Chemical Studies on the Regulation
of DNA Structures
and Z-DNA Specific Reactions

Kiyohiko Kawai

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Preface

The study presented in this thesis has been carried out under the direction of Professor Isao Saito at the Department of Synthetic Chemistry and Biological Chemistry of Kyoto University during April 1993 to March, 1999. The study is concerned with chemical studies on the regulation of DNA structures and Z-DNA specific reactions.

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January, 1999

Contents

General Introduction

As for its flexible nature as a biopolymer, DNA is able to form a number of local conformations. In addition to canonical right handed B-form structure, DNA has been demonstrated to adopt A-form DNA, left handed Z-form DNA, cruciform, parallel stranded DNA, bent DNA, triplex and quadruplex DNA.[1] Such polymorphism of DNA structure has been suggested to play an important role in a number of transcriptional and replicative processes.[2] None the less, when and how these local conformations form remain unresolved, and their detail biological functions are still unclear.

Understanding the forces responsible for DNA conformational change would be of fundamental value in exploring the biological roles of alternative DNA conformations. One useful method to study the contribution of various thermodynamic forces on DNA conformations is to examine the effects of chemical modification on the thermal stability and structures. The chemical modification at the base,[3] sugar moiety[4] and DNA back bone[5] have been extensively studied. For example, Dias et al. used C8 brominated guanine as a probe for glycosidic conformation in telomeric DNA.[3a] Strauss et al. substituted one of the nonbridging phosphate oxygens with methyl group for the neutralization of the charge and demonstrated that a DNA bend is caused by neutralization of one face of DNA helix.[5a] From these results, they have proved the hypothesis that proteins with cationic surfaces induce substantial DNA bending by neutralizing phosphates on one DNA face.[6]

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Among the DNA conformational changes, transition from the canonical right handed B-form DNA to left handed Z-form DNA is perhaps the most dramatic DNA conformational change. Z-form DNA was named for the jagged appearance of its left handed backbone (Figure 1). Although almost twenty years have past since the first determination of its structure from single-crystal X-ray diffraction,[7] the physiological role of Z-DNA remains a topic of active debate.[8] There are some reports for chemical modifications which have been shown to stabilize Z-conformation, such as C5-methylation or C5-bromination[9] of cytosine or C8-bromination of guanine.[10] However, previous thermodynamic works reveal little for the effect of these substituents on the B- to Z-DNA transition in solution, hence, the precise molecular mechanism of B- to Z-DNA transition is still well not understood.
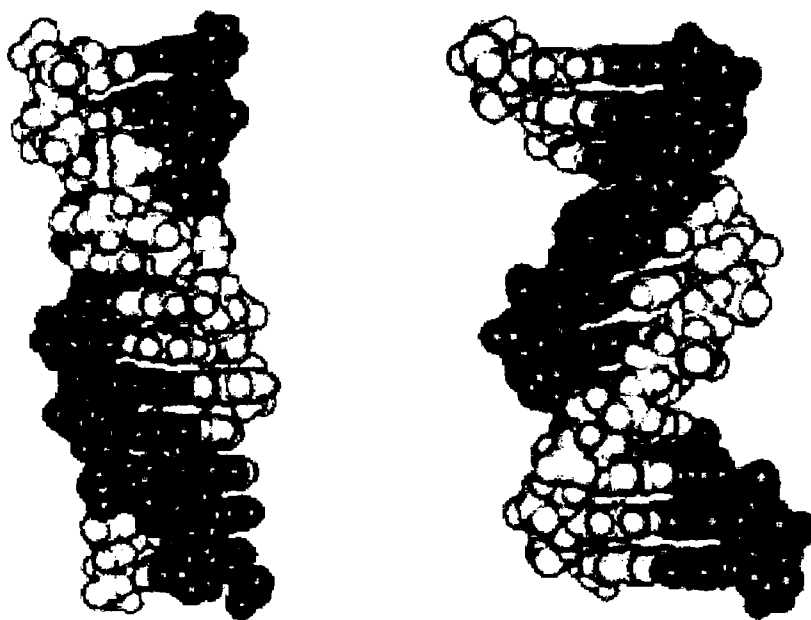
Figure 1. Structural model for right-handed B-form DNA (right) and left handed Z-form DNA (left).

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Chemical modification which can stabilize a certain DNA conformation provides opportunities for investigating its conformation dependent thermodynamic properties, detail structure and reactivities. Recently, our group reported the construction of stable parallel stranded DNA by incorporation of 5-methylisocytosine and isoguanine and the detail structure of parallel stranded DNA was determined by NMR.[11] Since the reactivities of Z-form DNA and several other local conformations have not been thoroughly investigated, it is highly desirable to develop a chemical modification to stabilize such DNA conformations in appropriate short oligonucleotides in a solution.

Another way to understand the precise biological functions of DNA local conformations is to have an appropriate detection system for DNA local structures in a living cell system. Since the DNA local structures are assumed to appear in a very short period of time in a living cell, methods which can trap the intermediate structure is useful. Meyer *et al.* attached reactive bromoacetoamidopropyl group to the end of oligonucleotides and trapped the formation of displacement loop (D-loop) with a covalent cross-link.[12] However, such reactive groups are not suitable for a biological system and therefore development of photo-triggered chemical probe is anticipated. Previously, our group investigated the photoreactions of 5-halouracil-containing B-form DNA[13] and DNA-RNA hybrid[14] and demonstrated that photo-generated uracilyl radical undergoes hydrogen abstraction in a highly conformation dependent manner. These results suggested that 5-halouracil may provide important informations on DNA conformations of biological interest. Therefore, we examined 5-halouracil as a photochemical probe for the left handed Z-form DNA. However, since previously reported chemical modifications which can stabilizes the Z-conformations

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have limited power for inducing the B- to Z-DNA transition or they are chemically unstable,[8d,e,9,10] we faced the difficulties of obtaining 5-halouracil-containing Z-form oligonucleotides in a solution. Therefore, in order to study the detail photoreaction of 5-halouracil in Z-DNA we first targeted to construct 5-halouracil-containing Z-form oligonucleotides in solution by introducing photochemically inert methyl group into guanine C8.

This thesis consists of 6 chapters on the chemical studies on the regulation of DNA structures and Z-DNA specific reactions. First three chapters describe the synthesis of purine C8 substituted base analogs and its effects on thermodynamics and DNA structures.

In chapter 1, synthesis, structure and thermodynamic properties of 8-methylguanine-containing oligonucleotides are reported. It was shown that incorporation of 8-methylguanine stabilizes the Z-conformation of short oligonucleotides of a variety of sequences.

In chapter 2, effects of two kinds of methylation on B- to Z-DNA transition are described. The effects of cytosine C5 methylation and guanine C8 methylation on the B- to Z-DNA transition was discussed in terms of thermodynamics and changes in solvent accessible surface areas.

In chapter 3, stabilization of Hoogsteen base pairing by introduction of NH_2 group at the C8 position of adenine is described. It was shown that incorporation of 8-aminoadenine stabilizes the Hoogsteen base pairing in triplex formation.

In the latter three chapters, photoreaction of 5-halouracil containing DNA is discussed.

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In chapter 4, conformational dependent photochemistry of 5-halouracil-containing DNA was discussed. Stereospecific 2'α-hydroxylation of deoxyribose in Z-form DNA is described.

In chapter 5, intrastrand 2'β Hydrogen Abstraction of 5'-Adjacent Deoxyguanosine by Deoxyuridin-5-yl in Z-form DNA is discussed. Incorporation of deuterium into uracil C5 during the C2' hydroxylation is described.

In chapter 6, photochemical halogen-exchange reaction of 5-iodouracil-containing oligonucleotides is described. It was found that 5-iodouracil undergoes efficient photo-halogen-exchange reaction in the presence of halide anions to produce corresponding C5 halogen exchanged uracils.

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Chapter 1

Synthesis, Structure and Thermodynamic Properties of 8-Methylguanine-Containing Oligonucleotides: Z-DNA under Physiological Salt Conditions

Abstract

Various oligonucleotides containing 8-methylguanine (m^8G) have been synthesized and their structures and thermodynamic properties investigated. Introduction of m^8G into DNA sequences markedly stabilizes the Z conformation under low salt conditions. The hexamer $d(CGCm^8GCG)_2$ exhibits a CD spectrum characteristic of the Z conformation under physiological salt conditions. The NOE-restrained refinement unequivocally demonstrated that $d(CGCm^8GCG)_2$ adopts a Z structure with all guanines in the *syn* conformation. The refined NMR structure is very similar to the Z form crystal structure of $d(CGCGCG)_2$, with a root mean square deviation of 0.6 between the two structures. The contribution of m^8G to the stabilization of Z-DNA has been estimated from the mid-point NaCl concentrations for the B-Z transition of various m^8G-containing oligomers. The presence of m^8G in $d(CGCm^8GCG)_2$ stabilizes the Z-conformation by at least $\Delta G = -0.8$ kcal/mol relative to the unmodified hexamer. The Z conformation was further stabilized by increasing the number of m^8Gs incorporated and destabilized by incorporating *syn*-A and *syn*- T, found respectively in the (A,T)-containing alternating and non-alternating pyrimidine-purine sequences. The results suggest that the chemically less

reactive m^8G base is a useful agent for studying molecular interactions of Z-DNA or other DNA structures that incorporate *syn*-G conformation.

Introduction

It has been well established that DNA structure has a remarkable conformational heterogeneity.[1,2]   Not only does the biologically relevant B-DNA exhibit considerable local heterogeneity, dramatically different DNA structures such as Z-DNA have also been discovered.   While the precise biological functions of Z-DNA have yet to be identified, its role in regulating DNA supercoiling has been amply demonstrated.[3,4]   A recent study by Rich and colleagues has shown that chicken double-stranded RNA adenosine deaminase has strong Z-DNA binding properties.[5]   This enzyme is known to work near the transcription apparatus, where a high negative supercoiling density along the DNA chain exist in front of the site of polymerase action.[2]   Thus far most of the thermodynamic properties of Z-DNA have been obtained through the use of supercoiled DNA plasmids containing various alternating $d(C \cdot G)_n$ inserts or their variants.[2-4] However, other aspects of Z- DNA have not been thoroughly investigated, presumably due to the difficulty of obtaining stable Z form oligonucleotides in a physiological salt solution.   Much of the available experimental data are limited to $d(C \cdot G)_n$ oligomers under non-physiological conditions of high alcohol or high salt concentrations.[6-8]   While some chemical modifications, such as C^5-methylation or C^5-bromination of cytosine[9] or C^8-bromination of guanine,[10,11] have been shown to stabilize the Z conformation in linear DNA oligomers, they have either limited power for inducing the B-Z transition or they are chemically unstable.   Therefore, it is desirable to have a more convenient and reliable way to stabilize Z

form oligomers under low salt conditions by incorporating chemically and photochemically inert modified bases. We report herein that the introduction of methyl group at the guanine C^8 position produces a stable m^8-modified guanine base and markedly stabilizes the Z conformation of short oligonucleotides of a variety of sequences under physiological salt conditions.

Results and Discussion

Although theoretical calculations suggested that methylation at the guanine C^8 position greatly stabilizes the Z conformation by favoring the *syn* glycosyl conformation,[12] such a property associated with m^8G-modified DNA has not been examined experimentally. While introduction of the bulky bromine atom at the C^8 position has been used previously,[10,11] the brominated DNA suffered the problem of chemical/photochemical instability. It would be desirable to use the more stable m^8G in DNA to investigated the molecular basis of a variety of Z conformation-specific reactions at the oligonucleotide level.

Scheme 1

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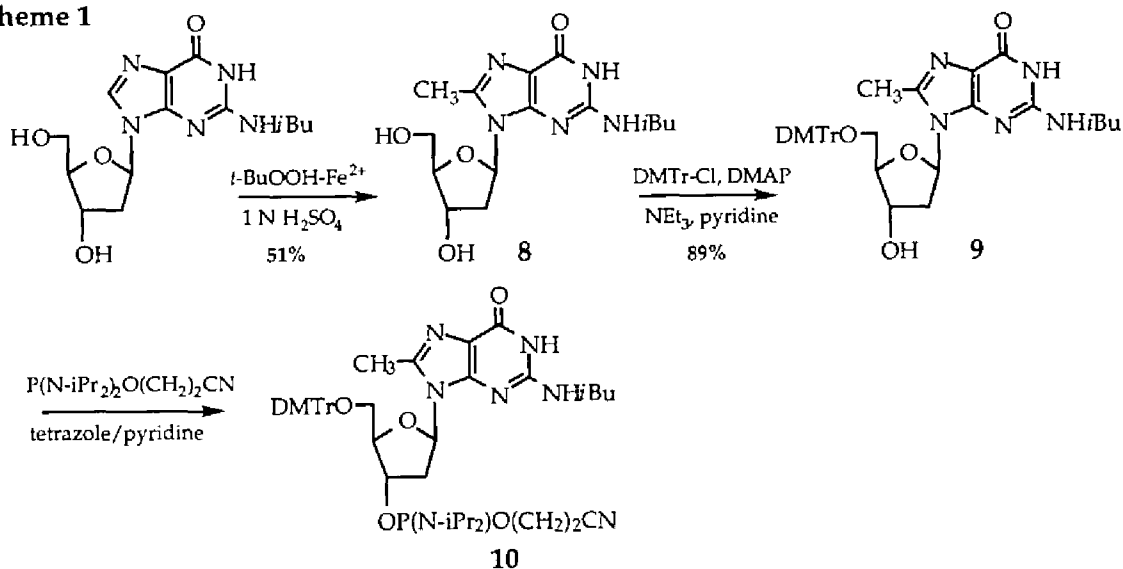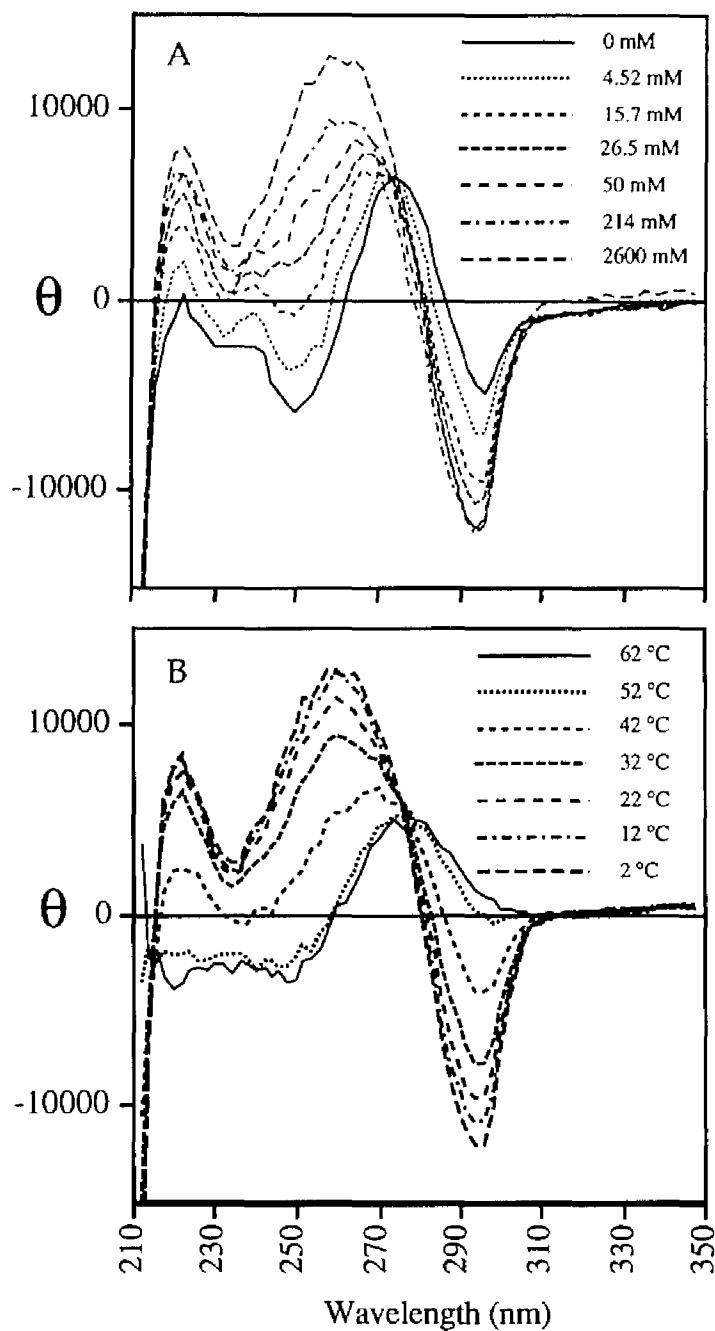
Figure 1. (A) CD spectra of d(Cm^{8}GCATm^{8}GCG) (**4**, 0.15 mM base concentration) in 5 mM Na cacodylate buffer, pH 7.0, at 10 °C at various NaCl concentrations. (B) CD Spectra of d(CGCm^{8}GCG) (**1**, 0.15 mM base concentration) in 5 mM Na cacodylate buffer, pH 7.0, under various temperatures.

The CD spectra of d(CGCm^{8}GCG)$_2$ (1) at different salt concentration are shown in Figure 1A at 10 °C.   The hexamer in a 50 mM NaCl solution has the characteristic CD spectrum of Z-DNA. Without added salt it is in the SS form, as judged by UV and CD spectroscopy, and is converted to the Z form by increasing salt concentration, with a mid-point at 45 mM NaCl.   Since the respective mid-point NaCl concentration for d(CGCGCG)$_2$ and d(m^{5}CGCGm^{5}CG)$_2$ are 2.6 M[13] and 2.0 M,[7] it is evident that the C^8-methylation of guanine greatly stabilizes the Z conformation.

NMR refinement of Z-DNA.   In order to unequivocally demonstrate that the structure of d(CGCm^{8}GCG)$_2$ (1) at 30 mM salt concentration is Z-DNA, NOE-restrained refinement has been carried out. 2D-NOESY and TOCSY in D$_2$O were used to assign the resonances of all non-exchangeable protons.   Since the structure is expected to be Z-DNA, as judged from the CD spectrum, the usual sequential assignment procedure would not be applicable.   Indeed, the aromatic-H$^{1'}$ and m^{8}G$_4$ methyl-H$^{1'}$ cross peak region of the 2D-NOESY spectrum (Figure 2) showed only strong intranucleotide G$_2$H$^{1'}$-G$_2$H^8, G$_4$H$^{1'}$-G$_4$Me and G$_6$H$^{1'}$-G$_6$H^8 cross-peaks, indicative of the *syn* conformation of guanine residues.   As has been noted before,[6,14] there is no internucleotide connectivity in Z-DNA, in contrast to that in right-handed B-DNA.   The assignment was subsequently extended to the aromatic-H$^{2'}$/H$^{2''}$ region and finally to all regions of the spectrum.   The TOCSY data supported the assignment (data not shown).

The chemical shifts of all resonances are tabulated in Table 1. Note that all cytidine H$^{2'}$ and H^5 resonances are unusually upfield (~1.7 and 2.6 p.p.m. respectively), analogous to those seen before.[6,14] The upfield shifts are due to the orientation of the sugar moiety of the dC nucleotide in Z-DNA, which places the H$^{2'}$ and H^5 protons

Table 1. Chemical Shifts (p.p.m.) for $d(CGCm^8GCG)_2$ at 2 °C

	H5/Me	H8/6	H1'	H2'	H2"	H3'	H4'	H5'	H5"	H1	H2a/4a	H2b/4b
C1	5.76	7.45	5.81	1.63	2.40	4.57	3.65	2.54	3.12		8.31	7.05
G2		7.75	6.22	2.77	2.77	5.07	4.19	4.14	4.11	13.24	8.36	6.52
C3	5.07	7.39	5.74	1.70	2.60	4.80	3.80	2.61	3.78		8.38	6.51
m⁸G4	2.54		6.28	2.79	2.79	5.00	4.19	4.16	4.13	13.17	8.26	6.49
C5	5.20	7.47	5.90	1.75	2.64	4.82	3.90	2.69	3.82		8.52	6.60
G6		7.79	6.25	3.22	2.44	4.85	4.20	4.33	4.11	13.38	na	na

H2a and H4a are base-pair hydrogen bonded amino protons, H2b and H4b are not.

directly under the ring current of the neighboring 5'- and 3'-dG guanine bases, respectively.
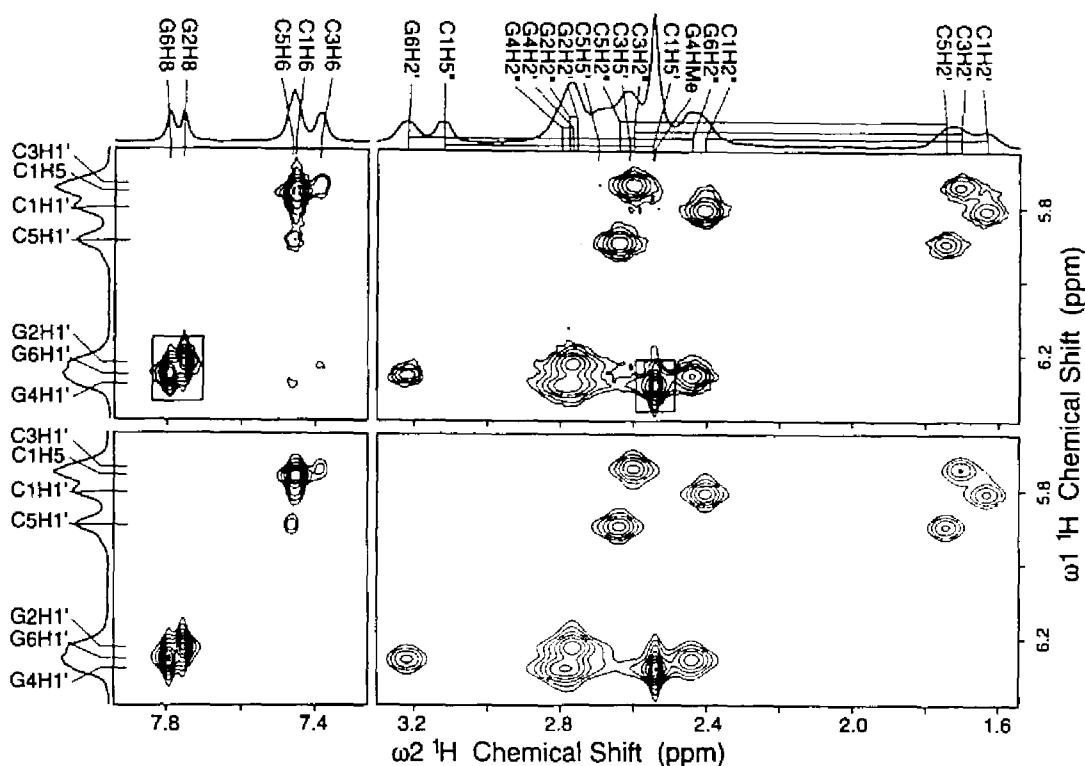
Figure 2. The experimental and simulated 2D NOESY spectra of the $H^{1'}$/aromatic/$H^{2'}$/methyl region of the native d(CGCm^{8}GCG)$_2$ at 2 °C. The refined NMR R-factor was 15.2%. Note the strong GH$^{1'}$-GH^8/GMe^8 cross-peaks due to the *syn* conformation and C$_3$H^5 C$_5$H^5 are unusually upfield shifted to 5.07 and 5.20 p.p.m. (not shown in this figure) respectively, due to the ring current influence of the guanine base on the 3' side in Z-DNA.

