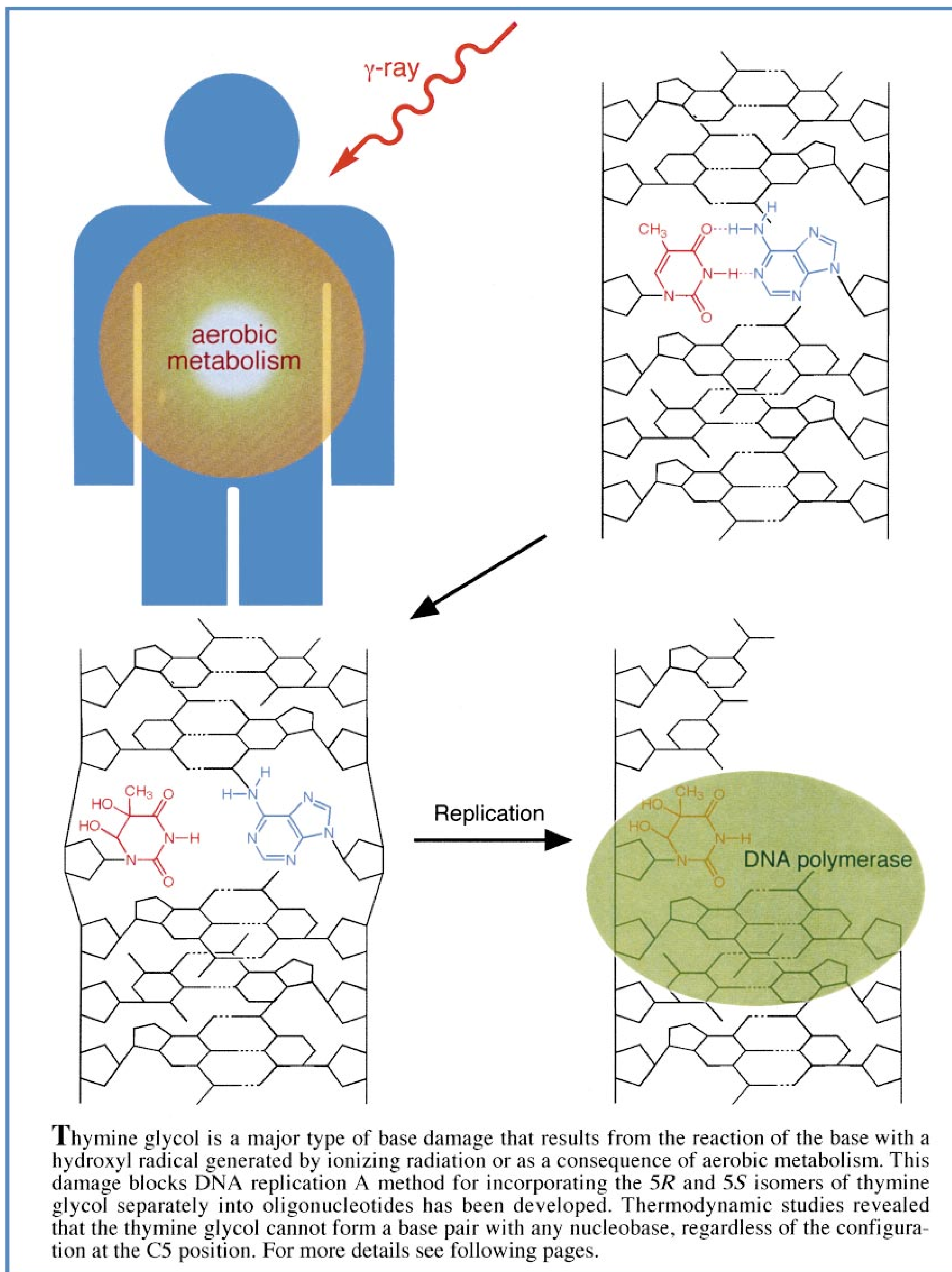


Thymine glycol is a major type of base damage that results from the reaction of the base with a hydroxyl radical generated by ionizing radiation or as a consequence of aerobic metabolism. This damage blocks DNA replication A method for incorporating the 5*R* and 5*S* isomers of thymine glycol separately into oligonucleotides has been developed. Thermodynamic studies revealed that the thymine glycol cannot form a base pair with any nucleobase, regardless of the configuration at the C5 position. For more details see following pages.

Synthesis and Thermodynamic Studies of Oligonucleotides Containing the Two Isomers of Thymine Glycol

Shigenori Iwai*[a, b]

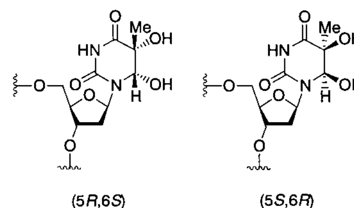
Abstract: Thymine glycol is a major type of base damage, which is formed in DNA by reactive oxygen species. I describe the synthesis of oligonucleotides containing the 5*S* isomer of thymine glycol, which was not obtained by the oxidation of the oligonucleotides. Before the 5*S* isomer was synthesized, a building block without the protection of the tertiary hydroxy function at the C5 position of thymine glycol was tested by the use of the previously reported 5*R* isomer. In the presence of imidazole, migration of the silyl group between the C5 and C6 positions was observed, while the result of the oligonucleotide synthesis was identical to the case of the fully protected building block. Therefore, oligonucleotides containing the (5*S*)-thymine glycol were synthesized with the disilylated building block. In contrast to the 5*R* derivative, two products were detected in the HPLC analysis of the crude mixture after deprotection. Analysis by matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry revealed that the larger peak was the desired oligonucleotide, and it was found that the by-product was completely degraded by a short treatment with ammonium hydroxide at room temperature. I also report the application of oligonucleotides containing each isomer of thymine glycol to thermodynamic analyses of base-pair formation. The thermodynamic parameters obtained for the duplexes containing either the (5*R*)- or (5*S*)-thymine glycol indicated that the thymine glycol cannot form a base-pair with any nucleobase, regardless of the configuration at the C5 position.

Keywords: chirality · DNA damage · oligonucleotides · solid-phase synthesis · thermodynamics

Introduction

Reactive oxygen species can damage DNA.[1] Thymine glycol (5,6-dihydro-5,6-dihydroxythymine) is a major type of base damage that results from the reaction of the base with a hydroxyl radical generated by ionizing radiation[2] or as a consequence of aerobic metabolism.[3] While it was reported that thymine glycol lacks mutagenicity[4] or causes mutations only at a low frequency,[5] it effectively blocks DNA replication.[6] This damage is repaired by means of the base-excision repair pathway initiated by enzymes, such as endonuclease III[7] and endonuclease VIII,[8] in cells. The nucleotide-excision repair[9] and the transcription-coupled repair[10] of this damage have also been shown.

On account of the two chiral carbon atoms at the C5 and C6 positions, there are four possible diastereomers of thymine glycol, namely (5*R*,6*S*), (5*R*,6*R*), (5*S*,6*R*), and (5*S*,6*S*) (Scheme 1). In solution, however, thymine glycol exists as either the (5*R*) *cis*–*trans* pair ((5*R*,6*S*) and (5*R*,6*R*)) or the (5*S*) *cis*–*trans* pair ((5*S*,6*R*) and (5*S*,6*S*)), as a result of epimerization at the C6 position.[11] It has been reported that the 5*R* and 5*S* isomers are formed in equal amounts in γ-irradiated DNA,[12] whereas oxidation of thymidine or thymidine-containing oligonucleotides preferentially yields (5*R*)-thymine glycol.[13]



Scheme 1. Structures of the two isomers of thymine glycol.

