Synthesis and properties of a novel molecular beacon containing a benzene-phosphate backbone at its stem moiety

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Received 26th January 2009, Accepted 6th April 2009
First published as an Advance Article on the web 12th May 2009
DOI: 10.1039/b901631g

This paper describes the synthesis and properties of a novel molecular beacon (MB) containing a benzene-phosphate backbone at its stem moiety. The fluorescence intensity of MBs was found to stabilize by the introduction of the benzene-phosphate backbone at its stem moiety. Furthermore, an MB containing the benzene-phosphate backbone was more resistant to DNase I (endonuclease) than an MB comprising natural DNA and 2′-*O*-methyl-RNA. These results indicate that the MB with the benzene-phosphate backbone is superior as a molecular beacon as compared to the MB composed of natural DNA and 2′-*O*-methyl-RNA.

Introduction

Nucleic acid probes modified with fluorescent dyes are widely used to detect specific DNA or RNA molecules. Molecular beacons (MBs) are dual-labeled nucleic acid probes that fluoresce upon hybridization with a complementary target sequence.[1–13] The nucleic acid is labeled at one end with a fluorescent reporter dye and at the opposite end with a fluorescence quencher. MBs are designed to form a stem–loop hairpin structure in the absence target DNA or RNA, forcing the fluorescence reporter group in proximity with the quencher group. In this conformation, fluorescence is quenched. In the presence of a complementary target molecule, the MB opens due to formation of the more stable probe–target duplex, increasing the distance between the reporter and quencher, and restoring fluorescence. The competing reaction between hairpin formation and target hybridization improves specificity of MBs compared with linear probes. However, designing MBs is not as simple as attaching arbitrary arm sequences to previously designed linear probes. The stem arms can also interact with the flanking region of the target RNAs, changing the hybridization specificity; adapting the arms to avoid such undesired interactions increases design complexity.[14–17]

We have recently reported the synthesis and properties of a nucleic acid analog consisting of a benzene-phosphate backbone.[18] The building blocks of the nucleic acid analog are composed of bis(hydroxymethyl)benzene residues connected to nucleobases *via* a biaryl-like axis. A thermal denaturation study of duplexes revealed that a nucleic acid analog with a benzene-phosphate backbone forms a thermally and thermodynamically stable duplex in itself, while it does not form a thermally stable duplex with complementary natural DNA or RNA. We thus envisioned that if we could introduce the analog into the stem moieties of an MB, the fluorescence intensity of the MB would stabilize because unnecessary interactions between the target RNA and stem moieties would be avoided, allowing highly sensitive detection of the target RNA. In this paper, we report the synthesis and properties of novel MBs containing the benzene-phosphate backbone at their stem moieties (Fig. 1). A preliminary account of this work has appeared.[19]

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Results

Synthesis of amidite units

We have previously reported the synthesis of phosphoramidite units of nucleoside analogs **1**, **2**, **3**, and **4** (Fig. 2) based on heterocyclization (Fig. 3a).[18] However, it was not an efficient access because of the many tedious steps involved. Recently, it has been reported that N^1-arylpyrimidines or N^9-arylpurines can be efficiently synthesized from an arylboronic acid derivative and nucleobases by the Chan–Lam–Evans reaction using Cu(II) as a catalyst (Fig. 3b).[20–26] Thus, we employed this reaction for the synthesis of our compounds.

First, we synthesized an arylboronic acid derivative **9** for the coupling reaction (Scheme 1). Dimethyl 5-aminoisophthalate (**5**) was diazotized with $NaNO_2$ in the presence of KI to give a 5-iodo derivative **6** in 48% yield. After reducing the ester moieties of **6** with $LiBH_4$, the resulting hydroxyl groups were protected with a *tert*-butyldimethylsilyl (TBDMS) group to give a bis(TBDMS) derivative **8** in 97% yield. The treatment of **8** with *n*-BuLi at −78 °C in Et_2O and then $B(OCH_3)_3$ afforded **9** in 60% yield.

The arylboronic acid derivative **9** was coupled with adenine (**10**) in the presence of $Cu(OAc)_2$ and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) in aqueous MeOH solution to give an N^9-aryladenine derivative **11** in 61% yield (Scheme 2).

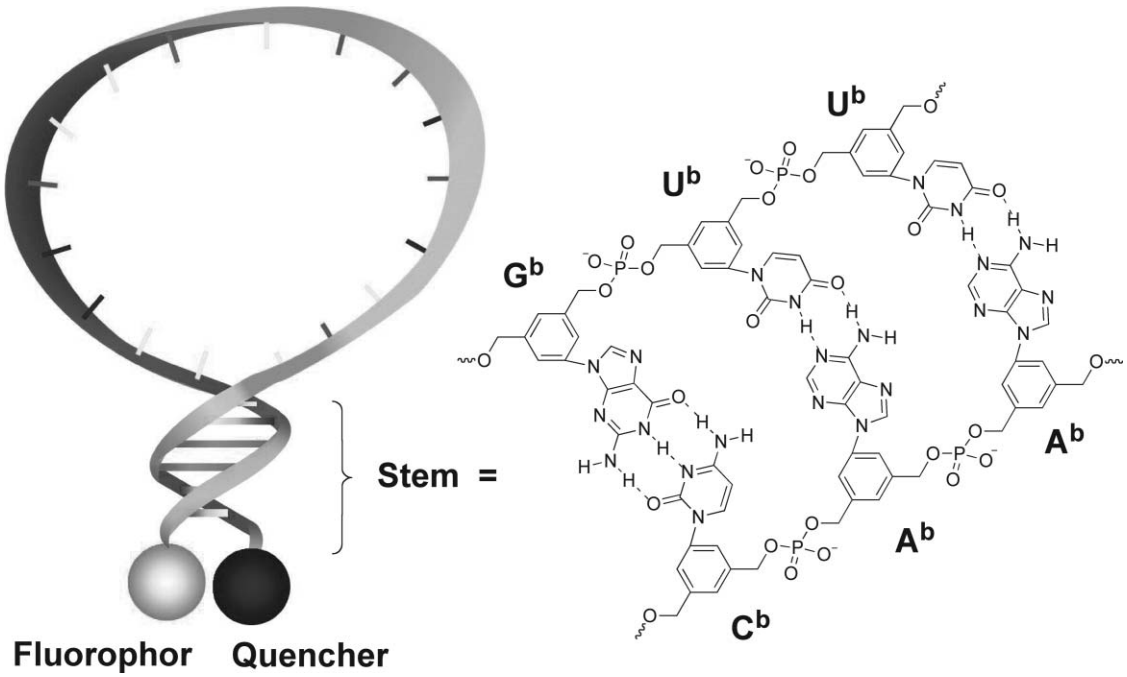


Fig. 1 Structure of the molecular beacon.

