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Molecular Design for Reversing the Photoswitching Mode of Turning ON and OFF DNA Hybridization

Xingguo Liang,[a] Nobutaka Takenaka,[a] Hidenori Nishioka,[a] and Hiroyuki Asanuma*[a, b]

Abstract: A new photoswitch for DNA hybridization involving *para*-substituted azobenzenes (such as isopropyl- or *tert*-butyl-substituted derivatives) with L-threoninol as a linker was synthesized. Irradiation of the modified DNA with visible light led to dissociation of the duplex owing to the destabilization effect of the bulky substituent on the *trans*-azobenzene. In contrast, *trans*-to-*cis* isomerization (UV light irradiation) facilitated duplex formation. The direction of this photoswitching mode was entirely reversed relative to the previous system with an unmodified azobenzene on D-threoninol whose *trans* form turned on the hybridization, and *cis* form turned it off. Such reversed and reversible photoswitching of DNA hybridization was directly demonstrated by using fluorophore- and quencher-attached oligonucleotides. Furthermore, it was revealed that the *cis*-to-*trans* thermal isomerization was greatly suppressed in the presence of the complementary strand owing to the formation of the more-stable duplex in the *cis* form.

Keywords: azobenzene · DNA · hybridization · photochemistry · photoswitch

Introduction

Photocontrol of DNA hybridization can be used as a robust tool for elucidating DNA-involving biological processes, as well as for constructing photon-fuelled DNA nanomechanical molecular devices. A large number of biofunctions have been photoregulated by covalently attaching light-responsive molecules to nucleic acids.[1] In our previous work, azobenzene was conjugated with DNA through a D-threoninol linker as the photoswitch and DNA hybridization was reversibly turned on (*trans*-azobenzene) and off (*cis*-azobenzene) by irradiation with light.[2,3] The photoregulation mechanism can be demonstrated as follows: the planar *trans*-azobenzene intercalates between the two adjacent base pairs and stabilizes the DNA duplex by stacking interactions, whereas the nonplanar *cis*-azobenzene destabilizes the duplex by steric hindrance.[3] On the basis of the above strategy, several model systems have also been constructed for photoswitching DNA transcription, DNA primer extension, and other enzymatic reactions.[4] On the other hand, the photoregulation efficiency was not high enough in some cases owing to the difficult *trans*→*cis* photoisomerization caused by the strong stacking interaction between *trans*-azobenzene and the DNA base pairs. For more-efficient photoregulation of DNA functions both in vitro and in vivo, novel molecular design is highly desirable.

Recently, we found that modification of azobenzene could remarkably affect the photoregulatory efficiency.[5] Methylation of azobenzene at the *ortho* position greatly enhanced the change in melting temperature (ΔT_m) induced by *cis*↔*trans* isomerization. The *trans*-2′,6′-dimethylazobenzene stabilized the duplex fairly well, whereas its *cis* form destabilized it more than the unsubstituted azobenzene did.[5] In contrast, the methyl group introduced at the *para* position of azobenzene destabilized the duplex in the *trans* form and stabilized it in the *cis* form relative to the corresponding isomers of unmodified azobenzene. Indeed, *para* substitution rather lowered the photoregulatory efficiency of the normal photoswitching mode. These results prompted us to design a reversed photoswitch that turns on duplex formation by UV

[a] Dr. X. Liang, N. Takenaka, H. Nishioka, Prof. Dr. H. Asanuma
Department of Molecular Design and Engineering
Graduate School of Engineering
Nagoya University
Furo-cho, Chikusa-ku
Nagoya 464-8603 (Japan)
Fax: (+81) 52-789-2528
E-mail: asanuma@mol.nagoya-u.ac.jp

[b] Prof. Dr. H. Asanuma
Core Research for Evolution Science and Technology (CREST)
Japan Science and Technology Agency (JST)
Kawaguchi, Saitama 332-0012 (Japan)

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light irradiation (*cis*-azobenzene) and turns it off by irradiation with visible light (*trans*-azobenzene). We expect that a completely reversed photoswitch will not only greatly extend our approach for photoregulating DNA functions, but will also diversify the design of light-fuelled DNA nanomachines,[6] especially when it is used with the combination of the normal photoswitch. In this study, we synthesized photoswitches of the reverse mode by tethering *para*-substituted azobenzene with a bulky group on L-threoninol. The reversed photoregulation of DNA hybridization was assayed by T_m measurement, and a fluorophore–quencher system was used for further evaluation of the reverse photoswitch. The *trans*→*cis* photoisomerization of the azobenzene derivatives conjugated with DNA and the thermal stability of their *cis* forms were also investigated.

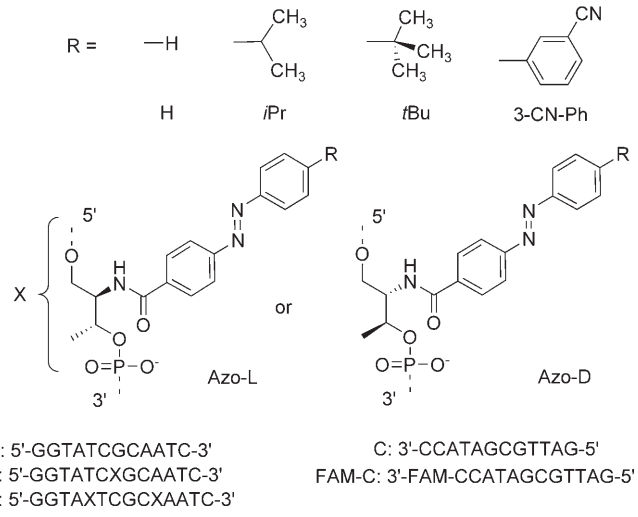
Results and Discussion

Molecular Design for Reversing the Direction of the Photoswitching Mode

To achieve the reversed photoregulation of hybridization, the duplex should be destabilized by *trans*-azobenzene as largely as possible, and destabilization by *cis* isomerization should be minimized. For destabilization by the *trans* form, a bulky group, such as isopropyl or *tert*-butyl, was attached at the *para* position of azobenzene to enlarge the steric hindrance of the DNA backbone.[5] To minimize the destabilization of *cis*-azobenzene, L-threoninol was used as the linker instead of D-threoninol.[7] According to our previous study, D-threoninol was shown to be favorable for a normal photoswitch because it facilitated the hybridization of modified DNA involving *trans*-azobenzene and largely destabilized the duplex involving the *cis* form.[2] In the case of the L form, the azobenzene moiety tethered on this linker stabilized the duplex less in the *trans* form and suppressed the destabilizing effect of the *cis* form,[2] indicating that L-threoninol should fit for reversed photoswitching mode. On the basis of the above molecular design, we synthesized azobenzene moieties with a isopropyl (*i*Pr), a *tert*-butyl (*t*Bu), or a 3-cyanophenyl (3-CN-Ph) group at the *para* position and introduced them into DNA through the D- or L-threoninol linker (Scheme 1).

