J|A|C|S
ARTICLES
Published on Web 10/08/2002

Squaryl Group as a New Mimic of Phosphate Group in Modified Oligodeoxynucleotides: Synthesis and Properties of New Oligodeoxynucleotide Analogues Containing an Internucleotidic Squaryldiamide Linkage

Kousuke Sato, Kohji Seio, and Mitsuo Sekine*

Contribution from the Department of Life Science, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 226-8501, Japan

Received June 1, 2002. Revised Manuscript Received August 1, 2002

Abstract: This paper describes the synthesis and properties of a new type of modified oligodeoxynucleotide containing a neutral but highly polarized squaryl group as a novel mimic of the phosphate group. A modified thymidine dimer derivative (TsqT) having a squaryldiamide linkage was synthesized in almost quantitative yield by a two-step substitution of diethyl squarate with 3′-amino-5′-*O*-(4,4′-dimethoxytrityl)-3′-deoxythymidine and 5′-amino-5′-deoxythymidine. The CD and UV studies of TsqT suggest that this dimer has basically a structure similar to that of TpT. The NMR studies of TsqT show a unique property, namely, that the squaryl group of TsqT is influenced by Mg^{2+} concentration. The ab initio calculations of TsqT showed a highly polarized structure resembling that of a phosphate group. This dimer structural motif was finally incorporated into oligodeoxynucleotides by use of the phosphoramidite approach. The hybridization affinity of these modified oligodeoxynucleotides for the complementary and mismatched oligodeoxynucleotides was studied in detail by using T_m experiments. Consequently, it turned out that in a matched duplex of 5′-d(CGCATsqTAGCC)-3′/5′-d(GGCTAATGCG)-3′ the A−T base pairs at the modified site can be preserved, but instead thermal destabilization of the overall structure was observed. To estimate the structure of the duplex, two kinds of fluorescein chromophores (fluorescein (FL) and cyanine 3 (Cy3)) were introduced into the 5′-terminal site of 5′-d(GACGCATsqTAGCCGAT)-3′ and 5′-d(ATCGGCTAATGCGTC)-3′, respectively. The fluorescence resonance energy transfer experiments using these functionalized oligodeoxynucleotides suggest that the matched duplexes have a bent structure at the modified site. This conclusion was also strongly supported by computational MM and MD simulations.

Introduction

As shown in Figure 1, squaric acid **1** is a dibasic acid that has two acidic hydroxyl groups with pK_a values of 0.54 and 3.48 as well as two highly polarized carbonyl groups.[1] This unique structure provides not only proton acceptor sites[2] at the carbonyl function for hydrogen bonding with other molecules but also binding sites to divalent metal ions.[3,4] All carbon atoms of this compound become equivalent upon complete dissociation.[4−6] Such a dissociated species can be stabilized by formation of a typical 2π aromatic system according to the Hückel rule in terms of the mesomeric effect between the carbonyl and endocyclic double bonds, as shown in Figure 1.

Reaction of **1** with alcohols giving rise to esters **2** easily occurs in the presence of an acid catalyst like the usual esterification of carboxylic acids. Nucleophilic substitution of the squaric acid esters **2** with amines gave the corresponding diamides **3**. These properties have been applied to enhance the biological activity of several drugs and to functionalize organic molecules. For example, phosphonosquaric acid has been designed in a manner where the squaryl group was used as a carboxylic acid equivalent.[7] The skeleton of squaryldiamides was also utilized as a linker of sugar−protein conjugates[8,9] by nucleophilic substitution at the 3- and 4-positions.

On the basis of these precedents, we came up with a new idea that disubstituted squaric acid derivatives **2** and **3** might exhibit chemical behavior similar to that of the phosphate group as far as the charge distribution is concerned, as depicted in Figure 1.[10] Our interest was focused on this inherent property.

On the other hand, there have been reported many analogues of internucleotidic linkages that do not have the phosphorus atom. Matteucci and Veeneman reported the synthesis of a methylene-bridged oligodeoxynucleotide,[11−13] and Dempcy

* Address correspondence to this author. E-mail: msekine@bio.titech.ac.jp.

(1) Schwartz, L. M.; Howard, L. O. *J. Phys. Chem.* **1971**, *75*, 1798.
(2) Terao, H.; Sugawara, T.; Kita, Y.; Sato, N.; Kaho, E.; Takeda, S. *J. Am. Chem. Soc.* **2001**, *123*, 10468−10474.
(3) Schmidt, A. H. *Synthesis* **1980**, 961−994.
(4) West, R.; Niu, H. Y. *J. Am. Chem. Soc.* **1963**, *85*, 2589−2590.
(5) Maahs, G.; Hegenberg, P. *Angew. Chem.* **1966**, *78*, 927−931.
(6) Cohen, S.; Lacher, J. R.; Park, J. D. *J. Am. Chem. Soc.* **1959**, *81*, 3480.
(7) Kim, C. U.; Misco, P. F. *Tetrahedron Lett.* **1992**, *33*, 3961−3962.
(8) Zhang, J.; Kovác, P. *Carbohydr. Res.* **1999**, *321*, 157−167.
(9) Blixt, O.; Norberg, T. *Carbohydr. Res.* **1999**, *319*, 80−91.
(10) Ehrhardt, H.; Hünig, S. *Chem. Ber.* **1977**, *110*, 2506−2523.
(11) Matteucci, M. *Tetrahedron Lett.* **1990**, *31*, 2385−2388.
(12) Agrawal, S., Ed. *Methods in Molecular Biology*; Humana Press: New York, 1993; Vol. 20.
(13) Veeneman, G. H.; van der Marel, G. A.; van den Elst, H.; van Boom, J. H. *Tetrahedron* **1991**, *47*, 1547−1562.

10.1021/ja027131f CCC: $22.00

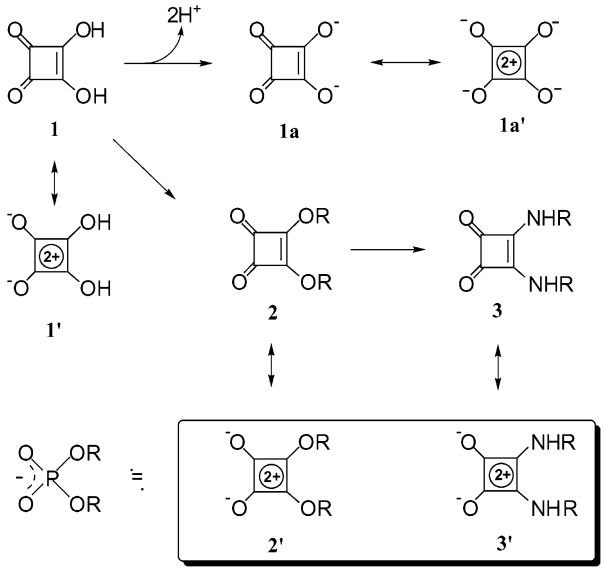


Figure 1. Structures of squaric acid (**1**), squarates (**2**), and squaryldiamides (**3**); and mesomeric effects of squaric acid derivatives **2** and **3** showing the high similarity between their mesomeric structures **2′** and **3′** and a dissociated dialkyl phosphate as the model of an internucleotidic phosphodiester residue.

reported positively charged guanidinium linkages.[14−16] Petersen and Wengel reported a piperazine linkage having one additional atom as compared to the normal phosphodiester linkage. The thermal stability increased upon changing 5′-C from sp^3 to sp^2.[17] An ISIS group also reported a wide variety of non-phosphorus linkages in connection with antisense and antigene strategies.[18−20] These backbone-modified oligodeoxynucleotides exhibited intriguing properties such as an increase of binding affinity for the target nucleic acid, a resistance to degradation by nucleases, and an increase of membrane permeability.

In this paper, we report the hitherto unprecedented synthesis of new types of oligodeoxynucleotide analogues, 5′-d(CGCATsqTAGCC)-3′ and 5′-d(GACGCATsqTAGCCGAT)-3′, containing a squaryldiamide internucleotidic linkage and their interesting, inherent structural properties that were found by CD, T_m experiments, fluorescence resonance energy transfer (FRET), and computational simulations.

Results and Discussion

Synthesis of Thymidine Dimer Derivative (8: TsqT) Containing a Squaryl Group. Various ester derivatives of squaric acid have been synthesized[3,21,22] and used as reagents for the formation of polycyclic compounds,[23] as precursors for the linker of sugar−protein conjugates,[8,9] and as the drug of alopecia areata.[24,25] However, our preliminary experiments suggested that ester derivatives of squaric acid are too reactive toward nucleophiles such as amines so that it seems to be impossible to use squaryl esters as the backbone structure of modified DNAs. Therefore, we designed a thymidine dimer derivative (**8**: TsqT) as the smallest modified structural motif in which two thymidine derivatives are linked via a squaryldiamide linkage (Scheme 1).[3,21] Reaction of 3′-amino-5′-*O*-(4,4′-dimethoxytrityl)-3′-deoxythymidine (**4**)[26−28] with 1.0 equiv of diethyl squarate in ethanol gave easily the amidoester derivative **5** in an excellent yield of 98%. The remaining ethoxy group of **5** was next substituted by treatment with 1.0 equiv of 5′-amino-5′-deoxythymidine (**6**) to give quantitatively the squaryldiamide derivative **7**. The 3′,5′-*O*-free dimer **8** was obtained in 87% yield by detritylation of **7**.