All our data point to the inevitable conclusion that d(CGCm^{8}GCG)$_2$ has a structure consistent with Z-DNA. We constructed a model of the d(CGCm^{8}GCG)$_2$ **1** by appropriate methylation of the Z-DNA d(CGCGCG)$_2$ crystal structure[15] and subjected it to a combined SPEDREF[16] and NOE-constrained refinement.[17] We measured 710 NOE integrals as the input for the

NOE-restrained refinement.   The refined structure, which has an NMR R factor of 15.2%, is shown in Figure 3.   The NOE-refined structure is very similar to the d(CGCGCG)$_2$ Z-DNA structure determined by X-ray crystallography.[15]   The r.m.s. difference between the two structures is only 0.6 Å.   The cytidine residues are in the $anti$/C2'-$endo$ conformation, whereas the guanosine residues are in the syn/C3'-$endo$ conformation (except for the 3'-terminal guanosines, which have a mixed C2'-$endo$/C3'-$endo$ sugar pucker). In the m^{8}G-modified Z-DNA structure the hydrophobic C^8-methyl groups are located in the periphery of the helix and prominently exposed to the solvent region.   In contrast, in the m^{5}C-modified Z-DNA structure the C^5-methyl groups form hydrophobic patches in the small recessed area of the concave 'major groove' (Figure 3). The simulated NOESY spectra based on the refined model agree with the observed data (Figure 2).   To the best of our knowledge this is the first example of a refined structure of Z-form DNA by NMR under physiological salt conditions without added organic solvent or divalent cation.

Dynamics of Z-DNA.   Z-DNA has been shown to have unusual rigidity.[2]   The measured T$_1$ relaxation inversion recovery time (T$_1$IR) of 2.7 s for the d(CGCm^{8}GCG) helix supports this notion. For a B-DNA hexamer the averaged T$_1$IR is ~1.7 s.   The stiffness of the Z-DNA double helix is also reflected in the remarkably slow exchange rate of its various exchangeable protons, including the G imino, G amino and C amino protons.   It has been shown that the G imino and C amino protons exchange with water in 30 and 50 min, respectively, whereas the G amino protons exchange in 330 min, at 5 °C and pH 7.[18]   Our ability to obtain a stable Z-DNA structure

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under physiological conditions affords a unique opportunity to investigate the behavior of the exchangeable protons.

The exchangeable proton NMR spectrum in H_2O (Figure 4) revealed three clear imino proton resonances at 13.17 (G_4), 13.24 (G_2) and 13.38 (G_6) p.p.m., suggesting Watson-Crick-type base pairs. The assignment was aided by the 2D NOESY cross-peaks between the imino protons and the other protons (Figure 4). The cross-peaks associated with the exchangeable protons are again consistent with Z-DNA. For example, we note that C_5-NH^4 amino protons have cross-peaks (peaks a and b) to the C_1-H^5 proton. Such cross-peaks can only happen between the two interstrand cytosines in the $C_{1p}G_2$:$C_{5p}G_6$ step of the Z-DNA hexamer, due to its extreme sheared base pair stacking pattern.
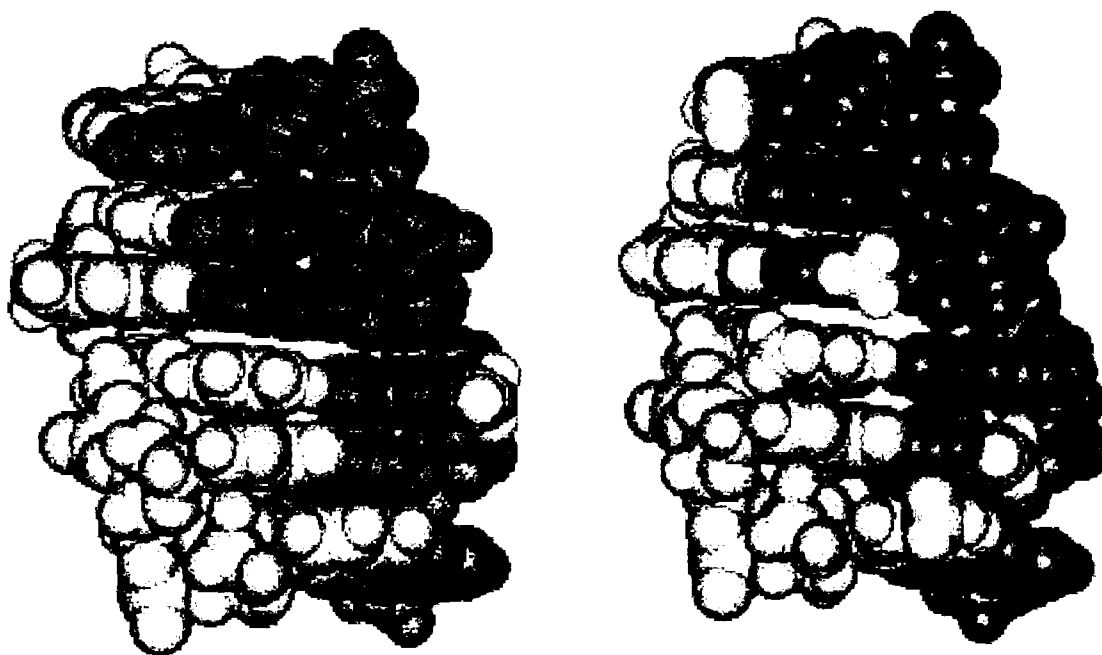
Figure 3. The refined model for d(CGCm^{8}GCG)$_2$ Z-DNA structure (left) and the model of d(m^{5}C-G)$_3$ right.

Note that cross-peaks between the geminal G amino protons are also observed, despite their broad resonances. The chemical shifts of the G amino protons (~8.4 and ~6.5 p.p.m.) are the same as those observed in $(C \cdot G)_{12}$ at 5 °C.[18] The fact that we observed two separate resonances for each G amino group suggests that the rotation around the C^2-N^2 bond of G in Z-DNA is slower than B-DNA on the NMR time scale. This is likely due to the *syn*-G conformation, which allows the N^2 amino group to hydrogen bond with the phosphate oxygen either directly or through bridging water molecules.

Kochoyan *et al.*[18] have determined that the base pair lifetime for Z-DNA is ~3 s at 5°C. We have measured the half-time ($t_{1/2}$) of the exchange process for the imino protons and obtained values of 60, 300 and 700 ms respectively for G_6, G_4 and G_2. It is clear that at the hexamer level the exchange rate of imino protons in a solution containing only 40 mM phosphate buffer, pH 7.0, is significantly faster than the base pair lifetime.
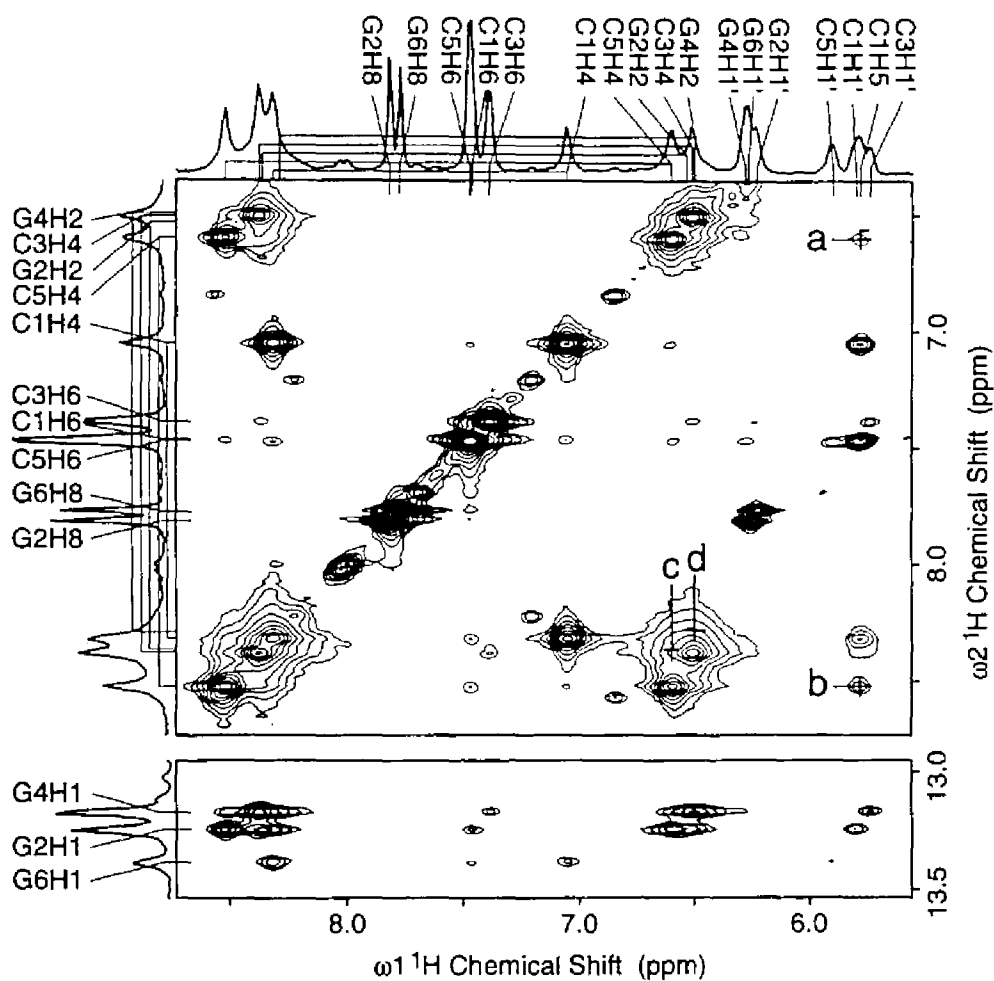
Figure 4. The exchangeable proton 2D NOESY spectra of the H^1-aromatic region of $d(CGCm^8GCG)_2$ at 2 °C. There are clear NOE cross-peaks (peaks a and b) between C_1H^4 amino protons and C_5H^5 protons from the opposite strand. Such cross-peaks can only happen in a Z-DNA structure. Note that the guanine N^2 amino geminal protons have a broad cross-peak (peaks c and d).

Thermodynamic properties.   The effect of m^8G substitution on the thermodynamic stability of the Z conformation was examined by measuring the proportions of the Z, B, and SS forms at various temperatures.   Figure 1B shows the CD spectra of $d(CGCm^8GCG)_2$ (1) in 2.6 M NaCl solution at various temperatures.   At 2°C it is nearly 100% Z-DNA.   The proportion of B increased with increasing temperature. For comparison, $d(CGCGCG)_2$ under the same salt conditions consisted of a 1:1 mixture of B and Z.   The proportions of Z, B, and SS for a m^8G-containing oligomer were determined at various temperatures by means of CD and UV spectroscopy as previously reported.[19]   A similar temperature-dependent B:Z equilibrium has also been observed for $d(CGCm^8GCG)_2$ at 30 mM salt concentration by NMR spectroscopy as shown in Figure 5.

2°	7°	12°	17°	22°
6.2%	8.1%	10.8%	14.4%	20.8%
8.0 7.7	8.0 7.7	8.0 7.7	8.0 7.7	8.0 7.7

^1H Chemical Shift (ppm)
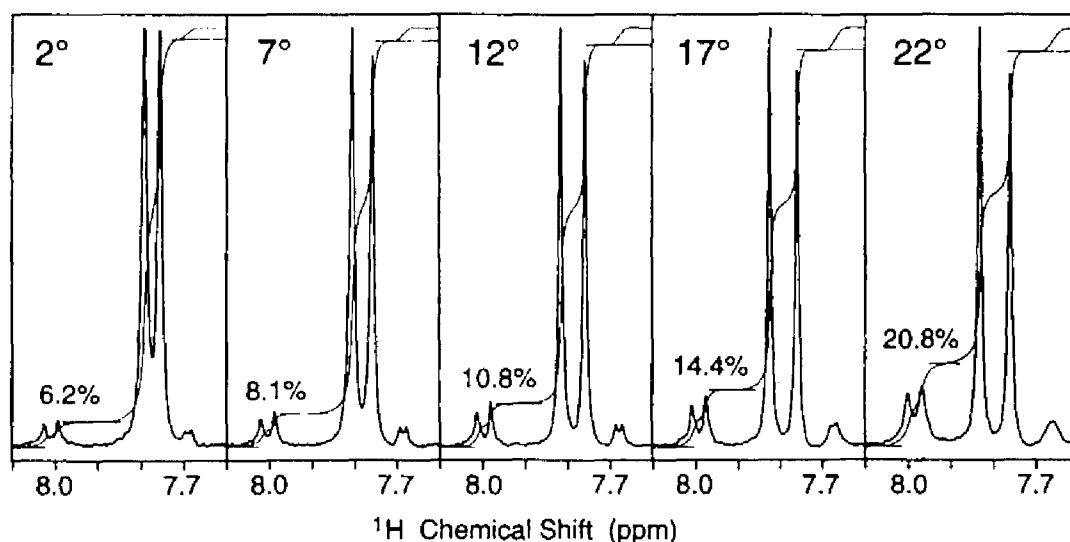
Figure 5. Proton 1D NMR spectra showing the temperature-dependent equilibrium of the B-Z transition as monitored by the G_2H^8 (7.75 p.p.m. at 2 °C) and G_6H^8 (7.79 p.p.m. at 2 °C) protons.   The population of the B form increases from 6.2% at 2 °C to 20.8% at 22 °C.

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The results for 1 and d(CGCGCG) at 2.6 M NaCl as a function of temperature are shown in Figure 6a and b. The thermodynamic parameters for the Z-B transition of the hexamer were determined from the data below 27 °C, where >97% of the hexamers are in either the Z or B form.

A van't Hoff plot for the Z-B transition of $d(CGCGCG)_2$ and $d(CGCm^8GCG)_2$ and the resulting ΔH and ΔS are shown in Figure 6c and Table 2 respectively. It is evident that the large stabilization of the Z conformation by introducing a methyl group at the guanine C^8 position is enthalpic in origin and that the methyl substitution stabilizes the Z form by at least 0.8 kcal/mol, which roughly corresponds to half of the reported free energy ($\sim$1-2 kcal/mol)[20-22] required to shift the equilibrium to the *syn* conformation of C^8-substituted deoxyguanosines. A similar extent of stabilization of *syn* conformation by incorporating 8-bromodeoxyguanosine into oligomers has recently been reported for a G quartet structure.[23]

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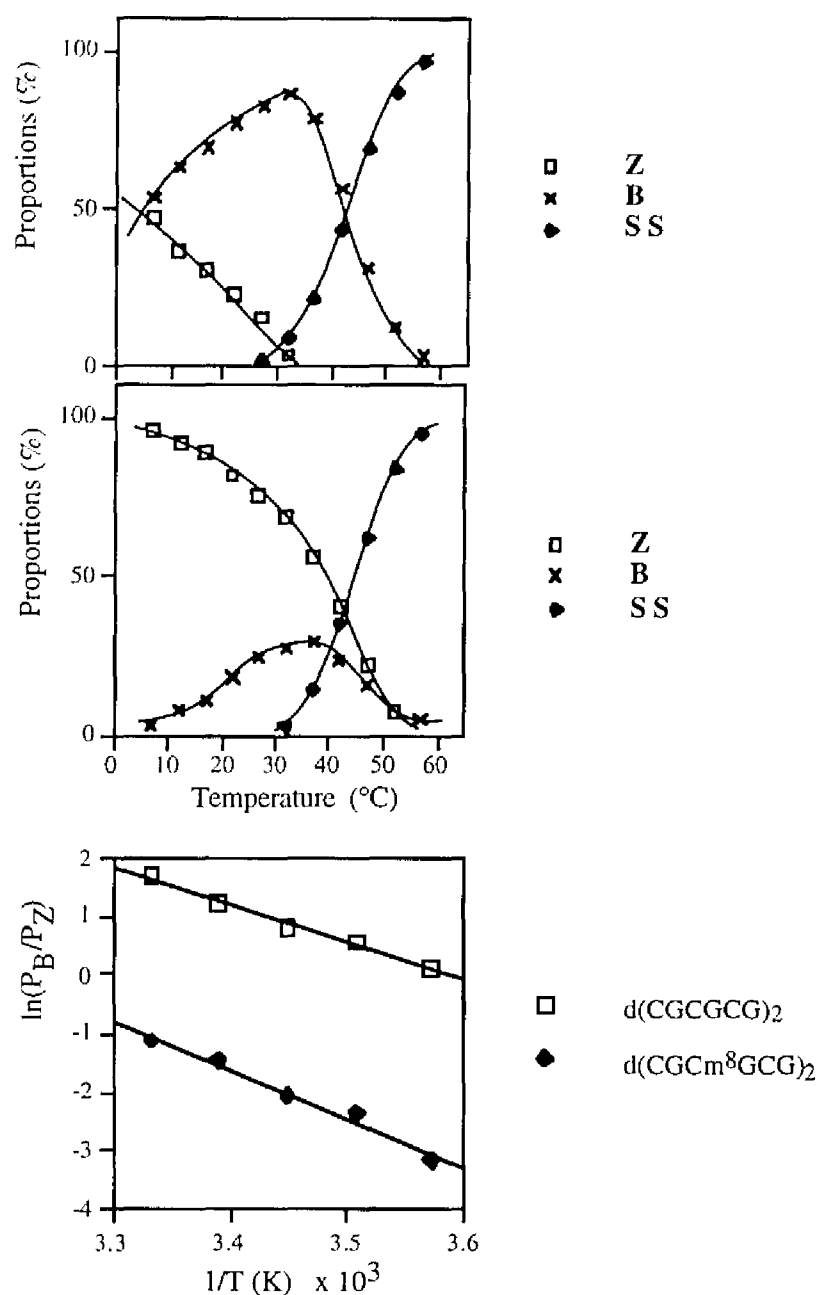
Figure 6. Proportions of Z, B, and SS conformations of (a) d(CGCGCG) and (b) d(CGCm⁸GCG) (1) as a function of temperature. Sample solutions contained 0.15 mM hexanucleotide (base concentration) in 2.6 M NaCl, 5 mM Na cacodylate buffer, pH 7.0. Proportions of Z, B, and SS were obtained by a combination of UV and CD spectroscopy. (c) van't Hoff plot for the Z-B conformational transition of d(CGCGCG) and d(CGCm⁸GCG) (1) obtained from the optical data.

Table 2. Thermodynamic Parameters for Z-B Transition of $d(CGCm^8GCG)_2$ (**1**) and $d(CGCGCG)_2$ at 2.6 M NaCl[a]

oligonucleotide	ΔG^{297K} (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (eu)
$d(CGCm^8GCG)_2$(**1**)	0.8	16.7 ± 1.0	53.7 ± 4.8
$d(CGCGCG)_2$	-0.8	12.8 ± 0.7	45.8 ± 2.3

[a] Z-B conformational transition was analyzed by two-state model from the data below 27 °C. Thermodynamic parameters were obtained by plotting ln(fraction Z / fraction B) versus 1/T.

When A·T base pairs are inserted into alternating $(C·G)_n$ sequences the B-Z transition is known to become more difficult.[3,4,24] For instance, the 5-methylcytosine (m^5C)-containing octadecamer $d(G-m^5C)\ A^{Br}U(G-m^5C)_4$ (**2**) was found to retain the typical B form even at 4.0 M NaCl. Thus, we examined the properties of various types of m^8G-containing oligomers having an A·T base pair in order to evaluate Z form stabilization induced by incorporation of m^8G (Table 3). In general the Z conformation was further stabilized by increasing the number of m^8G incorporated and destabilized by incorporating *syn*-A and *syn*-T. The CD spectrum of $d(Cm^8GCATm^8GCG)_2$ (**3**) indicates that this oligomer is converted from coil to Z with a mid-point at 45 mM NaCl. Oligomer **4**, which is obtained by replacing the terminal G·C base pairs of **3** with A·T base pairs, maintained the Z conformation with a mid-point at 470 mM

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Table 3. Midpoint NaCl Concentration in B-Z Transition of Various 8-Methylguanine-Containing Oligonucleotides

oligonucleotide[a]	number of residue			NaCl
	m^8G	*syn* A[b]	*syn* T[c]	(mM)
d(CGCm^{8}GCG)$_2$ (1)	2	0	0	30[d]
d(CGCGCG)$_2$	0	0	0	2600[e]
d(^{5}mCGCG^{5}mCG)$_2$	0	0	0	2000[f]
d[(Gm^{5}C)$_4$A^{Br}U(Gm^{5}C)$_4$]$_2$ (2)	0	0	0	-
d(Cm^{8}GCATm^{8}GCG)$_2$ (3)	4	2	0	45[d]
d(Tm^{8}GCATm^{8}GCA)$_2$ (4)	4	4	0	470
d(Cm^{8}GCATm^{8}GTG) (5) d(GCGTACAC)	2	3	0	2450
d(Cm^{8}GCTCm^{8}GCG) (6) d(GCm^{8}GAGCm^{8}GC)	4	0	1	120

[a] m^8G = 8-Methyl-2'-deoxyguanosine, m^5C = 5-methyldeoxycytidine; [b] *syn*-A conformation; [c] *syn*-T conformation; [d] transition from single strand; [e] reference 13; [f] reference 7. The data were taken at 10 °C.

NaCl. The incorporation of m^8G into only one strand is also capable of stabilizing the Z conformation considerably (oligomer 5). A non-alternating pyrimidine-purine sequence has been shown to destabilize the Z conformation due to the energetically disfavored *syn* conformation of pyrimidine nucleosides.[3,4] One of the central G·C base pair of d(CGCGCGCG)$_2$ can be replaced by a T·A base pair without significantly increasing the mid-point NaCl concentration, if the

duplex incorporates two m^{8}G into each strand (oligomer 6).   Such a low salt concentration requirement of 120 mM for an imperfect Z-DNA (out-of-alternation pyrimidine-purine sequence) is remarkable. Our results suggest that we can now study many heretofore inaccessible DNA conformations involving Z-DNA, e.g. the B-Z junction and the Z-Z junction.

Conclusion

The substitution of a methyl group at the guanine C^8 position dramatically stabilizes the Z conformation of short oligonucleotides of a variety of base sequences.   Some of these m^{8}G-modified oligomers exist as a stable Z form under physiological salt conditions without added organic solvent or divalent metal.[3-7]   While significant information on specific chemical reactions for DNA local structures has been accumulated during the past several years,[25-28] considerably less is known about the origin of these specificities. Incorporation of the m^{8}G moiety into DNA oligomers could be a powerful tool to examine the molecular basis for many types of Z conformation-specific reactions at the oligomer level under physiological salt conditions.

Experimental Section

Materials and methods. Pyridine and acetonitrile (HPLC grade) were dried over calcium hydride. 2'-Deoxyguanosine was purchased from YAMASA Corporation. Nucleoside β-cyanoethylphosphoramidite reagents (A, G, C, T) were obtained from Applied Biosystems. Calf intestine alkaline phosphatase (AP, 1000 unit/mL) and snake venom phosphodiesterase (s.v. PDE, 3 unit/mL) were purchased from Boehringer Mannheim. Silica gel column chromatography was carried out on Wakogel C-200. Thin layer chromatography was carried out on a Merck silica gel 60 PF_{254} plate. N-Isobutyryl-2'-deoxyguanosine was prepared according to the published procedure.[29] ^1H NMR spectra were recorded on a JEOL-JNM-GX400 spectrometer and a Varian-Gemini 200 spectrometer. FAB mass were obtained on a JEOL-JMS-SX102A.