Abstract in Japanese:

リンカーにL-トレオニノールを用いてパラ位置換（例えば*tert*-ブチルやイソプロピル置換）アゾベンゼンをDNAに導入することで、新規な光スイッチを合成した。この光スイッチに可視光を照射すると、*trans*-アゾベンゼン上の嵩高い置換基による立体障害で二重鎖は解離した。一方UV光を照射して*trans*から*cis*-体へ異性化させたところ、二重鎖の形成が促進された。この光スイッチは、未修飾のアゾベンゼンとD-threoninolを用いて合成したこれまでの光スイッチ（*trans*-体で二重鎖形成し*cis*-体で二重鎖が解離）と全く逆の方向を示した。今回合成した光スイッチが従来のものと逆であることは、蛍光色素とクエンチャーを導入したオリゴヌクレオチドを用いて直接的に示すことが出来た。更に、*cis*-体から*trans*-体への熱異性化が、対応する相補鎖が存在すると大きく抑制されることも明らかにした。



S_{Na}: 5'-GGTATCGCAATC-3'
S_{a1}: 5'-GGTATCXGCAATC-3'
S_{a2}: 5'-GGTAXTCGCXAATC-3'

C: 3'-CCATAGCGTTAG-5'
FAM-C: 3'-FAM-CCATAGCGTTAG-5'

S_{a2}-Dab: 5'-Dabcyl-GGTAXTCGCXAATC-3' (X = *t*Bu-Azo-L)

Scheme 1. Structures of azobenzene moieties introduced into DNA and DNA sequences used in this study. Sequence C is complementary to S_{Na}. S_{a1} and S_{a2} are the modified versions of S_{Na} involving azobenzene derivatives. In FAM-C, a fluorescein moiety is attached to C as the fluorophore. In S_{a2}-Dab, a dabcyl moiety is attached to S_{a2} as the quencher.

The azobenzene derivatives with various substituents were synthesized by coupling of ethyl 4-nitrosobenzoate with the corresponding aniline, followed by hydrolysis with sodium hydroxide (Scheme 2a). The resulting azobenzenes were then tethered on D- or L-threoninol and incorporated into a 12-nt-long DNA by using standard phosphoramidite chemistry as reported previously (Scheme 2b).[2] All the modified DNAs were purified by reversed-phased HPLC and characterized by MALDI-TOF MS. All the azobenzene residues in DNA were sufficiently stable under the conditions employed. The DNA sequences are shown in Scheme 1.

Change in Melting Temperature of DNA Duplex Induced by *trans–cis* Isomerization of Azobenzene Derivatives

At first, we investigated the destabilization effect of *para* substitution of azobenzene with a bulky group on DNA-duplex formation. As a control, unsubstituted *trans*-azobenzene tethered on D-threoninol (H-Azo-D), a normal-mode photoswitch, stabilized the duplex by 1.2 °C relative to the native one (S_{Na}/C, T_m = 47.7 °C, see Table 1).[2,8] In contrast, *para* modification (the *trans* form) lowered the T_m of the duplex by 4–7 °C: The T_m values of duplex S_{a1}/C for 3-CN-Ph-Azo-D and *i*Pr-Azo-D were 43.6 and 41.0 °C, respectively. Evidently, *para* substitution with a bulky group was effective for destabilizing the duplex in the *trans* form. For the *cis* form, however, *para* modification did not cause much change of T_m, which was around 43 °C for both substituents. As a result, T_m for *cis*-*i*Pr-Azo-D exceeded that of its *trans* form by 1.9 °C ($\Delta T_m = -1.9$ °C) when D-threoninol was used. The photoswitch direction was reversed, although the ΔT_m was not large enough.

CHEMISTRY
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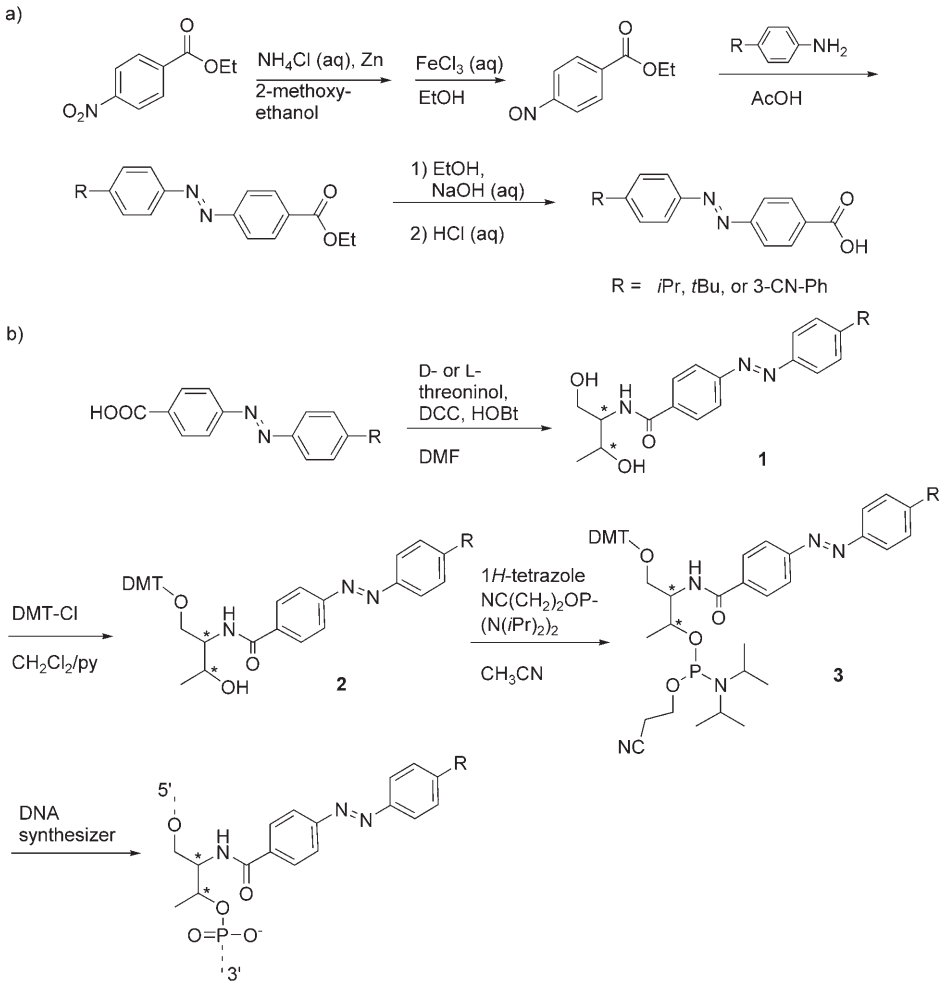

