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PAPER

Short-RNA selective binding of oligonucleotides modified using adenosine and guanosine derivatives that possess cyclohexyl phosphates as substituents†

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We have developed new artificial oligonucleotides which distinguish short RNA targets from long ones. The modification of the 5′ termini of oligonucleotides by using adenosine derivatives that possess a bulky cyclohexyl phosphate moiety at their base moiety and a phosphate group at the position of their 5′-hydroxyl group maximized their short RNA selectivity. The 2′-*O*-methyl-RNA (5′-X$C_mA_mA_mC_mC_mU_mA_mC_mU_m$) having these modifications exhibits *ca.* 10 °C higher T_m in the duplexes with the complementary short RNA (3′-GUUGGAUGA-5′) than with the long RNA (3′-AUUAUAUGUUGGAUGAUGGUUA-5′). The oligodeoxynucleotides having the same modification exhibited similar selectivity. Such short-RNA selective binding of terminally modified oligonucleotides can be employed to distinguish between mature microRNAs and pre-microRNAs.

Introduction

MicroRNAs (miRNAs) are noncoding RNAs approximately 22 nt in length.[1] Since the discovery of the miRNA let-7 in 1993,[2] the presence of miRNAs in animals, plants, and other living organisms has been revealed.[3] Moreover, their involvement in various biological processes such as development, differentiation, cell proliferation, apoptosis, and stress responses has been revealed.[1] Recent bioinformatic data indicate that approximately one third of all protein-coding genes are regulated by miRNAs,[4] and that they have been used as biomarkers by means of miRNA expression profiling for various diseases such as rheumatic disease,[5] cancer,[6–9] and cardiovascular disease.[10]

It is well known that miRNAs are first transcribed from genomic DNAs as pri-miRNAs, which are cleaved by the endogeneous nuclease Drosha to give pre-miRNAs that are 60–70 nt in length.[1] After being transported to the cytoplasm, pre-miRNAs are cleaved by the nuclease Dicer to give mature miRNAs. Mature miRNAs thus generated are the biologically active forms of miRNAs that bind to target mRNAs to suppress translation processes.

Studies have recently shown that pri-miRNA and pre-miRNA processing is regulated in various ways. For example, the expression levels of some mature miRNAs do not correlate with those of pri-miRNA.[11–13] In such cases, functionally inactive miRNA precursors are sometimes more abundant than functionally mature miRNAs. Moreover, the biochemical mechanisms by which the processing of pre-miRNAs to mature miRNAs is regulated have been revealed in some cases.[14–17]

Because both pre-miRNAs and mature miRNAs co-exist in the cytoplasm, distinguishing one from the other requires the use of both sequence-specific and size-specific techniques.[11,17]

In order to selectively detect biologically active mature miRNAs, several technologies have been developed.

The detection by using padlock probes with rolling circle amplification[18] and RNA-primed array-based Klenow enzyme (RAKE) assay[19] are the methods employed to selectively detect mature miRNAs by using enzymatic primer extension.

A more convenient strategy for the selective detection of mature miRNAs is to use oligonucleotides that demonstrate low affinity toward long pre-miRNAs, but high affinity toward short mature miRNAs. Such short-RNA selective binding can be achieved by the use of hairpin oligonucleotides that interfere with the binding of the long pre-miRNAs due to the steric repulsion of the stem-loop region. Such hairpin probes are used in the RT-PCR technique[20] as well as in microarray devices.[21]

Yet another differentiation strategy, which we reported previously, is to develop oligonucleotide probes capable of short-RNA selective binding by the simple modification of the 3′ and/or 5′ termini of the oligonucleotides.[22] We developed deoxyadenosine derivatives dA^{Chcm} and dA^{ChcmP}, possessing a cyclohexane ring and a phosphorylated cyclohexane ring at the base moiety and as the modification at the 3′ and 5′ termini (Fig. 1). The oligonucleotide 11mer, having two dA^{ChcmP} residues at the 5′ and 3′ termini, demonstrated higher affinity toward the complementary shorter RNA 9mer than toward the complementary longer RNA 15mer (ΔT_m = +9 °C). In addition, it was also proved that even the

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† Electronic supplementary information (ESI) available: Partial atomic charges of dG^{CmcmP}, synthetic schemes of **ON-7**, **ON-9** and **ODN-1**, and NMR charts of newly synthesized compounds. See DOI: 10.1039/c1ob06580g

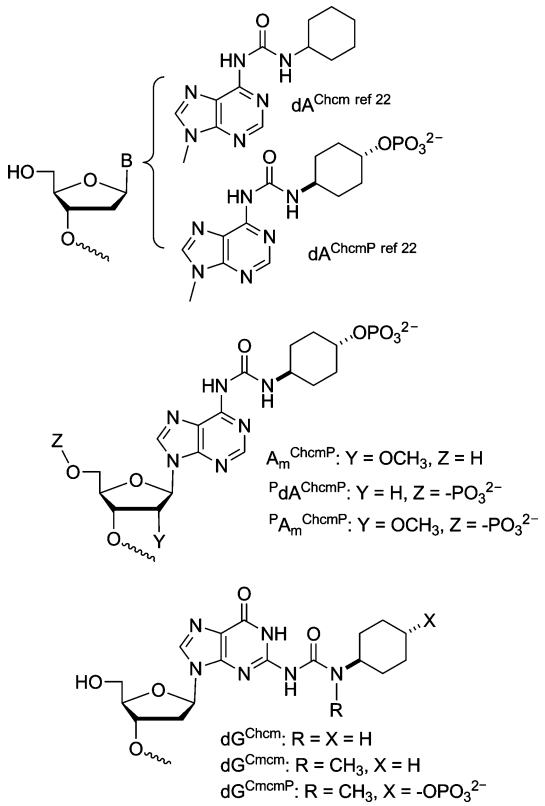


Fig. 1 Structures of adenosine-type and guanosine-type nucleoside residues modified using *N*-cyclohexylcarbamoyl groups. Chcm, Cmcm and P mean cyclohexylcarbamoyl, cyclohexyl(methyl)carbamoyl and phosphate groups, respectively.

introduction of a single dA^{ChcmP} residue at the 5′ terminus scarcely affected this behavior ($\Delta T_m = +7\,^{\circ}C$). These results suggest that the modification of the oligonucleotide termini by bulky, negatively charged residues such as dA^{ChcmP} is an effective way to develop oligonucleotides capable of short-RNA selective binding because of the steric and electrostatic repulsion between long RNAs (Fig. 2).

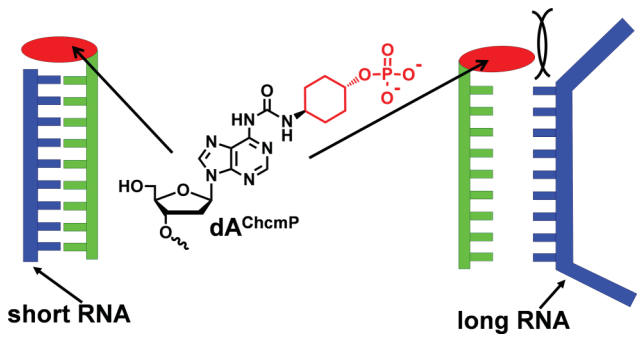


Fig. 2 Schematic illustration of the short-RNA selective binding of an oligonucleotide modified using dA^{ChcmP}.

On the basis of previous results, we approached the task of improving the short-RNA selectivity of terminally modified oligonucleotides by changing the structures of the terminal residues. We synthesized and examined the short-RNA selective binding of oligonucleotides possessing guanosine-type terminal modifications such as dG^{Chcm}, dG^{Cmcm}, and dG^{CmcmP} (Fig. 1), and compared their hybridization properties with those of previously reported dA^{ChcmP}-modified oligonucleotides. We also examined the influence of the phosphorylation at the 5′-hydroxyl group and of the 2′ methoxylation on the short-RNA selectivity by synthesizing new adenosine-type modifications such as A$_m^{ChcmP}$, ^{P}dA^{ChcmP}, and ^{P}A$_m^{ChcmP}$ (Fig. 1). We found the following: 1) Adenosine derivatives improve the short-RNA selectivity more than guanosine derivatives; and 2) ^{P}dA^{ChcmP} possessing an additional phosphate group at the 5′-position of dA^{ChcmP} improves the short-RNA selectivity even further ($\Delta T_m > 10\,^{\circ}C$).

Results and discussion

Preparation of oligonucleotides incorporating phosphoramidites of dG^{Chcm} and dG^{Cmcm}

We first designed two deoxyguanosine derivatives, dG^{Chcm} and dG^{Cmcm}, having carbamoyl and *N*-methylcarbamoyl linkages, respectively, between the 2-amino group and the cyclohexyl group (Fig. 1). It is well known that the 2-*N*-carbamoylguanine base can assume two stable conformations: an open form and a closed form (Fig. 3).[23] In the case of *N*-alkylcarbamoylguanines such as the base moiety of dG^{Chcm}, the carbonyl oxygen of the carbamoyl group can form a hydrogen bond with the imino proton at position 1, causing the carbamoyl group to block Watson–Crick-type hydrogen bonding (Fig. 3A, closed form). In addition, the carbamoyl group can form another type of hydrogen bond between the terminal NH group and the nitrogen atom at position 3, causing the carbamoyl group to extrude in the minor groove (Fig. 3A, open form).

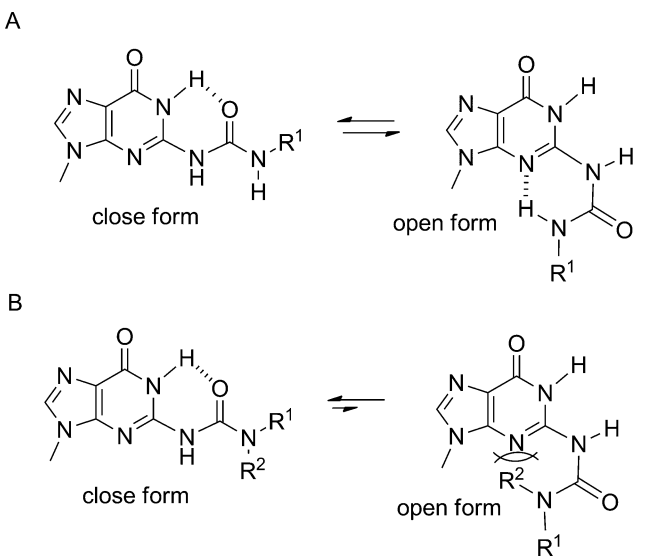


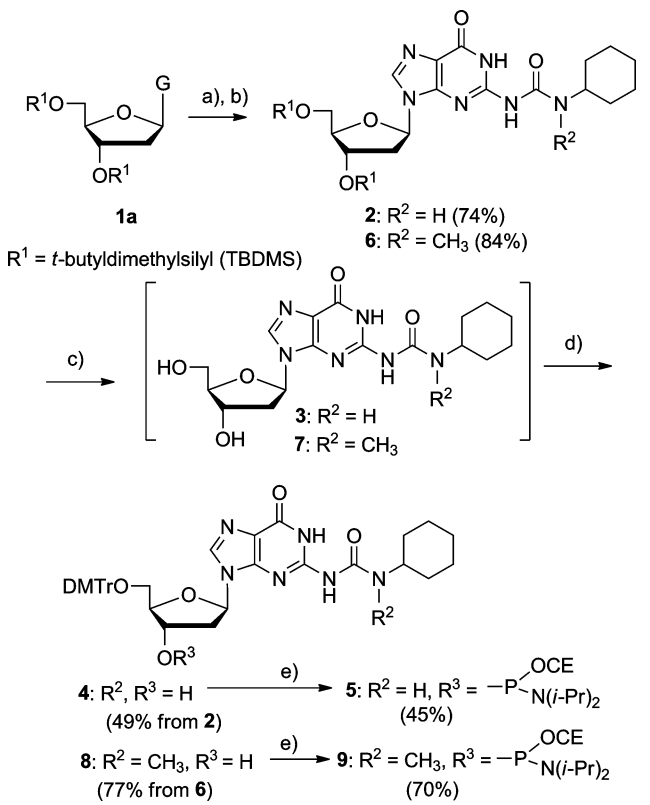
Fig. 3 Conformers: (A) Of 2-*N*-(*N*-alkylcarbamoyl)guanine; (B) Of 2-*N*-(*N*,*N*-dialkylcarbamoyl)guanine.