Studies on thymine glycol have been performed on chemically oxidized oligonucleotides. Although $KMnO_4$ has frequently been used as an oxidizing agent, identification and purification of the desired product by high-performance liquid chromatography (HPLC) are extremely difficult, because $KMnO_4$ oxidation gives various products, even when

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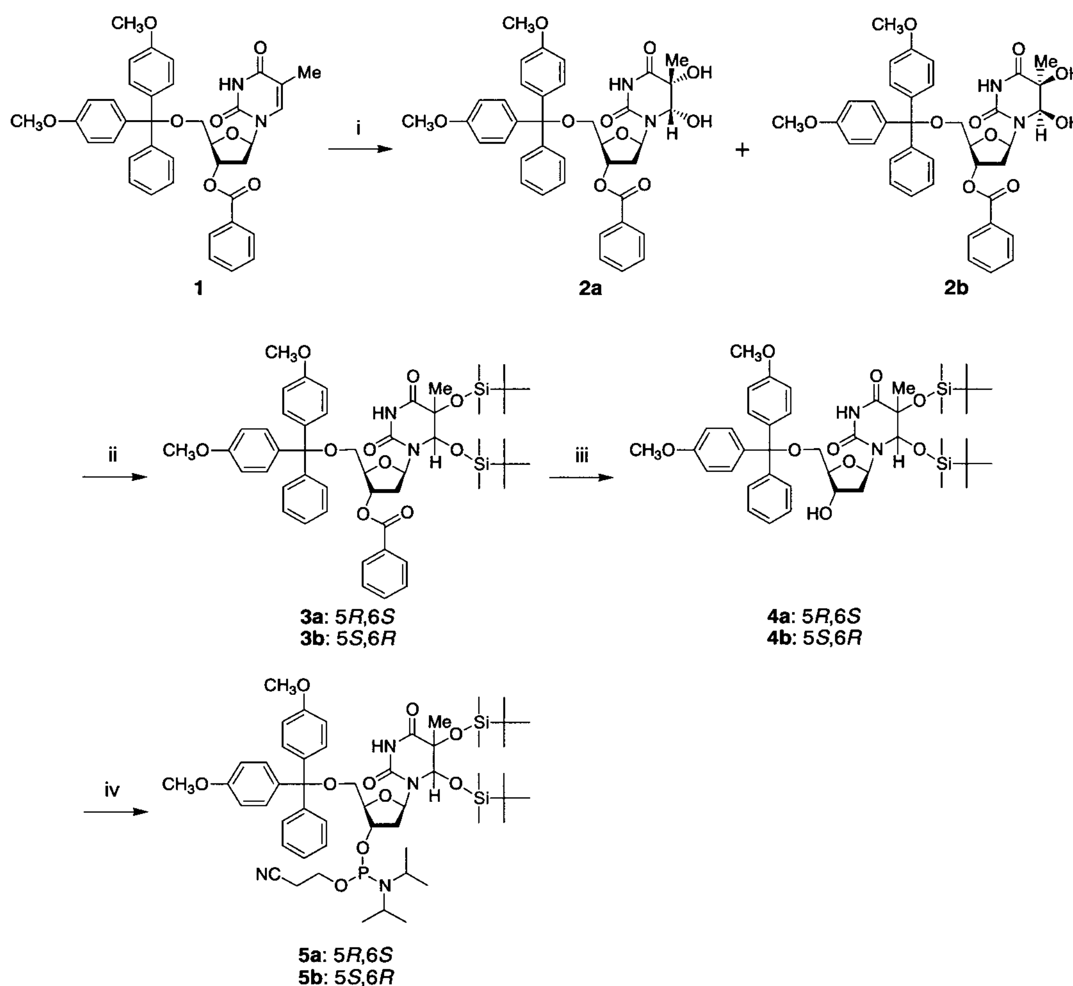
the oligonucleotide is short and contains only one thymine base.[5, 13b,c, 14] Alternatively, OsO_4 can be used; however, the yield of thymine glycol is very low.[5] The 5*R* isomer of thymine glycol is usually obtained by this postsynthetic oxidation method.[13b,c]

The chemical synthesis of oligonucleotides that uses phosphoramidite building blocks is superior to the postsynthetic methods for the preparation of modified or damaged DNA because the sequence and the chain length are not restricted.[15] This direct incorporation method also has advantages in large-scale preparations, especially for structural biology. Matray and Greenberg described the synthesis of oligonucleotides containing (5*R*)-5,6-dihydro-5-hydroxythymine, the C6-reduced form of thymine glycol.[16] I recently reported the synthesis of a building block of (5*R*)-thymine glycol and its incorporation into oligonucleotides.[17] This study was undertaken because the chemical structure and the alkali lability of thymine glycol were very similar to those of the (6–4) photoproduct in my previous work.[18] In this paper, I describe the examination of the silyl protection of the hydroxy functions of thymine glycol and the synthesis of oligonucleotides containing the 5*S* isomer of this damaged base. The oligonucleotides containing each isomer were used for thermodynamic studies of duplex formation, in order to gain insight into the in-vivo effect of thymine glycol.

Results and Discussion

Silyl protection of the hydroxy functions of thymine glycol: The original synthetic scheme for the (5*R*,6*S*)-thymine glycol building block (**5a**), reported in a communication,[17] is shown in Scheme 2. Thymine glycol has a secondary hydroxy function at the C6 position, which requires protection in the oligonucleotide synthesis. The protecting group for this hydroxy function needs to be intact during chain assembly and the removal of other protecting groups, and to be removed in the final step without damaging the thymine glycol moiety. Consequently, the *tert*-butyldimethylsilyl (TBDMS) group, which is generally used for the 2′-protection in oligoribonucleotide synthesis,[19] was chosen for this purpose. The hydroxy function at the C5 position was simultaneously protected when an excess amount of the reagent was used;[17] however, this tertiary hydroxy does not seem to require protection, analogous to the (6–4) photoproduct, in which the tertiary hydroxy group was not protected for the oligonucleotide synthesis.[18] Therefore, a phosphoramidite building block of thymine glycol without the protection of the tertiary hydroxy function was prepared, and its usage in the oligonucleotide synthesis was tested.

The reaction of 5′- and 3′-protected (5*R*,6*S*)-thymidine glycol ((5*R*,6*S*)-5,6-dihydro-5,6-dihydroxythymidine, **2a**) with



Scheme 2. Reagents and conditions: i) OsO_4, pyridine, room temperature, 2 h; ii) TBDMS-Cl (5 equiv), imidazole, DMF, 37 °C, 24 h; iii) K_2CO_3, MeOH, room temperature, 2 h; iv) $[(CH_3)_2CH]_2NP(Cl)OCH_2CH_2CN$, $[(CH_3)_2CH]_2NC_2H_5$, THF, room temperature, 30 min.

1.2 molar equivalents of *tert*-butyldimethylchlorosilane gave two monosilylated compounds in the ratio of 4:1. In the NOESY spectra, both products gave strong crosspeaks between H6 and the methyl group at the C5 position and between H6 and H2′, which indicated that the two hydroxy functions were in the *cis* orientation and that the base moiety was in the high *anti* conformation around the glycosyl bond, respectively. The difference between the two products was that only the minor product gave crosspeaks between the butyl proton of the TBDMS group and the aromatic and methoxy protons of the 4,4′-dimethoxytrityl (DMT) group protecting the 5′-hydroxy function. These results indicated that the major and minor products were the O^6- and O^5-silylated derivatives (**6a** and **6b** in Scheme 3), respectively. It was unusual that the tertiary hydroxy function was protected with the TBDMS group so easily. I considered the imidazole used in the silylation reaction as the cause of this phenomenon. Isolated **6a** and **6b** were treated with imidazole separately, and this treatment caused isomerization of each compound to a mixture of **6a** and **6b** in the ratio of 4:1, as determined by ^1H NMR spectroscopy. The stability of the thymine glycol against imidazole was confirmed by the same treatment of **2a**. I concluded that the TBDMS group was first introduced to the secondary hydroxy function at the C6 position, and then migrated to the tertiary hydroxy in the presence of imidazole. Migration of the TBDMS group has been reported in ribonucleoside[20] and carbohydrate[21] chemistry. The preparation of the fully protected thymidine glycol (**3a**) in my previous study[17] must have been enabled by this migration.

