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Structural Study of Antisense Dimers, Modified Adenosine-Thymidine Phosphorothioate

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Abstract Antisense molecules are structurally simple linear oligomers of nucleotides. They can recognize a complementary sequence by base pairing, therefore, antisense drugs composed of 15–16 bases are potentially useful, unlike drugs such as protein agonists, antagonists, and inhibitors. Since antisense oligomers are classified as nucleotides, they are subject to attack by nucleases. In order to be antisense drugs resistant to degradation by nucleases, the structural modifications in the linkages, bases, and sugars to satisfy this requirement are considerable. We attempted in this study, to synthesize 16-mer antisenses with a modified linkage and adenosine. When studying on the three-dimensional structure of the oligomer, however, the existence of isomers may complicate the interpretation of the NMR data. Therefore, an attempt was made to eliminate the above problem, thus, two dimers were synthesized and their structural studies were carried out.

Keywords: Antisense, nucleotide, dimer, NMR

During the past several decades, most chemotherapeutic strategies have been focused on proteins. However, since the late 1970s, nucleic acids have become important chemotherapeutic agents [11]. The synthetic oligonucleotide targeted on mRNA is called an antisense molecule, whereas that targeted on DNA is called an antigene molecule. An antisense molecule can prevent mRNA translation, resulting in the inhibition of protein synthesis [6]. Because of this phenomenon, antisense molecules can be used as antiviral and anticancer therapeutic drugs. An antisense molecule is highly selective because it can recognize a complementary mRNA based on sequence base pairing. In order to interfere mRNA translation, a short complementary strand of mRNA is sufficient. The binding of small segments to mRNA can inhibit the expression of the corresponding protein that causes diseases. Traditional drugs have been designed based on the three-dimensional structure of the target protein [4]. Although three-dimensional structures of approximately 12,000 proteins are known, many more are yet to be determined. The development of antisense-oriented drugs is easier than protein-oriented drugs [9]. Therefore, this concept is currently the subject of intensive study.

The primary advantage of an antisense molecule is its simplicity. It recognizes a complementary sequence by base pairing of adenine-uracil (thymine) and guanine-cytosine. As a result, antisense drugs composed of 15 bases can be useful, unlike protein agonists, antagonists, and inhibitors. Even though antisense drugs have an advantage compared to traditional drugs, there are still several requirements that need to be resolved before their successful application. In order to produce an inhibitory effect on translation, an antisense molecule must be tightly associated with mRNA [11]. The stability of the hybrid duplex is determined by measuring on the dissociation temperature by UV absorption. That is, the higher the hyperchromic effect, the more stable the hybrid duplex. Since antisense oligomers are oligonucleotides, they are subject to attack by nucleases. Therefore, antisense drugs must be able to resist degradation by nucleases. The structural modifications in the linkages, bases, and sugars that are required to meet this condition are considerable. In this study, we investigated two adenosine-thymidine dimers based on modifying the constituents of

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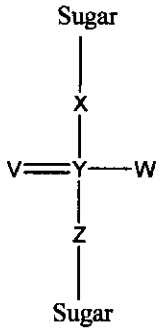

X	Y	Z	V	W
O	P	O	O	S^-
O	P	O	O	NHR
NH_2	P	O	O	O^-
O	P	O	O	Me
O	P	O	O	OR
O	P	O	S	S^-
CH_2	NMe	O	None	None
S	CH_2	O	None	None
CH_2	NH	C=O	None	None
CH_2	CH_2	NH	None	None
O	SiR_2	O	None	None
CH_2	CH_2	S	None	None

Fig. 1. Modifications of sugar-phosphate linkage.

the phosphodiester bonds and sugars before developing new antisense oligomers.

Sulfur, amine, and alkyl groups were used for the substitution into the phosphodiester bonds (Fig. 1) [11]. The oligomers thus modified exhibited a greater resistance against nucleases and less stability than oligomers with natural linkages. A new antisense molecule was obtained by modification, however, the phosphorothioate linkage had two isomers, because the thioxo group was either directed to the left or the right side.

