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Synthesis and antisense properties of oligodeoxyribonucleotides containing C5-substituted arabinofuranosyluracil

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Abstract—An oligodeoxyribonucleotide (ODN) containing three C5-substituted arabinofuranosyluracils was synthesized by the post-synthetic modification method from the ODN containing three C5-substituted 2,2′-anhydrouridines. The stability of the modified ODN/DNA duplex was lower than that of the corresponding normal duplex but that of the modified ODN/RNA duplex showed little change. The modified ODN could induce RNase H activity and was resistant against nuclease.

1. Introduction

Antisense oligonucleotide (AON) regulates gene expression by hybridization with the target mRNA.[1] AON has potential therapeutic application and provides an approach to drug discovery. There are several requirements for gene regulation by AON such as sequence-specific recognition, nuclease resistance, and cell penetration. In addition, the degradation of mRNA by ribonuclease H (RNase H), which degrades the RNA of the DNA/RNA duplex, is efficient for suppressing gene expression. It was reported that arabinonucleic acid (ANA) forms a duplex with RNA and induces RNase H degradation of the target RNA.[2] These properties of ANA apparently demonstrate that the sugar puckering of the ANA strand in an ANA/RNA duplex adopts an O4′-*endo* conformation.[3] However, the binding affinity of the modified oligodeoxyribonucleotide (ODN) containing arabinonucleosides with RNA is lower than that of the corresponding natural DNA.

We developed a post-synthetic modification for the synthesis of C2- and C5-substituted arabinofuranosyluracil from 2,2′-anhydro-β-D-arabinofuranosyl-5-methoxycarbonylmethyluracil.[4] In these experiments, we found the deamination at the C-2 position to reproduce a 2,2′-ether bond. It was considered that this reaction was caused by attack of the 2′-hydroxy group on C-2 as reported in the literature.[5] The obtained C5-substituted 2,2′-anhydro-β-D-arabinofuranosyluracil can be converted to C5-substituted β-D-arabinofuanosyluracil by alkaline hydrolysis.

In this paper, we report the synthesis of the ODN containing C5-substituted arabinufuranosylpyrimidines by a new post-synthetic modification method. Tris(2-aminoethyl)amine was attached as C5-substituent because it has positive charges in a neutral solution and increasing duplex stability was expected by the attachment of tris(2-aminoethyl)amine.[6] We also studied the ability to induce RNase H degradation of the RNA target by the ODN containing C5-substituted arabinufuranosylpyrimidines. Substitution of arabinofuranosyluracil at the C-5 position, which is placed in the major groove of a duplex, seems not to inhibit the RNase H activity because RNase H does not interact with a DNA/RNA duplex in the major groove but in the minor groove.[7]

2. Synthesis of C5-substituted arabinofuranosyluracil from 2,2′-anhydro-5-methoxycarbonylmethyluridine

2,2′-Anhydro-5-methoxycarbonylmethyluridine (**1**) was synthesized by the reported method.[8] This nucleoside was treated with 50% tris(2-aminoethyl)amine in ethanol at 37 °C for 40 h. The obtained C2- and C5-substituted arabinopyrimidine nucleoside (**2**) was incubated in sodium acetate buffer (pH 4.6) at 37 °C for 60 h to convert to C5-substituted 2,2′-anhydro-β-D-arabinofuranosyluracil (**3**). This product was subsequently treated with 0.1 M sodium hydroxide aqueous solution at 37 °C

