NUCLEOSIDES AND NUCLEOTIDES 111. THERMAL STABILITY OF OLIGODEOXYRIBONUCLEOTIDE DUPLEXES CONTAINING N^6-HYDROXYADENINE IN SUBSTITUTION FOR ADENINE[1)]

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An oligodeoxyribonucleotide containing N^6-hydroxyadenine (**H**) has been synthesized. An order of Tms of duplexes consisting of 5'd(CCTGGTA**H**CAGGTCC)3' : 5'd(GGACCTGNTACCAGG) (**N** = A, G, T, C) was **H** : T > **H** : G > **H** : A > **H** : C.

KEYWORDS N^6-hydroxyadenine; oligonucleotide; thermal denaturation; mutation

Treatment of cells with methoxyamine and hydroxylamine (a presumed intermediate in nitrate reduction *in vivo*) may induce N^6-methoxyadenine (**M**) and N^6-hydroxyadenine (**H**) besides other modified bases in DNA and plasma.[2)] Changing the tautomeric form,[3)] the adenine analogues may form relatively stable base pairs with not only thymine residues but also cytosine residues in DNA (Chart 1). Incorporation of dCTP by DNA polymerases into DNA strands at the site opposite to **M** or **H** may cause A to G transitions, which may be one of the steps leading to mutation by the amines.[4)]

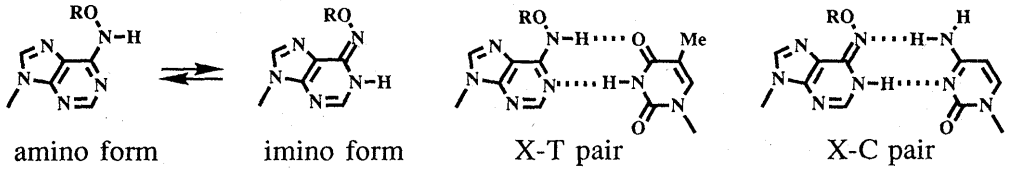

Chart 1. **X** = N^6-methoxyadenine (**M**, R = Me), **X** = N^6-hydroxyadenine (**H**, R = H).

Therefore, synthesis of oligonucleotides containing **M** or **H** and use of the oligonucleotides for biological and biophysical studies can be used to study molecular mechanisms of mutation. Recently, we reported a synthesis and properties of oligonucleotides containing **M**.[5)] **M** formed fairly stable base pairs with **N** (four normal bases, A, G, T, and C) in duplexes consisting of oligodeoxyribonucleotides.[5c)] Also, TTP and dCTP were efficiently incorporated into DNA strands at the sites opposite to **M** by Klenow DNA polymerase.[5c)]

However, oligonucleotides containing **H** have not been synthesized since the exocyclic hydroxyamino group is not chemically stable.[6)] We have examined methods for protection of the hydroxylamino group of **H** for oligonucleotide synthesis and found that the *tert*-butyldiphenylsilyl (TBDPS) group of 2'-deoxy-N^6-TBDPSoxyadenosine (**4**) (Chart 2) is stable during the reactions used in DNA synthesis and is removed easily by treatment with conc. ammonium hydroxide after **4** is introduced into oligonucleotides. A scheme for synthesis of N^6-hydroxy-2'-deoxyadenosine (d**H**) and its amidite unit is shown in Chart 2. 2'-Deoxyinosine was converted into 3',5'-di-*O*-acetyl-O^6-triisopropylbenzenesulfonyl-2'-deoxyinosine (**2**),[7)] which was treated with [(*tert*-butyldiphenylsilyl)oxy]amine[8)] in dioxane to give 3',5'-di-*O*-acetyl-N^6-TBDPSoxy-2'-deoxyadenosine (**3**). The acetyl group of **3** was removed selectively by treatment with K_2CO_3 in anhydrous methanol to give **4**,[9)] which was converted into an amidite unit **6** according to the reported methods.[10)]

Using the amidite unit **6**, d**H** was introduced into an oligonucleotide, 5'd(CCTGGTA**H**CAGGTCC)3', on a DNA synthesizer (Applied Biosystem 381A) according to the reported methods[10)] with a minor modification. That is, 1% dichloroacetic acid in anhydrous CH_2Cl_2 solution was used for deprotection of the dimethoxytrityl (DMTr) group since the TBDPS group of **6** was partially removed by treatment with 3% trichloroacetic acid in anhydrous CH_2Cl_2. The fully protected oligonucleotide containing d**H** was deprotected and purified by the same procedure as that for deprotection and purification of normal oligodeoxyribonucleotides.[10)] First, the fully protected oligonucleotide attached to a resin was incubated with ammonium hydroxide at 55 °C for 5 h. The TBDPS group was completely removed by this treatment. The oligonucleotide, protected by a DMTr group at the 5'-end, was purified by C-18 column chromatography (Ø 1 x 7 cm. Waters, U.S.A.) with a linear gradient of acetonitrile from 10% to 30% in 0.1 M triethylammonium acetate buffer (pH 7.0). Treatment of the main peak with 80% acetic acid gave the oligonucleotide, which had a single peak by HPLC analysis with a C-18 column

