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Crucial role of linker portion in acridine-bearing oligonucleotides for highly efficient site-selective RNA scission

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Abstract—Through a series of linkers, 9-amino-2-methoxy-6-nitroacridine and 9-amino-6-chloro-2-methoxyacridine were tethered to the middle of oligonucleotide, and the abilities of these conjugates for site-selective activation of RNA (inducing site-selective scission by Lu(III)) were compared. The RNA-activating ability was strongly dependent on the structures of both acridine and linker. By tethering 9-amino-2-methoxy-6-nitroacridine with a rigid and chiral linker, derived from L-threoninol, quite fast site-selective RNA scission was accomplished.

Development of artificial ribonucleases, which hydrolyze RNA at predetermined site, has been a subject of growing interest. In most cases, chemical scissors were tethered to DNA oligomers as sequence-recognizing moiety.[1–5] Recently, we proposed a new approach for site-selective RNA scission utilizing DNA–acridine conjugates.[6–9] When substrate RNA form heteroduplexes with these conjugates, its phosphodiester linkage opposite the acridine is efficiently activated, and thus selectively hydrolyzed by metal ions such as lanthanide(III), Zn(II), and Mn(II). This site-selective activation is completely 'noncovalent', since the molecular scissors are not linked to any sequence-recognizing moiety.

In order to promote this site-selective RNA scission still more, the acridine part in the conjugates was modified in various ways, and it was found that the RNA-activating ability increases with increasing acidity of acridine (9-amino-2-methoxy-6-nitroacridine is the most efficient).[10] However, the structure of linker portion that connects acridine and oligonucleotide has never been sufficiently optimized. Since the present site-selective RNA activation originates from both the acid catalysis by the acridine and the conformational change of RNA backbone due to its intercalation, the linker structure should be also critical. In this study, we use four linkers and attach either 9-amino-2-methoxy-6-nitroacridine ($pK_a = 8.8$) or 9-amino-6-chloro-2-methoxyacridine ($pK_a = 10.5$) into the middle of oligonucleotide. Effects of the linkers on site-selective RNA activation have been systematically studied.

The DNA–acridine conjugates (DNA_1–X) used in this study are presented in Figure 1. Linkers **1** and **2** are obtained from L- and D-threoninol as starting material, respectively, and their side chains are less flexible due to the amide linkage (linker **4** is also from L-threoninol but has a shorter side chain).[11] The configuration of the branching point in these linkers is retained throughout the synthetic procedure. On the other hand, linker **3** consists of a flexible tetramethylene side chain, and has a prochiral carbon center at the branching point. Thus, DNA_1–**3n** and DNA_1–**3c** are used as mixtures of diastereomers. Through these linkers, 9-amino-2-methoxy-6-nitroacridine (**1n**, **2n**, and **3n**) or 9-amino-6-chloro-2-methoxyacridine (**1c**, **2c**, **3c**, and **4**) is bound to the middle of 36-mer DNA_1. When RNA_1 (labeled with FAM at the 5′-end) is hybridized to these DNA_1–X conjugates, only U19 remains unpaired and it is the target site for site-selective scission.

The phosphoramidite monomer **9** for the incorporation of **1n** was prepared according to Scheme 1. First, L-threoninol[12] was coupled with *N*-Fmoc-protected 4-aminobutyric acid **5**, and then the primary alcohol in the adduct **6** was protected with dimethoxytrityl (DMTr) group to produce **7**. After the Fmoc group was removed,