We first attempted to synthesize 16-mer antisense with a modified linkage and adenosine. When studying the three-dimensional structure of the oligomer, however, the existence of isomers disturbs the interpretation of the NMR data. Therefore, an attempt was made to solve the above problem before studying the three-dimensional structure of the oligomer. As such, two dimers were synthesized (Fig. 2), one with a modified linkage (AT-I) and the other with a modified linkage and adenosine (AT-II). AT-I was synthesized using an ABI 392 DNA/RNA synthesizer on a 1 μmol scale from commercial 2'-deoxynucleoside phosphoramidites. The purification was performed by a reverse phase HPLC (Hamilton PRP-1, 18–28% CH_3CN/100 mM TEAA for 20 min, pH 7, monitored at 260 nm) [3, 8]. The modified adenosine phosphoramidite was then synthesized as previously described [5].

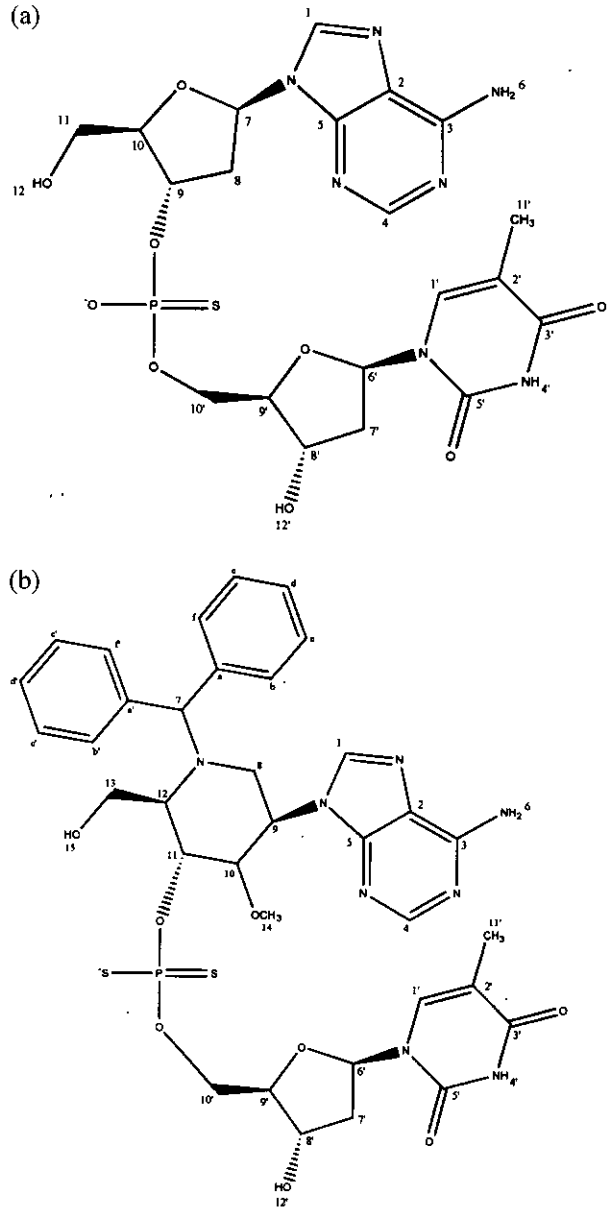

Fig. 2. Structures of adenosine-thymidine modified (a) in phosphorothioate linkage (AT-I) and (b) in both linkage and adenosine (AT-II).

The AT-II dimer was synthesized as follows: The assembly of AT-II was accomplished using an ABI 392 DNA/RNA synthesizer on a 1 μmol scale with modified adenosine phosphoramidite and commercial 2'-deoxythymidine phosphoramidite. The standard protocol for phosphorothioate oligodeoxynucleotides was used with an exception of a 10 min coupling time for the incorporation of the modified nucleotide. The solid supports and protecting groups were cleaved by treatment with concentrated NH_4OH at 55°C for 17 h. The solution was then lyophilized with additional triethylamine every hour to prevent detritylation.