Synthesis of 8-methyl-2'-deoxyguanosine 7. Introduction of methyl group at C8 position of guanine was performed by free radical methylation method.[30] To a solution of 2'-deoxyguanosine (500 mg, 1.89 mmol) and $FeSO_4 \cdot 7H_2O$ (2.1 g, 7.48 mmol) in 100 mL of 1 N H_2SO_4, an aqueous solution (100 mL) containing 0.81 mL of 70% t-butyl hydroperoxide (5.81 mmol) was added dropwise over a period of 5 min. After stirring at 0 °C for 40 min, the reaction mixture was neutralized with saturated KOH solution. The resulting brownish solution was filtered off and the filtrate was concentrated to dryness. The residue was triturated with 60 mL of 28% NH_4OH and 150 mL of methanol and filted. The filtrate was concentrated to dryness and subjected to silica gel column chromatography. Elution with 28% NH_4OH-methanol (2 : 8) afforded 7 as a white powder: yield 176 mg (34 %). mp 230 °C (dec.); ^1H NMR (D_2O, 400 MHz) δ 2.20 (ddd, 1 H, J = 14.0, 6.4, 2.6 Hz, 2"), 2.35 (s, 3 H, -8CH₃), 2.84 (ddd, 1 H, J = 14.0, 8.0, 6.4 Hz, 2'), 3.69 (dd, 1 H, J = 12.6, 4.3 Hz, 5'), 3.75 (dd, 1 H, J = 12.6, 3.1 Hz,

5''), 3.98 (ddd, 1 H, J = 4.3, 3.4, 3.1 Hz, 4'), 4.53 (ddd, 1 H, J = 6.4, 3.4, 2.6 Hz, 3'), 6.13 (dd, 1 H, J = 8.0, 6.4 Hz, 1'); FABMS (positive ion) m/z 282 $(M+H)^+$; UV (H_2O) 252.2 nm (ε 12,700).

Synthesis of 8-methyl-N-isobutyryl-2'-deoxyguanosine 8. To a solution of N-isobutyryl-2'-deoxyguanosine (1.0 g, 2.97 mmol) and $FeSO_4 \cdot 7H_2O$ (6.7 g, 24.1 mmol) in 160 mL of 1 N H_2SO_4, an aqueous solution (100 mL) containing 2.6 mL of 70% t-butyl hydroperoxide (19.0 mmol) was added dropwise over a period of 5 min. After stirring at 0 °C for 60 min, the reaction mixture was neutralized with saturated KOH solution. After centrifugation of the brown slushy mixture, the supernatant was separated. The supernatant was concentrated to dryness and the resulting brownish solid was triturated three times with 200 mL of methanol. The combined methanol solution was concentrated and the residue was subjected to silica gel column chromatography. Elution with CH_2Cl_2-methanol (9 : 1) afforded 8-methyl-N-isobutyryl-2'-deoxyguanosine 8 as a white powder: yield 527 mg (51%). 8-Methyl-N-isobutyryl-2'-deoxyguanosine: mp 195 °C (dec.); ^{1}H NMR (D_2O) δ 1.21 (d, 6 H, J = 6.9 Hz, -CH(CH$_3$)$_2$), 2.25 (ddd, 1 H, J = 13.7, 6.9, 3.6 Hz, 2''), 2.56 (s, 3 H, -8CH$_3$), 2.72 (sep, 1 H, J = 6.9 Hz, -CH(CH$_3$)$_2$), 3.17 (ddd, 1 H, J = 13.7, 7.4, 6.9 Hz, 2'), 3.72 (dd, 1 H, J = 11.9, 5.4 Hz, 5''), 3.74 (dd, 1 H, J = 11.9, 4.1 Hz, 5'), 3.92 (ddd, 1 H, 5.4, 4.1, 3.6 Hz, 4'), 4.59 (ddd, 1 H, J = 6.9, 3.6, 3.6 Hz, 3'), 6.32 (dd, 1 H, J = 7.4, 6.9 Hz, 1'); FABMS (positive ion) m/z 352 $(M+H)^+$.

Synthesis of 8-methyl-N-isobutyryl-5'-O-(dimethoxytrityl)-2'-deoxyguanosine 9. 8 (430 mg, 1.22 mmol) was dried by co-evaporation with dry pyridine three times. The residue was dissolved in 13 mL of dry pyridine, and then 632 mg (1.83 mmol) of

4,4'-dimethoxytrityl chloride (632 mg, 1.83 mmol), 4-dimethylaminopyridine (3.8 mg, 0.03 mmol) and triethylamine 257 μL (1.83 mmol) were added to the reaction mixture. The solution was stirred for 6 h at room temperature. The reaction mixture was concentrated, and the residue was extracted with ethyl acetate. White power was precipitated out from the organic layer and filtered off. Washing of the precipitate with ethyl acetate and n-hexane provide 9 as a white powder: yield 713 mg (89%). mp 180 °C (dec.); ^{1}H NMR (CD$_3$OD, 200 MHz) δ 1.15 (s, 6 H, J = 6.8 Hz, -CH(CH$_3$)$_2$), 1.19 (s, 3 H, J = 6.8 Hz, -CH(CH$_3$)$_2$), 2.29 (ddd, 1 H, J = 13.5, 7.2, 4.4 Hz, 2''), 2.55 (s, 3H, -8CH$_3$), 2.63 (sep, 1 H, J = 6.8 Hz, -CH(CH$_3$)$_2$), 3.17 (dd, 1 H, J = 10.1, 3.2 Hz, 5'), 3.25-3.45 (m, 2 H, 2', 5''), 3.72 (s, 3 H, -OCH$_3$), 3.73 (s, 3 H, -OCH$_3$), 4.09 (ddd, 1 H, J = 7.2, 3.4, 3.2 Hz, 4'), 4.64 (ddd, 1 H, J = 7.2, 4.4, 3.8 Hz, 3'), 6.32 (dd, 1 H, J = 7.2, 6.4 Hz, 1'), 6.63-6.73 (m, 4 H, aromatic), 7.11-7.36 (m, 9 H, aromatic); HR FABMS (positive ion) m/z 654.2972 (M+H, 645.2928 calcd for C$_{36}$H$_{40}$O$_7$N$_5$).

Synthesis of 8-methyl-N-isobutyryl-3'-O-[2-cyanoethoxy-(N,N-diisopropylamino)phosphino]-5'-O-(dimethoxytrityl)-2'-deoxyguanosine 10. 9 (500 mg, 0.76 mmol) was dried by co-evaporation with pyridine (three times) and redissolved in 5.7 mL of pyridine. To this solution were added 275 μL of 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.95 mmol) and 1.9 mL of 0.5 M tetrazole in acetonitrile and the mixture was stirred over night at room temperature under argon. The reaction mixture was poured into ice-cooled water and extracted with ethyl acetate. The organic layer was washed with saturated NaHCO$_3$ and dried over anhydrous Na$_2$SO$_4$ and concentrated. Crude 10 (580 mg) was used for automated DNA synthesizer without further purification. ^{1}H NMR (CD$_3$OD) δ 1.17 (d, 12 H, J = 6.5 Hz, -NCH(CH$_3$)$_2$), 1.26 (dd, 6 H, J =

6.8, 3.4 Hz, -CH($\underline{\text{CH}}_3$)$_2$), 2.41-2.64 (m, 3 H, 2', -NC$\underline{\text{H}}$(CH$_3$)$_2$), 2.57 (s, 3 H, -8CH$_3$), 2.63-2.77 (m, 1 H, -C$\underline{\text{H}}$(CH$_3$)$_2$), 2.69 (t, 1 H, J = 5.9, -OCH$_2$-), 2.84 (t, 1 H, J = 5.9, -OCH$_2$-), 3.28-3.54 (m, 3 H, 2', -CH$_2$CN), 3.72 (s, 3 H, -OCH$_3$), 3.73 (s, 3 H, -OCH$_3$), 3.54-3.71 (m, 2 H, 5', 5''), 4.07-4.34 (m, 1 H, 4'), 4.09 (ddd, 1 H, J = 7.2, 3.4, 3.2 Hz, 4'), 4.64-4.83 (m, 1 H, 3'), 6.36 (t, 1 H, J = 7.3 Hz, 1'), 6.60-6.80 (m, 4 H, aromatic), 7.06-7.44 (m, 9 H, aromatic); ^{31}P NMR (CD$_3$OD) δ 148.48; FABMS (positive ion) m/z 854 (M+H)$^+$.

Synthesis of deoxynucleotides 1-6. Oligonucleotides were prepared by the β-(cyanoethyl)phosphoramidite method on controlled pore glass supports (1 μmol) by using ABI 381 A DNA synthesizer. After automated synthesis, the oligomer was detached from the support by soaking in conc. aqueous ammonia for 1 h at room temperature. Deprotection was conducted by heating the conc. aqueous ammonia for 12 h at 55 °C. Aqueous ammonia was then removed by evaporation, and the crude oligomer was purified by reverse phase HPLC and lyophilized. Purity and concentration of all oligonucleotides were determined by complete digestion with s.v. PDE and AP to 2'-deoxymononucleosides.

NMR analysis. The NMR solution (1 mM duplex with 0.04 M phosphate buffer, thus 0.06 M Na$^+$, pH 7.0, in D$_2$O) of d(CGCm^{8}GCG)$_2$ was prepared using the established procedure.[16] NMR spectra were collected on a Varian VXR500 500 MHz spectrometer and processed with FELIX v1.1 on Silicon Graphics IRIS workstations. The temperature was controlled to be accurate within 0.01 °C. T$_1$ relaxation experiments were carried out with

the standard 180-t-90° inversion-recovery sequence and the average T_1 relaxation time was 2.7 s. The non-exchangeable proton 2D NOESY spectra were collected at 2°C with a mixing time of 100 ms and a total recycle delay of 7.0 s. The data were collected by the States/TPPI technique[31] with 512 t_1 increments and 2048 t_2 complex points each the average of 16 transients. Apodization of the data in the t_1 and t_2 dimensions consisted of 8 Hz exponential multiplication with half of a sine-squared function for the last fourth of the data to reduce truncation artifacts. Integrals from the non-exchangeable 2D NOE dataset were extracted by evaluation with the observed cross-peak shapes of each spin in the f_1 and f_2 dimensions. These shapes were determined by spectral analysis using the program MYLOR.[16] The exchangeable proton 2D NOESY experiment was carried out in 90% H_2O/10% D_2O solution using the 1-1 pulse sequence[31] as the read sequence, with a mixing time of 100 ms and a recycle delay of 2.7 s, each data point the average of 24 transients. The starting model was constructed by Midasplus (UCSF). Forty cycles of refinement of the starting model were then carried out by the sequence of procedures comprising the SPEDREF package.[16] This includes a full matrix relaxation calculation of the NOEs for the model with comparison of the experimental and simulated spectra to deconvolute overlapped areas of the spectra. Minimization of the residual errors within the program X-PLOR[17] was then performed using conjugate gradient minimization of the NOE-derived force-springs together with the chemical force field. A refined structure was obtained with the NMR R factor (Σ $|N_O - N_c|$/ΣN_o, where N_O and N_c are the experimental and calculated NOE cross-peak intensities respectively) is 15.2%. The optimal rotational correlation time was determined to be 6 ns using the procedure described before.[16] The coordinates and related molecular

constraints of the refined structure have been deposited in the Brookhaven Protein Databank (identifiers ITNE and RITNEMR).

CD measurements. Circular dichroism (CD) spectra were recorded on Jasco J-700 spectrophotometer equipped with a Peltier temperature controller. CD spectra of oligonuclotide solutions (0.1 mM duplex in 30 mM phosphate, pH 7.0) were recorded using 1 cm path length cell. CD spectra at different temperatures were recorded at intervals of 5 °C with a 1 min equilibration period.

Measurement of melting temperature. Thermal denaturation profiles were obtained with Jasco V-550 spectrophotometer equipped with a Peltier temperature controller. Absorbance of the samples was monitored at 260 nm from 2 to 80 °C with a heating rate of 1 °C/min. Experiments with a heating rate of 0.5 °C/min gave the same results, suggesting that thermodynamic equilibrium had been achieved. The data were normalized to percent denaturation. A linear least squares analysis of the data gave a slope of transition and the y-intercept, from which the melting temperature was calculated.

Analysis of the thermodynamic data. The proportions of Z, B, and singlestranded (SS) forms in a m^8G-containing oligomer were determined by means of CD and UV spectroscopy as reported.[16] Since the molar extinction coefficients of the B and Z forms of the hexamer were found to be approximately the same, the proportions of SS relative to that of B and Z at each temperature were estimated by UV melting experiments at 260 nm. The relative ratio of the amount of B and Z was determined by the CD ellipticity at 295 nm and by NMR (*vide infra*).

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Chapter 2

Effects of Cytosine C5 and Guanine C8 Methylation on the B- to Z-DNA Transition

Abstract

The effects of methylation at the cytosine C5 and guanine C8 positions on the B- to Z-DNA transition were investigated. Thermodynamic parameters for the B- to Z-DNA transition of the hexamer $d(CGCGCG)_2$, 5-methylcytosine (m^5C) containing $d(m^5CGCGm^5CG)_2$ and 8-methylguanine (m^8G) containing $d(CGCm^8GCG)_2$ were determined. It was clearly demonstrated that stabilization of the Z-conformation by introduction of a methyl group at the cytosine C5 position is of entropic origin, whereas the methyl group at the guanine C8 position is enthalpic in origin.

Introduction

It is now well established that DNA structure is polymorphic, and that many sequence-specific non-B-DNA conformations exist, often times in response to changes in the environmental conditions. These DNA local structures are suggested to play an important role in a number of transcriptional and replicative processes.[1] An understanding of how DNA conformations are affected by the various substituent groups of the nucleotide bases would help to extend our understanding of the various mechanism available to

control cellular functions of DNA local structures.   One of the most dramatic structural transition observed in DNA is that between right-handed B- and left handed Z-DNA.[2]   A variety of substituents are known to stabilizes the Z-conformation including methylation of cytosine C5 position[3] and bromination of guanine C8.[4]   In particular, 4-5 % of cytosine residues are methylated in human DNA, and it now appears that DNA methylation, which causes a stabilization of Z-conformation, plays an important part in gene expression during development.[5]   However, molecular mechanism of the effects of methyl groups on the stability of the Z-conformation is not well understood.   Recently we have reported that a methyl group at the guanine C8 position dramatically stabilizes the Z conformation of short oligonucleotides with a variety of sequences.[6]   In this chapter, we focused on the thermodynamic effects of the guanine C8 and cytosine C5 methyl substituent on the B- to Z-DNA transition (Figure 1).

Results and Discussion

As Z-conformation is more favorable at low temperature, B- to Z-DNA transition was demonstrated to accompanied by favorable increase in enthalpy and unfavorable decrease in entropy.   For example, the enthalpy and the entropy for B- to Z-DNA transition of $d(CGCGCG)_2$ at NaCl 2.6 M condition have been determined to be $\Delta H = -12.8$ kcal mol^{-1} and $\Delta S = -46$ eu mol^{-1}, respectively (Table 1).[6] Although the origin of enthalpic stabilization is not known, it may arise from base stacking or hydrogen bonding between the guanine N^2 amino group and the phosphate oxygen through bridging water molecules.[6]   Unfavorable change in entropy may assigned to the

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stiffness of Z-DNA or otherwise to the relatively favorable solvation in B-DNA.
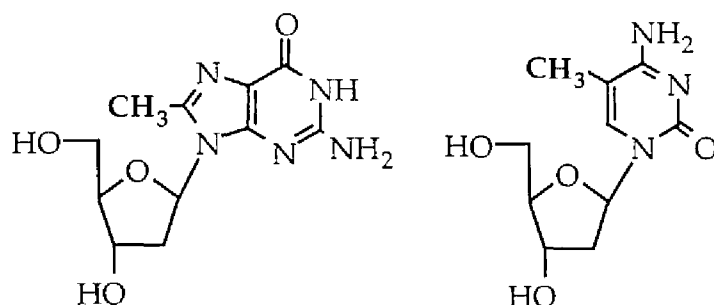
Figure 1. Structure of 8-methylguanine (m^{8}G) left and 5-methylcytosine (m^{5}C) right.

Table 1. Thermodynamic Parameters for B-Z Transition of d(CGCGCG)$_2$, d(m^{5}CGCGm^{5}CG)$_2$ and d(CGCm^{8}GCG)$_2$ at 2.6 M NaCl[a]

oligonucleotide	ΔG^{297K} (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (eu)
d(CGCGCG)$_2$	0.8	-12.8 ± 0.7	-45.8 ± 2.3
d(m^{5}CGCG m^{5}CG)$_2$	-0.15	-12.8 ± 0.4	-42.6 ± 1.5
d(CGCm^{8}GCG)$_2$	-0.8	-16.7 ± 1.0	-53.7 ± 4.8

[a]B-Z conformational transition was analyzed by two-state model from the data below 27 °C. Thermodynamic parameters were obtained by plotting ln(fraction Z / fraction B) versus 1/T.

C5-methylation of cytosine have been shown to stabilize the Z conformation.[3] This stabilization effect of methylation was suggested to result from the methyl group actually filling in a hydrophobic pocket at the major groove.[2,7] However, previous works revealed only little about the entropy component of the free

energy of B- to Z-DNA transition and therefore precise molecular mechanism of the effects of methyl groups on the stability of the Z-conformation is not well understood.[7]   Therefore, we studied the thermodynamics of B- to Z-DNA transition of d(m^{5}CGCGm^{5}CG)$_2$ at NaCl 2.6 M conditions and compared with unmodified d(CGCGCG)$_2$. While ΔH values for these two hexamer were proven to be almost the same, significant increase in the entropy was observed for d(m^{5}CGCGm^{5}CG)$_2$.   This result clearly demonstrated that the stabilization of Z-conformation by cytosine C5 methylation is of entropic origin.

On the other hand, thermodynamic analyses of B- to Z-DNA transition of d(CGCm^{8}GCG)$_2$ at NaCl 2.6 M conditions indicated that the stabilization effects of guanine C8 methyl group results from a large favorable decrease in enthalpy (ΔH=-3.9 kcal mol^{-1}) and an unfavorable significant decrease in entropy (ΔS=-11.1 eu mol^{-1}). The enthalpic origin of 1.9 kcal/mol by each methyl group at the guanine C8 position corresponds to the reported free energy (1-2 kcal mol^{-1}) required to shift the equilibrium to the *syn* conformation of C8-substituted deoxyguanosines.[8]

The contribution of solvent interactions to the stability of alternative DNA conformations has been previously discussed in qualitative terms.   A useful semiempirical approach to estimating the contribution of solvent interactions on molecular structures has been to calculate the solvent accessible surface (SAS) of the molecule and of its component parts.[7]   The SAS of the hexamer d(CGCGCG)$_2$ were calculated in their canonical B- and Z-DNA conformations. Analogous sets of B-form and Z-form coordinates of d(m^{5}CGCGm^{5}CG)$_2$ and d(CGCm^{8}GCG)$_2$ were generated by adding standard geometry methyl group to the four cytosine bases at the C5 position, and to two guanine bases at the C8 position respectively.

The calculated SAS of the three hexamers in their B- and Z-DNA conformations are summarized in Table 2 and   Figure 2.   The difference in exposed surfaces of the hexamers in B- versus Z-DNA is higher for $d(m^5CGCGm^5CG)_2$ (79 $Å^2$) than for $d(CGCGCG)_2$ (37 $Å^2$). This increase of SAS is interpreted as increase in the number of released water molecules which is consistent with the entropy driven stabilization of Z-conformation   by cytosine C5 methylation. However, in the case of guanine C8 methyl groups, the difference in SAS between B- and Z-form becomes significantly small, which is reflected in an unfavorable change in entropy.

Table 2. Calculated Area of Solvent Accessible Surface (SAS) of $d(CGCGCG)_2$, $d(m^5CGCGm^5CG)_2$ and $d(CGCm^8GCG)_2$ in their B- and Z-form Conformations[a]

oligonucleotide	B-form ($Å^2$)	Z-form ($Å^2$)	ΔSAS_{B-Z}
$d(CGCGCG)_2$	1775	1738	37
$d(m^5CGCG\,m^5CG)_2$	1838	1759	79
$d(CGCm^8GCG)_2$	1788	1784	4

[a]Solvent accessible surface area was calculated according to the reported method.[9]

B   Z   B   Z   B   Z
                    1838 $Å^2$
1775 $Å^2$ 1788 $Å^2$ 1784 $Å^2$

                                   1759 $Å^2$
         1738 $Å^2$

ΔSAS = 37 $Å^2$ ΔSAS = 79 $Å^2$ ΔSAS = 4 $Å^2$

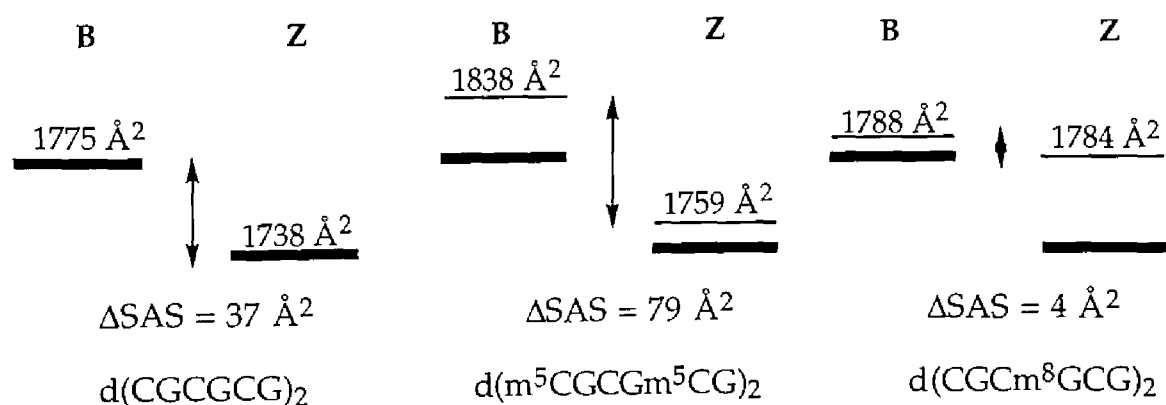$d(CGCGCG)_2$ $d(m^5CGCGm^5CG)_2$ $d(CGCm^8GCG)_2$

Figure 2. Simplified diagram of calculated surface areas.

Ho *et al.* have pointed out that potentation of Z-DNA by cytosine methylation is a solvent effect. They have calculated the solvent free energies of DNA structures by converting each surface type to a free energy by applying an atomic solvation parameter that describes energy required to transfer that surface type from an organic phase to an aqueous solvent phase.[7] According to their method, increase in SAS of 42 Å^2 and decrease in 33 Å^2 were estimated to be -1.8 kcal mol^{-1}, and 1.4 kcal mol^{-1} respectively, which roughly agreed with our experimental results in this chapter.

Conclusion

The availability of this type of experimentally derived structural and thermodynamic information on the stabilities of sequences with various modifications as B- *versus* Z-DNA makes this transition an excellent system for studying the contribution of various thermodynamic forces on macromolecular conformations. In this chapter, the overall stabilizing effect of the methyl group was demonstrated to be quite different for cytosine C5 from that for guanine C8. The stabilization of Z-conformation by a cytosine C5 methyl group is primary of entropic origin that arises from the increase in the solvent accessible surface area in the B- to Z-DNA transition, while methylation at guanine C8 enthalpically stabilize the Z-conformation as a consequence of stabilization of *syn* conformation.