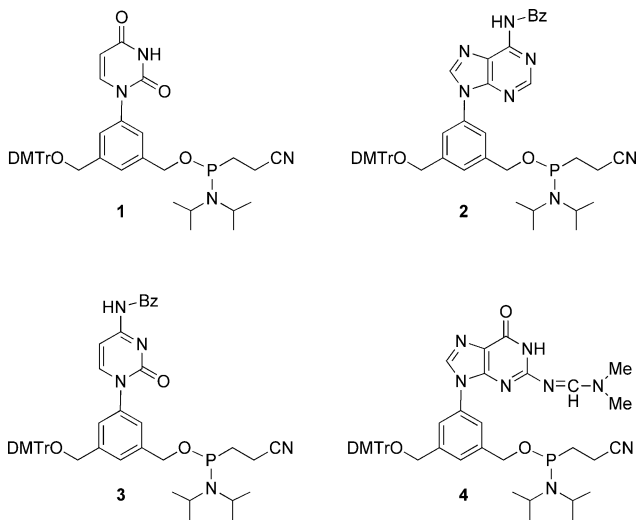


Fig. 2 Structures of amidite units.

The reaction of **9** with 2-bis(*tert*-butoxycarbonyl)amino-6-chloropurine (**12**) gave a 2-amino-N^9-aryl-6-chloropurine derivative **13** in 33% yield (Scheme 3). The treatment of **13** with aqueous TFA solution afforded an N^9-arylguanine derivative **14** in 87% yield. Similarly, the reaction of **9** with cytosine (**15**) gave an N^1-arylcytosine derivative **16** in 91% yield (Scheme 4). The treatment of **16** with 6 M HCl and then aqueous $NaNO_2$ produced N^4-aryluracil (**17**) in 85% yield. These compounds were converted to the phosphoramidites **1**, **2**, **3**, and **4** according to the reported method. From these results, it was found that **11** and **13** were efficiently synthesized by the cross-coupling reaction using $Cu(OAc)_2$ as the catalyst although the yield of the coupling reaction of **12** was lower than that of other compounds.

Synthesis of MBs

We selected an mRNA of a human RNase H (position 140–156) as a target sequence. The sequences of MBs and target RNAs used in this study are shown in Table 1. Fluorescein was attached to the 5′-terminus of each probe, while dabcyl was attached to the opposite terminus. MB**1** comprised natural DNA and 2′-*O*-methyl-RNA, while MB**2** comprised 2′-*O*-methyl-RNA and the nucleic acid analog with the benzene-phosphate backbone. RNA**1** had a natural sequence, whereas RNA**2** included a complementary sequence to the stem moiety of the MBs. RNA**3** contained 4 mismatched bases.

UV melting studies of MBs

The stability of hairpin conformations of the MBs was studied by thermal denaturation (Fig. 4). Although the T_m value (51.6 °C in 1.0 M NaCl) of MB**2** containing the benzene-phosphate backbone was slightly lower than that of MB**1** composed of natural DNA and 2′-*O*-methyl-RNA (56.3 °C in 1.0 M NaCl), the benzene-phosphate moieties of MB**2** could form a thermally stable hybrid under test conditions.

Detection of target RNAs by MBs

Each MB was allowed to anneal with the target RNAs. The fluorescence intensity increased as a function of the target RNA concentration (Figs. 5a and b). When RNA**1** and RNA**2** with complementary sequences were used as targets, the fluorescence intensity plateaued near 0.5 μM of target RNA. On the other hand, when RNA**3** containing 4 mismatched bases was used as the target, the fluorescence intensity increased gradually and did not reach a plateau even at 3.0 μM of target RNA. This indicates that these MBs can distinguish the target RNAs from the 4-nucleotide

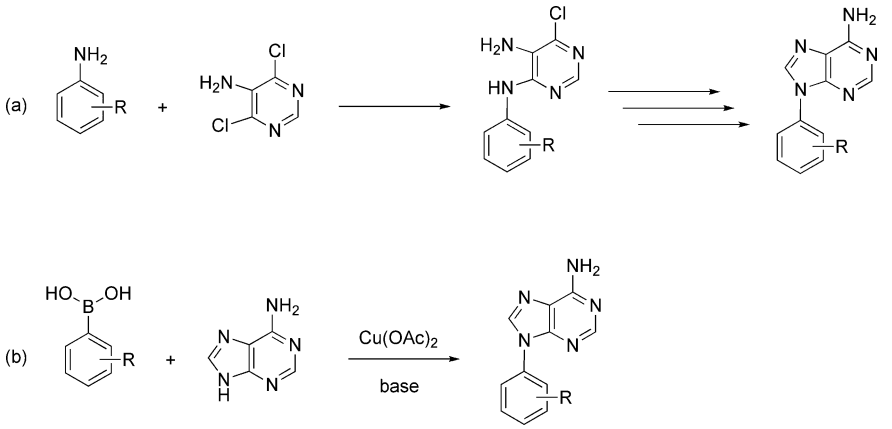
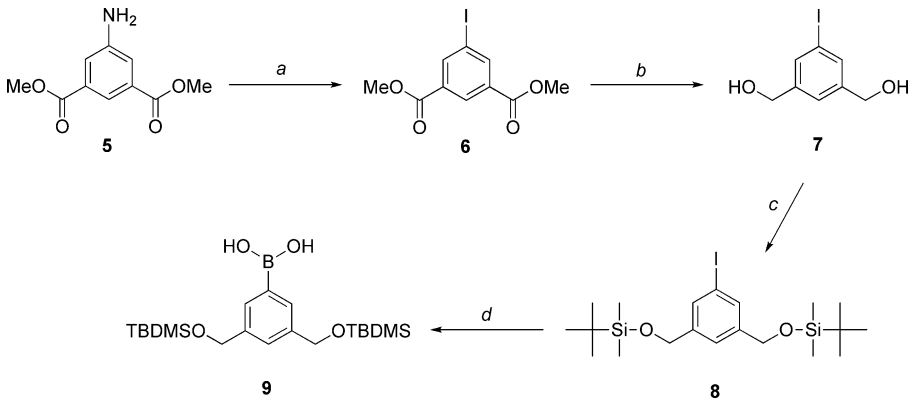
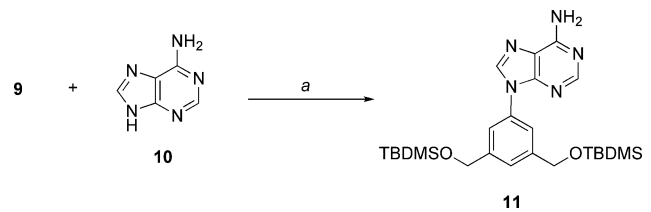


Fig. 3 Synthetic routes.



