

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

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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

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'*-arylpurimides or *N'*-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 NaNO₂ in the presence of KI to give a 5-iodo derivative **6** in 48% yield. After reducing the ester moieties of **6** with LiBH₄, 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 Et₂O and then B(OCH₃)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'*-aryladenine derivative **11** in 61% yield (Scheme 2).

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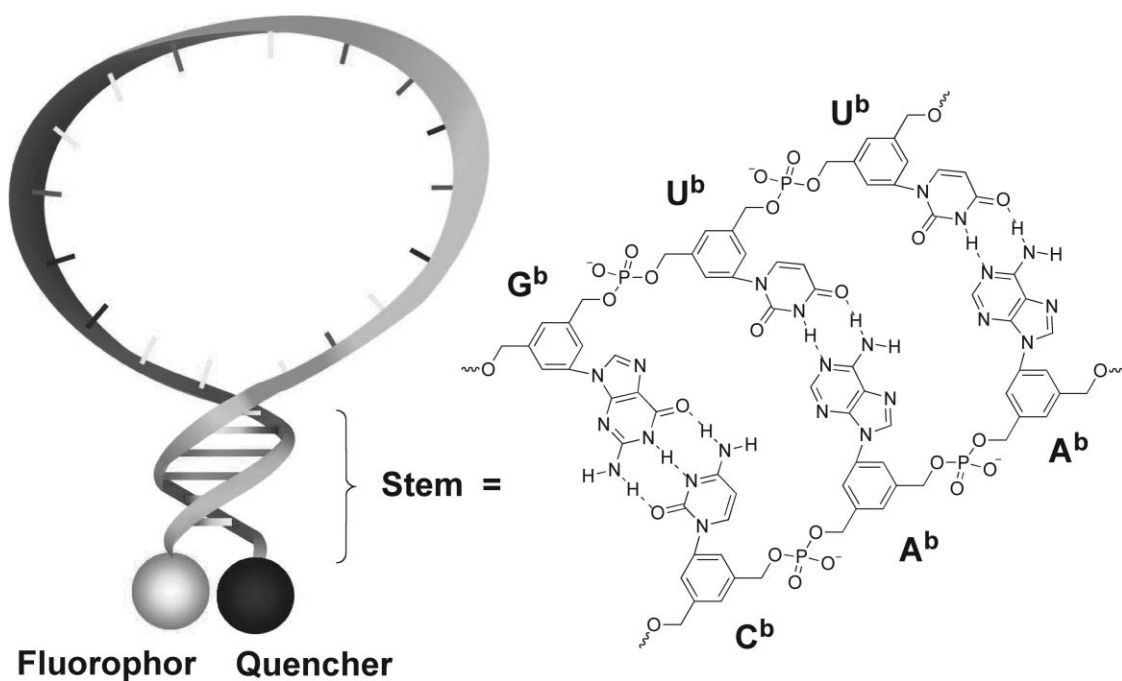


Fig. 1 Structure of the molecular beacon.

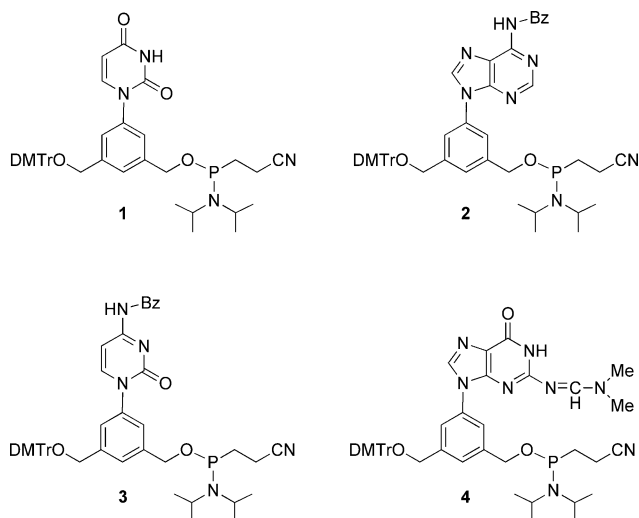


Fig. 2 Structures of amidite units.