Experimental Section

Materials and methods. Pyridine and acetonitrile (HPLC grade) were dried over calcium hydride. 2'-Deoxyguanosine was purchased from YAMASA Corporation. Nucleoside β-cyanoethylphosphoramidite reagents (A, G, C, T, m^{5}C) were obtained from Applied Biosystems. Cyanoethyl phosphoramidite of 8-methyl-2'-deoxyguanosine was prepared by the reported procedure.[6] Calf intestine alkaline phosphatase (AP, 1000 unit/mL) and snake venom phosphodiesterase (s.v. PDE, 3 unit/mL) were purchased from Boehringer Mannheim.

Synthesis of deoxyhexanucleotides. Oligonucleotides were prepared by the β-(cyanoethyl)phosphoramidite method on controlled pore glass supports (1 μmol) by using ABI 381 A DNA synthesizer. After automated synthesis, the oligomer was detached from the support by soaking in conc. aqueous ammonia for 1 h at room temperature. Deprotection was conducted by heating the conc. aqueous ammonia for 12 h at 55 °C. Aqueous ammonia was then removed by evaporation, and the crude oligomer was purified by reverse phase HPLC and lyophilized. Purity and concentration of all oligonucleotides were determined by complete digestion with s.v. PDE and AP to 2'-deoxymononucleosides.

CD Measurements. Circular dichroism (CD) spectra were recorded on Jasco J-700 spectrophotometer equipped with a Peltier temperature controller. CD spectra of oligonuclotide solutions (0.1 mM duplex in 2.6 M NaCl, 5 mM Na cacodylate, pH 7.0) were recorded using 1 cm path length cell. CD spectra at different

temperatures were recorded at intervals of 5 °C with a 1 min equilibration period.

Measurement of melting temperature. Thermal denaturation profiles were obtained with Jasco V-550 spectrophotometer equipped with a Peltier temperature controller. Absorbance of the samples was monitored at 260 nm from 2 to 80 °C with a heating rate of 1 °C/min. Experiments with a heating rate of 0.5 °C/min gave the same results, suggesting that thermodynamic equilibrium had been achieved. The data were normalized to percent denaturation. A linear least squares analysis of the data gave a slope of transition and the y-intercept, from which the melting temperature was calculated.

Analysis of the thermodynamic data. The proportions of Z, B, and singlestranded (SS) forms in a m^8G-containing oligomer were determined by means of CD and UV spectroscopy as reported.[16] Since the molar extinction coefficients of the B and Z forms of the hexamer were found to be approximately the same, the proportions of SS relative to that of B and Z at each temperature were estimated by UV melting experiments at 260 nm. The relative ratio of the amount of B and Z was determined by the CD ellipticity at 295 nm.

Calculation of Surface Areas. The method involves first building models for hexanucleotide sequences in either the B- or the Z-conformations. The atomic coordinates of sequences as B-DNA were generated using standard helical parameters for B-DNA. The

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atomic coordinates of the same sequences as Z-DNA were generated from the crystal structures of previously crystallized sequences. Models of m^{5}C and m^{8}G containing sequences were constructed by adding a sp^3 methyl group at the C5 carbon of a cytosine base and at the C8 carbon of guanine base respectively, using standard distances and geometries. The solvent accessible surface areas of the DNA models were calculated by using the Connally rolling ball method probe radius of 1.4 Å.[9]

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Chapter 3

Stabilization of Hoogsteen Base-Pairing by Introducing NH$_2$ Group at the C8 Position of Adenine

Abstract

The synthesis and thermodynamic properties of 8-aminodeoxyadenosine (8-amdA)-containing oligonucleotides has been described.   In order to examine the effects of 8-amdA on Hoogsteen base pair formation, we investigated the stabilities of 8-amdA-containing triplexs.   It was found that introduction of 8-amino group leads to the increase in Tm of third strand dissociation. The results indicate that the incorporation of an amino group at the C8 position of adenine greatly stabilized the Hoogsteen base pairing in triplex formation.

Introduction

In addition to the standard Watson-Crick type hydrogen bonding, nucleoside bases can recognize its complementary bases by Hoogsteen-type base pairing,[1] which is typically known to take place in a triplex between the purine strand of the Watson-Crick base pair and a pyrimidine third strand.[2]   A variety of base modifications was demonstrated to stabilize the Hoogsteen base pairing in a triplex, but most modifications were made on the third strand of the triplex for their potential therapeutic application.[3]   Since ^{1}H-NMR studies on

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8-aminodeoxyadenosine (8-amdA) and thymidine (dT) derivatives have shown that they effectively form Hoogsteen base pairs in $CDCl_3$ through an additional H-bonding between the 8-amino group of 8-amdA and the C2 carbonyl of dT,[4] the introduction of an amino group of dA on the purine strand is expected to stabilize Hoogsteen base pairing as shown in Figure 1. In this chapter, we describe that introduction of an amino group at the C8 position remarkably stabilizes the Hoogsteen-type base pairing in triplex DNA.
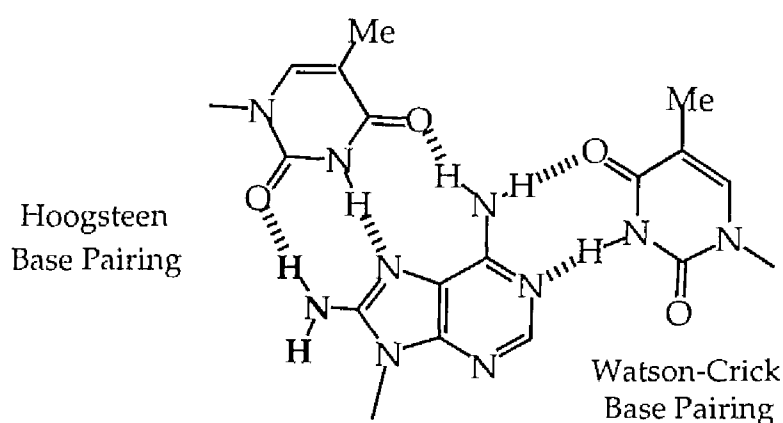
Figure 1. Base triplet T:8-amdA•T.

Results and Discussion

The 8-amdA-containing deoxyoligonucleotides (ODNs) were synthesized according to the standard phosphoramdite chemistry on a DNA synthesizer. The phosphoramidite of protected 8-amdA (9) was prepared in 8 steps from deoxyadenosine as shown in Scheme 1. Introduction of an amino group was performed by nucleophilic displacement of 8-bromoadenosine with NaN_3 followed by hydrogenation according to the reported method.[5] After protecting the N6 and N8 amino groups as N,N-dimethylformamidine derivative, the *tert*-butyldimethylsilyl groups were removed by

treatment with KF in DMF. Standard dimethoxytritylation and phosphophitylation of 7 yielded phosphoramidite 9. Incorporation of 8-amdA into oligonucleotides was confirmed by enzymatic digestion and electrospray mass spectrometry.

Scheme 1

a
1: R_1, R_2, R_3=H

2: R_1=Br, R_2, R_3=H
b

3: R_1=Br, R_2, R_3=TBDMS
c
4: R_1=N_3, R_2, R_3=TBDMS

5: R_2, R_3=TBDMS

f
6: R_2, R_3=TBDMS

7: R_2, R_3=H
g

8: R_2=DMTr, R_3=H
h

9: R_2=DMTr
R_3=P(N-iPr$_2$)O(CH$_2$)$_2$CN
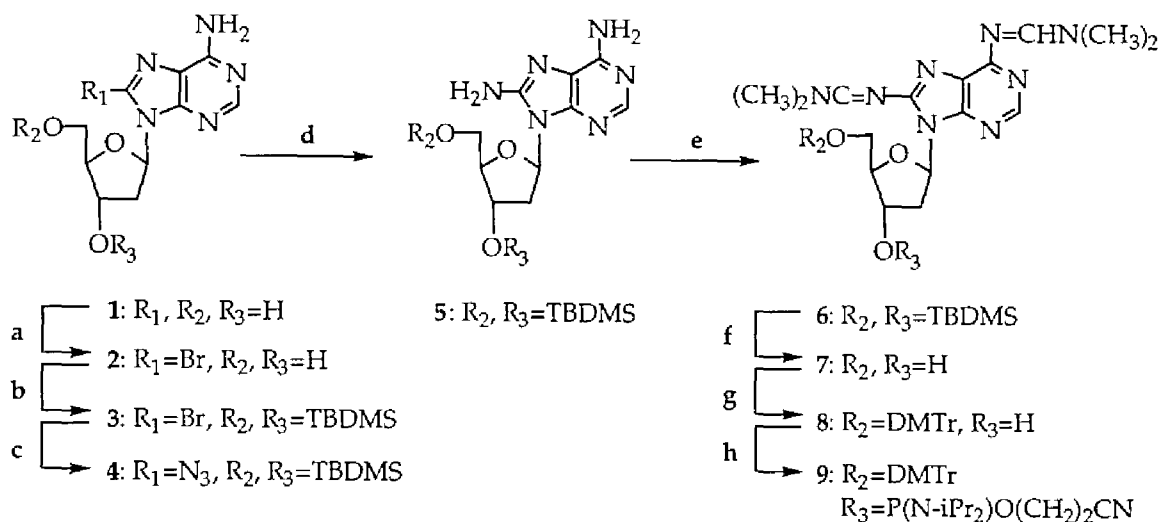
(a) Br$_2$ saturated water, pH 4.2 NaOAc buffer, 3 h, 34%; (b) TBDMS-Cl, imidazole, pyridine, 12 h, 81%; (c) NaN$_3$, DMF, 14 h; (d) H$_2$, 10% Pd/C, MeOH, 3 h, 85% (2-step yield); (e) DMF-dimethylacetal, pyridine, 3 days, 90%; (f) 18-crown-6, KF, DMF, 15 h, 68%; (g) DMTrCl, NEt$_3$, pyridine, 15 h, 63%; (h) P(N-iPr)$_2$O(CH)$_2$CN, tetrazole, pyridine, 5 h, 98%.

PM3 semi-empirical calculations suggested that Hoogsteen-type base pairing in T:(8-amdA•T) (-6.9 kcal) triplet is more stable than unmodified T:(A•T) (-5.8 kcal) triplet.[6] In order to examine the effects of 8-amdA in a triplex formation, the stabilities of both duplexes and triplexes under neutral (pH 7.0) and acidic conditions (pH 6.0) were investigated. Melting transition data for duplexes and

third-strand dissociation of ODNs (10:11•12, 10:11•13, 10:11•14) are summarized in Table 1. Representative melting curves at pH 7.0 are depicted in Figure 2. The third strand transition temperatures exhibit a marked pH dependence, reflecting protonation of C residues. Introduction of one amino group (10:11•13) leads to the increase in Tm of third-strand dissociation relative to the unmodified ODN (10:11•12). The triplex was further stabilized by increasing the number of incorporated 8-amdA (10:11•14), which contains three 8-amdA residues; this system shows a dramatic increase in Tm of third-strand dissociation. These results clearly indicate that the introduction of an 8-amino group stabilizes triplexes by Hoogsteen base pairing and slightly destabilizes Watson-Crick base pairing in duplex formation.

5'-d(TTCTCTTTCT)-3'   1 0

3'-d(CGA$\underline{TTCTCTTTCT}$AGC)-5'   1 1

5'-d(GCT$\underline{AAGAGAAAGA}$TCG)-3'   1 2

5'-d(GCT$\underline{AAGAGA^a{}^m AAGA}$TCG)-3'   1 3

5'-d(GCT$\underline{A^a{}^m AG^a{}^m A GA^a{}^m A AGA}$TCG)-3' 1 4

5'-d(A$^a{}^m$AG$^a{}^m$AGA$^a{}^m$AAGA)-3'   1 5
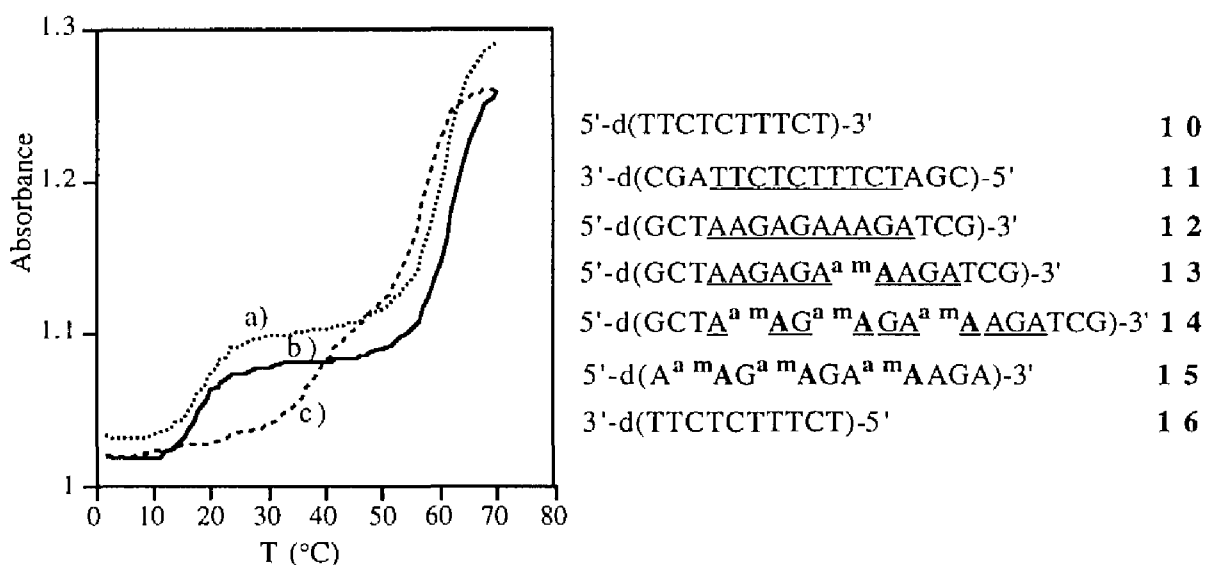
3'-d(TTCTCTTTCT)-5'   1 6

Figure 2. UV mixing curves of (a)10:11·12, (b)10:11·13, (c)10:11·14. Measurements were conducted at 260 nm in 100 mM Na cacodylate buffer (pH 7.0) containing oligomers (4 µM each strand) and NaCl (1.0 M).

Table 1. Tm Values [°C] for the Dissociation of the Third Strand and Duplex[a]

Triplex	pH 6.0 Third strand	Duplex	pH 7.0 Third strand	Duplex
10:11•12	33.0	62.2	17.5	62.5
10:11•13	36.5	60.8	18.8	61.0
10:11•14	57.5[b]	57.5[b]	38.3	57.7
10:15•16	43.0[b]	43.0[b]	nd[c]	nd[c]

[a]UV-melting curves were obtained in a 100 mM Na cacodylate buffer containing 1.0 M NaCl at a strand concentration of 4 µM. [b]Cooperative single melting behavior of triplex to single strand was observed. [c]Not determined.
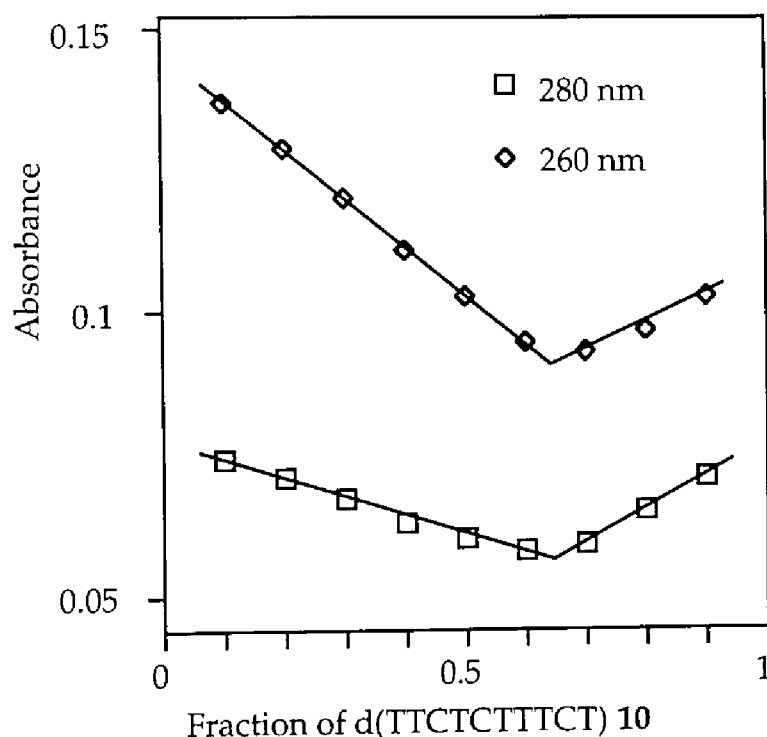

Figure 3. UV mixing curves of 10·15. Measurements were conducted at 260 nm and 280 nm in 100 mM Na cacodylate buffer (pH 6.0) containing oligomers (0.02 mM total base conc.) and NaCl (1.0 M).

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Parallel-stranded duplex with a Hoogsteen base pair was reported in homopurine-homopyrimidine strands[7] and 3'-3' and 5'-5' linked system,[8] therefore, we examined the existence of parallel-stranded duplex formation of 10-15 which forms a stable triplex in the presence of 16. It was found that 10-15 showed a cooperative single melting behavior (Tm = 38.2 °C); however, UV mixing curve experiment indicated that 10-15 forms a 2:1 complex as shown in Figure 3.[9] The results suggest that 15-16 would forms a partially mismatched triplex. Wang *et al.* recently showed that 2'-deoxy-3-isoadenine (iA)-containing oligonucleotide d(CG[iA]TCG) forms a B-type anti-parallel duplex with central Hoogsteen-type iA:T base pairs.[10] We next studied the Hoogsteen-type base pairing in an anti-parallel duplex. Interestingly, octanucleotide d(8-amAT)$_4$ showed a remarkably higher thermal stability (Tm = 25.3 °C) compared to the unmodified oligonucleotide (Tm = 8.3 °C). Considering that (i) triplex formation is difficult in this purine-pyrimidine alternating sequence and (ii) an 8-amino group itself destabilizes the Watson-Crick base-pairing, the present d(8-amAT)$_4$ system may form an anti-parallel Hoogsteen-type duplex.

Conclusion

The present results clearly demonstrated that one amino group at the adenine C8 position greatly stabilizes the Hoogsteen base pairing in triplex formation by an additional hydrogen bonding. Incorporation of 8-amdA into DNA will provide a useful tool for stabilizing nucleic acid local structures which would be of structural and biological significance.

Experimental Section

Materials and methods. Calf intestine alkaline phosphatase (AP, 1000 units/mL), snake venom phosphodiesterase (s.v. PDE, 3 units/mL) and P1 Nuclease (P1, 100 units/mL) were purchased from Boehringer Mannheim. 2'-Deoxyadenosine was purchased from Yamasa Shoyu Co. Ltd. Bromine saturated water, 10% palladium on carbon and N,N-dimethylformamide dimethyl acetal was purchased from Nacalai Tesque Co. Sodium azide and dimethyl amino pyridine were obtained from Wako Pure Chemical Co. *tert*-Butyl dimethylchlorosilane and 4,4'-dimethoxytrityl chloride was purchased from Tokyo Kasei Co. 2-Cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite was purchased from Aldrich. The reagents for DNA synthesizer such as I_2 solution (I_2/H_2O/pyridine/tetrahydrofuran, 0.5:9.5:20:70) were purchased from Applied Biosystems. A-, G-, C-, and T-β-(cyanoethyl)phosphoramidites were purchased from Glen Research. 1H-tetrazole were purchased from Dojin Laboratories. Pyridine was dried over BaO and distilled before use. DMF was dried over CaH_2 and dried before use. Silica gel column chromatography was carried out on Wakogel C-200. Thin layer chromatography was carried out on a Merck silica gel 60F$_{254}$ plate. ^{1}H-NMR spectra were recorded on Varian GEMINI-200 (200 MHz) or Varian Mercury-400 (400 MHz). HPLC analysis was carried out using PU-980 HPLC system (JASCO, Tokyo) equipped with a CHEMCOBOND 5-ODS-H (4.6 x 150 mm) or a Cosmosil 5C18-MS column (4.6 x 150 mm). Detection was carried out at 254 nm.

Synthesis of 8-bromodeoxyadenosine. 2'-Deoxyadenosine (10 g, 0.04 mol) was suspended in a NaOAc buffer (0.5 M, pH 4.2, 150 mL) with stirring. Then bromine water (200 mL) was added and fully dissolved solution was obtained. The resulting solution was stirred

for 3 hr at room temperature; after 2 hr precipitate began to form. The color of the solution was discharged by addition of $NaHSO_3$ and the pH of the solution was adjusted 7 with 5N NaOH. The mixture was filtered and washed with cold water (100 mL), then with methanol (100 mL) and dried in vacuo to provide 4.42g (34% yield) of 8-bromodeoxyadenosine as a yellow powder. ^1H-NMR (400 MHz) (D_2O) δ 2.37 (ddd, 1 H, J=14.4, 8.6, 6.0, 2''), 3.00 (ddd, 1 H, J=14.4, 10.1, 4.6, 2'), 3.78 (dd, 1 H, J=9.2, 3.6, 5'), 3.86 (dd, 1 H, J=9.2, 2.4, 5''), 4.16 (m, 1 H, 4'), 6.48 (dd, 1 H, J=10.1, 6.0, 1'), 8.10 (s, 1 H, A2). FAB MASS (positive ion): m/e 330 (M+H). Exact mass calcd for $C_{10}H_{12}O_3N_5{}^{79}Br$ 330.0202, found 330.0186.

Synthesis of 2',5'-bis-O-tert-butyldimethylsiliyl-8-bromodeoxyadenosine. 8-Bromodeoxyadenosine (5.9 g, 17.9 mmol) was dried by coevaporation with pyridine (20 mL, two times) and suspended in 60 mL of dry pyridine. To this solution, tert-Butyl dimethylchlorosilane (13.5 g, 89.5 mmol) and imidazole (6.09 g, 89.5 mmol) were added and solution was stirred at room temperature for 12 hr. After evaporation of the solvent, the residue was extracted with ethyl acetate followed by silica gel chromatography (hexane: diethyl ether =1:1) to yield 2',5'-bis-O-tert-butyldimethylsiliyl-8-bromodeoxyadenosine as a white powder (6.94 g, 81 %). ^1H-NMR (200 MHz) ($CDCl_3$) δ -0.07 (s, 3 H, Si-CH3), 0.03 (s, 3 H, Si-CH3), 0.12 (s, 3 H, Si-CH3), 0.80 (s, 9 H, t-Bu), 0.91 (s, 9 H, t-Bu), 2.21 (ddd, 1 H, J=11.2, 7.0, 4.2, 2''), 3.57-3.71 (m, 2 H, 4', 2'), 3.89 (dd, 1 H, J=16.2, 3.6, 5'), 3.92 (dd, 1 H, J=16.2, 3.4, 5''), 4.85 (ddd, 1 H, J=7.4, 5.8, 3.6, 3'), 5.62 (s, 2 H, A6NH2), 6.32 (dd, 1 H, J=7.0, 6.6, 1'), 8.23 (s, 1 H, A2). FAB MASS (positive ion): m/e 558 (M+H). Exact mass calcd for $C_{22}H_{41}O_3N_5{}^{79}BrSi$ 558.2009, found 558.1910.