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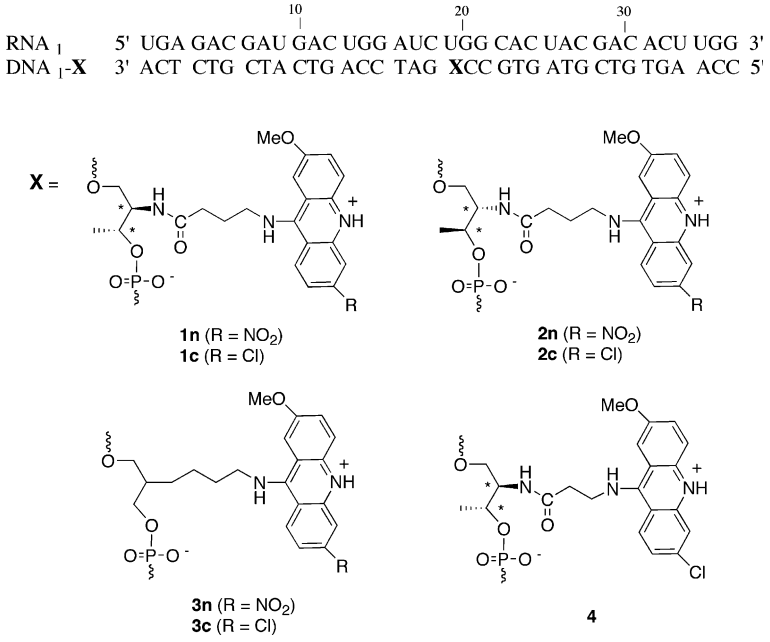
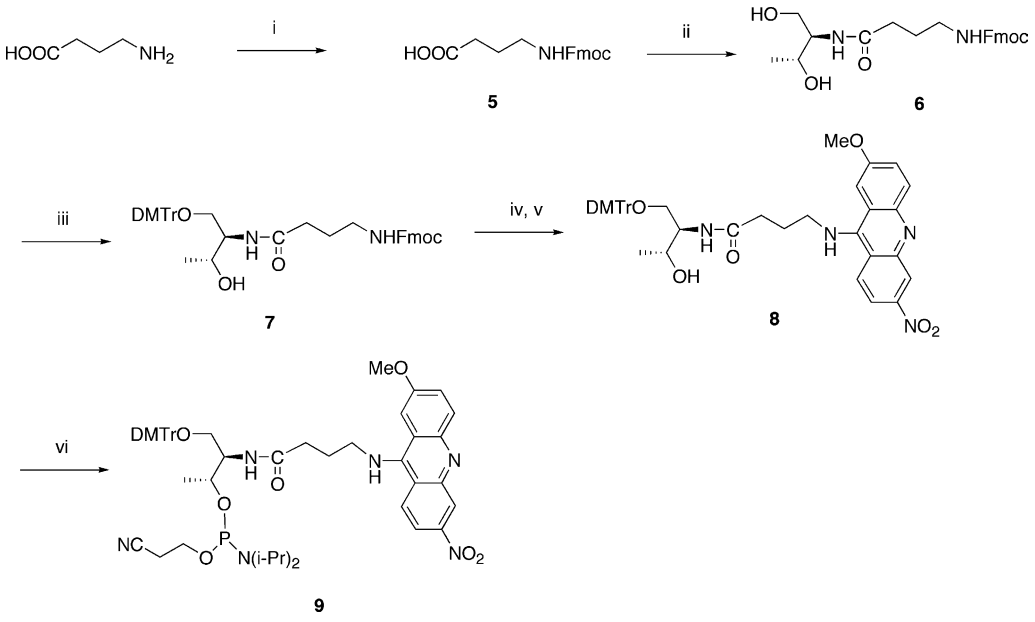


Figure 1. Structures of substrate RNA and DNA–acridine conjugates used in the present study.



Scheme 1. Synthesis of the phosphoramidite monomer **9** for the incorporation of **1n**. Reagents and conditions: (i) dioxane, $NaHCO_3$, Fmoc–Cl, rt, 2 h; (ii) L-threoninol, 1,3-dicyclohexylcarbodiimide, 1-hydroxybenzotrizole, *N*,*N*-dimethylformamide (DMF), rt, overnight; (iii) DMTr–Cl, pyridine, CH_2Cl_2, rt, overnight; (iv) 5% piperidine/DMF, rt, 5 min; (v) 9-(*p*-chlorophenoxy)-2-methoxy-6-nitroacridine, diisopropylethylamine, DMF, 80 °C, overnight; (vi) $(i\mathrm{Pr}_2\mathrm{N})_2\mathrm{PO}(\mathrm{CH}_2)_2\mathrm{CN}$, 1*H*-tetrazole, acetonitrile, rt, 1 h.

the product was reacted with 9-(*p*-chlorophenoxy)-2-methoxy-6-nitroacridine.[10] Finally, the resulting acridine derivative **8** was reacted with 2-cyanoethyltetraisopropylphosphorodiamidite. Other phosphoramidite monomers for **1c**, **2n**, **2c**, and **4** were synthesized by similar procedure. The monomer for **3n** was prepared from diethyl malonate and 4-bromobutyronitrile by the method of Nelson et al.[13] The DNA syntheses and purification were carried out as described in a previous paper.[7] These conjugates were characterized by MALDI-TOF/MS analyses and also by HPLC determination of base composition on their digests by snake venom phosphodiesterase and alkaline phosphatase.

Figure 2 shows typical polyacrylamide gel electrophoresis patterns for the scission of RNA_1 by Lu(III) at pH 8.0 and 37 °C. In the absence of oligonucleotide additives, RNA_1 was randomly cleaved by Lu(III) (lane 1). When the DNA–acridine conjugates were added to the system, however, the phosphodiester linkage in the

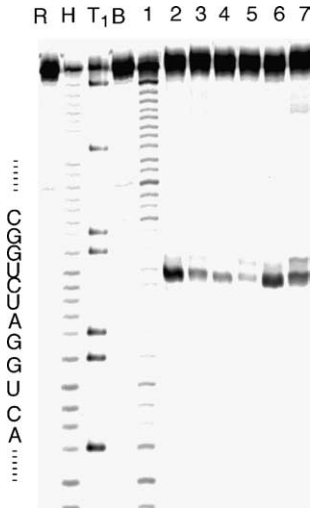


Figure 2. Site-selective RNA scission by Lu(III) in the presence of DNA–acridine conjugates. Lane 1, Lu(III) only; lane 2, DNA_1–**1n**; lane 3, DNA_1–**1c**; lane 4, DNA_1–**2n**; lane 5, DNA_1–**2c**; lane 6, DNA_1–**3n**; lane 7, DNA_1–**3c**. At pH 8.0 and 37 °C for 2 h; $[RNA_1] = 5\,\mu M$; $[DNA_1–X] = 10\,\mu M$; $[Lu(III)] = 100\,\mu M$; [Tris–HCl] = 10 mM; [NaCl] = 200 mM. R, RNA_1 only; H, alkaline hydrolysis; T_1, RNase T_1 digestion; B, control reaction in the buffer solution.