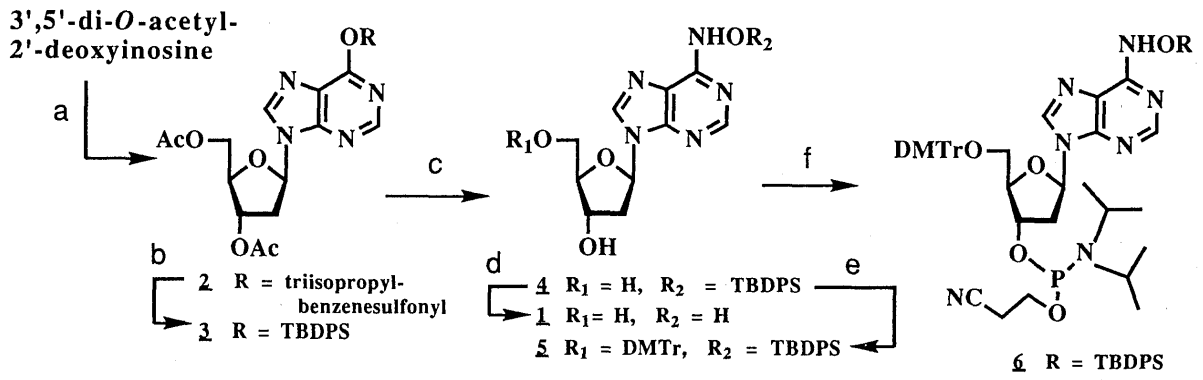

Chart 2. a; TPSCl (2 eq), triethylamine (3 eq), dimethylaminopyridine (0.01 eq), CH_2Cl_2, r.t., 2 h, 61 %. b; *tert*-butyldiphenylsilyloxyamine (5 eq), dioxane, reflux, 12 h, 42%. c; K_2CO_3 (2 eq), MeOH, r.t., 30 min., 78%. d; *n*-tetrabutylammonium fluoride (2 eq), THF, r.t., 1h, 52%. e; DMTrCl (1.5 eq), pyridine, r.t., 2 h, 91%. f; 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (2 eq), *N*, *N*-diisopropylamine (5 eq), CH_2Cl_2, r.t., 1 h, 78%.

(Chemcosorb ODS-2, Chemco Scientific Co., Ltd., Japan) (Fig. 1a) or with a DEAE column (TSK gel DEAE-2SW, Toso, Japan) (Fig. 1b). After hydrolysis of the oligonucleotide by snake venom phosphodiesterase and bacterial alkaline phosphatase to nucleosides, the nucleoside composition of the oligonucleotide was confirmed by HPLC (4.6 x 250 mm, Chemcosorb 5-ODS-H) (Fig. 1c). A peak corresponding d**H** was confirmed by coelution with a sample of d**H** (**1**)[11] synthesized from **4**.

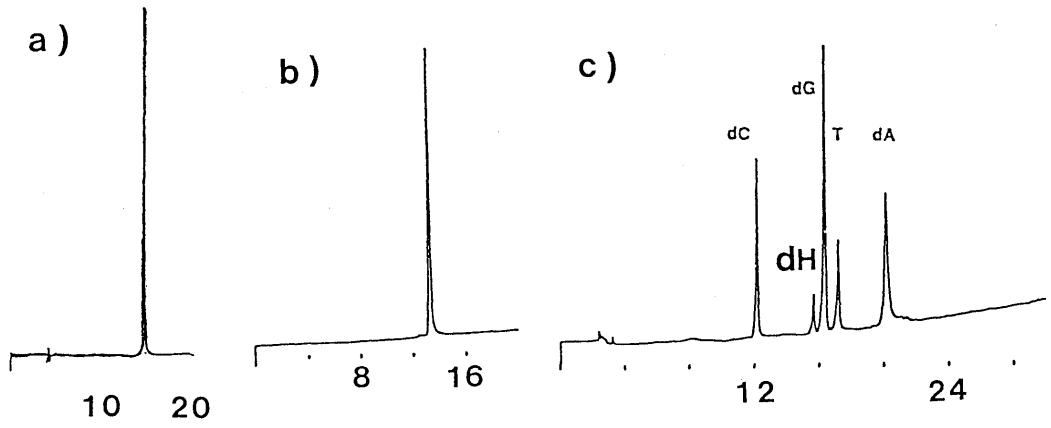

Fig 1. Profiles of HPLC Analysis a; C-18 column, a linear gradient of acetonitrile from 5% to 25% in 0.1 M triethylammonium acetate buffer (pH 7.0) in 20 min. b; DEAE column, a linear gradient of ammonium formate from 0.5 M to 1.5 M in 20% acetonitrile (pH 7.0). c; C-18 column, a linear gradient of methanol from 0% to 50% in water in 30 min.