The 3′-hydroxy function of the desired monosilylated thymidine glycol (**6a**) was deprotected and phosphitylated to give the building block for the oligonucleotide synthesis (**8**), as shown in Scheme 3, and a 13-mer, d(ACGCGATg-ACGCCA), in which Tg represents (5*R*)-thymine glycol, was synthesized. Figure 1 shows a comparison of the HPLC chromatograms of crude mixtures of the 13-mers synthesized

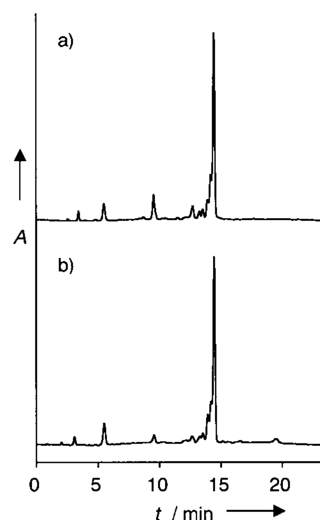
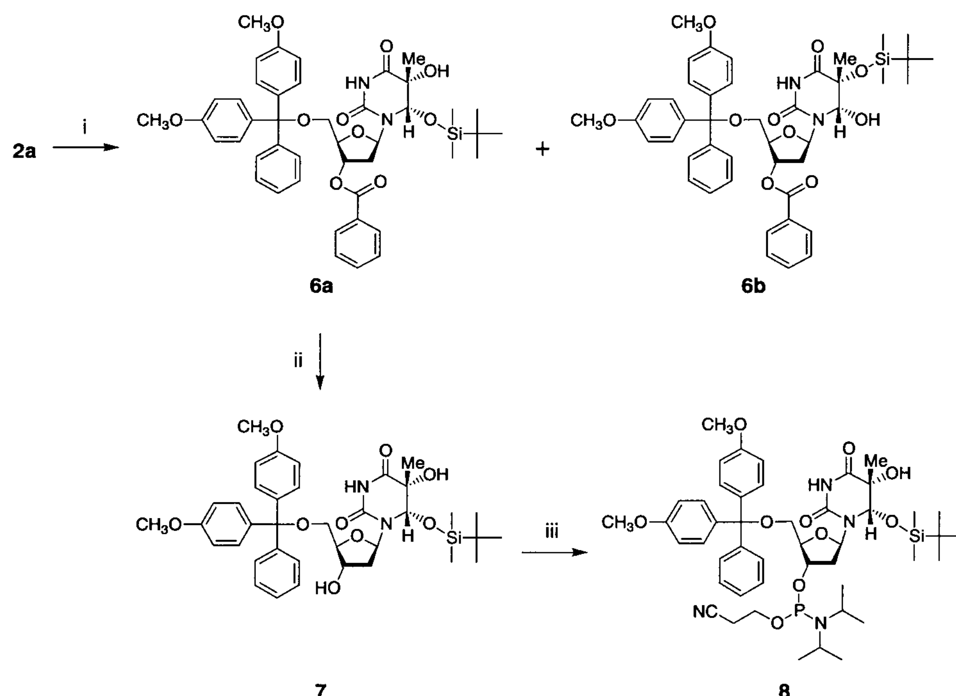


Figure 1. HPLC analysis of the crude mixtures of the 13-mers synthesized with **5a** (a) and **8** (b).

with the disilylated and monosilylated building blocks (**5a** and **8**, respectively). The 13-mer synthesized with **5a** has already been characterized,[17] and the two chromatograms are identical to each other. This indicates that the protection of the tertiary hydroxy function is not required for oligonucleotide synthesis by the phosphoramidite chemistry. Considering the 20% loss by the silyl migration in the preparation of the building block, however, the disilylated building block (**5a**) is recommended for the synthesis of oligonucleotides that contain thymine glycol.



Scheme 3. Reagents and conditions: i) TBDMS-Cl (1.2 equiv), imidazole, DMF, room temperature, 20 h; ii) K_2CO_3, MeOH, room temperature, 2 h; iii) $[(CH_3)_2CH]_2NP(Cl)OCH_2CH_2CN$, $[(CH_3)_2CH]_2NC_2H_5$, THF, room temperature, 30 min.

Synthesis of oligonucleotides containing (5*S*)-thymine glycol: As described previously, (5*S*,6*R*)-thymidine glycol (**2b**) was separated on silica gel from the (5*R*,6*S*) isomer (**2a**) after the oxidation of the protected thymidine with OsO_4.[17] Neither of the isomers gave a NOESY crosspeak between H6 and H1′, and a strong NOE was detected between H6 and H2′ only in the case of **2b**. These observations indicated the (5*S*,6*R*) configuration of **2b**, as described by Vaishnav et al.[13a] Barvian and Greenberg have described a diastereoselective synthesis of (5*S*,6*R*)-thymidine glycol,[22] but an advantage of the present method is that both isomers can be obtained at the same time.

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For the reason described above, the disilylated building block (**5b**) was prepared to synthesize oligonucleotides containing (5*S*)-thymine glycol. As shown in Scheme 2, the procedures were the same as those for the (5*R*,6*S*) isomer. The structure of **3b** was analyzed by NOESY again. An NOE observed between H6 and H1′ showed that the conformation around the glycosyl bond was *syn*. This is reasonable because the bulky TBDMS groups must be kept away from the sugar moiety. As expected, there was a strong crosspeak between H6 and the methyl group at the C5 position, which indicated the *cis* orientation of the two TBDMS-protected hydroxy functions. The absence of the crosspeaks between H6 and the methyl proton of the O^5-TBDMS group and between the C5 methyl proton and the methyl proton of the O^6-TBDMS group also showed the *cis* orientation, which indicated that the configuration of (5*S*,6*R*)-thymine glycol was retained. The fully protected compound (**3b**) was converted to the phosphoramidite building block (**5b**), as shown in Scheme 2.

From **5b**, an 11-mer, d(CGTACTg*CATGC), in which Tg* represents (5*S*)-thymine glycol (since the deprotection of thymine glycol results in the epimerization at the C6 position,[11, 22] I describe the stereochemistry only at the C5 position in the case of deprotected thymine glycol, and (5*S*)-thymine glycol means a mixture of the (5*S*,6*R*) and (5*S*,6*S*) isomers, which are not separated by HPLC), and a 30-mer, d(CTCGTCAGCATCTTg*CATCATACAGTCAGTG), were synthesized. The procedures were the same as those for the oligonucleotides containing the (5*R*)-thymine glycol.[17] For comparison, the Tg 11-mer and the Tg 30-mer, containing (5*R*)-thymine glycol, were synthesized. After deprotection with ammonium hydroxide at room temperature, followed by treatment with tetrabutylammonium fluoride (TBAF), the crude mixtures of the Tg and Tg* 11-mers were analyzed by reversed-phase HPLC. While the Tg 11-mer gave only a single main peak, two large peaks (peaks 1 and 2 in Figure 2) were obtained for the Tg* 11-mer. The ratio of the two products changed, depending on the deprotection methods, as shown in Figure 2. To designate the desired product, the two products were isolated by HPLC and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The results are listed in Table 1. This analysis demonstrated that peak 1 was the desired 11-mer containing (5*S*)-thymine glycol. The smaller *m*/*z* value obtained for peak 2 suggested that this byproduct contained 5-hydroxy-5-methylhydantoin (HMH) instead of thymine glycol, although the (5*S*)-thymine glycol in the oligonucleotide was not converted to HMH by the procedure described for the released thymine glycol.[23] The difference in the reactivity of the (5*R*)- and (5*S*)-thymine glycols during deprotection may be attributed to the difference in the spatial arrangement of their functional groups. Since an analysis of the products by mass spectrometry is not always possible, I searched for a simple method to discriminate between the desired product containing (5*S*)-thymine glycol and the by-product. Treatment with ammonium hydroxide at room temperature for 2 h resulted in the complete conversion of the by-product to a new product, whereas most of the thymine glycol-containing oligonucleotide remained intact after the same treatment (Figure 3). The new product generated from the by-product