Scheme 1 *Reagents and conditions*: (a) (1) $NaNO_2$, 0.37 M HCl, 0 °C, 1 h, (2) KI, 0 °C, 1 h, 48%; (b) $LiBH_4$, THF, rt, 24 h, 76%; (c) TBDMSCl, imidazole, DMF, rt, 2 h, 97%; (d) (1) n-BuLi, THF, −78 °C, 1 h, (2) $B(OMe)_3$, −78 °C, 1 h, and then rt, 12 h, 60%.

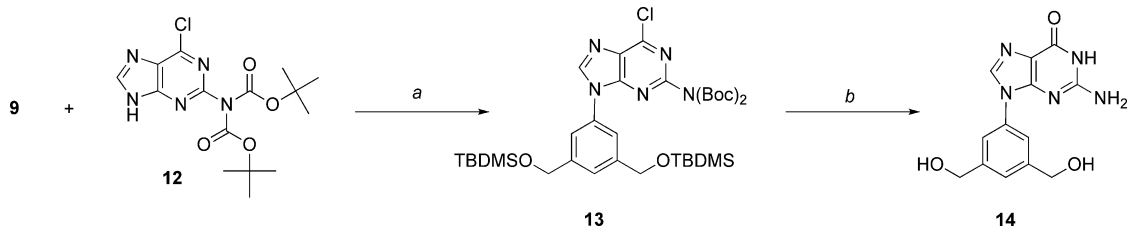


Scheme 2 *Reagents and conditions*: (a) adenine, $Cu(OAc)_2 \cdot H_2O$, TMEDA, MeOH:H_2O (4:1 v/v), rt, 2 h, 61%.

mismatched RNA. Furthermore, the difference in the fluorescence intensities between MB**2**:RNA**1** and MB**2**:RNA**2** hybrids with excess amounts of RNA targets turned out to be smaller than that between MB**1**:RNA**1** and MB**1**:RNA**2** hybrids.

Nuclease resistant property of MBs

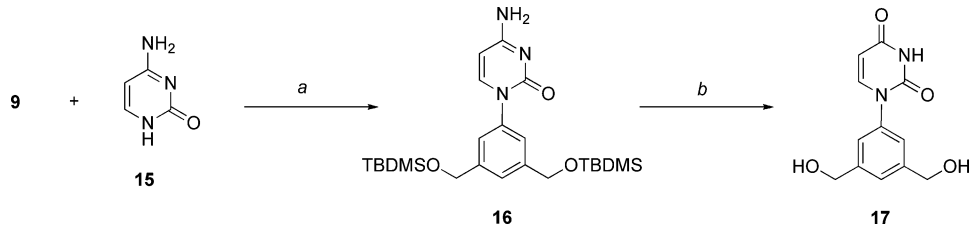
The stability of the MBs against nucleolytic degradation was examined using DNase I (endonuclease). MB**1** and MB**2** were incubated with DNase I at 37 °C, and the reactions were analyzed by carrying out polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. As shown in Figs. 6a and b, MB**1** comprising natural DNA and 2′-*O*-methyl-RNA was hydrolyzed randomly after 1 min of incubation; MB**2** comprising 2′-*O*-methyl-RNA and the nucleic acid analog with the benzene-phosphate backbone was highly resistant to the enzyme. Therefore, it was apparent that MB**2** containing the benzene-phosphate backbone was more resistant to DNase I than MB**1**. Figs. 7a and b show the change in the fluorescence intensities of the reaction mixtures containing MBs and DNase I. The fluorescence intensity of the mixture containing



Scheme 3 *Reagents and conditions*: (a) 2-amino-*N,N*-bis(*tert*-butoxycarbonyl)-6-chloropurine, $Cu(OAc)_2 \cdot H_2O$, TMEDA, MeOH:H_2O (4:1 v/v), rt, 2 h, 33%; (b) 50% TFA, H_2O,rt, 48 h, 87%.

Table 1 Sequences of oligomers used in this study. Me, r, d, and b represent 2′-*O*-methylribonucleoside, ribonucleoside, 2′-deoxyribonucleoside, and the benzene-type analog, respectively. The underlined letters indicate complementary sequences to the loop regions of MBs. The bold letters indicate a complementary sequence to the stem region of MB. The italicized letters represent the mismatched bases. **Flu** and **Dab** indicate fluorescein and dabcyl, respectively

No. of ON	sequence
MB**1**	**Flu**-5′-d(GCAAGC)-2′-*O*-Me(CCGGUCCACUUGUGCUC)-d(GCUUGC)-3′-**Dab**
MB**2**	**Flu**-5′-b(GCAAGC)-2′-*O*-Me(CCGGUCCACUUGUGCUC)-b(GCUUGC)-3′-**Dab**
RNA**1**	3′-r(GUCGUCCUUUGGCCAGGUGAACACGAGACGUGAGUAA)-5′
RNA**2**	3′-r(GUCG**CGUUCG**GGCCAGGUGAACACGAGACGUGAGUAA)-5′
RNA**3**	3′-r(GUCGUCCUUUGG*A*CAG*C*UGA*U*CAC*C*AGACGUGAGUAA)-5′



Scheme 4 *Reagents and conditions*: (a) cytosine, $Cu(OAc)_2 \cdot H_2O$, TMEDA, MeOH:H_2O (4:1 v/v), rt, 2 h, 91% (b) (1) 0.16 M HCl, rt, 1 h; (2) aqueous $NaNO_2$, 0 °C, 3 h, and then 110 °C, 3 days, 85%.