A triethylammonium acetate solution (TEAA 100 mM, 1 ml, pH 7) was added and the residue was purified using a reverse phase HPLC (Hamilton PRP-1, 300 × 7 mm, 18–48% CH_3CN/100 mM TEAA, pH 7, monitored at 260 nm). The desired fractions were lyophilized to dryness, water (2 × 1 ml) was then added and the fractions were re-lyophilized to remove any TEAA salt. The treatment of the remaining residue with 0.3 ml of AcOH for 20 min cleaved the trityl group. After lyophilization with ethanol (0.3 ml), the residue was taken up in 1 ml of water, extracted with diethyl ether (3 × 1 ml), and then lyophilized to dryness. After the addition of 1 ml of water to dry the DNA pellet, the solution was quantified by UV absorbance at 260 nm and 70°C. The extinction coefficients of the natural nucleotides were used (dAMP, 15200: dTMP, 8830). PAGE and an HPLC confirmed the purity of the dimer.

Table 1. Assignments of NMR data of AT-1 (top) and AT-II (bottom) determined by one and two dimensional NMR experiments.

δ^1H (J/Hz)	COSY	Assignment
1.55(d, 1.1)	H11'/H1'	H11'
2.14(m)	H7'/H6', H7'/H8'	H7'
2.68(m)	H8/H7, H8/H9	H8
3.71(m)	H11/H10	H11
3.99(m)	H9'/H10', H9'/H8'	H9'
4.03(m)	H10'/H9'	H10'
4.18(m)	H10/H9, H10/H11	H10
4.40(m)	H8'/H9', H8'/H7'	H8'
4.90(m)	H9/H8, H9/H10	H9
6.05(t, 6.7)	H6'/H7'	H6'
6.24(t, 6.7)	H7/H8	H7
7.40(d, 1.1)	H1'/H11'	H1'
8.02(s)*	-	H4
8.14(s)*	-	H1
1.80(d, 1.26)	H11'/H1'	H11'
1.90(m)	H7'b/H7'a	H7'b
2.01(m)	H7'a/H7'b, H7'a/H8', H7'a/H6'	H7'a
2.38(m)	H10/H9, H10/H11	H10
2.53(m)	H8b/H8a, H8b/H9	H8b
2.67(m)	H13/H12	H13
2.84(m)	H8a/H8b, H8a/H9	H8a
3.68(s)	H14/H10*	H14
3.81(m)	H9'/H8', H9'/H10'	H9'
3.93(m)	H11/H10, H11/H12	H11
4.04(m)	H10'/H9'	H10'
4.25(m)	H8'/H9', H8'/H7'a	H8'
4.38(m)	H12/H13	H12
4.42(m)	H9/H8a, H9/H10	H9
5.55(s)	H7/Hb	H7
6.16(m)	H6'/H7'a, H6'/H7'b	H6'
6.80(m)	Hc/Hb, Hc/Hd	Hc
7.02(m)	Hd/Hc	Hd
7.18(m)	Hb/Hc, Hb/H7	Hb
7.82(d, 1.26)	H1'/H11'	H1'
7.88(s)*	H4/H10*	H4
8.39(s)*	H1/H9*	H1
11.18(s)	-	H4'

*denotes correlations obtained from NOE data.
*denotes signals shown in Fig. 3.

