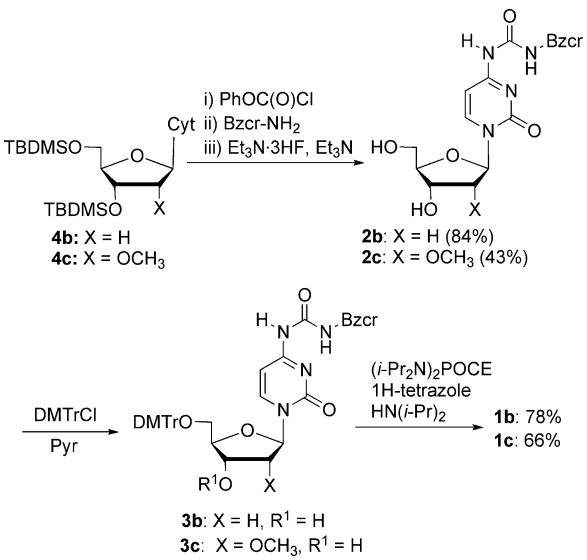
The preparation of **1a** is shown in Scheme 2. Deoxycytidine hydrochloride was treated with cyclohexyl isocyanate to give 4-*N*-(*N*-cyclohexylcarbamoyl)deoxycytidine (**2a**, dC^{Chcm}) in 89% yield,



Scheme 2

which was subjected to the usual tritylation to give **3a**. Subsequently, compound **3a** was converted to the phosphoramidite **1a** in 41% yield, according to the usual procedure.[19]

Next, we synthesized the nucleoside precursor **3b** containing Bzcr, as shown in Scheme 3. 3′,5′-*O*-Bis(*t*-butyldimethylsilyl)-deoxycytidine (**4b**)[20] was treated with phenyl chloroformate in the presence of pyridine to give the carbamate intermediate, which was coupled with 4-(3,6,9-trioxaundecylenedioxy)phenylamine at 70 °C. Subsequently, the TBDMS groups were removed to give the nucleoside **2b** (dC^{Bzcr}) in 84% yield. Treatment of **2b** with DMTrCl gave the DMTr derivative **3b** in 82% yield. The 5′-protected derivative **3b** was then phosphitylated to give the phosphoramidite unit **1b** in 78% yield. Similarly, the 2′-*O*-methyl derivatives **1c**, **2c** (C_m^{Bzcr}) and **3c** were synthesized from **4c** (Scheme 3).[21]



Scheme 3

By use of these phosphoramidites and commercially available universal support II,[22] we synthesized the various 2′-*O*-methyl-RNA probes such as **probes 2–4** having 5′-Y-$C_mA_mA_mC_mC_mU_mA_mC_mU_m$-Z-3′ sequences, where Y and Z represents the modified cytidine residue incorporated by the use of **1a–1c**. The sequences and the structures of the probes are shown in Table 1 and Scheme 4A, respectively.

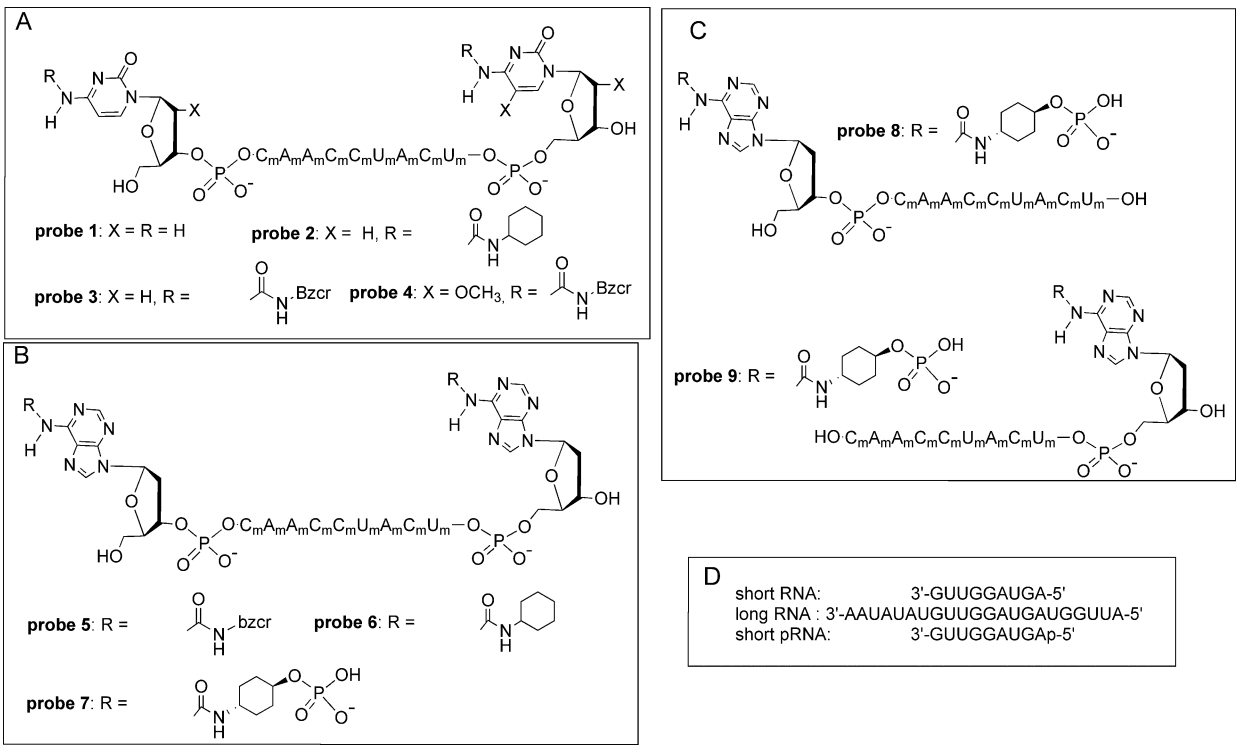
Hybridization properties of probes 1–4 to the short and long RNAs

The hybridization properties of **probes 2–4** toward **short RNA** and **long RNAs** (Scheme 4D) were studied by measuring UV-melting temperatures (T_m) and compared with those of **probe 1** (Y, Z = dC) without the modifications at the terminal deoxycitidine residues. Here, **short RNA** is the 9-mer RNA having 3′-GUUGGAUGA-5′ sequence complementary to the 5′-$C_mA_mA_mC_mC_mU_mA_mC_mU_m$-3′ sequence of **probes 2–4**, and **long RNA** is the 22-mer RNA having additional 7-mer and 6-mer sequences at the 3′- and 5′-sites, respectively. As shown in Table 1, **probe 1** (having unmodified deoxycytidines at both termini) bound to **short** and **long RNAs** with identical affinities, both T_m values being 45 °C. In contrast, the introduction of the *N*-cyclohexylcarbamoyl group (**probe 2**: Y, Z = dC^{Chcm}) increased the T_m of the duplex with **short RNA** by 4 °C, keeping T_m of the duplex with **long RNA** unchanged. As the result,

Table 1 T_m (°C) of the duplexes formed between the 2′-*O*-methyl-RNA probes: 5′-Y-$C_mA_mA_mC_mC_mU_mA_mC_mU_m$-Z-3′ and the **short RNA** or **long RNA** targets

	Short RNA (9-mer)	Long RNA (22-mer)	ΔT_m [a]
Probe 1 (Y, Z = dC)	45	45	±0
Probe 2 (Y, Z = dC^{Chcm})	49	45	+4
Probe 3 (Y, Z = dC^{Bzcr})	57	53	+4
Probe 4 (Y, Z = C_m^{Bzcr})	58	55	+3
Probe 5 (Y, Z = dA^{Bzcr})	53	49	+4
Probe 6 (Y, Z = dA^{Chcm})	50	47	+3
Probe 7 (Y = dA^{ChcmP})	52 (51)[b]	43	+9 (+8)[b]
Probe 8 (Y = dA^{ChcmP}, Z = none)	47	40	+7
Probe 9 (Y = none, Z = dA^{ChcmP})	45	42	+3

[a] ΔT_m = (T_m of short-RNA) − (T_m of long-RNA). [b] The T_m and ΔT_m of the experiment using **short pRNA**.



Scheme 4

probe 2 (Y, Z = dC^{Chcm}) showed higher affinity toward **short RNA** than **long RNA**, as indicated by the ΔT_m value of +4 °C, which was calculated by subtracting the T_m value with **long RNA** from that with **short RNA**. These results indicated that the terminally modified oligonucleotide probes showed selectivity toward short RNA, and that the mechanism of the selectivity was not due to the destabilization of the duplex with **long RNA** by steric hindrance, but due to the stabilization of the duplex with **short RNA** by the attractive interactions at the termini, as shown in Fig. 1b.

The steric effects of **probe 3** (Y, Z = dC^{Bzcr}) with the larger Bzcr groups were also examined. As shown in Table 1, the introduction of the Bzcr group increased the affinity toward **short** and **long RNAs** significantly by 12 °C and 8 °C, respectively, compared with those of **probe 1** (Y, Z = dC). This is probably because of the stacking effects of the terminal benzene rings toward the opposite bases. However, the ΔT_m value did not improve compared with that of the **probe 2**, which has smaller cyclohexane rings.