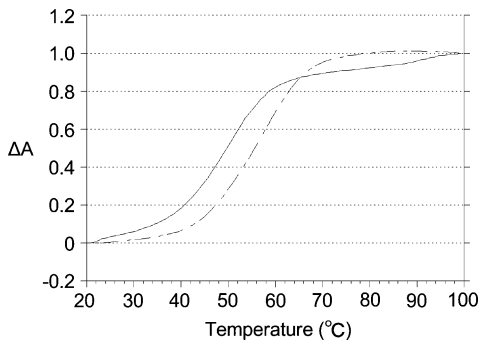


Fig. 4 UV melting profiles. Solid line: MB**2**. Dashed line: MB**1**.

MB**1** increased with time, whereas that of the mixture containing MB**2** hardly changed.

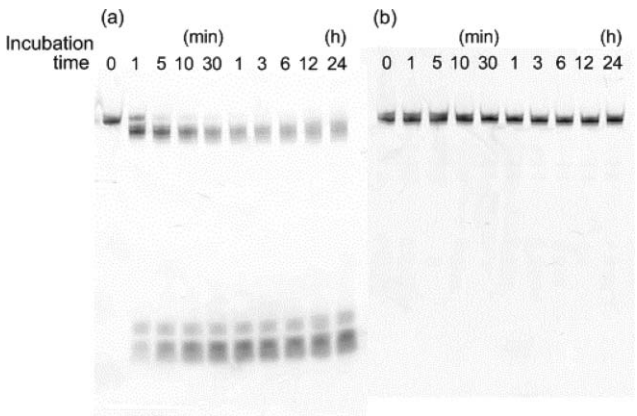


Fig. 6 20% PAGE of MBs hydrolyzed by DNase I. (a) MB**1**. (b) MB**2**.

Discussion

In a previous paper, we reported the synthesis of nucleoside analogs based on heterocyclization. However, it was not an efficient access because of the many tedious steps involved.[18] Recently, it was reported that N^1-arylpyrimidines or N^9-arylpurines can be efficiently synthesized from an arylboronic acid derivative and nucleobases by the Chan–Lam–Evans reaction using Cu(II)

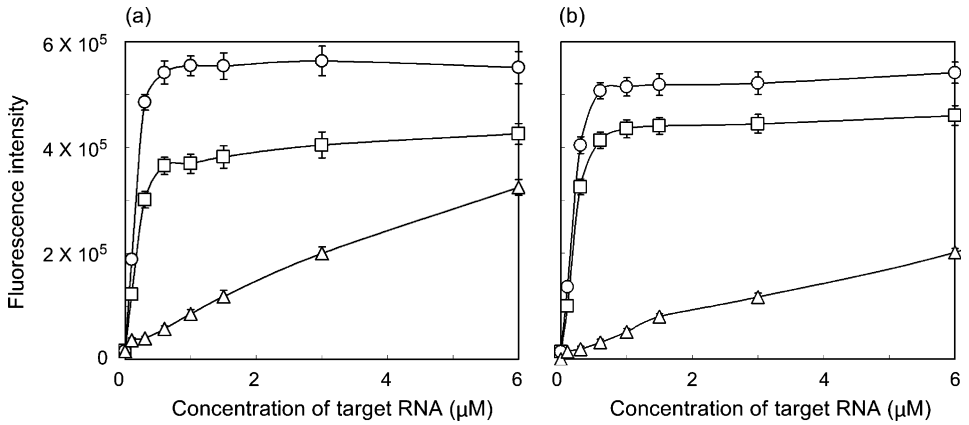


Fig. 5 Fluorescence intensity measurement (1). (a) The profiles of MB**1**. (b) The profiles of MB**2**. Circle: RNA**2**. Square: RNA**1**. Triangle: RNA**3**.

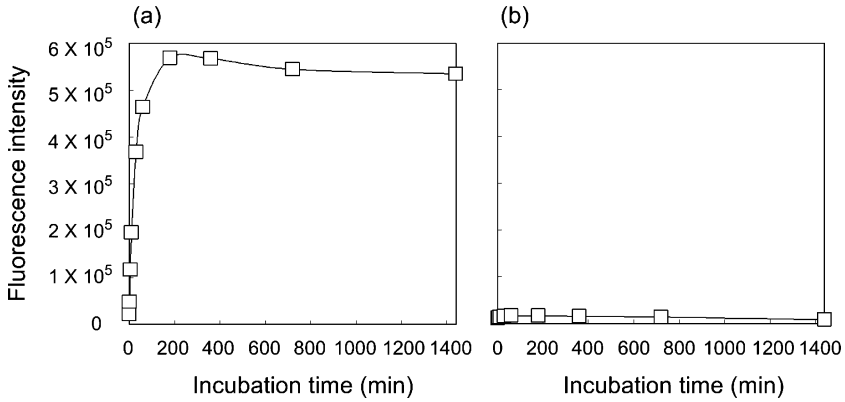


Fig. 7 Fluorescence intensity measurement (2). (a) MB**1**. (b) MB**2**.

as a catalyst.[20–26] Thus, we employed the reaction for the synthesis of our compounds.

The arylboronic acid derivative was coupled with cytosine in the presence of $Cu(OAc)_2$ and TMEDA in aqueous MeOH solution to give an N^1-arylcytosine derivative in a good yield. The N^1-arylcytosine derivative was efficiently converted to N^1-aryluracil. Similarly, the reaction of the arylboronic acid derivative with adenine gave an N^9-aryladenine derivative in a moderate yield. The yield of the coupling reaction of a 2-amino-6-chloropurine derivative with the arylboronic acid derivative was lower than that of other compounds. However, the total synthetic yield of the amidite unit was better than the previous one.

MBs are designed to form a stem–loop hairpin structure in the absence of a target, quenching the fluorophore reporter. However, designing MBs is not as simple as attaching arbitrary arm sequences to previously designed linear probes. The stem arms can also interact with the flanking regions of target RNAs, changing the hybridization specificity; adapting the arms to avoid such undesired interactions increases design complexity.[14–17] In our previous research, we found that the nucleic acid analog with the benzene-phosphate backbone forms a thermally and thermodynamically stable duplex in itself but does not form a thermally stable duplex with complementary natural DNA or RNA.[18] Thus, we expected that the fluorescence intensity of the MBs would stabilize by the introduction of the analog into their stem moieties, as unnecessary interactions between the target RNA and stem moieties would be avoided.

