Research Article

Photoswitching of Site-Selective RNA Scission by Sequential Incorporation of Azobenzene and Acridine Residues in a DNA Oligomer

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Photoresponsive systems for site-selective RNA scission have been prepared by combining Lu(III) ions with acridine/azobenzene dual-modified DNA. The modified DNA forms a heteroduplex with substrate RNA, and the target phosphodiester linkages in front of the acridine residue is selectively activated so that Lu(III) ion rapidly cleaves the linkage. Azobenzene residue introduced adjacent to the acridine residue acts as a photoresponsive switch, which triggers the site-selective scission upon UV irradiation. A *trans* isomer of azobenzene efficiently suppresses the scission, whereas the cis isomer formed by UV irradiation hardly affects the scission. As a result, 1.7–2.4-fold acceleration of the cleavage was achieved simply by irradiating UV for 3 min to the mixture prior to the reaction. Considering the yield of photoisomerization, the intrinsic activity of a *cis* isomer is up to 14.5-fold higher than that of the *trans* isomer.

1. Introduction

In this couple of decades, significant attention has been focused on site-selective RNA scission, since it is indispensable for future molecular biology and therapy [1–3]. Discovery of important roles of short RNA in living cells further promoted this [4]. We have recently developed efficient artificial systems for site-selective RNA scission by combining a metal ion (lanthanide ions or some transition metal ions) as molecular scissor and an acridine-modified DNA as a sequence selective RNA activator [5]. Either of the 5'- or 3'-phosphodiester linkage of the target nucleotide in front of the acridine moiety, which is in protonated form under neutral condition, is site-selectively activated through general acid catalysis [6]. When Lu(III) ion is used as the catalyst, the general acid catalysis preferentially promotes the cleavage at the 3'-side linkage. In addition, conformational change of RNA backbone caused by acridine intercalation is thought to be another important factor in

the activation. The other portions of RNA are protected from metal ion-induced hydrolysis by duplex formation with DNA additives. Sequence of the target site can be freely chosen, and the reaction is selective and efficient enough to achieve simultaneous tandem scission in close proximity as small as 10 nucleotides [7]. This technique has been applied to new genotyping methods for single-nucleotide (SNP) or insertion-deletion (indel) polymorphisms [8].

One significant advantage of this system is that any desired function can be added to it by additional modification to the acridine-modified DNA. One such example is the addition of a ligand to DNA to localize the catalyst near the target site [9]. Introduction of an iminodiacetate ligand to the point over the major groove results in more than 3-fold acceleration of the site-selective cleavage. Photoswitching of the cleavage is another attractive functionalization to the system, since light can trigger the reaction at any desired timing without changing chemical or physical conditions [10]. Azobenzene is one of the most popular photoresponsive
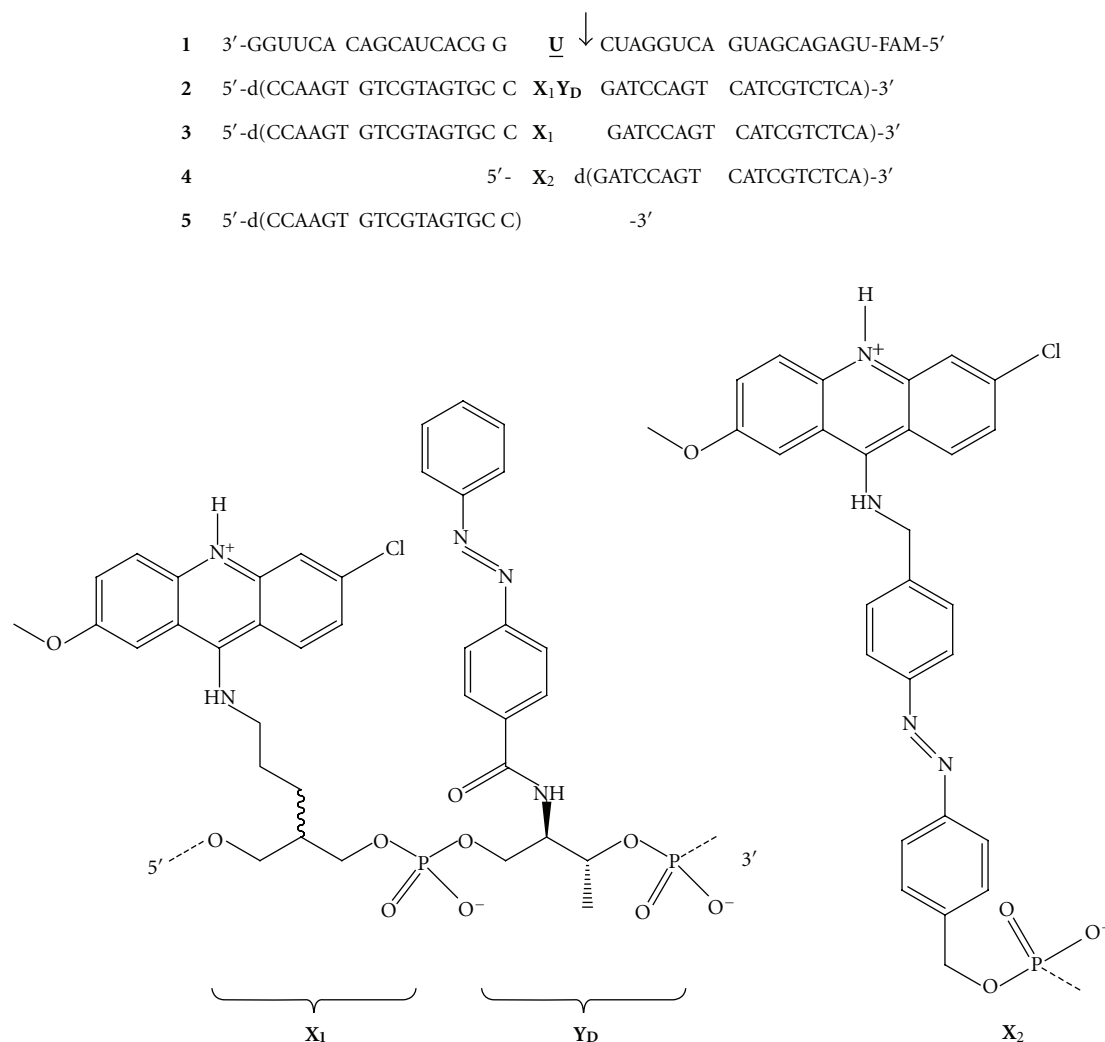
1   3′-GGUUCA CAGCAUCACG G   **U** ↓ CUAGGUCA GUAGCAGAGU-FAM-5′

