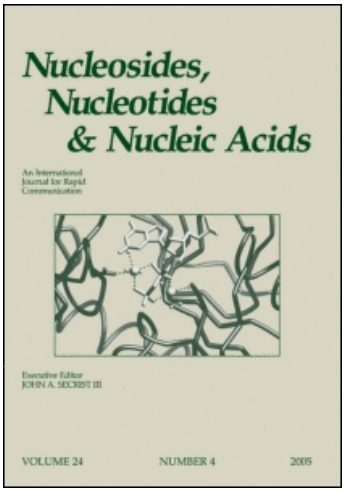

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SYNTHESIS OF PHOTO-RESPONSIVE ACRIDINE-MODIFIED DNA AND ITS APPLICATION TO SITE-SELECTIVE RNA SCISSION

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□ *Photo-responsive phosphoramidite monomers, which bear an azobenzene between acridine and the phosphoramidite unit, were synthesized, and incorporated into oligonucleotides. Upon UV irradiation, the azobenzene in the modified DNA efficiently isomerized from the trans isomer into the cis isomer. Although the T_m values of their duplexes with complementary DNA were not much changed by the isomerization, site-selective RNA scission was significantly accelerated by the UV irradiation when Mn(II) ion was used as the catalyst for RNA scission.*

Keywords Photo-responsive; acridine; modified DNA

INTRODUCTION

To date, various conjugates of oligonucleotides with functional molecules such as intercalating agents, fluorescent dyes, and metal complexes have been prepared because of their attractive potentials for biological and biochemical applications.[1–4] Acridine is known as an intercalating agent and has been widely used for fluorescent labelling, duplex stabilization, and so on.[5–12] Recently, we incorporated an acridine to oligonucleotides and used these conjugates for site-selective scission of RNA.[13–15] There, the phosphodiester linkages of the RNA in front of the acridine were activated by noncovalent interactions such as local perturbation of RNA-backbone conformations and promptly hydrolyzed by metal ions. One attractive functionalization of such biochemical tool is to add photo-responsibility, because light can then trigger RNA scission at

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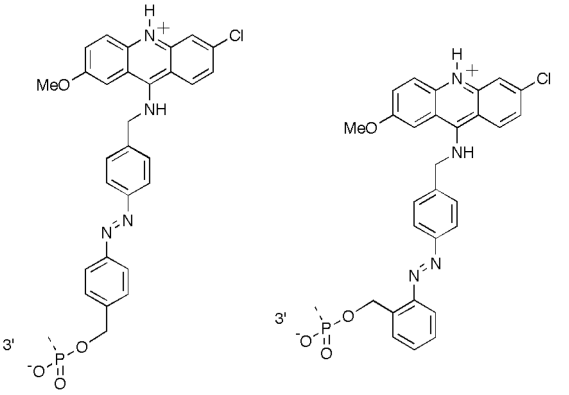
1 5′ -FAM-UGA GAC GAU GAC UGG AUC **U**GG CAC UAC GAC ACU UGG -3′
2 3′ -ACT CTG CTA CTG ACC TAG **X**
3 3′ -ACT CTG CTA CTG ACC TAG **Y**
4 3′ -CC GTG ATG CTG TGA ACC -5′
5 3′ -TTT **X**
6 5′ -TGA GAC GAT GAC TGG ATC -3′



X **Y**

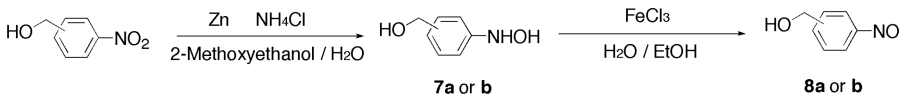
FIGURE 1 The structures of azobenzene-bearing acridine monomer residues (**X**, **Y**) and the oligonucleotides used in the present work.