The fluorescence intensities of MBs increased as a function of the target RNA concentrations. When excess amounts of target RNAs were added to solutions containing MBs, the fluorescence intensity of the solution containing the MB**1**:RNA**2** hybrid was greater than that of the solution containing the MB**1**:RNA**1** hybrid. The target RNA**2** includes a sequence, that is complementary to the stem moiety of MB**1**, at the flanking region. Thus, the difference in the fluorescence intensities of the hybrids is considered to be a result of the interaction between the flanking region of target RNA**2** and the stem moiety of MB**1**. When MB**2** containing the benzene-phosphate backbone at the stem moiety was used, the fluorescence intensity of the solution containing the MB**2**:RNA**2** hybrid was slightly greater than that of the solution containing the MB**2**:RNA**1** hybrid. However, the difference in the fluorescence intensities between the MB**2**:RNA**1** and MB**2**:RNA**2** hybrids was apparently smaller than that between the MB**1**:RNA**1** and MB**1**:RNA**2** hybrids. The result indicates that the sequence dependency of the fluorescence intensity of MBs can be reduced by the introduction of the benzene-phosphate backbone at their stem moieties.

The resistance of MBs to nucleolytic hydrolysis by nucleases is an important factor to be considered when they are used in living cells.[27–30] MBs form stem–loop hairpin structures in the absence of target RNAs. Under these conditions, fluorescence is not observed because the fluorescence of fluorophore is quenched by the quencher. However, if MBs are the substrate of nucleases in the cells, they are digested by the nucleases, and fluorophore is spatially separated from the quencher so that fluorescence is observed. This is one of the reasons for the high fluorescence background observed when MBs are used in living cells.

Thus, the stability of MBs against nucleolytic degradation was examined using DNase I (endonuclease). MB**1** comprising natural DNA and 2′-methyl-RNA was hydrolyzed randomly after 1 min of incubation; MB**2** composed of 2′-*O*-methyl-RNA and the nucleic acid analog with the benzene-phosphate backbone was highly resistant to the enzyme. Therefore, it was found that MB**2** containing the benzene-phosphate backbone was more resistant to DNase I than MB**1**. The fluorescence intensities of the reaction mixtures were also measured. The fluorescence intensity of the reaction mixture containing MB**1** increased with time, whereas that of the reaction mixture containing MB**2** hardly changed. Thus, from these results, it was found that MB**2** has superior properties as a molecular beacon as compared to MB**1**.

In the present study, novel MBs containing a benzene-phosphate backbone at the stem moiety were synthesized, and their properties were studied. The sequence dependency of the fluorescence intensity of MBs was found to reduce by the introduction of the benzene-phosphate backbone at the stem moiety. Furthermore, it was found that MB**2** containing the benzene-phosphate backbone was more resistant to DNase I (endonuclease) than MB**1** composed of natural DNA and 2′-*O*-methyl-RNA. The use of these MBs in living cells is now under investigation.

Experimental

General remarks

The NMR spectra were recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C) and were reported in ppm downfield from tetramethylsilane.

The coupling constants (J) are expressed in Hertz. The mass spectra were obtained by the electron ionization (EI) or fast atom bombardment (FAB) method. Thin-layer chromatography was carried out on Merck coated plates $60F_{254}$. Silica gel column chromatography was carried out on Wakogel C-300.

Dimethyl 5-iodoisophthalate (6)

To a solution of dimethyl 5-aminoisophthalate (1.00 g, 4.78 mmol) and $NaNO_2$ (0.66 g, 9.56 mmol) in H_2O (5.7 mL) was added 2 M HCl (1.3 mL) at 0 °C, and the whole was stirred at 0 °C for 1 h. To the mixture was added a solution of KI (1.59 g, 9.56 mmol) in H_2O (23 mL), and the whole was stirred at room temperature for 1 h. The mixture was partitioned between $CHCl_3$ and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 2% MeOH in $CHCl_3$) to give **6** (0.737 g as a pale yellow solid, 48%): LRMS (EI) m/z 320 (M^+); ^{1}H NMR ($CDCl_3$) δ 8.63 (t, 1H, J = 1.5, H-2), 8.55 (d, 2H, J = 1.5, H-4 and H-6), 3.95 (s, 6H, CH_3); ^{13}C NMR ($CDCl_3$) δ 164.8, 142.5, 132.2, 129.9, 93.4, 52.7; HRMS (EI) calcd for $C_{10}H_9IO_4$ 319.9553, found 319.9546.

1,3-Bis(hydroxymethyl)-5-iodobenzene (7)

A mixture of **6** (0.46 g, 1.44 mmol) and $LiBH_4$ (0.16 g, 7.35 mmol) in THF (8 mL) was stirred at room temperature for 24 h. To the mixture was added aqueous $NaHCO_3$ (1 mL) at 0 °C, and the whole was stirred at room temperature for 1 h. The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 30–100% EtOAc in hexane) to give **7** (0.29 g as a white solid, 76%): LRMS (EI) m/z 264 (M^+); ^{1}H NMR ($CDCl_3$) δ 7.65 (m, 2H, H-4 and H-6), 7.33 (m, 1H, H-2), 4.66 (d, 4H, J = 5.2, CH_2), 1.71 (t, 2H, J = 5.2, OH); ^{13}C NMR (DMSO-d_6) δ 145.0, 133.1, 123.8, 94.4, 62.1; HRMS (EI) calcd for $C_8H_9IO_2$ 263.9647, found 263.9637.

1,3-Bis(*tert*-butyldimethylsilyloxymethyl)-5-iodobenzene (8)

A mixture of **7** (0.27 g, 1.03 mmol), TBDMSCl (0.34 g, 2.27 mmol), and imidazole (0.31 g, 4.53 mmol) in DMF (4 mL) was stirred at room temperature for 2 h. EtOH (1 mL) was added to the mixture, and the whole was stirred for 10 min. The mixture was partitioned between EtOAc and H_2O. The organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 30–100% EtOAc in hexane) to give **8** (0.50 g as a pale oil, 97%): ^{1}H NMR ($CDCl_3$) δ 7.48 (s, 2H, H-4 and H-6), 7.20 (s, 1H, H-2), 4.62 (s, 4H, CH_2), 0.89 (s, 18H, *t*-BuSi), 0.05 (s, 12H, CH_3Si); ^{13}C NMR ($CDCl_3$) δ 143.6, 133.5, 122.9, 94.1, 64.1, 25.9, 18.4, −5.3.