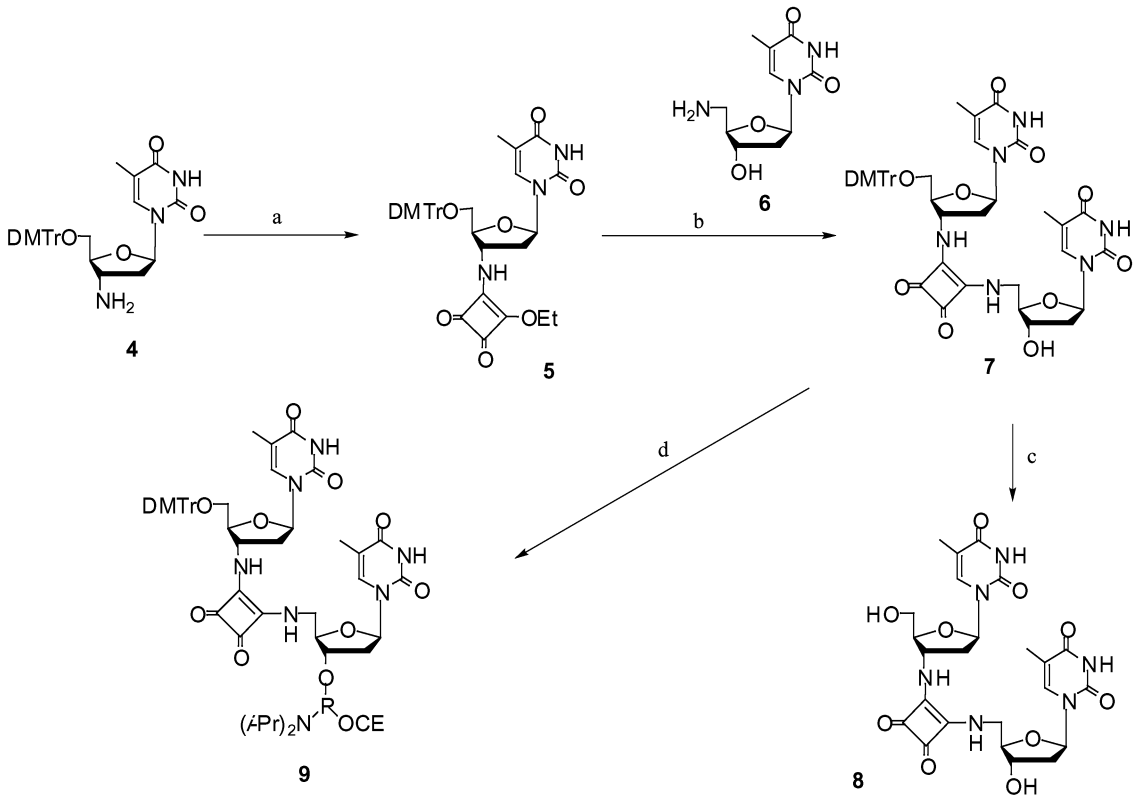
UV Spectra of TsqT (8) and Its Model Compound 10. The UV spectra of TsqT (**8**) and 3-isopropylamino-4-methylaminocyclobuten-1,2-dione (**10**) (i.e., a model compound of **8**) were measured. As shown in Figure 2a, TsqT (**8**) has a λ_{max} peak at 270 nm with a shoulder at 292 nm. Compound **10** has a λ_{max} peak at 293 nm with a shoulder at 276 nm, as shown in Figure 2b. Therefore, the longer wavelength peak (292 nm) of **8** is due to the peak of **10** at 293 nm. It seems that the shoulder peak of **10** at 276 nm was hidden under the eminent peak of **8** at 270 nm. Since **10** has a large ϵ (λ_{max}) value of 4.69×10^4 at 292 nm in water that is 3−4 times greater than those of the canonical nucleosides, it is surmised that the squaryldiamide group contributes substantially to the overall CD spectra of **8**.

CD Spectra of TsqT. The CD spectra of TsqT and TpT were measured at 5 °C. These results are shown in Figure 3. The CD spectrum of TsqT exhibits a wider positive Cotton effect around 285 nm (Figure 3a) than that of TpT around 279 nm (Figure 3b). This difference is due to the contribution of the squaryldiamide group that has a strong UV absorption at the region of 260−300 nm to induce a new CD spectrum based on the surrounding chirality. The intensity of the positive Cotton effects of both TsqT and TpT is very similar to the molar ellipticity value [θ] of 3.2×10^4 (deg cm^2/dmol), while a negative Cotton effect around 260 nm of TsqT is about half that of TpT. As shown in Figure 3b, the CD spectrum of TsqT approaches that of TpT at 85 °C. The intensity of maximum absorption (θ) of the TsqT dimer was reduced by 45%, and that of the TpT dimer was reduced by 53%. It is not possible to conclude that these results imply that TsqT is more rigid than TpT, since it is unclear at the present time how much the squaryldiamide group contributes to the overall CD spectra.

Changes in ^{1}H and ^{13}C Chemical Shifts by Addition of $MgCl_2$. Effects of the Mg^{2+} ion on the squaryldiamide group of TsqT (**8**) were studied by NMR. Figure 4 shows NMR charts of TsqT (**8**) obtained by use of the Mg^{2+} ion of different equivalents of 0, 1.0, and 5.0. In Figure 4a (in the absence of Mg^{2+}) the squaryldiamide protons showed broad signals at 7.54 and 8.03 ppm in DMF-d_7. With addition of 1.0 equiv of Mg^{2+}, these protons were shifted significantly to a low magnetic field at 9.31 and 9.77 ppm (Figure 4b), and the broad signals slightly sharpened.

(14) Dempcy, R. O.; Almarsson, Ö.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 7864−7868.
(15) Dempcy, R. O.; Browne, K. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1995**, *117*, 6140−6141.
(16) Dempcy, R. O.; Browne, K. A.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6097−6101.
(17) Petersen, G. V.; Wengel, J. *Tetrahedron* **1995**, *51*, 2145−2154.
(18) Bhat, B.; Swayze, E. E.; Wheeler, P.; Dimock, S.; Perbost, M.; Sanghvi, Y. S. *J. Org. Chem.* **1996**, *61*, 8186−8199.
(19) von Matt, P.; Lochmann, T.; Altmann, K.-H. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1549−1551.
(20) Mesmaeker, A. D.; Lesueur, C.; Bèvierre, M.-O.; Fritsch, V.; Wolf, R. M. *Angew. Chem., Int. Ed. Engl.* **1997**, *35*, 2790-2794.
(21) Schmidt, A. H. *Synthesis* **1978**, 869−880.
(22) Schmidt, A. H.; Ried, W. *Synthesis* **1978**, 1−23.
(23) Koo, S.; Liebeskind, L. S. *J. Am. Chem. Soc.* **1995**, *117*, 3389−3404.
(24) Papadopoulos, A. J.; Schwartz, R. A.; Janniger, C. K. *Am. J. Clin. Dermatol.* **2000**, *1*, 101−105.
(25) Pardasani, A. G.; Turner, E.; McMichael, A. J. *Arch. Dermatol.* **2001**, *137*, 970−972.
(26) Lavandera, I.; Fernández, S.; Ferrero, M.; Gotor, V. *J. Org. Chem.* **2001**, *66*, 4079−4082.
(27) Imazawa, M.; Eckstein, F. *J. Org. Chem.* **1978**, *43*, 3044−3048.
(28) Horwitz, J. P.; Chua, J.; Noel, M. *J. Org. Chem.* **1964**, *29*, 2076−2078.

Scheme 1 [a]



[a] Key: (a) Diethylsquarate (1.0 equiv), *i*-Pr_2NEt (0.5 equiv), and EtOH; 98%. (b) 5′-Amino-5′-deoxythymidine (**6**) (1.0 equiv), *i*-Pr_2NEt (0.5 equiv), EtOH, 40 °C; 99%. (c) 80% AcOH aq; 87%. (d) Chloro(2-cyanoethoxy)(*N*,*N*′-diisopropylamino)phosphine (1.2 equiv), NEt_3 (3.0 equiv), THF; 72%.

This increase in the acidity of the amide protons strongly suggests that Mg^{2+} can bind to the carbonyl groups by coordination. Further addition of more Mg^{2+} did not affect the chemical shifts of the squaryldiamide, as shown in Figure 4c, but these broad signals became sharper than those in the presence of less than 1.0 equiv of Mg^{2+}. On the other hand, the NH proton of the thymine base that was observed as a broad signal at 11.19 ppm (Figure 4a) was little shifted by addition of Mg^{2+}, but the signal turned sharp like that of the squaryldiamide protons. The ^{13}C NMR chemical shifts of the two carbonyl groups of the squaryldiamide group were shifted slightly to a high magnetic field from 188.62 up to 188.28 ppm upon addition of 1.0 equiv of Mg^{2+} (data not shown). These shifts were less than expected, but it seems that the carbonyl groups of the squaryldiamide were sufficiently polarized so that formation of the metal complex with Mg^{2+} does not affect the electron density of the carbonyl carbons.

Most Stable Energy-Minimized Structure of 8 and Charge Localization of the Squaryldiamide Derivative 10 and Dimethyl Phosphate. Squaryldiamide derivatives have four rotamers because of the sp^2 character of the nitrogen atom of the amide group having a planar structure (Figure 5). Calculation of *N*-isopropyl-*N*′-methylsquaryldiamide (having *i*-Pr and Me groups as the 5′-upstream and 3′-downstream residues, respectively, as the model compound of TsqT) was carried out by using the Gaussian 98 program[29] at the level of MP2/6-31G*//HF/6-31G*.

