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A highly acidic acridine for efficient site-selective activation of RNA leading to an eminent ribozyme mimic

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Abstract—9-Amino-2-methoxy-6-nitroacridine (**1a**) is conjugated with oligonucleotide for site-selective RNA hydrolysis. When this conjugate forms a duplex with complementary RNA, the phosphodiester linkage of the RNA in front of **1a** is activated and selectively hydrolyzed by Lu(III) ion. Covalent fixation of the metal ion to sequence-recognizing moiety is unnecessary. The site-selective hydrolysis by this conjugate is 2.2 times as fast as that by the oligonucleotide bearing 9-amino-6-chloro-2-methoxyacridine (**1b**), which hitherto has been the most active for the RNA activation. The acridine derivative **1a** is more acidic than **1b**, and thus is more effective as acid catalyst for the RNA hydrolysis.

Preparation of site-selective artificial ribonucleases has been a subject of growing interest. In almost all those hitherto reported, a catalyst for RNA hydrolysis was tethered to oligonucleotide as sequence-recognizing moiety.[1] Recently, we have developed an entirely new strategy, which involves no covalent fixation of catalyst to oligonucleotide.[2,3] The target phosphodiester linkage in substrate RNA is activated through non-covalent interactions with oligonucleotide bearing an acridine, and differentiated from the other linkages in terms of reactivity. Thus, this linkage is selectively hydrolyzed even when catalysts (e.g. lanthanide(III) ions) are not fixed anywhere. The site-selective scission is highly efficient, mainly because the catalysts are free from complexation with strong ligands. Consistently, rather poor active catalysts such as Zn(II) ion and Mn(II) ion also showed sufficient catalysis. Previous kinetic study indicated that these non-covalent site-selective hydrolyses involve the acid catalysis by the acridine in its protonated form.[3] Among all the acridine derivatives investigated, 9-amino-6-chloro-2-methoxyacridine (**1b**), which has the smallest pK_a (=8.5),[4] was the most active. According to this result, still faster site-selective RNA hydrolysis should be possible if more acidic acridine derivative is tethered to oligonucleotide. Here we report that oligonucleotide bearing 9-amino-2-methoxy-6-nitroacridine (**1a**: pK_a=7.3)[4] shows enormously fast site-selective RNA hydrolysis. This result confirms the previously proposed acid catalysis by the acridines, providing a strong basis for the molecular design towards still more efficient RNA processing.

Oligonucleotides used in this study are presented in Fig. 1. The acridine–DNA conjugate (**DNA$_1$–1a**) bears 9-amino-2-methoxy-6-nitroacridine (**1a**) at the 5′-end of 18-mer oligonucleotide which is complementary to the 5′-side of the 36-mer substrate RNA. The unmodified 17-mer oligonucleotide (**DNA$_2$**) is complementary to the remaining portion of the RNA. When the RNA is hybridized to both of **DNA$_1$–1a** and **DNA$_2$**, only U19 remains unpaired as the target site. Another acridine–DNA conjugate (**DNA$_1$–1b**) involves 9-amino-6-chloro-

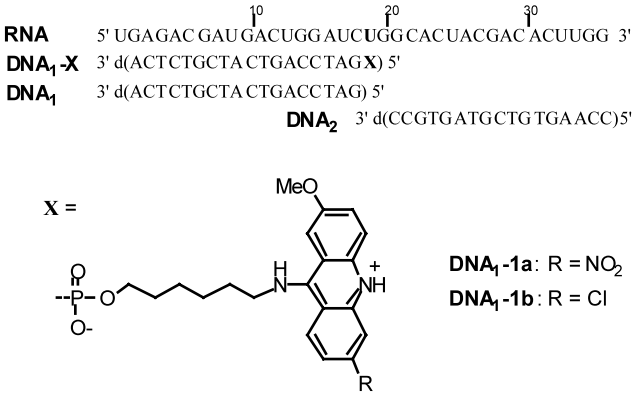

Figure 1. Oligonucleotides used in this study.

Keywords: acridine; oligonucleotides; site-selective scission; lanthanide; acid catalysis.