For the purpose of developing short-RNA selective hybridization probes, we assumed that the closed form is stabilized for the effective repulsive interaction with the counter strand because such interaction is not expected from the open form conformation.

In addition, the formation of the open form conformation is prohibited in *N*,*N*-dialkylcarbamoyl guanines such as the base

moiety of dG^{Cmcm} because of the steric repulsion between the alkyl group and the nitrogen atom at position 3 (Fig. 3B).

Thus, we speculated that *N*,*N*-dialkylcarbamoyl-type dG^{Cmcm} would be more suitable to achieve the short-RNA selective binding. To confirm this speculation, we synthesized two guanosine derivatives, dG^{Chcm} **3** and dG^{Cmcm} **7**, and their respective phosphoramidites **5** and **9** (Scheme 1). dG^{Chcm} **3** was synthesized from 3′,5′-*O*-bis(*tert*-butyldimethylsilyl)deoxyguanosine **1a**.[24] The 6-*O* position was protected by treatment with trimethylsilyl chloride, and then the amino group of **1a** was acylated with phenyl chloroformate. The phenoxycarbonyl intermediate was converted by treatment with cyclohexylamine to the fully protected dG^{Chcm} derivative **2** (74%). The TBDMS groups were removed using triethylamine trihydrofluoride[25] to give dG^{Chcm} **3** as the intermediate. The 5′-hydroxyl group of **3** was protected with a DMTr group to give **4** (49%). Finally, phosphitylation of the 3′-hydroxyl group[26] gave the phosphoramidite of dG^{Chcm} **5** (45%).



Scheme 1 Preparation of phosphoramidites of dG^{Chcm} (R^2 = H) and dG^{Cmcm} (R^2 = CH$_3$). a) Trimethylsilyl chloride, pyridine, then PhOC(O)Cl, pyridine; b) cyclohexylamine for **2** or *N*-methylcyclohexylamine for **6**; c) NEt$_3$·3HF, NEt$_3$, pyridine; d) DMTrCl, pyridine; e) 1*H*-tetrazole, diisopropylamine, CEO-P(N-*i*Pr$_2$)$_2$.

Similarly, the *N*-methylcyclohexyl derivative **6** (84%), intermediate **7**, DMTr derivative **8** (77%), and its phosphoramidite **9** (70%) were prepared using *N*-methylcyclohexylamine in place of the cyclohexylamine.

In order to study the affinities of oligonucleotides toward the shorter **RNA-9mer** and the longer **RNA-22mer**, we used phosphoramidite units of dG^{Chcm} (**5**) and dG^{Cmcm} (**9**) to synthesize terminally modified 2′-*O*-methyl-RNAs (**ON-2**, **-3**, and **-4**) having the common sequence 5′-XC$_m$A$_m$A$_m$C$_m$C$_m$U$_m$A$_m$C$_m$U$_m$Y-3′ (Fig. 4A), where X and Y represent 5′- and 3′-terminal nucleoside residues incorporated by **5** or **9**. **ON-2** incorporates dG^{Chcm} residues at both the X and Y positions, **ON-3** incorporates a dG^{Cmcm} residue at these same positions, and **ON-4** incorporates a dG^{Cmcm} residue at only the 5′ X position. We incorporated dG^{Chcm} and dG^{Cmcm} residues at the 3′ termini by using an automated DNA/RNA synthesizer by means of the usual procedure with universal support II.[27] For comparison, we also synthesized **ON-1** having unmodified deoxyguanosine at the X and Y positions.

A) 5′-X-C$_m$A$_m$A$_m$C$_m$C$_m$U$_m$A$_m$C$_m$U$_m$-Y-3′

ON-1: X = Y = dG

ON-2: X = Y = dG^{Chcm}

ON-3: X = Y = dG^{Cmcm}

ON-4: X = dG^{Chcm}, Y = none

ON-5: X = dG^{CmcmP}, Y = none

ON-6: X = dA^{ChcmP}, Y = none [ref22]

ON-7: X = A$_m$^{ChcmP}, Y = none

ON-8: X = ^{P}A$_m$^{ChcmP}, Y = none

ON-9: X = ^{P}dA^{ChcmP}, Y = none

ODN-1: 5′-^{P}dA^{ChcmP}d(CAACCTACT)-3′

B)

RNA-9mer: 3′-GUUGGAUGA-5′

RNA-22mer: 3′-AUUAUAU[GUUGGAUGA]UGGUUA-5′

Fig. 4 Sequences: (A) Of oligonucleotides **ON-1** to **ON-9** and **ODN-1**; (B) Of the RNAs to be hybridized with them. C$_m$, A$_m$, and U$_m$ denote the corresponding 2′-*O*-methylnucleotide residues; dC, dA, and dT denote deoxynucleotide residues.

Hybridization properties of oligonucleotides incorporating dG^{chcm} and dG^{cmcm}

We investigated the hybridization properties of **ON-2** (X, Y = dG^{Chcm}, dG^{Chcm}) and **ON-3** (X, Y = dG^{Cmcm}, dG^{Cmcm}) by measuring the UV melting temperature of their duplexes with the shorter **RNA-9mer** and the longer **RNA-22mer** (Fig. 4B). The results are shown in Table 1 together with associated T_m data of terminally unmodified **ON-1** (X, Y = dG, dG).

Unmodified **ON-1** (X, Y = dG, dG) showed higher affinity toward **RNA-22mer** than toward **RNA-9mer** (T_m = 49 °C and 46 °C, respectively). In contrast, modified **ON-2** (X, Y = dG^{Chcm}, dG^{Chcm}) showed yet higher affinity toward both **RNA-22mer** and **RNA-9mer** (T_m = 53 °C and 50 °C, respectively). These results show that the modification by dG^{Chcm} increased the thermal stability of the duplexes with both **RNA-9mer** and with **RNA-22mer** in comparison with the modification by dG. Neither **ON-1** (X, Y = dG, dG) nor **ON-2** (X, Y = dG^{Chcm}, dG^{Chcm}) showed selectivity toward the short **RNA-9mer** (ΔT_m = –3 °C in both cases).

In the case of the dG^{Cmcm} modified **ON-3** (X, Y = dG^{Cmcm}, dG^{Cmcm}), the T_m with **RNA-9mer** was almost identical to that of **ON-2** (X, Y = dG^{Chcm}, dG^{Chcm}) with **RNA-9mer** giving T_m = 49 °C.

Table 1 T_m [°C] for the duplexes of modified oligonucleotides with **RNA-9mer** and **RNA-22mer**

	RNA-9mer	RNA-22mer	ΔT_m[a]
ON-1 (X, Y = dG, dG)	46	49	−3
ON-2 (X, Y = dG^{Chcm}, dG^{Chcm})	50	53	−3
ON-3 (X, Y = dG^{Cmcm}, dG^{Cmcm})	49	47	+2
ON-4 (X = dG^{Cmcm}, Y = none)	46	45	+1
ON-5 (X = dG^{CmcmP}, Y = none)	47	42	+5
ON-6[22] (X = dA^{ChcmP}, Y = none)	47	40	+7
ON-7 (X = A_m^{ChcmP}, Y = none)	50	41	+9
ON-8 (X = $^{P}A_m^{ChcmP}$, Y = none)	51	40	+11
ON-9 (X = $^{P}dA^{ChcmP}$, Y = none)	50	41	+9
ODN-1 (X = $^{P}dA^{ChcmP}$, Y = none)	38	29	+9

[a] ΔT_m = T_m of **RNA-9mer** − T_m of **RNA-22mer** .

In contrast, the T_m of the duplex of **ON-3** (X, Y = dG^{Cmcm}, dG^{Cmcm}) with **RNA-22mer** was significantly decreased from 53 °C to 47 °C. It should be noted that the structural difference between **ON-2** (X, Y = dG^{Chcm}, dG^{Chcm}) and **ON-3** (X, Y = dG^{Cmcm}, dG^{Cmcm}) is just a methyl group at the guanine moiety at each terminus. This result clearly suggests that this small difference, the presence of a methyl group on the carbamoyl nitrogen, is essential for selective destabilization of the duplex with **RNA-22mer**. Accordingly, low selectivity (ΔT_m = +2 °C) toward **RNA-9mer** was observed for **ON-3** (X, Y = dG^{Cmcm}, dG^{Cmcm}).

In the case of **ON-1** (X, Y = dG, dG), its duplex with **RNA-22mer** was more stable than its duplex with **RNA-9mer**, probably because of the formation of G–U base pairs[28,29] at the 5′ and 3′ termini. Similar base pairing is possible in the duplex of **ON-2** (X, Y = dG^{Chcm}, dG^{Chcm}) with **RNA-22mer**. In contrast, dG^{Cmcm} of **ON-3** (X, Y = dG^{Cmcm}, dG^{Cmcm}) cannot pair with U in **RNA-22mer** due to the closed conformation (Fig. 3B), which decreased the T_m.

In the cases of the previously reported dA^{Chcm} and dA^{ChcmP} modification, the modification at the 5′ terminus contributed more to the selective hybridization toward **RNA-9mer**.[22] Therefore, we next measured the T_m of **ON-4** (X = dG^{Cmcm}, Y = none) having a single dG^{Cmcm} residue incorporated at the 5′ terminus. Its value for the duplex with **RNA-9mer**, T_m = 46 °C, is identical to the value for the duplex of **ON-1** (X, Y = dG, dG) with **RNA-9mer**. In contrast, the value for **ON-4** (X = dG^{Cmcm}, Y = none)/**RNA-22mer** duplex, T_m = 45 °C, is 4 °C lower than the value for the **ON-1** (X, Y = dG, dG)/**RNA-22mer** duplex. This result indicates that **ON-4** with a single modification at the 5′ termini by dG^{Cmcm} can still destabilize the duplex with **RNA-22mer**.

Interestingly, the comparison of **ON-3** (X, Y = dG^{Cmcm}, dG^{Cmcm}) with **ON-4** (X = dG^{Cmcm}, Y = none) suggests that dG^{Cmcm} at the 3′ termini contributes to the stabilization of both duplexes with **RNA-9mer** and **RNA-22mer** to a similar extent, *i.e.*, 2–3 °C. A contributing factor might be the hydrophobic interaction of the Cmcm substituent and nucleobases in the counter strands.