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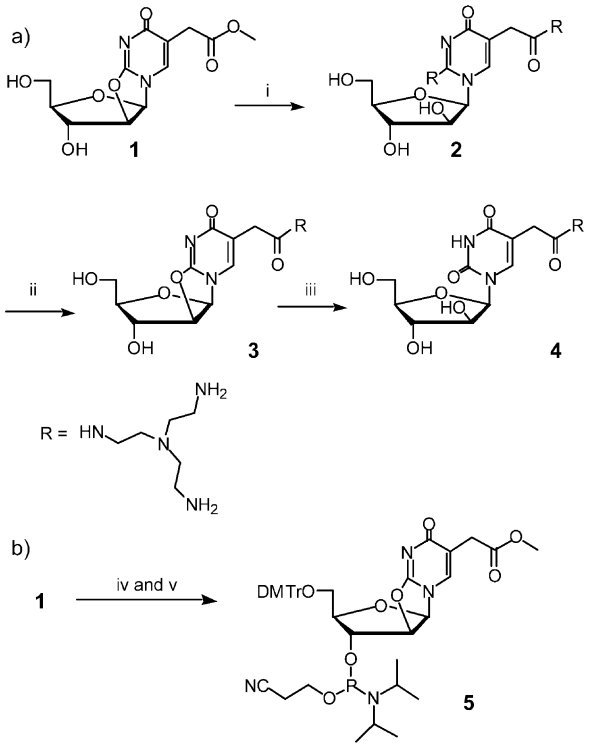
for 24 h to give β-D-arabinofuranosyl-5-[*N*-[2-[*N,N*-bis(2 - aminoethyl)amino]ethyl]carbamoylmethyl]uracil (**4**). This conversion route is shown in Scheme 1a. The reaction mixtures at each step were analyzed and purified by reversed-phase HPLC. The purified products were identified by ESI-MS. The yields and ESI-MS data are presented in Table 1.

3. Synthesis of the ODN containing a single modified nucleoside

To incorporate 2,2′-anhydro-5-methoxycarbonylmethyluridine (**1**) into ODN, **1** was protected by a dimethoxytrityl group at the 5′-position and phosphitylated by chlorophosphoramidite at the 3′-position as shown in Scheme 1b. This nucleoside phosphoramidite (**5**) was incorporated into ODN by an automated DNA synthesizer. Sequences and structures are shown in Scheme 2 (**ODN1**–**4**). The protected ODN on CPG (**ODN1**) was allowed to react with tris(2-aminoethyl)amine overnight at 55 °C. The 5′-DMTr-ODN was purified by HPLC and then the 5′-DMTr group of the ODN was removed by an acid treatment to give **ODN2**. **ODN2** was slowly converted to **ODN3** containing C5-substituted 2,2′-anhydrouridine in an acetate buffer (pH 4.6) at 37 °C for 5 days. The 2,2′-ether bond was hydrolyzed by aqueous sodium hydroxide to obtain **ODN4**. The obtained ODNs at each step were purified by HPLC and identified by ESI-MS. Yields and ESI-MS data are shown in Table 2. These results indicate that C5-methoxycarbonylmethyl-2,2′-anhydrouridine could be converted to C5-substituted β-D-arabinofuranosyluracil in DNA. This post-synthetic method does not require protection of the 2′-hydroxyl group.



Scheme 1. (i) Tris(2-aminoethyl)amine in ethanol; (ii) acetate buffer (pH 4.6); (iii) 0.1 M NaOH aq soln.; (iv) DMTr-Cl, pyridine; (v) $(Pr^i{}_2N)(CNCH_2CH_2O)PCl$, $Pr^i{}_2NEt$, CH_2Cl_2.

Table 1. Yields and MS data of nucleoside analogues

Compd	Yield[a] (%)	m/z ($[M+H]^+$)	
		Found	Calc.
2	74.4	559.4	559.37
3	97.9	435.2[b]	435.20[b]
4	97.5	453.1[b]	453.21[b]

[a] Yields were estimated from the areas of the HPLC peaks.
[b] The signals were detected as $[M+Na]^+$.

4. Synthesis of the ODN containing three modified nucleosides

To know the effect of several modified nucleosides in one ODN on the stability and structure of a duplex, three **4** were incorporated into ODN. The sequence and structure of ODN are shown in Scheme 2 (**ODN5**–**8**). **ODN8** was synthesized from **ODN5**, which has three C5-methoxycarbonylmethyl-2,2′-anhydrouridine in ODN, by the same method as the synthesis of **ODN4**. This product was analyzed by nuclease digestion. Figure 1 shows the HPLC chromatogram of the hydrolysate of **ODN8** by snake venom phosphodiestrase, nuclease P1, and alkaline phosphatase. The product eluted at 3.4 min was collected and identified by ESI-MS. From this result, this compound was **4** (m/z ($[M+H]^+$) Found, 431.3; calc., 431.2. The nucleoside composition was **4**:dC:dG:T:dA = 2.7:7.0:2.2:2.1:1.0 for 3:7:2:2:1 by theoretical calculation. The yield of **ODN8** was 72% from **ODN6**.