5′-side of U19 was selectively and efficiently hydrolyzed (lanes 2–7). For all the conjugates, the scission band is notably stronger than the corresponding band in the random cleavage of RNA_1 in lane 1. Apparently, RNA_1 is selectively activated just in front of the acridine residue in the conjugates.[14]

The pseudo-first-order rate constants (k_{obs}) of the scission at the 5′-side of U19 in the presence of the DNA–acridine conjugates are presented in Figure 3. The conjugate DNA_1–**1n** is remarkably active for the site-selective RNA scission ($k_{obs} = 11.0 \times 10^{-2}\,h^{-1}$). Here, 9-amino-2-methoxy-6-nitroacridine is bound to DNA_1, using the linker **1** that possesses amide-based and rather rigid side chain. This conjugate is 4.8 times as active as DNA_1–**1c** bearing 9-amino-6-chloro-2-methoxyacridine through the same linker. Similarly, DNA_1–**2n** ($k_{obs} = 4.4 \times 10^{-2}\,h^{-1}$) is 4.6-fold more active than DNA_1–**2c**. These differences in the ability for RNA activation (and thus for the resultant RNA scission) are ascribed to the stronger acidity of 9-amino-2-methoxy-6-nitroacridine than 9-amino-6-chloro-2-methoxyacridine ($pK_a = 8.8$ vs 10.5). It is also noteworthy that DNA_1–**1n** is even more active than DNA_1–**3n**, which involves a flexible tetramethylene linker. In order to link highly acidic 9-amino-2-methoxy-6-nitroacridine to sequence-recognizing DNA_1, rather rigid linker **1** is superior to flexible linker **3**, since it firmly fixes the acridine to a suitable position for the acid catalysis.

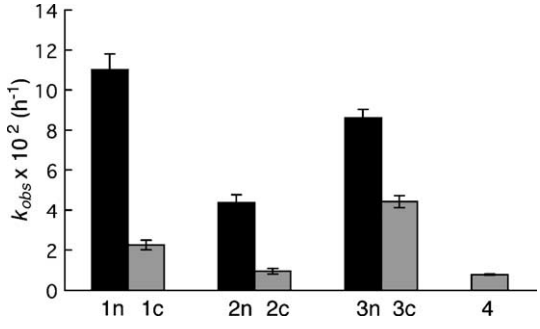


Figure 3. Dependence of RNA-activating ability of DNA–acridine conjugate on the structure of linker.

When 9-amino-6-chloro-2-methoxyacridine is bound to DNA_1, however, flexible linker **3** is more favorable than linkers **1** and **2**. Thus, DNA_1–**3c** ($k_{obs} = 4.4 \times 10^{-2}\,h^{-1}$) is twice as active as DNA_1–**1c** ($2.3 \times 10^{-2}\,h^{-1}$), and four times as active as DNA_1–**2c** ($0.95 \times 10^{-2}\,h^{-1}$). Assumably, this acridine is less acidic so that the major factor for the site-selective RNA activation is the conformational change of RNA backbone due to the intercalation, rather than its acid catalysis. This 'conformational catalysis' is never optimized when the acridine is placed appropriately for the acid catalysis. Accordingly, rigid linkers **1** and **2** are not very effective here.

Furthermore, the stereochemistry of the branching carbon atom in the linker portions must be precisely controlled. Thus, DNA_1–**1n** (derived from L-threoninol) is 2.5 times as active as DNA_1–**2n** (from D-threoninol), while DNA_1–**1c** is also more active than DNA_1–**2c**. The stereochemistry of this carbon greatly affects the position of acridine in the DNA/RNA heteroduplexes, giving rise to the difference in the RNA-activating ability. Consistently, the circular dichroism induced on the acridine in the middle portion of DNA_1–**1n**/RNA_1 duplex is notably different from that for the DNA_1–**2n**/RNA_1 duplex (data not shown). As expected, the length of linker is also crucially important. Thus, DNA_1–**4**, which is derived from L-threoninol but has a shorter side chain, shows only one-third ability of DNA_1–**1c**.

In conclusion, a quite efficient RNA activator has been synthesized by attaching 9-amino-2-methoxy-6-nitroacridine into the middle of oligonucleotide through chiral linker derived from L-threoninol. Precise design of both acridine and linker is crucially important here.

Acknowledgements

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