Scheme 2. Synthesis of a) the azobenzene derivatives and b) the corresponding phosphoramidite monomers for their incorporation into DNA.

Table 1. Melting temperatures of the duplex involving azobenzene and its derivatives in the *trans* and *cis* forms.[8]

Azobenzene	Duplex	T_m [°C][a]		
		cis	*trans*	ΔT_m[b]
H-Azo-D	S_{a1}/C	43.2	48.9	5.7
3-CN-Ph-Azo-D	S_{a1}/C	43.6	43.6	0.0
*i*Pr-Azo-D	S_{a1}/C	42.9	41.0	−1.9
3-CN-Ph-Azo-L	S_{a1}/C	44.5	43.6	−0.9
*i*Pr-Azo-L	S_{a1}/C	46.9	40.2	−6.7
*t*Bu-Azo-L	S_{a1}/C	45.9	38.6	−7.3
3-CN-Ph-Azo-L	S_{a2}/C	33.7	32.1	−1.6
*i*Pr-Azo-L	S_{a2}/C	39.0	28.1	−10.9
*t*Bu-Azo-L	S_{a2}/C	38.6	25.3	−13.3
S_{Na}/C (native)		47.7		

[a] Experimental conditions: $[S_{a1}]=[S_{a2}]=[C]=5\,\mu M$, $[NaCl]=100\,mM$, pH 7.0 (10 mM phosphate buffer). [b] Change of T_m induced by the *cis*→*trans* isomerization.

When L-threoninol was used for tethering isopropyl-modified azobenzene (*i*Pr-Azo-L), the degree of the reverse effect was greatly improved: $-\Delta T_m$ became as large as 6.7 °C in the case of the S_{a1}/C duplex. This value was much larger than that of *i*Pr-Azo-D ($-\Delta T_m = 1.9$ °C). It should also be noted that it was even larger than the ΔT_m of H-Azo-D ($\Delta T_m = 5.7$ °C) with the previous normal switching mode, showing that *i*Pr-Azo-L could be used as an efficient reverse mode of photoswitch (see Supporting Information, Figure S1 for the melting curves). This remarkable improvement was mainly attributed to the increase in T_m in the *cis* form: the T_m for *cis*-*i*Pr-Azo-L (46.9 °C) became very close to that of the native one (47.7 °C), indicating that the destabilization effect of the nonplanar *cis* structure was fairly offset by the stabilization effect of the introduced isopropyl group. On the other hand, *cis*-3-CN-Ph-Azo-L failed to stabilize the duplex.[9]

Among the substituents we examined, the *tert*-butyl group showed the largest $-\Delta T_m$ value (see Table 1). The order of $-\Delta T_m$ for the S_{a1}/C duplex involving single azobenzene derivative was *t*Bu-Azo-L > *i*Pr-Azo-L ⩾ 3-CN-Ph-Azo-L. Introduction of multiple azobenzenes demonstrated the superiority of *t*Bu-Azo-L. When two azobenzenes were introduced into the same 12-nt-long DNA, $-\Delta T_m$ for *t*Bu-Azo-L in duplex S_{a2}/C was as large as 13.3 °C, which was 2.4 °C larger than that of *i*Pr-Azo-L (Table 1).

Observation of Photoswitching of DNA Hybridization by the Use of a Fluorophore–Quencher System

To prove the reversed photoswitching of hybridization with *t*Bu-Azo-L more directly, duplex formation was monitored from the change of fluorescence by using a fluorophore–quencher system (see Scheme 1). In FAM-C, a (6-fluorescein-6-carboxamido)hexanoate (FAM) was attached to the 3′-terminal of sequence C as the fluorophore. In S_{a2}-Dab, a 4-(4-dimethylaminophenylazo)benzoic acid (Dabcyl) moiety was attached at the 5′-end of sequence S_{a2} as the quencher. Once the duplex S_{a2}-Dab/FAM-C is formed, the fluorescence from FAM should be quenched by the Dabcyl. As shown in Figure 1 (see the dotted line), single-stranded FAM-C exhibited a strong emission at around 520 nm at 30 °C. When S_{a2}-Dab, the modified DNA that involves two *trans*-*t*Bu-Azo-L residues, was added, only 27 % of the total fluorescence from FAM was quenched (compare the dotted

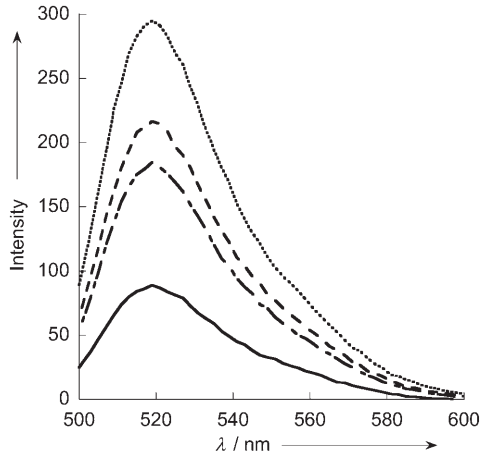

Figure 1. Photoinduced intensity change of the fluorescence (excitation at 490 nm) from FAM-C at 30 °C. The dotted line shows the fluorescence spectra of single-stranded FAM-C, whereas the broken and solid lines show the spectra of FAM-C in the presence of S_{a2}-Dab before (*trans* form) and after (*cis* form) irradiation with UV light for 1 min (with an interference bandpass filter centered at 341.5 nm), respectively. The dotted–broken line is the spectrum after the solution was irradiated with visible light (through an interference bandpass filter centered at 449.5 nm) for 30 s. Solution conditions: [S_{a2}-Dab] = 1.0 μM, [FAM-C] = 0.5 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

line with the broken one in Figure 1). But when this solution was irradiated with UV light, about 70 % of the fluorescence was quenched (see the solid line in Figure 1). Clearly, *trans*→*cis* isomerization of azobenzenes facilitated the hybridization of S_{a2}-Dab with FAM-C and the fluorescence from FAM was quenched by Dabcyl more efficiently. Upon irradiation of this solution with visible light, the fluorescence recovered owing to the dissociation of the duplex caused by *cis*→*trans* isomerization (compare the dotted–broken line with the solid one in Figure 1). Thus, the reverse switching of the duplex formation by *t*Bu-Azo-L was further evidenced by the fluorescence change induced by irradiation with light.