The most stable structure obtained was that having the *E* conformation around the *N*-*i*-Pr amide linkage and the *Z* conformation around the *N*-methyl amide linkage [designated as (*E, Z*)]. This conformer was more stable than the (*E, E*), (*Z, E*), and (*Z, Z*) rotamers by 1.77, 2.10, and 2.27 kcal/mol, respectively. These results suggest that the most stable (*E, Z*) rotamer exists predominantly to a degree of ca. 93% at 25 °C.

The charge localization of **10** and a dissociated dimethyl phosphate species as the model of the internucleotidic phosphodiester linkage was calculated by the SPARTAN 5.0 program, and the atomic charges were obtained by a procedure based on fitting the so-called electrostatic potentials.[30] The charge density map and charge distribution of the dissociated dimethyl phosphate are shown in parts a and b of Figure 6, respectively. The dissociated dimethyl phosphate has negative charges of −0.84 each on the oxygen atoms, since it has an explicit dissociated oxygen anion. On the other hand, the oxygen atoms of the carbonyl groups of **10** have proven to have relatively high charge units of −0.47 and −0.51, as expected. These results are shown in Figure 6c and d. Comparing the electron density map of the oxygen atom of the dissociated dimethyl phosphate with those of the oxygen atoms of **10**, these polarizing patterns are similar. It is surmised that electrostatic repulsion between the squaryldiamide group in modified DNA duplexes and the phosphate group in the complementary strand

(29) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Zakrzewski, V. G.; Montgomery, J. A., Jr.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J. M.; Daniels, A. D.; Kudin, K. N.; Strain, M. C.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B. G.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A. *Gaussian 98*; Gaussian, Inc.: Pittsburgh, PA, 1998.

(30) Chirlian, L. E.; Francl, M. M. *J. Comput. Chem.* **1987**, *8*, 894−905.

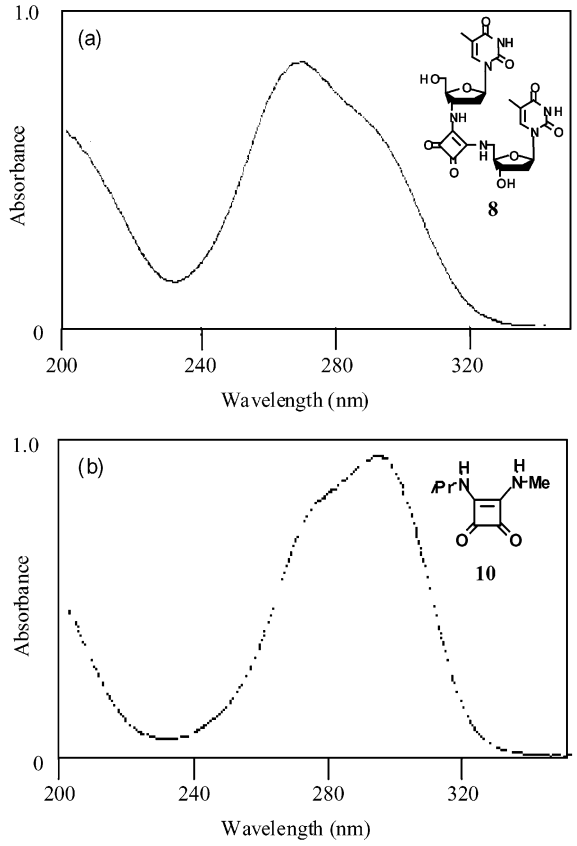


Figure 2. UV spectra of (a) TsqT (**8**) and (b) *N*-isopropyl-*N*′-methylsquaryldiamide (**10**).

is smaller than that of two confronting phosphate groups in unmodified DNA duplexes. It is highly suggested that the squaryldiamide linkage can be used as a new mimic of the phosphate group in oligodeoxynucleotides.

Synthesis of Phosphoramidite Building Unit 9 and Its Incorporation into Oligodeoxynucleotides. To synthesize oligodeoxynucleotides having the modified dimer block **8**, the thymidine dimer phosphoramidite unit **9** was synthesized, as shown in Scheme 1. Phosphitylation of **7** in the usual way gave the phosphoramidite building unit **9** in 72% yield. The standard solid-phase synthesis using **9** in the phosphoramidite method was performed to obtain 5′-CGCATsqTAGCC-3′ and 5′-GACGCATsqTAGCCGAT-3′. The coupling time prescribed for the building unit **9** was 15 min. The coupling yield for the building unit was more than 99%. Under the usual conditions (55 °C, 8 h) required for the deprotection of *N*-acyl groups on the base residues, the squaryldiamide linkage proved to be somewhat sensitive to hydrolysis. The half-life time of TsqT (**8**) in concentrated NH_3 at 55 °C was 4 h (TLC analysis). Therefore, the protecting groups of dC, dA, and dG were removed by treatment with concentrated NH_3 at room temperature. Thus, two modified oligodeoxynucleotides 5′-d(CGCATsqTAGCC)-3′ and 5′-d(GACGCATsqTAGCCGAT)-3′ were successfully isolated in 19 and 24%, respectively. These isolated yields are the same level as those of unmodified oligonucleotides in the usual DNA synthesis.

Enzyme digestion of 5′-d(CGCATsqTAGCC)-3′ and 5′-d(GACGCATsqTAGCCGAT)-3′ with snake venom phosphodiesterase and calf intestine alkaline phosphatase gave four deoxynucleosides and TsqT (**8**) in the correct ratios. This result also suggested that TsqT (**8**) is apparently resistant to venom phosphodiesterase and alkaline phosphatase.

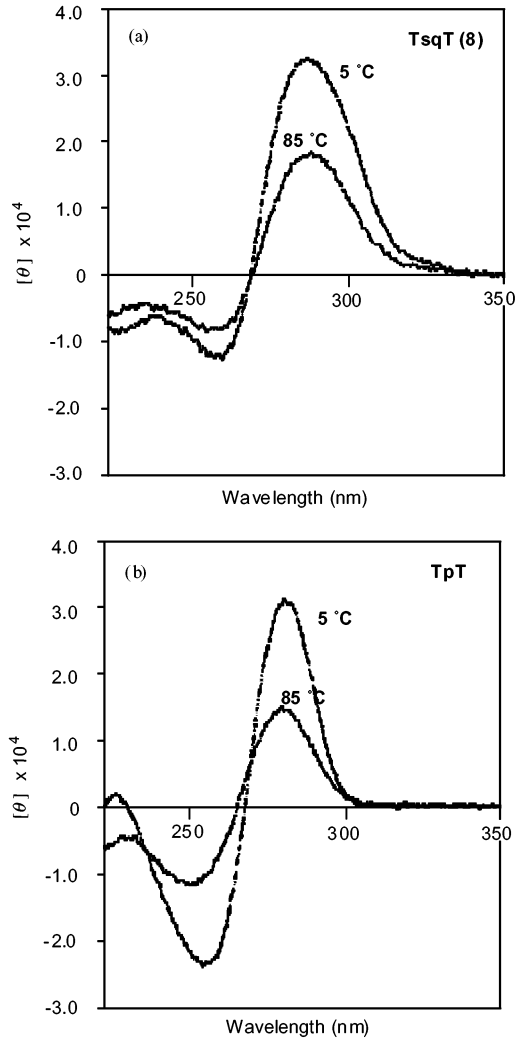


Figure 3. CD spectra of TpT and TsqT (**8**): (a) TsqT (**8**) spectra at 5 and 85 °C and (b) TpT spectra at 5 and 85 °C.

Thermal Stability and Thermodynamic Parameters of the Duplex Formed between Modified Oligodeoxynucleotides and the Complementary Oligodeoxynucleotides. The thermal stability of a duplex formed between 5′-d(CGCATsqTAGCC)-3′ and 5′-d(GGCTAATGCG)-3′ was measured (Figure 7a). We selected this relatively short chain length and base sequence for measurement of T_m values, since one mismatch can be detected clearly within the range of 12.2−17.0 °C, as shown in entries 2 and 3 of Table 1. This enables us to discuss a delicate change in the duplex. As shown in Table 1, the T_m value (30.4 °C) of the modified duplex (entry 4) was lower by 11.3 °C than that of the control unmodified duplex (41.7 °C) (entry 1). This result suggested a possibility that the central TsqT dimer block does not form a hydrogen bond with the dimer sequence of d(ApA) at the opposite site. To see if the A−T base pairs at the modified sites can be formed, the T_m values of the duplexes of 5′-d(CGCATsqTAGCC)-3′ with mismatched complementary strands (i.e., 5′-d(GGCTAGTGCG)-3′ and 5′-d(GGCTGATGCG)-3′), in which dA at the middle site was replaced with dG, were measured in the same way (Figure 7b, Table 1). These mismatched duplexes reduced significantly the T_m values by 9−10 °C more than those of the matched duplex (entries 4−6

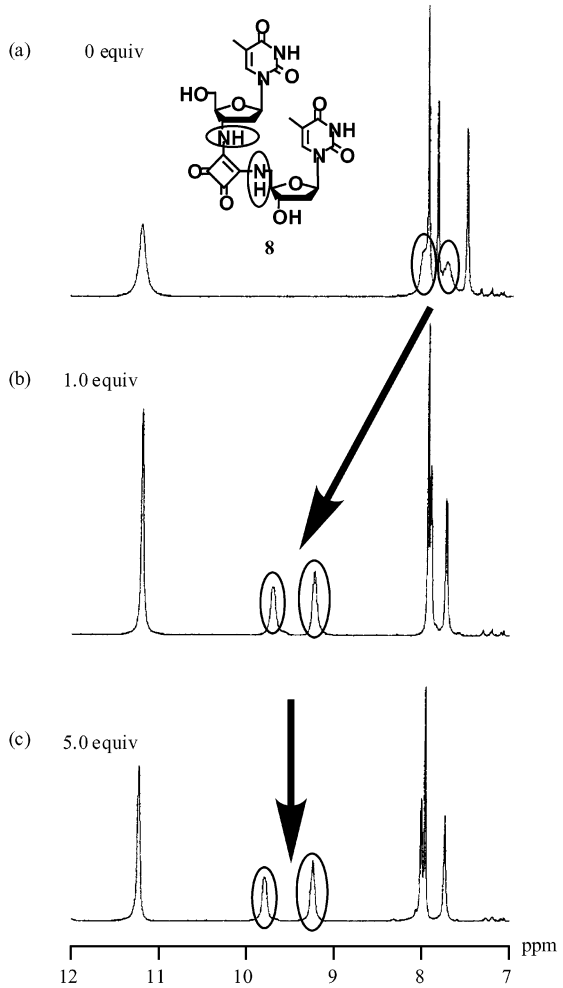


Figure 4. ^{1}H NMR spectra of TsqT (**8**) in the presence of $MgCl_2$: (a) 0 equiv of $MgCl_2$, (b) 1.0 equiv of $MgCl_2$, and (c) 5.0 equiv of $MgCl_2$. Open circles refer to two squaryldiamide protons.