5. T_m of the ODN/DNA and ODN/RNA duplexes

The thermal stability of an ODN/DNA duplex comprising **ODN4** or **ODN8** was measured by an UV melting curve. The results are summarized in Table 3.

ODN1-4: 5'-CGC TTC TXC CTG CCA-3'
ODN5-8: 5'-CGC XTC TXC CXG CCA-3'

ODN	X	5'-terminal
ODN1 and **5**	**1**	DMTrO-
ODN2 and **6**	**2**	HO-
ODN3 and **7**	**3**	HO-
ODN4 and **8**	**4**	HO-

Scheme 2.

Table 2. Yields and ESI-MS data

Compd	Yield (%)	ESI-MS data	
		Found	Calc.
ODN2	8.9	4768.0	4769.1
ODN3	68	4622.1	4622.9
ODN4	91	4640.9	4640.9

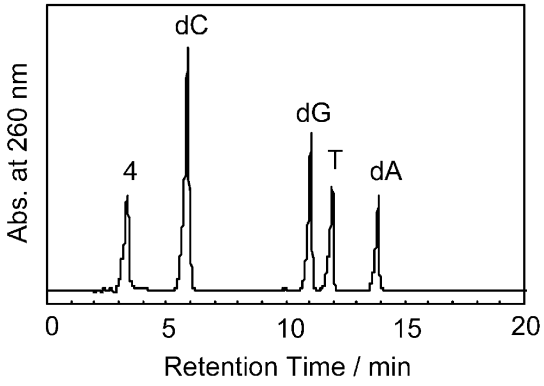


Figure 1. HPLC profile of the hydrolysate from nuclease digestion of **ODN8**. HPLC condition: column, wakosil 5C18 (4×250 mm); eluent A, 50 mM triethylammonium acetate (pH 7.2); B, 50 mM triethylammonium acetate in 70% acetonitrile; gradient, 3–53% B in 35 min; flow rate, 1 mL/min.

The **ODN4**/DNA duplex has similar stability with the **N-ODN**/DNA duplex. Noranha et al. reported that the ODN containing arabinonucleosides formed a less stable duplex with DNA than normal ODN.[2b] We also reported that T_m of the ODN containing a single arabinofuranosylthymine (5′-CGCTTCTaraTCCTGCCA-3′) with DNA was 57.6 °C in the previous paper.[4] Therefore, ΔT_m of **ODN4**/DNA against that of the ODN containing a single arabinofuranosylthymine is 3.6 °C. This increasing stability arises from a positively charged C5-substituent. The positively charged C5-substituent may stabilize an ODN/DNA duplex by an interaction with negatively charged phosphate groups in the duplex. On the other hand, **ODN8** that contained three modified nucleosides showed lower T_m than **ODN4**. This result suggests the conformational change of the duplex by three arabinofuranoses had a large effect on T_m over the stabilization by positively charged amino groups when the ODN has several arabinonucleosides. For the duplexes with RNA, **ODN4** formed a slightly more stable duplex compared with **N-ODN**, and **ODN8** formed the duplex with only a small loss of stability. This property of forming a moderate stable duplex with RNA is suitable as an antisense oligonucleotide.

6. RNase H induced activity

Induction of RNase H activity by the modified ODN was investigated in comparison with unmodified ODN. 5′-Fluorescein-labeled RNA 21mer and two-fold excess of the ODNs (**N-ODN**, **ODN8**, and random DNA) were annealed in 60 mM KCl, 2.5 mM Mg_2Cl, 2mM DTT, and a ribonuclease inhibitor. The reactions were started by the addition of *E. coli* RNase H and the reaction mixtures were incubated at rt for 30 or 90 min. The RNA fragments were analyzed by denatured polyacrylamide gel electrophoresis (Fig. 2). *E. coli* RNase H was able to degrade the RNA in the **ODN8**/RNA duplex. The reaction rate ($t_{1/2}$ = 46 h for **ODN8**) was almost the same as that for the **N-ODN**/RNA duplex ($t_{1/2}$ = 44 h). This result indicates that the C5-substituted arabinofuranosyluracil residue did not affect the induction of RNase H activity.