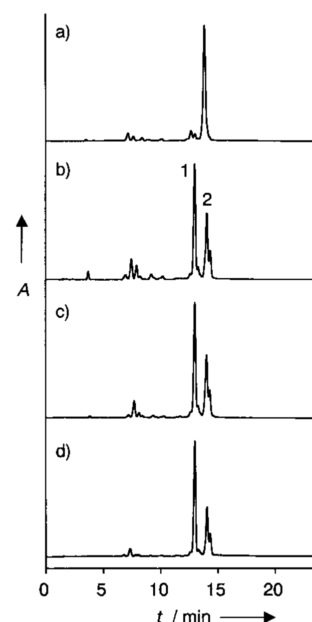

Figure 2. HPLC analysis of the crude mixtures of the Tg 11-mer (a) and the Tg* 11-mer (b, c, and d). The Tg* 11-mer was deprotected with NH_4OH (b), NH_3/MeOH (c), or K_2CO_3/MeOH (d), and the two products (peaks 1 and 2) were isolated for characterization by MALDI-TOF mass spectrometry and by the ammonia treatment.

Table 1. MALDI-TOF MS analysis of the 11-mers.

Oligonucleotide		Sequence	*m*/*z* observed	*m*/*z* theoretical[a]
T 11-mer		d(CGTACTCATGC)	3287.6	3289.6
Tg 11-mer		d(CGTACTgCATGC)	3323.2	3323.6
Tg* 11-mer	peak 1	d(CGTACTg*CATGC)	3323.2	3323.6
	peak 2		3295.5	3293.6[b]

[a] $[M - H]^-$. [b] An 11-mer containing 5-hydroxy-5-methylhydantoin

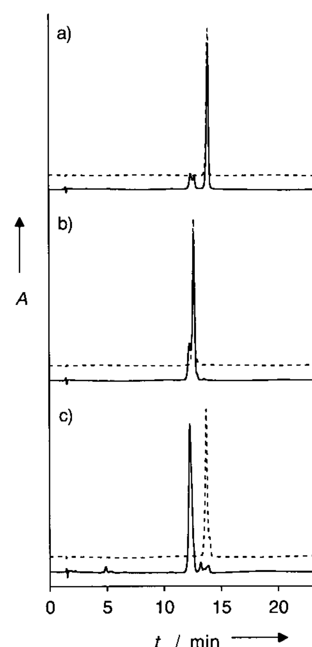

Figure 3. Ammonia treatment of the deprotected oligonucleotides. The Tg 11-mer (a) and the two products of the Tg* 11-mer (b, peak 1; c, peak 2) were treated with 28% ammonia water at room temperature for 2 h and analyzed by reversed-phase HPLC. The elution profiles of the untreated oligonucleotides are shown by broken lines.

by this treatment was supposed to be an 11-mer in which the base moiety was decomposed to urea.

A similar result was obtained for the 30-mer (Figure 4). Since the smaller peak was completely shifted to the other side of the larger peak by the ammonia treatment, the larger peak was isolated as the desired product. The presence of (5*S*)-thymine glycol in the 30-mer was demonstrated by the analysis of the nucleoside composition,[14b] as shown in Figure 5. This analysis also showed that epimerization at the C5 position of thymine glycol did not occur during the synthesis and the deprotection of these oligonucleotides.

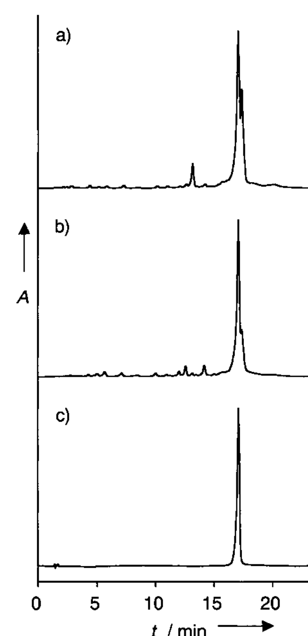

Figure 4. HPLC analysis of the crude (a and b) and purified (c) Tg* 30-mer. Deprotection was performed with NH_4OH (a) or K_2CO_3/MeOH (b).

Thermodynamic analysis of duplex formation: Thermodynamic analyses are useful to gain insight into the mechanisms of the damage-induced mutations and the damage recognition of repair enzymes.[24] The oligonucleotides containing each isomer of thymine glycol, which were synthesized as described above, were used for the thermodynamic analyses. Two types of duplexes were designed, as shown in Table 2. The target base-pairs are usually placed in the center of a duplex in thermodynamic studies,[24a] and the first set, d(CGTAC*X*CATGC) d(GCATGAGTACG), can be used to yield clues about the recognition mechanisms of the repair enzymes. The second set, in which the damaged base is located at the 5′ end of the double-stranded region, is a model for replication,[25] and the four normal bases were placed opposite the undamaged thymine or each isomer of the thymine glycol, to study their base-pair formation. Although NMR studies have been reported,[13b,c] the duplex destabilization cannot be quantified from the structure, and the latter type of duplexes cannot be analyzed by NMR spectroscopy.

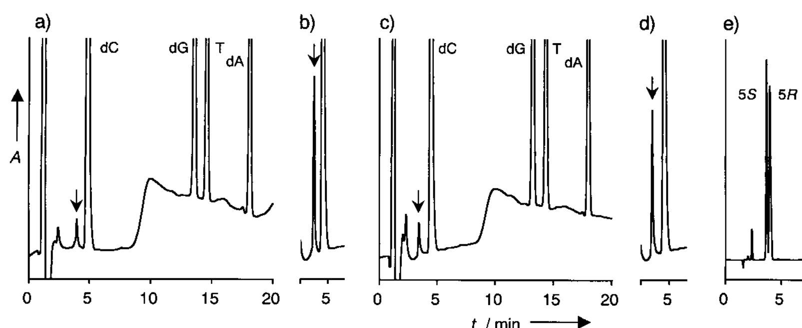

Figure 5. Detection of thymidine glycol after enzyme digestion. a) and c) The Tg and Tg* 30-mers were treated with nuclease P1, phosphodiesterase I, and alkaline phosphatase, and the products were separated by HPLC. Thymidine glycol in each sample is indicated by an arrow. b) and d) The authentic (5*R*)- and (5*S*)-thymidine glycols were added to the Tg 30-mer and Tg* 30-mer mixtures, respectively, to show the identity. e) Separation of the (5*R*)- and (5*S*)-thymidine glycol was demonstrated by co-injection. The monitoring wavelength was 230 nm in all cases.