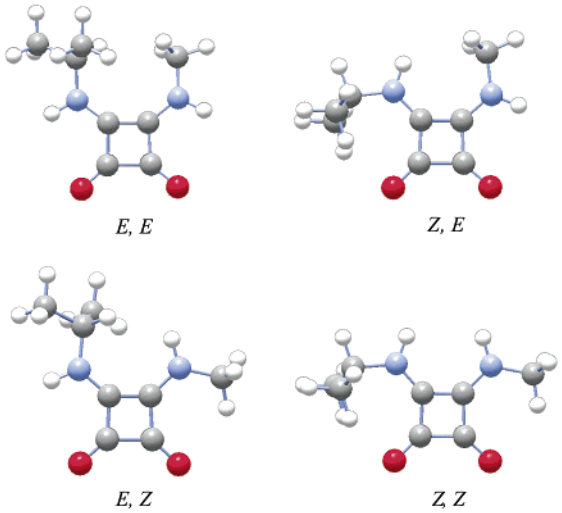


Figure 5. Ab initio calculation of four energy-optimized rotamers of *N*-isopropyl-*N*′-methylsquaryldiamide (**10**).

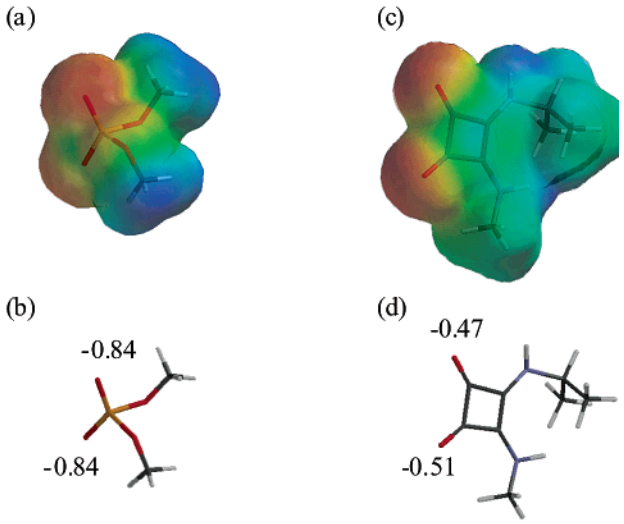


Figure 6. Electrostatic charge distribution calculated by ab initio calculation: (a) charge density map of *N*-isopropyl-*N*′-methylsquaryldiamide (**10**), (b) charge localization on the oxygen atoms of dissociated dimethyl phosphate, (c) charge density map of *N*-isopropyl-*N*′-methylsquaryldiamide (**10**), and (d) charge localization on the oxygen atoms of *N*-isopropyl-*N*′-methylsquaryldiamide (**10**).

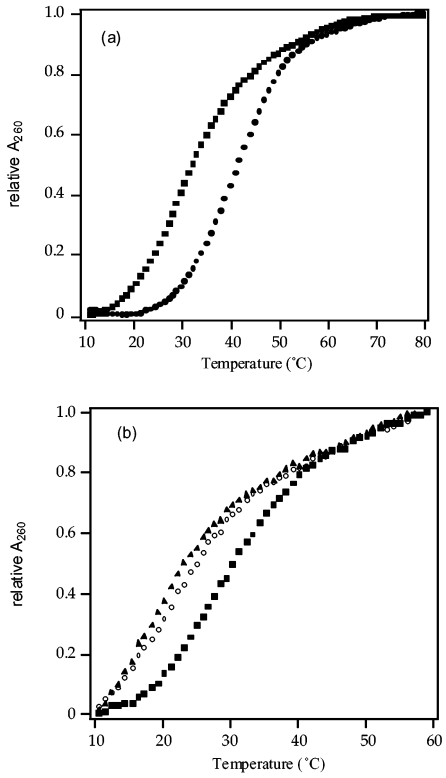


Figure 7. T_m curves of 10mer duplexes: (a) 5′-d(CGCATTAGCC)-3′/5′-d(GGCTAATGCG)-3′ (filled circle) and 5′-d(CGCATsqTAGCC)-3′/5′-d(GGCTAATGCG)-3′ (filled square); and (b) 5′-d(CGCATsqTAGCC)-3′/5′-d(GGCTAATGCG)-3′ (filled square), 5′-d(CGCATsqTAGCC)-3′/5′-d(GGCTAGTGCG)-3′ (open circle), and 5′-d(CGCATsqTAGCC)-3′/5′-d(GGCTGATGCG)-3′ (filled triangle).

of Table 1). These results showed that *the matched duplex that has a squaryldiamide group can form hydrogen bonds at the central A−T base pair sites*. Thermodynamic parameters of the matched and mismatched duplexes were calculated based on their T_m values at various concentrations of the duplexes (Table 1).

The $-\Delta H°$, $-\Delta S°$, and $-\Delta G°$ values of the unmodified duplex (entry 1) (49.3 kcal/mol, 129 × 10^{-3} kcal/K·mol, and 10.8 kcal/mol, respectively) were the highest of all. On the other hand, the $-\Delta S°$ value of the matched modified duplex (entry 4) was 70.9 cal/K·mol and was the lowest of all. The $-\Delta H°$ value was also the lowest, and $-\Delta G°$ was the second highest

Table 1. T_m Values and Thermodynamic Parameters of 10mer Duplexes

5′-CGCA*XX*′AGCC-3′ / 3′-GCGT*YY*TCGG-5′		T_m values			thermodynamic parameters		
entry	XX′/YY′	T_m (°C)	ΔT_m (°C)	$\Delta\Delta T_m$ (°C)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/K·mol)	$-\Delta G°$ (kcal/mol)
1	TpT/AA	41.7			49.3	129.0	10.8
2	TpT/GA	24.7	−17.0		37.1	96.5	8.3
3	TpT/AG	29.5	−12.2		33.1	82.8	8.4
4	TsqT/AA	30.4	−11.3		29.9	70.9	8.8
5	TsqT/GA	21.4	−20.3	−9.0	34.2	88.1	7.9
6	TsqT/AG	20.1	−21.6	−10.3	35.6	94.4	7.4

Table 2. T_m Values and Thermodynamic Parameters of 15mer Duplexes

5′-GACGCA*XX*′AGCCGAT-3′ / 3′-CTGCGTYYTCGGCTA-5′		T_m values		thermodynamic parameters		
entry	XX′/YY′	T_m (°C)	ΔT_m (°C)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/K·mol)	$-\Delta G°$ (kcal/mol)
1	TpT/AA	60.8		79.4	209.6	16.9
2	TsqT/AA	56.4	−4.4	68.3	179.6	14.7

with 8.8 kcal/mol. These results imply that $-\Delta S°$ compensates the unfavorable disadvantage in $-\Delta H°$. The unmodified duplexes of 5′-d(CGCATTAGCC)-3′/5′-d(GGCTGATGCG)-3′ and 5′-d(CGCATTAGCC)-3′/5′-d(GGCTAGTGCG)-3′ having a mismatched base pair G−T have lower T_m values by 12.2 and 17.0 °C, respectively, than that of the matched duplex, as shown in Table 1. Therefore, incorporation of TsqT into the 10mer duplex, which exhibited ΔT_m 11.3 °C, affects the duplex stability to the same or lesser degree as observed in the one-mismatched duplex.

To investigate the trends in T_m values and thermodynamic parameters between modified and unmodified duplexes, the T_m values of 5′-d(GACGCATsqTAGCCGAT)-3′/5′-d(ATCGGCTAATGCGTC)-3′ and 5′-d(GACGCATTAGCCGAT)-3′/5′-d(ATCGGCTAATGCGTC)-3′ were also measured at various concentrations (Table 2).

As shown in Tables 1 and 2, the ΔT_m value of the 15mer duplex (−4.4 °C, entry 2 of Table 2) was smaller by 6.9 °C than that of the 10mer duplex (−11.3 °C, entry 4 of Table 1). The $-\Delta\Delta H°$ and $-\Delta\Delta S°$ values of the 15mer duplexes were smaller by 8.3 kcal/mol and 28.1 cal/K·mol, respectively, than those of the 10mer duplexes. Namely, the enthalpic loss by incorporation of the squaryldiamide group decreased as the chain length was lengthened. However, this effect was competitive by the increased entropy loss. In total, the $-\Delta\Delta G°$ values of both 15mer and 10mer duplexes were almost the same (15mer, 2.2 kcal/mol and 10mer, 2.0 kcal/mol). Therefore, the replacement of the phosphate group to a squaryldiamide group can be estimated to destabilize the duplex stability by ca. 2.0 kcal/mol. This degree is within the range acceptable for various hybridization-mediated studies in life science.