Hybridization properties of modified oligonucleotides incorporating 5′-terminal dG^{CmcmP}

To increase the selectivity of the dG^{Cmcm}-modified oligonucleotide toward the shorter **RNA-9mer**, we investigated the effect of introducing a phosphate group on the cyclohexane ring, expecting to observe a similar improvement in the short-RNA selectivity, as was observed for phosphorylation of dA^{ChcmP}.[22] In order to incorporate the phosphorylated dG^{Cmcm} derivative dG^{CmcmP} (Fig. 1), we synthesized the corresponding phosphoramidite **13a** (Scheme 2) as follows.

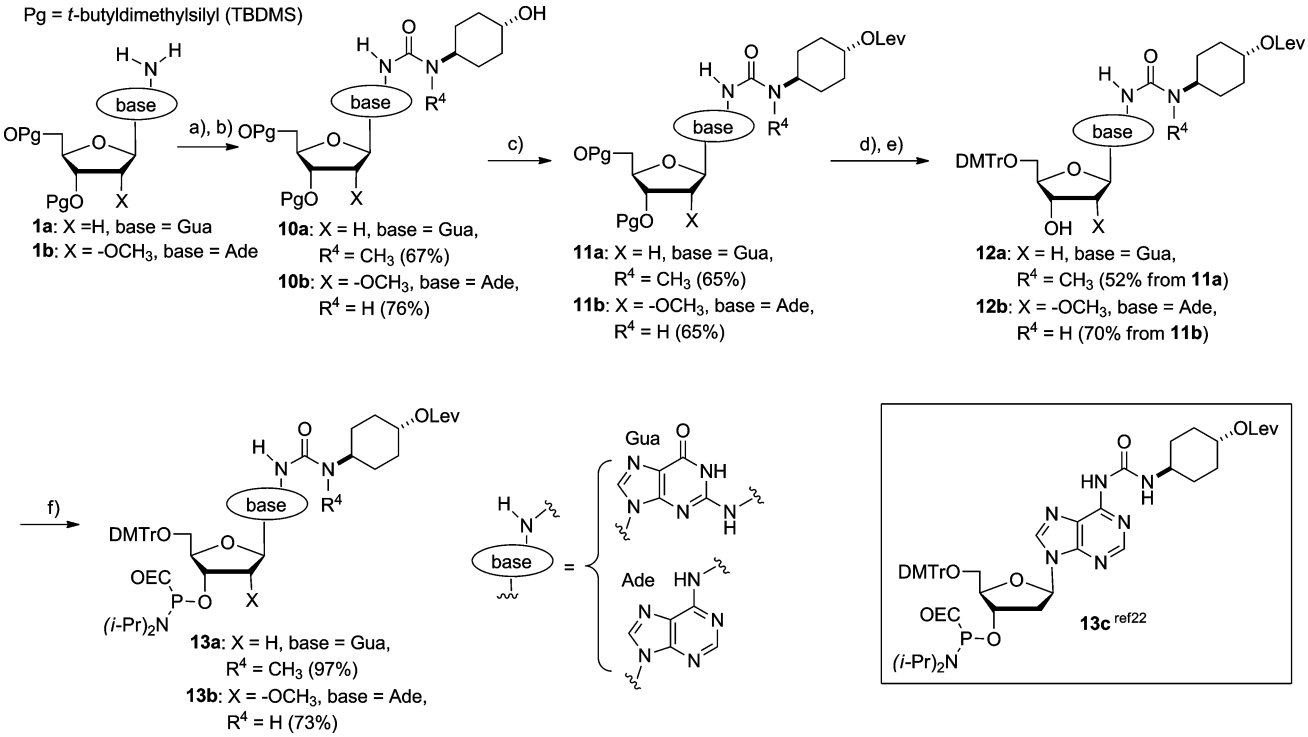
Compound **1a** was treated with phenyl chloroformate, and subsequently with *trans*-4-hydroxy-*N*-methylcyclohexylamine to give the carbamoyl derivative **10a** (67%). The protection of the hydroxyl group by the levulinoyl (Lev) group gave **11a** (65%). The removal of the TBDMS group by $NEt_3 \cdot 3HF$ and subsequent protection of the 5′-hydroxyl group by the DMTr group gave **12a** (52%). Finally, the usual phosphitylation of the 3′-hydroxyl group[26] gave the phosphoramidite **13a** (97%).

We synthesized modified oligonucleotide **ON-5** (X = dG^{CmcmP}, Y = none) having a dG^{ChcmP} residue at the 5′ terminus (Scheme 3). The protected 2′-*O*-methyl-RNA precursor with its nucleobases and the internucleotidic phosphates protected by acyl-type and cyanoethyl protecting groups, respectively, was synthesized on controlled pore glass (CPG). The phosphoramidite **13a** was coupled to the 5′-hydroxyl group of the protected 2′-*O*-methyl-RNA precursor, and then oxidized in a 0.1 M I_2 solution to give the terminally modified oligonucleotide **14a**. The levulinoyl group of **14a** was removed by treatment with hydrazine–acetic acid solution to give **15a**. The liberating hydroxyl group was phosphorylated using a phosphoramidite-type phosphorylating agent[30] to give the fully protected precursor **16a** of the 2′-*O*-methyl-RNA having dG^{CmcmP} at the 5′ termini. Finally, all protecting groups were removed by successive treatment with aqueous NH_3 and 2% aqueous CF_3COOH to give **ON-5** (X = dG^{CmcmP}, Y = none).

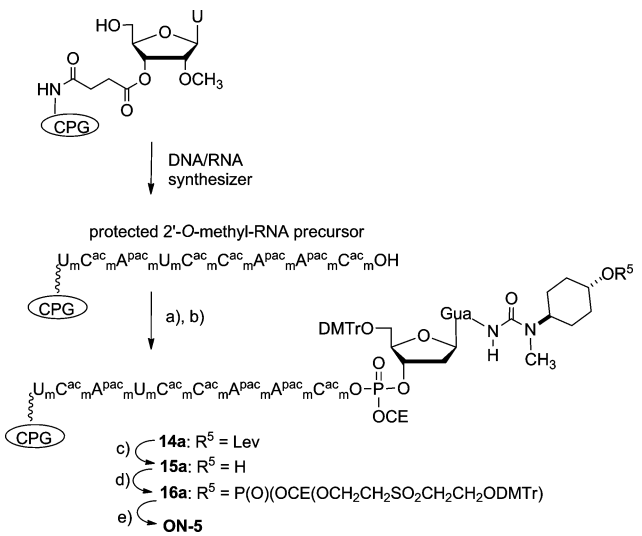
We studied the hybridization behavior of **ON-5** (X = dG^{CmcmP}, Y = none) with the shorter **RNA-9mer** and the longer **RNA-22mer**. Comparison with **ON-4** (X = dG^{Cmcm}, Y = none) (Table 1) shows that incorporation of a phosphate group on the cyclohexane ring reduced the T_m for the duplex with **RNA-22mer** (from T_m = 45 °C to 42 °C) but had essentially no effect on the T_m for the duplex with **RNA-9mer** (T_m = 47 °C in both cases). As a result, the short-RNA selectivity was improved (ΔT_m = +5 °C).

Qualitatively, this trend is in accordance with that reported previously for **ON-6** (X = dA^{ChcmP}, Y = none) having a dA^{ChcmP} at the 5′ termini.[22] Thus, we have clarified that the incorporation of a phosphate group on the cyclohexane ring improves short-RNA selective binding not only for adenosine-type dA^{ChcmP} modification but also for guanosine-type dG^{CmcmP} modification. However, as shown in Table 1, selectivity is higher for **ON-6** having dA^{ChcmP} than that for **ON-5** (X = dG^{CmcmP}, Y = none) whose ΔT_m were +7 °C and +5 °C, respectively. Thus, the deoxyadenosine derivative dA^{ChcmP} is superior to the deoxyguanosine derivative dG^{CmcmP} for improving the short-RNA selectivity.

The low affinities of **ON-5** (X = dG^{CmcmP}, Y = none) and **ON-6** (X = dA^{ChcmP}, Y = none) toward the longer **RNA-22mer** can be explained by considering the structure of the 5′ termini of the modified oligonucleotides in the duplex state. Shown in Fig. 5 are the structures of the terminal residues of the previously reported



Scheme 2 Preparation of phosphoramidites of dG^{ChcmP} and A_m^{ChcmP}. a) Trimethylsilyl chloride, pyridine, then PhOC(O)Cl, pyridine; b) *trans*-4-hydroxy-*N*-methylcyclohexylamine; c) DCC, levulinic acid, DMAP, CH_2Cl_2; d) $NEt_3 \cdot 3HF$, THF; e) DMTrCl, pyridine; f) 1*H*-tetrazole, diisopropylamine, CEO-P(N-*i*Pr_2)$_2$, CH_2Cl_2 for **13a** or diisopropylethylamine, CEO-P(Cl)(N-*i*Pr_2), CH_2Cl_2, for **13b**.



Scheme 3 Preparation of **ON-5** incorporating dG^{CmcmP}. a) **13a**, 1*H*-tetrazole, CH_3CN, then 0.1 M DMAP, Ac_2O–pyridine (1 : 9, v/v); b) 0.1 M I_2, pyridine–H_2O; c) 0.5 M NH_2NH_2/pyridine–AcOH (3 : 2, v/v); d) (*i*-Pr)$_2$N-P(OCE)(OCH$_2$CH$_2$SO$_2$CH$_2$CH$_2$ODMTr), 1*H*-tetrazole, CH_3CN, then 0.1 M I_2/pyridine–H_2O (9 : 1, v/v); e) 28% aq. NH_3, then 2% aq. TFA on a C-18-cartridge column.

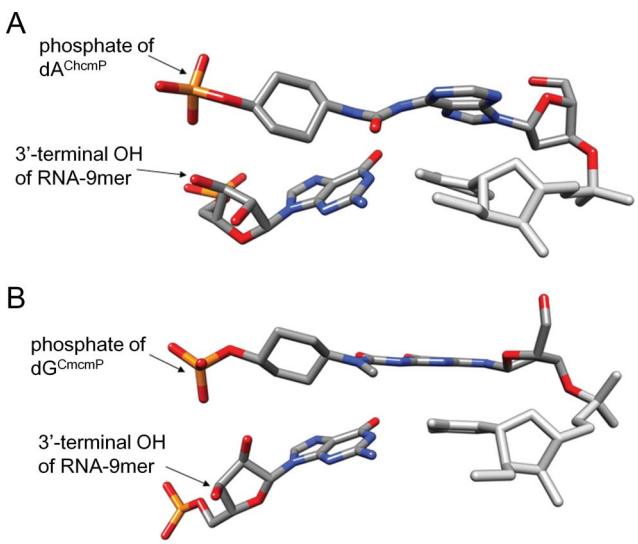


Fig. 5 Structure of the terminal residues of the modified oligonucleotide/**RNA-9mer** duplexes obtained by molecular dynamics simulations: A) dA^{ChcmP}-modified **ON-6**/**RNA-9mer** duplex; B) dG^{CmcmP}-modified **ON-5**/**RNA-9mer** duplex.