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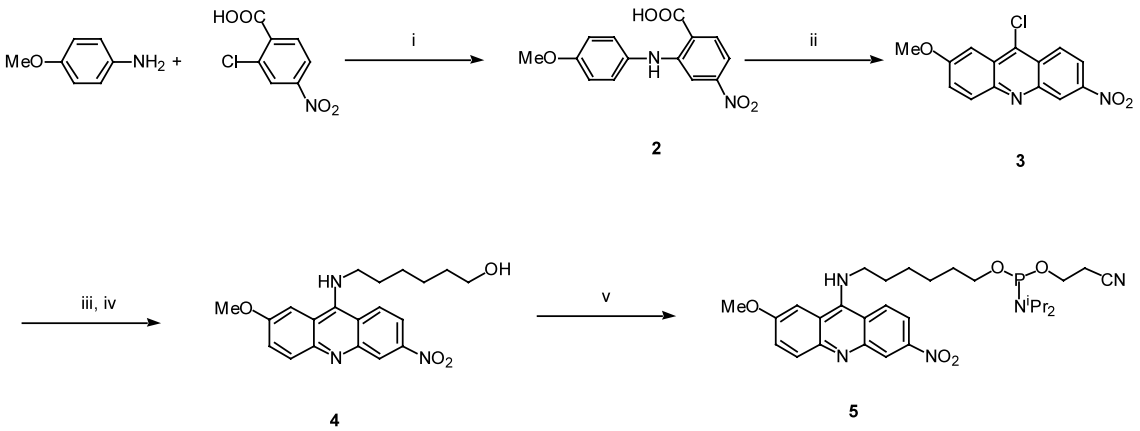

Scheme 1. Reagents and conditions for the synthesis of the phosphoramidite monomer **5** bearing **1a**: (i) Cu, CuO, K_2CO_3, H_2O, reflux, 12 h; (ii) $POCl_3$, reflux, 2 h; (iii) *p*-chlorophenol, 115°C, 12 h; (iv) 6-amino-1-hexanol, DMF, 70°C, 12 h; (v) $(^iPr_2N)_2POEtCN$, 1*H*-tetrazole, DMF, rt, 1 h.

2-methoxyacridine (**1b**) in place of **1a**, and, as described above, has been the most effective RNA activator until the present study.

All the oligonucleotides were synthesized using standard phosphoramidite chemistry on an automated synthesizer. The synthetic route for the phosphoramidite monomer bearing **1a** (**5**) is shown in Scheme 1. Commercially available 2-chloro-4-nitrobenzoic acid was coupled with *p*-methoxyaniline to give **2**, which was cyclized by using phosphoryl chloride. The resultant **3** was heated in *p*-chlorophenol, and then with 6-amino-1-hexanol in DMF to give the linker-bearing acridine **4**,[5] which was quantitatively converted to the phosphoramidite monomer **5**. Attachment of **5** to oligonucleotide was performed in the final cycle of the DNA synthesis with an extended coupling time of 10 min. After the synthesis, **DNA$_1$–1a** was detached from the support and deprotected with 0.4 M methanolic NaOH (4:1 MeOH/H_2O) at room temperature for 16 h, and the mixture was desalted. **DNA$_1$–1a** was purified by a reversed-phase HPLC equipped with an RP-C_{18} column, and characterized by MALDI-TOF MS analyses (*m*/*z*, 5862.9; calcd $[M-H]^-$, 5862.0). In addition, the acridine unit **4** (*m*/*z*, 370) was detected by ESI MS, when **DNA$_1$–1a** was digested with snake venom phosphodiesterase and alkaline phosphatase. The digest was further analyzed by the HPLC, providing the expected base composition (A:T:G:C = 4:5:3:6).

The site-selective RNA hydrolyses using the modified oligonucleotides as RNA activators were performed with 100 μM $LuCl_3$ at pH 8.0 and 37°C in the presence of 200 mM NaCl.[6] After 2 h, the reactions were quenched by 10 mM EDTA-2Na and analyzed by denaturing PAGE. The scission efficiency was evaluated by a Fuji Film FLA-3000G fluorescent imaging analyzer. As shown in lane 4 of Fig. 2, the substrate RNA is site-selectively and efficiently hydrolyzed by **DNA$_1$–1a/DNA$_2$**/Lu(III) system. The hydrolysis occurs mainly at the 5′-side of U19 as the target site (the minor scission is perceived at its 3′-side).[7] By comparing the intensity of this major band with that of the corresponding band in lane 1 for the random scission by Lu(III), the site-selective activation of the target phosphodiester linkage by the **DNA$_1$–1a/DNA$_2$** combination is evident. Another acridine–DNA conjugate **DNA$_1$–1b**, which bears 9-amino-6-chloro-2-methoxyacridine (**1b**), also induces site-selective RNA hydrolysis (lane 3). It is noteworthy that the site-selective hydrolysis by the **DNA$_1$–1a/DNA$_2$**/Lu(III) system is significantly faster than that by the **DNA$_1$–1b/DNA$_2$**/Lu(III) system (Fig. 3). Pseudo-first-order rate constants are 0.22 and 0.10