CD Spectra of the Modified Duplex. To study the duplex structure, we measured CD spectra of the duplex of 5′-d(CGCATsqTAGCC)-3′/5′-d(GGCTAATGCG)-3′ having TsqT at 5 and 85 °C. At 5 °C, it is clear that the modified and unmodified control strands form duplexes, judged from the T_m experiments. The CD spectrum of the modified duplex showed a positive Cotton effect at 288 nm, which was shifted significantly to a longer wavelength region as compared with that of the control duplex (Figure 8a). This shift effect seems to be more significant than expected from the CD spectra of TsqT and TpT, since the contribution of the modified dimer to the total CD spectra should be diluted in the oligodeoxynucleotides. Interestingly, it was observed that upon heating at 85 °C the two CD spectra were almost overlapped with each other (Figure 8b). At this temperature both the modified and the control duplexes are dissociated to single strands as mentioned before from the results of the T_m experiments. Therefore, this spectral change would result from not only TsqT itself but also the global conformational change of the duplex that can use the two A−T base pairs.

It seems to us that this modified duplex should have a unique structure, such as bent or twist, to reduce the steric or electric repulsion caused by introduction of the squaryldiamide linkage in a manner where the two A−T base pairs can be formed.

FRET Experiment of Modified Duplex. FRET experiments have been used to estimate the distance between two certain positions within 80 Å in biomolecules.[31−33] To study in more detail the possibility that the modified duplex has a bent structure, we set up FRET experiments using 5′-**FL**-d(GACGCATsqTAGCCGAT)-3′ and its complementary strand 5′-**CY**-d(ATCGGCTAATGCGTC)-3′ having a fluorescein group (FL) and a Cy3 dye (CY) as a donor and an acceptor, respectively, at the 5′-terminal site. As the reference material, 5′-**FL**-d(GACGCATTAGCCGAT)-3′ was also synthesized in a similar manner. These fluorescence-linked oligodeoxynucleotides were synthesized according to the standard methods using commercially available units at the 5′-terminal position.

FRET of the duplex formed between the two fluorescein-linked 15mers was measured at 10 °C by excitation at 490 nm after annealing. As shown in Figure 9, fluorescence intensity of the modified and unmodified duplexes (5′-**FL**-d(GACGCATsqTAGCCGAT)-3′/5′-d(ATCGGCTAATGCGTC)-3′ and 5′-**FL**-d(GACGCATTAGCCGAT)-3′/5′-d(ATCGGCTAATGCGTC)-3′, 5′-d(GACGCATsqTAGCCGAT)-3′/5′-**CY**-d(ATCGGCTAATGCGTC)-3′ and 5′-d(GACGCATTAGCCGAT)-3′/5′-**CY**-d(ATCGGCTAATGCGTC)-3′) was negligible. Compared with these results, the double-labeled duplexes (5′-**FL**-d(GACGCATsqTAGCCGAT)-3′/5′-**CY**-d(ATCGGCTAATGCGTC)-3′, 5′-**FL**-d(GACGCATTAGCCGAT)-3′/5′-**CY**-d(ATCGGCTAATGCGTC)-3′) exhibited FRET effects significantly. As

(31) Tuschl, T.; Gohlke, C.; Jovin, T. M.; Westhof, E.; Eckstein, F. *Science* **1994**, *266*, 785−789.
(32) Bassi, G. S.; Murchie, A. I. H.; Walter, F.; Clegg, R. M.; Lilley, D. M. J. *EMBO J.* **1997**, *16*, 7481−7489.
(33) Walter, N. G.; Hampel, K. J.; Brown, K. M.; Burke, J. M. *EMBO J.* **1998**, *17*, 2378−2391.

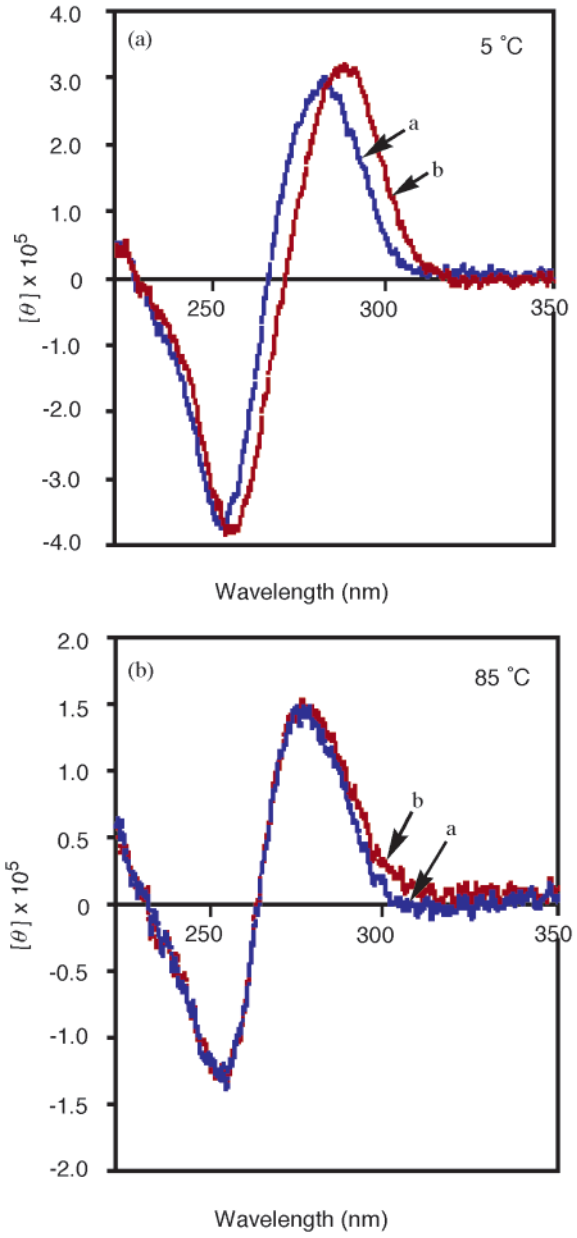


Figure 8. CD spectra of 5′-d(CGCATTAGCC)-3′/5′-d(GGCTAATGCG)-3′ (a, blue) and 5′-d(CGCATsqTAGCC)-3′/5′-d(GGCTAATGCG)-3′ (b, red) at 5 and 85 °C.

shown in Figure 9a, the fluorescence spectrum of the latter control duplex showed 31% reduction of FL fluorescence at 515 nm. This reduction shows the emission energy transfer from FL to CY. On the other hand, the modified duplex exhibited a larger FRET effect of 44% at 515 nm (Figure 9b). The absolute distances between the two 5′-terminals of the sense and antisense oligodeoxynucleotides in the modified and unmodified duplexes were calculated from the Förster distance (R_0) of fluorescein and cyanine 3 to be 62 and 56 Å, respectively.[34] These results strongly imply that the 5′-terminal position of the modified duplex containing the squaryldiamide group becomes closer to the 5′-terminal site of the complementary strand owing to a bent structure.

Computational Simulations. To ascertain if the modified duplex having a squaryldiamide linkage has a unique structure such as bent, we did computer simulations of 5′-d(CGCATsqTAGCC)-3′/5′-d(GGCTAATGCG)-3′ using MacroModel version 6.0. In this calculation the squaryldiamide residue having the most stable (*E*, *Z*) conformation was inserted into the above duplex. The energy minimization and molecular dynamics calculation of the modified and unmodified duplexes were done under the same conditions by using an AMBER* force field[35−37] and a GB/SA solvent model.[38,39] As shown in Figure 10, a slide of the A−A and T−T stacking and a twist of the two A−T base pairs can be seen in the modified site. The whole bent angle of the modified duplex was estimated to be 18.6° larger than that of the unmodified duplex, and the twist angle of the modified duplex is 4.6° larger than that of the unmodified duplex. These conformational changes arising from the squaryldiamide linkage may induce such a bent structure with a somewhat twisted form. Many bent structures of double-stranded DNA have been discovered in their complexes with various proteins.[40−42] Therefore, this type of analogue could provide a new tool for studies of three-dimensional interaction of nucleic acids−nucleic acids and nucleic acids−proteins.

Conclusion

In our studies, the squaryldiamide skeleton was found to distort the DNA duplex to an acceptable degree but to preserve the base pairing ability at the modified dimer site, and the overall structure changed with a bend of the backbone structure in a manner where disturbance induced by incorporation of TsqT can be rationally released. Since the base-recognition ability can be maintained in the modified oligodeoxynucleotides, they could be used for a wide variety of studies that require precise hybridization with the complementary target molecules such as mRNA.