The reaction of **9** with 2-bis(*tert*-butoxycarbonyl)amino-6-chloropurine (**12**) gave a 2-amino-*N'*-aryl-6-chloropurine derivative **13** in 33% yield (Scheme 3). The treatment of **13** with aqueous TFA solution afforded an *N'*-arylguanine derivative **14** in 87% yield. Similarly, the reaction of **9** with cytosine (**15**) gave an *N'*-arylcytosine derivative **16** in 91% yield (Scheme 4). The treatment of **16** with 6 M HCl and then aqueous NaNO₂ produced *N'*-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 dabcyI was attached to the opposite terminus. MB1 comprised natural DNA and 2'-*O*-methyl-RNA, while MB2 comprised 2'-*O*-methyl-RNA and the nucleic acid analog with the benzene-phosphate backbone. RNA1 had a natural sequence, whereas RNA2 included a complementary sequence to the stem moiety of the MBs. RNA3 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 MB2 containing the benzene-phosphate backbone was slightly lower than that of MB1 composed of natural DNA and 2'-*O*-methyl-RNA (56.3 °C in 1.0 M NaCl), the benzene-phosphate moieties of MB2 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 RNA1 and RNA2 with complementary sequences were used as targets, the fluorescence intensity plateaued near 0.5 μM of target RNA. On the other hand, when RNA3 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

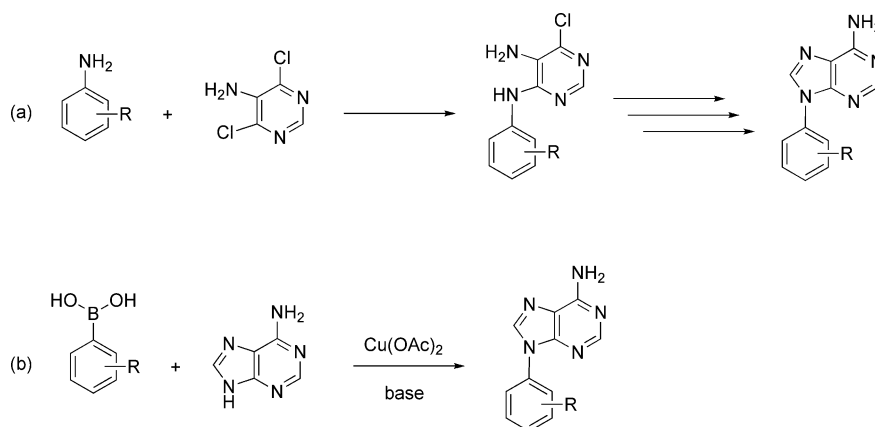
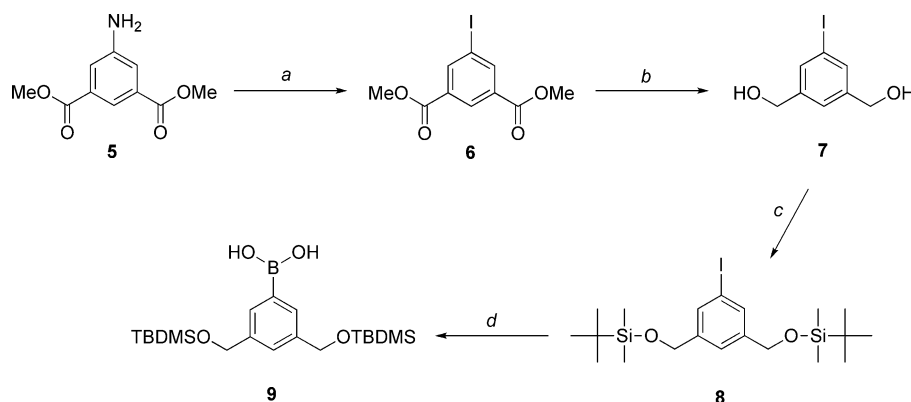
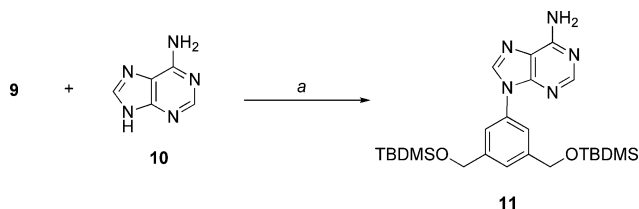


Fig. 3 Synthetic routes.



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) $n\text{-BuLi}$, THF, -78 °C, 1 h, (2) B(OMe)_3 , -78 °C, 1 h, and then rt, 12 h, 60%.