2   5′-d(CCAAGT GTCGTAGTGC C **X₁Y_D** GATCCAGT   CATCGTCTCA)-3′

3   5′-d(CCAAGT GTCGTAGTGC C **X₁**   GATCCAGT   CATCGTCTCA)-3′

4   5′- **X₂** d(GATCCAGT   CATCGTCTCA)-3′

5   5′-d(CCAAGT GTCGTAGTGC C)   -3′

FIGURE 1: Structures of the substrate RNA (**1**) and the modified DNA (**2–4**). The target phosphodiester linkage (the 5′-phosphate of U-19) is indicated by the arrow.

molecules used in such studies. Azobenzene isomerizes from *trans* isomer, which is planer and rather hydrophobic, into bulky and polar *cis* isomer upon UV ($\lambda \approx 320$ nm) irradiation and goes back to *trans* isomer upon visible light ($\lambda \approx 450$ nm) irradiation.

We have reported the first photoresponsive system of site-selective RNA scission by using acridine-modified DNA and free Mn(II) ion as a cleaving catalyst [11]. There, an azobenzene group was inserted between the acridine ring and the backbone of DNA as a linker (see **X₂** in Figure 1). Although phototriggered acceleration of site-selective RNA scission was achieved in the system, the cleavage activity was quite marginal (the yield of selective cleavage after 18 h was 4%), and the magnitude of acceleration was only 40% after UV irradiation. Accordingly, more active and clear-cut photocontrol of site-selective RNA scission using free Lu(III) ion as the catalyst has been desired. In this study, new photocontrollable site-selective RNA activator has been prepared by introducing an independent azobenzene residue to DNA oligomer in combination with an acridine residue

(Figure 1). Such azobenzene residues in *trans* form stack on the acridine in the adjacent residue and efficiently inhibit the site-selective RNA activation. When the solution is irradiated with UV light and the azobenzene isomerizes into *cis* isomer, the acridine is released from stacking, and the cleavage reaction is significantly accelerated. Clear-cut photo-control of efficient site-selective RNA scission has been successfully accomplished.

2. Materials and Methods

2.1. Spectroscopy. ¹H NMR spectra were obtained on a 500 MHz NMR spectrometer (Bruker Biospin). For electronic spray ionization (ESI) mass spectroscopy, an HITACHI M-8000 LC/3DQMS mass spectrometer was used. MALDI-TOF/MS was measured on a Bruker Daltonics Auto-FLEX mass spectrometer.