After hybridization of the two strands, thermal melting curves, monitored at $\lambda =$ 260 nm, were measured at total oligonucleotide concentrations (C_t) varying between 2.0 and 20.0 μM. Thermodynamic parameters for each duplex were determined from the T_m data by the van't Hoff method, assuming a two-state model for duplex melting (Figure 6).[26] The results, as well as the T_m values at $C_t = 2.0$ μM, are listed in Table 2. When thymine glycol was placed in the center of the 11-mer duplex, a large destabilization was observed compared to the undamaged

Table 2. Thermodynamic parameters of duplex formation.

Duplex	T_m[a] [°C]	$\Delta H°$ [kcal mol^{-1}]	$\Delta S°$ [cal mol^{-1} K^{-1}]	$\Delta G°$ (25 °C) [kcal mol^{-1}]	$\Delta\Delta G°$[b] (25 °C) [kcal mol^{-1}]
5′-CGTAC*X*CATGC-3′					
3′-GCATGAGTACG-5′					
X = T	44.0	– 79.2 ± 3.1	– 221 ± 10	– 13.31 ± 0.20	
X = Tg	24.7	– 67.7 ± 0.1	– 198 ± 0	– 8.52 ± 0.00	4.79 ± 0.20
X = Tg*	25.1	– 68.3 ± 0.6	– 200 ± 2	– 8.60 ± 0.01	4.71 ± 0.21
5′-CA*X*AGCACGAC-3′					
3′- *Y*TCGTGCTG-5′					
X = T, *Y* = A	39.8	– 67.3 ± 2.4	– 186 ± 8	– 11.76 ± 0.13	
X = T, *Y* = G	38.1	– 63.3 ± 2.3	– 175 ± 7	– 11.21 ± 0.13	
X = T, *Y* = C	36.0	– 58.7 ± 0.7	– 161 ± 2	– 10.72 ± 0.04	
X = T, *Y* = T	37.0	– 62.2 ± 0.4	– 172 ± 1	– 11.01 ± 0.02	
X = Tg, *Y* = A	37.9	– 61.6 ± 2.2	– 169 ± 7	– 11.13 ± 0.10	0.63 ± 0.23
X = Tg, *Y* = G	38.7	– 61.5 ± 1.5	– 169 ± 5	– 11.28 ± 0.07	– 0.07 ± 0.20
X = Tg, *Y* = C	36.4	– 58.3 ± 1.9	– 159 ± 6	– 10.78 ± 0.10	– 0.06 ± 0.14
X = Tg, *Y* = T	37.4	– 60.4 ± 1.7	– 166 ± 6	– 10.99 ± 0.08	0.02 ± 0.10
X = Tg*, *Y* = A	37.7	– 57.5 ± 0.9	– 156 ± 3	– 10.94 ± 0.04	0.82 ± 0.17
X = Tg*, *Y* = G	38.6	– 62.9 ± 1.5	– 173 ± 5	– 11.36 ± 0.08	– 0.15 ± 0.21
X = Tg*, *Y* = C	36.4	– 59.2 ± 2.1	– 162 ± 7	– 10.81 ± 0.10	– 0.09 ± 0.14
X = Tg*, *Y* = T	37.1	– 60.3 ± 1.2	– 166 ± 4	– 10.94 ± 0.05	0.07 ± 0.07

[a] $C_t = 2.0$ μM. [b] $\Delta G°$(Tg) – $\Delta G°$(T)

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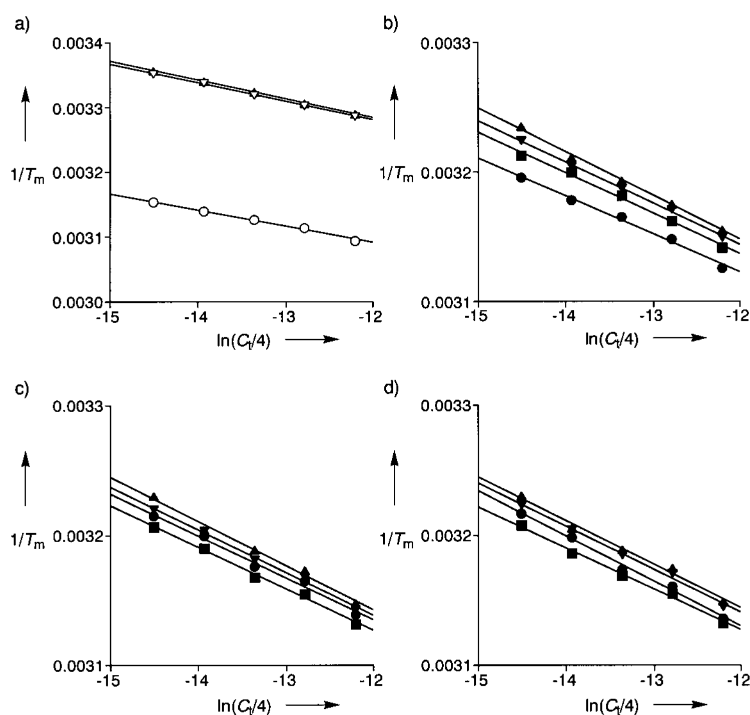

Figure 6. Plots of $1/T_m$ versus $\ln C_t/4$ for the duplexes containing thymine glycol. a) d(CGTAC*X*CATGC) d(GCATGAGTACG) where X = T (○), Tg (△), and Tg* (▽). b), c), and d) d(CA*X*AGCACGAC) d(GTCGTGCT*Y*) where X = T (b), Tg (c), and Tg* (d); Y = A (●), G (■), C (▲), and T (▼).

duplex, regardless of the C5 configuration of the oxidized base. In the replication model, in which thymine glycol was located at the end of the double-stranded region, the difference in the free energy (ΔG°) for the duplexes with the correct and mismatched base-pairs was small, as reported previously,[25] but the correct T–A pair distinctly showed stabilization of the duplex ($\Delta\Delta G^\circ$ = 0.55 to 1.04 kcal mol^{-1} at 25 °C, compared to the mismatches). When thymine was replaced by thymine glycol, the absolute values of ΔG° obtained for the duplexes containing the thymine glycol–adenine pair were reduced to the level of the mismatched pairs, while those for the other duplexes were not altered. No obvious difference was observed between the 5*R* and 5*S* isomers of thymine glycol.

As shown by the thermodynamic analysis of the first set of the duplexes, both (5*R*)- and (5*S*)-thymine glycols incorporated into the center of the duplex greatly reduced the thermodynamic stability. This observation may seem strange because the functional groups for base-pair formation are apparently intact after the oxidation of the thymine base. However, this result supports the structural features of (5*R*)-thymine glycol-containing duplexes studied by NMR spectroscopy. In the NMR studies by Bolton and co-workers,[13b,c] thymine glycol induced a highly localized alteration in the DNA structure, and the oxidized base as well as the opposite adenine were reported to be extrahelical. The reduced transition entropy change (ΔS°) measured in the present study for the duplexes containing the Tg–A and Tg*–A pairs may reflect the flexibility of the extrahelical bases. As described previously,[13b,c] this structural feature may be recognized by repair proteins. Base-pair formation was not observed between thymine glycol and any of the four normal bases when thymine glycol was located at the end of the duplex. It is possible that this lack of base-pairing ability, together with the nonplanar structure of thymine glycol, causes the replication block. It may be noteworthy that the thermodynamic characteristics, as well as the reduced thermal stability (ΔT_m), of the thymine glycol-containing duplexes obtained in this study are quite similar to those reported for duplexes containing 3-hydroxy-2-(hydroxymethyl)tetrahydrofuran, that is an abasic site analogue.[25b, 27]

Experimental Section

General methods: All solvents and reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan), except for *tert*-butyldimethylchlorosilane (Shin-Etsu Chemical, Tokyo, Japan), (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (Digital Specialty Chemicals, Ontario, Canada), and TBAF (Tokyo Chemical Industry, Tokyo, Japan). Reagents for the DNA synthesizer were purchased from Applied Biosystems Japan (Tokyo, Japan). Nuclease P1 and alkaline phosphatase were purchased from Roche Diagnostics (Mannheim, Germany), and phosphodiesterase I from Worthington Biochemical (Lakewood, NJ, USA).