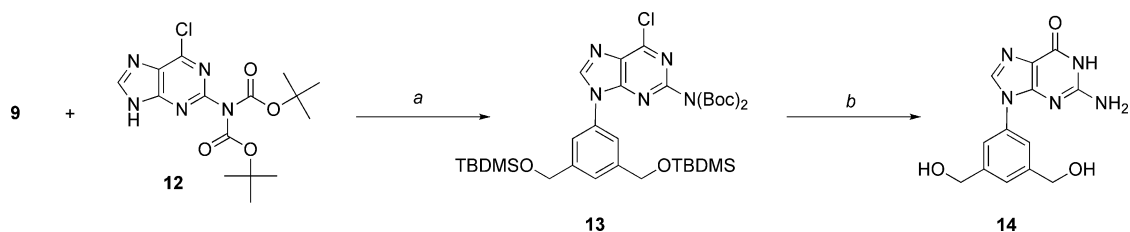


Scheme 2 Reagents and conditions: (a) adenine, $\text{Cu(OAc)}_2 \cdot \text{H}_2\text{O}$, TMEDA, $\text{MeOH:H}_2\text{O}$ (4:1 v/v), rt, 2 h, 61%.

mismatched RNA. Furthermore, the difference in the fluorescence intensities between MB2:RNA1 and MB2:RNA2 hybrids with excess amounts of RNA targets turned out to be smaller than that between MB1:RNA1 and MB1:RNA2 hybrids.

Nuclease resistant property of MBs

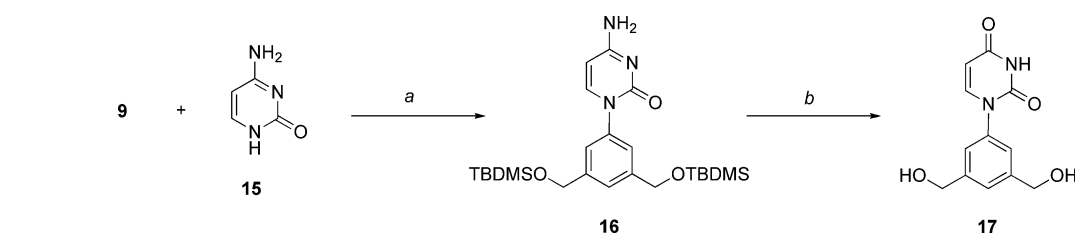
The stability of the MBs against nucleolytic degradation was examined using DNase I (endonuclease). MB1 and MB2 were incubated with DNase I at 37 °C, and the reactions were analyzed by carrying out polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. As shown in Figs. 6a and b, MB1 comprising natural DNA and 2'-*O*-methyl-RNA was hydrolyzed randomly after 1 min of incubation; MB2 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 MB2 containing the benzene-phosphate backbone was more resistant to DNase I than MB1. 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, $\text{Cu(OAc)}_2 \cdot \text{H}_2\text{O}$, TMEDA, $\text{MeOH:H}_2\text{O}$ (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
MB1	Flu -5'-d(GCAAGC)-2'- <i>O</i> -Me(CCGGUCCACUUGUGCUC)-d(GCUUGC)-3'- Dab
MB2	Flu -5'-b(GCAAGC)-2'- <i>O</i> -Me(CCGGUCCACUUGUGCUC)-b(GCUUGC)-3'- Dab
RNA1	3'-r(GUCGUCCUUUGGCCAGGUGAACACGAGACGUGAGUAA)-5'
RNA2	3'-r(GUCGCGUUCGGGCCAGGUGAACACGAGACGUGAGUAA)-5'
RNA3	3'-r(GUCGUCCUUUGG <u>ACAGCUGAU</u> <u>CAC</u> ACGAGACGUGAGUAA)-5'



Scheme 4 Reagents and conditions: (a) cytosine, Cu(OAc)₂·H₂O, TMEDA, MeOH:H₂O (4:1 v/v), rt, 2 h, 91% (b) (1) 0.16 M HCl, rt, 1 h; (2) aqueous NaNO₂, 0 °C, 3 h, and then 110 °C, 3 days, 85%.

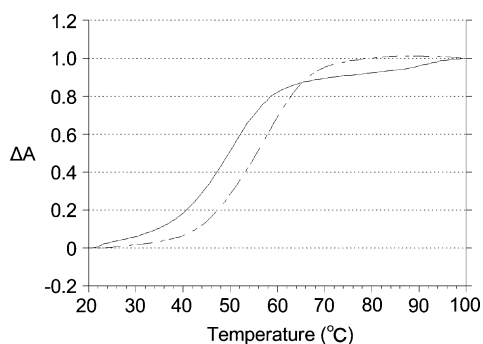


Fig. 4 UV melting profiles. Solid line: MB2. Dashed line: MB1.