The T_m of duplex S_{a2}-Dab/FAM-C was also measured under the solution conditions used for fluorescence measurement, which was determined as 26.4 °C for the *trans* form and 39.2 °C for the *cis* form. Because the fluorescence measurement was carried out at 30 °C, which was only 9.2 °C lower than the T_m for the *cis* form, the duplex was not completely formed. That might be one of the reasons that about 30 % of the fluorescence remained after irradiation with UV light (solid line in Figure 1). Another possible reason is the incomplete *trans*→*cis* photoisomerization. Only about 62 % of S_{a2}-Dab was isomerized to the *cis* form under these conditions (data not shown). For similar reasons, the duplex could not completely dissociate after irradiation with visible light at 30 °C (more than 90 % was isomerized to the *trans* form), which is only 3.6 °C higher than the T_m of the *trans* form. The difference in fluorescence between the broken (100 % of the *trans* form) and the dotted–broken lines could be explained by the incomplete *cis*→*trans* isomerization under visible light irradiation.

By using the fluorophore–quencher system, we also investigated the reversibility of the photoregulation of hybridization by *t*Bu-Azo-L. As shown in Figure 2, the fluorescence

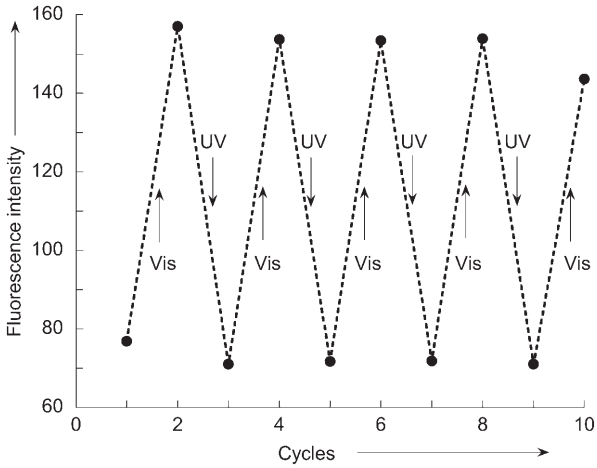

Figure 2. Photoinduced change of the fluorescence from FAM at 519 nm (excitation at 490 nm) at 30 °C for the solution containing S_{a2}-Dab/FAM-C. The arrows indicate irradiation with either UV or visible light at 30 °C: UV light centered at 341.5 nm for 1 min and visible light centered at 449.5 nm for 30 s. Solution conditions: [S_{a2}-Dab] = 1.0 μM, [FAM-C] = 0.5 μM, [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer).

change was induced by repeated irradiation of UV or visible light without obvious deterioration of photoregulatory efficiency, indicating that the formation and dissociation of the duplex could be reversibly photoswitched.[10] Notably, irradiation with UV or visible light did not affect the fluorescence intensity at all at 60 °C, at which temperature the duplex completely dissociated both in the *trans* and *cis* forms (see Supporting Information, Figure S2). Thus, the reversed photoswitching of hybridization was unambiguously substantiated.

Isomerization of an Azobenzene in the Presence and Absence of the Complementary Strand and Molecular Modeling of the Duplex

Photoisomerization of H-Azo-D, *i*Pr-Azo-L, and *t*Bu-Azo-L in S_{a1} as well as the thermal isomerization of their *cis* forms were measured at various temperatures either in the absence or presence of the complementary strand C (Figure 3). It was found that the thermal stability of *cis*-*t*Bu-Azo-L (or *cis*-*i*Pr-Azo-L) was greatly improved by the duplex formation (see Supporting Information, Figure S3 for the spectra). At 25 °C, for example, the half-life of *cis*-*t*Bu-Azo-L was 495 h in the presence of C, which was about five times longer than that in the absence of C ($t_{1/2}$ = 102 h). For *cis*-H-Azo-D, in duplex S_{a1}/C, however, its half-life was only 1.4-fold longer than that in the single-stranded S_{a1} (Figure 3a). At 60 °C, the half-lives of *cis*-*t*Bu-Azo-L and *cis*-H-Azo-D are 1.6 h and 2.4 h, respectively, which were independent of the presence of the complementary strand (see

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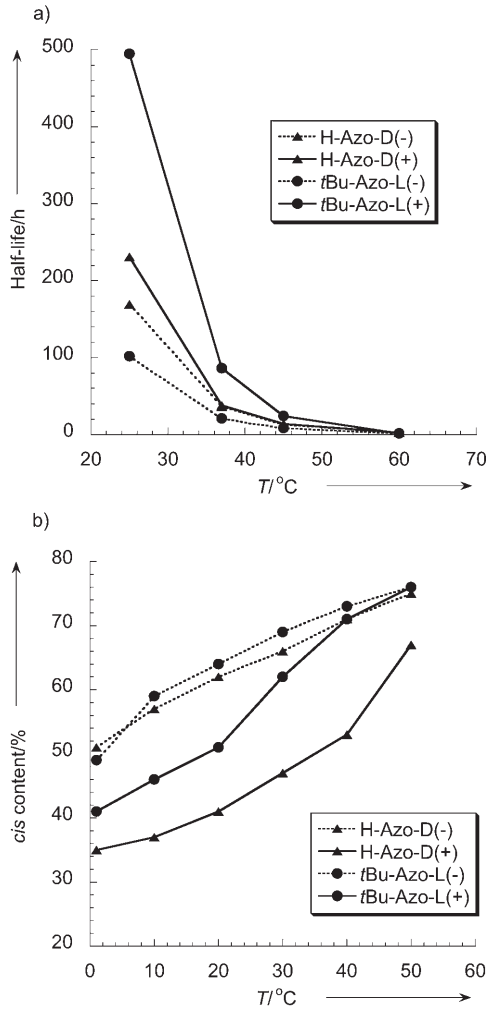

Figure 3. Thermal isomerization and photoisomerization of azobenzene derivatives at various temperatures. a) Half-life of *cis*-H-Azo-D (triangles) and *cis*-*t*Bu-Azo-L (circles) in S_{a1} in the absence ((−), dotted lines) or in the presence ((+), solid lines) of the complementary strand C. b) Content of *cis*-H-Azo-D (triangles) and *cis*-*t*Bu-Azo-L (circles) after irradiation with UV light in the absence (dotted lines) or in the presence (solid lines) of the complementary strand C.