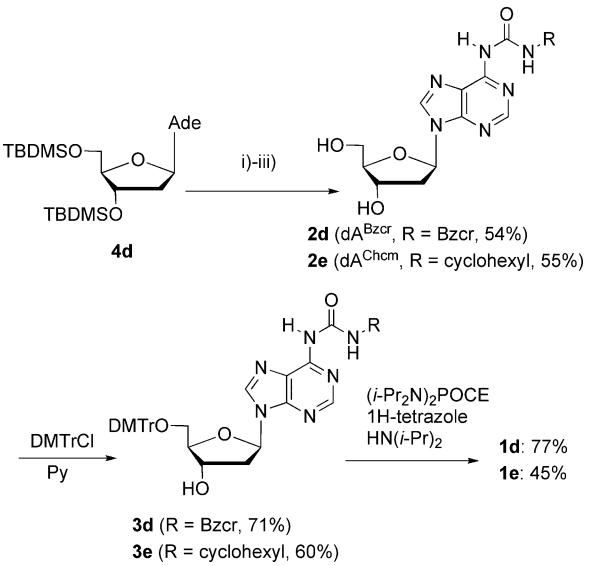
Next, the properties of **probe 4** (Y, Z = C_m^{Bzcr}) having 2′-*O*-methylribose structure were examined to see the effects of the conformational restraint on the sugar moiety.[23] However, no significant improvement was observed, either in the hybridization property (T_m) or selectivity (ΔT_m) relative to **probe 3**. Therefore, we decided to optimize the structure of the modified nucleosides at the terminus by using 2′-deoxynucleotides.

Preparation of deoxyadnosine 3′-phosphoramidite derivatives 1d and 1e and oligonucleotides incorporating them

In order to increase the selectity toward **short RNA** of the 2′-*O*-methyl-RNA probes, we designed **probes 5** (Y, Z = dA^{Bzcr}) and **6** (Y, Z = dA^{Chcm}) with the adenine bases modified with the Bzcr and cyclohexane rings, respectively, and **probe 7** (Y, Z = dA^{ChcmP}) having the phosphate groups on the cyclohexane ring (Scheme 4B). **Probes 5** and **6** are designed in this manner since it was expected

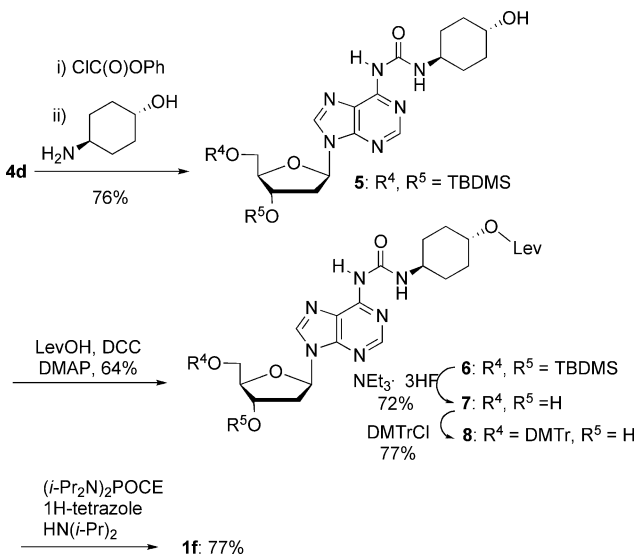
that the larger purine base could more effectively interact with the opposite strands than the modified cytosine bases in **probes 2** and **3**. We also designed **probe 7** with the terminal phosphate groups, expecting that the anionic phosphate group could destabilize the duplex with **long RNA** by the electrostatic interactions with the phosphate backbone, as shown in Fig. 1a.

For these purposes, we prepared the phosphoramidites **1d** and **1e**, as shown in Scheme 5. Briefly, the protected deoxyadenosine **4d** was converted to **2d** and **2e** according to the procedure for **2b** and **2c**, respectively. Subsequently, 5′-protection and 3′-phosphitylation were performed in the usual manner to give **1d** and **1e**.



Scheme 5 *Reagents*: i) PhOC(O)Cl, ii) cyclohexylamine or Bzcr-NH_2, iii) $Et_3N{\cdot}3HF$, Et_3N.

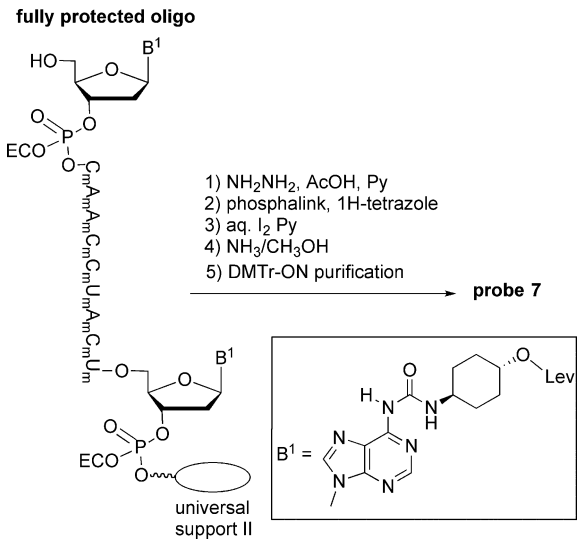
We also prepared the phosphoramidite **1f** for the synthesis of **probe 7** having dA^{ChcmP} at both ends (Scheme 6). Compound **4d** was converted to the *N*-(4-hydroxycyclohexyl)carbamoyl derivative **5** in 76% yield. The hydroxyl group of **5** was protected by a levulinoyl group (Lev) to give **6** in 64% yield. Desilylation followed by tritylation gave the nucleoside **8**, which was converted to the phosphoramidite **1f** in 77% yield by a similar procedure to that used for **1a–c**.[19]



Scheme 6

Using phosphoramidites **1d**, **1e** and **1f**, we prepared **probes 5**, **6**, and **7**, respectively. **Probes 5** (Y, Z = dA^{Bzcr}) and **6** (Y, Z = dA^{Chcm}) were synthesized according to the standard phosphoramidite chemistry protocol.

The phosphorylated oligonucleotide **probe 7** (Y, Z = dA^{ChcmP}) was synthesized, as schematically shown in Scheme 7, using the phosphoramidite **1f** and 2-cyanoethyl 2-(4,4′-dimethoxytrityloxyethylsulfonylethyl) *N*,*N*-diisopropylphosphoramidite (Phosphalink™) as the *in situ* phosphorylating agent during the solid-phase DNA synthesis.[24] First, the fully protected oligonucleotide was synthesized on the CPG having the universal linker. After the chain elongation, the Lev groups were removed by treatment with hydrazine acetate, and the liberated hydroxyl group was phosphorylated by treatment with Phosphalink. Finally, the cleavage of the partially protected oligonucleotide from the solid supports and the deprotection of the base and the phosphate residues were performed by treatment with methanolic ammonia. The following standard DMTr-ON purification gave the desired **probe 7**.



Scheme 7

Hybridization properties of probes 5–7 to short and long RNAs

The T_m values of the duplex of **probes 5–7** with **short RNA** and **long RNA** were measured. The results are also shown in Table 1. As shown in the table, T_m of the duplex **probe 5**/**short RNA** was 53 °C and that with **long RNA** was 49 °C, each of which was lower than corresponding T_m of the cytidine derivative **probe 3**/**short RNA** and **probe 3**/**long RNA**. Despite the decrease in these T_m values, ΔT_m of **probe 5** was 4 °C, which was identical to that of **probe 3**. In the case of **probe 6**, the T_m values of the duplexes with **short RNA** and **long RNA** were 50 °C and 47 °C, respectively. The observed ΔT_m (3 °C) was close to 4 °C (49–45 °C) of the corresponding cytidine derivative **probe 2**. Although the modified deoxyadenosine residues at the terminus of **probe 5** and **probe**

6 could potentially form Watson–Crick hydrogen bonds with the uridine resiudes at the opposite sites of **long RNA**, the lower affinity of these probes toward **long RNA** indicated that these modified adenosine residues did not form such hydrogen bonds probably due to the steric hindrance by the substituents.

Interestingly, anionic modification of the oligonucleotide probe improved the selectivity toward **short RNA** of **probe 7** to give a ΔT_m value of +9 °C. The effect of the phosphate groups could be extracted by comparing with the data of **probe 6**. Phosphorylation slightly increased the stability with **short RNA**, as shown by T_m values of **probe 6/short RNA** (50 °C) and **probe 7/short RNA** (52 °C). On the other hand, the T_m for the duplex of **probe 7/long RNA** (43 °C) was destabilized by 4 °C by phosphorylation relative to **probe 6/long RNA** (47 °C). These results indicated that the increase in the selectivity toward **short RNA** could be due to the destabilization of the duplex with **long RNA** probably because of anionic repulsion between the phosphate groups and the phosphodiester backbone of the **long RNA** target. Thus, it was suggested that the presence of the anionic substituents at the proper position of the probe's termini could improve the size selectivity of the oligonculeotide probes.

Considering the fact that the 5′-hydroxyl group of matured miRNA is generally phosphorylated, we also tested the hybridization of **probe 7** on **short pRNA** (Scheme 4D) with 5′-pAGUAGGUUG-3′ sequence in which the 5′-hydroxyl group was phosphorylated. As a result, the T_m of **probe 7/short pRNA** duplex was 51 °C, which is almost identical to that of the **probe 6/short RNA** duplex. This result indicates that the presence of the 5′-phosphate on the target RNA does not affect the duplex stability.