Table I. Tms (°C)[14]

(X)\(N)	T	C	A	G
H	51	42	42	50
M[15]	50	46	52	48
A[15]	54	44	45	53
G[15]	48	59	54	52

Next, we examined the stability of duplexes containing **H** and **M** by thermal denaturation. Tms of a set of duplexes consisting of 5'd(CCTGGTA**X**CAGGTCC)3' and 5'd(GGACCTG**N**TACCAGG) (**X** = **H**, **M**, A, and G. **N** = T, C, A, and G) are listed in Table I. The Tm of the duplex containing the H : T pair (the **H** : T duplex) (Tm = 51 °C) is similar to the Tm of the **M** : T duplex (50 °C) but lower than the Tm of the A : T duplex (54 °C), probably owing to the steric hindrance between N^7 of **M** or **H** and the methoxy group or the hydroxy group, respectively.[12] On the other hand, the **M** : C duplex (46 °C) is more stable than the A : C duplex (44 °C) since the imino form of **M**, a predominant form in polar solvent such as DMSO, water, etc.,[13] is suitable for formation of the **M** : C pair (Fig. 1). In contrast, the Tm

of the **H** : C duplex (42 °C) is lower than Tms of the **M** : C duplex (46 °C) and the A : C duplex (44 °C). It can be surmised from the result that **H** prefers to be in the amino form, which is not suitable for formation of the **H** : C pair.

However, the difference in Tms of the **H** : T duplex and the **H** : C duplex (9 degree difference) was smaller than the difference in Tms of the A : T duplex and the A : C duplex (11 degree difference). Namely, the difference in the stability of the **H** : C pair and the **H** : T pair was smaller than the difference in the stability of the A : C pair and A : T pair. The result may not contradict the reported result that dHTP (dH triphosphate) was enzymatically incorporated into DNA strands in substitution for not only dATP but also dGTP, probably forming the **H** : T and the **H** : C pairs.[16] A study of enzymatic incorporation of dNTPs into DNA strands at the site opposite to **H** is of prime interest since the study may contribute to clarifying the molecular mechanism leading to mutation.[5c]

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9) Methanol (30 ml) and K_2CO_3 (398 mg, 2.9 mmol) were added to **3** (855 mg, 1.45 mmol) and the mixture was stirred at room temperature for 30 min. Then, the mixture was neutralized by adding 1 N HCl and the solvent was evaporated *in vacuo*. The residue was chromatographed over a silica gel column (Ø 3.2 x 13 cm) with a mixture of $CHCl_3$ and ethanol as an eluent to give **4** as a foam (560 mg, 78%). Mass (*m/z*) : 505 (M^+), 389 (base + 1). ^1H NMR ($CDCl_3$) dppm : 9.56 (br s, 1H, N^6-H), 7.84 - 7.32 (m, 12H, H-2, and H-8, and Ph), 6.16 (d d, 1H, H-1', $J_{1',2'}$ = 9.5 Hz), 5.49 (d, 1H, 3'-OH) 4.67 (m, 1H, 5'-OH), 4.16 - 4.08 (m, 2H, H-3' and H-4'), 3.91 - 3.70 (m, 2H, 5'-Ha,b), 2.79 - 2.77 (m, 1H, H-2'a), 2.29 - 2.27 (m, 1H, H-2'b), 1.28 - 1.18 (m, 9H, Me_3 of TBDPS).
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11) Compound **4** (0.2 mmol) was incubated with tetrabutylammonium fluoride (0.4 mmol) in THF (10 ml) at room temperature for 1 h. Then the solvent was evaporated *in vacuo* and the residue was chromatographed over a column of C-18 silica gel (Ø 1.0 x 10 cm) with a mixture of methanol and water as an eluent to give **1** (0.1 mmol, 52%). Physical properties of **1** was similar to a reported result; M. J. Robins and G. L. Basom, *Can. J. Chem.*, **51**, 3136 (1973).
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14) Each oligonucleotide (6 μmole) was dissolved in a buffer of 0.1 M NaCl, 0.01 M Na cacodylate (pH 7.0, 3 ml) and the solution was heated at 70 °C for 20 min; then the solution was cooled gradually to 5°C and used in the thermal denaturation study. Thermally induced transitions of the duplexes were monitored at 254 nm. Sample temperature was increased one degree per one min. Each Tm is given an average of 3 measurements.
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