TLC analyses were carried out on Merck silica gel 60F_{254} plates, which were visualized by UV illumination at λ = 254 nm and spraying of an anisaldehyde/sulfuric acid solution, followed by heating. For column chromatography, either Wakogel C-200 or C-300 (Wako Pure Chemical Industries) was used. ^{1}H NMR spectra were measured on a Bruker DPX300 spectrometer, with tetramethylsilane as the internal standard. COSY and NOESY spectra were used for the signal assignment and the configuration determination, respectively. ^{31}P NMR spectra were measured on the same spectrometer at 121.5 MHz, with trimethyl phosphate as the internal standard. Mass spectra were obtained on a JEOL HX-110, VG ZAB-HF, or Hitachi M-4000H spectrometer. HPLC analyses were carried out on a Gilson gradient-type analytical system equipped with a Waters 996 photodiode array detector. A μBondasphere C18 5 μm 300 Å column (3.9 × 150 mm, Waters Corporation, Milford, MA, USA) was used with a linear gradient of acetonitrile (0–10%, 5–13%, and 7–13% for the 11-, 13-, and 30-mers, respectively) in 0.1 M triethylammonium acetate (TEAA, pH 7.0). Anion-exchange HPLC was performed on a TSK-GEL DEAE-2SW column (4.6 × 250 mm, Tosoh Corporation, Tokyo, Japan), with a linear gradient of ammonium formate (0.3–0.8 M and 0.4–1.0 M for the 11- and 30-mers, respectively) in 20% acetonitrile. MALDI-TOF mass analyses of purified oligonucleotides were carried out on a PerSeptive Biosystems Voyager Elite spectrometer, with a mixture of 3-hydroxypicolinic acid and picolinic acid (9:1, w/w) as a matrix.

Compounds containing the (5*R*,6*S*)-thymine glycol (**2a**, **3a**, **4a**, and **5a**) were prepared as described previously.[17]

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*S*,6*R*)-5,6-dihydro-5,6-dihydroxythymidine (2b): Protected thymidine (**1**) was oxidized with OsO_4 in pyridine, and **2b** was separated from **2a** on silica gel, as described previously.[17] R_f = 0.62 ($CHCl_3$/MeOH 10/1); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.0 (d, ^3J(H,H) = 7 Hz, 2H; Bz), 7.9 (s, 1H; NH), 7.6 (t, ^3J(H,H) = 7 Hz, 1H; Bz), 7.5–7.2 (m, 11H; Bz, DMT), 6.8 (d, ^3J(H,H) = 9 Hz, 4H; DMT), 6.5 (t, ^3J(H,H) = 7 Hz, 1H; H1′), 5.7–5.6 (m, 1H; H3′), 5.0 (s, 1H; H6), 4.3–4.2 (m, 1H; H4′), 3.8 (s, 6H; OCH_3), 3.7 (br, 1H; 5-OH), 3.5 (dd, ^3J(H,H) = 11, 4 Hz, 1H; H5′), 3.4 (dd, ^3J(H,H) = 11, 3 Hz, 1H; H5′), 3.3 (br, 1H; 6-OH), 2.5–2.4 (m, 2H; H2′, H2″), 1.4 (s, 3H; CH_3); HRMS: (FAB): found: 681.2462 $[M-H]^-$; calcd: 681.2448.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*S*,6*R*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]thymidine (3b): A solution of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-benzoyl-(5*S*,6*R*)-5,6-dihydro-5,6-dihydroxythymidine (**2b**, 299 mg, 438 μmol), imidazole (298 mg, 4.38 mmol), and *tert*-butyldimethylchlorosilane (330 mg, 2.19 mmol) in *N*,*N*-dimethylformamide (DMF, 2.5 mL) was stirred at 37 °C for 24 h. This mixture was diluted with

chloroform (20 mL) and washed with 0.5 M sodium phosphate (pH 5.0). The organic layer was dried with Na_2SO_4, and after evaporation and coevaporation with toluene, the residue was chromatographed on silica gel (10 g) with a step gradient of ethyl acetate in hexane. The product was eluted with 15% ethyl acetate in hexane. Yield: 334 mg (366 μmol, 84%). R_f = 0.71 (hexane/ethyl acetate 3/2); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.0 (d, ^3J(H,H) = 7 Hz, 2H; Bz), 7.6 (t, ^3J(H,H) = 7 Hz, 1H; Bz), 7.5–7.4 (m, 4H; Bz, DMT), 7.4–7.2 (m, 7H; DMT), 7.1 (s, 1H; NH), 6.8 (d, ^3J(H,H) = 9 Hz, 4H; DMT), 5.7 (dd, ^3J(H,H) = 8, 5 Hz, 1H; H1′), 5.6 (m, 1H; H3′), 4.9 (s, 1H; H6), 4.3 (dd, ^3J(H,H) = 8, 5 Hz, 1H; H4′), 3.8 (s, 6H; OCH_3), 3.5 (dd, ^3J(H,H) = 10, 5 Hz, 1H; H5′), 3.3 (dd, ^3J(H,H) = 10, 5 Hz, 1H; H5′), 2.7–2.6 (m, 1H; H2′), 2.5–2.4 (m, 1H; H2″), 1.3 (s, 3H; CH_3), 0.9, 0.8 (s, 18H; TBDMS), 0.2 (s, 6H; TBDMS), 0.1, 0.0 (s, 6H; TBDMS); HRMS: (FAB): found: 909.4149 $[M-H]^-$; calcd: 909.4178.

5′-*O*-(4,4′-Dimethoxytrityl)-(5*S*,6*R*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]thymidine (4b): 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*S*,6*R*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]thymidine (**3b**, 322 mg, 353 μmol) was dissolved in a 50 mM solution of K_2CO_3 in methanol (15 mL). After 2 h, sodium phosphate (0.5 M, pH 5.0, 20 mL) was added. The mixture was extracted with chloroform (30 mL in total). The organic layer was dried with Na_2SO_4, and after evaporation, the residue was chromatographed on silica gel (8 g) with a step gradient of ethyl acetate in hexane. The product was eluted with 25–30% ethyl acetate in hexane. Yield: 286 mg (354 μmol, 100%); R_f = 0.37 (hexane/ethyl acetate 3/2); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.4 (d, ^3J(H,H) = 7 Hz, 2H; DMT), 7.3–7.2 (m, 7H; DMT), 7.0 (s, 1H; NH), 6.8 (d, ^3J(H,H) = 9 Hz, 4H; DMT), 5.5 (t, ^3J(H,H) = 6 Hz, 1H; H1′), 4.8 (s, 1H; H6), 4.5–4.4 (m, 1H; H3′), 3.9 (dd, ^3J(H,H) = 11, 5 Hz, 1H; H4′), 3.8 (s, 6H; OCH_3), 3.4 (dd, ^3J(H,H) = 10, 5 Hz, 1H; H5′), 3.2 (dd, ^3J(H,H) = 10, 6 Hz, 1H; H5′), 2.5–2.3 (m, 2H; H2′, H2″), 1.9 (d, ^3J(H,H) = 3 Hz, 1H; 3′-OH), 1.3 (s, 3H; CH_3), 0.9, 0.8 (s, 18H; TBDMS), 0.2 (s, 6H; TBDMS), 0.1, 0.0 (s, 6H; TBDMS); HRMS: (FAB): found: 805.3933 $[M-H]^-$; calcd: 805.3916.