2.2. Preparation of Oligonucleotides. The phosphoramidite of **X₃** was synthesized starting from the coupling between
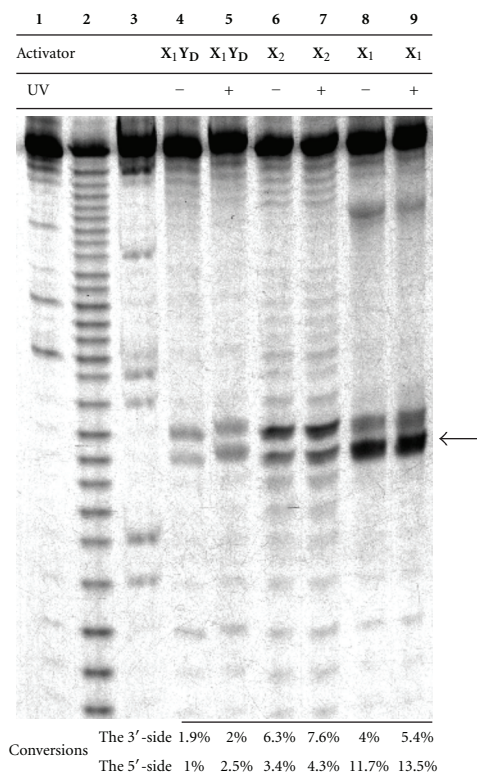
FIGURE 2: A denaturing 20% PAGE pattern for site-selective scission of **1** activated by **2** or (**4** + **5**) in the presence of Lu(III) with or without UV irradiation. Lane 1, control in a buffer solution; lane 2, treatment with Lu(III) alone; lane 3, RNase T1 digestion; lane 4, **2** and Lu(III) without UV irradiation; lane 5, **2** and Lu(III) with UV irradiation; lane 6, **4, 5,** and Lu(III) without UV irradiation; lane 7, **4, 5,** and Lu(III) with UV irradiation; lane 8, **3** and Lu(III) without UV irradiation; lane 9, **3** and Lu(III) with UV irradiation. The 5′-side cleavage of U-19 is indicated by the arrow. Conversions of each scission are presented in the bottom. Reaction conditions; [RNA] = $5\,\mu$M, [DNA] = $10\,\mu$M, [LuCl$_3$] = $100\,\mu$M, [Tris] = 10 mM, [NaCl] = 200 mM, pH 7.5, 37°C, 4 h.

2-chloro-4-nitrobenzoic acid and 4-isopropoxyaniline as described in [6, 12]. The synthetic route for the phosphoramidite of X_2 is described in [11]. The phosphoramidites for Y_D and Y_L were synthesized according to [13]. All the oligonucleotides were synthesized on an ABI 3400 DNA Synthesizer in $1\,\mu$mol scale. Reagents for automated DNA syntheses were purchased from Glen Research Co. (VA, USA). For the synthesis of modified DNA, an extended coupling time of 10 min was adopted for the coupling of azobenzene- and acridine-phosphoramidite monomers. The DMTr-off oligonucleotides were cleaved from the support and deprotected by a treatment with methanolic sodium hydroxide (1 mL, 0.4 M, methanol : water = 4 : 1) at room temperature for 16 h.

2.3. Purification and Characterization of the Modified Oligonucleotides.
The oligonucleotides were first desalted by Poly-Pak II cartridges (Glen Research Co.), and the resulting crude products were then purified by denaturing 20% PAGE.
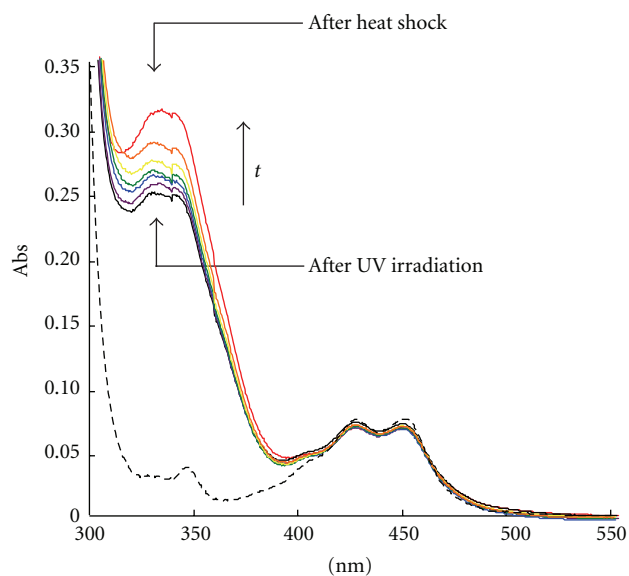

FIGURE 3: Time dependence of UV-VIS absorption of **1/2** complex. The spectra were measured 0 (black), 1 (purple), 6 (blue), 11 (green), 16 (yellow), and 21 h (orange) after UV irradiation at pH 7.5 and 37°C. The spectrum after heat shock (*trans*-dominant) is drawn in red. The dashed line represents the spectrum of **1/3** complex without azobenzene.

TABLE 1: MALDI-TOF/MS analyses of the conjugates.