desired timing and place without changing chemical or physical conditions. In this study, we have synthesized two new phosphoramidite monomers that bear an azobenzene between an acridine and the phosphoramidite group (**12a** and **12b**), and incorporated them into oligonucleotides (**2**, **3,** and **5** in Figure 1). Azobenzene, which is isomerized from its *trans*-isomer into *cis*-isomer upon UV irradiation ($\lambda \approx 320$ nm) and goes back to the *trans*-isomer upon visible light irradiation ($\lambda \approx 450$ nm), is one of the most popular photo-responsive molecules used in biological and biochemical studies, because of its large photo-isomerization yield, high stability of the isomers, and satisfactory reversibility. Here, these modified oligonucleotides, which involve both an acridine (site-selective RNA activator) and a photo-responsive azobenzene, are combined with Mn(II) ion for site-selective RNA scission. The RNA scission, which is based on site-selective RNA activation strategy, has been photo-controlled.

RESULTS AND DISCUSSION

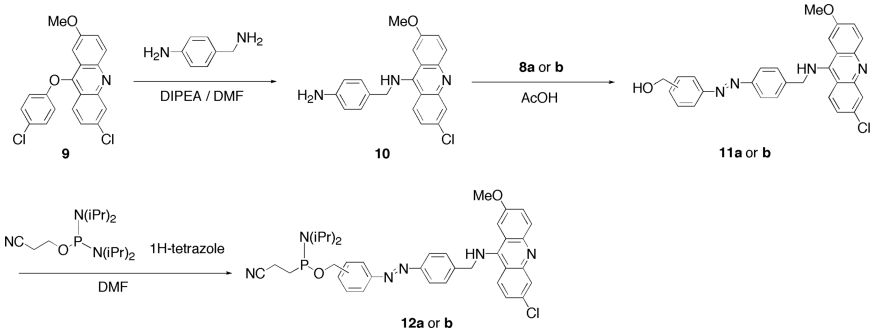
Synthesis of Phosphoramidite Monomers and DNA Conjugates

Two kinds of photo-responsive monomers bearing azobenzene between the acridine and the phosphoramidite unit (**12a** and **12b**) were synthesized. With **12a**, an azobenzene of *para*-geometry is introduced to oligonucleotides (X in Figure 1), whereas the azobenzene from **12b** takes *ortho*-geometry (Y in Figure 1). This difference should give rise to subtle but significant change in the position of the acridine



SCHEME 1

when the oligonucleotides bind with the RNA substrate for site-selective scission. First, the 6-chloro-9-(*p*-chlorophenoxy)-2-methoxyacridine (**9**) was reacted with 4-aminobenzylamine to give 9-(*p*-aminobenzylamino)-6-chloro-2-methoxyacridine (**10**). Then **10** was coupled with 4- or 3-nitrosobenzyl alcohol (**8a** or **b**), which was obtained from Scheme 1, to give acridine-azobenzene conjugate (**11a** and **b**). Finally, a 2-cyanoethyl-N,N-diisopropylphosphoramidite group was introduced to **11a** and **b** to provide the phosphoramidite monomers **12a** and **12b**.



SCHEME 2

The photo-responsive acridine-modified oligonucleotides (**2**, **3**, and **5** in Figure 1) were synthesized on an automated DNA synthesizer. In the final step involving **12a** or **b**, the coupling time was extended to 30 minutes. These modified oligonucleotides were purified as usual and characterized with MALDI-TOFMS (Table 1). The short oligonucleotide **5** was also analyzed with electronic spray ionization (ESI) mass spectroscopy. In site-selective RNA scission, **2** (or **3**) is hybridized to substrate RNA **1** in combination with another unmodified DNA (**4**). As the result, the acridine-*p*-azobenzene residue (**X**) from **12a** (or the acridine-*o*-azobenzene residue **Y** from **12b**) is placed just in front of the target U-19 residue in the RNA **1** (see Figure 1).