5′-*O*-(4,4′-Dimethoxytrityl)-(5*S*,6*R*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]thymidine 3′-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (5b): *N*,*N*-Diisopropylethylamine (242 μL, 1.39 mmol) and (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (155 μL, 696 μmol) were added to a solution of 5′-*O*-(4,4′-dimethoxytrityl)-(5*S*,6*R*)-5,6-dihydro-5,6-di[(*tert*-butyl)dimethylsilyloxy]thymidine (**4b**, 281 mg, 348 μmol) in tetrahydrofuran (THF, 3.5 mL). This mixture was stirred for 30 min, diluted with ethyl acetate, and washed with 2% $NaHCO_3$ and water. The organic layer was dried with Na_2SO_4, and after evaporation, the residue was chromatographed on silica gel (7 g) with a step gradient of ethyl acetate in hexane containing 0.1% pyridine. The product was eluted with 15% ethyl acetate in hexane, and after evaporation, the pyridine was removed by coevaporation with acetonitrile. Yield: 215 mg (214 μmol, 61%); R_f = 0.66, 0.58 (hexane/ethyl acetate 3/2); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.9 (s, 1H; NH), 7.5–7.4 (m, 2H; DMT), 7.4–7.2 (m, 7H; DMT), 6.9–6.8 (m, 4H; DMT), 5.6–5.5 (m, 1H; H1′), 4.9, 4.8 (s, 1H; H6), 4.6–4.5 (m, 1H; H3′), 4.2–4.1 (m, 1H; H4′), 3.9–3.7 (m, 7H; OCH_3, OCH_2CH_2CN × ½), 3.7–3.5 (m, 3H; OCH_2CH_2CN × ½, $CH(CH_3)_2$), 3.3–3.2 (m, 2H; H5′), 2.6 (t, ^3J(H,H) = 6 Hz, 1H; OCH_2CH_2CN × ½), 2.4 (t, ^3J(H,H) = 6 Hz, 1H; OCH_2CH_2CN × ½), 2.6–2.3 (m, 2H; H2′, H2″), 1.4, (s, 3H; CH_3), 1.2–1.1 (m, 12H; $CH(CH_3)_2$), 0.8 (s, 18H; TBDMS), 0.2 (s, 6H; TBDMS), 0.1, 0.0 (s, 6H; TBDMS); ^{31}P NMR (121.5 MHz, $CDCl_3$, 25 °C, trimethyl phosphate): δ = 147, 146; HRMS: (SI): found: 1005.4992 $[M-H]^-$; calcd: 1005.4990.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*R*,6*S*)-5,6-dihydro-5-hydroxy-6-(*tert*-butyl)dimethylsilyloxythymidine (6a) and 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-benzoyl-(5*R*,6*S*)-5,6-dihydro-5-(*tert*-butyl)dimethylsilyloxy-6-hydroxythymidine (6b): A solution of 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-benzoyl-(5*R*,6*S*)-5,6-dihydro-5,6-dihydroxythymidine (**2a**, 2.03 g, 2.97 mmol), imidazole (485 mg, 7.12 mmol), and *tert*-butyldimethylchlorosilane (537 mg, 3.56 mmol) in DMF (10 mL) was stirred at room temperature for 20 h. This mixture was diluted with chloroform (100 mL) and washed with 0.5 M sodium phosphate (pH 5.0). The organic layer was dried with Na_2SO_4, and after evaporation and coevaporation with toluene, the residue was chromatographed on silica gel (50 g) with a step gradient of ethyl acetate in hexane. The two isomers were separately eluted out at 25% ethyl acetate, and the configurations were determined by the NOESY experiments.

6a: Yield: 988 mg (1.24 mmol, 42%); R_f = 0.45 (hexane/ethyl acetate 3/2); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.0 (d, ^3J(H,H) = 7 Hz, 2H; Bz), 7.8 (s, 1H; NH), 7.6 (t, ^3J(H,H) = 7 Hz, 1H; Bz), 7.5–7.4 (m, 4H; Bz, DMT), 7.4–7.2 (m, 7H; DMT), 6.8 (d, ^3J(H,H) = 9 Hz, 4H; DMT), 6.2 (dd, ^3J(H,H) = 9, 6 Hz, 1H; H1′), 5.6–5.5 (m, 1H; H3′), 4.9 (s, 1H; H6), 4.2 (dd, ^3J(H,H) = 8, 5 Hz, 1H; H4′), 3.8 (s, 6H; OCH_3), 3.4 (dd, ^3J(H,H) = 10, 5 Hz, 1H; H5′), 3.3 (dd, ^3J(H,H) = 10, 5 Hz, 1H; H5′), 3.3 (s, 1H; -OH), 2.4–2.2 (m, 2H; H2′, H2″), 1.4 (s, 3H; CH_3), 0.8 (s, 9H; TBDMS), 0.1 (s, 3H; TBDMS), 0.0 (s, 3H; TBDMS); HRMS: (FAB): found: 795.3311 $[M-H]^-$; calcd: 795.3313.

6b: Yield: 228 mg (0.29 mmol, 10%); R_f = 0.51 (hexane/ethyl acetate 3/2); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 8.1 (d, ^3J(H,H) = 8 Hz, 2H; Bz), 7.7 (s, 1H; NH), 7.6 (t, ^3J(H,H) = 7 Hz, 1H; Bz), 7.5–7.4 (m, 4H; Bz, DMT), 7.4–7.2 (m, 7H; DMT), 6.8 (d, ^3J(H,H) = 9 Hz, 4H; DMT), 6.4 (dd, ^3J(H,H) = 10, 5 Hz, 1H; H1′), 5.7 (d, ^3J(H,H) = 6 Hz, 1H; H3′), 5.1 (s, 1H; H6), 4.2 (br, 1H; H4′), 3.8 (s, 6H; OCH_3), 3.5 (dd, ^3J(H,H) = 10, 3 Hz, 1H; H5′), 3.4 (dd, ^3J(H,H) = 10, 3 Hz, 1H; H5′), 3.1 (s, 1H; -OH), 2.8–2.7 (m, 1H; H2′), 2.5 (dd, ^3J(H,H) = 14, 5 Hz, 1H; H2″), 1.5 (s, 3H; CH_3), 0.7 (s, 9H; TBDMS), 0.2 (s, 3H; TBDMS), 0.1 (s, 3H; TBDMS); HRMS: (FAB): found: 795.3319 $[M-H]^-$; calcd: 795.3313.

5′-*O*-(4,4′-Dimethoxytrityl)-(5*R*,6*S*)-5,6-dihydro-5-hydroxy-6-(*tert*-butyl)-dimethylsilyloxythymidine (7): 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-benzoyl-(5*R*,6*S*)-5,6-dihydro-5-hydroxy-6-(*tert*-butyl)dimethylsilyloxythymidine (**6a**, 988 mg, 1.24 mmol) was dissolved in a 50 mM solution of K_2CO_3 in methanol (50 mL). This mixture was stirred for 2 h, and after the solution was cooled in an ice bath, sodium phosphate (0.5 M, pH 5.0, 100 mL) was added. The mixture was extracted with chloroform (150 mL in total). The organic layer was dried with Na_2SO_4, and after evaporation, the residue was chromatographed on silica gel (20 g) with a step gradient of ethyl acetate in hexane. The product was eluted with 30–35% ethyl acetate in hexane and obtained as a powder by precipitation in hexane (60 mL) from a chloroform solution (3 mL). Yield: 626 mg (904 μmol, 73%); R_f = 0.22 (hexane/ethyl acetate 3/2); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.4 (d, ^3J(H,H) = 7 Hz, 2H; DMT), 7.4 (s, 1H; NH), 7.3–7.2 (m, 7H; DMT), 6.8 (d, ^3J(H,H) = 9 Hz, 4H; DMT), 6.1 (dd, ^3J(H,H) = 8, 7 Hz, 1H; H1′), 4.8 (s, 1H; H6), 4.4 (br, 1H; H3′), 3.9 (dd, ^3J(H,H) = 9, 5 Hz, 1H; H4′), 3.8 (s, 6H; OCH_3), 3.4 (dd, ^3J(H,H) = 10, 5 Hz, 1H; H5′), 3.3 (s, 1H; -OH), 3.2 (dd, ^3J(H,H) = 10, 6 Hz, 1H; H5′), 2.3–2.1 (m, 2H; H2′, H2″), 1.9 (br, 1H; 3′-OH), 1.4 (s, 3H; CH_3), 0.8 (s, 9H; TBDMS), 0.1 (s, 3H; TBDMS), 0.0 (s, 3H; TBDMS); HRMS: (FAB): found: 691.3063 $[M-H]^-$; calcd: 691.3051.