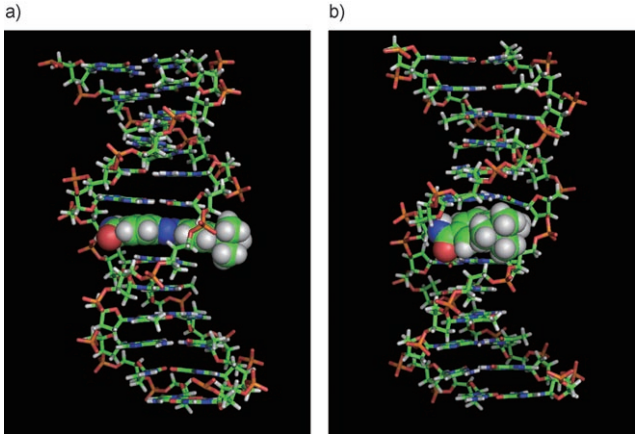

Figure 4. Energy-minimized structures of S_{a1}/C duplex involving *t*Bu-Azo-L (CPK part) either in the a) *trans* or b) *cis* form calculated with Insight-II/Discover.

Supporting Information, Table S1).[11] We assumed that a stable complex was formed between *cis*-*t*Bu-Azo-L and the DNA, duplex and thereafter the thermal isomerization was greatly suppressed.

The results of molecular modeling showed that the *tert*-butyl group of *cis*-*t*Bu-Azo-L fitted fairly well in the minor groove (Figure 4b). Thus, the stabilization effect of *cis*-*t*Bu-Azo-L for duplex formation could be explained by the strong binding of the *tert*-butyl group with the minor groove of the DNA duplex. Similar results were also obtained for *cis*-*i*Pr-Azo-L (see Supporting Information, Table S2 and Figure S4). The stabilization effect by *cis*-3-CN-Ph-Azo-L was rather poor, probably because the phenyl group at the *para* position could not bind well in the groove. Destabilization of the DNA duplex by *trans*-*t*Bu-Azo-L (or *trans*-*i*Pr-Azo-L) could be explained as follows: steric hindrance occurred between the *tert*-butyl (or isopropyl) group and the complementary strand so that the azobenzene moiety partially flipped out from the duplex, which prevented its effective stacking with base pairs and distorted the duplex. As reported previously, the *trans*→*cis* photoisomerization became difficult when the *trans*-azobenzene stacked well between the base pairs.[3] Accordingly, we could estimate the degree of stacking from the *trans*→*cis* photoisomerization efficiency. Actually, for both H-Azo-D and *t*Bu-Azo-L, the ratio of the *cis* form after irradiation with UV light became lower owing to duplex formation (Figure 3b, Supporting Information, Table S2). For H-Azo-D at 10 °C, for example, the *cis* content was 37 % in the presence of C, which was 20 % less than that without C.[12] Similarly, for *t*Bu-Azo-L, the *cis* content changed from 59 % to 46 % under the same conditions, indicating that *trans*-*t*Bu-Azo-L also intercalated into the duplex and was not completely flipped out (see Supporting Information, Figure S5 for the spectra). However, it is also notable that the *cis* fraction for *t*Bu-Azo-L in the duplex state (46 %) was about 10 % more than that for H-Azo-D (37 %) under the same conditions. This higher efficiency of *trans*→*cis* photoisomerization is favorable for its application in photoregulating DNA functions at lower temperature. *trans*-*t*Bu-Azo-L probably does not stack as well as H-Azo-D owing to the steric hindrance of the *tert*-butyl group. The results of molecular modeling also showed that a part of *trans*-*t*Bu-Azo-L was forced to be pushed out from the base pairs to the major groove side owing to the repulsion between the *tert*-butyl group and the ribose or phosphate in the complementary strand (Figure 4a). Thus, we can conclude that the destabilization effect of *trans*-*t*Bu-Azo-L was primarily attributed to the steric hindrance between the *tert*-butyl group and the backbone of the complementary strand C. Further evidence for this conclusion comes from the similar results obtained for *cis*-*i*Pr-Azo-L (see Supporting Information, Figure S4).

Conclusions

In conclusion, an efficient reverse mode of photoswitching was achieved by introducing a bulky group at the *para* position of azobenzene with L-threoninol as a linker. The destabilization effect by the *trans* form was attributed to the steric hindrance between the bulky group and the backbone of the complementary DNA. On the other hand, the stabilization effect by the *cis* form was attributed to the groove binding of the introduced bulky group. Both the thermal stability of *cis* form and the efficiency of the *trans*→*cis* photoisomerization, which are important for the photoregulation of DNA functions, were better for this reverse mode than for the normal mode. With these photoresponsive DNAs, more-efficient photoregulation of bioprocess is expected. At present, both the photoregulation of biological functions and the construction of light-fuelled DNA molecular machines are underway by using the combination of these two kinds of photoswitches working in different directions.

Experimental Section

Materials

Ethyl 4-nitrobenzoate, 4-isopropylaniline, and 4-*tert*-butylaniline were purchased from Tokyo Chemical Industry (Tokyo). D-Threoninol, L-threoninol, and 3-(4-aminophenyl)benzonitrile were purchased from Sigma–Aldrich. Azobenzene-tethered oligonucleotides were synthesized by using typical phosphoramidite chemistry as described previously.[2,4] All the conventional phosphoramidite monomers, 5′-Dabcyl phosphoramidite, CPG columns, other reagents for DNA synthesis, and Poly-Pak cartridges were purchased from Glen Research Co. (Sterling, VA). FAM-C labeled with (6-fluorescein-6-carboxamido)hexanoate (FAM) at the 3′-end was purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The synthesized oligonucleotides were purified by reversed-phase HPLC (ODS column: Merck LiChrospher 100 RP-18(e)) and characterized by positive-mode of MALDI-TOF MS (Autoflex Linear, Bruker Daltonics, Yokohama).