It should be emphasized that a two-step substitution of diethyl squarate with two different amino-deoxynucleoside derivatives proceeded very smoothly in quantitative yields under mild conditions, even in ethanol. Moreover, the squaryldiamide linkage has proven to be stable during deprotection of base-protecting groups as well as removal of the DMTr group. These inherent chemical properties of squaric acid derivatives as synthetic intermediates would provide easy synthesis of a wide variety of oligodeoxynucleotide analogues. In addition, the squaryldiamide group can be used as a new mimic of a wide variety of phosphate groups that are attached to biomolecules involving nucleosides, and there is a potential possibility that various kinds of base−squaric acid and nucleoside−squaric acid conjugates could be used as key skeletons for creation of a new

(34) Lilley, D. M. J.; Wilson, T. J. *Curr. Opin. Chem. Biol.* **2000**, *4*, 507−517.
(35) Weiner, S. J.; Kollman, P. A.; Case, D. A.; Singh, U. C.; Ghio, C.; Alagona, G.; Profeta, S., Jr.; Weiner, P. *J. Am. Chem. Soc.* **1984**, *106*, 765−784.
(36) Weiner, S. J.; Kollman, P. A.; Nguyen, D. T.; Case, D. A. *J. Comput. Chem.* **1986**, *7*, 230−252.
(37) McDonald, D. Q.; Still, W. C. *Tetrahedron Lett.* **1992**, *33*, 7743−7746.
(38) Still, W. C.; Tempczyk, A.; Hawley, R. C.; Hendrickson, T. *J. Am. Chem. Soc.* **1990**, *112*, 6127−6129.
(39) For the use of MacroModel software, see: Tsuruoka, H.; Shohda, K.; Wada, T.; Sekine, M. *J. Org. Chem.* **2000**, *65*, 7479−7494. Sekine, M.; Kurasawa, O.; Shohda, K.; Seio, K.; Wada, T. *J. Org. Chem.* **2000**, *65*, 6515−6524. Sekine, M.; Kurasawa, O.; Shohda, K.; Seio, K.; Wada, T. *J. Org. Chem.* **2000**, *65*, 3571−3578. Seio, K.; Wada, T.; Sekine, M. *Helv. Chim. Acta* **2000**, *83*, 162−180. Seio, K.; Wada, T.; Sakamoto, K.; Yokoyama, S.; Sekine, M. *J. Org. Chem.* **1998**, *63*, 1429−1443. Seio, K.; Wada, T.; Sakamoto, K.; Yokoyama, S.; Sekine, M. *J. Org. Chem.* **1996**, *61*, 1500−1504. Seio, K.; Wada, T.; Sakamoto, K.; Yokoyama, S.; Sekine, M. *Tetrahedron Lett.* **1995**, *36*, 9515−9518.
(40) Zhurkin, V. B.; Lysov, Y. P.; Ivanov, V. I. *Nucleic Acids Res.* **1979**, *6*, 1081−1096.
(41) Travers, A. A. *Cell* **1990**, *60*, 177−180.
(42) Beese, L. S.; Derbyshire, V.; Steitz, T. A. *Science* **1993**, *260*, 352−355.

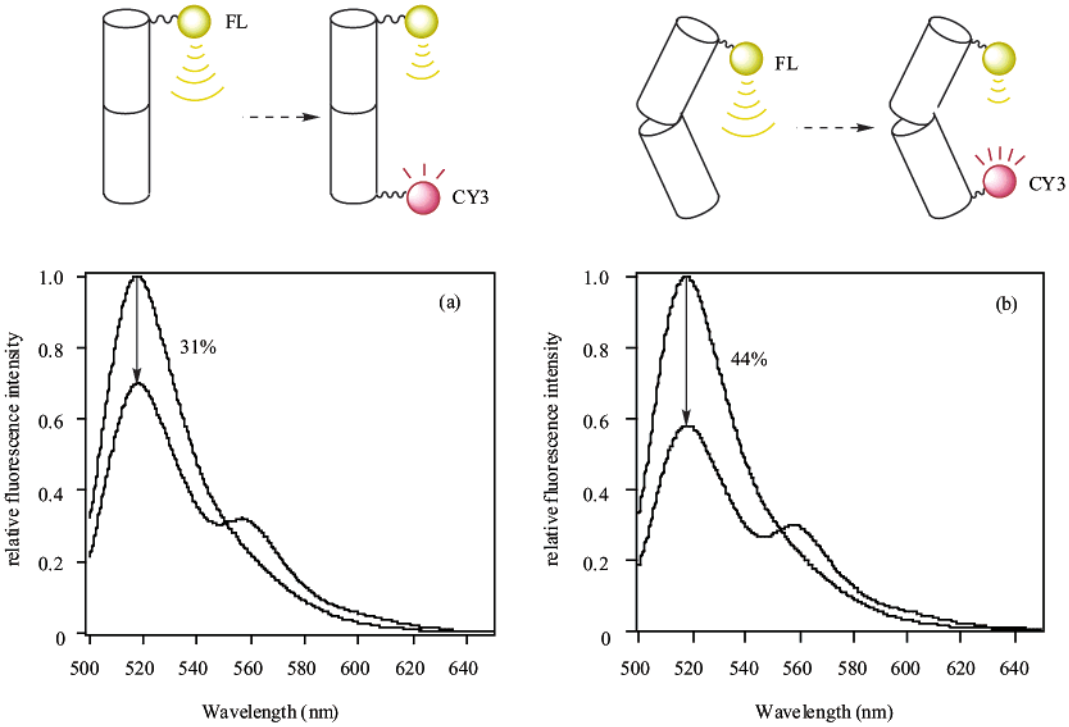


Figure 9. Fluorescence spectra of 15mer duplexes, only fluorescein (donor) and fluorescein (donor)-cyanine 3 (acceptor): (a) 5′-**FL**-d(GACGCATTAGC-CGAT)-3′/5′-**CY**-d(ATCGGCTAATGCGTC)-3′ and (b) 5′-**FL**-d(GACGCATsqTAGCCGAT)-3′/5′-**CY**-d(ATCGGCTAATGCGTC)-3′. The reduced ratio of fluorescein emission at 515 nm is described in the chart.

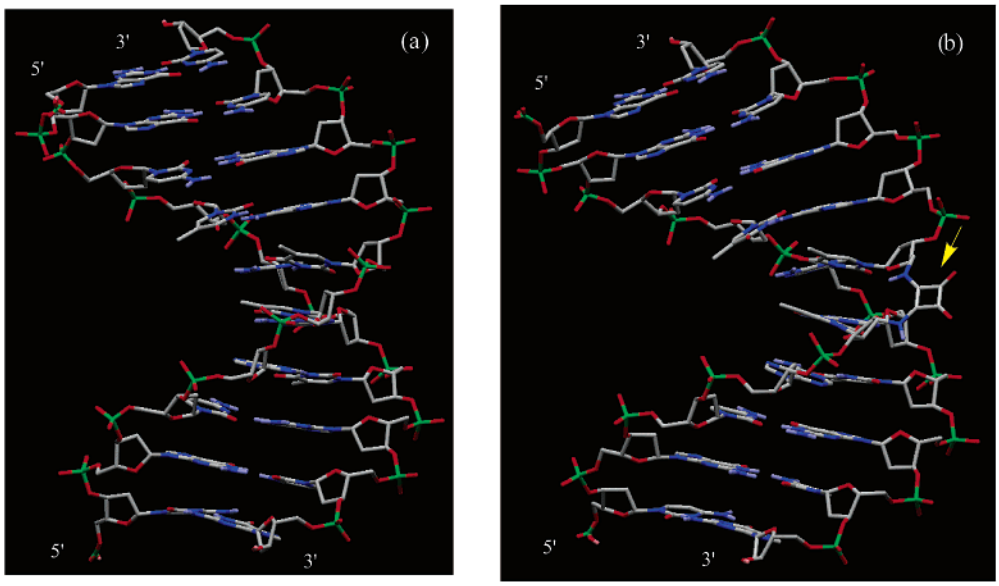


Figure 10. Structures obtained after MD simulations: (a) averaged structure of 5′-d(CGCATTAGCC)-3′/5′−d(GGCTAATGCG)-3′ obtained after 2 ns MD simulation and (b) averaged structure of 5′-d(CGCATsqTAGCC)-3′/5′−d(GGCTAATGCG)-3′ obtained after 2 ns MD simulation. The yellow arrow shows the squaryldiamide group.

class of antisense/antigene molecules and nucleotide analogues. We have reported here the first example that suggests this kind of possibility in nucleotide chemistry. Further studies are now under way in this direction.

Experimental Section

General Methods. TLC was performed with Merck silica gel 60 (F_{254}) plates. ^{1}H, ^{13}C, and ^{31}P NMR spectra were obtained on a JEOL GX-270 apparatus at 270, 68, and 109 MHz, respectively. The chemical shifts were measured from tetramethylsilane (0 ppm) or DMSO-d_6 (2.49 ppm) for ^{1}H NMR, $CDCl_3$ (77.0 ppm), DMSO-d_6 (39.7 ppm), or DMF-d_7 (2.74 ppm) for ^{13}C NMR, and 85% phosphoric acid (0 ppm) for ^{31}P NMR. Column chromatography was performed with Wako silica gel C-200. Reverse-phase HPLC was performed on a combination of Waters 2690 and 996 systems and a SHIMADZU 6A system using μBondasphere and μBondapak C-18 columns (Waters Co., Ltd., 3.9 mm × 150 mm and 7.8 mm × 300 mm, respectively) with a linear gradient of 0−15% CH_3CN/H_2O containing 0.1 M NH_4OAc (pH 7.0) at a flow rate of 1.0 and 3.0 mL/min, respectively. Anion-exchange HPLC was performed on a SHIMADZU 10A system using a Gen-Pak FAX column (Waters Co., Ltd., 4.6 mm × 100 mm) with a linear gradient of 0−0.6 M NaCl/10% CH_3CN and 25 mM phosphate buffer