5′-*O*-(4,4′-Dimethoxytrityl)-(5*R*,6*S*)-5,6-dihydro-5-hydroxy-6-(*tert*-butyl)-dimethylsilyloxythymidine 3′-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (8): *N*,*N*-Diisopropylethylamine (105 μL, 600 μmol) and (2-cyanoethyl)-*N*,*N*-diisopropylchlorophosphoramidite (67 μL, 300 μmol) were added to a solution of 5′-*O*-(4,4′-dimethoxytrityl)-(5*R*,6*S*)-5,6-dihydro-5-hydroxy-6-(*tert*-butyl)dimethylsilyloxythymidine (**7**, 104 mg, 150 μmol) in THF (1.5 mL). This mixture was stirred for 30 min, diluted with ethyl acetate, and washed with 2% $NaHCO_3$ and water. The organic layer was dried with Na_2SO_4, and after evaporation, the residue was chromatographed on silica gel (5 g) with a step gradient of ethyl acetate in hexane containing 0.1% pyridine. The product was eluted with 30% ethyl acetate in hexane, and after evaporation, the pyridine was removed by precipitation of the amidite in pentane (10 mL) from a chloroform solution (0.5 mL). Yield: 116 mg (130 μmol, 87%); R_f = 0.34 (hexane/ethyl acetate 3/2); ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C, TMS): δ = 7.5–7.2 (m, 10H; DMT, NH), 6.9–6.8 (m, 4H; DMT), 6.2–6.1 (m, 1H; H1′), 4.9, 4.8 (s, 1H; H6), 4.5–4.4 (m, 1H; H3′), 4.1–4.0 (m, 1H; H4′), 3.8–3.7 (m, 7H; OCH_3, OCH_2CH_2CN × ½), 3.7–3.5 (m, 3H; OCH_2CH_2CN × ½, $CH(CH_3)_2$), 3.3–3.1 (m, 3H; -OH, H5′), 2.6 (t, ^3J(H,H) = 7 Hz, 1H; OCH_2CH_2CN × ½), 2.4 (t, ^3J(H,H) = 7 Hz, 1H; OCH_2CH_2CN × ½), 2.3–2.0 (m, 2H; H2′, H2″), 1.4 (s, 3H; CH_3), 1.2–1.0 (m, 12H; $CH(CH_3)_2$), 0.8 (s, 9H; TBDMS), 0.0 (s, 6H; TBDMS); ^{31}P NMR (121.5 MHz, $CDCl_3$, 25 °C, trimethyl phosphate): δ = 147, 146; HRMS: (FAB): found: 891.4121 $[M-H]^-$; calcd: 891.4129.

Oligonucleotide synthesis: Oligonucleotides were synthesized on either a 0.2 or 1.0 μmol scale on an Applied Biosystems Model 394 DNA/RNA synthesizer. The thymine glycol building blocks (**5a**, **5b**, and **8**) were dissolved in anhydrous acetonitrile at a concentration of 0.1 M. Phosphoramidites bearing the (4-*tert*-butylphenoxy)acetyl group for the protection of the exocyclic amino function were used for dA, dG, and dC. The reaction time for the coupling of the thymine glycol building blocks was prolonged to 5 min. After chain assembly and removal of the terminal 5′-DMT group

on the synthesizer, the solid supports containing the oligonucleotides were treated with 28% aqueous ammonia (2 mL) at room temperature for 2 h. The resulting ammoniac solutions were concentrated to dryness on a rotary evaporator equipped with a vacuum pump. In the case of the (5*S*)-thymine glycol, the solid supports containing the oligonucleotides were treated with 50 mM K_2CO_3 in methanol (1 mL) at room temperature for 4 h.[28] The supports were removed by filtration, and the solutions were mixed with sodium phosphate (0.5 M, pH 5.0, 1 mL). After concentration, these mixtures were passed through a NAP10 column (Amersham Pharmacia Biotech, Uppsala, Sweden), and the eluates were concentrated to dryness. The residues were dissolved in a 1.0 M solution of TBAF in THF (0.1 mL), and the mixtures were left at room temperature. After 16 h, TEAA (0.1 M, pH 7.0, 0.4 mL) was added, and the solutions were desalted on a NAP10 column. For the deprotection of oligonucleotides synthesized on the 1.0 μmol scale, larger volumes of the reagents and NAP25 columns were used. The deprotected oligonucleotides were purified by both reversed-phase and anion-exchange HPLC.

Detection of thymidine glycol in oligonucleotides: Aliquots (0.5 A_{260} units) of the purified 30-mers were mixed with nuclease P1 (40 μg) in ammonium acetate (30 mM, pH 5.3, 400 μL) and incubated at 37 °C. After 20 h, water (466 μL), Tris-HCl (0.5 M, pH 9.0, 100 μL), $MgCl_2$ (1 M, 10 μL), phosphodiesterase I (0.4 units, 4 μL), and alkaline phosphatase (20 units, 20 μL) were added, and the mixtures were incubated again at 37 °C for 2 h. After concentration, the products were subjected to HPLC analysis on an Inertsil ODS-2 column (4.0 × 150 mm, GL Sciences, Tokyo, Japan), first by an isocratic elution with TEAA (0.1 M, pH 7.0) for 5 min and then by the use of an acetonitrile gradient (0–10%) over a period of 20 min. The elution profiles, monitored at $\lambda = 230$ nm, are shown in Figure 5. The authentic samples of (5*R*)- and (5*S*)-thymidine glycols were obtained by deprotection of **2a** and **2b**, respectively, with K_2CO_3/MeOH and then with 80% acetic acid.

Thermodynamic studies: Duplexes were formed by cooling the solutions of the two strands (9 nmol) in water (90 μL) from 70 °C to 10 °C, and melting curves were measured at the total oligonucleotide concentrations (C_t) of 2.00, 3.56, 6.32, 11.2, and 20.0 μM in a buffer (pH 7.0, 350 μL) containing 10 mM sodium phosphate, 100 mM NaCl, and 0.1 mM ethylenediaminetetraacetic acid on a Beckman DU-600Tm spectrophotometer. T_m values were obtained by the two-point average method with the data processing software supplied by the manufacturer. The reciprocal of T_m was plotted against $\ln C_t/4$, and the thermodynamic parameters were obtained from the equations, $1/T_m = (R/\Delta H^\circ)\ln C_t/4 + \Delta S^\circ/\Delta H^\circ$ and $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, in which R is the gas constant (= 1.987 cal mol^{-1} K^{-1}) and T is the absolute temperature.[26] The error analysis was carried out as previously reported.[29]

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