Table 3. T_m of ODN/DNA and ODN/RNA duplexes

Compd	T_m (°C)	ΔT_m (°C)
For DNA		
N-ODN	61.5	0
ODN4	61.2	−0.3
ODN8	52.4	−9.1
For RNA		
N-ODN	68.6	0
ODN4	70.2	1.6
ODN8	67.2	−1.4

T_m were measured by UV melting curves in 150 mM sodium chloride/10 mM sodium phosphate (pH 7.0)/10 μM EDTA. Concentration of DNA or RNA was 2 μM for each strand. ΔT_m refers to [T_m(modified duplex) − T_m(normal duplex)].

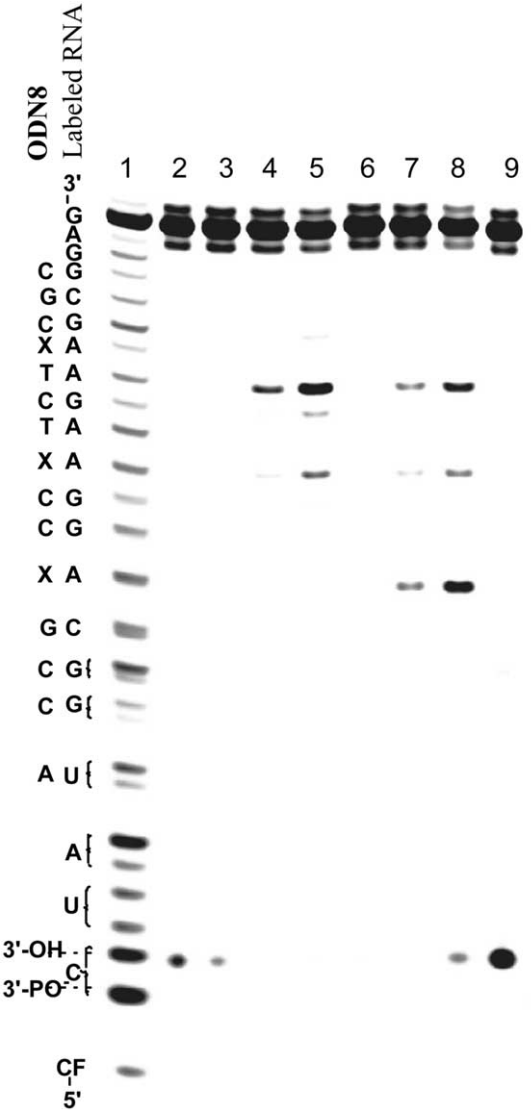


Figure 2. RNase H mediated cleavage of target RNA. 21-nt 5′-fluorescein-labeled RNA (5′-F-CUA UGG CAG GAA GAA GCGGAG-3′) was cmplementary to ODN at underlined base. Lane 1, alkaline hydrolysis; lane 2, 21-nt RNA; lane 3, incubated **N-ODN**/RNA in absence of RNase H; lane 4 and 5, incubated **N-ODN**/RNA in presence of RNase H for 30 and 90 min, respectively; lane 6, incubated **ODN8**/RNA in absence of RNase H; lane 7 and 8, incubated **ODN8**/RNA in presence of RNase H for 30 and 90 min, respectively; lane 9, incubated randam ODN/RNA in presence of RNase H for 90 min (randam DNA; 5′-CAT AGGAGA TGC CAT-3′).

7. Nuclease resistance

ODN8 or **N-ODN** was incubated with snake venom phosphodiesterase to know the nuclease resistance of the modified ODN. The digested ODNs were analyzed by HPLC and the amount of full-length ODN remaining was quantitated by the ratio of peak areas on the HPLC chromatogram. 38% of the full-length **ODN8** remained after 30 min although the full-length **N-ODN** had disappeared after 30 min. The half-life times ($t_{1/2}$) of **ODN8** and **N-ODN** were 20 min and 4 min, respectively. Although the modified nucleoside was not placed at a terminal position of the ODN, **ODN8** had resistance for snake venom phosphodiesterase, which was exonuclease. This result suggests that the introduction of C5-substituted arabinofuranosylurcil in ODN led to nuclease resistance even with a few residues present in the ODN.

8. Conclusion

The ODN containing three C5-substituted arabinofuranosyluracils was synthesized by the post-synthetic modification method from the ODN containing three C5-substituted 2,2′-anhydrouridines. This procedure was convenient for the synthesis of arabinopyrimidine nucleoside-containing ODN compared with a conventional pre-synthetic modification method. The modified ODN could induce RNase H activity and impart stability against nuclease. These properties are suitable for an antisense molecule.

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