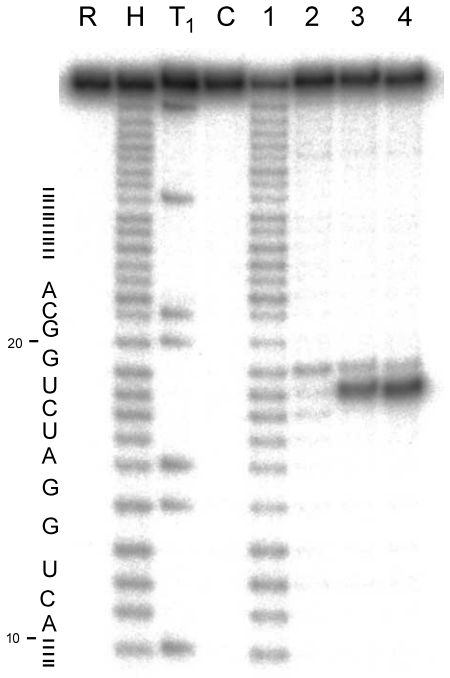

Figure 2. Site-selective scission of the RNA by the combinations of modified or unmodified **DNA$_1$**, **DNA$_2$**, and Lu(III) at 37°C and pH 8.0 in 2 h; $[RNA]_0 = 1$, $[DNA] = 10$ and $[Lu(III)] = 100$ μM. Lane 1, treatment with Lu(III) alone; lane 2, **DNA$_1$/DNA$_2$**/Lu(III); lane 3, **DNA$_1$–1b/DNA$_2$**/Lu(III); lane 4, **DNA$_1$–1a/DNA$_2$**/Lu(III). R, no treatment of the RNA; H, alkaline hydrolysis; T_1, RNase T_1 digestion; C, control in buffer solution.

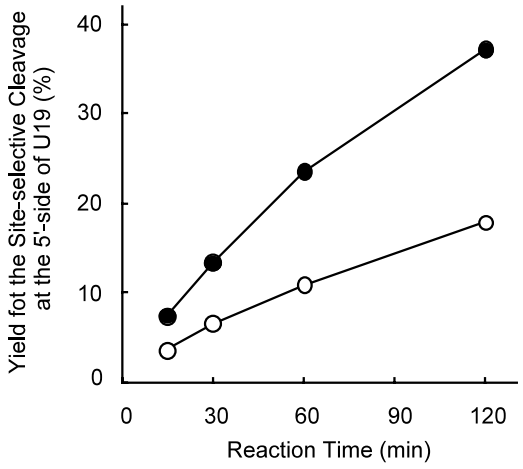

Figure 3. Time-course of the site-selective RNA hydrolysis at U19. Filled circles, hydrolysis with **DNA$_1$–1a**/**DNA$_2$**/Lu(III); open circles, hydrolysis with **DNA$_1$–1b**/**DNA$_2$**/Lu(III).

h^{-1}, respectively. Site-selective RNA activation by **1a**, synthesized according to Scheme 1, sufficiently surpasses that by commercially available **1b**.

This notably high activity of the **DNA$_1$–1a**/**DNA$_2$**/Lu(III) system is attributable to the high acidity of **1a**, as substantiated by the following results. The pK_a values of the acridine residues in **DNA$_1$–1a** and **DNA$_1$–1b** were directly evaluated by using their absorbances (Fig. 4). Curve fitting of the experimental points gives the pK_a values of 8.8±0.1 for **DNA$_1$–1a**[8] and 10.5±0.1 for **DNA$_1$–1b**. Upon the attachment of acridines to oligonucleotides, their pK_a values are elevated by 1.5–2.0 units, compared to that of the corresponding free acridine. These pK_a values show that almost 100% of **1b** in **DNA$_1$–1b** should be protonated under the reaction conditions (pH 8.0) and potent for the acid catalysis. On the other hand, the fraction of protonated form for **1a** in **DNA$_1$–1a** is smaller (86%). Apparently, **DNA$_1$–1a** is more active for the present RNA hydrolysis than