ON-6 (X = dA^{ChcmP}, Y = none)/**RNA-9mer** (Fig. 5A) and **ON-5** (X = dG^{CmcmP}, Y = none)/**RNA-9mer** duplex (Fig. 5B) obtained by molecular dynamics simulation by using the AMBER[31] program and the parameters for the 2′-*O*-methyl backbone.[32] The phosphate group of the dA^{ChcmP} interacts with the 3′-terminal hydroxyl group of **RNA-9mer** (Fig. 5A). Thus, in the duplex with **RNA-22mer** having additional phosphodiester backbone extended from this 3′-hydroxyl group, strong steric and electrostatic repulsions are expected between the phosphate of dA^{ChcmP} and the phosphodiester backbone of **RNA-22mer**. In contrast, in the case of the **ON-5** (X = dG^{CmcmP}, Y = none)/**RNA-9mer** duplex modified by dG^{CmcmP}, the phosphate group interacts more strongly with the 2′-OH of the terminal residue (Fig. 5B). Comparison of Fig. 5A and 5B

suggests that we should expect dA^{ChcmP} to destabilize the duplex with **RNA-22mer** more effectively than dG^{CmcmP} because of the effective repulsion between the phosphate on the cyclohexane ring and the phosphodiester backbone of **RNA-22mer** extended from the terminal 3′-hydroxyl group.

Improvement of short-RNA selective binding by 2′-methoxylation and 5′-phosphorylation of dA^{ChcmP}

As mentioned above, we clarified that adenosine-type dA^{ChcmP} effectively improves the short-RNA selectivity more than guanine-type dG^{CmcmP}. Therefore, we investigated further the effect on the short-RNA selectivity of oligonucleotides modified with dA^{ChcmP} derivatives.

We designed A$_m$^{ChcmP} (Fig. 1) having the 2′-*O*-methylribose instead of the 2′-deoxyribose of dA^{ChcmP}. In addition, we designed the 5′-phosphate derivatives ^{P}dA^{ChcmP} and ^{P}A$_m$^{ChcmP} (Fig. 1).

It is well known that 2′-*O*-methyladenosine assumes predominantly the N-type sugar conformation.[33,34] It has also been suggested that the phosphorylation of the 5′-hydroxyl group of adenosine reduces the flexibility of the glycosidic conformation.[35] Thus, we expected that the 5′-terminal nucleotide residues such as A$_m$^{ChcmP}, ^{P}dA^{ChcmP}, and ^{P}A$_m$^{ChcmP}, having relatively inflexible sugar and/or glycosidic conformations, should interact effectively with the counter strand.

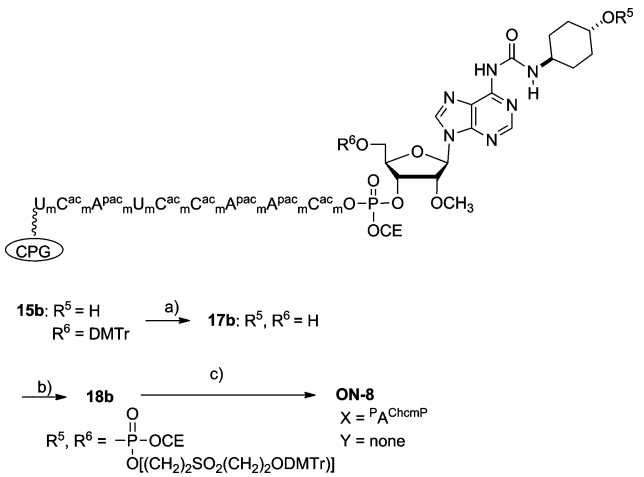
In order to introduce A$_m$^{ChcmP} and ^{P}A$_m$^{ChcmP}, we prepared the 2′-*O*-methyladenosine phosphoramidite **13b** (Scheme 2) in a manner that is almost identical to that for the guanosine derivative **13a**. Starting from 3′,5′-*O*-bis-TBDMS-2′-*O*-methyladenosine **1b**,[36] a (*trans*-4-hydroxylcyclohexylamino)carbonyl group was introduced to the amino group of the adenine moiety to give **10b**. The protection of the hydroxyl group by the Lev group gave **11b**. Further conversion to the DMTr-protected derivative **12b** and phosphitylation with 2-cyanoethyl-*N*,*N*-diisopropylphosphorochloridite gave the phosphoramidite derivative **13b**.

We prepared the oligonucleotide **ON-7** (X = A$_m$^{ChcmP}, Y = none) as described for **ON-5** (X = dG^{ChcmP}, Y = none) but by using phosphoramidite **13b** in place of **13a** (Scheme S1†).

We also synthesized 5′-phosphorylated **ON-8** (X = ^{P}A$_m$^{ChcmP}, Y = none) as follows (Scheme 4). Starting from the 5′-DMTr oligonucleotide **15b**, which is the synthetic intermediate of **ON-7**, the DMTr group was removed by treatment with 3% DCA/CH_2Cl_2 to give **17b**. The hydroxy groups at the 5′ position and the cyclohexane ring were phosphitylated simultaneously by using the aforementioned phosphoramidite-type phosphitylating agent to give **18b**. Finally, deprotection and cleavage from the solid supports were carried out by treatment with 3% DCA/CH_2Cl_2 and aqueous ammonia to give **ON-8** (X = ^{P}A$_m$^{ChcmP}, Y = none).

We prepared **ON-9** (X = ^{P}dA^{ChcmP}, Y = none) having 5′-phosphorylated deoxyadenosine-type modification at the 5′ terminus (Scheme S2†), as described for **ON-8**, but using previously reported phosphoramidite of dA^{ChcmP} (**13c** in Scheme 2).[22]

We evaluated the hybridization properties of **ON-7** (X = A$_m$^{ChcmP}, Y = none), **ON-8** (X = ^{P}A$_m$^{ChcmP}, Y = none), and **ON-9** (X = ^{P}dA^{ChcmP}, Y = none) by examining their duplexes with the shorter **RNA-9mer** and the longer **RNA-22mer** (Table 1). Comparison of the data for **ON-6** (X = dA^{ChcmP}, Y = none) and **ON-7** (X = A$_m$^{ChcmP}, Y = none) shows that the introduction of the 2′-methoxy group to the ribose moiety increased the stability of the duplex with **RNA-9mer** by 3 °C (from T_m = 47 °C to T_m = 50 °C) but left the stability of the duplex with **RNA-22mer** essentially unchanged (T_m = 40–41 °C). As a result, the short-RNA selectivity of **ON-7** (X = A$_m$^{ChcmP}) (ΔT_m = +9 °C) increased.



Scheme 4 Preparation of base and 5′-bisphosphorylated **ON-8**. a) 3% dichloroacetic acid/CH_2Cl_2; b) (*i*-Pr)$_2$N-P(OCE)(OCH$_2$CH$_2$SO$_2$-CH$_2$CH$_2$ODMTr), 1*H*-tetrazole, CH_3CN, then 0.1 M I_2/pyridine–H_2O (9 : 1, v/v); c) 28% aq. NH_3, then 2% aq. TFA on a C-18-cartridge column.

We then evaluated the effect of 5′ phosphorylation by comparing **ON-7** (X = A$_m$^{ChcmP}, Y = none) and **ON-8** (X = ^{P}A$_m$^{ChcmP}, Y = none). For **ON-7** (X = A$_m$^{ChcmP}, Y = none) and **ON-8** (X = ^{P}A$_m$^{ChcmP}, Y = none), the 5′ phosphorylation of the 2′-*O*-methyladenosine residue changed the T_m of their duplexes with both **RNA-9mer** and **RNA-22mer** slightly within 1 °C; the short-RNA selectivity for the 5′-phosphorylated **ON-8** (X = ^{P}A$_m$^{ChcmP}, Y = none) was comparable to or somewhat larger than that for **ON-7** (X = A$_m$^{ChcmP}, Y = none) with ΔT_m = +11 °C and +9 °C, respectively. In contrast, in the comparison of **ON-6** (X = dA^{ChcmP}, Y = none) and **ON-9** (X = ^{P}dA^{ChcmP}, Y = none), the 5′ phosphorylation of the deoxyadenosine-type terminal residue increased the T_m of their duplexes with **RNA-9mer** by 3 °C (from T_m = 47 °C to T_m = 50 °C) but had little effect on the T_m of their duplexes with **RNA-22mer**. Thus, the short-RNA selectivity of **ON-9** (X = ^{P}dA^{ChcmP}, Y = none) (ΔT_m = +9 °C) became comparable to that of **ON-7** (X = A$_m$^{ChcmP}, Y = none) and **ON-8** (X = ^{P}A$_m$^{ChcmP}, Y = none) modified with 2′-*O*-methyladenosine derivatives.

Among A$_m$^{ChcmP}-, ^{P}A$_m$^{ChcmP}-, and ^{P}dA^{ChcmP}-modified oligonucleotides, all of which demonstrated comparable short-RNA selectivity, we selected ^{P}dA^{ChcmP} for further studies because its starting material is 2′-deoxyadenosine which is more conveniently available than 2′-*O*-methyladenosine, the starting material of A$_m$^{ChcmP} and ^{P}A$_m$^{ChcmP}.

First, we evaluated the short-RNA selectivity of ^{P}dA^{ChcmP} incorporated into the DNA backbone. We synthesized the oligodeoxynucleotide **ODN-1** having the ^{P}dA^{ChcmP}d-(CAACCTACUT) sequence (Scheme S2†) in a manner similar to that described for **ON-8** and **ON-9** but by using the canonical deoxynucleoside phosphoramidites in place of the 2′-*O*-methyl-RNA phosphoramidites.

As shown in Table 1, the T_m of the duplexes of oligodeoxynucleotide **ODN-1** (X = ^{P}dA^{ChcmP}, Y = none) with **RNA-9mer**

(T_m = 38 °C) and **RNA-22mer** (T_m = 29 °C) are lower than those of the 2′-*O*-methyl-RNA derivative **ON-9** (X = $^{P}dA^{ChcmP}$, Y = none) by more than 10 °C. However, the short-RNA selectivity of **ODN-1** (X = $^{P}dA^{ChcmP}$, Y = none) is identical to that of **ON-9** (X = $^{P}dA^{ChcmP}$, Y = none) (ΔT_m = +9 °C in both cases). Thus, the $^{P}dA^{ChcmP}$ modification is clearly useful for the development of both 2′-*O*-methyl-RNA-type and DNA-type oligonucleotides that are capable of short-RNA selective binding.

Sequence dependence of short-RNA selective binding for oligodeoxynucleotides modified by $^{P}dA^{ChcmP}$

In order to expand the applicability of the $^{P}dA^{ChcmP}$-modified oligonucleotide, we evaluated the sequence dependence of the short-RNA selective binding by using oligodeoxynucleotides modified by $^{P}dA^{ChcmP}$. We focused on the two base pairs adjacent to the terminal $^{P}dA^{ChcmP}$, expecting that the structures and stabilities of these base pairs should affect interactions between $^{P}dA^{ChcmP}$ and the counter strands. We synthesized **ODN-2** through to **ODN-7** (Fig. 6A) by changing the one or two bases adjacent to $^{P}dA^{ChcmP}$ and investigated the hybridization properties with the corresponding target **9mer-X$_1$X$_2$** and **22mer-X$_1$X$_2$** (Fig. 6B), where X$_1$X$_2$ were designed to be complementary to each ODN.