3,5-Bis(*tert*-butyldimethylsilyloxymethyl)phenylboronic acid (9)

To a solution of **8** (1.40 g, 2.82 mmol) in Et_2O (14 mL) was slowly added n-BuLi (1.6 M in hexane, 3.52 mL, 5.64 mmol) at −78 °C, and the whole was stirred at −78 °C for 1 h. To the mixture was slowly added trimethyl borate (1.24 mL, 11.1 mmol) at −78 °C, and the whole was slowly warmed to room temperature and stirred for 12 h. The mixture was partitioned between EtOAc and 0.5 M HCl. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 0–2% MeOH in $CHCl_3$) to give **9** (0.69 g as a white solid, 60%): ^{1}H NMR ($CDCl_3$) δ 8.03 (s, 2H, H-2 and H-6), 7.61 (s, 1H, H-4), 4.87 (s, 4H, CH_2), 0.10 (s, 18H, *t*-BuSi), 0.16 (s, 12H, CH_3Si); ^{13}C NMR ($CDCl_3$) δ 141.0, 131.7, 129.5, 128.0, 65.0, 26.0, 18.5, −5.2. Anal. Calcd for $C_{20}H_{39}BO_4Si_2$: C, 58.52; H, 9.58. Found: C, 58.24; H, 9.34.

9-[3,5-Bis(*tert*-butyldimethylsilyloxymethyl)phenyl]adenine (11)

A mixture of **9** (81 mg, 0.20 mmol), adenine (32 mg, 0.24 mmol), TMEDA (30 μL, 0.20 mmol), $Cu(OAc)_2 \cdot H_2O$ (20 mg, 0.10 mmol) in MeOH (1.6 mL) and H_2O (0.4 mL) was vigorously stirred under an atmosphere of air at room temperature for 2 h. The mixture was partitioned between $CHCl_3$ and brine. The organic layer was dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 20–100% EtOAc in hexane) to give **11** (60 mg, 61%): LRMS (EI) m/z 499 (M^+); ^{1}H NMR ($CDCl_3$) δ 8.40 (s, 1H, adenine), 8.08 (s, 1H, adenine), 7.54 (s, 2H, aromatic H), 7.36 (s, 1H, aromatic H), 5.85 (s, 2H, NH_2), 4.83 (s, 4H, CH_2), 0.96 (s, 18H, *t*-BuSi), 0.13 (s, 12H, CH_3Si); ^{13}C NMR ($CDCl_3$) δ 155.6, 153.6, 150.0, 143.6, 139.7, 134.7, 122.9, 120.1, 119.3, 64.4, 25.9, 18.4, −5.3; HRMS (EI) calcd for $C_{25}H_{41}N_5O_2Si_2$ 499.2799, found: 499.2803. Anal. Calcd for $C_{25}H_{41}N_5O_2Si_2 \cdot 1/2H_2O$: C, 59.01; H, 8.32; N, 13.76. Found: C, 59.19; H, 8.17; N, 13.76.

2-Amino-*N*^{2}-bis(*tert*-butoxycarbonyl)-9-[3,5-bis(*tert*-butyldimethylsilyloxymethyl)phenyl]-6-chloropurine (13)

A mixture of **9** (0.58 g, 1.42 mmol), 2-amino-*N,N*-bis(*tert*-butoxycarbonyl)-6-chloropurine (0.63 g, 1.70 mmol), TMEDA (0.21 mL, 1.39 mmol), and $Cu(OAc)_2 \cdot H_2O$ (0.14 g, 0.70 mmol) in MeOH (4 mL) and H_2O (1 mL) was vigorously stirred under an atmosphere of air at room temperature for 2 h. The mixture was partitioned between $CHCl_3$ and brine. The organic layer was dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 25% EtOAc in hexane) to give **13** (0.34 g, 33%): LRMS (FAB) m/z 734 (MH^+); ^{1}H NMR ($CDCl_3$) δ 8.43 (s, 1H, H-8), 7.53 (s, 2H, aromatic H), 7.43 (s, 1H, aromatic H), 4.83 (s, 4H, CH_2), 1.44 (s, 18H, *t*-BuO), 0.96 (s, 18H, *t*-BuSi), 0.13 (s, 12H, CH_3Si); ^{13}C NMR (DMSO-d_6) δ 152.5, 152.1, 151.7, 150.5, 144.9, 143.9, 133.7, 130.5, 123.6, 119.1, 83.6, 64.3, 27.9, 25.9, 18.4, −5.3; HRMS (FAB) calcd for $C_{16}H_{19}N_6O_3$ 734.35360, found 734.35432. Anal. Calcd for $C_{35}H_{57}N_5O_6$ Si_2Cl: C, 57.23; H, 7.69; N, 9.54. Found: C, 57.48; H, 7.43; N, 9.19.

9-[3,5-Bis(hydroxymethyl)phenyl]guanine (14)

A mixture of **13** (0.50 g, 0.68 mmol), TFA (5.2 mL, 70 mmol), and H_2O (5.2 mL) was stirred at room temperature for 48 h. The solvent was evaporated in vacuo. The resulting residue was filtered and washed with H_2O to give **14** (0.17 g, 87%): LRMS (FAB) m/z 288 (MH^+); ^{1}H NMR (DMSO-d_6) δ 10.67 (s, 1H, NH), 7.93 (s, 1H, H-8), 7.42 (s, 2H, aromatic H), 7.35 (s, 1H, aromatic H), 6.48 (s, 2H, NH_2), 5.31 (t, 2H, J = 5.4, OH), 4.55 (d, 4H, J = 5.4, CH_2); ^{13}C NMR (DMSO-d_6) δ 157.5, 154.1, 151.5, 144.2, 137.4, 135.0, 124.2, 120.8, 117.3, 62.8; HRMS (FAB) calcd for $C_{13}H_{14}N_5O_3$ 288.1097, found 288.1101. Anal. Calcd for $C_{13}H_{13}N_5O_3 \cdot 1/7H_2O$: C, 53.87; H, 4.62; N, 24.16. Found: C, 54.11; H, 4.64; N, 24.07.