Synthesis of 2',5'-bis-*O-tert*-butyldimethylsiliyl-8-aminodeoxyadenosine. 2',5'-Bis-*O-tert*-butyldimethylsiliyl-8-bromodeoxyadenosine (1.85 g, 3.32 mmol) was dried by coevaporation with pyridine (10 mL, two times) and dissolved in 15 mL of dry DMF. To this solution, NaN_3 (735 mg, 11.31 mmol) was added and the reaction mixture was heated for 17 hr at 75 °C. After evaporation of the solvent, the residue was extracted with diethyl ether. The residue was dissolved in methanol (100 mL) containing water (10 mL) and was added 1.0 g of 10% palladium on carbon. This solution was exposed to hydrogen for 3 hr at 50 psi with stirring at room temperature. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The crude solid was purified by silica gel chromatography, eluting with a 2-4% gradient of MeOH in diethyl ether to yield 1.17g (yield 85 %) of 2',5'-bis-*O-tert*-butyldimethylsiliyl-8-aminodeoxyadenosine as a white powder. ^{1}H-NMR (200 MHz) (CDCl$_3$) δ 0.06 (s, 6 H, Si-CH$_3$), 0.08 (s, 3 H, Si-CH$_3$), 0.09 (s, 3 H, Si-CH$_3$), 0.88 (s, 9 H, *t*-Bu), 0.90 (s, 9 H, *t*-Bu), 2.18 (ddd, 1 H, J=13.2, 6.4, 2.8, 2''), 2.59 (ddd, 1 H, J=13.2, 8.5, 7.4, 2'), 3.77-4.00 (m, 3 H, 4', 5', 5''), 4.55 (m, 1 H, 3'), 5.13 (s, 2 H, A8NH$_2$), 5.45 (s, 2 H, A6NH$_2$), 6.32 (dd, 1 H, J=8.2, 6.4, 1'), 8.16 (s, 1 H, A2). FAB MASS (positive ion): m/e 495 (M+H). Exact mass calcd for $C_{22}H_{43}O_3N_6Si_2$ 495.2934, found 495.2944.

Synthesis of 2,8-[bis-*N*-(dimethylamino)methylene]- 2',5'-bis-*O-tert*-butyldimethylsiliyl-8-aminodeoxyadenosine. 2',5'-Bis-*O-tert*-butyldimethylsiliyl-8-aminodeoxyadenosine (1.17 g, 2.37 mmol) was dried by coevaporation with pyridine (10 mL, two times) and dissolved in 10 mL of dry pyridine. To this solution, *N,N*-dimethylformamide dimethyl acetal (8 mL, excess) was added. After stirring the reaction mixture at room temperature for 3 days, the solvent was evaporated and the residue was purified by silica gel

chromatography (dichloromethane: methanol =97:3) to afford 1.29 g (yield 90 %) of 2,8-[bis-N-(dimethylamino)methylene]-2',5'-bis-O-$tert$-butyldimethylsiliyl-8-aminodeoxyadenosine.   ^{1}H-NMR (200 MHz) (CDCl$_3$) δ -0.02 (s, 3 H, Si-CH$_3$), 0.01 (s, 3 H, Si-CH$_3$), 0.10 (s, 6 H, Si-CH$_3$), 0.85 (s, 9 H, t-Bu), 0.90 (s, 9 H, t-Bu), 2.10 (ddd, 1 H, J=13.0, 7.0, 3.2, 2''), 3.09 (s, 3 H, N-CH), 3.10 (s, 3 H, N-CH$_3$), 3.12 (s, 3 H, N-CH$_3$), 3.20 (s, 3 H, N-CH$_3$), 3.52-3.75 (m, 2 H, 4', 2'), 3.88-4.00 (m, 3 H, 5', 5''), 4.78 (m, 1 H, 3'), 6.69 (t, 1 H, J=7.4, 1'), 8.31 (s, 1 H, A2), 8.72 (s, 1 H, N=CH), 8.75 (s, 1 H, N=CH).   FAB MASS (positive ion): m/e 605 (M+H).   Exact mass calcd for C$_{28}$H$_{53}$O$_3$N$_8$Si$_2$ 605.3779, found 605.3797.

Synthesis   of   2,8-[bis-N-(dimethylamino)methylene]-8-aminodeoxyadenosine.   2,8-[Bis-N-(dimethylamino)methylene]-2',5'-bis-O-$tert$-butyldimethylsiliyl-8-aminodeoxyadenosine (1.29 g, 2.14 mmol) and 18-crown-6 (2.26 g, 8.6 mmol) and KF (497 mg, 8.6 mmol) were added to dry DMF (10 mL) and the reaction mixture was stirred for 16 hr.   Removal of the solvent under reduced pressure followed by silica gel chromatography (dichloromethane: methanol =92:7)   to   provied   2,8-[bis-N-(dimethylamino)methylene]-8-aminodeoxy-adenosine as a white powder (0.55 g, 68 % yield).   ^{1}H-NMR (200 MHz) (D$_2$O) δ 2.14 (ddd, 1 H, J=12.0, 6.4, 1.9, 2''), 2.85 (m, 1 H, 2'), 2.96 (s, 3 H, N-CH$_3$), 2.97 (s, 3 H, N-CH$_3$), 3.05 (s, 6 H, N-CH$_3$), 3.65 (dd, 1 H, J=9.7, 3.4, 5''), 3.73 (dd, 1 H, J=9.7, 3.0, 5'), 4.00 (m, 1 H, 4'), 4.52 (m, 1 H, 3'), 6.50 (dd, 1 H, J=9.0, 6.4, 1'), 8.03 (s, 1 H, A2), 8.28 (s, 1 H, N=CH), 8.38 (s, 1 H, N=CH).   FAB MASS (positive ion): m/e 377 (M+H).   Exact mass calcd for C$_{16}$H$_5$O$_3$N$_8$ 377.2049, found 377.2034.

Synthesis of 8-aminodeoxyadenosine. 2,8-[Bis-*N*-(dimethylamino)methylene]-8-aminodeoxyadenosine (20 mg, 0.05 mmol) was deprotected with 28 % NH$_4$OH for 8 hr at 55 °C and purified with HPLC to yield 8-aminodeoxyadenosine. (14 mg, quant.). ^{1}H-NMR (200 MHz) (D$_2$O) δ 2.21 (ddd, 1 H, J=15.0, 6.4, 2.6, 2''), 2.67 (ddd, 1 H, J=15.0, 8.4, 6.6, 2'), 3.73 (m, 2 H, 5', 5''), 4.00 (m, 1 H, 4'), 4.49 (m, 1 H, 3'), 6.24 (dd, 1 H, J=8.7, 6.3, 1'), 7.96 (s, 1 H, A2). FAB MASS (positive ion): m/e 267(M+H). Exact mass calcd for C$_{10}$H$_{15}$O$_3$N$_6$ 267.1205, found 267.1201.

Synthesis of 5'-*O*-(4,4'-dimethoxytrityl)-2,8-[bis-*N*-(dimethylamino)methylene]-8-aminodeoxyadenosine. 2,8-[Bis-*N*-(dimethylamino)methylene]-8-aminodeoxyadenosine (530 mg, 1.41 mmol) and dimethyl amino pyridine (5 mg. cat.) were dried by coevaporation with pyridine (10 mL, three times). To this solution was added 4,4'-dimethoxytrityl chloride (665 mg, 1.96 mmol), followed by pyridine (8 mL) and triethylamine (283 μL, 1.96 mmol). The reaction mixture was stirred at room temperature for 15 hr. After evaporation of the solvent, the residue was extracted with ethyl acetate followed by silica gel chromatography (dichloromethane: methanol =95:5) gave 0.6 g (63 % yield) of 5'-*O*-(4,4'-dimethoxytrityl)-2,8-[bis-*N*-(dimethylamino)methylene]-8-aminodeoxyadenosine as a white powder. ^{1}H-NMR (200 MHz) (CDCl$_3$) δ 2.25 (ddd, 1 H, J=13.2, 7.6, 4.6, 2''), 3.04 (s, 3 H, N-CH$_3$), 3.08 (s, 3 H, N-CH$_3$), 3.12 (s, 3 H, N-CH$_3$), 3.34 (s, 3 H, N-CH$_3$), 3.38-3.51 (m, 3 H, J=9.7, 3.4, 2', 5', 5''), 4.01 (ddd, 1 H, J=6.8, 6.0, 5.2, 4'), 4.95 (ddd, 1 H, J=6.6, 4.4, 4.0, 3'), 6.64-6.78 (m, 5 H, methoxyphenyl-o, 1'), 7.11-7.42 (m, 9 H, phenyl, methoxyphenyl-m), 8.14 (s, 1 H, A2), 8.70 (s, 1 H, N=CH), 8.73 (s, 1 H, N=CH). FAB MASS (positive ion): m/e 679 (M+H). Exact mass calcd for C$_{37}$H$_{43}$O$_5$N$_8$ 679.3356, found 679.3381.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2,8-[bis-N-(dimethylamino)methylene]-8-aminodeoxyadenosine-3'-O-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]. 5'-O-(4,4'-dimethoxytrityl)-2,8-[bis-N-(dimethylamino)methylene]-8-aminodeoxyadenosine (400 mg, 0.59 mmol) was coevaporated with pyridine (3 mL, three times) and dissolved in dry pyridine (3 mL). To this solution was added 2-cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite (212 μL, 0.73 mmol), and 0.5 M 1H-tetrazole (1.45 mL, solution in dry CH₃CN) under an argon atmosphere. After 5 hr, the reaction mixture was diluted with ethyl acetate (50 mL) and organic phase was washed with saturated sodium hydrogen carbonate solution (50 mL, two times) and then with water (50 mL, two times). The organic layer was dried over with sodium sulfate and evaporated to dryness to yield 5'-O-(4,4'-dimethoxytrityl)-2,8-[bis-N-(dimethylamino)methylene]-8-aminodeoxyadenosine-3'-O-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] (480 mg, 98 %). ^{1}H NMR (400 MHz, CDCl₃) δ 1.10-1.29 (m, 12 H, CH($\underline{CH_3}$)₂), 2.23-2.38 (m, 1 H, 2''), 2.44-2.63 (m, 2 H, OCH₂$\underline{CH_2}$CN), 3.05 (s, 3 H, N(CH₃)₂), 3.09 (s, 3 H, N(CH₃)₂), 3.13 (s, 3 H, N(CH₃)₂), 3.20 (s, 3 H, N(CH₃)₂), 3.35-3.76 (m, 7 H, C$\underline{H}$(CH₃)₂), O$\underline{CH_2}$CH₂CN, 2', 5', 5''), 3.74 (s, 6 H, OCH₃), 4.15-4.26 (m, 1 H, 4'), 4.84-4.97 (m, 1 H, 3'), 6.67-6.77 (m, 5 H, methoxyphenyl-o, 1'), 7.12-7.43 (m, 9 H, phenyl, methoxyphenyl-m), 8.11 (s, 1 H, H2), 8.70 (s, 1 H, NC$\underline{H}$N(CH₃)₂), 8.75 70 (s, 1 H, NC$\underline{H}$N(CH₃)₂). ^{31}P NMR (121 MHz, CDCl₃) δ 149.41, 148.98 (diastereomers). FAB MASS (positive ion): m/e 879 (M+H). Exact mass calcd for C₄₆H₆₀O₆N₁₀P 879.4434, found 879.4458.

Synthesis of oligonucleotides. Oligonucleotides were prepared by the β-(cyanoethyl)phosphoramidite method on controlled pore glass supports (1 μmol) by using ABI 381 A DNA

synthesizer.   After automated synthesis, the oligomer was detached from the support, deprotected and purified by HPLC.   After lyophilization, approximately 20 OD of pure oligomer was isolated. Purity and concentrations of all oligonucleotides were determined by complete digestion with s.v. PDE, P1 nuclease and AP to 2'-deoxymononucleosides.   The 8-aminodeoxyadenosine containing oligonucleotides were also identified by electrospray mass spectrometry: calcd for 13: 4978.3, found 4977.9; calcd for 14: 5008.4, found 5007.9; calcd for 17: 3163.2, found 3162.4.

Measurement of melting temperature.   Thermal denaturation profiles were obtained with Jasco V-550 spectrophotometer equipped with a Peltier temperature controller. The absorbance of the samples was monitored at 260 nm from 2 °C to 80 °C with a heating rate of 1 °C per minute.   Experiments with a heating rate of 0.5 °C per minute gave the same results as those at 1 °C per minutes, suggesting that thermodynamic equilibrium was achieved.   The data were normalized to percent denaturation.   A linear least-squares analysis of the data gave a slope of transition and y intercept from which the melting temperature were calculated. Representative melting curves are depicted in Figure 2.

UV mixing experiments.   The oligonucleotide (15 and 17) solutions were prepared in 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90 molar ratios in 1 M NaCl containing 100 mM Na cacodylate buffer (pH 6.0).   Two samples containing only one of the two strands were prepared (100:0 or 0:100) in the same buffer.   The final concentration of the oligonucleotide was 0.02 mM (base conc.).

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The UV measurements were carried out as described above after equilibrating each sample at 15 °C for 10-15 min. The UV mixing curve was shown in Figure 3.

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Chapter 4

Conformation Dependent Photochemistry of 5-Halouracil-Containing DNA: Stereospecific 2'α-Hydroxylation of Deoxyribose in Z-form DNA

Abstract

The photoreaction of Z-form ^IU-containing octanucleotide d(CGCG^{I}UGCG) was investigated. It was revealed that the stereospecific C2' α-hydroxylation occurs in the Z-form DNA together with the formation of C1' and C2' oxidation products and a halogen exchanged product. Under O_2-limiting conditions, the yield of both C1' and C2' oxidation products in the Z-form oligomers decreased considerably. When the photoreaction was carried out under $^{18}O_2$ atomosphere, the electrospray MS demonstrated the incorporation of ^{18}O atom into the C2' hydroxylated product. These results indicate that atmospheric O_2 is the source for the C2' hydroxyl group. Upon addition of NaI as a reductant of hydroperoxide, a dramatic increase in the hydroxylated product was observed. These results suggest that both the C2' oxidation product and hydroxylated product are produced via a common hydroperoxide intermediate. Upon treatment with ribonuclease T1, the C2' hydroxylated product was quantitatively hydrolyzed to d(CGC)rGp and d(UGCG). These results suggest that this photochemical and enzymatic procedure can be used as a specific probe for the existence of the local Z-form structure in longer DNA.

Introduction

It is well-recognized that DNA has a remarkable structural heterogeneity.[1]   Such local DNA conformations are thought to play an important biological role in processes such as gene expression by altering DNA-protein interactions.[2]   Although the left-handed Z-form DNA is one of the characteristic and significant local structures of DNA,[3] the precise biological functions of Z-DNA have not been fully understood presumably due to the lack of an appropriate detection method in a living cell system.   Recently, Rich and colleagues have reported the creation of a Z-form-specific endonuclease as a probe for Z-DNA.[4]   The Z-form region is assumed to appear in a very short period of time in a living cell, and therefore, utilization of a photochemical reaction which directly reflects the DNA local conformation would be infinitely useful. Previously, we have demonstrated that photoirradiation of 5-halouracil-containing   B-form   and   DNA-RNA   hybrid oligonucleotides results in a conformation dependent H abstraction, $i.$ $e.$, the competitive C1' and C2' hydrogen abstractions were observed in the B-form DNA, whereas predominant C1' hydrogen abstraction occurred in the DNA-RNA hybrid.[5]   Inspection of the molecular model of Z form DNA suggested that the 5-halouracil in the Z-form DNA would have a unique photoreactivity compared with B-form DNA due to differences in the manner that purine bases stacked on the 3' pyrimidine base, $e. g.$,   the ribose C2β hydrogen of the 5' side purine is very close to C5 of uracil, whereas C1' hydrogen is very far from C5 of uracil.   In addition, the increase in the UV absorption at 308 nm by Z-DNA was expected to enhance the reactivity.   In this study, we examined the photoreaction of the [I]U-containing Z-form octanucleotide and found that a novel stereospecific 2'α-hydroxylation occurs efficiently in the Z-form DNA.

Results and Discussion

To date, only few chemical or photochemical reactions that are specific to the Z form DNA have been developed. Most of the available experimental data are limited to the investigation on poly(dG-dC) which exists in a Z-form only at a high salt condition.[3a,6] We already demonstrated that the incorporation of a methyl group at the guanine C8 position (m^8G) dramatically stabilizes the Z-conformation of short oligonuceotides in a variety of sequences.[7] For example, ^IU-containing octanucleotide d($CGCG^IUGCG$) (ODN 1) mixed with complementary m^8G-containing oligonucleotide d(Cm^8GCACm^8GCG) (ODN 3) forms a typical Z-conformation at a 2 M NaCl concentration as evidenced by the CD spectrum shown in Figure 1. On the other hand, when unmodified oligonucleotide 2 was used as a complement for 1, ODN 1-2 showed a typical B-form CD spectrum. These two systems, ODN 1-2 and ODN 1-3, were used to investigate their photoreactivity.

Figure 2(a) shows a HPLC profile of the photoirradiated B-form ODN 1-2, showing the formation of 4 and 5 as the C1' and C2' oxidation products, respectively. The peak eluted at 22.7 min was found to be the halogen exchanged product 6 which was produced from the photoreaction of ^IU with $NaCl$.[8] In the photoirradiation of the Z-form ODN 1-3, the product 4 resulting from C1' oxidation was significantly suppressed and the formation of a new product eluted at 24.0 min was observed as shown in Figure 2(b). Enzymatic digestion of the new product showed the formation of dG, dC, rG and dU in a ratio of 3 : 3 : 1 : 1, indicating that this product is the ribonucleotide containing octamer 7 as summarized in Scheme 1. The careful HPLC analysis indicated that 2'-arabinosylguanosine was not detected in the enzymatic digestion of the photoirradiated

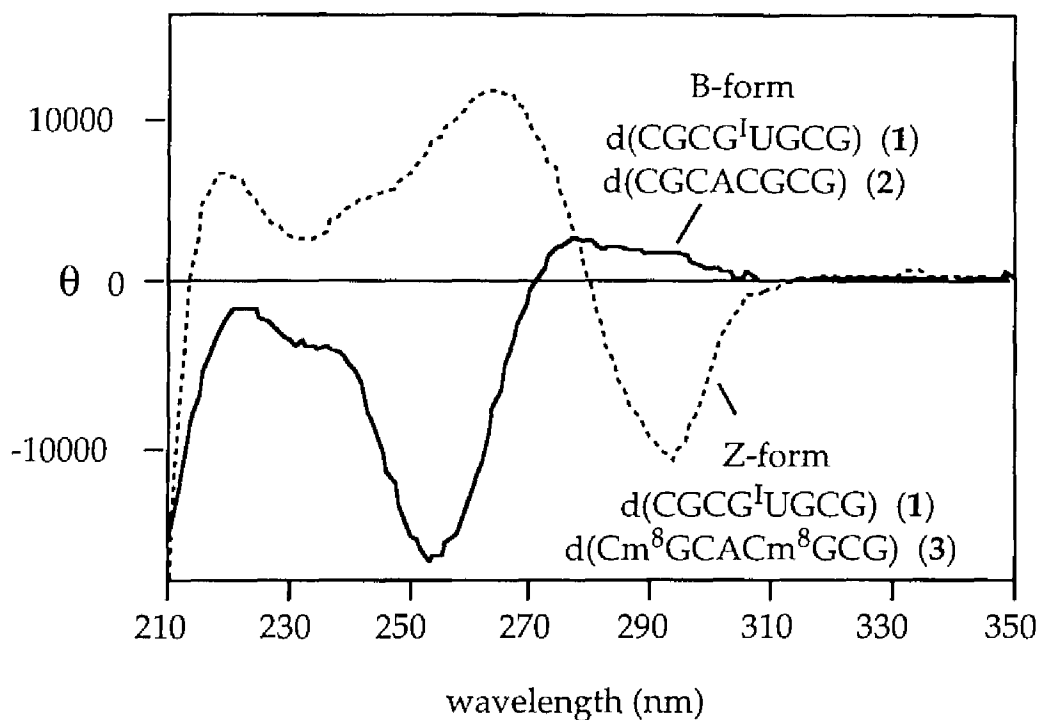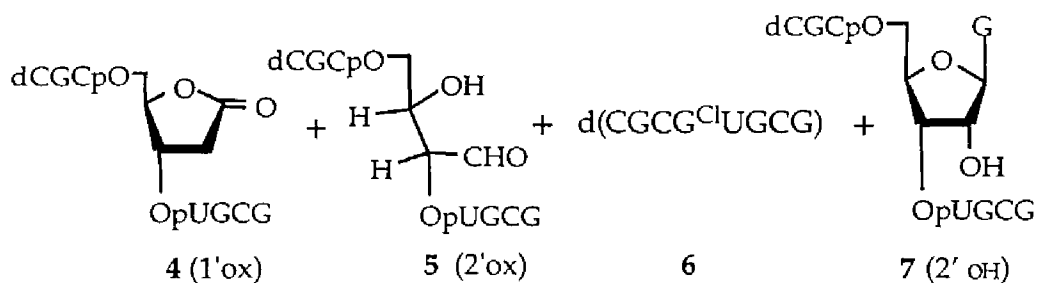reaction mixture, indicating that stereospecific α-hydroxylation occurs at C2′ carbon of deoxyribose.

Figure 1. CD-spectrum of 5-iodouracil-containing oligonucleotides **1-2** and **1-3**. Sample solution contained 0.1 mM oligonucleotides (base conc.), 2 M NaCl, and 50 mM Na cacodylate (pH 7.0) at 2 °C.