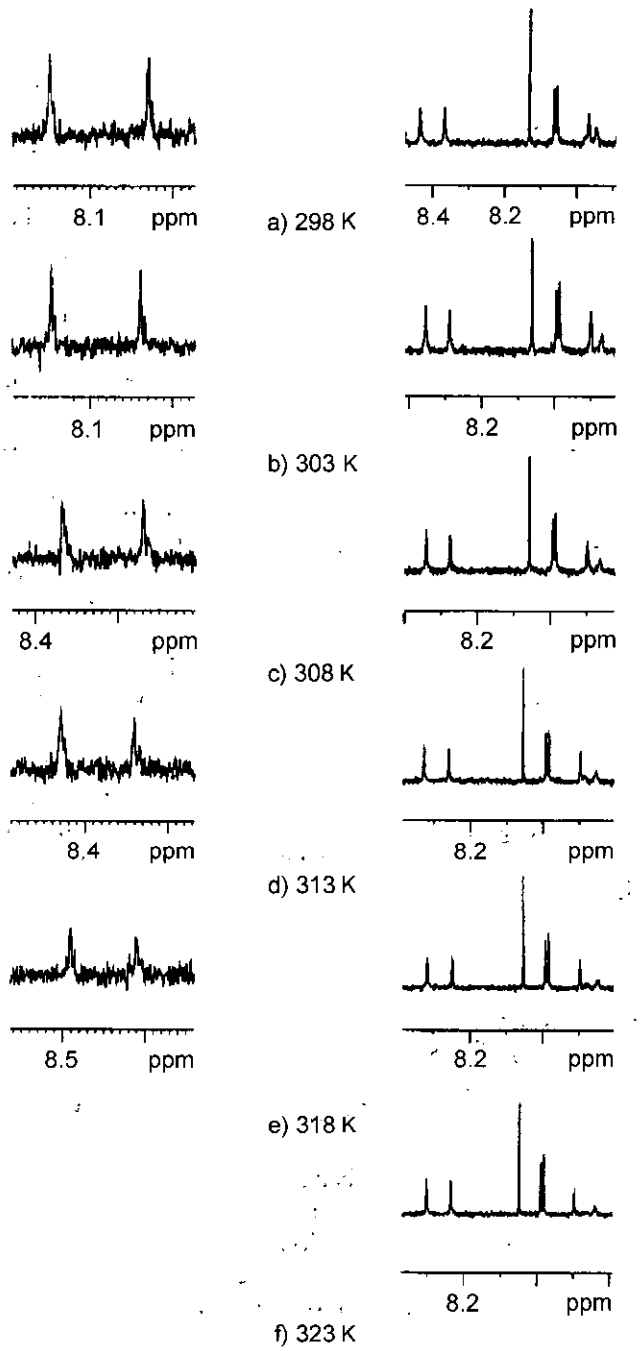

Fig. 3. ^{1}H NMR spectra of AT-I obtained at various temperatures ranging from 298 K to 318 K (left column), and AT-II obtained at various temperatures ranging from 298 K to 323 K (right column).

Before determining the existence of isomers, the structure of the compound was determined. An NMR analysis was performed on a Bruker ARX400 NMR spectrometer (9.4 T) [7, 10, 12]. The NMR spectra of ^{1}H NMR, Correlated Spectroscopy (COSY) [2], Homonuclear Hartmann Hahn Spectroscopy (HOHAHA) [1], and Nuclear Overhauser Exchanged Spectroscopy (NOESY) [13] were collected in DMSO-d_6. The concentration of the samples was 50 mM. For the ^{1}H-NMR analysis, 32 transients were acquired with a 1 sec relaxation delay using 32 K data points. The 90° pulse was 9.7 μsec with a spectral width of 6,500 Hz. The spectra were obtained at temperatures ranging from 298 K to 323 K at 5 K intervals. Two-dimensional spectra were acquired with 2048 data points for t2 and 256 for t1 increments.

The ^{1}H signals of adenosine and thymidine were assigned based on previous data [14]. The assignments of the NMR data from AT-I and AT-II as determined by the one and two-dimensional NMR analyses are listed in Table 1. In the ^{1}H NMR spectrum of AT-I, the ^{1}H signals at 8.02 and 8.14 ppm denoted H4 and H1, respectively. As shown in Fig. 3a, the signals at 298 K were doublets, however, they should have been singlets because they were not correlated with any other protons. Therefore, the doublets were considered to be isomer signals caused by the direction of the thioxo group. Under higher temperatures (298 K to 318 K) the doublets became singlets. Accordingly, although the doublets observed at 298 K resulted from the existence of isomers, they did not complicate the interpretation of the NMR data.

Likewise, in the ^{1}H NMR spectrum of AT-II, the ^{1}H signals at 7.88 and 8.34 ppm were assigned to H4 and H1, respectively. The signals at 298 K were clearly doublets, even though they should have been singlets. At higher temperatures (298 K to 323 K) the doublets did not become singlets (Fig. 3b). In the case of AT-II, the modified adenosine included two benzene rings, therefore, steric hindrance prevented the linkage from rotating. However, since the signals showing doublets could still be distinguished from the doublet signals caused by correlations with other protons, the interpretation of the NMR data was not affected.

Synthesized oligomers of modified adenosine are valuable because of the novel structure of adenosine. Therefore, new antisense oligomers with phosphorothioate linkages and modified adenosine are now being synthesized, and the dissociation temperatures of these molecules will soon be measured and also structure was studied by NMR.

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