Contribution of the 5′ and 3′ modifications to the ΔT_m value

As described above, our results suggested that the 11-mer oligonucleotides with both terminal bases modified by bulky anionic substituents could selectively recognize short 9-mer RNA over long 22-mer RNA. In order to clarify the contribution of 5′-terminal and 3′-terminal modifications on the duplex stability and the short RNA selectivity, we synthesized **probes 8** (Y = dA^{ChcmP}, Z = none) and **9** (Y = none, Z = dA^{ChcmP}) lacking the 3′- and 5′-terminal modifications, respectively (Scheme 4C). The T_m values of the duplexes of **probe 8** or **9** with **short RNA** and **long RNA** are also shown in Table 1. The duplex of the **probe 8** and **short RNA** was more stable (T_m 47 °C) than the duplex with **long RNA** (T_m 40 °C), with a ΔT_m value of +7 °C. In the case of the 3′-terminal-modified **probe 9**, the stability with **short RNA** was lower (T_m 45 °C), while that with **long RNA** was higher (T_m 42 °C), with a consequent decrease in selectivity (ΔT_m +3 °C). This result clearly shows that 5′-terminal modification contributes more than 3′-terminal modification to the **short RNA** selectivity. Interestingly, the sum of ΔT_m of the 5′-modified **probe 8** and the 3′-modified **probe 9** was +10 °C, which is close to ΔT_m = +9 °C of **probe 7**.

Molecular modeling of the duplex

In order to visualize the positions of the phophorylated cyclohexane ring at the 5′- and 3′-termini, we performed 15 ns molecular dynamic simulation of the **probe 7/short RNA** duplex. Shown in Fig. 2 is the average structure for the last 5 ns. As shown in Fig. 2, the cyclohexane ring at the 5′-terminus of **probe 7** stacked with

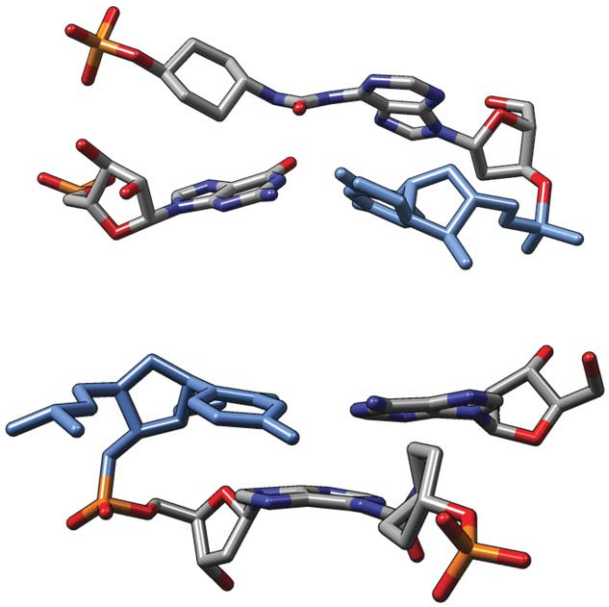


Fig. 2 Model structures of the (top) 5′-terminal and (bottom) 3′-terminal regions of **probe7** in **probe7/short RNA** duplex.

the 3′-terminal nucleobase of **short RNA**. These results clearly show that substituents attached to the base moiety can effectively interact with the opposite strand. In addition, the phosphate group on the cyclohexane ring is in close proximity (<4 Å) to the 3′-hydroxyl group of **short RNA**, from which the additional 5′-UAUAUUA-3′ sequence would extend to **long RNA**. In contrast, the cyclohexane ring at the 3′-terminus of **probe 7** did not stack on the 5′-terminal nucleobase of **short RNA**, and the distance between the phosphate group and the 5′-hydroxyl group of **short RNA** was rather long (*ca.* 10 Å). Thus, effects arising from the weak steric and electrostatic repulsion were expected even when the additional 5′-AUUGGUA-3′ sequence was extended from the 5′-hydroxyl group. This structural model obtained by the MD calculation suggests a possible explanation for the contribution of the 5′-substituent to the **short RNA** selectivity being larger than the 3′-substituent.

Conclusions

In this paper, we have proposed a new design for artificial oligonucleotide probes, enabling them to selectively recognize a shorter RNA target over a longer RNA target. Our results suggest that the modification of the terminus of the oligonucleotide probes by bulky substituents such as cyclohexane and Bzcr groups improved the selectivity of the probes toward short RNA targets. The selectivity was further improved by the addition of the phosphate group to the cyclohexane ring. We compared the contribution of the 5′- and 3′-terminal modifications toward the short RNA selectivity and proved that 5′-modification contributed more.Similar selectivity over the longer RNA target was also observed even when the 5′-end of the target RNA was phosphorylated.

The maximum selectivity toward the short RNA target (as measured by the ΔT_m value) was 9 °C in the case of the probe modified at the both ends. However, because we used very short 9-mer RNA as the model compound of matured miRNA, this

value of 9 °C would not be sufficient for real ~25-mer miRNAs targets. Interestingly, we also found that **probe 8** lacking 3′-modification showed a rather large ΔT_m of 7 °C. This result indicated the possibility of using 5′-modified probes such as **probe 8** to discriminate the 3′-end of matured miRNA from the unprocessed pre-miRNAs. Although improved selectivity toward short RNA targets might be necessary, such short-RNA selective binding of terminally modified oligonucleotides seems to be applicable for the selective detection of matured-miRNA over pre-miRNAs. Currently, such discrimination is performed using hairpin-type oligonucleotide probes in combination with PCR[12] and oligonucleotide microarray[25] technologies. Although such a hairpin design has been reported to be effective to discriminate matured miRNAs from pre-miRNAs, the formation of the hairpin structures required rather long stem–loop regions in the probes, which are not necessary if size discrimination could be achieved by use of simple 5′- and 3′-terminal modifications. Improvement in the selectivity toward short RNA targets of terminally modified oligonucleotides and development of applications to miRNA detection and microarray techniques are underway, and will be reported elsewhere.

Experimental section

General

^{1}H, ^{13}C and ^{31}P NMR spectra were obtained at 500, 126 and 203 MHz, respectively. The chemical shifts were measured relative to tetramethylsilane (0.0 ppm) and DMSO-d_6 (2.49 ppm) for ^{1}H NMR, $CDCl_3$ (77.0 ppm) and DMSO-d_6 (39.7 ppm) for ^{13}C NMR and 85% phosphoric acid (0.0 ppm) for ^{31}P NMR.

4-*N*-(*N*-Cyclohexylcarbamoyl)deoxycytidine (2a)

Deoxycytidine hydrochloride (5.0 g, 19.0 mmol) was dissolved in anhydrous *N*,*N*-dimethylformamide (190 mL). To this solution, triethylamine (2.6 mL, 19.0 mmol) and cyclohexyl isocyanate (2.7 mL, 21 mmol) were added, and the resulting solution was stirred at 50 °C for 1 d. After the completion of the reaction, the solvents were removed under reduced pressure and the residue was diluted with a mixture of chloroform (150 mL) and isopropyl alcohol (50 mL). The organic layer was washed three times with water (200 mL each), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (100 g) by use of chloroform-methanol (8:1, v/v) to give **2a** (5.94 g, 89%); δ_H (DMSO, 500 MHz) 1.21–1.34 (5H, m), 1.50–1.52 (1H, br), 1.63–1.66 (2H, br), 1.78–1.81 (2H, br), 1.96–2.01 (1H, m), 2.20–2.25 (1H, m), 3.52–3.62 (3H, m), 3.82 (1H, dd, J = 3.7 Hz, J = 7.3 Hz), 4.18–4.22 (1H, m), 5.02 (1H, t, J = 5.2 Hz), 5.25 (1H, d, J = 4.2 Hz), 6.09 (1H, t, J = 6.3 Hz), 6.24 (1H, br), 8.15 (1H, d, J = 7.3 Hz), 9.79 (1H, br); δ_C (DMSO) 23.9, 25.2, 23.4, 40.7, 47.7, 61.0, 70.0, 85.9, 87.8, 94.6, 143.3, 152.7, 153.4, 162.3; HRMS (ESI) m/z calcd for $C_{16}H_{24}N_4NaO_5^+$: 375.1639, found 375.1663.

4-*N*-(*N*-Cyclohexylcarbamoyl)-5′-*O*-(4,4′-dimethoxytrityl)deoxycytidine (3a)

Coumpound **2a** (1.0 g, 2.84 mmol) was co-evaporated three times with anhydrous pyridine and dissolved in anhydrous pyridine (28 mL). To this solution, 4,4′-dimethoxytrityl chloride (1.15 g, 3.41 mmol) was added and the resulting solution was stirred at ambient temperature for 3 h. After the reaction was quenched by addition of methanol (3 mL), the solvents were removed under reduced pressure. The residue was dissolved in ethyl acetate (30 mL) and the solution was washed three times with saturated aq. $NaHCO_3$ (30 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on silica gel column with chloroform-methanol (10:1, v/v) containing 0.5% pyridine to give **3a** (1.45 g, 78%); δ_H ($CDCl_3$, 500 MHz) 1.18–1.33 (5H, br), 1,57–1.59 (1H, br), 1.72 (2H, br), 1.90 (2H, br), 2.16–2.21 (1H, m), 2.57–2.60 (1H, br), 3.39 (1H, dd, J = 3.2, 10.6), 3.47 (1H, dd, J = 3.7 Hz, 10.6 Hz), 3.62 (1H, br), 3.79 (6H, s), 4.09 (1H, d, J = 3.9 Hz), 4.44 (1H, br), 6.23 (1H, t, J = 5.7 Hz), 6.85 (4H, d, J = 8.3 Hz), 7.21–7.44 (9H, m), 8.01 (1H, d, J = 7.6 Hz), 8.57 (1H, br), 10.86 (1H, br); δ_C ($CDCl_3$) 25.2, 25.7, 33.0, 41.9, 49.4, 55.4, 62.9, 71.3, 86.2, 86.7, 87.1, 113.5, 127.3, 128.2, 128.3, 129.2, 130.1, 135.5, 135.6, 142.3, 144.3, 158.8; HRMS (ESI) m/z calcd for $C_{37}H_{42}N_4NaO_7^+$: 677.2946, found 677.2994.