Synthesis of the Azobenzene Derivatives

A common synthetic pathway for synthesizing all three azobenzene derivatives (Scheme 2) was as follows:[13] First, ethyl 4-nitrosobenzoate was synthesized from ethyl 4-nitrobenzoate. After ethyl 4-nitrobenzoate (3.0 g, 15.4 mmol) was dissolved in 2-methoxyethanol (42 mL), a solution of NH_4Cl (1.28 g, 24.0 mmol) in water (11 mL) was added. Finely powdered zinc dust (2.7 g, 41.3 mmol) was added in small portions to the stirred solution at a constant temperature of 33–35 °C. The reaction mixture was stirred overnight at room temperature, and the solid part was removed by filtration. The filtrate was added dropwise, under a nitrogen atmosphere with rapid stirring over a period of 30 min, to a solution of $FeCl_3{\cdot}6H_2O$ (10.5 g, 38.8 mmol) in water (75 mL) and ethanol (18 mL) at a temperature below 0 °C in an ice/NaCl bath. After an additional 30 min of stirring, the reaction mixture was poured into cold water (150 mL). The product was recrystallized in ethanol/water after filtration to give ethyl 4-nitrosobenzoate (1.95 g, 71 %).

Typical Procedure for Coupling Ethyl 4-Nitrosobenzoate with Anilines

Ethyl 4-(4-isopropyl-phenylazo)benzoate (ethyl-*i*Pr-Azo): In a flask shielded from light 4-isopropylaniline (0.68 g, 5.0 mmol) and ethyl 4-nitrosobenzoate (0.90 g, 5.0 mmol) were dissolved in glacial acetic acid (30 mL) under nitrogen. The reaction mixture was stirred overnight at room temperature, and the solution was then poured into water and extracted with ethyl acetate. The organic layer was washed with water, a saturated aqueous solution of $NaHCO_3$, and a saturated aqueous solution of NaCl and then dried over $MgSO_4$. After removal of the solvent, the crude mixture was subjected to silica gel column chromatography (hexane/AcOEt 8:1) to afford ethyl-*i*Pr-Azo (1.46 g, 99 %). In the case of ethyl-3-CN-Ph-Azo, the product was precipitated from the reaction mixture of ethyl 4-nitrosobenzoate and 3-(4-aminophenyl)benzonitrile followed by recrystallization in AcOH/water (1:1).

*i*Pr-Azo: In a flask shielded from light, ethyl-*i*Pr-Azo (1.44 g, 4.86 mmol) was dissolved in ethanol (20 mL). For hydrolysis, aqueous NaOH (2.0 M, 4.0 mL) was added, and the mixture was stirred overnight in the dark. After the pH was adjusted to below 7.0 by adding aqueous HCl (1.0 M, ca. 8 mL), the product was extracted with ethyl acetate. The organic layer was washed with water and a saturated aqueous solution of NaCl and then dried over $MgSO_4$. After removal of the solvent, 4-(4-isopropyl-phenylazo)benzoic acid (*i*Pr-Azo) (1.23 g, 94 %) was obtained and was used directly in the next step without further purification.

Ethyl 4-nitrosobenzoate: ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.36–8.13 (m, 4H, Ar-H), 4.38 (q, ^3J(H,H) = 7.0 Hz, 2H, -O-CH_2-CH$_3$), 1.35 (t, ^3J(H,H) = 7.0 Hz, 3H, -O-CH$_2$-CH_3).

Ethyl-*i*Pr-Azo: ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.20–7.52 (m, 8H, Ar$_{azo}$-H), 4.41 (q, ^3J(H,H) = 7.0 Hz, 2H, -OCH_2CH$_3$), 3.04 (sept, ^3J(H,H) = 7.0 Hz, 1H, -CH(CH$_3$)$_2$), 1.39 (t, ^3J(H,H) = 7.5 Hz, 3H, -OCH$_2$CH_3), 1.29 ppm (d, ^3J(H,H) = 7.0 Hz, 6H, -CH(CH_3)$_2$).

*i*Pr-Azo: ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.19–7.52 (m, 8H, Ar$_{azo}$-H), 3.04 (sept, ^3J(H,H) = 7.0 Hz, 1H, -CH(CH$_3$)$_2$), 1.29 ppm (d, ^3J(H,H) = 7.0 Hz, 6H, -CH(CH_3)$_2$).

Ethyl-3-CN-Ph-Azo (recrystallized from H_2O/AcOH 1:1, 69.6 %): ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.32–7.72 (m, 12H, Ar-H), 4.38 (q, ^3J(H,H) = 7.0 Hz, 2H, -O-CH_2-CH$_3$), 1.37 ppm (t, ^3J(H,H) = 7.0 Hz, 3H, -O-CH$_2$-CH_3).

3-CN-Ph-Azo (78.4 %): ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.32–7.72 ppm (m, 12H, Ar-H).

Ethyl-*t*Bu-Azo (78.6 %): ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.21–7.67 (m, 8H, Ar$_{azo}$-H), 4.40 (q, ^3J(H,H) = 7.0 Hz, 2H, -OCH_2CH$_3$), 1.39 (t, ^3J(H,H) = 7.0 Hz, 3H, -OCH$_2$CH_3), 1.38 ppm (s, 9H, -C(CH_3)$_3$).

*t*Bu-Azo (98.2 %): ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.19–7.67 (m, 8H, Ar$_{azo}$-H), 1.38 ppm (s, 9H, -C(CH_3)$_3$).

Synthesis of the Phosphoramidite Monomers

The phosphoramidite monomers involving azobenzene derivatives were synthesized according to the Scheme 2.[2,4] Typical procedure (phosphoramidite monomer with *i*Pr-Azo): L-Threoninol (0.16 g, 1.50 mmol) and *i*Pr-Azo (0.40 g, 1.50 mmol) were added in dry DMF (40 mL) containing dicyclohexylcarbodiimide (0.37 g, 1.80 mmol) and 1-hydroxybenzotriazole (0.24 g, 1.80 mmol). After the reaction mixture was stirred at room temperature for 24 h, the solvent was removed, and the remaining oil was subjected to silica gel column chromatography ($CHCl_3$/MeOH 7:1) to afford **1** (*i*Pr-Azo) (0.56 g, quant.).