	Calcd. m/z	
	[M + H]	Found
2	11511.1	11509.5
3	11136.8	11116.4
6	11592.8	11566.0
7	11592.8	11563.6
8	11592.8	11563.8
9	11592.8	11543.5
10	11218.5	11250.5

Final purification was carried out on a reversed phased HPLC equipped with an RP-C18 column (Cica-Merck LiChro-CART 125-4; a linear gradient of 0%–25% acetonitrile with ammonium formate (50 mM) over 25 min; flow rate 0.5 mL/min). They were fully characterized by MALDI-TOF/MS in the positive ion mode (Table 1). Concentration of the stock solution of each oligonucleotide was determined both with UV absorption of DNA at 260 nm and HPLC quantification of each nucleosides formed by digestion with snake venom phosphodiesterase and alkaline phosphatase.

2.4. RNA Cleavage Assay.
A mixture of the substrate RNA (18 μL, 5.5 μM) and modified DNA (11 μM) in pH 7.5 Tris buffer (11 mM) containing NaCl (220 mM) was prepared and divided into halves. Both portions were heated to 90°C for 1 min and slowly cooled to room temperature. One of the portions was irradiated with UV light from UV Spot Light Source (Hamamatsu Photonics) through a UV-D36C
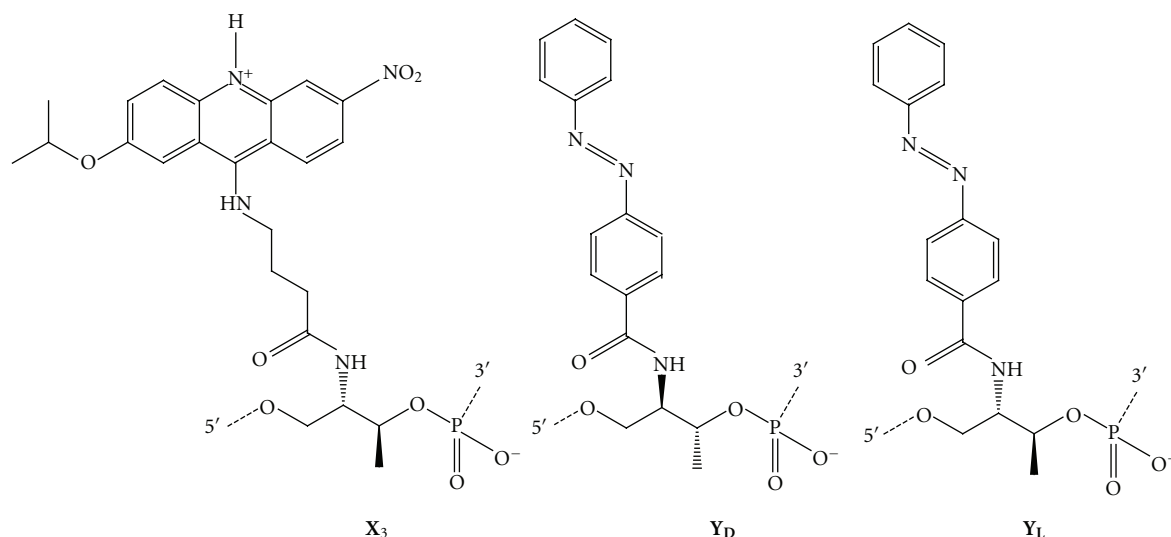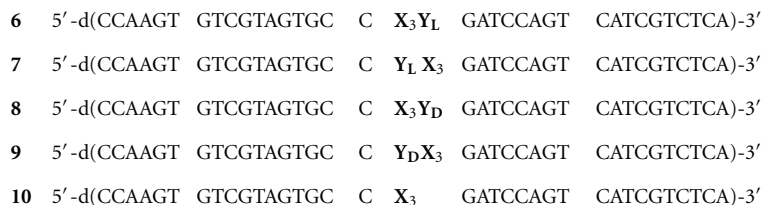
6 5′-d(CCAAGT GTCGTAGTGC C **X₃Y_L** GATCCAGT CATCGTCTCA)-3′

7 5′-d(CCAAGT GTCGTAGTGC C **Y_L X₃** GATCCAGT CATCGTCTCA)-3′

8 5′-d(CCAAGT GTCGTAGTGC C **X₃Y_D** GATCCAGT CATCGTCTCA)-3′

9 5′-d(CCAAGT GTCGTAGTGC C **Y_DX₃** GATCCAGT CATCGTCTCA)-3′

10 5′-d(CCAAGT GTCGTAGTGC C **X₃**   GATCCAGT CATCGTCTCA)-3′

FIGURE 4: Structures of the modified DNA bearing more active acridine (X_3) and the two azobenzene residues (Y_D and Y_L).

(Asahi Technoglass) for 3 min to isomerize *trans*-azobenzene into its *cis* isomer. Selective cleavage reaction was initiated by adding 1 μL of aqueous LuCl₃ solution (1 mM, final concentration was 100 μM) to the mixture. After incubation for a predetermined reaction time at 37°C, the reaction was quenched by 1 μL of EDTA·2Na solution (100 mM) and analyzed on 20% denaturing PAGE. All the reactions were carried out in black tubes so that the *cis* isomer did not isomerize into *trans* isomer by ambient visible light.