Scheme 1

5′-d(CGCG^IUGCG)-3′ (1)
5′-d(CX CACX CG)-3′

$\xrightarrow[2\ h]{h\nu\ (302\ nm)}$

2 ; X = G   (B-form)
3 ; X = m^8G (Z-form)

dCGCpO ⌐O⌐=O   dCGCpO⌐—OH   dCGCpO⌐—O—G
OpUGCG   H—⌐   OpUGCG
                     H—⌐—CHO
        +   +   d(CGCG^ClUGCG)   +
OpUGCG   OpUGCG   OpUGCG

4 (1′ox)   **5** (2′ox)   **6**   **7** (2′ oH)

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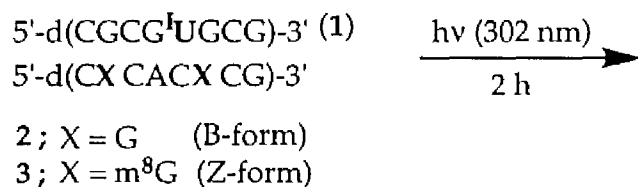
Figure 2. HPLC profiles of UV irradiated (a) d(CGCG^{I}UGCG) (ODN 1)/d(CGCACGCG) (ODN 2) and (b) ODN 1/d(Cm^{8}GCACm^{8}GCG) (ODN 3). The reaction mixture was anal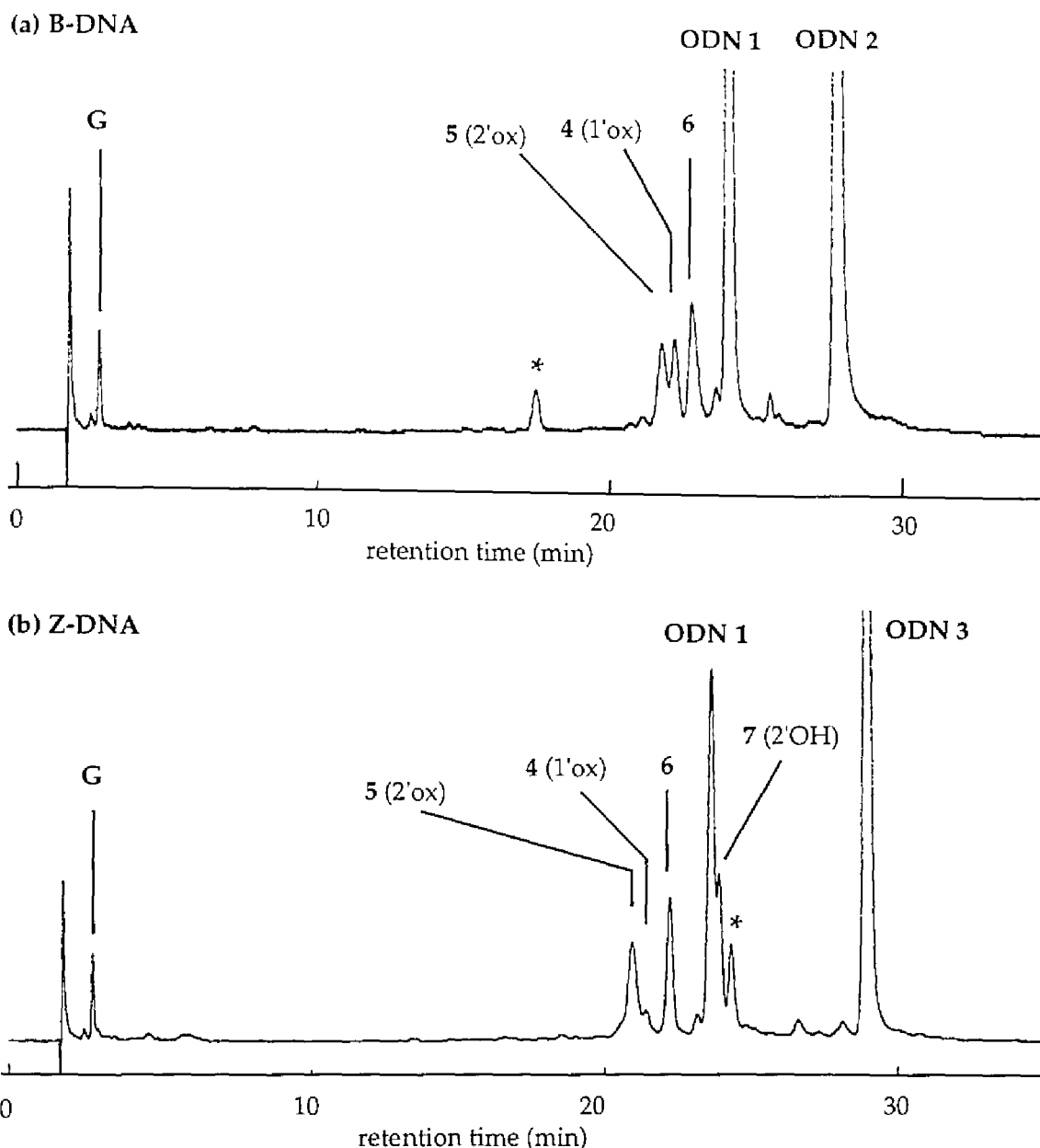yzed by HPLC on Cosmosil 5C18 MS column (4.6 x 150 mm) detected at 254 nm; elution was with 0.05 M ammonium formate (pH 6.5) containing 2 - 8 % acetonitrile, linear gradient, 30 min, at a flow rate of 1.0 mL/min, at 30 °C. Identification of each peak (4-7) was confirmed by electrospray MS (ESMS) and enzymatic digestion. The peaks with asterisk were found to be thermally unstable, therefore further characterizations of the products were unsuccessful.

Of special importance is that the C2'α hydroxylated product **7** was obtained preferentially from the Z-form ODN **1-3**, whereas the C1' oxidation product **4** decreased considerably in the Z-form. These results indicate the partitioning between C1' and C2' product pathways is different for the two conformations as expected. As shown in Table 1, higher conversions in the photoreaction under identical conditions were always observed in the Z-form compared with B-form. This result can be interpreted by the increased absorption at a longer wavelength by changing from the B- to Z-form DNA.[3] For example, the absorbance at 308 nm is approximately two times greater in Z-form ODN **1-3** than in B-form ODN **1-2**. A similar Z-form dependent stereospecific C2'α hydroxylation was also observed in ^{Br}U-containing ODNs.

Under O_2-limiting conditions, the yields of both **5** and **7** decreased in the Z-form as shown in Table 1, implying that the reactions giving **5** and **7** are O_2 dependent processes. When the photoreaction was carried out under $^{18}O_2$ atomosphere, the electrospray MS (ESMS) demonstrated the incorporation of ^{18}O atom into the C2' hydroxylated product **7** as shown in Figure 3. On the other hand, when the photoreaction of Z-form ODN **1-3** was carried out in $H_2^{18}O$, ESMS of the isolated product **7** showed no incorporation of the ^{18}O atom into the C2' hydroxylation product. These results indicate that O_2 is the source of the C2' hydroxy group. Upon addition of NaI, which is known as a reductant for hydroperoxides, a dramatic increase of **7** was observed with a concomitant decrease of **5**. These results indicate that both **5** and **7** are produced via a common hydroperoxide intermediate **8** as shown in Scheme 2. However, in the case of the photoreaction of the B-form ODN **1-2**, the formation of **5** was preferred over **7** even in the presence of 50 mM NaI. Therefore, the preferential reduction of **8**

to 7 (path a) compared to the Criegie type rearrangement leading to 5 via 9 (path b) would partly contribute to the specific C2'α hydroxylation observed in the Z-form DNA. The molecular basis for the preferential hydroxylation in the Z-form DNA is not fully understood, however, the structural characteristics of Z-form DNA such as C3' endo sugar puckering and alternative syn-anti conformation might effect the partitioning of the decomposition paths of hydroperoxide intermediate 8.
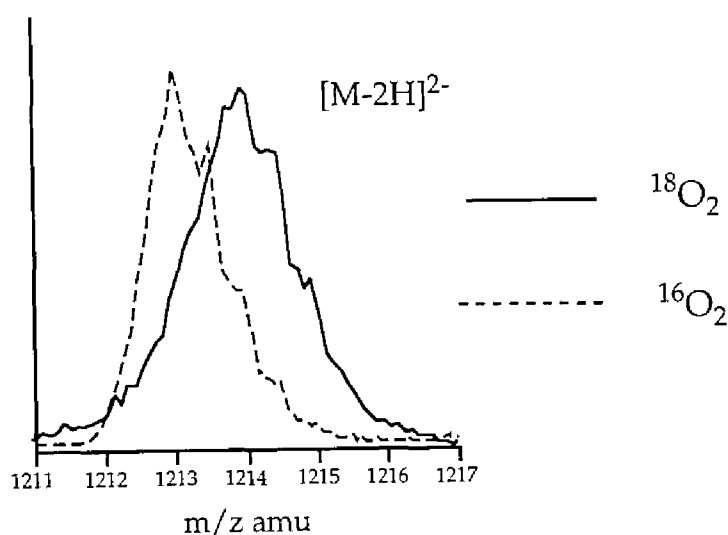

Figure 3. Electrospray MS of 7 formed under $^{18}O_2$ atmosphere (solid line) and $^{16}O_2$ atmosphere (dashed line).
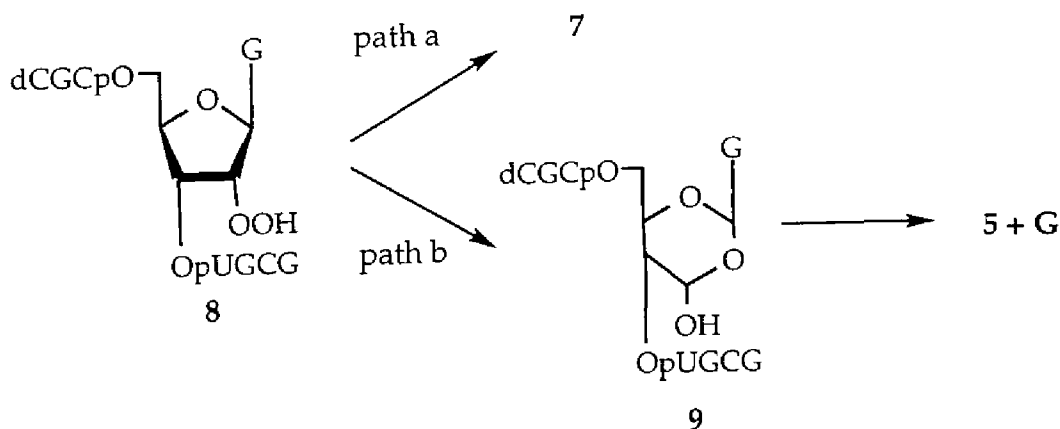
Scheme 2

Table 1. Product Analysis in the Photoirradiation of 5-Iodouracil-Containing B- and Z-Form Deoxyoctanucleotides[a]

	ODN condition	NaI (mM)	conversion (%)	G (μM)	products (%)[b] 4 (1'ox)	5 (2'ox)	6	7 (2'oH)
1	1-2	0	43	7.8	11	14	17	-
2	1-2 O$_2$-limiting	0	52	7.8	13	5.8	18	-
3	1-2	50	36	10	16	33	19	6.7
4	1-3	0	70	11	3.1	19	16	14
5	1-3 O$_2$-limiting	0	71	2.9	1.1	0.8	17	1.1
6	1-3	50	62	1.8	-	1.0	11	68

[a]The reaction mixture (total volume, 100 μL) contained deoxyoctanucleotide (0.5 mM total base concentration), 50 mM sodium cacodylate (pH 7.0), 2.0 M NaCl and NaI as indicated. Irradiation was performed with a transilluminator (302 nm) for 2 h at 0 °C from a distance of 5 cm. A 10-μL aliquot was analyzed by HPLC as described in Figure 2. For quantitation of 7, 10-μL aliquot was directly subjected to enzymatic digestion with snake venom PDE (0.3 unit/mL) and calf intestine alkaline phosphatase (100 unit/mL) and was analyzed by reverse phase HPLC. [b]Yields were determined by a comparison of the peak area of the authentic material after enzymatic digestion which were calculated based on the consumed ODNs.

Interestingly, upon treatment with ribonuclease T1, the C2' hydroxylation product 7 was quantitatively hydrolyzed to d(CGC)rG> (> : cyclic phosphate) and d(UGCG), which were identified by comparison with authentic samples after enzymatic digestion. Figure 4 shows the HPLC profiles of photoirradiated ODN 1-3 in the presence of NaI at different concentrations of NaCl after ribonuclease T1 treatment, indicating that the amount of d(UGCG) increased proportionally with increasing Z-DNA ratio. These results suggest that this photochemical and enzymatic procedure can be used as a specific detection method for the Z-form region in longer DNA.
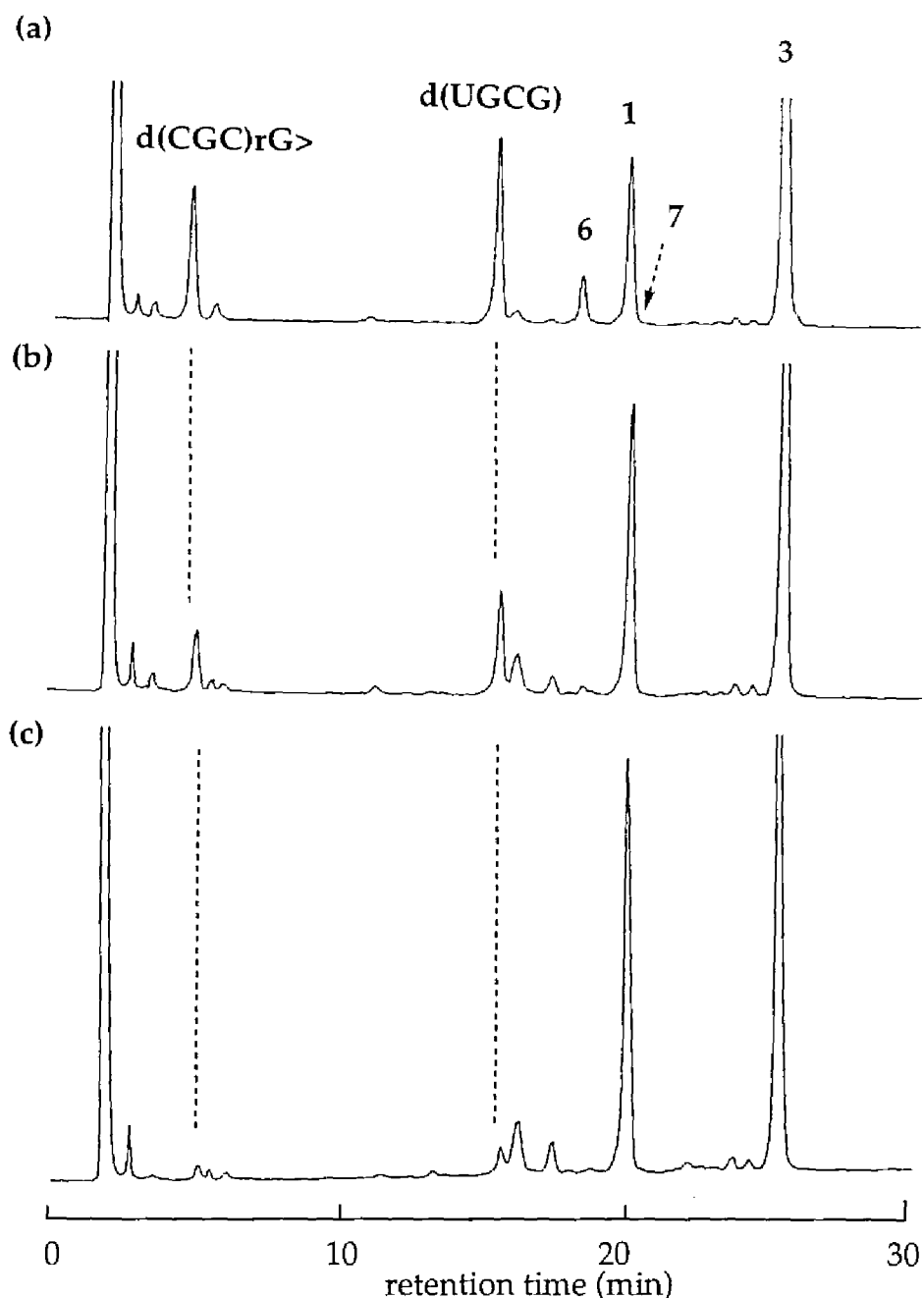
Figure 4. HPLC profiles of photoirradiated d(CGCG^{I}UGCG) (ODN 1)/d(Cm^{8}GCACm^{8}GCG) (ODN 3) after ribonuclease T1 treatment in the presence of (a) 1.5 M NaCl, (b) 0.4 M NaCl, and (c) 0.1 M NaCl. Each of the reaction mixture (50 μL) containing octamer (0.5 mM total base concentration) NaI (50 mM) and NaCl (0.1, 0.4 and 1.5 M) in 50 mM Na cacodylate buffer (pH 7.0) in a quartz capillary cell was irradiated for 1 h at 0 °C with transilluminator (302 nm). To 10 μL aliquot was added 0.5 μL of

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ribonuclease T1 (10 unit/μL), and the solution was incubated at 37 °C for 4 h. The reaction mixture was analyzed by HPLC on Cosmosil 5C18 MS column (4.6 x 150 mm) detected at 254 nm; elution was with 0.05 M ammonium formate (pH 6.5) containing 2 - 7 % acetonitrile, linear gradient, 30 min, at a flow rate of 1.0 mL/min, at 40 °C.   Under these condition all hydroxylated product 7 were hydrolyzed to d(CGC)rG> and d(UGCG) judged by HPLC analysis.
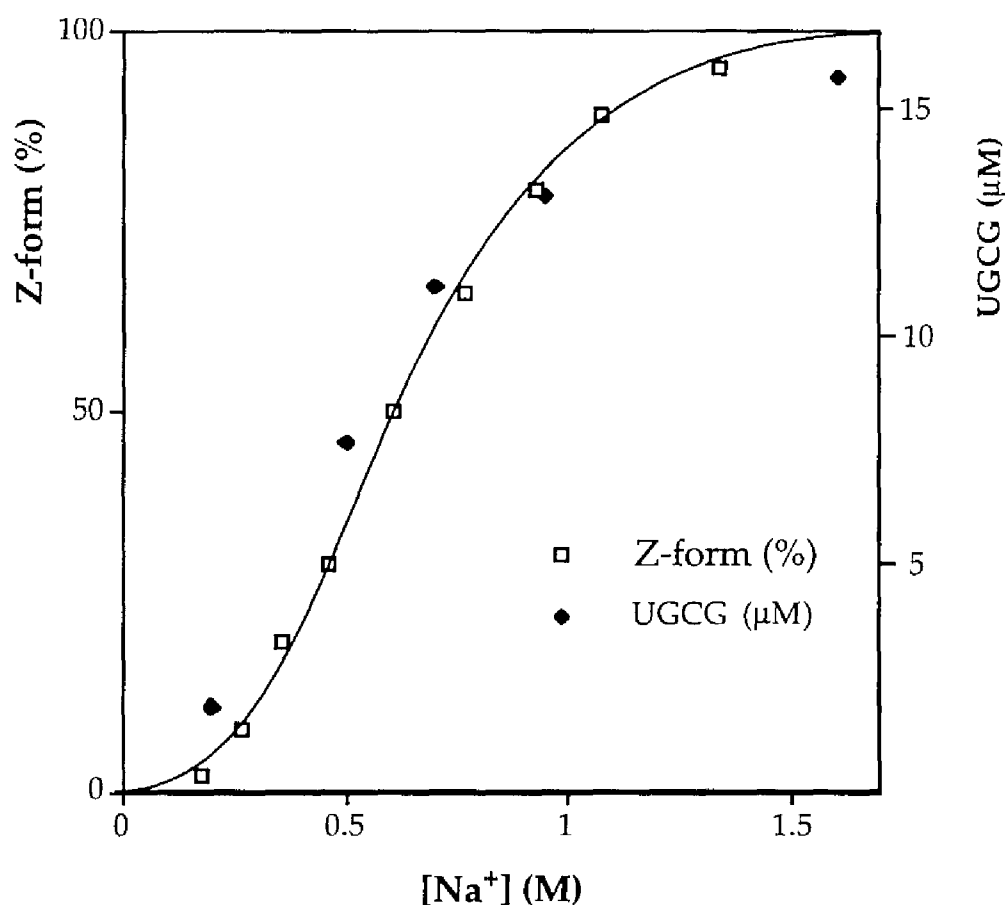

Figure 5.   Formation of d(UGCG) and the proportion of Z-conformation of ODN 1-3 as a function of [Na+].   The proportion of Z-conformation was estimated by CD and UV spectroscopy as previously described.[7,9]   The amounts of d(UGCG) from photoirradiated ODN 1-3 at different NaCl concentrations after ribonuclease T1 treatment were determined by HPLC as described in Figure 4.

Conclusion

In the present study, we found that the stereospecific C2'α hydroxylation efficiently occurred at the 5' side of the 5-halouracil in Z-form DNA. 5-Halouracil-substituted DNA is known to be functional *in vivo*. For instance, all thymine residues in *Ecoli* genomic DNA can be substituted with ^{Br}U. Since the C2'α hydroxylation sites in DNA can be easily detected by ribonuclease T1, this photochemical and enzymatic method would be useful to detect the Z-form region in DNA as shown in Scheme 3.

Scheme 3
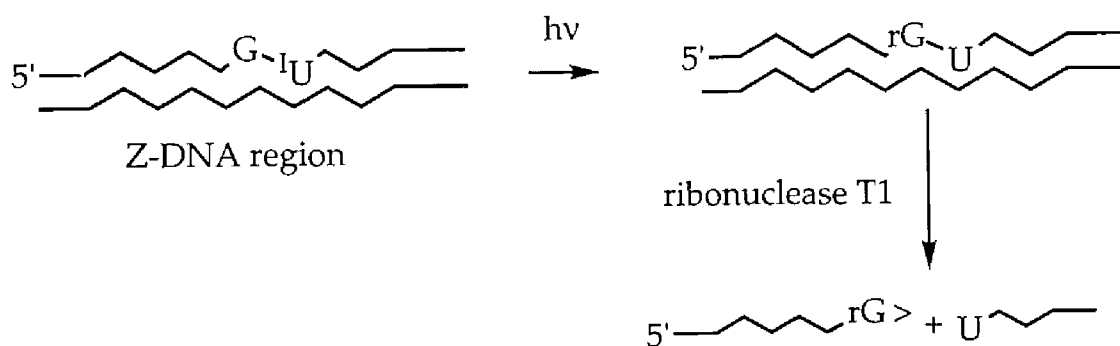
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Experimental Section

Materials and methods. Calf intestine alkaline phosphatase (AP, 1000 units/mL) and snake venom phosphodiesterase (s.v. PDE, 3 unit/mL) were purchased from Boehringer Mannheim. Nuclease P1 (P1, 1000 unit/mL), 5-iodo-2'-deoxyuridine and 5-bromo-2'-deoxyuridine was purchased from Yamasa Shoyu Co. Ltd. Ribonuclease T1 (Rnase T1, 10,000 unit/mL) was purchased from Pharmacia Biotech. 2-Cyanoethyl N,N,N',N'-tetraisopropyl-phosphorodiamidite was purchased from Aldrich. The reagents for DNA synthesizer such as I_2 solution (I_2/H_2O/pyridine/tetrahydrofuran, 0.5:9.5:20:70) A-, G-, C-, and T-β-(cyanoethyl)phosphoramidites were purchased from Applied Biosystems. 1H-tetrazole was purchased from Dojin Laboratories. HPLC analysis was carried out using PU-980 HPLC system (JASCO, Tokyo) equipped with a Chemcobond 5-ODS-H (4.6 x 150 mm) or a Cosmosil 5C18-MS column (4.6 x 150 mm). Detection was carried out at 254 nm. UV irradiation was carried out with a transilluminator (302 nm, Funakoshi FTI-36M). The ice cooled reaction mixture in quartz capillary cell (7 mm diameter) was irradiated from the distance of 5 cm under described conditions. Electrospray mass spectra were recorded on Perkin-Elmer Sciex API 165 systems.

Synthesis of oligodeoxynucleotides. Oligonucleotides were prepared by the β-(cyanoethyl)phosphoramidite method on controlled pore glass supports (1 µmol) by using ABI 381 A DNA synthesizer. Cyanoethyl phosphoramidite of 5-iodo-2'-deoxyuridine and 2'-deoxyuridine was prepared according to the

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standard phosphoramidite chemistry on a DNA synthesizer. Cyanoethyl phosphoramidite of 8-methyl-2'-deoxyguanosine were prepared by the reported procedure.[7] After automated synthesis, the oligomer was detached from the support, deprotected and purified by HPLC as described previously. After lyophilization, approximately 20 OD of pure oligomer was isolated. Purity and concentrations of all oligonucleotides were determined by complete digestion with s.v. PDE and AP to 2'-deoxymononucleosides.

CD measurements. Circular dichroism (CD) spectra were recorded on Jasco J-700 spectrophotometer equipped with a Peltier temperature controller. CD spectra of oligonuclotide solutions (0.1 mM duplex and 2 M NaCl in 50 mM sodium cacodylate, pH 7.0) were recorded using 1 cm path length cell.

Characterization of photoproducts (4), (5) and (6). Photoproduct (4) and (5) were characterized according to the published procedure.[5b] Halogen exchanged product (6) was characterized according to the published procedure.[9] Formation of photoproducts (4), (5) and (6) was futher confirmed by electrospray MS. Electrospray MS (negative); (4): calcd 2277.5, found 2277.1., (5): calcd 2265.5, found 2264.8., (6): calcd 2447.1, found 2446.2.