A)

5′ ⟶ 3′

$^{P}dA^{ChcmP}d(N_1N_2ACCTACT)$

ODN-1: N_1N_2 = CA **ODN-5**: = CC
ODN-2: = TA **ODN-6**: = TT
ODN-3: = CT **ODN-7**: = TG
ODN-4: = CG

B)

RNA-9mer-X$_1$X$_2$: 3′-X$_1$X$_2$UGGAUGA-5′
RNA-22mer-X$_1$X$_2$: 3′-AUUAUAU[X$_1$X$_2$GGAUGA]UGGUUA-5′

X$_1$X$_2$ = GU for **ODN-1** GG for **ODN-5**
AU for **ODN-2** AA for **ODN-6**
GA for **ODN-3** AC for **ODN-7**
GC for **ODN-4**

Fig. 6 Sequences for evaluation of the sequence dependence of short-RNA selectivity: A) Of ODNs modified with $^{P}dA^{ChcmP}$; B) Of target RNAs. Nucleotide residues different from those of **ODN-1** are underlined.

The T_m data are shown in Table 2; for comparison, they include data for **ODN-1**, already shown in Table 1. First, we examined the influence of the nucleotide residue N$_1$ adjacent to $^{P}dA^{ChcmP}$. When N$_1$ changed from dC to T, as in the case of **ODN-2** {N$_1$N$_2$ = d(TA)}, T_m of the duplexes with **9mer-AU** and **22mer-AU** decreased (from T_m = 30 °C to 25 °C), reducing the short-RNA selectivity (ΔT_m = +5 °C). This result clearly suggests that the base pair at the N$_1$-X$_1$ position affects both the T_m and the ΔT_m values.

Next, we examined the influence of N$_2$, the second neighbor of $^{P}dA^{ChcmP}$, by fixing N$_1$ to the dC of **ODN-1**. The data of **ODN-3** {N$_1$N$_2$ = d(CT)}, **-4** {N$_1$N$_2$ = d(CG)}, and **-5** {N$_1$N$_2$ = d(CC)} (ΔT_m = +7, +8, and +9 °C, respectively) show that duplexes having different base pairs at the second neighbor base–base pair N$_2$–X$_2$ exhibited significant short-RNA selectivities.

The data for **ODN-6** {N$_1$N$_2$ = TT} and **ODN-7** {N$_1$N$_2$ = d(TG)} show comparable short-RNA selectivities (ΔT_m = +7 °C). Thus, the collective data for **ODN-1** to **ODN-7** show that their short-RNA selectivities, as measured by ΔT_m, clearly depend on the sequence at least at the N$_1$N$_2$ position, and are all significantly large, between +5 °C and +9 °C.

Table 2 T_m [°C] of the duplexes of modified oligodeoxynucleotides, 5′-$^{P}dA^{ChcmP}N_1N_2ACCTACT$-3′, and the corresponding **RNA-9mer-X$_1$X$_2$** or **RNA-22mer-X$_1$X$_2$** having the common sequence 3′-X$_1$X$_2$UGGAUGA-5′

	RNA-9mer (X$_1$X$_2$)	**RNA-22mer** (X$_1$X$_2$)	ΔT_m[a]
ODN-1	38	29	+9
N$_1$N$_2$ = d(CA)	(GU)	(GU)	
ODN-2	30	25	+5
N$_1$N$_2$ = d(TA)	(AU)	(AU)	
ODN-3	40	33	+7
N$_1$N$_2$ = d(CT)	(GA)	(GA)	
ODN-4	44	36	+8
N$_1$N$_2$ = d(CG)	(GC)	(GC)	
ODN-5	47	38	+9
N$_1$N$_2$ = d(CC)	(GG)	(GG)	
ODN-6	33	26	+7
N$_1$N$_2$ = d(TT)	(AA)	(AA)	
ODN-7	40	33	+7
N$_1$N$_2$ = d(TG)	(AC)	(AC)	

[a] ΔT_m = T_m of **RNA-9mer**-X$_1$X$_2$ − T_m of **RNA-22mer**-X$_1$X$_2$.

Conclusion

We investigated the effect on the oligonucleotide binding of guanosine-type terminal modifications by dG^{Chcm} and dG^{Cmcm}. The incorporation of a single methyl group on the 2-N position of the guanine ring significantly reduced the oligonucleotide affinity toward the longer **RNA-22mer** but had essentially no effect on its affinity toward the shorter **RNA-9mer**. This result suggests the importance of conformational restriction around the carbamoyl group of dG^{Cmcm}.

We also investigated the effect on the oligonucleotide binding of guanosine-type terminal modifications by dG^{CmcmP} with a phosphate moiety on the cyclohexane ring. dG^{CmcmP} improved the short-RNA selectivity of the modified oligonucleotide in comparison with dG^{Cmcm}, but lowered the short-RNA selectivity to a value lower than that previously reported for adenosine-type dA^{ChcmP}.

We further modified the termini by introducing a 2′-methoxy or 5′-phosphate group onto the adenosine-type modification. The 5′-phosphate derivative $^{P}dA^{ChcmP}$ significantly improved the short-RNA selectivity of the modified oligonucleotide in comparison with dA^{ChcmP}, raising the short-RNA selectivity to as high as ΔT_m = 10 °C.

The modification by $^{P}dA^{ChcmP}$ at the 5′ terminus of both 2′-*O*-methyl-RNA and oligodeoxynucleotide increases the relative binding affinity toward short RNA. Currently, the chain length of the 5′-modified oligonucleotides is 9nt. Oligonucleotide probes of this length should be capable of recognizing the 9 nucleotide residues of the 3′ terminal of miRNAs.

It is well known that the 5′-terminal sequence of a miRNA family is the seed sequence that binds to a target mRNA and is common to all family members.[37] Thus, oligonucleotide probes that recognize the 3′-terminal region are expected to be useful for detection of mature miRNAs in a variant-specific manner. Because

both 2′-*O*-methyl-RNA and oligodeoxynucleotides modified by ^{P}dA^{ChcmP} maintain the value of short-RNA selectivity, these probes should be useful in the development of microarray-related and RT-PCR related techniques for detecting miRNA. Such applications of our probes will be reported elsewhere.

Experimental section

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2-*N*-cyclohexylcarbamoyldeoxyguanosine (2)

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)deoxyguanosine (**1a**)[24] (2.5 g, 5.0 mmol) was rendered anhydrous by repeated co-evaporation three times with dry pyridine, and finally dissolved in dry pyridine (50 mL). Trimethylsilyl chloride (1.9 mL, 15 mmol) was added and the solution was stirred at ambient temperature for 10 min. Phenyl chloroformate (953 μL, 7.6 mmol) was added and the solution was stirred at ambient temperature for 3 h. Subsequently, cyclohexylamine (3.4 mL, 30 mmol) was added and the mixture was stirred for 2 h. The solvents were removed under reduced pressure, and the residue was diluted with ethyl acetate (150 mL), washed three times with saturated aq. $NaHCO_3$ (100 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a column of silica gel using chloroform–methanol (30 : 1, v/v) as eluent to give **2** (2.3 g, 74%). ^{1}H NMR (DMSO-d_6, 500 MHz): δ = 0.03–0.09 (12H, m), 0.85–0.87 (18H, m), 1.21–1.23 (3H, m), 1.31–1.34 (2H, m), 1.51 (1H, m), 1.63 (2H, m), 1.80–1.82 (2H, m), 2.28–2.30 (1H, m), 2.65–2.68 (1H, m), 3.54 (1H, br), 3.63–3.71 (2H, m), 3.84 (1H, m), 4.48 (1H, m), 6.12–6.13 (1H, m), 7.14 (1H, s br), 8.09 (1H, s), 9.76 (1H, s br), 11.79 (1H, s br); ^{13}C NMR (DMSO-d_6, 126 MHz): δ = –5.5, –5.5, –5.0, –4.8, 17.7, 18.0, 24.0, 25.1, 25.6, 25.8, 32.3, 47.9, 62.6, 72.0, 82.7, 87.2, 115.2, 119.2, 129.4, 136.4, 148.8, 153.8; HRMS (ESI): *m*/*z* calcd for $C_{29}H_{52}N_6NaO_5Si_2^+$ [M+Na]$^+$: 643.3430, found 643.3428.

5′-*O*-(4,4′-Dimethoxytrityl)-2-*N*-cyclohexylcarbamoyldeoxyguanosine (4)

Compound **2** (1.8 g, 2.9 mmol) was rendered anhydrous by repeated co-evaporation three times with dry pyridine, and finally dissolved in dry pyridine (14 mL). Triethylamine trihydrofluoride (1.90 mL, 11.6 mmol) and triethylamine (1.60 mL, 11.6 mmol) were added, and the solution was stirred at ambient temperature for 23 h. After the solvents were removed under reduced pressure, the residue was chromatographed on a column of silica gel using chloroform–methanol (9 : 1, v/v) as eluent to give the crude intermediate **3** (1.95 g). A portion (480 mg) of crude **3** was collected and dissolved in dry pyridine (12 mL). 4,4′-Dimethoxytrityl chloride (624 mg, 1.84 mmol) was added and the mixture was stirred at ambient temperature for 1.5 h. The reaction was quenched by addition of methanol (3 mL), and the mixture was diluted with ethyl acetate (60 mL). The solution was washed three times with saturated aq. $NaHCO_3$ (40 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a column of silica gel using chloroform–methanol (49 : 1, v/v) as eluent to give **4** (742 mg, 49%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.34 (5H, m), 1.52 (1H, m), 1.68 (2H, m), 1.93 (2H, m), 2.61 (2H, m), 3.28 (2H, m), 3.69 (3H, s), 3.73–3.74 (1H, m), 4.16 (1H, m), 4.64 (1H, m), 6.27 (1H, m), 6.70–6.72 (4H, m), 7.10–7.24 (7H, m), 7.34 (2H, m), 7.79 (1H, s); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 24.9, 25.6, 29.7, 32.9, 41.0, 49.0, 55.1, 64.1, 71.6, 77.4, 84.2, 86.3, 113.0, 119.4, 126.8, 127.8, 128.2, 130.0, 135.7, 144.6, 149.2, 155.1, 158.4; HRMS (ESI): *m*/*z* calcd for $C_{38}H_{42}N_6NaO_7^+$ [M+Na]$^+$: 717.3007, found 717.3027.