4-*N*-[*N*-[3,4-(3,6,9-Trioxaundecylenedioxy)phenyl]carbamoyl]deoxycytidine (2b)

3′,5′-*O*-Bis(*t*-butyldimethylsilyl)deoxycytidine (**4b**: 1.0 g, 2.20 mmol) was dissolved in dichloromethane (22 mL). To this solution, pyridine (266 μL, 3.30 mmol) and phenyl chloroformate (330 μL, 2.64 mmol) were added, and the resulting solution was stirred at ambient temperature for 30 min. The reaction mixture was diluted with chloroform (30 mL) and washed three times with saturated $NaHCO_3$ (30 mL each). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was co-evaporated three times with anhydrous pyridine and then dissolved in anhydrous pyridine (22 mL). To this solution, 4′-aminobenzo-15-crown[5]ether (1.87 g, 6.60 mmol) was added and the resulting mixture was stirred 3 h at 70 °C. After being cooled to room temperature, the solvent was removed under reduced pressure and the residue was diluted with chloroform (30 mL). The solution was washed three times with saturated aq. $NaHCO_3$ solution (30 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was co-evaporated with three times with anhydrous pyridine and then dissolved in anhydrous pyridine (22 mL). To this solution, $3HF{\cdot}NEt_3$ (1.8 mL, 11 mmol) and NEt_3 (1.5 mL, 11 mmol) were added. The solution was stirred at ambient temperature for 1 d and the solvent removed under reduced pressure. The residue was triturated with a mixture of chloroform (10 mL) and ethyl acetate (30 mL) to give **2b** (992 mg, 84%). δ_H (DMSO, 500 MHz) 2.02–2.04 (1H, m), 2.25–2.28 (1H, m), 3.54–3.61 (10H, m), 3.74–3.78 (4H, m), 3.84–3.85 (1H, br), 4.01–4.04 (4H, br), 4.21–4.22 (1H, br), 5.07 (1H, t, J = 5.2 Hz), 5.30 (1H, d, J = 4.2 Hz), 6.12 (1H, t, J = 6.3 Hz), 6.36–6.37 (1H, br), 6.91–6.93 (2H, br), 7.18 (1H, s), 8.23 (1H, d, J = 7.6 Hz), 10.08 (1H, s), 11.16 (1H, br); δ_C (DMSO) δ 40.8, 45.8, 61.0, 68.5, 68.8, 69.0, 69.8, 69.9, 70.0, 70.4, 86.1, 87.9, 94.9, 106.6, 112.0, 114.6, 131.9, 143.9, 144.9, 148.8, 151.2, 153.5, 162.3; HRMS (ESI) m/z calcd for $C_{24}H_{32}N_4NaO_{10}^+$: 559.2011, found 559.2006.

5′-*O*-(4,4′-Dimethoxytrityl)-4-*N*-[*N*-[3,4-(3,6,9-trioxaundecylenedioxy)phenyl]carbamoyl]deoxycytidine (3b)

Compound **2b** (1.2 g, 2.24 mmol) was co-evaporated three times with anhydrous pyridine and then dissolved in anhydrous pyridine (22 mL). To this solution, 4,4′-dimethoxytrityl chloride (909 mg, 2.68 mmol) was added and the solution was stirred at ambient temperature for 3 h. After the reaction was quenched by the addition of methanol (3 mL), the solution was diluted with ethyl acetate (20 mL), washed three times with saturated aq. $NaHCO_3$ (20 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (16 g) with chloroform-methanol (99:1, v/v) to give **3b** (1.15 g, 82%). δ_H ($CDCl_3$, 500 MHz) 2.13 (1H, br), 2.51 (1H, br), 3.29–3.32 (1H, dd, $J = 3.2$ Hz, $J = 10.6$ Hz), 3.39–3.42 (1H, dd, $J = 3.7$ Hz, $J = 10.6$ Hz), 3.64–3.71 (8H, m), 3.73 (6H, s), 3.78–3.84 (4H, m), 3.98–4.13 (5H, m), 4.38 (1H, br), 6.19 (1H, t, $J = 5.9$ Hz), 6.73–6.80 (4H, m), 7.16- 7.40 (11H, m), 8.01 (1H, d, $J = 7.3$ Hz), 10.91–10.95 (2H, br); δ_C ($CDCl_3$) 41.8, 55.4, 63.1, 68.5, 69.5, 69.6, 69.8, 70.2, 70.4, 70.7, 70.8, 86.0, 86.8, 97.6, 106.0, 111.7, 113.4, 114.9, 127.1, 128.1, 128.3, 130.1, 133.9, 135.7, 135.8, 142.4, 144.4, 144.5, 149.1, 151.5, 156.5, 158.7, 164.8; HRMS (ESI) m/z calcd for $C_{45}H_{50}N_4NaO_{12}^+$: 861.3317, found 861.3315.

2′-*O*-Methyl-4-*N*-[*N*-[3,4-(3,6,9-trioxaundecylenedioxy)-phenyl]carbamoyl]cytidine (2c)

3′,5′-*O*-Bis(*t*-butyldimethylsilyl)-2′-*O*-methylcytidine (**4c**: 1.0 g, 2.1 mmol) was dissolved in dichloromethane (21 mL). To this solution, pyridine (250 μL, 3.1 mmol) and phenyl chloroformate (309 μL, 2.5 mmol) were added, and the resulting solution was stirred at ambient temperature for 30 min. The reaction mixture was diluted with chloroform (20 mL) and washed three times with saturated $NaHCO_3$ (20 mL each). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was co-evaporated three times with anhydrous pyridine and then dissolved in anhydrous pyridine (21 mL). To this solution, 4′-aminobenzo-15-crown[5]ether (2.9 g, 10.3 mmol) was added and the resulting mixture was stirred 3 h at 70 °C. After being cooled to room temperature, the solvent was removed under reduced pressure and the residue was diluted with chloroform (20 mL). The solution was washed three times with saturated aq. $NaHCO_3$ solution (20 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was co-evaporated three times with anhydrous pyridine and then dissolved in anhydrous pyridine (21 mL). To this solution was added $3HF{\cdot}NEt_3$ (1.7 mL, 10.3 mmol) and NEt_3 (1.0 mL, 10.3 mmol). The solution was stirred at ambient temperature for 1 d and the solvent removed under reduced pressure. The residue was triturated with a mixture of chloroform (10 mL) and ethyl acetate (30 mL) to give **2b** (503 mg, 43%). δ_H (DMSO, 500 MHz) 3.45 (3H, s), 3.59–3.63 (9H, m), 3.72–3.79 (6H, m), 3.88–3.89 (1H, m), 4.01–4.07 (5H, m), 5.11 (1H, d, $J = 6.8$ Hz), 5.20 (1H, t, $J = 5.0$ Hz), 5.86 (1H, d, $J = 2.7$ Hz), 6.36 (1H, br), 6.90–6.94 (2H, m), 7.16 (1H, s), 8.37 (1H, d, $J = 7.6$ Hz), 10.10 (1H, br), 11.17 (1H, br); δ_C (DMSO) 57.8, 59.5, 67.5, 68.5, 68.8, 68.9, 69.8, 69.9, 70.4, 83,6, 84.2, 87.8, 94.7, 106.5, 112.0, 114.6, 131.7, 143.9, 144.9, 148.8, 151.2, 153.2, 162.3; HRMS (ESI) m/z calcd for $C_{25}H_{34}N_4NaO_{11}^+$: 589.2116, found 589.2122.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-4-*N*-[*N*-[3,4-(3,6,9-trioxaundecylenedioxy)phenyl]carbamoyl]-deoxycytidine (3c)

Compound **2c** (450 mg, 0.79 mmol) was co-evaporated three times with anhydrous pyridine and then dissolved in anhydrous pyridine (7.9 mL). To this solution, 4,4′-dimethoxytrityl chloride (323 mg, 0.95 mmol) was added and the solution was stirred at ambient temperature for 2.5 h. After the reaction was quenched by the addition of methanol (1 mL), the solution was diluted with ethyl acetate (10 mL), washed three times with saturated aq. $NaHCO_3$ (10 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (30 g) with chloroform-hexane (5:1, v/v) to give **3c** (655 mg, 95%). δ_H ($CDCl_3$, 500 MHz) 2.51 (1H, d, $J = 10.0$ Hz), 3.53–3.55 (1H, m), 3.61–3.63 (1H, m), 3.70 (3H, s), 3.76–3.83 (18H, m), 3.90–3.92 (4H, m), 4.03–4.05 (1H, m), 4.12 (2H, t, $J = 4.5$ Hz), 4.18 (2H, t, $J = 4.5$ Hz), 4.43–4.48 (1H, m), 6.07 (1H, s), 6.78 (1H, d, $J = 8.8$ Hz), 6.86–6.88 (4H, m), 7.05- 7.08 (1H, m), 7.24–7.42 (10H, m), 8.48 (1H, d, $J = 7.8$ Hz), 10.86 (1H, s), 11.26 (1H, s); δ_C ($CDCl_3$) 55.4, 58.9, 59.0, 61.1, 68.1, 69.3, 69.8, 69.9, 70.7, 70.8, 71.1, 71.2, 83.3, 84.2, 87.3, 88.1, 88.2, 98.2, 107.9, 112.9, 113.5, 114.9, 127.3, 128.2, 128.5, 130.1, 130.3, 133.3, 135.5, 135.8, 143.2, 143.9, 145.5, 149.4, 151.6, 156.7, 158.8, 158.9, 165.2. HRMS (ESI) m/z calcd for $C_{46}H_{52}N_4NaO_{13}^+$: 891.3423, found 891.3406.