TABLE 1 Mass spectral data of the photo-responsive acridine-modified oligonucleotides.

	calcd (M+H)	found
2	5980.5	5978.2
3	5980.5	5986.4
5	1393.5	1396.3

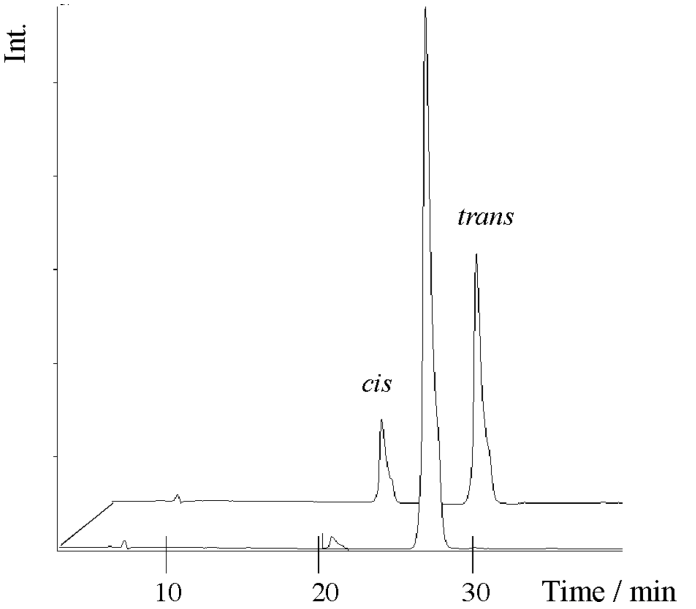


FIGURE 2 HPLC patterns of **5** before (front) and after (back) UV irradiation. Irradiation time is 3 minutes (details are presented in Experimental section).

Efficiency of the Photo-Isomerization of the Azobenzene in the Modified Oligonucleotides

In order to evaluate the yield of the photo-isomerization of the azobenzene unit, the short oligonucleotide **5** was used and analyzed by RP-HPLC before and after the UV irradiation (3 minutes). As seen in Figure 2, clearly separated two peaks were observed. The faster peak (retention time = 21 minutes), whose area significantly increased after the UV irradiation, is assignable to **5** involving *cis*-form azobenzene, while the slower one (27 minutes) to the *trans*-isomer. The ratio of mole absorption coefficient of the *cis*-isomer at the detecting wavelength ($\lambda = 332$ nm) to that of the *trans*-isomer was independently determined to be 1:5.3. Before the UV irradiation, the azobenzene in **5** exists mostly in the *trans*-form (the *cis*:*trans* ratio = 1:10). After the UV irradiation for 3 minutes, 55% of the *trans*-azobenzene was isomerized into the *cis*-form. The population of *cis*-azobenzene in the photo-stationary state was 59% under the conditions employed (the *cis*:*trans* ratio = 3:2).

The thermal isomerization rate of the reverse isomerization was determined from the time dependence of UV-VIS spectra of **2** (data not shown). The pseudo-first order rate constant of *cis*- to *trans*-isomerization is 0.013 h^{-1} ($t_{1/2} = 53$ hours).

TABLE 2 The melting temperatures of the duplexes between acridine-modified DNA conjugate and complementary DNA.

	T_m (°C)	
	Before UV irradiation	After UV irradiation
2/6	46.9	46.1
3/6	43.4	43.5

Duplex Forming Activity of the Modified Oligonucleotides

The T_m values of the duplexes of the photo-responsive acridine-modified oligonucleotides **2** and **3** with their complementary oligonucleotide (**6**) are shown in Table 2. The **3**/**6** duplex involving *ortho*-azobenzene has similar T_m values with that (43.9°C) of the unmodified duplex without azobenzene unit, whereas the **2**/**6** duplex, in which the azobenzene takes *para*-geometry, has significantly higher T_m. It is presumed that the acridine successfully stacks on the adjacent base pairs when the *para*-azobenzene unit is introduced, but the acridine attached to the *ortho*-azobenzene does not. These T_m values hardly changed upon photo-induced *cis-trans* isomerization of the azobenzenes, thus the isomerization does not much alter the stacking modes of the acridines.