5′-*O*-(4, 4′-Dimethoxytrityl)-2-*N*-cyclohexylcarbamoyl-deoxyguanosine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (5)

Compound **4** (400 mg, 0.58 mmol) was rendered anhydrous by repeated co-evaporation three times each with dry pyridine, dry toluene and dry CH_2Cl_2 and finally dissolved in dry CH_2Cl_2 (5.8 mL). 1*H*-Tetrazole (25 mg, 0.35 mmol), diisopropylamine (50 μL, 0.35 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (203 μL, 0.64 mmol) were added, and the solution was stirred at ambient temperature for 22 h. The reaction was quenched by addition of methanol (2 mL), and the solvents were removed under reduced pressure. The residue was dissolved in diethylether (30 mL), and washed three times with 0.1 M NaOH (20 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a column of silica gel using chloroform–methanol (200 : 1, v/v) to give **5** (230 mg, 45%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 0.97–1.08 (12H, m), 1.23–1.34 (5H, m), 1.47 (1H, m), 1.61 (2H, m), 1.87 (2H, m), 2.25 (1H, m), 2.32 (1H, m), 2.51 (1H, m), 2.60 (1H, m), 3.11–3.12 (2H, m), 3.17–3.25 (1H, m), 3.33–3.56 (1H, m), 3.48–3.49 (2H, m), 3.67 (6H, m), 4.12–4.16 (1H, m), 4.63 (1H, m), 6.20 (1H, m), 6.69–6.71 (4H, m), 7.09–7.21 (7H, m), 7.29–7.31 (2H, m), 7.72–7.75 (1H, m); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 20.3, 20.3, 20.5, 20.5, 24.6, 24.7, 24.7, 25.8, 29.8, 33.3, 40.1, 43.3, 43.3, 43.4, 43.4, 45.9, 48.6, 55.3, 55.4, 58.1, 58.3, 63.5, 73.2, 73.7, 73.8, 84.0, 85.6, 85.8, 86.5, 113.2, 113.2, 117.9, 120.0, 127.0, 127.9, 128.2, 128.3, 129.1, 130.1, 130.2, 130.2, 135.7, 135.7, 135.8, 144.6, 144.6, 148.7, 148.9, 154.5, 158.6; ^{31}P NMR ($CDCl_3$, 203 MHz): δ = 150.0; HRMS (ESI): *m*/*z* calcd for $C_{47}H_{58}N_8O_8^-$ [M-H]$^-$: 893.4121, found 893.4094.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2-*N*-(*N*-cyclohexyl-*N*-methylcarbamoyl)deoxyguanosine (6)

Compound **1a** (2.5 g, 5.0 mmol) was rendered anhydrous by repeated co-evaporation three times with dry pyridine, and finally dissolved in dry pyridine (50 mL). Trimethylsilyl chloride (1.6 mL, 13 mmol) was added and the solution was stirred at ambient temperature for 1 min. Phenyl chloroformate (953 μL, 7.6 mmol) was added and the solution was stirred at ambient temperature for 4 h. Subsequently, *N*-methylcyclohexylamine (6.7 mL, 50 mmol) was added and the mixture was stirred for 24 h. The solvents were removed under reduced pressure, and the residue was diluted with ethyl acetate (100 ml), washed three times with saturated aq. $NaHCO_3$ (100 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a column of silica gel using hexane–ethyl acetate (3 : 2, v/v) as eluent to give **6** (2.7 g, 84%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = –0.07 to –0.05 (12H, m), 0.75–0.77 (18H, m), 0.95–0.97 (1H, m), 1.27 (4H, m), 1.53–1.55 (3H, m), 1.69 (2H, m), 2.18–2.21 (1H, m), 2.28–2.32 (1H, m), 2.82 (3H, s), 3.62–3.67 (2H, m), 3.78–3.79

(1H, m), 3.93 (1H, br), 4.42–4.43 (1H, m), 6.01–6.03 (1H, t, J = 6.0 Hz), 7.79 (1H, s), 8.13 (1H, s br), 12.38 (1H, s br); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = –5.7, –5.6, –5.1, –4.9, 17.7, 18.2, 25.1, 25.3, 25.5, 25.7, 28.5, 29.9, 41.1, 54.1, 62.3, 71.0, 77.4, 83.0, 83.0, 87.3, 120.2, 135.9, 148.4, 148.9, 154.0, 155.7; HRMS (ESI): m/z calcd for $C_{30}H_{54}N_6NaO_5Si_2^+$ $[M+Na]^+$: 657.3586, found 657.3604.

5′-*O*-(4,4′-Dimethoxytrityl)-2-*N*-(*N*-cyclohexyl-*N*-methylcarbamoyl)deoxyguanosine (8)

According to a similar procedure for **4**, compound **6** (2.0 g, 3.15 mmol) was first converted to the crude intermediate **7** (1.42 g) by treatment with triethylamine trihydrofluoride (2.1 mL, 12.6 mmol) for 6.5 h, and column chromatography by using chloroform–methanol (50 : 1, v/v). Subsequently, **7** (1.0 g) was converted to **8** (1.2 g, 77%) by treatment with 4,4′-dimethoxytrityl chloride (1.12 g, 3.31 mmol) for 1 h and purification by column chromatography using chloroform–methanol (99 : 1, v/v).[1] ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.04 (1H, m), 1.31 (4H, m), 1.62 (3H, m), 1.76 (2H, m), 2.49–2.55 (2H, br), 2.64 (3H, s), 3.26–3.30 (2H, br), 3.69 (7H, m), 4.08 (1H, m), 4.23 (1H, m), 4.67 (1H, m), 5.43 (1H, m), 6.20 (1H, m), 6.72–6.74 (4H, br), 7.10–7.38 (9H, br), 7.76 (1H, s), 12.76 (1H, s); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 25.2, 25.4, 28.3, 29.8, 40.6, 54.2, 55.0, 63.9, 71.6, 77.4, 83.9, 86.1, 86.5, 113.0, 120.0, 123.8, 126.7,127.7, 129.8, 135.6, 136.2, 136.7,144.6, 149.0, 149.2, 149.3, 154.6, 156.1, 158.3; HRMS (ESI): m/z calcd for $C_{39}H_{44}N_6NaO_7^+$ $[M+Na]^+$: 731.3164, found 731.3177.

5′-*O*-(4,4′-Dimethoxytrityl)-2-*N*-(*N*-cyclohexyl-*N*-methylcarbamoyl)deoxyguanosine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (9)

According to a similar procedure for **5**, compound **8** (710 mg, 1.0 mmol) was converted to the phosphoramidite **9** (642 mg, 70%) by treatment with 1*H*-tetrazole (42 mg, 0.60 mmol), diisopropylamine (85 µL, 0.60 mmol) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (350 µL, 1.1 mmol) for 5 h, and column chromatography using chloroform–methanol (200 : 1, v/v). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.10–1.17 (12H, m), 1.20–1.42 (5H, m), 1.60–1.65 (3H, m), 1.68 (3H, s), 1.79–1.81 (2H, m), 2.17 (1H, m), 2.39 (1H, m), 2.44 (1H, m), 2.62–2.64 (1H, m), 3.17–3.22 (1H, m), 3.32–3.41 (1H, m),3.55–3.62 (2H, m), 3.68–3.73 (1H, m), 3.77 (6H, s), 3.83–3.92 (1H, m), 4.00 (1H, br), 4.22–4.27 (1H, m), 4.68–4.72 (1H, m), 6.20 (1H, m), 6.80 (4H, m), 7.19–7.38 (7H, m), 7.44–7.48 (2H, m), 7.73 (1H, s), 12.34 (1H, br); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 20.1, 20.3, 21.3, 22.4, 22.8, 23.5, 23.8, 24.4, 25.2, 27.6, 27.8, 29.5, 29.8, 39.5, 39.7, 43.0, 54.0, 54.5, 55.0, 55.3, 55.7, 58.0, 58.2, 63.0, 63.3, 73.3, 73.9, 84.0, 84.3, 84.5, 85.6, 86.2, 112.9, 113.2, 117.5, 117.7, 120.8, 121.0, 126.7, 126.9, 127.9, 129.9, 135.6, 136.3, 136.5, 136.7, 144.6, 148.5, 148.8, 153.8, 154.0, 155.8, 158.4; ^{31}P NMR ($CDCl_3$, 203 MHz): δ = 149.4, 149.7; HRMS (ESI): m/z calcd for $C_{48}H_{62}N_8O_8P^+$ $[M+H]^+$: 909.4423, found 909.4424.

Synthesis of modified 2′-*O*-methyl-RNA (ON-2, ON-3 and ON-4)

The phosphoramidites of dG^{Chcm} (**5**) or dG^{Cmcm} (**9**) were dissolved in dry acetonitrile (0.1 M) and loaded on the automated RNA synthesizer. The synthesis was initiated from the universal support II (1.0 µmol) for **ON-2** and **ON-3** or from the solid support of 2′-*O*-methyluridine (0.1 µmol) for **ON-4**. The standard DMTr-ON RNA synthesis protocol consisting of chain elongation, removal of the DMTr group, capping and oxidation was used. The oligonucleotides were first cleaved from the solid support by using 2.0 M ammonia/methanol (2 mL) for 1 h. After the solution was collected, the solvents were removed under reduced pressure and the residue was treated with 28% aqueous ammonia (2 mL) for 8 h. The ammonia was removed under reduced pressure, and the solution was diluted by the addition of 0.1 M ammonium acetate (10 mL). The solution was charged on a C-18 cartridge column, washed with 10% acetonitrile–0.1 M ammonium acetate, treated with aqueous 2% TFA and washed with 0.1 M ammonium acetate. After an additional wash with water, the oligonucleotides were eluted by using 30% acetonitrile–water. The pure oligonucleotides were obtained by purification on an anion-exchange HPLC (gradient of 0–62% of 1 M NaCl in 25 mM NaH_2PO_4, pH 6.0 in 35 min). Finally, the phosphate salts were removed on a C-18 cartridge column by using water as eluent. Finally, the pure oligonucleotides were eluted by using 30% acetonitrile–water. The yields of oligonucleotides were calculated assuming that the extinction coefficients of the oligonucleotides were identical to those of oligoribonucleotides having ribonucleotide residues in place of 2′-*O*-methyl-ribonucleotide residues and guanosine in place of the terminal modified deoxyguanosines. **ON-2**: ε_{260} = 104 200, MALDI-TOF mass $[M + H^+]$ calcd 3792.8, found 3794.7; **ON-3**: ε_{260} = 104 200, MALDI-TOF mass $[M + H^+]$ calcd 3820.9, found 3821.0; **ON-4**: ε_{260} = 94 100; MALDI-TOF mass $[M + H^+]$ calcd 3352.7, found 3354.0.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2-*N*-[*N*-(*trans*-4-hydroxycyclohexyl)-*N*-methylcarbamoyl]deoxyguanosine (10a)