MB1 increased with time, whereas that of the mixture containing MB2 hardly changed.

Discussion

In a previous paper, we reported the synthesis of nucleoside analogs based on heterocyclization. However, it was not an

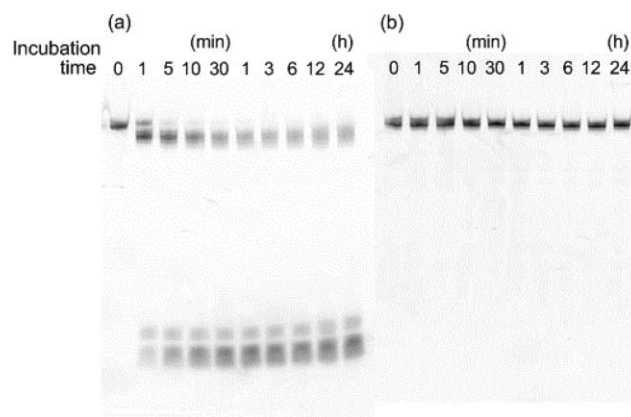


Fig. 6 20% PAGE of MBs hydrolyzed by DNase I. (a) MB1. (b) MB2.

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)

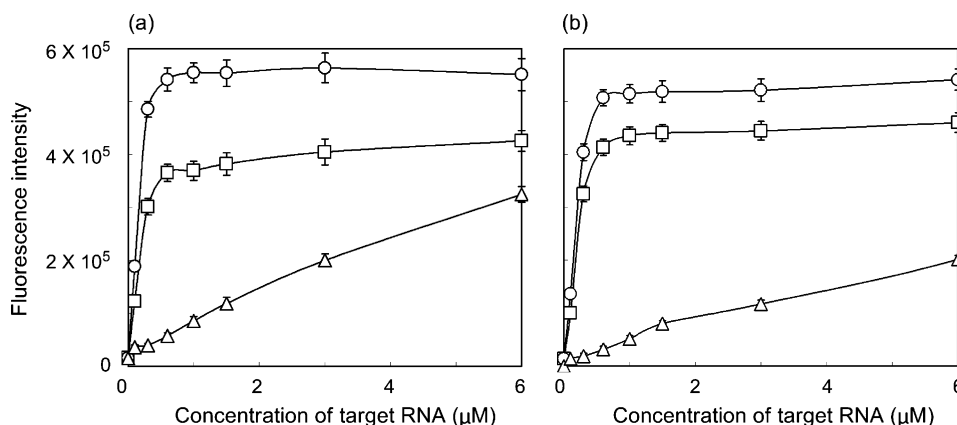


Fig. 5 Fluorescence intensity measurement (1). (a) The profiles of MB1. (b) The profiles of MB2. Circle: RNA2. Square: RNA1. Triangle: RNA3.

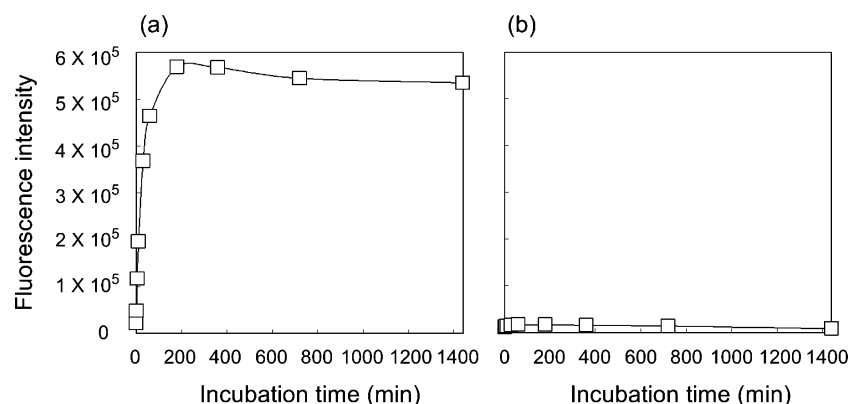


Fig. 7 Fluorescence intensity measurement (2). (a) MB1. (b) MB2.