3. Results and Discussion

3.1. Photoresponsive Site-Selective RNA Scission by a Combination of Lu(III) Ion and Acridine-Azobenzene Dual-Modified DNA. To achieve efficient photo-control of site-selective RNA scission catalyzed by Lu(III) ion, new acridine/ azobenzene dual-modified DNA was synthesized (**2** in Figure 1). This DNA has an acridine residue (X_1) as the 18th residue. The acridine ring in X_1 is 9-amino-6-chloro-2-methoxyacridine. This residue is the most popular acridine residue, which is often used as a duplex stabilizer or a fluor-escent label [14, 15]. The linker moiety in X_1 is a flexi-ble alkyl chain, and the stereochemistry of the branching point is not controlled. Adjacent to X_1, an azobenzene resi-due (Y_D) was also introduced as the 19th residue. This conjugate of D-threoninol and azobenzene is known to be a good photoswitch of duplex formation [13]. When the corresponding simple acridine-modified DNA **3** is
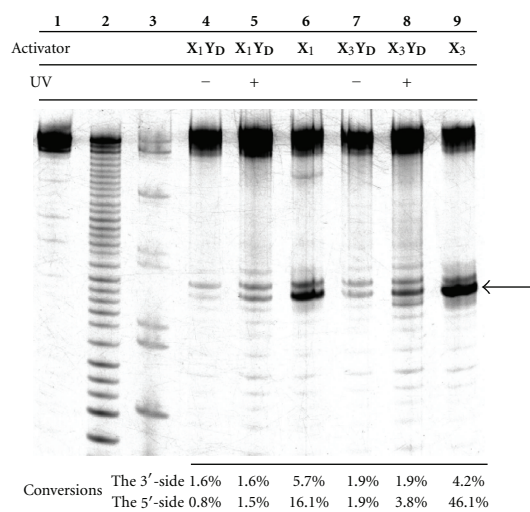

FIGURE 5: A denaturing 20% PAGE pattern for site-selective scission of **1** activated by **2**, **3**, **8**, or **10**. Lane 1, control reaction in a buffer solution; lane 2, treatment with Lu(III) alone; lane 3, RNase T1 digestion; lane 4, **2** and Lu(III) without UV irradiation; lane 5, **2** and Lu(III) with UV irradiation; lane 6, **3** and Lu(III) without UV irradiation; lane 7, **8** and Lu(III) without UV irradiation; lane 8, **8** and Lu(III) with UV irradiation; lane 9, **10** and Lu(III) without UV irradiation. The 5′-side cleavage of U-19 is indicated by the arrow. Conversions of each scission are presented in the bottom. Reaction conditions; [RNA] = 5 μM, [DNA] = 10 μM, [LuCl₃] = 100 μM, [Tris] = 10 mM, [NaCl] = 200 mM, pH 7.5, 37°C, 4 h.

	1	2	3	4	5	6	7	8
Activator	X_3Y_L	X_3Y_L	Y_LX_3	Y_LX_3	X_3Y_D	X_3Y_D	Y_DX_3	Y_DX_3
UV	−	+	−	+	−	+	−	+

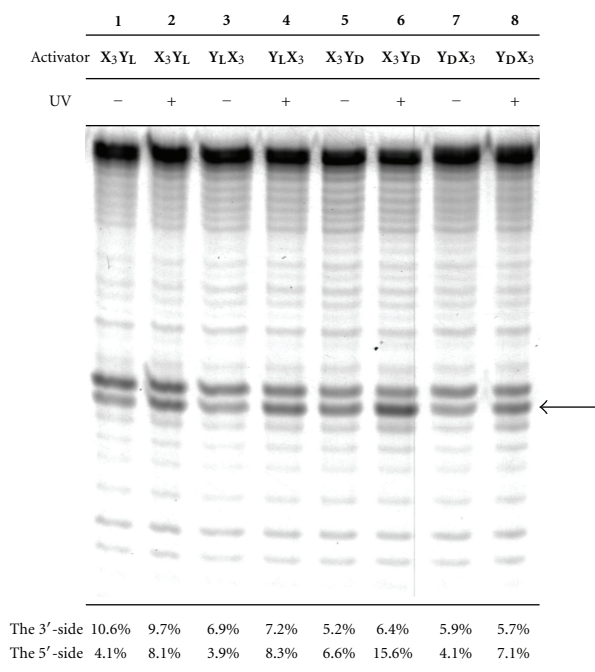|	1	2	3	4	5	6	7	8
The 3′-side	10.6%	9.7%	6.9%	7.2%	5.2%	6.4%	5.9%	5.7%
The 5′-side	4.1%	8.1%	3.9%	8.3%	6.6%	15.6%	4.1%	7.1%