Compound **1a** (2.5 g, 5.0 mmol) was rendered anhydrous by repeated co-evaporation three times with dry pyridine, and finally dissolved in dry pyridine (100 mL). Trimethylsilyl chloride (5.1 ml, 40 mmol) was added and the solution was stirred at ambient temperature for 20 min. Phenyl chloroformate (953 µL, 7.5 mmol) was added and the solution was stirred at ambient temperature for 3 h. The solution was diluted with ethyl acetate (200 mL) and then washed once with saturated aq. $NaHCO_3$ (150 mL). The organic layer was collected and concentrated under reduced pressure. The residue was dissolved in pyridine (100 mL), and *trans*-4-hydroxy-*N*-methylcyclohexylamine (4.9 g, 38 mmol) was added. After being stirred at ambient temperature for 16 h, the solvent was removed under reduced pressure and the residue was diluted with ethyl acetate (200 ml), washed three times with saturated aq. $NaHCO_3$ (150 ml), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with chloroform–methanol (50 : 1, v/v) to give **10a** (6.57 g, 67%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 0.03 (12H, m), 0.84–0.86 (18H, m), 1.48 (4H, m), 1.65 (2H, m), 2.04 (2H, m), 2.28–2.30 (1H, m), 2.35–2.41 (1H, m), 2.85 (3H, s), 3.55 (1H, br), 3.71 (2H, m), 3.89–3.90 (1H, m), 4.02 (1H, br), 4.50 (1H, m), 6.13–6.15 (1H, t, J = 6.3 Hz), 7.87 (1H, s); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = –5.5, –5.4, –4.8, –4.7, 18.0, 18.4, 25.7, 25.8, 26.0, 28.2, 34.2, 41.4, 53.5, 62.7, 69.4, 71.7, 77.4, 83.4, 87.8, 120.4, 136.2, 148.7, 149.0, 154.2, 156.0; HRMS (ESI): m/z calcd for $C_{30}H_{54}N_6NaO_6Si_2^+$ $[M+Na]^+$: 673.3536, found 673.3526.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-2-*N*-[*N*-(*trans*-4-levulinyloxycyclohexyl)-*N*-methylcarbamoyl]deoxyguanosine (11a)

Compound **10a** (4.2 g, 6.45 mmol) was rendered anhydrous by repeated co-evaporation with dry pyridine, and the residue was dissolved in dry CH_2Cl_2 (65 mL). Levulinic acid (1.31 mL, 12.9 mmol), *N*, *N*′-dicyclohexylcarbodiimide (2.66 g, 12.9 mmol) and 4-dimethylaminopyridine (79 mg, 0.645 mmol) were added and the resulting solution was stirred at ambient temperature for 1 h. The precipitates were removed by filtration and washed with CH_2Cl_2 (50 mL). The filtrate was washed three times with saturated aq. $NaHCO_3$ (100 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 (99 : 1, v/v) to give **11a** (3.15 g, 65%). ^{1}H NMR (CDCl3, 500 MHz): δ = 0.01–0.04 (12H, m), 0.84–0.85 (18H, m), 1.51 (4H, m), 1.71 (2H, m), 2.02 (2H, m), 2.15 (3H, s), 2.26–2.31 (1H, m), 2.35–2.40 (1H, m), 2.49–2.52 (2H, m), 2.69–2.71 (2H, m), 2.86 (3H, s), 3.70 (2H, m), 3.88 (1H, d, J = 3.0 Hz), 4.12 (1H, br), 4.50 (1H, m), 4.58 (1H, br), 6.11 (1H, t, J = 6.3 Hz), 7.85 (1H, s); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = −5.5, −5.4, −4.8, −4.7, 18.0, 18.4, 25.7, 25.9, 27.5, 28.3, 28.7, 29.9, 30.3, 37.9, 41.3, 52.8, 62.6, 71.6, 72.0, 77.4, 83.3, 87.8, 120.5, 136.2, 148.4, 148.8, 154.1, 155.8, 172.3, 206.8; HRMS (ESI): m/z calcd for $C_{35}H_{60}N_6NaO_8Si_2^+$ $[M+Na]^+$: 771.3903, found 771.3906.

5′-*O*-(4,4′-Dimethoxytrityl)-2-*N*-[*N*-(*trans*-4-levulinyloxycyclohexyl)-*N*-methylcarbamoyl]deoxyguanosine (12a)

Compound **11a** (3.0 g, 4.0 mmol) was dissolved in tetrahydrofuran (20 mL). Triethylamine trihydrofluoride (2.6 mL, 16 mmol) was added and the resulting solution was stirred at ambient temperature for 20 h. The solvents were removed under reduced pressure, and the residue was dissolved in chloroform–methanol (60 mL; 5 : 1, v/v). The solution was washed once with 5% aq. ammonium chloride (40 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with chloroform–methanol (19 : 1, v/v) to give the crude material of the 3′,5′-unprotected intermediate (1.23 g). This material was used for the synthesis of **12a** without further purification. 540 mg of the crude material was rendered anhydrous by repeated co-evaporation with dry pyridine, and finally dissolved in dry pyridine (10 mL). 4,4′-Dimethoxytrityl chloride (491 mg, 1.45 mmol) was added and the solution was stirred at ambient temperature for 2 h. The reaction was quenched by addition of water (2 ml), and the solution was diluted with ethyl acetate (50 mL). The solution 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 a silica gel column with chloroform–methanol (99 : 1, v/v) to give the crude material of **12a** (751 mg, 52% from **11a**). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.45 (4H, m), 1.65 (2H, m), 1.99 (2H, m), 2.17 (3H, s), 2.34 (4H, m), 2.51–2.54 (2H, t, J = 6.0 Hz), 2.61 (1H, m), 2.71–2.73 (2H, t, J = 6.3 Hz), 3.20–3.21 (1H, d, J = 6.0 Hz), 3.29–3.30 (1H, d, J = 8.0 Hz), 3.71 (6H, s), 4.14 (1H, m), 4.30 (1H, m), 4.57 (1H, m), 4.63 (1H, m), 6.14 (1H, m), 6.73–6.74 (4H, m), 7.10–7.13 (1H, m), 7.17–7.19 (2H, m), 7.26 (4H, m), 7.38–7.40 (2H, m), 7.71 (1H, s), 12.49 (1H, s br); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 27.4, 28.1, 28.4, 29.0, 30.0, 30.4, 38.0, 38.8, 40.6, 52.8, 55.3, 64.0, 68.2, 72.0, 72.1, 77.4, 84.2, 86.3, 86.5, 113.3, 120.7, 127.0, 128.0, 128.1, 128.9, 130.1, 131.0, 135.8, 135.9, 137.1, 144.9, 149.0, 154.5, 156.1, 158.6, 172.3, 206.9; HRMS (ESI): m/z calcd for $C_{44}H_{50}N_6NaO_{10}^+$ $[M+Na]^+$: 845.3481, found 845.3476.

5′-*O*-(4,4′-Dimethoxytrityl)-2-*N*-[*N*-(*trans*-4-levulinyloxycyclohexyl)-*N*-methylcarbamoyl]deoxyguanosine 3′-(2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite) (13a)

Compound **12a** (740 mg, 0.90 mmol) was rendered anhydrous by repeated co-evaporation three times each with dry pyridine, dry toluene, dry CH_2Cl_2 and finally dissolved in dry CH_2Cl_2 (9 mL). To this solution, 1*H*-tetrazole (38 mg, 0.54 mmol), diisopropylamine (76 μL, 0.54 mmol) and 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (314 μL, 0.99 mmol) were added, and the solution was stirred at ambient temperature for 3.5 h. After the reaction was quenched with methanol (2 mL), the solution 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 dissolved in chloroform (2 mL) and then poured into diisopropyl ether–hexane (20 mL; 1 : 3, v/v). The resulting precipitates were collected by filtration to give **13a** (895 mg, 97%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.10–1.24 (12H, m), 1.37–1.71 (7H, m), 1.95–2.08 (3H, m), 2.20 (3H, s), 2.22–2.32 (1H, br), 2.41–2.44 (1H, m), 2.48–2.56 (2H, m), 2.60–2.65 (1H, m), 2.74–2.90 (2H, m), 3.16–3.22 (1H, m), 3.31–3.43 (1H, m), 3.54–3.60 (2H, m), 3.68–3.73 (1H, m), 3.76 (6H, s), 3.81–3.90 (1H, m), 4.08 (1H, br), 4.21–4.25 (1H, m), 4.58–4.60 (1H, m), 4.65–4.70 (1H, m), 6.18 (1H, m), 6.80 (4H, m), 7.20–7.39 (7H, m), 7.42–7.50 (2H, m), 7.75 (1H, s), 12.25 (1H, br); ^{13}C NMR ($CDCl_3$ 126 MHz): δ = 20.4, 20.6, 24.7, 27.4, 27.5, 27.9, 28.4, 30.0, 30.4, 38.1, 39.6, 40.0, 43.3, 43.4, 52.7, 55.4, 58.1, 58.3, 58.4, 63.6, 72.1, 73.6, 73.7, 74.3, 74.5, 84.7, 84.9, 85.8, 86.0, 86.4, 86.5, 113.4, 117.5, 117.7, 121.6, 121.9, 127.1, 128.1, 130.1, 135.8, 135.9, 136.1, 136.9, 137.3, 145.0, 145.2, 148.5, 148.6, 148.8, 153.9, 154.2, 155.9, 158.8, 172.4, 206.8; ^{31}P NMR ($CDCl_3$, 203 MHz): δ = 149.2, 149.6; HRMS (ESI): m/z calcd for $C_{53}H_{68}N_8O_{11}P^+$: 1023.4740, found 1023.4745.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-6-*N*-[*N*-(*trans*-4-hydroxycyclohexyl)carbamoyl]-2′-*O*-methyladenosine (10b)

Compound **1b**[36] (2.6 g, 5.2 mmol) was rendered anhydrous by repeated co-evaporation three times with dry pyridine, and finally dissolved in dry pyridine (50 mL). Phenyl chloroformate (1.4 mL, 11.4 mmol) was added and the solution was stirred at ambient temperature for 45 min, and *trans*-4-hydroxycyclohexylamine (3.0 g, 25.8 mmol) was added. After being stirred at 85 °C for 2 h, the solvent was removed under reduced pressure and the residue was diluted with chloroform (50 ml). The filtrate was washed three times with saturated aq. $NaHCO_3$ (30 ml), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with chloroform–methanol (100 : 1, v/v) to give **10b** (2.6 g, 76%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 0.03–0.07 (12H, m), 0.85–0.87 (18H, m), 1.34–1.46 (4H, m), 1.98–2.00 (2H, br), 2.09–2.11 (2H, br), 3.22 (1H, s), 3.43 (3H, s), 3.67–3.72 (1H, m), 3.74–3,77 (2H, m), 3.92–3.95 (1H, m), 4.06–4.07 (1H, m), 4.16 (1H, t, J = 4.5 Hz), 4.50 (1H, t, J = 5.0 Hz), 6.14 (1H, d, J = 4.0 Hz), 8.43 (1H, s), 8.46 (1H, s), 8.98 (1H, s),

9.45 (1H, d, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = −5.3, −5.2, −4.6, −4.5, 18.3, 18.6, 25.9, 26.2, 31.0, 33.9, 43.6, 58.6, 62.1, 69.6, 70.0, 83.9, 85.3, 86.9, 120.9, 141.8, 150.1, 150.8, 151.2, 153.7; HRMS (ESI): m/z calcd for $C_{30}H_{53}N_6O_6Si_2^-$ [M–H]$^-$: 649.3571, found 649.3562.