6-*N*-[*N*-[3,4-(3,6,9-Trioxaundecylenedioxy)phenyl]carbamoyl]-deoxyadenosine (2d)

3′,5′-*O*-Bis-(*t*-butyldimethylsilyl)deoxyadenosine (100 mg, 0.208 mmol) was dissolved in pyridine (2.1 mL). To this solution phenyl chloroformate (57 μL, 0.458 mmol) was added and the resulting solution was stirred at ambient temperature for 2 h. 4′-Aminobenzo-15-crown[5]ether (295 mg, 1.04 mmol) was added and the solution was stirred at 85° for 30 min. After being cooled to room temperature, the solvent was removed under reduced and the residue was dissolved in chloroform (5 mL) and washed three times with saturated aq. $NaHCO_3$ (5 mL each). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was co-evaporated twice with anhydrous pyridine and then dissolved in anhydrous pyridine (2.1 mL). To this solution $3HF{\cdot}NEt_3$ (169 μL, 1.04 mmol) and NEt_3 (144 μl, 1.04 mmol) were added. After being stirred at ambient temperature for 1 d, the solvent was evaporated under reduced pressure. The residue was dissolved in chloroform (5 mL) and the solution was washed three times with saturated aq. $NaHCO_3$ (5 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with chloroform-methanol (200:1.5, v/v) to give **2d** (63 mg, 54%). δ_H (DMSO, 500 MHz) 2.35–2.37 (1H, m), 2.74–2.77 (1H, m), 3.54–3.56 (1H, m), 3.62–3.64 (9H, m), 3.76–3.80 (4H, m), 3.90 (1H, dd, $J = 4.4$ Hz, $J = 7.6$ Hz), 4.03–4.08 (4H, m), 4.43–4.45 (1H, m), 5.02 (1H, t, $J = 5.6$ Hz), 5.35 (1H, d, $J = 4.1$ Hz), 6.45 (1H, t, $J = 6.8$ Hz), 6.94 (1H, d, $J = 8.5$ Hz), 7.10 (1H, dd, $J = 2.3$ Hz, $J = 8.5$ Hz), 7.29 (1H, d, $J = 2.3$ Hz), 8.67 (2H, s), 10.03 (1H, s), 11.61 (1H, s); δ_C (DMSO) 61.6, 68.5, 68.8, 69.0, 69.8, 70.4, 70.6, 83.8, 88.0, 106.7, 112.0, 114.6, 120.5, 132.2, 142.2, 144.7, 148.8, 150.0, 150.3, 150.8. 150.9; HRMS (ESI) m/z calcd for $C_{25}H_{32}N_6NaO_9^+$: 583.2123, found 583.2107.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*N*-[*N*-[3,4-(3,6,9-trioxaundecylenedioxy)phenyl]carbamoyl]deoxyadenosine (3d)

Compound **2d** (500 mg, 0.892 mmol) was co-evaporated three times with anhydrous pyridine and then dissolved in anhydrous pyridine (8.9 mL). To this solution DMTrCl (363 mg, 1.07 mmol) was added and the resulting solution was stirred at ambient temperature for 1 d. After the reaction was quenched by addition of methanol (1 mL), the solution was diluted with chloroform (10 mL). The solution was washed three times with saturated aq. $NaHCO_3$ (10 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on an NH–silica gel column (50 g) with chloroform-methanol (100:1, v/v) to give **3d** (547 mg, 71%). δ_H ($CDCl_3$, 500 MHz) 2.56–2.61 (1H, m), 2.83–2.88 (1H, m), 3.36–3.44 (2H, m), 3.77 (14H, s), 3.90–3.92 (4H, m), 4.12–4.19 (5H, m), 4.69–4.70 (1H, br), 6.45 (1H, t, $J = 6.3$ Hz), 6.78–6.85 (5H, m), 6.97 (1H, dd, $J = 2.4$ Hz, $J = 8.5$ Hz), 7.20–7.39 (10H, m), 8.07 (1H, br), 8.11 (1H, s), 8.51 (1H, s), 11.55 (1H, s); δ_C ($CDCl_3$) 40.1, 45.7, 55.3, 63.9, 68.8, 69.5, 69.7, 70.4, 70.5, 70.9, 72.2, 84.6, 86.3, 106.9, 112.5, 113.2, 114.8, 120.9, 127.0, 127.9, 128.2, 130.1, 130.2, 132.3, 135.8, 141.7, 144.7, 145.3, 149.3, 149.9, 150.2, 150.8, 151.4, 158.6; HRMS (ESI) *m/z* calcd for $C_{46}H_{50}N_6NaO_{11}^+$: 885.3430, found 885.3413.

6-*N*-(*N*-Cyclohexylcarbamoyl)deoxyadenosine (2e)

Compound **2e** was synthesized from **4d** (2.9 g, 4.2 mmol) in 55% yield (864 mg) according to the procedure for **2d** by use of cyclohexylamine (2.4 mL, 21 mmol) in place of 4′-aminobenzo-15-crown[5]ether. δ_H (DMSO, 500 MHz) 1.25–1.38 (5H, br), 1.54–1.56 (1H, br), 1.67–1.69 (2H, br), 1.87–1.89 (2H, br), 2.31–2.35 (1H, m), 2.71–2.76 (1H, m), 3.51–3.53 (1H, br), 3.60–3.66 (2H, br), 3.88 (1H, dd, $J = 4.4$ Hz, $J = 7.6$ Hz), 4.42–4.43 (1H, m), 5.01 (1H, br), 5.35 (1H, br), 6.42 (1H, t, $J = 6.7$ Hz), 8.55 (1H, s), 8.61 (1H, s), 9.41 (1H, d, $J = 7.6$ Hz), 9.50 (1H, s); δ_C (DMSO) 24.2, 25.3, 32.6, 48.1, 61.6, 70.7, 83.8, 88.1, 120.2, 142.1, 150.0, 150.4, 150.9, 152.6; HRMS (ESI) *m/z* calcd for $C_{17}H_{24}N_6NaO_4^+$: 399.1751, found 399.1786.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*N*-(cyclohexylcarbamoyl)-deoxyadenosine (3e)

Compound **3e** was synthesized from **2e** (300 mg, 0.80 mmol) in 60% yield (324 mg) according to the procedure for **3d**. δ_H ($CDCl_3$, 500 MHz) 1.26–1.44 (5H, br), 1.60–1.63 (1H, br), 1.73 (2H, br), 2.00–2.02 (2H, br), 2.55–2.59 (1H, m), 2.82–2.87 (1H, m), 3.36–3.43 (2H, m), 3.75 (6H, s), 3.82–3.84 (1H, br), 4.18–4.20 (1H, m), 4.68–4.69 (1H, br), 6.47 (1H, t, $J = 6.4$ Hz), 6.77 (4H, d, $J = 8.1$ Hz), 7.16–7.38 (9H, m), 8.10 (1H, s), 8.23 (1H, s), 8.43 (1H, s), 9.44 (1H, d, $J = 7.8$ Hz); δ_C ($CDCl_3$) 24.8, 25.8, 33.2, 40.3, 49.0, 55.3, 63.8, 72.4, 84.6, 86.3, 86.7, 113.3, 120.9, 127.0, 128.0, 130.1, 135.7, 135.8, 141.1, 144.6, 149.9, 150.5, 151.1, 153.2, 158.7; HRMS (ESI) *m/z* calcd for $C_{38}H_{42}N_6NaO_6^+$: 701.3058, found 701.3044.

