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SYNTHESIS AND HYBRIDIZATION PROPERTIES OF OLIGONUCLEOTIDES CONTAINING 6-MEMBERED AZASUGAR NUCLEOTIDES

Kyeong-Eun Jung*, Kichul Kim, Mirim Yang, Kwangjun Lee, and Hong Lim

AgroPharma Research Institute, Dongbu Research Council, 103-2 Yusung-Gu, Moonji-Dong, Taejon, Korea 305-380

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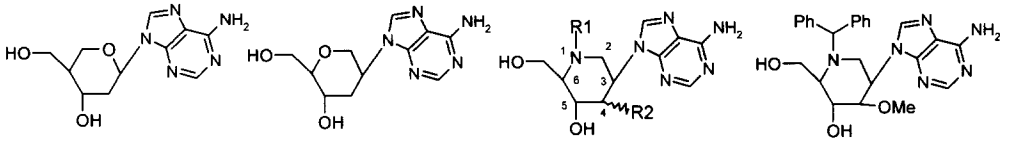
Abstract: A modified nucleoside was synthesized with adenine and a 6-membered azasugar, and it was converted to the phosphoramidite which was used for the incorporation into oligonucleotides. The hybridization properties of the modified oligonucleotides with DNA and RNA were studied.

After decades of research for antisense oligonucleotides (ONs), the first antisense drug, Vitravene® as a phosphorothioate, is now available for cytomegalovirus retinitis.[1] However, phosphorothioates still have undesired properties such as immune responses probably due to protein binding.[2] A number of structural modifications of ON have been explored extensively in order to overcome the limitations of phosphorothioates and improve the characteristics of ONs such as nuclease stability, binding affinity, cell delivery, and decrease toxicity.[3] Sugar modifications have drawn much attention because of their promising features. 2'-*O*-Modified nucleosides[4] and conformationally locked nucleosides[5] were incorporated into ONs affording better RNA affinity and higher nuclease stability. Hexose nucleosides[6] were also introduced into ONs and some of them[7] showed strong affinities to RNA.



A **B** **C** **D**

Recently we have explored variety of antisense ONs focused on sugar-modified nucleosides.[8] Even though ONs consisting of hexose nucleoside **A** have been found to bind insufficiently to complementary DNA or RNA, ONs constructed with 1', 5'-anhydrohexitol nucleoside **B** showed high affinity with RNA as well as DNA. We chose six-membered azasugar nucleosides **C** with base at the 3-C position of sugar instead of the aminal position expecting both to have high affinities to DNA and RNA as seen in 1', 5'-anhydrohexitol ON and nuclease resistance as carbocyclic nucleosides. Moreover, the introduction of nucleic base at this carbon is expected to make easier the preparation without isomerizations during a glycosylation. Various substituents including lipophilic groups can be positioned at nitrogen of azasugar easily without harsh reaction conditions. Poor cellular uptakes of ONs have been a big hurdle for the development of antisense therapeutics. The introduction of a lipophilic moiety on nitrogen of azasugar can enhance the cellular delivery of ON.

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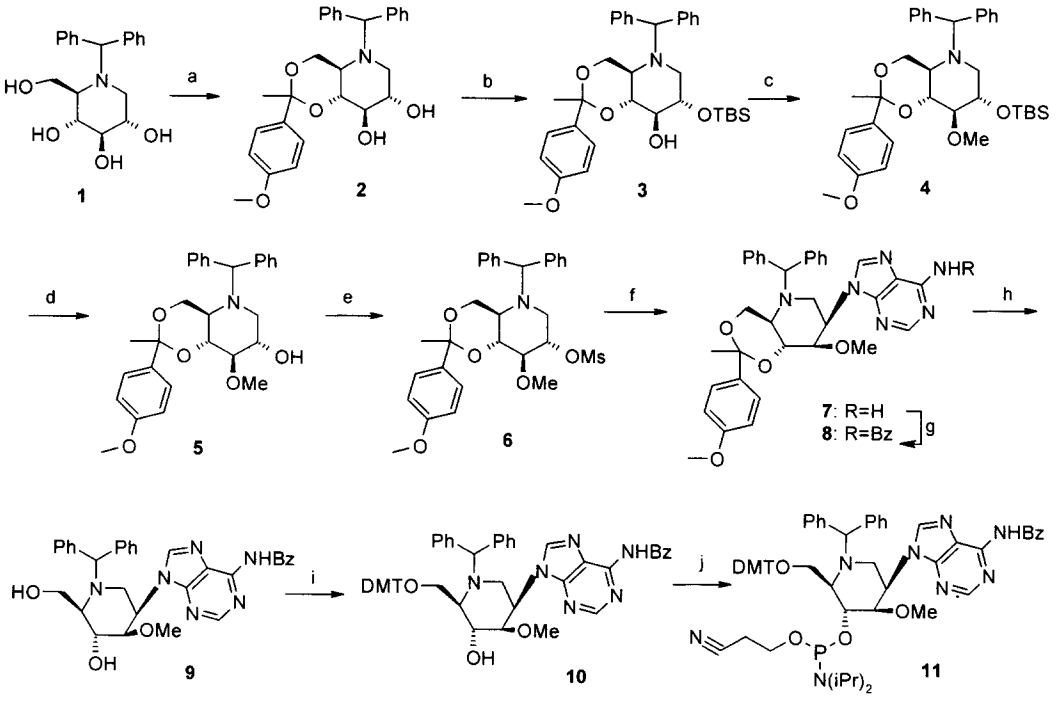
Synthesis of the adenosine building block **D** containing a 6-membered azasugar is illustrated in Scheme I. *N*-Benzhydryl-1-deoxynojirimycin **1** was prepared by the procedure published by Baxter.[9] 1, 3-Diol of **1** was protected using α, *p*-dimethoxystyrene and pyridinium *p*-toluenesulfonate to give **2**.[10] The use of other diol protecting reagents such as 2-methoxypropene and benzaldehyde did not give good results. The treatment of protected sugar **2** with TBSCl, silver nitrate and pyridine provided the selective protection of 3-hydroxyl moiety with the very minor product of disilylation. The structure of alcohol **3** was confirmed by 2D-COESY and NOESY NMR spectroscopy.