Characterization of product 7. The reaction mixture (total volume 200 μL) contained deoxyoligonucleotides d(CGCG^{I}UGCG) (1)/ d(Cm^{8}GCACm^{8}GCG) (3) (0.5 mM total base concentration) and 2 M NaCl in 50 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary

cell. After irradiation at 0 °C for 3 h, the resulting mixture was subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column. Elution was with 0.05 M ammonium formate containing 2 - 8 % acetonitrile, linear gradient (30 min) at a flow rate of 1.0 mL/min, at 30 °C. The HPLC peak at 24.0 min was collected and the fraction was concentrated. The residue was dissolved in water and then subjected to enzymatic digestion with s.v. PDE (0.3 unit/mL) and calf intestine AP (100 unit/mL), and the mixture was subjected to HPLC analysis. HPLC conditions; Cosmosil 5C18-MS column; 0.05M ammonium formate containing 2 - 10 % acetonitrile, linear gradient, 20 min; flow rate of 1.0 mL/min. Formation of rG, dC, dU and dG in a raito of 1:3:1:3 was observed. Product 7 was further confirmed by electrospray MS. Electrospray MS (negative); calcd 2428.6, found 2428.2.

Quantitative analysis of photoirradiated [1]U-containing B-form and Z-form oligodeoxynucleotides. The reaction mixture (total volume 100 μL) contained 0.5 mM (base concentration) of ODN and described sodium salt in 50 mM sodium cacodylate buffer (pH 7.0) in quartz capillary cell. After irradiated at 0 °C for 1 h, 10 μL of the aliquot was taken up and subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column; elution was with 0.05 M ammonium formate containing 2 - 8 % acetonitrile, linear gradient (30 min) at a flow rate of 1.0 mL/min, at 30 °C. Yield of C1'-oxidation (4), C2'-oxidation products (5), halogen exchanged product (6) and C2'-hydroxylation product (7) were determined by comparison of their HPLC peak areas with the authentic samples.

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Chapter 5

Intrastrand 2'β Hydrogen Abstraction of 5'-Adjacent Deoxyguanosine by Deoxyuridin-5-yl in Z-form DNA

Abstract

In order to identify the site of H abstraction by the deoxyuridin-5-yl radical in Z-form DNA, we examined the photoreaction of d(CGCG(2'β-D)^{I}UGCG) **2-d** / d(Cm^{8}GCACm^{8}GCG) **3** whose G_4 residue was deuterated with the diastereoselectivity of 2'R:2'S=91:9. Electrospray MS analysis of ribonuclease T1 digested fragments of the 2'α-hydroxylation product **4** demonstrated that 2'α-hydroxylation results from the abstraction of the 2'β-H atom by the deoxyuridin-5-yl in Z-form DNA.

Introduction

DNA local conformations such as A form, Z form, triplex and DNA-RNA hybrid have been suggested to play an important role in biological processes.[1]   However, the biological relevance of DNA local conformations is still not well understood due to the lack of a reliable detection method which is applicable to a living cell.   Since the DNA local structures are assumed to appear in a very short period of time, utilization of a photoreaction as a DNA conformational probe would provide important structural information on a short-lived DNA local structure.   Recently, we found that the 2'-deoxyuridin-

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5-yl generated in the fixed geometry of a DNA duplex undergoes H abstraction in a highly DNA conformation-dependent manner. For example, the competitive 1'- and 2'α-H abstractions occurred in the B-form DNA,[2] whereas the predominant 1'-H abstraction occurred in a DNA-RNA hybrid.[3] More recently, we found that preferential 2'α-hydroxylation occurred efficiently in Z-form DNA.[4] Inspection of the X-ray structure of the purine-pyrimidine step in Z-DNA indicated that the 2'β H of purine is close to the C5 position of the pyrimidine, suggesting a selective abstraction of 2'β-H by the deoxyuridin-5-yl (Figure 1).[5] In this chapter, we examined the photoreaction of d(CGCG(2'β-D)^{I}UGCG) **2-d**/ d(Cm^{8}GCACm^{8}GCG) **3** in which 2'β-H of the G$_4$ residue was stereoselectively deuterated, and demonstrated that the 2'α-hydroxylation results from the abstraction of the 2'β-H by the deoxyuridin-5-yl.
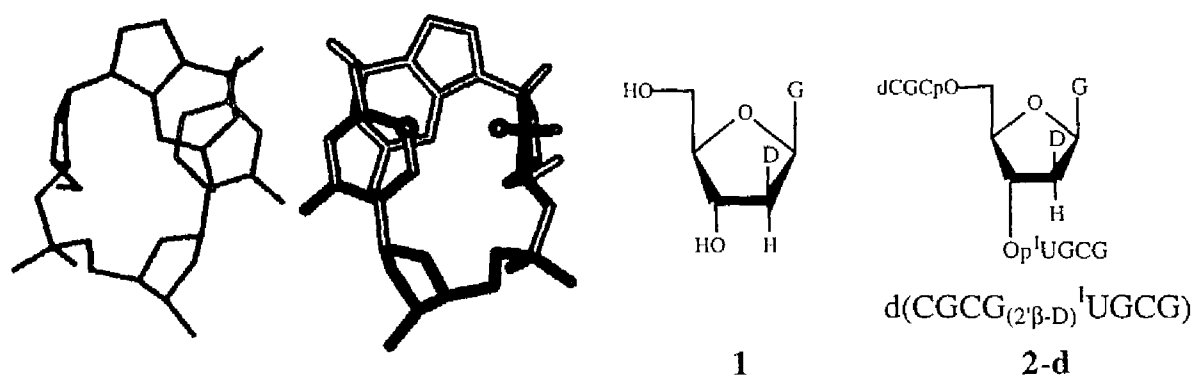
Figure 1. Purine-pyrimidine step of Z-DNA. C2′β hydrogen and C5 carbon are represented by balls. The complementary strand is shown as a thin line.

Result and Discussion

The stereoselectively deuterated nucleoside, (2'S)-[2'-^{2}H]-2'-deoxyguanosine **1** with the diastereoselectivity of 2'R:2'S=91:9, was synthesized according to the previous method[6] and incorporated into the oligonucleotide via the standard phosphoramidite method. The octanucleotide d(Cm^{8}GCACm^{8}GCG) **3** as a complement for

d($CGCG^IUGCG$) **2** and d($CGCG(2'\beta$-D$)^IUGCG$) **2-d** was prepared as previously reported.[4,7] The structure of the octamers was confirmed by enzymatic digestion and by electrospray MS (ESMS). The 5-iodouracil-containing Z-form duplex **2-d/3** was irradiated at 302 nm with a transilluminator. The 2'α-hydroxylated product **4** produced was isolated and treated with ribonuclease T1. As previously reported,[4] the 2'α-hydroxylated product **4** was quantitatively hydrolyzed to d(CGC)rG> (> : cyclic phosphate) **5** and d(UGCG) **6** (Scheme 1).

Scheme 1

5'-d($CGCG^IUGCG$)-3' **2**

5'-d(Cm^8GCACm^8GCG)-3' **3**   hv (302 nm)

m^8G = 2'-deoxy-8-methylguanosine

dCGCpO—O G

ribonuclease T1

OH
OpUGCG
4

d(CGC)rG> + d(UGCG)

5   **6**
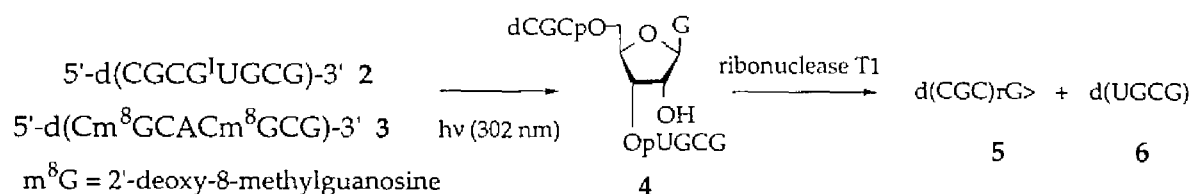
In order to identify the position of deuterium (D) after the 2'α-hydroxylation reaction, the hydrolyzed fragments **5** and **6** were subjected to ESMS. Figure 2 shows the ESMS of **5** and **6** obtained from the photoreaction of **2-d-3** and the unlabeled octamers (control), indicating that 2'β-D is specifically abstracted and incorporated into **6**. These results clearly indicate that 2'β-H of G_4 was abstracted by the 5'-adjacent deoxyuridin-5-yl as an initiation step for the formation of the 2'α-hydroxylated product **4** (Scheme 2). The intramolecular kinetic isotope effects (KIE) [(k_H/k_D)=(%4/%4-d)] obtained for the 2'α-hydroxylation by photoirradiation of the [I]U-containing Z-form oligonucleotide was found to be 1.2±0.1. The magnitude of KIE obtained in the present study was in the range of 1.0 to 5.8 which were previously reported for 1', 4', and 5' H abstraction by several antitumor antibiotics.[8] However, the KIE value of 1.2±0.1 is

substantially lower than the value 7.2 which was observed for the 2'α-H abstraction in B-form DNA.[2c]
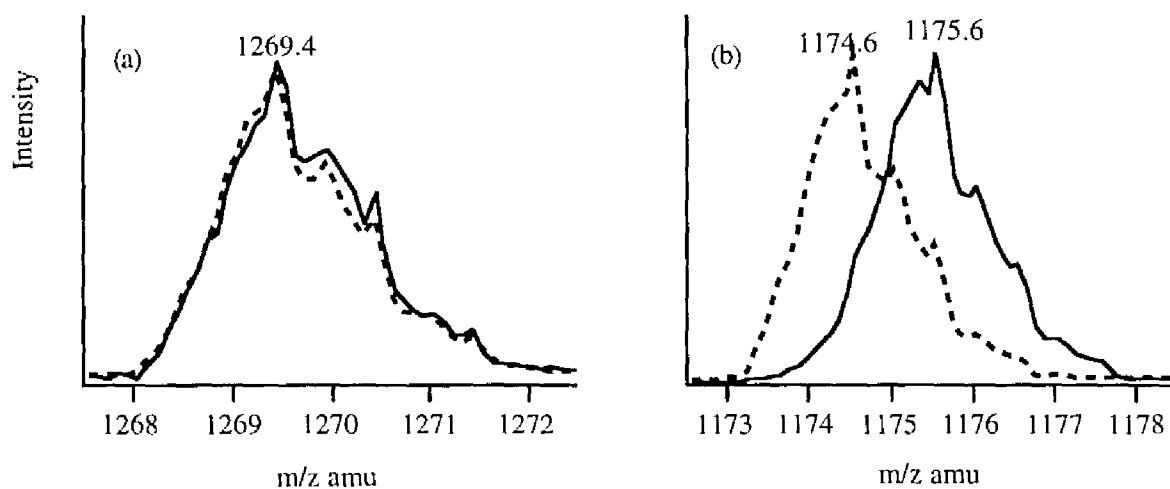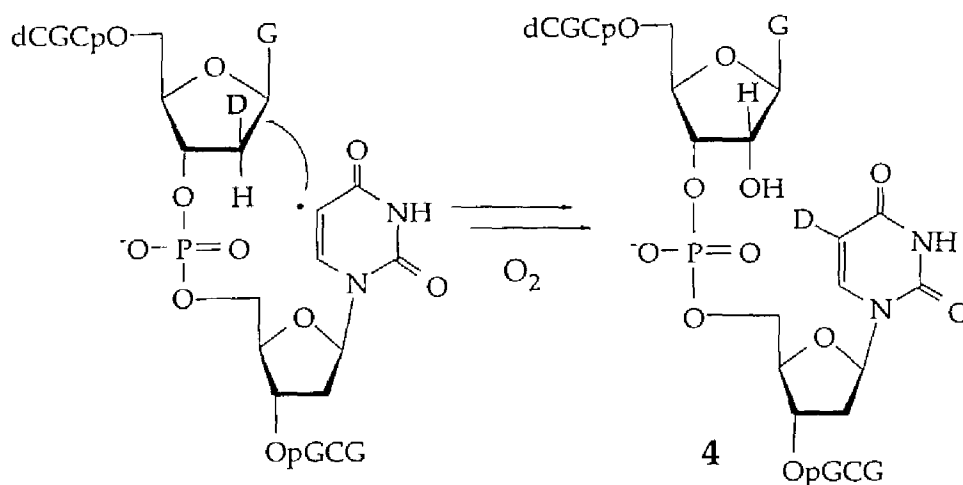
Scheme 2

Figure 2. ESMS of (a) d(CGC)rG> **5** and (b) d(UGCG) **6.** MS of the products obtained from the deuterated and unlabeled octamers are indicated by the solid and dashed lines, respectively.

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We previously developed a simple method to evaluate the conformational energy needed to achieve the transition state for H abstraction in DNA.[3] The calculation provided a qualitative explanation for the competitive 1'- and 2'α-H abstractions and the selective 1'-H abstraction by the deoxyuridin-5-yl in DNA duplex and DNA-RNA hybrid, respectively. Based on this method, the transition states for the 1'-, 2'α- and 2'β-H abstractions in Z-form DNA were calculated and are summarized in Table 1. These results suggest that the AMBER energy of the 2'β H-abstraction in Z-form DNA is significantly low compared to the other transition states. The smaller KIE observed in Z-DNA would be a consequence of the lower energy for 2'β-H abstraction in Z-form DNA.

Table 1. Minimized AMBER Total Energies (kcal/mol) of Octamer Containing Transition States for Ribose Hydrogen Abstraction by Uracilyl-5-yl Radical[a]

| Conformation | Transition state | | | Unmodified |
	C1'	C2'α	C2'β	
Z-form	-91.1 (14.6)[b]	-85.5 (20.2)[b]	-95.2 (10.5)[b]	-105.7

[a]Starting structures were generated from standard Z DNA in a builder module of Insight II (MSI, San Diego, CA). The models were energy minimized by conjugate gradient (0.001 kcal/molÅ). To prevent unusual distortion of the helix, the terminal residues were tethered to the initial structures (100 kcal/molÅ). Total energies of the molecules were evaluated using AMBER force field and distance-dependent dielectric of 4r. [b]The value in parentheses is the difference in energy from the corresponding unmodified octamers d(CGCGUGCG)/d(CGCACGCG).

Conclusion

In this chapter, using stereoselectively deuterated octanucleotide, we demonstrated that the C2' α-hydroxylation was a consequence of the reaction of O_2 with the C2' radical which was resulted from 2' β-H abstraction by the deoxyuridin-5-yl formed in Z-form DNA.

Experimental Section

Materials and methods. Calf intestine alkaline phosphatase (AP, 1000 units/mL) and snake venom phosphodiesterase (s.v. PDE, 3 unit/mL) were purchased from Boehringer Mannheim. 5-iodo-2'-deoxyuridine was purchased from Yamasa Shoyu Co. Ltd. Ribonuclease T1 (Rnase T1, 10,000 unit/mL) was purchased from Pharmacia Biotech. 2-Cyanoethyl N,N,N',N'-tetraisopropyl-phosphorodiamidite was purchased from Aldrich. The reagents for DNA synthesizer such as I_2 solution (I_2/H_2O/pyridine/tetrahydrofuran, 0.5:9.5:20:70) A-, G-, C-, and T-β-(cyanoethyl)phosphoramidites were purchased from Applied Biosystems. 1H-tetrazole were purchased from Dojin Laboratories. HPLC analysis was carried out using PU-980 HPLC system (JASCO, Tokyo) equipped with a Cosmosil 5C18-MS column (4.6 x 150 mm). Detection was carried out at 254 nm. UV irradiation was carried out with a transilluminator (302 nm, Funakoshi FTI-36M). The ice cooled reaction mixture in quartz capillary cell (7 mm diameter) was irradiated from the distance of 5 cm under described conditions. Electrospray mass spectra were recorded on Perkin-Elmer Sciex API 165 systems.

Synthesis of oligodeoxynucleotides. Oligonucleotides were prepared by the β-(cyanoethyl)phosphoramidite method on controlled pore glass supports (1 μmol) by using ABI 381 A DNA synthesizer. Cyanoethyl phosphoramidite of 5-iodo-2'-deoxyuridine and 8-methyl-2'-deoxyguanosine were prepared by the reported procedure.[4] After automated synthesis, the oligomer was detached from the support, deprotected and purified by HPLC.

After lyophilization, approximately 20 OD of pure oligomer was isolated. Purity and concentrations of all oligonucleotides were determined by complete digestion with s.v. PDE and AP to 2'-deoxymononucleosides.

Quantitative analysis of photoirradiated ^{I}U-containing Z-form oligodeoxynucleotides. The reaction mixture (total volume 100 μL) contained 0.5 mM (base concentration) of ODN and 2 M NaCl in 50 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary cell. After irradiated at 0 °C for 2 h, 10-μL aliquot was directly subjected to enzymatic digestion with snake venom PDE (0.3 unit/mL) and calf intestine alkaline phosphatase (100 unit/mL) and was analyzed by reverse phase HPLC.

Rnase T1 digestion of 2'-hydoxylation products. To a solution containing HPLC purified 2'-hydroxylated product (4), Rnase T1, 10,000 unit/mL was added. After 2 h of incubation at 37 °C , the solution was subjected to HPLC analysis. HPLC analysis was carried out on a Cosmosil 5C18-MS column. Elution was with 0.05 M ammonium formate containing 2 - 10 % acetonitrile, linear gradient (20 min) at a flow rate of 1.0 mL/min, at 30 °C. HPLC peak at 12.4 min and 17.0 min were concentrated and subjected to ESMS analysis.

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Chapter 6

# Photochemical Halogen-Exchange Reaction of 5-Iodouracil-Containing Oligonucleotides

Abstract

Photoreactions of 5-iododeoxyuridine ($d^I U$) in aqueous solutions in the presence of various inorganic salts have been investigated. In the presence of NaCl and NaBr, $d^I U$ undergoes an efficient photochemical halogen-exchange reaction to give $d^{Cl} U$ and $d^{Br} U$, respectively.

Introduction

Photoreactions of 5-halouracils are important tools for investigating nucleic acid structures and nucleic acid-protein interactions.[1] Especially, 5-iodouridine ($d^I U$) has been widely used as a photo-cross-linking agent, and the photoreactions of $d^I U$ monomer[2] or the photo-cross-linking of $d^I U$-containing DNA with nucleic acid binding proteins have been extensively studied.[3] $d^I U$-containing DNA has also been known to cause DNA strand breaks via the creation of alkali labile sites.[4] We have been investigating the molecular mechanism of such DNA lesions and the effect of DNA conformational change on the photoreactivity of $d^I U$- and $d^{Br} U$-containing DNA.[5] During the careful product analysis of the photoreaction of $d^I U$-containing-oligonucleotides, I have accidentally

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found the formation of 5-chlorouridine (d^{Cl}U) from d^{I}U, which was resulted from the halogen exchange with NaCl used in an aqueous buffer solution. While many inorganic salts exist in a biological system, direct photoreactions of 5-halouracil-containing DNA or RNA with inorganic salts have not been recognized so far. In this chapter, I have investigated the photoreactions of 5-iodouridine in the presence of various inorganic salts and found that the 5-iodo of d^{I}U is efficiently replaced by other halogen atoms. We also applied this novel photo-halogen-exchange reaction for the post modification of 5-iodouracil-containing DNA.

R = deoxyribose, ODN   X = Cl, Br
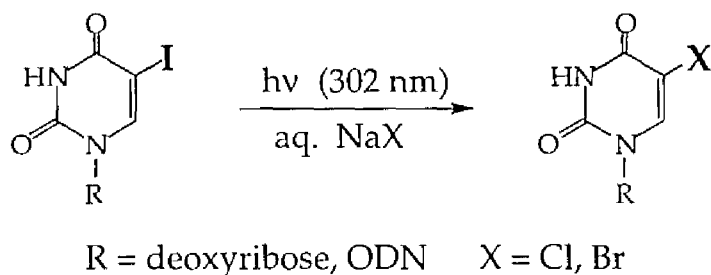
Results and Discussion

Photoirradiation of monomer d^{I}U with transilluminator (302 nm) in water in the presence of 100 mM 2-propanol as H-donor gave deoxyuridine (dU) as a sole product. Upon photoirradiation of d^{I}U in the presence of 4 M NaCl, d^{Cl}U was produced in high yields in addition to the formation of dU. Similarly, when photoirradiation of d^{I}U was conducted in the presence of 4 M NaBr, halogen exchanged product d^{Br}U was also obtained as a major photoproduct together with dU as shown in Table 1 and Figure 1. In both cases, the consumption of d^{I}U was remarkably enhanced by added salts without significant increase of dU. To verify the effect of halogen anions, photoreaction in the presence of NaI was compared with that in the presence of NaClO$_4$. Added NaI cosiderably accelerated the consumption of d^{I}U, whereas NaClO$_4$ gave rather a inhibitory effect

on the rate of the disappearance of d^{I}U. These results indicated that the presence of halogen anion dramatically accelerate the photoconversion of d^{I}U.