A solution (20 mL) of **1** (*i*Pr-Azo) (0.56 g, 1.50 mmol) in dry pyridine was cooled over ice under nitrogen, and 4,4′-dimethoxytrityl chloride (0.61 g, 1.80 mmol) in dry dichloromethane (8 mL) was added over 3–5 min. The reaction mixture was stirred for 30 min, the ice bath was removed, and the reaction mixture was stirred at room temperature for a further 5 h. The solvent was then removed by azeotropic distillation with toluene, followed by silica gel column chromatography (hexane/AcOEt/Et_3N 50:50:3) to afford **2** (*i*Pr-Azo) (0.70 g, 70.9 %).

The residual trivial amount of water in **2** (*i*Pr-Azo) and 1*H*-tetrazole were removed by azeotropic distillation with dry acetonitrile (twice). Then, 1*H*-tetrazole (20 mg, 0.29 mmol), **2** (*i*Pr-Azo) (0.16 g, 0.24 mmol), and 2-cyanoethyl *N,N,N′,N′*-tetraisopropylphosphordiamidite (80 μL, 0.27 mmol) were mixed in dry acetonitrile (4 mL) under nitrogen for 2 h. After removal of the solvent by evaporation, the crude mixture was dissolved in ethyl acetate. The solution containing **3** (*i*Pr-Azo) was washed with water, a saturated solution of $NaHCO_3$, and a saturated solution of NaCl. The solution was dried over $MgSO_4$, and then the acetonitrile was removed. The oily product (0.41 g, quant.) was obtained and immediately used for DNA synthesis in the next step without further purification.

1 (3-CN-Ph-Azo) (attached to D- or L-threoninol, quant.): ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.36–7.75 (m, 13H, Ar-H, -N*H*CO-), 4.69 (m, 2H, $CH_3CH(OH)$-, -(NH)CHCH$_2$O*H*), 3.97 (m, 2H, HOCH$_2$C*H*(NHCO-)-, -C*H*(OH)CH$_3$), 3.65 and 3.56 (m, 2H, HOC*H*$_2$CH(NHCO-)-), 1.13 ppm (d, ^3J(H,H) = 6.0 Hz, 3H, -CH(OH)C*H*$_3$).

2 (3-CN-Ph-Azo) (attached to D- threoninol (30.2%) or L-threoninol (42.7%)): ^{1}H NMR (500 MHz, CDCl$_3$, 20 °C, TMS): δ = 8.09–6.79 (m, 26H, Ar-H, Ar$_{azo}$-H, DMT, -N*H*CO-), 4.25 (m, 1H, -C*H*(OH)CH$_3$), 4.15 (m, 1H, HOCH$_2$C*H*(NHCO-)-), 3.77 and 3.76 (s, 6H, -C$_6$H$_4$OC*H*$_3$), 3.61 and 3.41 (dd, ^2J(H,H) = 9.5 Hz, ^3J(H,H) = 4.0 Hz, 2H, -C*H*$_2$ODMT), 1.24 ppm (d, ^3J(H,H) = 6.5 Hz, 3H, -CH(OH)C*H*$_3$)

1 (*i*Pr-Azo) (attached to D- or L-threoninol, quant.): ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.12–7.53 (m, 9H, Ar$_{azo}$-H, -N*H*CO-), 4.68 (m, 2H, CH$_3$CH(O*H*)-, -(CONH)CHCH$_2$O*H*), 3.97 (m, 2H, HOCH$_2$C*H*-(NHCO-)-, -C*H*(OH)CH$_3$), 3.64 and 3.56 (m, 2H, HOC*H*$_2$CH(NHCO-)-), 3.05 (sept, ^3J(H,H) = 7.0 Hz, 1H, -C*H*(CH$_3$)$_2$), 1.30 (d, ^3J(H,H) = 7.0 Hz, 6H, -CH(C*H*$_3$)$_2$), 1.12 ppm (d, ^3J(H,H) = 6.0 Hz, 3H, -CH(OH)C*H*$_3$).

2 (*i*Pr-Azo) (attached to D- or L-threoninol, 70.9%): ^{1}H NMR (500 MHz, CDCl$_3$, 20 °C, TMS): δ = 7.99–6.78 (m, 22H, Ar$_{azo}$-H, DMT, -N*H*CO-), 4.24 (m, 1H, -C*H*(OH)CH$_3$), 4.14 (m, 1H, -HOCH$_2$C*H*(NHCO-)-), 3.77 and 3.76 (s, 6H, -C$_6$H$_4$OC*H*$_3$), 3.60 and 3.40 (dd, ^2J(H,H) = 10.0 Hz, ^3J(H,H) = 4.0 Hz, 2H, -C*H*$_2$ODMT), 3.01 (sept, ^3J(H,H) = 7.0 Hz, 1H, -C*H*(CH$_3$)$_2$), 1.31 (d, ^3J(H,H) = 7.0 Hz, 6H, -CH(C*H*$_3$)$_2$), 1.23 ppm (d, ^3J(H,H) = 6.5 Hz, 3H, -CH(OH)C*H*$_3$).

1 (*t*Bu-Azo-L) (93.9%): ^{1}H NMR (500 MHz, [D$_6$]DMSO, 20 °C, TMS): δ = 8.13–7.67 (m, 9H, Ar$_{azo}$-H, -N*H*-CO-), 4.68 (m, 2H, CH$_3$CH(O*H*)-, -(NH)CHCH$_2$(O*H*)), 3.97 (m, 2H, -CH$_2$(OH)C*H*(NH)CO-)-, C*H*(OH)CH$_3$), 3.65 and 3.55 (dd, ^2J(H,H) = 10.0 Hz, ^3J(H,H) = 4.0 Hz, 2H, HOC*H*$_2$CH(NHCO-)-), 1.38 (s, 9H, -C(C*H*$_3$)$_3$), 1.12 ppm (d, ^3J(H,H) = 6.5 Hz, 3H, -CH(OH)C*H*$_3$).