Scheme I



a) α, *p*-dimethoxystyrene, PPTS, overnight, rt, 76%; b)TBSCl, $AgNO_3$, pyridine, 2hr, rt, 73%; c) MeI, NaH, 2hr, rt, 94%; d) TBAF, 2hr, rt, 86%; e) MsCl, Et_3N, 2hr, rt, 99%; f) adenine, NaH, 18-crown-6, 17hr, 100°C, 50%; g) BzCl, pyridine, NH_4OH, 66%; h) 80% AcOH, 4hr, 30 °C, 55%; i) DMTCl, pyridine, overnight, rt, 88%; j) Cl-P $(OCH_2CH_2CN)N(iPr)_2$, $EtN(iPr)_2$, 2hr, rt, 59%.

O-Alkylation of the alcohol **3** with methyl iodide gave compound **4** which underwent the deprotection of TBS group using TBAF in THF. The resulting alcohol **5** was sulfonated to give the mesylate **6** by methanesulfonyl chloride and triethylamine. Nucleophilic displacement of **6** with adenine was completed using NaH and 18-crown-6 in DMF affording **7** in 50% yield.[11] Adenine analogue **7** was then mono-benzoylated and the diol protecting group was cleaved by 80% acetic acid giving nucleoside **9** without the deprotection of N-benzhydryl moiety. A DMT group was then introduced to the primary hydroxyl group of **9**, and the use of 2-cyanoethyl diisopropylchlorophosphoramidite and diisopropylethylamine to nucleoside **10**

afforded the adenine building block **11**.[12]

Assemly of oligonucleotides **1-11** of Table I was accomplished by using ABI 392 DNA/RNA Synthesizer on a 1 μmol scale by the standard method except 10 min coupling time for the incorporation of modified nucleotide. The yields of modified phosphoramidite coupling was 90 - 98 %, and oligonucleotides prepared were purified by reverse-phase HPLC (Hamilton PRP column, 7x300 mm) and characterized by mass spectrometry (MALDI/TOF/MS) and PAGE. Hybridization characteristics of modified ONs were studied with complementary sequence of DNA and RNA by measuring melting temperatures (*T*m) in a buffer solution (pH 7.0) containing 100 mM NaCl, 10 mM NaH_2PO_4, 0.1 mM EDTA and 2.5 μM of each oligonucleotide.

Table I. *T*m , Δ*T*m and Δ*T*m /mod values of synthesized oligomer and natural oligomers (°C)

	sequence	DNA			RNA		
		*T*m	Δ*T*m	Δ*T*m/mod	*T*m	Δ*T*m	Δ*T*m/mod
1	5'-AGGGAGAGAAAG-3'	45	-	-	34	-	-
2	5'-AGGG**A**GAGAAAG-3'	37.8	-7.2	-7.2	35.9	+1.9	+1.9
3	5'-AGGGAG**A**GAAAG-3'	37.9	-7.1	-7.1	35.3	+1.3	+1.3
4	5'-**A**GGGAGAGAAAG-3'	46.9	+1.9	+1.9	33.3	-0.7	-0.7
5	5'-AGGGAGAGAA**A**G-3'	35	-10	-10	25	-9	-9
6	5'-AGGG**A**GAG**A**AAG-3'	39.9	-5.1	-2.6	39	+5	+2.5
7	5'-**A**GGG**A**GAGAAAG-3'	37	-8	-4	37.8	+3.8	+1.9
8	5'-**A**GGGAG**A**GAAAG-3'	35.3	-9.7	-4.9	34.2	+0.2	+0.1
9	5'-AGGG**A**G**A**GAAAG-3'	31.2	-13.8	-6.9	31.1	-2.9	-1.5
10	5'-**A**GGG**A**GAG**A**AAG-3'	28.7	-16.3	-5.4	35.3	+1.3	+0.4
11	5'-d**A**GGG**A**G**A**G**A**AAG-3'	19.8	-25.2	-6.3	27.5	-6.5	-1.6