Effects of Photo-Isomerization of the Azobenzene on Site-Selective RNA Scission Using the Photo-Responsive Acridine-Modified Oligonucleotides

Figure 3 shows a typical polyacrylamide gel electrophoresis pattern of site-selective RNA scission using the **2**/**4** or the **3**/**4** combinations. Mn(II) ion was used as the catalyst for RNA hydrolysis. In the experiments, the mixtures of these oligonucleotides and the substrate RNA **1** were divided into two portions and one portion was directly used for RNA scission by adding $MnCl_2$. Another portion was first irradiated with UV (λ = 300–400 nm) for 3 minutes, and then used for the RNA scission. In the presence of either the **2**/**4** combination or the **3**/**4** combination (without UV irradiation), **1** was cleaved site-selectively at the 3′-side of U-19 (lanes 4 and 6, respectively). These results are completely consistent with previous results in which modified oligonucleotides bearing only acridine (without the azobenzene between the acridine and the main chain) were used in place of **2** and **3**.[13] Even with the incorporation of the azobenzene, the phosphodiester linkage of RNA which is located in front of the acridine is also selectively activated and hydrolyzed by the metal ion.

With the use of the **2**/**4** combination, the scission efficiency was notably increased upon the UV irradiation (compare lane 4 with lane 5). As shown

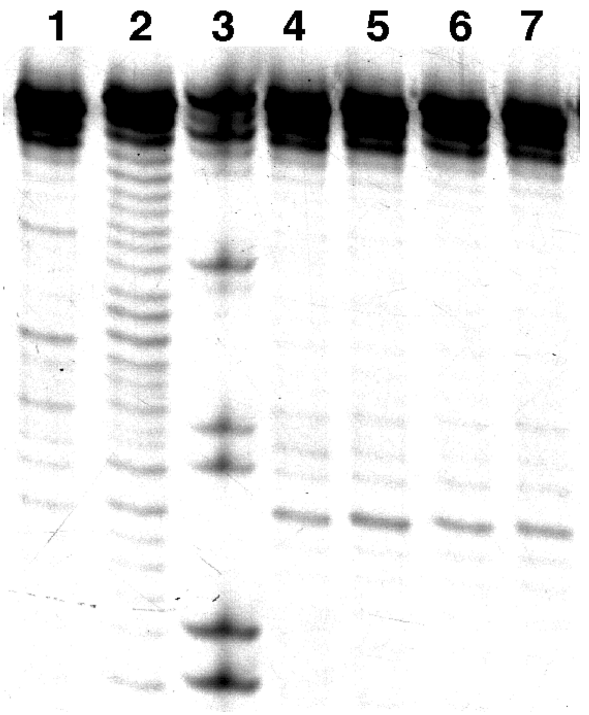


FIGURE 3 Gel electrophoresis patterns for site-selective scission of **1** by combining **2**/**4** or **3**/**4** with Mn(II) ion. Lane 1, control; lane 2, treatment with Mn(II) alone; lane 3, RNase T1 digestion; lane 4, **2**/**4** and Mn(II); lane 5, **2**/**4** and Mn(II) with UV irradiation; lane 6, **3**/**4** and Mn(II); lane 7, **3**/**4** and Mn(II) with UV irradiation. Reaction conditions; [RNA] = 5 μM, [DNA] = 10 μM, [$MnCl_2$] = 1 mM, [Tris] = 10 mM, [NaCl] = 200 mM, pH 8.0, 37°C, 18 hours.

in Table 3, the conversion of scission at the 3'-side of U-19 was 2.7% before the UV irradiation, but increased to 3.9% after the UV irradiation. Thus, 1.4-fold acceleration was achieved simply by irradiating UV for 3 minutes. The reproducibility of this acceleration has been confirmed by several separate reactions. With the **3**/**4** combination, however, the scission efficiency was not much changed by UV irradiation (lanes 6 versus 7). In lanes 4–7, a minor site-selective scission simultaneously occurred at the 5′-side of U-19 (the bands of larger mobility in the gel). This minor scission by the **2**/**4** combination was also promoted by 1.4 fold by the photo-induced isomerization of the azobenzene, whereas the corresponding scission by the **3**/**4** combination was little affected by the isomerization (see also Table 3). Although the results in Table 2 indicate that the interactions between the acridine and adjacent base pairs seem to be roughly the same before and after the UV irradiation, a slight difference of the orientation of the acridine in the DNA/RNA duplex probably causes notable change in the efficiency of site-selective RNA scission. As shown in a previous work,[15] the scission activities of DNAs bearing modified acridines were quite different even

TABLE 3 Conversions of the site-selective scissions of 1 at U-19 before and after UV irradiation.