3′,5′-*O*-Bis(*tert*-butyldimethylsilyl)-6-*N*-[*N*-(*trans*-4-levulinyloxycyclohexyl)carbamoyl]-2′-*O*-methyladenosine (11b)

Compound **10b** (2.1 g, 3.2 mmol) was rendered anhydrous by repeated co-evaporation with dry pyridine, and the residue was dissolved in dry CH_2Cl_2 (30 mL). Levulinic acid (0.72 mL, 6.3 mmol), *N*,*N*′-dicyclohexylcarbodiimide (1.3 g, 6.3 mmol) and 4-dimethylaminopyridine (40 mg, 0.32 mmol) were added and the resulting solution was stirred at ambient temperature for 21 h. The precipitates were removed by filtration and washed with chloroform (50 mL). The filtrate 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 a silica gel column with chloroform–methanol (4 : 1, v/v) to give **11b** (1.56 g, 65%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 0.10–0.11 (12H, m), 0.93–0.94 (18H, m), 1.45–1.53 (4H, m), 2.01–2.03 (2H, m), 2.15–2.17 (2H, m), 2.19 (3H, m), 2.57–2.58 (2H, m), 2.74–2.76 (2H, m), 3.48 (3H, s), 3.77–3.80 (1H, m), 3.84 (1H, m), 3.98–4.01 (1H, m), 4.12–4.15 (2H, m), 4.52 (1H, t, J = 4.9 Hz), 4.77–4.81 (1H, m), 6.17 (1H, d, J = 3.9 Hz), 7.84 (1H, s), 8.31 (1H, s), 8.51 (1H, s), 9.39 (1H, d, J = 7.6 Hz); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = −5.2, −4.6, −4.4, 18.4, 26.0, 26.2, 28.6, 29.9, 30.1, 30.6, 38.2, 58.7, 62.1, 69.9, 72.3, 85.5, 86.9, 101.4, 121.1, 141.3, 150.1, 151.4; HRMS (ESI): m/z calcd for $C_{35}H_{59}N_6O_8Si_2^-$ [M–H]$^-$: 747.3938, found 747.3943.

5′-*O*-(4, 4′-Dimethoxytrityl)-6-*N*-[*N*-(*trans*-4-levulinyloxycyclohexyl)carbamoyl]-2′-*O*-methyladenosine (12b)

Compound **11b** (1.6 g, 2.1 mmol) was rendered anhydrous by repeated co-evaporation with dry pyridine, and the residue was dissolved in pyridine (20 mL). Triethylamine trihydrofluoride (1.8 mL, 11 mmol) and triethylamine (1.5 mL, 11 mmol) were added and the resulting solution was stirred at ambient temperature for 3 days. The solvents were removed under reduced pressure, and the residue was dissolved in chloroform (50 mL). The solution was washed three times with aq. $NaHCO_3$ (30 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 (98 : 2, v/v) to give the crude material of the 3′,5′-free derivative (0.98 g, 98%). This material (0.98 g, 1.9 mmol) was rendered anhydrous by repeated co-evaporation with dry pyridine, and finally dissolved in dry pyridine (10 mL). 4,4′-Dimethoxytrityl chloride (0.77 g, 2.3 mmol) was added and the solution was stirred at ambient temperature for 3 h. The reaction was quenched by addition of water (1 mL), and the solution was diluted with chloroform (50 mL). 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 a silica gel column with hexane–chloroform (1 : 4, v/v) to give the crude material of **12b** (1.1 mg, 70% from **11b**).^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.47–1.53 (4H, m), 2.01–2.03 (2H, m), 2.15–2.17 (2H, m), 2.56–2.61 (3H, m), 2.74–2.77 (2H, m), 3.42–3.44 (1H, m), 3.51–3.52 (1H, m), 3.57 (3H, s), 3.79 (6H, s), 4.20 (1H, s), 4.39–4.40 (1H, m), 4.50–4.51 (1H, m), 4.79 (1H, m), 6.17 (1H, d, J = 3.42), 6.81 (4H, d, J = 8.30), 7.22–7.43 (9H, m), 7.87 (1H, s), 8.14 (1H, s), 8.45 (1H, s), 9.37 (1H, d, J = 7.57); ^{13}C NMR ($CDCl_3$, 126 MHz): δ = 28.6, 29.9, 30.1, 30.5, 38.2, 48.2, 55.4, 59.1, 63.2, 70.0, 72.3, 83.5, 84.4, 86.8, 86.9, 113.4, 121.1, 127.2, 128.1, 128.4, 130.3, 135.8, 135.9, 144.8, 150.1, 150.6, 151.3, 153.4, 158.8, 172.5, 206.9; HRMS (ESI): m/z calcd for $C_{44}H_{50}N_6NaO_{10}^+$ [M+Na]$^+$: 845.3481, found 845.3507.

5′-*O*-(4,4′-Dimethoxytrityl)-6-*N*-[*N*-(*trans*-4-levulinyloxycyclohexyl)carbamoyl]- 2′-*O*-methyladenosine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (13b)

Compound **12b** (250 mg, 0.30 mmol) was rendered anhydrous by repeated co-evaporation three times each with dry pyridine, dry toluene, dry CH_2Cl_2 and finally dissolved in dry CH_2Cl_2 (4.4 mL). To this solution, diisopropylethylamine (261 µL, 1.5 mmol) and chloro-2-cyanoethoxydiisopropylaminophosphine (177 mg, 0.75 mmol) were added, and the solution was stirred at ambient temperature for 6 h. The reaction was quenched by addition of water (1 mL), and the solution was diluted with chloroform (50 mL). The solution was washed three times with saturated aq. Na_2CO_3 (30 mL each), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was trituated with diisopropyl ether–diethyl ether (50 mL; 1 : 1, v/v), and the resulting precipitates were collected by filtration to give **13b** (223 mg, 73%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.06–1.25 (12H, m), 1.45–1.56 (4H, m), 2.01–2.03 (2H, m), 2.15–2.17 (2H, m), 2.20 (3H, m), 2.38 (1H, t, J = 6.10), 2.56–2.57 (2H, m), 2.64 (1H, t, J = 6.35), 2.74–2.76 (2H, m), 2.33–2.36 (1H, m), 2.37–2.51 (3H, m), 3.54–3.67 (4H, m), 3.78–3.79 (6H, m), 3.85–3.92 (1H, m), 4.34–4.40 (1H, m), 4.56–4.57 (1H, m), 4.60–4.64 (1H, m), 4.79 (1H, m), 6.11–6.13 (1H, m), 6.78–6.81 (4H, m), 7.22–7.44 (9H, m), 7.84 (1H, s), 8.08–8.13 (1H, m), 8.42–8.43 (1H, m), 9.37 (1H, d, J = 7.08); ^{13}C NMR ($CDCl_3$ 126 MHz): δ = 24.9, 28.6, 29.9, 30.1, 30.5, 38.2, 43.5, 48.3, 55.5, 63.3, 72.3, 84.1, 86.9, 113.4, 121.2, 127.2, 128.1, 128.4, 128.5, 130.3, 130.4, 130.4, 135.8, 144.7, 150.5, 151.3, 153.4, 158.8, 172.5, 206.9; ^{31}P NMR ($CDCl_3$, 203 MHz): δ = 151.5, 152.2; HRMS calcd. for $C_{53}H_{67}N_8NaO_{11}P^+$ [M+Na]$^+$: 1045.4559; found, 1045.4590.

Synthesis of ON-2, ON-3 and ON-4

The terminally modified 2′-*O*-methyl-oligoribonucleotides were synthesized on a DNA/RNA synthesizer in 1 µmol scale by using the commercially available 2′-*O*-methyl-RNA phosphoramidites, phosphoramidite **5** and **9**, and the solid supports. For **ON-2** and **ON-3**, the universal supports II (Glen Research Inc.) was used. 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 a C-18 cartridge column and the failure sequences were eluted by use of 10% CH_3CN/0.1 M ammonium acetate as an eluent. 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 purification on an anion-exchange HPLC column 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 C-18 cartridge column to give the pure oligonucleotide after being lyophilized to dryness. The yields were calculated by assuming the molar extinction coefficients of dG^{Chcm} and dG^{Cmcm} are identical to those of deoxyguanosine. **ON-2**: MALDI-TOF mass $[M+H]^+$ calcd 3792.8, found 3794.7; **ON-3**: MALDI-TOF mass $[M+H]^+$ calcd 3820.9, found 3821.0; **ON-4**: MALDI-TOF mass $[M+H]^+$ calcd 3352.7, found 3354.0.

Synthesis of ON-5, ON-7, ON-8, ON-9, and ODN-1 to ODN-10

By use of phosphoramidite **13a**, **13b** or **13c**, 2′-*O*-methyl-RNA phosphoramidites, the appropriate fully protected oligonucleotide $XC_mA_mA_mC_mC_mU_mA_mC_mU_m$ or $Xd(N_1N_2ACCTACT)$, was synthesized on the CPG supports. Here X represents the Lev protected nucleoside residue introduced by using **13a**, **13b** or **13c**. For the synthesis of ON-5 and ON-7, 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/pyridine–acetic acid (60 mL : 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) were 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/pyridine–H_2O (500 μL, 9 : 1, v/v) for 2 min. For the syntheses of **ON-8**, **ON-9** and **ODN**s, the terminal 5′-*O*-DMTr group was removed by treatment with 1% TFA/CH_2Cl_2 for 30 s prior to the NH_2NH_2 treatment.

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/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 further purified by anion-exchange HPLC. **ON-5**: MALDI-TOF mass $[M+H]^+$ calcd 3448.7, found 3448.8; **ON-7**: MALDI-TOF mass $[M+H]^+$ calcd 3448.68, found 3449.57; **ON-8**: MALDI-TOF mass $[M+H]^+$ calcd 3528.65, found 3530.32; **ON-9**: MALDI-TOF mass $[M+H]^+$ calcd 3498.63, found 3500.03; **ODN-1**: MALDI-TOF mass $[M+H]^+$ calcd 3256.57, found 3258.81; **ODN-2**: MALDI-TOF mass $[M+H]^+$ calcd 3271.57, found 3277.29; **ODN-3**: MALDI-TOF mass $[M+H]^+$ calcd 3247.56, found 3247.56; **ODN-4**: MALDI-TOF mass $[M+H]^+$ calcd 3272.57, found 3274.48; **ODN-5**: MALDI-TOF mass $[M+H]^+$ calcd 3232.56, found 3234.88; **ODN-6**: MALDI-TOF mass $[M+H]^+$ calcd 3262.56, found 3262.45; **ODN-7**: MALDI-TOF mass $[M+H]^+$ calcd 3287.57, found 3289.11.

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 the rate of 0.5 °C min^{-1} and heated to 85 °C at the same rate. During this annealing and melting, the absorption at 260 nm was recorded at 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. The T_m values are accurate within ±1 °C.

MD simulation

MD calculation was carried out by using the AMBER program[31] with parameters for 2′-*O*-methyl-RNA and backbone parameters of parm99.parmbsc0 force field.[32] 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 cyclohexylcarbamoyldeoxyguanosine (dG^{CmcmP}) residues to give the model structure of **ON-5/RNA-9mer** duplex. 29 Na^+ ions and 7 Cl^- ions were added by using the addions command, and the systems were surrounded by 3856 TIP3P model waters. The cut-off distance for the non-bonding interactions was set to 9.0 Å and the electrostatic interactions were treated with the PME method. After the 5000 steps of minimization, the systems were headed to 300 K within 0.1 ns with the positional restraints of 10 kcal mol^{-1} $Å^{-2}$ on the heavy atoms during which the volume was kept constant. Afterwards, the system was equilibrated under the 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 dG^{ChcmP} were calculated at the HF/6-31G* level.

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