***6*-*N*-[*N*-(*trans*-4-Hydroxycyclohexyl)carbamoyl]-3′,5′-*O*-bis(*tert*-butyldimethylsilyl)deoxyadenosine (5)**

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)deoxyadenosine (3.0 g, 6.25 mmol) and phenyl chloroformate (1.72 mL 13.8 mmol) were dissolved in pyridine (63 mL) and the solution was stirred at ambient temperature for 2 h. To this solution *trans*-4-aminocyclohexanol (3.6 g, 31.3 mmol) was added. After being stirred at 85 °C for 30 min, the mixture was cooled to ambient temperature, diluted with chloroform (50 mL), washed three times with saturated $NaHCO_3$ (50 mL), diried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was chromatographed on an NH-silica gel column (60 g) with hexane-chloroform (12 : 5, v/v) to give the title compound in 76% yield (3.0 g). δ_H (DMSO, 500 MHz) 0.08–0.11 (12H, m), 0.79–0.93 (18H, m), 1.26–1.34 (4H, m), 1.82–1.84 (2H, br), 1.94–1.96 (2H, br), 2.33–2.38 (1H, m), 2.91–2.96 (1H, m), 3.46–3.48 (1H, m), 3.55–3.57 (1H, m), 3.65 (1H, dd, $J = 4.4$ Hz, $J = 11.0$ Hz), 3.79 (1H, dd, $J = 5.9$ Hz, $J = 11.0$ Hz), 3.85–3.88 (1H, m), 4.57 (1H, dd, $J = 4.4$ Hz), 4.63–4.66 (1H, m), 6.40 (1H, t, $J = 6.6$ Hz); 8.52 (1H, s), 8.57 (1H, s), 9.35 (1H, d, $J = 7.6$ Hz), 9.54 (1H, s); δ_C (DMSO) -5.5, -5.0, -4.8, 17.7, 17.9, 25.7, 30.4, 33.5, 38.5, 48.0, 62.4, 67.8, 71.8, 83.5, 87.1, 120.3, 142.1, 150.0, 150.3, 150.7, 152.6; HRMS (ESI) *m/z* calcd for $C_{29}H_{52}N_6NaO_5Si_2^+$: 643.3430, found 643.3407.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-6-*N*-[*N*-(*trans*-4-levulinyloxycyclohexyl)carbamoyl]deoxyadenosine (6)

Compound **5** (2.2 g, 3.55 mmol) was rendered anhydrous by repeated coevaporation with anhydrous pyridine and finally dissolved in anhydrous dichloromethane (36 mL). To this solution levulinic acid (729 μg, 7.09 mmol), *N*,*N*′-dicyclohexylcarbodiimide (1.46 g, 7.09 mmol) and 4-dimethylaminopyridine (43 mg, 0.34 mmol) were added, and the resulting solution was stirred at ambient temperature for 1 h. The precipitate was removed by filtration and washed with dichloromethane (50 mL). The filtrate was washed three times with saturated aq. $NaHCO_3$ (50 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on an NH-silica gel column (50 g) with hexane-chloroform (2:1, v/v) to give **6** (1.62 g, 64%). δ_H ($CDCl_3$, 500 MHz) 0.08–0.10 (12H, m), 0.90–0.91 (18H, m), 1.47–1.57 (4H, m), 2.00–2.03 (2H, br), 2.14–2.17 (2H, br), 2.19 (3H, m), 2.43–2.48 (1H, m), 2.54–2.58 (2H, m), 2.60–2.65 (1H, m), 2.73–2.76 (2H, m), 3.76 (1H, dd, $J = 3.2$ Hz, $J = 11.2$ Hz), 3.84–3.88 (2H, m), 4.02 (1H, dd, $J = 3.4$ Hz, $J = 7.1$ Hz), 4.60–4.63 (1H, m), 4.76–4.80 (1H, m), 6.47 (1H, t, $J = 6.3$ Hz), 7.88 (1H, s), 8.27 (1H, s), 8.50 (1H, s), 9.40 (1H, d, $J = 7.6$ Hz); δ_C ($CDCl_3$) -5.4, -4.7, -4.6, 18.1, 18.5, 25.1, 25.7, 25.8, 26.0, 28.4, 29.8, 30.0, 34.0, 38.1, 41.1, 48.0, 62.8, 71.9, 72.1, 84.5, 88.1, 120.8, 141.5, 150.0, 150.4, 151.0, 153.4, 172.3, 206.8; HRMS (ESI) *m/z* calcd for $C_{34}H_{59}N_6O_7Si_2^+$: 719.3978, found 741.3752.

6-*N*-[*N*-(*trans*-4-Levulinyloxycyclohexyl)-carbamoyl]deoxyadenosine (7)

Compound **6** (1.5 g, 2.08 mmol) was rendered anhydrous by repeated coevaporation with anhydrous pyridine and finally dissolved in anhydrous pyridine (21 mL). To this solution $3HF{\cdot}NEt_3$ (1.70 mL, 10.4 mmol) and triethylamine (1.44 mL, 10.4 mmol) were added and the resulting solution was stirred at ambient temperature for 12 h. The reaction was quenched by addition of water (5 mL), the solution was concentrated under reduced pressure. The residue was dissolved in chloroform (20 mL) and washed three times with saturated aq. $NaHCO_3$ (20 mL each). The organic layer

was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a NH-silica gel column (60 g) with chloroform-methanol (200:3, v/v) to give **7** (730 mg, 72%); δ_H ($CDCl_3$, 500 MHz) 1.48–1.55 (4H, m), 2.01–2.04 (2H, br), 2.15–2.17 (2H, br), 2.20 (3H, s), 2.35–2.38 (1H, m), 2.56–2.58 (2H, t, $J = 6.6$ Hz), 2.74–2.77 (2H, t, $J = 6.6$ Hz), 3.02–3.08 (1H, m), 3.79–3.86 (2H, m), 3.96–3.99 (1H, m), 4.24 (1H, s), 4.76–4.82 (2H, m), 5.80 (1H, dd, $J = 2.2$ Hz, $J = 11.2$ Hz), 6.38 (1H, dd, $J = 5.6$ Hz, $J = 9.3$), 8.05 (1H, s), 8.09 (1H, s), 8.49 (1H, s), 9.30 (1H, d, $J = 7.3$ Hz); δ_C ($CDCl_3$) 28.4, 29.8, 30.0, 30.4, 38.1, 41.1, 48.3, 63.3, 72.1, 73.1, 87.5, 89.5, 121.9, 142.8, 149.4, 150.7, 151.0, 153.5, 172.4, 207.0; HRMS (ESI) *m/z* calcd for $C_{22}H_{30}N_6NaO_7^+$: 513.2068, found 513.2011.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*N*-[*N*-(*trans*-4-levulinyloxycyclohexyl)carbamoyl]deoxyadenosine (8)

Compound **7** (600 mg, 1.22 mmol) was rendered anhydrous by repeated co-evaporation with anhydrous pyridine and finally dissolved in anhydrous pyridine (12 mL). To this solution 4,4′-dimethoxytrityl chloride (497 mg, 1.47 mmol) was added and the solution was stirred at ambient temperature for 4 h. Methanol (3 mL) was added and the mixture was diluted with chloroform (10 mL) and washed three times with saturated aq. $NaHCO_3$ (10 mL each). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a NH-silica gel column (10 g) with hexane-chloroform (1:1, v/v) to give **8** (741 mg, 77%); δ_H ($CDCl_3$, 500 MHz) 1.45–1.60 (4H, m), 2.01–2.03 (2H, br), 2.14–2.18 (2H, br), 2.20 (3H, s), 2.54–2.58 (3H, m), 2.75 (2H, t, $J = 6.5$ Hz), 2.82–2.87 (1H, m), 3.36–3.43 (2H, m), 3.76 (6H, s), 3.84–3.86 (1H, m), 4.15–4.18 (1H, dd, $J = 4.4$ Hz, $J = 8.3$ Hz), 4.68–4.71 (1H, m), 4.77–4.81 (1H, m), 6.47 (1H, t, $J = 6.3$ Hz), 6.78 (4H, d, $J = 8.5$ Hz), 7.19–7.39 (9H, m), 8.09 (1H, s), 8.13 (1H, br), 8.43 (1H, s), 9.38 (1H, d $J = 7.6$ Hz); δ_C ($CDCl_3$) 28.5, 29.8, 30.0, 30.4, 38.1, 40.3, 48.2, 55.3, 63.8, 72.2, 72.5, 84.6, 86.3, 86.7, 113.3, 120.9, 127.1, 128.0, 128.2, 130.1, 135.7, 141.1, 144.6, 150.0, 150.4, 151.1, 153.3, 158.7, 172.4, 206.9; HRMS (ESI) *m/z* calcd for $C_{43}H_{48}N_6NaO_9^+$: 815.3375, found 815.3365.

General procedure for the synthesis of the phosphoramidite compounds: 5′-*O*-(4,4′-Dimethoxytrityl)-6-*N*-[*N*-[3,4-(3,6,9-trioxaundecylenedioxy)phenyl]carbamoyl]deoxyadenosine 3′-(2-cyanoethyl *N*,*N*-diisopropylphophoramidite) (1d)

Compound **3d** (547 mg, 0.63 mmol) was rendered anhydrous by co-evaporation with anhydrous pyridine (3 ×), anhydrous toluene (3 ×) and anhydrous CH_2Cl_2 (3 ×). The residue was then dissolved in anhydrous CH_2Cl_2 (6.3 mL). To this solution 1*H*-tetrazole (27 mg, 0.38 mol), diisopropylamine (54 μL, 0.380 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (221 μL, 0.97 mmol) were added. The solution was stirred at ambient temperature for 5 h. After the solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate-diethyl ether (10 mL, 1:10, v/v) and washed five times with 0.2 M aq. NaOH (10 mL each). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on an NH-silica gel column (20 g) with chloroform-methanol (200:1, v/v) to give **3d** (517 mg, 77%). δ_H ($CDCl_3$, 500 MHz) 1.12–1.21 (12H, m), 2.46- 2.49 (1H, m), 2.61–2.64 (1H, m), 2.70–2.74 (1H, m), 2.90–2.94 (1H, m), 3.33–3.44 (2H, m), 3.57–3.74 (4H, m), 3.77 (14H, m), 3.91–3.92 (4H, m), 4.15–4.21 (4H, m), 4.30–4.33 (1H, m), 4.76–4.81 (1H, m), 6.46–6.49 (1H, m), 6.77–6.80 (4H, m), 6.88 (1H, d, $J = 8.5$ Hz), 7.02–7.04 (1H, m), 7.20–7.40 (10H, m), 8.14 (1H, d, $J = 12.0$ Hz), 8.53 (1H, s), 11.55 (1H, br); δ_C ($CDCl_3$) 20.3, 20.4, 20.5, 20.6, 24.6, 24.7, 39.6, 43.3, 43.4, 55.3, 58.2, 58.4, 58.5, 63.4, 63.5, 69.1, 69.6, 69.8, 70.6, 70.7, 71.1, 71.2, 73.4, 73.5, 74.1, 74.2, 84.9, 86.0, 86.2, 86.6, 107.6, 113.1, 113.2, 115.1, 117.6, 117.7, 121.1, 127.0, 127.9, 128.2, 128.3, 130.2, 132.3, 135.7, 141.7, 144.6, 145.7, 149.6, 150.0, 150.2, 150.3, 150.9, 151.4, 158.6; ^{31}P NMR ($CDCl_3$) δ 149.9, 150.0. HRMS (ESI) *m/z* calcd for $C_{55}H_{67}N_8NaO_{12}P^+$: 1085.4508, found 1085.4510.