1-[3,5-Bis(*tert*-butyldimethylsilyloxymethyl)phenyl]cytosine (16)

A mixture of **9** (0.40 g, 0.97 mmol), cytosine (0.13 g, 1.12 mmol), TMEDA (0.15 mL, 0.97 mmol), and $Cu(OAc)_2 \cdot H_2O$ (97 mg, 0.49 mmol) in MeOH (4 mL) and H_2O (1 mL) was vigorously stirred under an atmosphere of air at room temperature for 2 h. The mixture was partitioned between $CHCl_3$ and brine. The organic layer was dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography (SiO_2, 0–10% MeOH in $CHCl_3$) to give **16** (0.42 g, 91%): LRMS (FAB) *m/z* 475 (MH^+); ^{1}H NMR ($CDCl_3$) δ 7.56 (d, 1 H, J = 7.4, H-6), 7.32 (s, 1H, aromatic H), 7.08 (s, 2H, aromatic H), 5.75 (d, 1 H, J = 7.4, H-5), 4.72 (s, 4H, CH_2), 0.90 (s, 18H, *t*-BuSi), 0.08 (s, 12H, CH_3Si); ^{13}C NMR ($CDCl_3$) δ 166.9, 156.7, 146.0, 143.5, 141.4, 123.8, 123.2, 95.5, 65.1, 26.6, 19.0, –4.6; HRMS (FAB) calcd for $C_{24}H_{42}N_3O_3Si_2$ 476.2778, found 476.2765.

1-[3,5-Bis(hydroxymethyl)phenyl]uracil (17)

A mixture of **16** (500 mg, 105 μmol), 6 M HCl (28 μL) and H_2O (1 mL) was stirred at room temperature for 1 h. A solution of $NaNO_2$ (15 mg, 210 μmol) in H_2O (130 μL) was added to the mixture at 0 °C. The whole was stirred at 0 °C for 3 h, and then at 110 °C for 3 days. The solvent was evaporated in vacuo, and the resulting residue was purified by column chromatography (SiO_2, 10–20% MeOH in $CHCl_3$) to give **17** (220 mg as a white solid, 85%): LRMS (EI) *m/z* 248 (M^+); ^{1}H NMR (DMSO-d_6) δ 11.42 (s, 1H, NH), 7.65 (d, 1H, J = 8.0, H-6), 7.32–7.17 (s, 3H, aromatic H), 5.65 (d, 1H, J = 8.0, H-5), 5.31 (t, 2H, J = 5.6, OH), 4.52 (d, 4H, J = 5.6, CH_2); ^{13}C NMR (DMSO-d_6) δ 163.7, 150.4, 145.5, 143.7, 138.7, 124.0, 122.8, 101.6, 62.7; HRMS (EI) calcd for $C_{12}H_{12}N_2O_4$ 248.0797, found 248.0790. Anal. Calcd for $C_{12}H_{12}N_2O_4$: C, 58.06; H, 4.87; N, 11.29. Found: C, 58.00; H, 4.87; N, 11.24.

6-*N*-Benzoyl-9-[3-(4,4′-dimethoxytrityloxymethyl)-5-(hydroxymethyl)phenyl]adenine (18)

Compound **11** was converted to **18** according to the reported method:[18] ^{1}H NMR ($CDCl_3$) δ 9.03 (s, 1H), 8.89 (s, 1H), 8.31 (s, 1H), 8.06 (d, 2H, J = 7.6), 7.69–6.82 (m, 19H), 4.84 (d, 2H, J = 5.6), 4.33 (s, 2H), 3.79 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 164.7, 158.6, 153.2, 151.8, 149.9, 149.5, 144.7, 143.3, 142.1, 142.0, 135.9, 134.4, 133.6, 132.8, 130.0, 128.9, 128.1, 128.0, 127.9, 126.9, 125.0, 123.3, 120.9, 120.3, 64.9, 64.4, 55.2, 53.6, 39.0, 20.7, 14.0; LRMS (FAB) *m/z* 678 (MH^+); HRMS (FAB) calcd for $C_{41}H_{36}N_5O_5$ 678.2716, found: 678.2711.

9-[3-(4,4′-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)phenyl]-2-*N*-[(dimethylamino)methylene]guanine (19)

Compound **14** was converted to **19** according to the reported method:[18] ^{1}H NMR ($CDCl_3$) δ 8.78 (s, 1H), 8.45 (s, 1H), 7.89–6.82 (m, 17H), 4.78 (s, 2H), 4.25 (s, 2H), 3.78 (s, 6H), 2.92 (s, 3H), 2.53 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 158.6, 158.1, 158.0, 156.8, 150.2, 144.9, 143.1, 141.2, 137.3, 136.0, 135.4, 129.9, 128.0, 126.9, 124.0, 121.0, 120.5, 120.1, 113.2, 86.5, 64.9, 64.4, 55.2, 40.7, 35.0; LRMS (FAB) *m/z* 645 (MH^+); HRMS (FAB) calcd for $C_{37}H_{37}N_6O_5$ 645.2747, found 645.2819. Anal. Calcd for $C_{37}H_{36}N_6O_5 \cdot 5/4H_2O$: C, 66.99; H, 5.99; N, 12.34. Found: C, 66.83; H, 5.73; N, 12.09.

4-*N*-Benzoyl-1-[3-(4,4′-dimethoxytrityloxymethyl)-5-(hydroxymethyl)phenyl]cytosine (20)

Compound **16** was converted to **20** according to the reported method:[18] ^{1}H NMR (DMSO-d_6) δ 11.35 (s, 1H), 8.17 (d, 1H, J = 7.2), 8.02 (d, 2H, J = 7.6), 7.65–6.90 (m, 20H), 5.37 (t, 1H, J = 6.0), 4.56 (d, 2H, J = 6.0), 4.12 (s, 2H), 3.73 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 158.1, 144.8, 143.9, 139.6, 135.5, 132.8, 129.7, 128.5, 128.0, 127.6, 126.8, 124.4, 123.1, 122.9, 113.3, 86.1, 79.2, 64.6, 62.3, 55.0; LRMS (FAB) *m/z* 654 (MH^+); HRMS (FAB) calcd for $C_{40}H_{36}N_3O_6$ 654.2604, found 654.2610. Anal. Calcd for $C_{40}H_{35}N_3O_6 \cdot 5/4H_2O$: C, 70.81; H, 5.61; N, 6.19. Found: C, 71.19; H, 5.67; N, 5.77.

1-[3-(4,4′-Dimethoxytrityloxymethyl)-5-(hydroxymethyl)phenyl]-uracil (21)

Compound **17** was converted to **21** according to the reported method:[18] ^{1}H NMR (DMSO-d_6) δ 11.41 (s, 1H), 7.67 (d, 1H, J = 8.0), 7.44–6.89 (m, 16H), 5.64 (d, 1H, J = 8.0), 5.32 (t, 1H, J = 5.4), 4.53 (d, 2H, J = 5.4), 4.09 (s, 2H), 3.72 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 163.7, 158.1, 150.4, 145.5, 144.8, 144.0, 139.6, 138.8, 135.5, 129.7, 128.0, 127.6, 126.8, 124.3, 123.4, 123.3, 113.4, 101.6, 86.0, 79.2, 64.6, 62.3, 55.0; LRMS (FAB) *m/z* 551 (MH^+); HRMS(FAB) calcd for $C_{33}H_{31}N_2O_6$ 551.2104, found 551.2176. Anal. Calcd for $C_{33}H_{30}N_2O_6 \cdot 1/2H_2O$: C, 70.83; H, 5.58; N, 5.01. Found: C, 70.62; H, 5.78; N, 4.74.