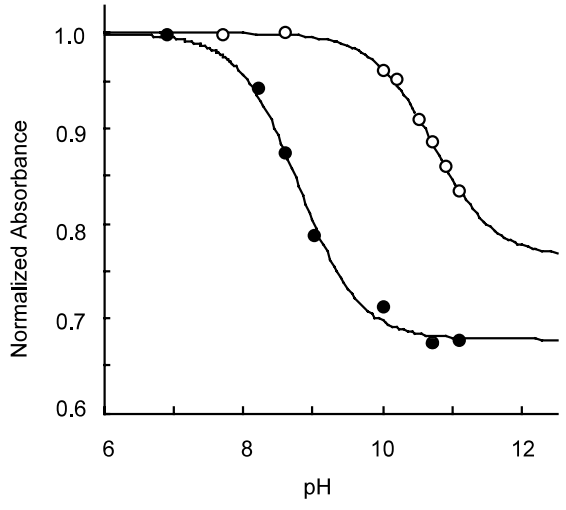

Figure 4. The pH dependence of the absorbance of acridines. Filled circles, **DNA$_1$–1a** (at 476 nm); open circles, **DNA$_1$–1b** (at 451 nm).

Table 1. Melting temperatures of the duplex between the **RNA** and modified or unmodified **DNA$_1$**[a]

	Tm (°C)	ΔTm (°C)[b]
DNA$_1$–1a	68.0	4.9
DNA$_1$–1b	69.1	6.0
DNA$_1$	63.1	–

[a] Conditions: [**RNA**] = 1.0 μM, [modified or unmodified **DNA$_1$**] = 1.0 μM, [NaCl] = 200 mM, and [Tris–HCl] = 10 mM (pH 8.0).
[b] Differences of Tm's from the value for the unmodified **DNA$_1$**.

DNA$_1$–1b, simply because its intrinsic activity as acid catalyst is greater as expected from the Brønsted rule. As shown in Table 1, the melting temperatures (Tm's) of the **RNA**/**DNA$_1$–1a** and the **RNA**/**DNA$_1$–1b** heteroduplexes are higher than that of the **RNA**/**DNA$_1$** duplex, and the duplex-stabilizing activity of the acridine in **DNA$_1$–1a** is smaller than that for **DNA$_1$–1b** (ΔTm = 4.9 and 6.0°C, respectively). It is unlikely that **DNA$_1$–1a** is more active for the RNA hydrolysis because **1a** interacts with the RNA more strongly than does **1b**.

In conclusion, highly efficient RNA activator has been synthesized by attaching 9-amino-2-methoxy-6-nitroacridine to oligonucleotide. The site-selective RNA hydrolysis by this conjugate is among the fastest ever reported. The present finding should serve as important information for the design of useful tools for biotechnology and molecular biology. Studies of their practical applications are currently underway in our laboratory.

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4. Albert, A. *The Acridines*, 2nd ed.; Edward Arnold Publishers: London, 1966; pp. 434–467. Note that these pK_a values are the ones for free acridine derivatives. As described in the text, they are elevated on their attachment to oligonucleotide due to electrostatic interactions with the anionic phosphates.

5. Spectral data of **4**: TLC R_f=0.22, 10:1 CH_2Cl_2/MeOH; ^{1}H NMR (DMSO-d_6) δ 8.67 (s, 1H), 8.53 (d, 1H), 7.98 (d, 1H), 7.93 (d, 1H), 7.68 (s, 1H), 7.49 (d, 1H), 4.32 (t, 1H), 3.96 (s, 3H, OMe), 3.78 (q, 2H), 1.73 (m, 2H), 1.36–1.25 (m, 6H). MS (ESI) m/z 370 $[M+H]^+$.
6. Light exposure was carefully avoided during the hydrolysis reaction.
7. In lane 4, the scission at the 3′-side of U19 is 10-fold less efficient than that at its 5′-side.
8. The pK_a of **1a** in **RNA**/**DNA**–**1a** heteroduplex is almost identical with this value.