4-*N*-(*N*-Cyclohexylcarbamoyl)-5′-*O*-(4,4′-dimethoxytrityl)deoxycytidine 3′-(2-cyanoethyl *N*,*N*-diisopropylphophoramidite) (1a)

The title compound was synthesized according to the same procedure as that used for **1d**, using **3a** (200 mg, 0.31 mmol), 1*H*-tetrazole (13 mg, 0.18 mol), diisopropylamine (26 μL, 0.18 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (107 μL, 0.34 mmol), to give the product in 41% yield (106 mg). δ_H ($CDCl_3$, 500 MHz) 1.08–1.18 (12H, m), 1.23–1.35 (5H, br), 1,59–1.61 (1H, br), 1.72 (2H, br), 1.92 (2H, br), 2.17–2.23 (1H, m), 2.44–2.46 (1H, m), 2.59–2.74 (2H, m), 3.34–3.37 (1H, m), 3.43–3.49 (1H, m), 3.55–3.66 (4H, m), 3.75–3.77 (1H, m), 3.81 (6H, s), 4.24 (1H, br), 4.56–4.61 (1H, m), 6.23–6.28 (1H, m), 6.83–6.86 (4H, m), 7.22–7.42 (9H, m), 7.98–8.07 (1H, m), 8.79 (1H, br), 10.88 (1H, br); δ_C ($CDCl_3$) 20.3, 20.4, 20.5, 24.6, 24.7, 24.8, 25.3, 25.8, 29.8, 33.0, 41.1, 41.3, 43.3, 43.4, 43.5, 49.3, 55.4, 58.3, 58.4, 58.5, 62.5, 62.9, 72.8, 73.2, 85.7, 85.9, 86.8, 86.9, 97.5, 113.4, 117.5, 127.2, 127.3, 128.1, 128.3, 130.1, 130.2, 130.3, 135.4, 135.5, 135.6, 142.1, 144.3, 153.5, 156.4, 158.8, 165.0; ^{31}P NMR ($CDCl_3$) δ 149.2, 150.2. HRMS (ESI) *m/z* calcd for $C_{46}H_{59}N_6NaO_8P^+$: 877.4024, found 877.4079.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*N*-[*N*-[3,4-(3,6,9-trioxaundecylenedioxy)phenyl]carbamoyl]-deoxycytidine 3′-(2-cyanoethyl *N*,*N*-diisopropylphophoramidite) (1b)

The title compound was synthesized according to the same procedure as that used for **1d**, using **3c** (600 mg, 0.72 mmol), 1*H*-tetrazole (30 mg, 0.43 mol), diisopropylamine (61 μL, 0.43 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (250 μL, 0.79 mmol) to give the product in 78% yield (580 mg). δ_H ($CDCl_3$, 500 MHz) 1.07–1.19 (12H, m), 2.31- 2.35 (1H, m), 2.44–2.47 (1H, m), 2.56 (1H, br), 2.66–2.70 (1H, m), 3.39–3.42 (1H, m), 3.53–3.66 (5H, m), 3.72–3.80 (14H, m), 3.91 (4H, m), 4.11–4.22 (5H, m), 4.64–4.67 (1H, m), 6.31 (1H,s), 6.81–6.87 (5H, m), 7.03-7.08 (1H, m), 7.23–7.42 (9H, m), 7.49–7.52 (1H, m), 8.18–8.25 (1H, m), 10.93 (1H, s), 11.29 (1H, s); δ_C ($CDCl_3$) 20.3, 20.4, 24.7, 24.8, 41.1, 43.4, 43.5, 55.4, 58.4, 58.5, 62.1, 68.9, 69.7, 69.9, 70.6, 70.8, 71.1, 71.2, 71.8, 71.9, 85.9, 86.8, 87.1, 97.9, 107.1, 112.4, 113.5, 115.2, 117.5, 117.7, 127.3, 128.2, 128.4, 130.2, 130.3, 133.7, 135.4, 135.6, 137.2, 142.9, 144.1, 145.1, 149.4, 151.7, 156.7, 158.8, 158.9, 165.0; ^{31}P NMR ($CDCl_3$) δ 149.6, 150.0. HRMS (ESI) *m/z* calcd for $C_{54}H_{67}N_6NaO_{13}P^+$: 1061.4396, found 1061.4366.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-methyl-6-*N*-[*N*-[3,4-(3,6,9-trioxaundecylenedioxy)phenyl]carbamoyl]deoxycytidine 3′-(2-cyanoethyl *N*,*N*-diisopropylphophoramidite) (1c)

The title compound was synthesized according to the same procedure as that used for **1d**, using **3c** (600 mg, 0.69 mmol), 1*H*-tetrazole (29 mg, 0.41 mol), diisopropylamne (59 μL, 0.41 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (241 μL, 0.76 mmol) to give the product in 66% yield (485 mg). δ_H ($CDCl_3$, 500 MHz) 1.00–1.20 (12H, m), 2.39- 2.41 (1H, m), 2.59–2.61 (1H, m), 3.46–3.70 (8H, m), 3.76–3.87 (16H, m), 3.90–3.94 (5H, m), 4.12–4.18 (4H, m), 4.28–4.29 (1H, m), 4.40–4.62 (1H, m), 6.07–6.10 (1H, m), 6.77–6.80 (1H, m), 6.84–6.88 (4H, m), 7.09- 7.18 (1H, m), 7.23–7.47 (10H, m), 8.47–8.58 (1H, m), 10.90 (1H, s), 11.29 (1H, s); δ_C ($CDCl_3$) 20.3, 20.4, 24.5, 24.7, 43.3, 43.4, 55.4, 58.2, 58.4, 58.6, 58.9, 60.1, 60.8, 68.9, 69.1, 69.3, 69.5, 69.7, 69.8, 70.6, 70.7, 71.1, 81.8, 81.9, 83.2, 83.9, 87.1, 87.2, 89.1, 89.4, 98.0, 107.4, 112.5, 113.4, 114.8, 117.6, 117.7, 127.4, 128.1, 128.7, 130.3, 133.5, 135.3, 135.4, 135.6, 143.1, 143.2, 143.7, 145.2, 149.3, 151.5, 156.6, 158.8, 165.0; ^{31}P NMR ($CDCl_3$) δ 151.5, 151.8. HRMS (ESI) *m*/*z* calcd for $C_{55}H_{69}N_6NaO_{14}P^+$: 1091.4502, found 1091.4510.

5′-*O*-(4,4′-Dimethoxytrityl) -6-*N*-[*N*-(cyclohexylcarbamoyl]-deoxyadenosine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (1e)

The title compound was synthesized according to the same procedure as that used for **1d**, using **3e** (250 mg, 0.37 mmol), 1*H*-tetrazole (16 mg, 0.22 mmol), diisopropylamine (31 μL, 0.22 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (129 μL, 0.41 mmol) to give the product in 45% yield (146 mg). δ_H ($CDCl_3$, 500 MHz) 1.11–1.20 (12H, m), 1.25–1.48 (5H, br), 1.61–1.64 (1H, br), 1.71–1.76 (2H, br), 2.02–2.04 (2H, br), 2.45–2.48 (1H, m), 2.59–2.70 (2H, m), 2.89–2.93 (1H, m), 3.31–3.43 (2H, m), 3.57–3.77 (4H, m), 3.78 (6H, s), 3.84–3.89 (1H, m), 4.28–4.32 (1H, m), 4.76–4.78 (1H, m), 6.44–6.47 (1H, m), 6.76–6.80 (4H, m), 7.19–7.40 (9H, m), 7.86 (1H, br), 8.09 (1H, s), 8.11 (1H, s), 8.45 (1H, s), 9.38 (1H, d, $J = 7.6$ Hz); δ_C ($CDCl_3$) 20.2, 20.4, 24.6, 24.7, 25.7, 29.7, 33.2, 39.2, 43.3, 43.4, 48.9, 55.2, 58.3, 58.5, 63.3, 63.5, 73.3, 73.5, 74.2, 84.8, 85.8, 86.0, 86.5, 113.1, 117.5, 121.0, 126.9, 127.8, 128.2, 130.1, 135.7, 141.6, 144.6, 150.0, 150.5, 151.0, 153.1, 158.5; δ_P ($CDCl_3$) 149.4, 149.5; HRMS (ESI) *m*/*z* calcd for $C_{47}H_{59}N_8NaO_7P^+$: 901.4137, found 901.4109.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*N*-[*N*-(*trans*-4-levurlnyloxycyclohexyl)carbamoyl]-deoxyadenosine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (1f)