	3′-side of U-19 (%).	5′-side of U19 (%)
2/4	2.7	1.0
2/4 + UV Irradiation	3.9	1.4
3/4	3.1	1.3
3/4 + UV Irradiation	3.4	1.4

though they have almost the same T_m values with the complementary RNA. It was accordingly suggested that subtle difference in the orientations of the acridine residues notably alters the scission activity.

The *trans*-**2** and the *cis*-**2** species never exist in 100% in the mixture, and thus the difference in the intrinsic activities of *trans*-**2**/**4** and *cis*-**2**/**4** should be still larger than observed above in the RNA hydrolysis. As estimated by the HPLC analysis using **5**, the ratio of the *trans*-isomer and the *cis*-isomer in the reaction mixture is 10:1 before the UV irradiation and 2:3 after the irradiation (the population of the *cis*-isomer was 59%). By analyzing the 1.4 fold difference of RNA hydrolysis rate using these two ratios, the intrinsic activity of the *cis*-**2**/**4** combination is at least twice as high as that of the *trans*-**2**/**4** combination. This ratio may be still greater, since the isomerization of azobenzene is suppressed in DNA-RNA duplexes because of steric hindrance and thus the population of the *cis*-**2** isomer could be still smaller even after the UV irradiation.

CONCLUSION

Acridine-modified oligonucleotides which bear photo responsive azobenzene between the acridine and the main chain have been synthesized. By combining one of them with Mn(II) ion, site-selective RNA scission has been photo-controlled. Intrinsic activity of the *cis*-isomer is at least twice as high as that of the *trans*-isomer. Except for previous reports on photo-responsive ribozymes or deoxyribozymes,[16–19] this is the first photo-responsive site-selective RNA scission system. In order to accomplish more clear-cut switching, still more efficient isomerization of the azobenzene is necessary. Such attempt is now under way in our laboratory.

EXPERIMENTAL

Materials

Water was deionized by Millipore Water Purification System (Billerica, MA, USA), and sterilized immediately before use. ^{1}H NMR spectra were obtained on a 500 MHz NMR spectrometer (Bruker Avance600). For

ESI-MS, a Hitachi M-8000 LC/3DQMS mass spectrometer was used. MALDI-TOF mass spectra were measured on an AutoFLEX (Bruker, Billerica, MA, USA). Photo-irradiation was achieved by using UV Spot Light Source (Hamamatsu Photonics, Hamamatsu, Japan; 200 W) through a UV-D36C filter (Asahi Technoglass, Tokyo, Japan). Accordingly, the light of 300–400 nm wavelength was irradiated onto the samples.

4-Hydroxyaminobenzyl alcohol (7a). To a solution of 4-nitrobenzyl alcohol (3.06 g, 19.7 mmol) and NH_4Cl (1.57 g, 29.4 mmol) in 2-methoxyethanol/H_2O (30 ml/10 ml), Zn powder (3.84 g, 58.8 mmol) was added by portions, and the mixture was stirred for 15 minutes at room temperature. After filtration of Zn powder, the solution was concentrated under reduced pressure. The product was purified by silica gel column chromatography (10:1 $CHCl_3$/MeOH) to afford 0.80 g of **7a** (29% yield) as a white-yellow solid. TLC: R_f = 0.20, 10:1 $CHCl_3$/MeOH, ^{1}H NMR (DMSO-d_6) δ 8.27 (d, 1H), 8.18 (s, 1H), 7.12 (d, 2H), 6.80 (d, 2H), 4.96 (t, 1H), 4.39 (d, 2H), MS (ESI): *m/z* 138 (M−H).

2-Hydroxyaminobenzyl alcohol (7b). The synthetic procedure was the same as described above for **7a**. ^{1}H NMR (DMSO-d_6) δ 8.28 (d, 1H), 7.90 (s, 1H), 7.18–7.09 (m, 3H), 6.95 (t, 1H), 5.13 (t, 1H), 4.34 (d, 2H), MS (ESI): *m/z* 138 (M–H).