1-[3-[[(2-Cyanoethoxy)(*N,N*-diisopropylamino)phosphinyl]-oxymethyl]-5-(4,4′-dimethoxytrityloxymethyl)phenyl]uracil (1)

Compound **21** (0.80 g, 1.45 mmol) was dissolved in THF (9.7 mL) containing *N,N*-diisopropylethylamine (1.46 mL, 8.72 mmol). Chloro(2-cyanoethoxy)(*N,N*-diisopropylamino)-phosphine (0.65 mL, 2.9 mmol) was added to the solution, and the mixture was stirred at room temperature for 1 h. Aqueous $NaHCO_3$ (saturated) and $CHCl_3$ were added to the mixture, and the separated organic layer was washed with aqueous $NaHCO_3$ (saturated) and brine, dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (a neutralized SiO_2, EtOAc) to give **1** (0.88 g, 81%): ^{31}P NMR ($CDCl_3$) δ 148.9.

4-*N*-Benzoyl-1-[3-[[(2-cyanoethoxy)(*N,N*-diisopropylamino)-phosphinyl]oxymethyl]-5-(4,4′-dimethoxytrityloxymethyl)phenyl]-cytosine (3)

Compound **20** (0.66 g, 1.01 mmol) was phosphitylated as described in the preparation of **1** to give **3** (0.83 g, 96%): ^{31}P NMR ($CDCl_3$) δ 149.4.

6-*N*-Benzoyl-9-[3-[[(2-cyanoethoxy)(*N,N*-diisopropylamino)-phosphinyl]oxymethyl]-5-(4,4′-dimethoxytrityloxymethyl)phenyl]-adenine (2)

Compound **18** (0.45 g, 0.66 mmol) was phosphitylated as described in the preparation of **1** to give **2** (0.51 g, 88%): ^{31}P NMR ($CDCl_3$) δ 149.4.

9-[3-[[(2-Cyanoethoxy)(*N*,*N*-diisopropylamino)phosphinyl]-oxymethyl]-5-(4,4′-dimethoxytrityloxymethyl)phenyl]-2-*N*-[(dimethylamino)methylene]guanine (4)

Compound **19** (0.78 g, 1.21 mmol) was phosphitylated as described in the preparation of **1** to give **4** (0.62 g, 60%): ^{31}P NMR ($CDCl_3$) δ 149.3.

Oligomer synthesis

The synthesis reagents, such as 3′-dabcyl (quencher) CPG, 6-fluorescein phosphoramidite (fluorophore), and 2′-*O*-Me-modified RNA phosphoramidite, were purchased from Glen Research Corporation (Sterling, VA). The synthesis was carried out with a DNA/RNA synthesizer by the phosphoramidite method. For the incorporation of the analogs into the oligomers, a 0.15 M solution of each analog phosphoramidite in THF and a coupling time of 20 min was used. Deprotection of the bases and phosphates was performed in concentrated NH_4OH at 55 °C for 16 h. The oligomers were purified by 20% PAGE containing 7 M urea to give the highly purified oligomers, MB**1** (20) and MB**2** (12). The yields are indicated in parentheses as OD units at 260 nm starting from 1.0 μmol scale. The extinction coefficients of the oligomers were calculated from those of the mononucleotides and dinucleotides according to the nearest-neighbor approximation method.[31] The extinction coefficient (at 260 nm) of the analogs used for calculations are as follows: A^B, 18500; C^B, 9050; G^B, 15300; U^B, 13100.[18]

MALDI-TOF/MS analyses of oligomers

Spectra were obtained with a time-of-flight mass spectrometer. MB**1**: calculated mass, 10279; observed mass, 10272. MB**2**: calculated mass, 10508; observed mass, 10507.

Thermal denaturation study

A solution containing the MBs in a buffer comprised of 10 mM sodium phosphate (pH 7.0) and 0.1 M NaCl was heated at 95 °C for 3 min, cooled gradually to an appropriate temperature, and then used for the thermal denaturation study. The thermal-induced transition of each mixture was monitored at 260 nm with a spectrophotometer. The sample temperature was increased by 0.5 °C/min.

Fluorescence measurement of MBs

Each solution containing each MB (0.3 μM) and the target RNA (0, 0.1, 0.3, 0.6, 1.0, 1.5, 3.0, or 6.0 μM) in a buffer comprised of 10 mM sodium phosphate (pH 7.0) and 0.1 M NaCl was heated at 95 °C for 3 min, cooled gradually to an appropriate temperature, and then used for the FRET analysis. Aliquots of the mixture were transferred into 96-well plates. The fluorescence in the well was read at 485 nm excitation and 535 nm emission using a micro-plate reader, Wallac 1420 ARVOsx (Oarkin Elmer Co. Ltd.).

Partial hydrolysis of MBs with DNase I

Each MB (600 pmol, final 3.0 μM) was incubated with DNase I (20 units) in a buffer containing 10 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$ (total 200 μL) at 37 °C. At appropriate periods, aliquots (5 μL) of the reaction mixture were separated and added to a solution of 9 M urea (15 μL). The mixtures were analyzed by electrophoresis on 20% polyacrylamide gel containing 7 M urea. The MB in the gel was visualized by a Typhoon system (Amersham Biosciences).

Fluorometry of MBs treated with DNase I

Each solution containing each MB (60 pmol, final 0.3 μM) in a buffer comprised of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$ was heated at 95 °C for 3 min, cooled gradually to an appropriate temperature. DNase I (20 units) was added to the mixture, and the whole (total 200 μL) was incubated at 37 °C. At appropriate periods, the fluorescence in the mixture was read at 485 nm excitation and 535 nm emission using a micro-plate reader, Wallac 1420 ARVOsx (Oarkin Elmer Co. Ltd.).

Acknowledgements

This study was supported by a Grant-in-Aid from PRESTO of JST (Japan Science and Technology) and was also supported in part by a Grant-in-Aid for Scientific Research (C) from JSPS (Japan Society for the Promotion of Science).

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