Figure 6: A denaturing 20% PAGE pattern for site-selective scission of **1** activated by **6–9**. Lane 1, **6** and Lu(III) without UV irradiation; lane 2, **6** and Lu(III) with UV irradiation; lane 3, **7** and Lu(III) without UV irradiation; lane 4, **7** and Lu(III) with UV irradiation; lane 5, **8** and Lu(III) without UV irradiation; lane 6, **8** and Lu(III) with UV irradiation; lane 7, **9** and Lu(III) without UV irradiation; lane 8, **9** and Lu(III) with UV irradiation. The 5′-side cleavage of U-19 is indicated by the arrow. Conversions of each scission are presented in the bottom. Reaction conditions; [RNA] = 5 μM, [DNA] = 10 μM, [LuCl$_3$] = 100 μM, [Tris] = 10 mM, [NaCl] = 200 mM, pH 7.5, 37°C, 7 h.

hybridized to the complementary substrate RNA **1**, the target U-19 residue alone remains unpaired, and the 5′-phosphate linkage (indicated by the arrow in Figure 1) of the target is efficiently cleaved by Lu(III) ion in the aid of acid catalysis by the acridine. The 3′-phosphate linkage is also marginally cleaved, but it is simply caused by conformational changes of RNA backbone and is not much affected by the acridine. As a control, previous dual-modified DNA (**4**) that bears an azobenzene as a linker moiety was also used for the cleavage experiment in combination with an unmodified DNA (**5**) and Lu(III).

Figure 2 is a typical denaturing 20% polyacrylamide gel electrophoresis (PAGE) pattern of the cleavage reactions. When **1** was hybridized to **2** and Lu(III) ion was added to the solution under dark, the 3′-phosphodiester of U-19 was only marginally cleaved, and the target 5′-phosphodiester was almost intact after 4 h reaction at 37°C (the lower band in lane 4). The cleavage yield was 1.9% and 1.0%, respectively. Compared with 11.7% for the 5′-side cleavage using simple acridine-modified DNA **3** (lane 8), addition of *trans*-azobenzene adjacent to the acridine almost completely suppressed the selective cleavage. When the annealed mixture was irradiated with UV prior to Lu(III) addition and the

azobenzene was isomerized into *cis*-form, on the other hand, the cleavage at the 5′-phosphodiester significantly recovered (lane 5). The cleavage yield at the 5′-phosphodieser was 2.5% here (the yield at the 3′-phosphodiester was 2.0% again). Similar cleavage yields were achieved in two more repeats of the reaction with independently prepared reaction mixtures. More than two times acceleration of the cleavage was achieved selectively at the target linkage simply with 3-min UV irradiation. In contrast, little change was observed in the cleavage in the presence of previous **4** + **5** combination (lanes 6 and 7). This combination preferentially promotes the 3′-side cleavage unlike the other acridine-modified DNA, but the ratio of the 3′-side cleavage to the 5′-side cleavage was both 1.8 irrespective of the UV irradiation. Previous X_2 is not capable of photo-control of Lu(III) catalyzed RNA hydrolysis. As expected, UV irradiation did not affect the cleavage using **3**, which does not bear azobenzene, as well (lanes 8 and 9). Effective photoregulation of site-selective RNA scission catalyzed by Lu(III) is achievable only with the present system where the acridine and the azobenzene moieties are separately introduced to DNA as individual residues.

3.2. Estimation of the Yield of Photoisomerization in DNA/ RNA Heteroduplex. According to the previous studies, photoisomerization of azobenzene introduced in DNA is somewhat suppressed when the DNA forms duplex with complementary DNA or RNA, probably because of stacking interaction with adjacent base pairs. The yield of *trans* to *cis* isomerization is reported to be only 20%–40% in DNA/DNA duplex [16]. The isomerization of azobenzene in **2** is also expected to be not perfect. In addition, it is known that *cis* to *trans* reverse isomerization is triggered not only by visible light irradiation, but thermal isomerization also occurs in a significant rate [17]. It is necessary to estimate these properties of Y_D introduced next to the acridine residue.

The population of each isomer can be estimated by monitoring the change of absorbance at 332 nm before and after UV irradiation. Figure 3 shows UV spectra of **1/2** complex before and after UV irradiation. After UV was irradiated to the solution, significant hypochromicity at 332 nm was observed. The absorbance gradually recovered thereafter, and the first-order rate constant of reverse isomerization was obtained from the time dependence of Abs$_{332}$ to be 0.032 h^{-1}. This number corresponds to the half-life of 16 h. It is fairly long enough for the reaction time employed in this study. Considering that $\varepsilon_{332, \text{trans}}/\varepsilon_{332, \text{cis}} = 9.15$ and the ratio of the population of *trans*- to *cis*-isomer is 94 : 6 after heat shock (90°C for 1 min) [18], the ratio just after UV irradiation is estimated to be 70 : 30. Almost no change was observed for the absorbance at 260 nm before and after UV irradiation (data not shown). Thus, isomerization of the azobenzene in such a long oligonucleotides does not affect the overall duplex formation under the conditions employed here.