4-Nitrosobenzyl alcohol (8a). In ice cold H_2O (60 ml), $FeCl_3$ (3.21 g, 19.8 mmol) was dissolved. To the stirring $FeCl_3$ solution was added dropwise ethanol solution (2 ml) of **7a** (0.50 g, 3.6 mmol), and the mixture was stirred for additional 20 minutes. After the solution was removed, the residue was re-dissolved in $CHCl_3$, and the organic layer was washed with H_2O. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford 0.30 g of **8a** as a crude product. TLC: R_f = 0.61, 10:1 CH_2Cl_2/MeOH, ^{1}H NMR (DMSO-d_6) δ 7.94 (d, 2H), 7.68 (d, 2H), 5.58 (t, 1H), 4.78 (d, 2H).

2-Nitrosobenzyl alcohol (8b). ^{1}H NMR (DMSO-d_6) δ 7.99 (d, 1H), 7.91 (t, 1H), 7.43 (t, 1H), 6.60 (d, 1H), 5.66 (s, 3H)

9-(*p*-aminobenzylamino)-6-chloro-2-methoxyacridine (10). To a solution of 6-chloro-9-(p-chlorophenoxy)-2-methoxyacridine **9** (2.0 g, 5.2 mmol) in DMF (10 ml) was added 4-aminobenzylamine (1.3 ml) and N,N-diisopropylethylamine (1 ml), and the mixture was stirred for 16 hours at 90°C. After the solvent was removed, $CHCl_3$ and saturated aqueous NH_4Cl were poured to make an emulsion. After filtration, washing the solids with methanol, DMF, aqueous sodium hydroxide (1 N), and H_2O gave 1.5 g of **10** (77% yield) as yellow solid. TLC: R_f = 0.25, 10:1 CH_2Cl_2/MeOH, ^{1}H NMR (DMSO-d_6) δ 8.34 (d, 1H), 7.86 (d, 1H), 7.82 (d, 1H), 7.62 (d, 1H), 7.48 (t, 1H), 7.40 (q, 1H), 7.28 (q, 1H), 7.07 (d, 2H), 6.52 (d, 2H), 4.99 (s, 2H), 4.80 (d, 2H), 3.79 (s, 3H), MS (ESI): *m/z* 364 (M + H).

Acridine-azobenzene conjugate (*p*-isomer; 11a). To a solution of **10** (0.25 g, 0.69 mmol) in acetic acid (10 ml) was added dropwise **8a** (0.19 g, 1.4

mmol) in acetic acid (10 ml), and the mixture was stirred for 32 hours at room temperature. After the solvent was removed, the product was purified by silica gel column chromatography (10:1 CH_2Cl_2/MeOH) to afford 30 mg of **11a** (9% yield) as a red solid. TLC: $R_f = 0.14$, 10:1 CH_2Cl_2/MeOH, ^1H NMR (DMSO-d_6) δ 8.32 (s, 1H), 7.90–7.84 (7H), 7.70 (d, 2H), 7.58 (s, 1H), 7.54 (d, 2H), 7.41 (d, 1H), 7.32 (d, 1H), 5.41 (t, 1H), 5.07 (s, 2H), 4.61 (d, 2H), 3.76 (s, 3H), MS (ESI): *m/z* 483 (M + H), 481 (M–H)

Acridine-azobenzene conjugate (*o*-isomer; 11b). ^1H NMR (DMSO-d_6) δ 8.35 (d, 1H), 7.91–7.88 (7H), 7.73–7.65 (4H), 7.58 (m, 2H), 7.46–7.34 (3H), 5.29 (s, 1H), 5.23 (s, 2H), 5.14 (s, 2H), 3.79 (s, 3H), MS (ESI): *m/z* 483 (M + H)