(pH 6.0) at a flow rate of 1.0 mL/min. ESI mass spectra were measured on Mariner (PerSeptive Biosystems Inc.). MALDI-TOF mass spectra were measured on Voyager RP (Applied Biosystems Inc.). UV spectra were measured by a U-2000 spectrophotometer (Hitachi Co., Ltd.). Thymidine was purchased from Yamasa Co., Ltd. Snake venom phosphodiesterase was purchased from Boehringer Mannheim Biochemica Co., Ltd., and alkaline phosphatase was purchased from Takara Shuzou Co., Ltd. Dry THF was purchased from Wako Pure Chemical Industries, Ltd. Triethylamine was distilled from CaH_2 and stored over Molecular Sieves 4A.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-(2-ethoxy-3,4-dioxocyclobuten-1-yl)amino-3′-deoxythymidine (5). A solution of 3′-amino-5′-*O*-(4,4′-dimethoxytrityl)-3′-deoxythymidine (**4**)[26−28] (0.1 g, 0.18 mmol) and diisopropylethylamine (16 μL, 92 μmol) in 2 mL of ethanol was stirred at room temperature (r.t.), and 3,4-diethoxy-3-cyclobuten-1, 2-dione (47 mg, 0.28 mmol) was added and stirred for 1 h. The reaction solution was evaporated, and the residue was purified by silica gel column chromatography (C-200, 6 g, ethyl acetate/hexane 0−80%) to give **5** as a foam (0.12 g, 98%). ^{1}H NMR ($CDCl_3$, δ) 1.21 (3H, t, CH_2CH_3, J = 6.76 Hz), 1.29 (3H, s, thymine CH_3), 2.48−2.62 (2H, m, 2′H), 3.42−3.59 (2H, m, 5′H), 3.79 (6H, s, OCH_3), 4.15 (1H, m, 4′H), 4.51, 4.54 (1H, 2H, m, q, 3′H, CH_2CH_3, J = 6.76 Hz), 6.78 (1H, t, 1′H, $J_{1′,2′}$ = 7.91 Hz), 6.84 (4H, m, DMTr), 7.26−7.40 (11H, m, DMTr), 7.67 (1H, s, 6H), 9.03, 9.07 (1H, br, 3′NH), 10.19, 10.41 (1H, br, thymine NH); ^{13}C NMR ($CDCl_3$, δ) 11.62, 15.94, 55.29, 56.62, 63.63, 70.02, 84.51, 86.17, 87.21, 113.01, 113.15, 127.27, 127.90, 128.14, 134.55, 134.73, 134.85, 143.73, 151.75, 158.68, 162.95, 170.40, 178.12, 181.10, 190.56. ESIMS Calcd: $C_{37}H_{36}N_3O_9Na$ $[M + Na]^+$ 689.6975. Found: 689.6871.

***N*-(Thymidin-5′-yl)-*N*′-[5′-*O*-(4,4′-dimethoxytrityl)]thymidin-3′-yl]-3,4-dioxocyclobuten-1,2-diamine (7).** To a solution of **5** (50 mg, 75 μmol) in 2 mL of ethanol was added 5′-amino-5′-deoxythymidine (**6**)[28,43−45] (18 mg, 75 μmol). The mixture was stirred at 40 °C for 24 h. The solution was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (C-200, 4 g, methanol/chloroform 0−8%) to give **7** (64 mg, 99%). ^{1}H NMR (DMSO-d_6, δ) 1.44 (3H, s, T5′-CH_3), 1.70 (3H, s, T3′-CH_3), 2.02−2.29 (4H, m, T5′-2′H, T3′-2′H), 3.12−3.73 (11H, m, OCH_3, T5′-5′H, T3′-5′H, T3′-4′H), 3.89 (1H, m, T5′-4′H), 4.11 (1H, m, T3′-3′H), 4.74 (1H, br, T5′-3′H), 5.36 (1H, br, OH), 6.14 (2H, t, T5′-1′H, T3′-1′H), 6.77, 6.80 (4H, m, DMTr), 7.15−7.37 (10H, m, DMTr, T3′-6H), 7.47 (1H, s, T5′-6H), 7.73 (1H, br, NH), 8.24 (1H, s, NH), 11.26 (2H, br, T5′-NH, T3′-NH); ^{13}C NMR (DMSO-d_6, δ) 11.87, 12.09, 45.58, 53.50, 54.98, 62.92, 70.46, 79.10, 83.25, 83.58, 84.94, 85.90, 109.54, 109.82, 113.05, 126.59, 127.50, 127.70, 129.56, 134.99, 135.16, 135.60, 135.85, 144.40, 150.12, 150.20, 157.90, 163.40, 181.81, 182.54. ESIMS Calcd: $C_{45}H_{45}N_6O_{12}Na$ $[M + Na]^+$ 884.8753. Found: 884.8174.

***N*-(Thymidin-5′-yl)-*N*′-(thymidin-3′-yl)-3,4-dioxocyclobuten-1,2-diamine (8).** Compound **7** (86 mg, 0.1 mmol) was dissolved in 2.0 mL of 80% acetic acid, and the mixture was stirred at r.t. for 1.5 h. The solution was evaporated under reduced pressure and coevaporated twice with water to remove the last traces of acetic acid. The residue was diluted with ethyl acetate and extracted with water. The aqueous layer was evaporated under reduced pressure to give **8** as a white solid (49 mg, 87%). ^{1}H NMR (DMF-d_7, δ) 1.78, 1.80 (each 3H, s, T5′-CH_3, T3′-CH_3), 2.14−2.52 (4H, m, T5′-2′H, T3′-2′H), 3.78−3.99 (6H, m, T5′-4′H, 5′H, T3′-4′H, 5′H), 4.37 (1H, m, T5′-3′H), 4.73 (1H, m, T3′-3′H), 5.31 (1H, br, 5′OH), 5.52 (1H, br, 3′OH), 6.27 (2H, t, t, 1′H, $J_{1′,2′}$ = 6.27, 6.60 Hz), 7.54 (1H, s, 6H), 7.76 (1H, br, NH of squaryl amide), 7.87 (1H, s, 6H), 7.97 (1H, br, NH of squaryldiamide), 11.19 (2H, br, NH, NH of thymines); ^{13}C NMR (DMF-d_7, δ) 17.89, 18.10, 44.76, 44.88, 52.02, 60.00, 67.27, 77.32, 89.90, 90.30, 91.70, 92.19, 115.82, 116.38, 127.18, 142.27, 156.82, 156.86, 169.87, 173.41, 174.94, 189.00. ESIMS Calcd: $C_{24}H_{29}N_6O_{10}$ $[MH]^+$ 561.5287. Found: 561.5207.

Thymidine Dimer Building Unit (9). To a stirred solution of **7** (250 mg, 0.29 mmol) and triethylamine (120 μL, 0.87 mmol) in 2.9 mL of dry THF was added chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphine (78 μL, 0.35 mmol) at r.t.. After being stirred for 3.5 h, the mixture was diluted with $CHCl_3$ and extracted with 5% $NaHCO_3$ aq. The organic layers were collected and dried over Na_2SO_4. After being filtered, the mixture was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (C-200, 5 g, methanol/chloroform 0−2%). The fractions containing the product were combined and evaporated under reduced pressure, and the residue was dissolved in 1.0 mL of $CHCl_3$ and precipitated into 20 mL of hexane to give **9** as a white solid (222 mg, 72%). ^{1}H NMR ($CDCl_3$, δ) 1.16, 1.18 (12H, d, CH_3 of *i*-Pr, J = 6.26 Hz), 1.49, 1.86 (each 3H, s, CH_3 of thymines), 2.31−2.73 (6H, m, 2′H, CE), 3.37−4.17 (18H, m, 3′H, 4′H, 5′H, OCH_3, *i*-Pr, CE), 4.49, 5.02 (each 1H, s, NH of squaryl amide), 6.01, 6.30 (each 1H, m, 1′H), 6.78−7.49 (15H, m, DMTr, 6H); ^{13}C NMR ($CDCl_3$, δ) 11.80, 12.26, 20.00, 20.37, 22.85, 22.94, 24.46, 24.52, 37.57, 39.00, 43.14, 43.32, 45.20, 45.29, 55.04, 55.15, 57.73, 58.13, 63.33, 84.00, 86.35, 86.88, 111.34, 112.69, 113.07, 117.86, 118.24, 126.83, 127.76, 127.95, 129.91, 135.01, 135.23, 144.11, 150.42, 150.72, 158.05, 158.35, 163.88, 166.93, 167.70, 182.24, 183.07; ^{31}P NMR ($CDCl_3$, δ) 149.34, 149.90. ESIMS Calcd: $C_{54}H_{62}N_8O_{13}PNa$ $[M + Na]^+$ 1085.0960. Found: 1085.0545.

UV Spectra. UV spectra of TsqT (**8**) and its model compound **10** were measured in aqueous solution at r.t. by a U-2000 spectrophotometer (Hitachi, Co., Ltd.). The λ_{max} values of **8** are 270 and 292 nm. Those of **10** are 276 and 293 nm.

NMR Spectra of 8 in the Presence of Mg^{2+}. NMR spectra were obtained on a JEOL GX-270. The ^{1}H NMR spectra of **8** were obtained at various concentrations of $MgCl_2$.