2 (*t*Bu-Azo-L) (64.2%): ^{1}H NMR (500 MHz, CDCl$_3$, 20 °C, TMS): δ = 8.00–6.78 (m, 22H, Ar$_{azo}$-H, DMT, -N*H*CO-), 4.24 (m, 1H, -C*H*(OH)CH$_3$), 4.14 (m, 1H, HOCH$_2$C*H*(NHCO-)-), 3.77 and 3.76 (s, 6H, -C$_6$H$_4$OC*H*$_3$), 3.60 and 3.40 (dd, ^2J(H,H) = 10.0 Hz, ^3J(H,H) = 4.0 Hz, 2H, -C*H*$_2$ODMT), 1.39 (s, 9H, -C(C*H*$_3$)$_3$), 1.23 ppm (d, ^3J(H,H) = 6.5 Hz, 3H, -CH(OH)C*H*$_3$).

Synthesis of DNA Modified with Azobenzene Derivatives

The oligonucleotides modified with azobenzene derivatives were synthesized on an ABI 3400 DNA/RNA Synthesizer by using the corresponding phosphoramidite monomer and other conventional precursors.[2,4] The dimethoxytrityl group at the 5′-end was retained before the synthesized DNA was cleaved from CPG. After deprotection, the DNA was purified first by using Poly-Pak cartridges and then by reversed-phase HPLC (Merck LiChrospher 100 RP-18(e) column, with a linear gradient by using mixture of acetonitrile and water containing ammonium formate (50 mM) as buffer (0.5 mL min^{-1}, detection at 260 nm). The purified DNAs were then characterized by using MALDI-TOF MS (positive mode): S$_{a1}$ with H-Azo-D: found: 4021 (calcd. for protonated form: 4020), 3-CN-Ph-Azo-D: found: 4121 (calcd.: 4121), *i*Pr-Azo-D: found: 4064 (calcd.: 4062), 3-CN-Ph-Azo-L: found: 4121 (calcd.: 4121), *i*Pr-Azo-L: found: 4062 (calcd.: 4062), *t*Bu-Azo-L: found: 4076 (calcd.: 4076); S$_{a2}$ involving 3-CN-Ph-Azo-L: found: 4598 (calcd.: 4597), *i*Pr-Azo-L: found: 4480 (calcd.: 4479), *t*Bu-Azo-L: found: 4507 (calcd.: 4507); S$_{a2}$-Dab: found: 4938 (calcd.: 4937).

Measurement of the Melting Temperatures

The melting curves of the duplexes were obtained by measuring the change in absorbance at 260 nm versus temperature with a spectrophotometer (model V-530 or V-550 (JASCO)) equipped with a programmable temperature controller. The T_m values were determined from the maxima in the first derivatives of the melting curves measured under the following conditions: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [DNA] = 5 μM. Both the heating and cooling curves were measured, and the obtained T_m values agreed within 2.0 °C. The T_m values presented here are an average of 2–4 independent experiments. The temperature ramp was 1.0 °C min^{-1}.

Photoisomerization of Azobenzene and Its Derivatives

The light source for the photoirradiation was a 150-W xenon lamp. For the *trans*→*cis* isomerization, a UV-D36C filter (Asahi Tech. Co.) was applied, and UV light (λ = 300–400 nm; 5.3 mW cm^{-2}) was used to irradiate the solution of the duplex at various temperatures for 3 min. The *cis*→*trans* isomerization was carried out by irradiation with visible light (λ > 400 nm) through an L-42 filter (Asahi Tech. Co.) at various temperatures for 1 min. In both cases, a water filter was used to eliminate infrared light. The spectra of *t*Bu-Azo-L in S$_{a1}$ changing with photoirradiation are shown in the (Supporting Information, Figure S5).

Half-life of Thermal Isomerization of cis-Azobenzene to the *trans* Form

The azobenzene tethered on DNA in a cuvette was irradiated with UV light to allow photoisomerization to the *cis* form under the following conditions: [NaCl] = 100 mM, pH 7.0 (10 mM phosphate buffer), [S$_{a1}$] = [C] = 25 μM, 60 °C, 5 min. The cuvette was then placed in the UV/Vis spectrometer, and the spectra were measured at constant temperature at a predetermined time interval. The rate constant of thermal isomerization (k) was obtained from the changes in the absorbance at the absorption maximum of *trans*-azobenzene (around 340 nm) with time. Considering that the thermal *cis*→*trans* isomerizations of azobenzene and its derivatives were first-order, the half-life was calculated as: $t_{1/2} = 0.693/k$. The spectra of *t*Bu-Azo-L in S$_{a1}$ changing with time are shown in the Supporting Information, Figure S3.

Measurement of Fluorescence

The fluorescence of the solution containing FAM-C only or both FAM-C and S$_{a2}$-Dab (pH 7.0 (10 mM phosphate buffer), 100 mM NaCl) was measured at 30 °C on a JASCO spectrofluorometer (FP-6500) equipped with a temperature controller. In this experiment, photoirradiation was carried out with a 300-W xenon lamp (MAX-301, Asahi Spectra Co., Ltd.) equipped with corresponding interference bandpass filters. For *trans*→*cis* isomerization, a solution of S$_{a2}$-Dab/FAM-C was irradiated with UV light from a bandpass filter centered at 341.5 nm (half bandwidth = 9 nm) at 30 °C for 1 min; for *cis*→*trans* isomerization, irradiation was carried out with visible light for 30 s through a bandpass filter centered at 449.5 nm.

Molecular Modeling

The Insight II/Discover 98.0 program package was used for molecular modeling to obtain the energy-minimized structures by conformation-energy minimization. The azobenzene residue was built by using the graphical program. The effects of water and counterions were simulated by a sigmoidal, distance-dependent, dielectric function. The B-type duplex was used as the initial structure, and the AMBER force field was used for calculation. Computations were carried out on a Silicon Graphics O2 workstation with the operating system IRIX64 Release 6.5. As an initial calculation structure, an azobenzene moiety (either *trans* or *cis* form) was placed between the base pairs.

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[8] About 75% of the total azobenzene was isomerized to the *cis* form whereas 95% was converted into the *trans* form.
[9] L-Threoninol was also effective for cyanophenyl-substituted azobenzene (3-CN-Ph-Azo-L), although the ΔT_m (−0.9 °C) was small.
[10] The photoswitching behaviour observed in Figure 2 was entirely reversed when unsubstituted azobenzene was tethered on D-threoninol (see reference [4a]).
[11] *para*-Substitution of the azobenzene accelerates thermal *cis*→*trans* isomerization (see reference [5]).
[12] At a temperature much higher than the T_m of the duplex (48.9 °C), strand C did not affect the *cis* content.
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