Phosphoramidite monomer of 11a (12a). To a solution of **11a** (30 mg, 0.062 mmol) and 2-cyanoethyl N,N,N′,N′-tetraisopropylphosphoramidite (21 mg, 0.068 mmol) in anhydrous DMF (1 ml) on ice was slowly added 1H-tetrazole (6 mg, 0.085 mmol) in anhydrous DMF (1 ml) under nitrogen, and the mixture was stirred for 1 hour at room temperature. After the solvent was removed, ethyl acetate (50 ml) was poured, and the organic layer was washed with saturated aqueous NaCl (50 ml × 2). This layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to afford 46 mg of **12a** (quant). This product was dissolved in anhydrous DMF (0.1 M) and used for automated DNA synthesis without further purification. TLC: $R_f = 0.57$, 10:1 CH_2Cl_2/MeOH, MS (ESI): *m/z* 683 (M + H),

Phosphoramidite monomer of 11b (12b). This was prepared in a similar manner as described for **12a**. MS (ESI): *m/z* 683 (M + H)

Preparation of modified oligonucleotides. The substrate **1** is 36mer-RNA (5′-end FAM-labeled) and the U19 was chosen as the target-site for the scission. The complementary A was replaced with the unit from either **12a** or **12b**. All the oligonucleotides were synthesized on an ABI 3400 DNA Synthesizer in 1 μmole scale. An extended coupling time of 30 minutes was adopted for **12a** and **12b**. The oligonucleotides were cleaved from the resin, and deprotected by a treatment with 0.4 M methanolic NaOH (methanol:water = 4:1, 1 ml) at room temperature for 16 hours.

Purification and characterization of the acridine-modified DNAs. The oligonucleotides were purified by Poly-Pak II cartridges (Glen Research Co., Sterling, VA, USA). The resulting crude product was then purified by denaturing 20% PAGE. Final purification was carried out by a reversed phased HPLC equipped with an RP-C18 column (Cica-Merck LiChroCART 125–4; a linear gradient of 0–25% acetonitrile in 50 mM ammonium formate over 25 minutes; flow rate 0.5 ml/minutes). They were characterized by MALDI-TOFMS (Bruker AutoFLEX) in the positive ion mode. **5** was also characterized with ESI-MS in the negative ion mode.

HPLC assay for photo-isomerization of the azobenzene. Aqueous solution of **5** (100 μM) was divided into two portions, and both were heated to 97°C for 1 minute and slowly cooled down to room temperature. One

portion was irradiated with UV for 3 minutes to isomerize *trans*-azobenzene into *cis*-form. 30 μl of each solution was analyzed by a reversed phased HPLC (a linear gradient of 15–50% acetonitrile in 50 mM ammonium formate over 40 minutes; flow rate 0.5 ml/minute; wavelength 332 nm).

Measurement of melting temperatures (T_m) of the duplexes between acridine-modified oligonucleotides and its complementary DNA. Melting profiles were measured on a JASCO V-530 UV/Vis spectrophotometer. The absorbance at 260 nm was measured from 5 to 60°C in a quartz cell of 1 cm path length with a rate of 1.0°C/minute. The specimens contained **2** or **3** (1 μM) and **6** (1 μM) in pH 8.0 Tris-HCl buffer. For the "*cis*-isomer" experiments, the specimens were irradiated with UV for 30 minutes before the T_m measurements, and soon used for the measurements.

RNA scission assay. The substrate RNA (5 μM), acridine modified DNA (**2** or **3**; 10 μM) and unmodified DNA (**6**; 10 μM) were added to Tris buffer (10 mM) containing NaCl (200 mM). The mixture was then divided, and both portions were heated to 97°C for 1 minute and slowly cooled down to room temperature. One of them was directly used for the RNA hydrolysis, while another was irradiated with UV light for 3 minutes to isomerize *trans*-azobenzene into *cis* isomer and then used for RNA scission. Selective cleavage reaction was initiated by adding aqueous solution of $MnCl_2$ to the mixture. After incubation for a predetermined reaction time at 37°C, the reaction was quenched by saturated EDTA·2Na solution and analyzed on 20% denaturing PAGE. All the reactions were carried out in shielded tubes to avoid the isomerization of the *cis*-azobenzene into *trans*-form by ambient light.

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