Synthesis of Oligonucleotides. TpT and all oligodeoxynucleotides were synthesized by ABI DNA/RNA synthesizer 392 on a 1.0 μmol scale and released from the CPG polymer support in the DMTr-on mode. The coupling time prescribed for the thymidine dimer building unit **9** was 15 min. The coupling using the dimer building unit proceeded in >99% yield, which was estimated by the DMTr cation analysis. The protecting groups of dC, dA, and dG used were acetyl, phenoxyacetyl, and isopropylphenoxyacetyl, respectively, and were deprotected by treatment with concentrated NH_3 (1.5 mL) at r.t. for 2 h. The ammonia solution was filtered and evaporated under reduced pressure. The residue was purified by a Sep-Pak reverse-phase column purchased from Waters Co., Ltd. After the column was immersed with CH_3CN (5.0 mL) and 0.1 M NH_4OAc (pH 7.0) (5.0 mL) for 1 min, the solution was removed by filtration. The sample was charged on the column. Subsequently, the failure sequences without the DMTr group were washed out by 10−12% CH_3CN/0.1 M NH_4OAc (pH 7.0) (10 mL). After that, the column was treated with 1.0% aqueous TFA (5.0 mL) at r.t. for 5 min to remove the DMTr group of DMTr−oligodeoxynucleotides bound to the column. Further elution was carried out by 0.1 M NH_4OAc (pH 7.0) (5.0 mL) and 15% CH_3CN in water (10 mL) to obtain the desired oligodeoxynucleotide. After removal of the solvents, the residue was purified by reverse-phase HPLC using a linear gradient of 0−15% CH_3CN in 0.1 M NH_4OAc (pH 7.0) for 20 min at the flow rate of 1 mL/min. If necessary, anion-exchange HPLC using a linear gradient of 0−60% buffer B in buffer A for 45 min at the flow rate 1 mL/min was used (buffer A, 10% CH_3CN/25 mM phosphate buffer (pH 6.0); buffer B, 10% CH_3CN/25 mM phosphate buffer (pH 6.0), 1.0 M NaCl).

The isolated yields were calculated by using the ϵ values obtained by the method of Cantor.[46] The ϵ value (=22386) of the TsqT dimer was calculated by the enzyme digestion of 5′-d(GACGCATsqTAGC-

(43) Hata, T.; Yamamoto, I.; Sekine, M. *Chem. Lett.* **1976**, 601−604.
(44) Yamomoto, I.; Sekine, M.; Hata, T. *J. Chem. Soc., Perkin Trans. 1* **1980**, 306−310.
(45) Lin, T.-S.; Prusoff, W. H. *J. Med. Chem.* **1978**, *21*, 109−112.
(46) Fasman, G. D., Ed. *Handbook of Biochemistry and Molecular Biology*, 3rd ed.; CRC Press: Cleveland, 1977; Vol. 1.

CGAT)-3′ with snake venom phosphodiesterase and calf intestine alkaline phosphatase. 5′-d(CGCATsqTAGCC)-3′: MALDI-TOF mass calcd for $C_{100}H_{123}N_{38}O_{56}P_8$, 3001.06; found, 3001.33. 5′-d(GACGCATsqTAGCCGAT)-3′: MALDI-TOF mass calcd for $C_{150}H_{184}N_{60}O_{85}P_{13}$, 4590.08; found, 4588.40.

Enzyme Analysis of Modified Oligodeoxynucleotides. The enzymatic digestion was performed by using a modified oligodeoxynucleotide (0.5 OD), snake venom phosphodiesterase (4 μL), and calf intestine alkaline phosphatase (2 μL) in 50 μL of alkaline phosphatase buffer (pH 9.0) at 37 °C for 2 h. After the enzymes were deactivated by heating to 100 °C for 1 min, the solution was diluted and filtered by a 0.45 mm filter (Millex-HV, MILLIPORE). This solution was analyzed by reverse-phase HPLC using a linear gradient of 0−20% CH_3CN in 0.1 M NH_4OAc (pH 7.0) for 30 min at the flow rate of 1 mL/min.

CD Spectra of TsqT, TpT, and Modified and Unmodified DNA Duplexes. CD spectra of TsqT, TpT, and modified and unmodified DNA duplexes were measured on a JASCO J-725 spectropolarimeter. The plots of molar ellipticity [θ] versus wavelength (nm) were obtained under the conditions of 10 mM phosphate buffer (pH 7.0), 150 mM NaCl, and 0.1 mM EDTA at 5, 25, 45, 65, and 85 °C. The blank experiments were also done under the same conditions. The concentration of each dimer was 1.0 OD/mL. The concentration of each oligodeoxynucleotide was 2.0 μM. Measurement was carried out in a 1.0 mL cell with a 0.5 cm light length. In a typical experiment, a solution of a duplex composed of two oligodeoxynucleotides was heated to 90 °C for 1 min and allowed to cool to r.t. overnight. The CD spectra of this solution were measured eight times over the range from 200 to 350 nm at 5 to 85 °C.

Thermal Denaturation Studies. Plots of A_{260} versus T (°C) for 5′-d(GACGCATsqTAGCCGAT)-3′ in the presence of 5′-d(ATCGGCTAATGCGTC)-3′ and 5′-d(CGCATsqTAGCC)-3′ in the presence of 5′-d(GGCTAATGCG)-3′, 5′-d(GGCTAGTGCG)-3′, or 5′-d(GGCTGATGCG)-3′ were obtained in 10 mM phosphate buffer (pH 7.0), 150 mM NaCl, and 0.1 mM EDTA using a BECKMAN DU 650 spectrophotometer. The blank experiments were done under the same conditions. A 2.0 μM solution of each oligodeoxynucleotide was put in a 400 μL cell with a 1 cm light length. In a typical experiment, a solution of a duplex composed of two oligodeoxynucleotides was heated to 60 °C and then allowed to cool to 5 °C over the period of 55 min. Data points were then collected as the temperature was increased at the rate of 1 °C/1 min. The first derivative of each curve was calculated by Igor Pro (WaveMetrics, Inc.).

Thermodynamic Parameters. Thermodynamic parameters $\Delta G°$, $\Delta S°$, and $\Delta H°$ were obtained by the measurement of Tm values at various oligodeoxynucleotide concentrations of 0.5, 1.0, 2.0, 3.0, and 6.0 μM.

FRET Experiments. Fluorescence spectra were obtained in 10 mM phosphate buffer (pH 7.0) containing 1.0 M NaCl using a SHIMADZU RF-5300PC spectrophotometer. The fluorescence dyes used as the donor and the acceptor were fluorescein and cyanine 3, which were introduced into the 5′-end of 5′-d(GACGCATsqTAGCCGAT)-3′ and 5′-d(ATCGGCTAATGCGTC)-3′, respectively. These oligonucleotides were synthesized using a DNA/RNA synthesizer by use of fluorescein and cyanine 3 phosphoramidite derivatives purchased from GLEN RESEARCH. The blank experiments were done under the same conditions. A 100 nM solution of each oligonucleotide was put in a 400 μL cell. In a typical experiment, a solution of a duplex composed of two oligodeoxynucleotides was heated in 10 mM phosphate buffer (pH 7.0) containing 1.0 M NaCl at 90 °C for 1 min and allowed to cool to r.t. overnight. The fluorescence spectra of these samples were measured at 10 °C on a fluorophotometer by excitation at 490 nm, and the emission was measured at 515 nm. The fluorescence of double strands containing only donor (F_d) 5′-**FL**-d(GACGCATsqTAGCCGAT)-3′/5′-d(ATCGGCTAATGCGTC)-3′, only acceptor (F_a) 5′-d(GACGCATsqTAGCCGAT)-3′/5′-**CY**-d(ATCGGCTAATGCGTC)-3′, and donor and acceptor (F_{da}) 5′-**FL**-d(GACGCATsqTAGCCGAT)-3′/5′-**CY**-d(ATCGGCTAATGCGTC)-3′ were measured, and FRET efficiency (F_E) was calculated by eq 1.

$$F_E = 1 - \left(\frac{F_{da} - F_a}{F_d}\right) \tag{1}$$

The absolute distances between the two 5′-terminals of the sense and antisense oligonucleotides in modified and unmodified duplexes were calculated from eq 2. R_0 refers to the Förster distance of donor−acceptor pairs of fluorophores, and R is the distance between the fluorophores.

$$F_E = \frac{1}{\left[1 + \left(\frac{R}{R_0}\right)^6\right]} \tag{2}$$

Computational Simulations. Four kinds of squaryldiamide rotamers (*Z, Z*), (*Z, E*), (*E, Z*), and (*E, E*) were optimized at the HF/6-31G* level, and the energies of the rotamers[29] were calculated at the MP2/6-31G* level by using the Gaussian 98 program package in ab initio calculations. The initial structure of TsqT (**8**) was constructed by using the structures of the most stable (*E, Z*) rotamer, and the thymidine residue was extracted from a library of MacroModel version 6.0. The force field used was the AMBER*[35−37] of MacroModel version 6.0, and the effect of the solvent was included by using the GB/SA[38] solvent model. The default values for the atomic partial charges were used in all the calculations. The initial structure was energy-minimized, and the optimized structure was used as the starting structure for the next simulation. The local minimum was searched by the 5000-step energy minimization, and global minimum structures were searched by 5000 trials by using the MCMM option. One of the low-energy structures was orientated in anti conformation where both thymine bases were introduced to the center of the 10mer duplex. The local minimum structure was searched for by a 5000-step energy minimization, and the optimized structure was used as the starting structure for the next MD simulation. MD simulation was carried out at 300 K in H_2O for 2000 ps where thousands of samples were collected. The averaged structure was obtained from the simulated structures in later 1000 ps. The bent angle was obtained from the three points in the duplexes; the first is the center of the two central A−T base pairs, and the second and the third are the centers of the base pairs of the two edges. The twist angle was obtained from the four points in the duplexes; they are the glycosyl nitrogen atoms in the base pairs of the two edges.

The distribution of the electrostatic charge of **10** and the dissociated species of dimethyl phosphate was calculated by the SPARTAN 5.0 program in ab initio calculations.

Acknowledgment. This work was supported by a Grant from "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS-RFTF97I00301) and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

JA027131F