Table 1. Effect of Inorganic Salts on the Photoreaction of 5-Iododeoxyuridine[a]

run	salt	consumption (%) (-d^{I}U)	products (%) dU	products (%) d^{Cl}U	products (%) d^{Br}U
1	no salt	29	29	-	-
2	NaCl 4 M	49	30	19	-
3	NaBr 4 M	87	39	-	47
4	NaI 4 M	61	61	-	-
5	NaClO$_4$ 2 M	17	13	-	-

[a]Each of the reaction mixtures (200 μL) containing 100 μM d^{I}U and 100 mM i-PrOH in a capillary cell was irradiated for 10 min at 0 °C with transilluminator (302 nm) under identical conditions.
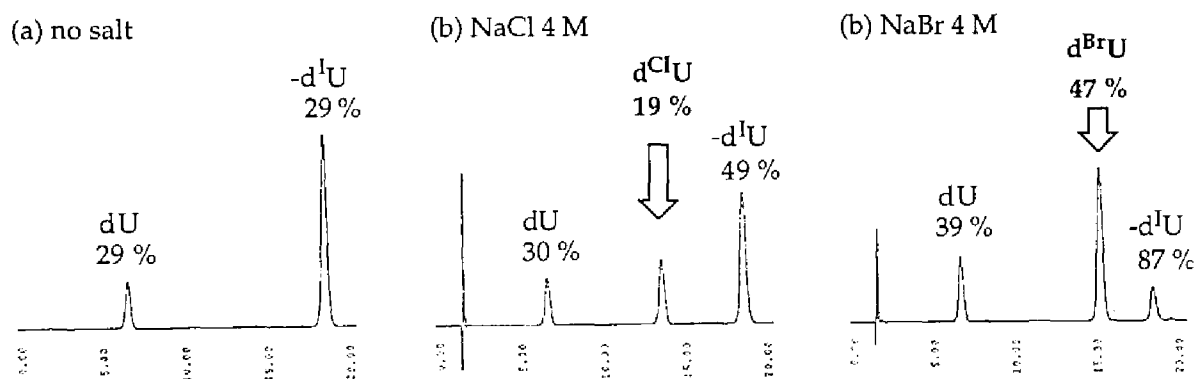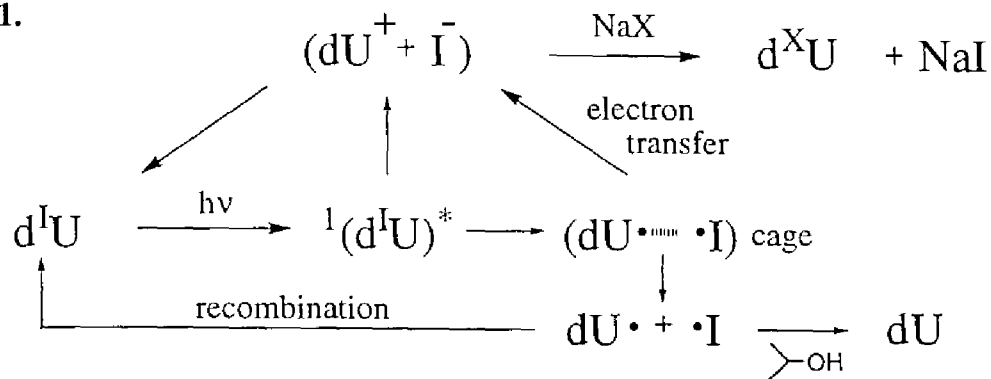

Figure 1. HPLC profiles for UV-irradiated d^{I}U in the presence of inorganic salts. The reaction mixture was analyzed by HPLC on a Chemcobond 5-ODS-H column (4.6 x 150 mm), detected at 280 nm. Elution was with 0.05 M ammonium formate containing 2 - 10 % acetonitrile linear gradient (20 min) at a flow rate of 1.0 mL/min. The reaction conditions are shown in Table 1.
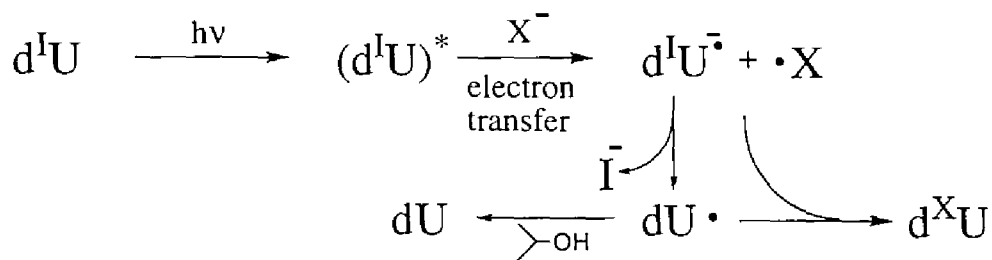
Previous studies on the photoreactions of vinyl iodides have indicated that in organic solvents vinyl iodides can afford photoproducts which are derived from carbocationic intermediates. Such carbocationic intermediates have been suggested to arise from an electron transfer within an initially formed caged radical pair.[6] Therefore, one possible mechanism for the present photo-halogen-exchange reaction may involve nucleophilic attack of halogen anion (X⁻) to the carbocation generated at uracil C5 as shown in Scheme 1 (a). However, such carbocationic intermediate has never been detected in the previous photoreactions of d^IU.[2,3] When the photoirradiation of d^IU was conducted in the presence of several nucleophiles such as methanol and ethane-1,2-diol,[6a] there were no indication for the formation of the products derived from the nucleophilic trapping of carbocationic intermediate dU^+.   Schuster *et al.*[7] have recently reported the photoinduced electron transfer from chloride anion to photoexcited anthraquinone to result in a formation of ·Cl.   An alternative mechanism for the present photo-halogen-exchange reaction may be a recombination of halogen radical ·X, generated by electron transfer with uracilyl radical which was also derived from electron transfer from X⁻ and subsequent release of I⁻ as shown in Scheme 1 (b).   Such mechanism would also explain the acceleration of the decomposition of d^IU in the presence halide anion.   Further study is necessary for the elucidation of the mechanism of this noble photoreaction.

Scheme 1.

(a)

$$(dU^{\cdot+} + \bar{I}) \xrightarrow{\text{NaX}} d^{X}U + NaI$$

$$d^{I}U \xrightarrow{h\nu} {}^{1}(d^{I}U)^{*} \longrightarrow (dU\cdot\!\!\cdots\cdot I)\ \text{cage}$$

electron transfer

recombination

$$dU\cdot{}^{+} \cdot I \xrightarrow[\text{>-OH}]{} dU$$

(b)

$$d^{I}U \xrightarrow{h\nu} (d^{I}U)^{*} \xrightarrow[\text{electron transfer}]{X^{-}} d^{I}U^{\cdot-} + \cdot X$$

$$dU \xleftarrow[\text{>-OH}]{} dU\cdot \xrightarrow{} d^{X}U$$

$\bar{I}$

We next examined the effect of halogen anion on the photoreaction of $d^{I}U$-containing oligonucleotides. Likewise, $d^{I}U$-containing oligonucleotides undergo an efficient photo-halogen-exchange reaction with NaCl and NaBr to yield $d^{Cl}U$- (1, 4) and $d^{Br}U$-containing oligonucleotides (2, 5), respectively, along with 2'-deoxyribonolactones (3 and 6) as a minor side product as shown in Table 2. These results clearly shows that $d^{Cl}U$- and $d^{Br}U$-containing DNA are readily available by photochemical post modification of $d^{I}U$-containing DNA in the presence of appropriate inorganic salts. Interestingly, when the photoirradiation of $d^{Br}U$-containing oligonucleotide was conducted in the presence of 1 M NaI, $d^{I}U$-containing oligonucleotide was obtained. Therefore, this photo-halogen-exchange reaction may also be very useful for the preparation of isotopically labeled $d^{I}U$-containing DNA by using $Na^{125}I$.
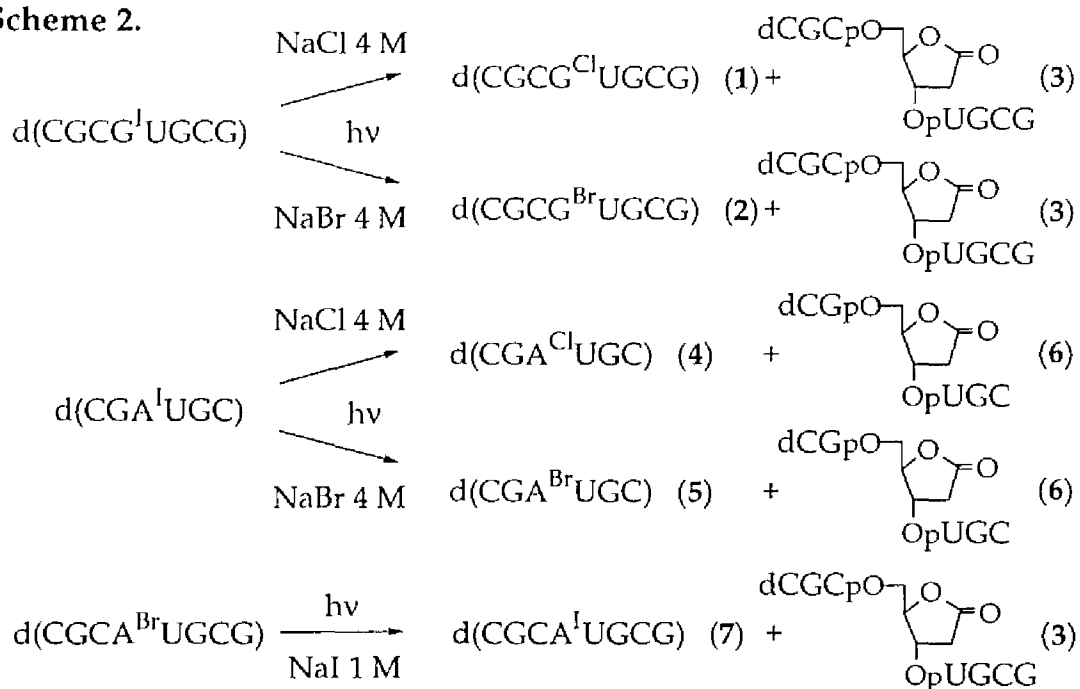
Scheme 2.

$$d(CGCG^I UGCG) \xrightarrow[\text{NaBr 4 M}]{\overset{\text{NaCl 4 M}}{\;h\nu\;}}$$

d(CGCG^{Cl}UGCG) (1) +

dCGCpO—OpUGCG (3)

d(CGCG^{Br}UGCG) (2) +

dCGCpO—OpUGCG (3)

$$d(CGA^I UGC) \xrightarrow[\text{NaBr 4 M}]{\overset{\text{NaCl 4 M}}{\;h\nu\;}}$$

d(CGA^{Cl}UGC) (4) +

dCGpO—OpUGC (6)

d(CGA^{Br}UGC) (5) +

dCGpO—OpUGC (6)

$$d(CGCA^{Br}UGCG) \xrightarrow[\text{NaI 1 M}]{h\nu} d(CGCA^I UGCG)\; (7) +$$

dCGCpO—OpUGCG (3)

Table 2. Product Analysis in the Photoirradiation of 5-Iodouracil-Containing Deoxyoligonucleotides[a]

ODN	run	salt	consumed ODN, %	halogen exchanged product (%)	ribonolactone (%)
d(CGCG^{I}UGCG)	1	no salt	10	-	3 (3>)
	2	NaCl 4 M	51	1 (20)	3 (3>)
	3	NaBr 4 M	93	2 (46)	3 (3>)
d(CGA^{I}UGC)	4	no salt	58	-	6 (13)
	5	NaCl 4 M	77	4 (22)	6 (9)
	6	NaBr 4 M	99	5 (48)	6 (7)
d(CGCA^{Br}UGCG)[b]	7	NaI 1 M	70	7 (15)	3 (23)

[a]Each of the reaction mixtures (100 μL) containing ODN (0.20 mM base conc.) in 50 mM sodium cacodylate buffer (pH 7.0) in a capillary cell was purged with argon for 2 min and irradiated for 1 h at 0 °C with transilluminator (302 nm) under the described salt conditions. [b]Photoirradiation was conducted for 10 minute.

Conclusion

In summary, we have demonstrated for the first time that d^IU-and d^IU-containing DNA undergo an efficient photo-halogen-exchange reaction in the presence of halide ions to produce C-5 halogen exchanged uracils. This efficient photoreaction of 5-halouracils in the presence of inorganic salts should be seriously taken into consideration when photoinduced crosslinking experiments using d^IU containing DNA are conducted in salt-containing solvent systems.

Experimental Section

Materials and methods. Calf intestine alkaline phosphatase (AP, 1000 units/mL) and snake venom phosphodiesterase (s.v. PDE, 3 unit/mL) were purchased from Boehringer Mannheim. 5-Iodo-2'-deoxyuridine and 5-bromo-2'-deoxyuridine was purchased from Yamasa Shoyu Co. Ltd. 5'-*O*-dimethoxytrityl-2'-deoxyuridine was prepared according to the published procedure.[5] 2-Cyanoethyl N,N,N',N'-tetraisopropylphosphorodiamidite and 5-chloro-2'-deoxyuridine were purchased from Aldrich. The reagents for DNA synthesizer such as I_2 solution (I_2/H_2O/pyridine/tetrahydrofuran, 0.5:9.5:20:70) A-, G-, C-, and T-β-(cyanoethyl)phosphoramidites were purchased from Applied Biosystems. 1H-tetrazole were purchased from Dojin Laboratories. NaCl, NaBr and NaI were of puriss grade and were used as received. HPLC analysis was carried out using PU-980 HPLC system (JASCO, Tokyo) equipped with a Chemcobond 5-ODS-H (4.6 x 150 mm) or a Cosmosil 5C18-MS column (4.6 x 150 mm). Detection was carried out at 280 nm for monomer and at 254 nm for ODN. UV irradiation was carried out with a transilluminator (302 nm, Funakoshi FTI-36M). The ice cooled reaction mixture in quartz capillary cell (7 m m diameter) was irradiated from the distance of 5 cm under described conditions. Electrospray mass spectra were recorded on Perkin-Elmer Sciex API 165 systems.

Synthesis of oligodeoxynucleotides. Oligonucleotides were prepared by the β-(cyanoethyl)phosphoramidite method on controlled pore glass supports (1 μmol) by using ABI 381 A DNA synthesizer. Cyanoethyl phosphoramidite of 5-iodo-2'-deoxyuridine was prepared by the reported procedure.[5] After automated

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synthesis, the oligomer was detached from the support, deprotected and purified by HPLC as described previously. After lyophilization, approximately 20 OD of pure oligomer was isolated. Purity and concentrations of all oligonucleotides were determined by complete digestion with s.v. PDE and AP to 2'-deoxymononucleosides.

Quantitative analysis of photoirradiated d^IU. The reaction mixture (total volume 200 µL) contained 0.1 mM of d^IU ,100 mM isopropanol and indicated sodium salt in a quartz capillary cell was irradiated with a transilluminator at 0 °C for 10 min. After irradiation, 10 µL of the aliquot was taken up and subjected to HPLC analysis. Analysis was carried out on a Chemcobond 5-ODS-H column; elution was with 0.05 M ammonium formate containing 2 - 10 % acetonitrile, linear gradient (20 min) at a flow rate of 1.0 mL/min. Yield of halogen-exchanged product $d^{Br}U$ and $d^{Cl}U$ were determined by comparison of HPLC peak areas of the authentic samples. HPLC profile of the photoirradiated d^IU was shown in Figure 1.

Characterization of photo-halogen exchanged product (1). The reaction mixture (total volume 200 µL) contained 0.2 mM (base concentration) $d(CGCG^IUGCG)$ and 4 M NaCl in 5 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary cell. After irradiated at 0 °C for 3 h, the resulting mixture was subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column. Elution was with 0.05 M ammonium formate containing 2 - 8 % acetonitrile, linear gradient (30 min) at a flow rate of 1.0 mL/min. The HPLC peak at 23.2 min was collected and the fraction was concentrated. The residue was dissolved in water and then subjected to enzymatic

digestion with s.v. PDE (0.3 unit/mL) and calf intestine AP (100 unit/mL), and the mixture was subjected to HPLC analysis: HPLC conditions; Cosmosil 5C18-MS column; 0.05M ammonium formate containing 2 - 10 % acetonitrile, linear gradient, 20 min; flow rate of 1.0 mL/min. The formation of 5-chlorodeoxyuridine together with dC, and dG was observed. The formation of halogen-exchanged products **1** was confirmed by electrospray MS. Electrospray MS (negative); **1**: calcd 2447.1, found 2446.2.

Characterization of photo-halogen exchanged product (2). The reaction mixture (total volume 200 μL) contained 0.2 mM (base concentration) d(CGCG^{I}UGCG) and 4 M NaBr in 5 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary cell. After irradiated at 0 °C for 3 h, the resulting mixture was subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column. Elution was with 0.05 M ammonium formate containing 2 - 8 % acetonitrile, linear gradient (30 min) at a flow rate of 1.0 mL/min. The HPLC peak at 23.6 min was collected and the fraction was concentrated. The residue was dissolved in water and then subjected to enzymatic digestion with s.v. PDE (0.3 unit/mL) and calf intestine AP (100 unit/mL), and the mixture was subjected to HPLC analysis: HPLC conditions; Cosmosil 5C18-MS column; 0.05M ammonium formate containing 2 - 10 % acetonitrile, linear gradient, 20 min; flow rate of 1.0 mL/min. The formation of 5-bromodeoxyuridine together with dC, and dG was observed. The formation of halogen-exchanged products **2** was confirmed by electrospray MS. Electrospray MS (negative); **2**: calcd 2491.5, found 2491.4.

Characterization of photo-halogen exchanged product (4).
The reaction mixture (total volume 200 μL) contained 0.2 mM (base concentration) d(CGA^{I}UGC) and 4 M NaCl in 5 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary cell.　After irradiated at 0°C for 3 h, the resulting mixture was subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column.　Elution was with 0.05 M ammonium formate containing 2 - 15 % acetonitrile, linear gradient (20 min) at a flow rate of 1.0 mL/min.　The HPLC peak at 14.9 min was collected and the fraction was concentrated. The residue was dissolved in water and then subjected to enzymatic digestion with s.v. PDE (0.3 unit/mL) and calf intestine AP　(100 unit/mL), and the mixture was subjected to HPLC analysis.　HPLC conditions; CHEMCOBOND 5-ODS-H column; 0.05M ammonium formate containing 2 - 10 % acetonitrile, linear gradient, 20 min; flow rate of 1.0 mL/min.　The formation of 5-chlorodeoxyuridine together with dC, dA and dG was observed.　The formation of halogen-exchanged products **4** was confirmed by electrospray MS. Electrospray MS (negative); **4**: calcd 1812.7, found 1811.7.

Characterization of photo-halogen exchanged product (5).
The reaction mixture (total volume 200 μL) contained 0.2 mM (base concentration) d(CGA^{I}UGC) and 4 M NaBr in 50 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary cell.　After irradiated at 0°C for 3 h, the resulting mixture was subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column.　Elution was with 0.05 M ammonium formate containing 2 - 15 % acetonitrile, linear gradient (20 min) at a flow rate of 1.0 mL/min.　The HPLC peak at 15.4 min was collected and the fraction was concentrated. The residue was dissolved in water and then subjected to enzymatic digestion with s.v. PDE (0.3 unit/mL) and calf intestine AP　(100

unit/mL), and the mixture was subjected to HPLC analysis. HPLC conditions; Chemcobond 5-ODS-H column; 0.05M ammonium formate containing 2 - 10 % acetonitrile, linear gradient, 20 min; flow rate of 1.0 mL/min. The formation of 5-bromodeoxyuridine together with dC, dA and dG was observed. The formation of halogen-exchanged products 5 was confirmed by electrospray MS. Electrospray MS (negative); 5: calcd 1857.1, found 1855.8.

Quantitative analysis of photoirradiated ^{I}U-containing oligodeoxynucleotide. The reaction mixture (total volume 100 μL) contained 0.2 mM (base concentration) of ODN and sodium salt indicated in 50 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary cell. After irradiated at 0 °C for 1 h, 10 μL of the aliquot was taken up and subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column; elution was with 0.05 M ammonium formate containing 2 - 15 % acetonitrile, linear gradient (20 min) for hexamer and 2 - 8 % acetonitrile, linear gradient (30 min) for octamer and at a flow rate of 1.0 mL/min. Yield of C1'-oxidation product and halogen-exchanged product were determined by comparison of HPLC peak areas of the authentic samples.

Characterization of photo-halogen exchanged product (7). The reaction mixture (total volume 200 μL) contained 0.2 mM (base concentration) d(CGGA^{Br}UGCG) and 1 M NaI in 50 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary cell. After irradiated at 0°C for 30 min, the resulting mixture was subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column. Elution was with 0.05 M ammonium formate containing 2 - 7 %

acetonitrile, linear gradient (30 min) at a flow rate of 1.0 mL/min, at 40 °C. The HPLC peak at 24.2 min was collected and the fraction was concentrated. The formation of halogen-exchanged products 7 was confirmed by coinjection with the authentic sample and by electrospray MS. Electrospray MS (negative); 5: calcd 2522.5, found 2521.7.

Quantitative analysis of photoirradiated ^{Br}U-containing oligodeoxynucleotide. The reaction mixture (total volume 100 μL) contained 0.2 mM (base concentration) of ODN and 1 M NaI in 50 mM sodium cacodylate buffer (pH 7.0) in a quartz capillary cell. After irradiated at 0°C for 10 min, 10 μL of the aliquot was taken up and subjected to HPLC analysis. Analysis was carried out on a Cosmosil 5C18-MS column; elution was with 0.05 M ammonium formate containing 2 - 7 % acetonitrile, linear gradient (30 min) at a flow rate of 1.0 mL/min, at 40 °C. Yield of C1'-oxidation product and halogen-exchanged product were determined by comparison of HPLC peak areas of the authentic samples.

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List of Publications

Chapter 1   Synthesis, Structure and Thermodynamic Properties
              of 8-Methylguanine-Containing Oligonucleotides: Z-
              DNA under Physiological Salt Conditions.
              Sugiyama, H.; Kawai, K.; Matsunaga, A.; Fujimoto, K.;
              Saito, I.; Robinson, H.; Wang, A. H.-J.   *Nucleic Acids
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Chapter 2   Effects of Methylation on B- to Z-DNA Transition.
              Kawai, K.; Sugiyama, H.; Saito, I.   *Nucleic Acids
              Symp. Ser.* **1996,** *35,* 87-88.

Chapter 3   Stabilization of Hoogsteen Base Pairing by
              Introduction of NH_2 Group at the C8 Position of
              Adenine.
              Kawai, K.; Saito, I.; Sugiyama, H.   *Tetrahedron Lett.*
              1998, *39,* 5221-5224.

Chapter 4   Conformation Dependent Photochemistry of 5-
              Halouracil Containing DNA: Stereospecific $2'\alpha$-
              Hydroxylation of Deoxyribose in Z-form DNA.
              Kawai, K.; Saito, I.; Sugiyama, H.   *J. Am. Chem. Soc.*
              in press

Chapter 5   Intrastrand $2'\beta$ Hydrogen Abstraction of 5'-Adjacent
              Deoxyguanosine by Deoxyuridin-5-yl in Z-form DNA.
              Kawai, K.; Saito, I.; Kawashima, E.; Ishido, Y.;
              Sugiyama, H.   *Tetrahedron Lett.*   in press

Chapter 6   Photochemical Halogen-Exchange Reaction of 5-
Iodouracil-Containing Oligonucleotides
Kawai, K.; Saito, I.; Sugiyama, H.   To be submitted.

List of Oral Presentations

1. "Regulation of B-Z Transition by Introduction of 8-Methyl-deoxyguanosine"
   K. Kawai, K. Fujimoto, A. Matsunaga, H. Sugiyama, I. Saito.
   69th Annual Meeting of Chemical Society of Japan, Kyoto, Japan, March, 1995.

2. "Photoreactivity of ^{Br}U-Containing Oligonucleotides in Z-form"
   K. Kawai, K. Fujimoto, H. Sugiyama, I. Saito.
   70th Annual Meeting of Chemical Society of Japan, Tokyo, Japan, March, 1996.

3. "Photoreactivity of Halouracil-Containing Oligonucleotides in Z-form"
   K. Kawai, H. Sugiyama, I. Saito.
   11th Symposium on Biofunctional Chemistry, Fukuoka, Japan, October, 1996.

4. "Photoreactivity of 5-Halouracil in Z-form DNA"
   K. Kawai, H. Sugiyama, I. Saito.
   72th Annual Meeting of Chemical Society of Japan, Tokyo, Japan, March, 1997.

5. "Photoreaction of 5-Halouracil-Containing Z-form DNA"
   K. Kawai, H. Sugiyama, I. Saito.
   19th Annual Meeting of The Japanese Society for Photomedicine and Photobiology, Kobe, Japan, July, 1997.

6   "Stabilization of Hoogsteen Base-Pairing by Introduction of NH_2 Group at Adenine C8"
K. Kawai, H. Sugiyama, I. Saito.
74th Annual Meeting of Chemical Society of Japan, Kyoto, Japan, March, 1998.

7.   "Photoreaction of 5-Halouracil-Containing Z-DNA: Mechanistic Study of Z-form Specific 2'-Hydroxylation"
K. Kawai, I. Saito, H. Sugiyama,
19th Annual Meeting of The Japanese Society for Photomedicine and Photobiology, Kumamoto, Japan, July, 1998.

8.   "Conformation-Dependent Photochemistry of 5-Halouracil-Containing DNA: Specific Ribose 2'-Hydroxylation in Z-form DNA"
K. Kawai, H. Sugiyama, I. Saito.
25th Symposium on Nucleic Acids Chemistry, Kobe, Japan, September, 1998.

Other Oral Presentation

1.   "Haloboration Polymerization between Boron Trichloride and Terminal Diynes"
K. Kawai, Y. Sasaki, N. Takizawa, Y. Chujo.
67th Annual Meeting of Chemical Society of Japan, Tokyo, Japan, March, 1994.