The title compound was synthesized according to the same procedure as that used for **1d**, using **8** (350 mg, 0.441 mmol), 1*H*-tetrazole (19 mg, 0.264 mmol), diisopropylamine (37 μL, 0.264 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (154 μL, 0.486 mmol), to give the product in 77% yield (303 mg). δ_H ($CDCl_3$, 500 MHz) 1.12–1.21 (12H, m), 1.48–1.60 (4H, m), 2.02–2.04 (2H, br), 2.15–2.17 (2H, br), 2.20 (3H, s), 2.46–2.48 (1H, m), 2.56–2.63 (3H, m), 2.74–2.77 (2H, m), 2.93–2.98 (1H, m), 3.35–3.43 (2H, m), 3.60–3.75 (4H, m), 3.77 (6H, s), 3.80–3.86 (1H, m), 4.29–4.32 (1H, m), 4.78–4.81 (2H, m), 6.43–6.46 (1H, m), 6.76–6.79 (4H, m), 7.18–7.40 (9H, m), 8.21–8.24 (1H, m), 8.34–8.36 (1H, br), 8.43 (1H, s), 9.48 (1H, d, $J = 7.6$ Hz); δ_C ($CDCl_3$) 20.2, 20.3, 20.4, 20.5, 24.5, 24.6, 24.7, 28.4, 29.7, 29.9, 30.3, 38.0, 39.3, 43.3, 43.4, 48.0, 48.2, 55.2, 55.3, 58.2, 58.3, 58.5, 63.3, 63.5, 72.1, 73.3, 73.4, 74.0, 84.8, 86.0, 86.5, 113.1, 117.5, 117.6, 121.0, 126.9, 127.9, 128.1, 128.2, 130.1, 135.7, 141.6, 144.6, 150.0, 150.4, 151.0, 153.3, 158.6, 172.3, 206.7; δ_P ($CDCl_3$) 149.7, 149.8; HRMS (ESI) *m*/*z* calcd for $C_{52}H_{65}N_8NaO_{10}P^+$: 1015.4453, found 1015.4482.

Synthesis of probes 2–6

The terminally modified 2′-*O*-methyloligoribonucleotides were synthesized on a DNA/RNA synthesizer on a 1 μmol scale by using the commercially available deoxycytidine phosphoramidite, 2′-*O*-methyl-RNA phosphoramidites, the universal supports II (Glen Research Inc.) and phosphoramidites **1a–1e**. The standard RNA synthesis protocol implemented in the DNA synthesizer was used. The cleavage of the synthesized 5′-DMTr-oligonucleotide from the solid support and the deprotection of the nucleobases were carried out by treatment with 28% aqueous ammonia at room temperature for 8 h. The solution was evaporated under reduced pressure at room temperature to remove ammonia, and the residue was diluted with 0.1 M ammonium acetate (50 mL). The solution was placed on the C18 cartridge column and the failure sequences were eluted by use of 10% CH_3CN/0.1 M ammonium acetate. After being washed with 0.1 M ammonium acetate and water, the column was treated with aqueous 2% TFA to remove the DMTr group, washed with 0.1 M ammonium acetate and water. The target oligonucleotide was eluted by use of 30% CH_3CN/water and the fractions containing the target were lyophilized to give the crude oligonucleotide. Pure material was obtained by being purified on an anion-exchange HPLC by use of 0–50% gradient of 1 M NaCl in 25 mM sodium phosphate–10% CH_3CN. The salts were removed by use of the C18 cartridge column to give the pure oligonucleotide after being lyophilized to dryness. The yields of the pure materials were 26% for the DNA and 33% for the 2′-*O*-methyl-RNA. These yields were calculated by assuming the molar extinction coefficients of dC^{chem}, dC^{bzcr}, dA^{chem} and dA^{bzcr} to be identical to those of deoxycytidine and deoxyadenosine, respectively.

Probe2: $\varepsilon_{260} = 98220$, yield: 11%, MALDI-TOF mass [M + H$^+$] calcd 3740.9, found 3738.1.

Probe3: $\varepsilon_{260} = 98220$, yield: 9%, MALDI-TOF mass [M + H$^+$] calcd 4080.9, found 4081.3.

Probe4: $\varepsilon_{260} = 98220$, yield: 1%, MALDI-TOF mass [M + H$^+$] calcd 4140.9, found 4138.1.

Probe5: $\varepsilon_{260} = 112400$, yield: 24%, MALDI-TOF mass [M + H$^+$] calcd 4128.9, found 4134.8.

Probe6: $\varepsilon_{260} = 112400$, yield: 24%, MALDI-TOF mass [M + H$^+$] calcd 3760.8, found 3766.0.

Synthesis of probes 7–9

By use of phosphoramidite **1e**, 2′-*O*-methyl-RNA phosphoramidites and universal support II, the fully protected oligonucleotide XCAACCUACUX, where X represents the Lev-protected nucleoside residue introduced by using **1f**, was elongated on the CPG supports. The CPG supports were dried under reduced

pressure and then transferred to a glass syringe equipped with a glass filter. 0.5 M NH_2NH_2 in a mixture of pyridine (60 mL) and acetic acid (40 μL) was added. After 15 min, the solution was eluted and the CPG supports were washed three times with anhydrous acetonitrile and then dried under reduced pressure. After 10 min argon gas was introduced to the syringe, and 1*H*-tetrazole (3.5 mg, 50 μmol), 2-cyanoethyl [2-(4,4′-dimethoxytrityloxy)ethyl]sulfonylethyl *N*,*N*-diisopropylphosphoramidite (32 mg, 50 μmol) and anhydrous acetonitrile (100 μL) was added. After 1 min, the solution was eluted and the CPG supports were washed three times with acetonitrile. Subsequently, the phosphite intermediate was oxidized by treatment with 0.1 M I_2 in a mixture of pyridine (450 μL) and H_2O (50 μL) for 2 min. The solution was eluted and the CPG supports were washed three times with acetonitrile, dried under reduced pressure and then treated with 2.0 M ammonia in methanol (2.0 ml) for 2 h. The solution was eluted and the eluent was concentrated under reduced pressure. The residue was dissolved in 28% aq. NH_3 and the reaction mixture was left to stand for 18 h. Ammonia was removed under reduced pressure and the material was purified on a C18 cartridge column with the standard DMTr-ON procedure. The material was further purified by an anion-exchange HPLC to give **probe7**. **Probes 8** and **9** were also synthesized by use of phosphoramidite **1f** for either the first or the last coupling.

Probe 7: $\varepsilon_{260} = 112400$, yield: 2%, MALDI-TOF mass [M + H$^+$] calcd 3950.8, found 3953.3.

Probe 8: $\varepsilon_{260} = 98600$, yield: 3%, MALDI-TOF mass [M + H$^+$] calcd 3417.7, found 3419.1.

Probe 9: $\varepsilon_{260} = 97700$, yield: 1%, MALDI-TOF mass [M + H$^+$] calcd 3417.7, found 3419.8.

T_m measurement

Each oligonucleotide was dissolved in 10 mM sodium phosphate (pH 7.0) containing 0.1 M NaCl and 0.1 mM EDTA so that the final concentration of each oligonucleotide was 1.0 μM. The solution was separated into quartz cells (10 mm) and incubated at 85 °C. After 800 s, the solution was cooled to 5 °C at a rate of 0.5 °C/min and then heated to 85 °C at the same rate. During this annealing and melting, the absorption at 260 nm was recorded every 1 °C and used to draw UV–melting curves. The T_m value was calculated as the temperature that gave the maximum of the first derivative of the UV–melting curve.

MD simulation

All MD calculation was carried out by using AMBER program[26] with the parm99.parmbsc0 force field.[26–28] First, the A-type duplex structure of 2′-*O*-methyl-(ACAACCUACUA)/r(UAGUAGGUUGU) was constructed by using the NUCGEN module. The 5′- and 3′-terminal U residues were removed and the 5′- and 3′-terminal 2′-*O*-methyl-A residues were changed to the phosphorylated cyclohexylcarbamoyldeoxyadenine (dA^{chcmp}) residues to give the model structure of the **probe7/short RNA** duplex. Twenty-nine Na^+ ions and seven Cl^- ion were added by using the addions command, and the systems were surrounded by 3856 TIP3P model waters. The cutoff distance for the non-bonding interactions was set to 9.0 Å and the electrostatic interactions were treated with the PME method.[29] After a 5000-step minimization, the systems were heated to 300 K within 0.1 ns with a positional restraint of 10 kcal/mol/Å^2 on the heavy atoms during which time the volume was kept constant. Afterwards, the system was equilibrated under a constant pressure of 1 atm for 0.2 ns at 300 K without any positional constraints. The simulation was continued for additional 10 ns at 300 K. The data of the last 5 ns were used for the analyses. The atomic charges of the aglycon part of dA^{chcmp} were calculated by using the Gaussian03 program[30] at the HF/6-31G* level.

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