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 $\text{Cu}(\text{OAc})_2$ and TMEDA in aqueous MeOH solution to give an N' -arylcytosine derivative in a good yield. The N' -arylcytosine derivative was efficiently converted to N' -aryluracil. Similarly, the reaction of the arylboronic acid derivative with adenine gave an N' -aryl adenine 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 MB1:RNA2 hybrid was greater than that of the solution containing the MB1:RNA1 hybrid. The target RNA2 includes a sequence, that is complementary to the stem moiety of MB1, 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 RNA2 and the stem moiety of MB1. When MB2 containing the benzene-phosphate backbone at the stem moiety was used, the fluorescence intensity of the solution containing the MB2:RNA2 hybrid was slightly greater than that of the solution containing the MB2:RNA1 hybrid. However, the difference in the fluorescence intensities between the MB2:RNA1 and MB2:RNA2 hybrids was apparently smaller than that between the MB1:RNA1

and MB1:RNA2 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). MB1 comprising natural DNA and 2'-methyl-RNA was hydrolyzed randomly after 1 min of incubation; MB2 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 MB2 containing the benzene-phosphate backbone was more resistant to DNase I than MB1. The fluorescence intensities of the reaction mixtures were also measured. The fluorescence intensity of the reaction mixture containing MB1 increased with time, whereas that of the reaction mixture containing MB2 hardly changed. Thus, from these results, it was found that MB2 has superior properties as a molecular beacon as compared to MB1.

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 MB2 containing the benzene-phosphate backbone was more resistant to DNase I (endonuclease) than MB1 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 (^1H) 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 60F₂₅₄. 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 NaNO₂ (0.66 g, 9.56 mmol) in H₂O (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 H₂O (23 mL), and the whole was stirred at room temperature for 1 h. The mixture was partitioned between CHCl₃ and H₂O. The organic layer was washed with aqueous NaHCO₃ (saturated) and brine, dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 2% MeOH in CHCl₃) to give **6** (0.737 g as a pale yellow solid, 48%): LRMS (EI) m/z 320 (M⁺); ¹H NMR (CDCl₃) 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, CH₃); ¹³C NMR (CDCl₃) δ 164.8, 142.5, 132.2, 129.9, 93.4, 52.7; HRMS (EI) calcd for C₁₀H₉IO₄ 319.9553, found 319.9546.