A = 9-[(3*R*, 4*R*, 5*R*, 6*R*)-*N*-benzhydryl-5-hydroxy-6-hydroxymethyl-4-methoxy piperidin-3-yl]adenine

Δ*T*m /mod: Δ*T*m per one modification

As shown in Table I, the replacement of adenosine with modified adenosine **A** enhances the affinity with RNA significantly, resulting up to a 5 °C increase of *T*m (oligomer **6**). However, the stability of duplexes was dependent on the position and number of modified nucleosides. The modified adenosine **A** in the middle region of the strands gave more thermal stability than in the 3'- and 5'-region. The oligomer **5**, which has **A** at near 3'-end of sequence especially reduced the thermal stability of ON/RNA duplexes significantly. The oligomer **6** and **7** containing **A** at an interval of three nucleosides increased the thermal stability with RNA the most. When modified nucleosides locate without a certain gap, *T*m goes down as seen in oligomer **9**. The steric influences of diphenylmethyl group on sugar ring can be the reason for this phenomenon. On the other hand, these modified ONs did not enhance *T*m with DNA except for the oligomer **4** which has a modified one at the 5'-end position. The other ONs showed decreased melting temperatures with complementary DNA in the range of -4 ~ -10 °C per modification. The cause of big differences of ON's affinities between DNA and RNA

remains to be studied.

The HPLC results of modified ONs are quite different from native ONs. Whereas natural dodecamer appears its peak at 15 min with elution of a linear gradient of 18% - 28% CH_3CN/100 mM TEAA within 10 min, these modified ONs usually do not show their peaks until 40 min in the same elution system. Their peaks appear around 15 min with elution of a linear gradient of 28 - 38% CH_3CN/100 mM TEAA within 10 min. The increase of hydrophobicity in these modified ONs can be advantageous for cellular uptake.

In summary, we report the synthesis of modified nucleoside containing a 6-membered azasugar and its phosphoramidites for oligonucleotide synthesis. Its incorporation into ONs exhibits strong hybridization with RNA depending on the location and number of substitutions. Further studies of derivatives with various groups of R1 and R2 of nucleoside **C** and other bases are underway in this laboratory.

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12. N^6-Benzoyl-9-{(3*R*,4*R*,5*R*,6*R*)-*N*-benzhydryl-5-[(2-cyanoethoxy) (*N*,*N*-diisopropylamino)phosphinoxy]-6-dimethoxytrityloxymethyl-4-methoxypiperidin-3-yl}adenine **11**. (diastereomeric mixtures) ^{1}H NMR ($CDCl_3$) δ 1.04 (d, 6H, *J*=6.7 Hz), 1.15 (d, 6H, *J* =6.5 Hz), 1.19 (d, 6H, *J* =6.5 Hz), 1.21 (d, 6H, *J* =6.7 Hz), 2.52 (t, 4H, *J* =6 Hz), 2.63 (d, 1H, *J* = 11.1 Hz), 2.73 (dd, 1H, *J* =2, 13 Hz), 2.84 (dd, 1H, *J* =4, 6 Hz), 2.92 (dd, 1H, *J* =3.5, 13.5 Hz), 3.27 (s, 3H, OMe), 3.33 (s, 3H, OMe), 3.36 - 3.80 (m, 11H), 3.834 (s, 6H, OMe), 3.838 (s, 6H, OMe), 3.84 (m, 2H), 4.34 (d, 2H, *J* =11.1 Hz), 4.40 (d, 2H, *J* =10.6 Hz), 4.68 (s, 1H), 4.70 (s, 1H), 4.77 (m, 2H), 6.83 (d, 8H, *J* =8.8 Hz), 7.12 - 7.39 (m, 30H), 7.55 (t, 4H, *J* =7 Hz), 7.62 (d, 2H, *J* =7.2 Hz), 8.07 (d, 4H, *J* =7.3 Hz), 8.72 (s, 1H), 8.75 (s, 1H), 9.06 (s, 1H), 9.32 (s, 1H). ^{31}P NMR ($CDCl_3$) δ 149.01, 150.07. FAB-MS m/z 1067.2, cacd 1067.2. HR FAB-MS m/z 1067.4940, cacd 1067.4949.