3.3. Still Faster Photocontrolled Cleavage by Use of More Active Acridine Residue. The acridine residue X_1 used in the
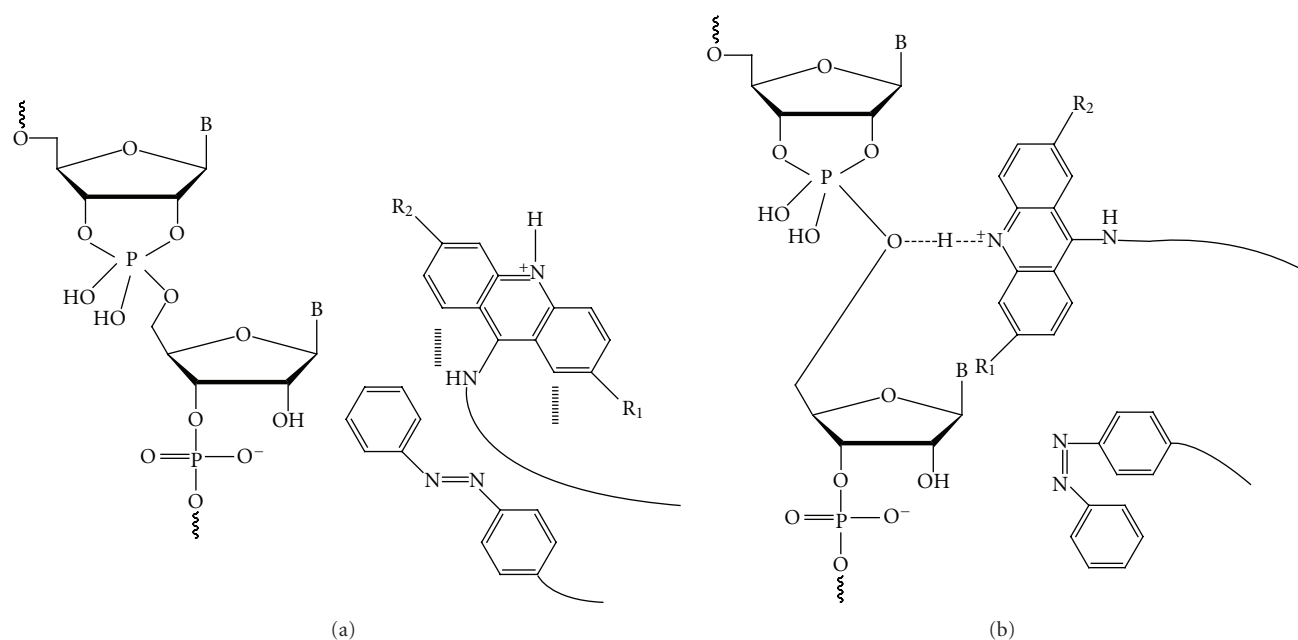
(a)

(b)

FIGURE 7: A proposed mechanism of the Photoswitching of site-selective RNA scission by azobenzene residues. (a) Azobenzene residues in *trans* form stack on the acridine in the adjacent residue and efficiently inhibit site-selective RNA activation. (b) When the solution is irradiated with UV light and the azobenzene isomerizes into *cis* isomer, the acridine is released from stacking and the cleavage reaction is significantly accelerated.

above experiments is commercially available and bears 9-amino-6-chloro-2-methoxyacridine. In our previous studies optimizing the structure of the acridine ring and the linker moiety, far more active acridine residue (X_3 in Figure 4) has been developed [12, 19]. This X_3 bears more acidic 9-amino-2-isopropoxy-6-nitroacridine via optically pure linker based on L-threoninol and 4-aminobuthanoic acid and shows nearly three times higher RNA activation ability than X_1. By using X_3, four kinds of dual-modified oligonucleotides were prepared. The dual-modified oligonucleotide **8** is a counter-part of **2** and bears X_3 in place of X_1 in a combination with Y_D in the 3′ side. The oligonucleotide **9** also bears X_3 and Y_D in the middle, but Y_D is inserted in the 5′ side of X_3. A previous study has shown that the steric effect caused by the isomerization of azobenzene strongly depends on the regiochemical conformation of the threoninol backbone [13]. For the purpose of comparison, Y_L, a diastereomer of Y_D, was prepared and incorporated into oligonucleotides **6** and **7**.