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

A mixture of **6** (0.46 g, 1.44 mmol) and LiBH₄ (0.16 g, 7.35 mmol) in THF (8 mL) was stirred at room temperature for 24 h. To the mixture was added aqueous NaHCO₃ (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 (SiO₂, 30–100% EtOAc in hexane) to give **7** (0.29 g as a white solid, 76%): LRMS (EI) m/z 264 (M⁺); ¹H NMR (CDCl₃) δ 7.65 (m, 2H, H-4 and H-6), 7.33 (m, 1H, H-2), 4.66 (d, 4H, J = 5.2, CH₂), 1.71 (t, 2H, J = 5.2, OH); ¹³C NMR (DMSO-*d*₆) δ 145.0, 133.1, 123.8, 94.4, 62.1; HRMS (EI) calcd for C₈H₉IO₂ 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 H₂O. The organic layer was washed with aqueous NaHCO₃ (saturated) and brine, dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 30–100% EtOAc in hexane) to give **8** (0.50 g as a pale oil, 97%): ¹H NMR (CDCl₃) δ 7.48 (s, 2H, H-4 and H-6), 7.20 (s, 1H, H-2), 4.62 (s, 4H, CH₂), 0.89 (s, 18H, *t*-BuSi), 0.05 (s, 12H, CH₃Si); ¹³C NMR (CDCl₃) 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 Et₂O (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 (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 0–2% MeOH in CHCl₃) to give **9** (0.69 g as a white solid, 60%): ¹H NMR (CDCl₃) δ 8.03 (s, 2H, H-2 and H-6), 7.61 (s, 1H, H-4), 4.87 (s, 4H, CH₂), 0.10 (s, 18H, *t*-BuSi), 0.16 (s, 12H, CH₃Si); ¹³C NMR (CDCl₃) δ 141.0, 131.7, 129.5, 128.0, 65.0, 26.0, 18.5, –5.2. Anal. Calcd for C₂₀H₃₀BO₄Si₂: 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·H₂O (20 mg, 0.10 mmol) in MeOH (1.6 mL) and H₂O (0.4 mL) was vigorously stirred under an atmosphere of air at room temperature for 2 h. The mixture was partitioned between CHCl₃ and brine. The organic layer was dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 20–100% EtOAc in hexane) to give **11** (60 mg, 61%): LRMS (EI) m/z 499 (M⁺); ¹H NMR (CDCl₃) 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, NH₂), 4.83 (s, 4H, CH₂), 0.96 (s, 18H, *t*-BuSi), 0.13 (s, 12H, CH₃Si); ¹³C NMR (CDCl₃) 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 C₂₅H₄₁N₅O₅Si₂ 499.2799, found: 499.2803. Anal. Calcd for C₂₅H₄₁N₅O₅Si₂·1/2H₂O: 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·H₂O (0.14 g, 0.70 mmol) in MeOH (4 mL) and H₂O (1 mL) was vigorously stirred under an atmosphere of air at room temperature for 2 h. The mixture was partitioned between CHCl₃ and brine. The organic layer was dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 25% EtOAc in hexane) to give **13** (0.34 g, 33%): LRMS (FAB) m/z 734 (MH⁺); ¹H NMR (CDCl₃) δ 8.43 (s, 1H, H-8), 7.53 (s, 2H, aromatic H), 7.43 (s, 1H, aromatic H), 4.83 (s, 4H, CH₂), 1.44 (s, 18H, *t*-BuO), 0.96 (s, 18H, *t*-BuSi), 0.13 (s, 12H, CH₃Si); ¹³C 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 C₁₆H₁₉N₆O₃ 734.35360, found 734.35432. Anal. Calcd for C₃₅H₅₇N₅O₆ Si₂Cl: 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 H₂O (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 H₂O to give **14** (0.17 g, 87%): LRMS (FAB) m/z 288 (MH⁺); ¹H 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, NH₂), 5.31 (t, 2H, J = 5.4, OH), 4.55 (d, 4H, J = 5.4, CH₂); ¹³C 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 C₁₃H₁₄N₅O₃ 288.1097, found 288.1101. Anal. Calcd for C₁₃H₁₃N₅O₃·1/7H₂O: 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·H₂O (97 mg, 0.49 mmol) in MeOH (4 mL) and H₂O (1 mL) was vigorously stirred under an atmosphere of air at room temperature for 2 h. The mixture was partitioned between CHCl₃ and brine. The organic layer was dried (Na₂SO₄), and concentrated. The residue was purified by column chromatography (SiO₂, 0–10% MeOH in CHCl₃) to give **16** (0.42 g, 91%): LRMS (FAB) *m/z* 475 (MH⁺); ¹H NMR (CDCl₃) 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, CH₂), 0.90 (s, 18H, *t*-BuSi), 0.08 (s, 12H, CH₃Si); ¹³C NMR (CDCl₃) 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 C₂₄H₄₂N₃O₃Si₂ 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 H₂O (1 mL) was stirred at room temperature for 1 h. A solution of NaNO₂ (15 mg, 210 μmol) in H₂O (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 (SiO₂, 10–20% MeOH in CHCl₃) to give **17** (220 mg as a white solid, 85%): LRMS (EI) *m/z* 248 (M⁺); ¹H 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, CH₂); ¹³C 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 C₁₂H₁₂N₂O₄ 248.0797, found 248.0790. Anal. Calcd for C₁₂H₁₂N₂O₄: 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 ¹H NMR (CDCl₃) 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); ¹³C NMR (CDCl₃) 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 C₄₁H₃₆N₅O₅ 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 ¹H NMR (CDCl₃) 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); ¹³C NMR (CDCl₃) 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 C₃₇H₃₇N₆O₅ 645.2747, found 645.2819. Anal. Calcd for C₃₇H₃₆N₆O₅·5/4H₂O: 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 ¹H 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); ¹³C 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 C₄₀H₃₆N₃O₆ 654.2604, found 654.2610. Anal. Calcd for C₄₀H₃₅N₃O₆·5/4H₂O: 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 ¹H 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); ¹³C 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 C₃₃H₃₁N₂O₆ 551.2104, found 551.2176. Anal. Calcd for C₃₃H₃₀N₂O₆·1/2H₂O: 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 NaHCO₃ (saturated) and CHCl₃ were added to the mixture, and the separated organic layer was washed with aqueous NaHCO₃ (saturated) and brine, dried (Na₂SO₄) and concentrated. The residue was purified by column chromatography (a neutralized SiO₂, EtOAc) to give **1** (0.88 g, 81%): ³¹P NMR (CDCl₃) 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%): ³¹P NMR (CDCl₃) 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%): ³¹P NMR (CDCl₃) 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, MB1 (20) and MB2 (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. MB1: calculated mass, 10279; observed mass, 10272. MB2: 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.).

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