Figure 5 shows a comparison of site-selective RNA scission using oligonucleotides bearing 9-amino-6-chloro-2-methoxyacridine (**2** and **3**) and 9-amino-2-isopropoxy-6-nitroacridine (**8** and **10**). As in lane 9, the oligonucleo-tide bearing 9-amino-2-isopropoxy-6-nitroacridine via L-threo-ninol linker (**10**) also efficiently activates RNA and promotes site-selective scission catalyzed by Lu(III). The efficacy is nearly three times as high as that of **3**. When an azobenzene residue with D-threoninol linker (Y_D) in *trans* form was inserted to the 3′ side of the acridine residue (**8**, lane 7), the site-selective cleavage was drastically suppressed just as in the system with 9-amino-6-chloro-2-methoxyacridine (**2**,

lane 4). However, the cleavage activity significantly recovered when UV was irradiated to the reaction mixture for 3 min prior to the initiation of cleavage reaction (lane 8). The yield of site-selective cleavage at the 5′-phosphate is again twice as high as that without UV irradiation.

To evaluate how the position of insertion and the regiochemical conformation of an azobenzene residue affect on its Photoswitching yield, site-selective RNA scission using **6–9** was performed (Figure 6). Note that the reaction time in this experiment was extended to 7 h to obtain more explicit difference in the cleavage yields. The recovery of the reaction activity after UV irradiation (=the yield of the 5′-phosphate cleavage with UV irradiation/that without UV irradiation) was 2.0-fold for **6**, 2.1-fold for **7**, 2.4-fold for **8**, and 1.7-fold for **9**. These results indicate that an azobenzene residue in *trans* form efficiently inhibits site-selective RNA activation irrespective of the position of its insertion and the regiochemical conformation of the linker moiety, and an azobenzene residue in *cis* form does not. In all of the cases, change in the 3′-side scission before and after UV irradiation was quite marginal. Considering that the general acid catalysis by protonated acridine has almost nothing to do with the 3′-side cleavage [6, 19], the present enhancement of the 5′-side cleavage may be mainly because of the recovery in general acid catalysis. It has been shown that an azobenzene in *trans* form is sufficiently planer and hydrophobic to stack on adjacent DNA base pairs when it is introduced into DNA duplex [20]. Strong stacking interaction in DNA duplex is also reported for various dyes bearing similar azobenzene rings [21]. The efficient inhibition of the function of acridines observed in

the present study may thus be attributed to the stacking interaction between *trans*-azobenzene and the acridines as well (Figure 7). This mechanism is also supported by an observation that incorporation of two acridine residues into the target site strongly decreases the cleavage yield [5]. The *trans* to *cis* isomerization triggered by UV irradiation releases the acridine from the stacking, and let it activates RNA. If this is the case, 1.7–2.4-fold recovery of the cleavage activity observed in Figure 6 after UV irradiation can be attributable mainly to the increase of the population of *cis* isomer from 6% to 30%. The ratio of the intrinsic activity of the *cis* isomer of **8** to the *trans* isomer can be calculated from

$$2.4(0.06x + 0.94y) = 0.30x + 0.70y, \qquad (1)$$

where x is the intrinsic activity of the *cis* isomer and y is that of the *trans* isomer. Thus, the activity of 100% *cis* isomer calculated from these numbers is as much as 14.5 times higher than that of 100% *trans* isomer for **8** and 4.5 times higher for **9**.

4. Conclusions

Efficient photo-control of site-selective RNA scission have been accomplished by combining free Lu(III) ion with dual-modified oligonucleotides which bear photoresponsive azobenzene residue adjacent to the acridine residue. Intrinsic activity of *cis* isomers of the dual-modified oligonucleotides is estimated to be up to 14.5 times as high as that of *trans* isomers. The suppressed activities by the introduction of *trans* azobenzenes were recovered by a factor of 2-3-fold simply with 3-min UV irradiation. Since the isomerization of azobenzene is reversible and *cis*- to *trans*-isomerization is with visible light, switching-off of the selective cleavage is also quite feasible with the present design. The most important advantage of the present design is the ease of individual optimization of the acridine and the azobenzene residues, as been presented for the acridine residue in this study. Various azobenzene derivatives with additional substituents of improved properties such as higher photoisomerization yield or enhanced thermal stability have been developed and introduced to DNA to date [22, 23]. Perfect on-off switching of RNA cleavage may be feasible by further promoting the isomerization using such improved azobenzene monomers. Development of further versatile tools in biochemistry is expected.
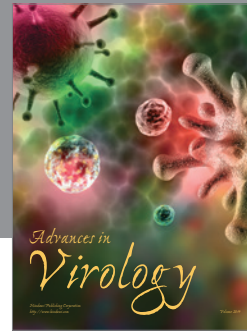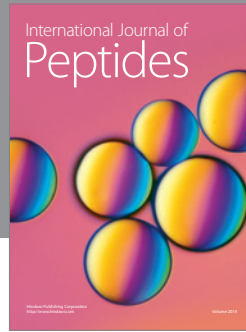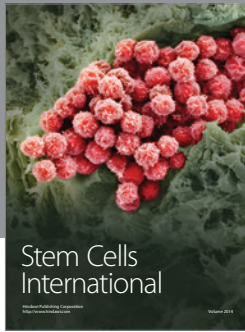
Acknowledgments

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