2′,6′-Dimethylazobenzene as an efficient and thermo-stable photoregulator for the photoregulation of DNA hybridization†

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The introduction of methyl groups into two *ortho* positions (2′ and 6′ positions) of the same benzene ring in an azobenzene remarkably raised both its photoregulation ability and the thermal stability of the *cis*-form.

The photoregulation of biologically active compounds can be used as a robust tool for the investigation of particular biological phenomena and mechanisms in living cells, which are otherwise very difficult to achieve.[1] The most commonly used approach is to covalently attach a light-responsive molecule to the target biological compound so that the light signal becomes an efficient trigger for the function under investigation. Recently, a large number of efficient light-switchable systems involving photoresponsive nucleic acids, proteins, cellular signalling molecules, or lipids have been constructed.[2,3]

We have previously introduced azobenzenes into DNA on D-threoninol as a linking molecule, and have efficiently photoregulated primer extension, transcription, and the RNase H reaction.[4–7] These photoregulatory bioreactions are mostly based on the reversible formation and dissociation of the DNA duplex by irradiating with either UV or visible light: planar *trans*-azobenzene (visible light irradiation) is intercalated between the adjacent base-pairs and thus stabilizes the duplex, whereas non-planar *cis*-azobenzene (UV light irradiation) destabilizes the duplex by steric hindrance.[8–12] Since photoregulatory efficiencies depended on the change in melting temperature (ΔT_m) induced by *trans–cis* isomerization, the enhancement of ΔT_m has been crucially important in achieving still more effective photoregulation. One of the methods for raising ΔT_m is to introduce multiple azobenzene moieties. We found that ΔT_m increased uniformly with the number of azobenzenes that were introduced, and even a 20-bp-long DNA duplex could be efficiently photoregulated by tethering 9 azobenzene moieties.[6,11,13] However, this causes great changes in the structure of the DNA duplex far from the B-form due to the enhanced asymmetry of the strands, and prevents its interaction with protein or enzymes both in the *trans*- and *cis*-forms. Obviously, the photoregulation of DNA hybridization with fewer azobenzenes is favorable for bio-applications. For this purpose, a new molecule that further stabilizes the duplex in the *trans*-form and destabilizes it in the *cis*-form is highly desirable. In the present paper, we found that the introduction of methyl groups to the *ortho* positions of azobenzene enhanced the value of ΔT_m. In particular, 2′,6′-dimethylazobenzene gave a three-times larger change in ΔT_m than the unmodified form used previously. Unexpectedly, we also found that this dimethylation significantly suppressed the thermal isomerization of *cis*-azobenzene to the *trans*-form.

Azobenzene was modified with methyl or ethyl groups with the aims of further stabilizing the duplex in the *trans*-form by stacking interactions and of destabilization of the *cis*-form due to steric hindrance. The modified azobenzene on D-threoninol was inserted at the centre of a 12 nt natural oligodeoxyribonucleotide.[14] The structures of the synthesized azobenzenes and the sequences of the azobenzene-modified oligodeoxyribonucleotides (**Da**) used in this study are shown in Fig. 1. Photo-isomerization of *trans*-azobenzene to the *cis*-form was performed by irradiating with UV light (300 nm $< \lambda <$ 400 nm) before the T_m measurement.‡§ By this procedure, 60–80% of the total azobenzene was isomerized to the *cis*-form.[15] When the *para*-position of the azobenzene was substituted with a methyl group (**4′-Me-Azo**), T_m of the *trans*-form became 46.8 °C, which was 2 °C lower than that of the non-substituted **Azo** (see Table 1). In contrast, T_m of the *cis*-form was increased by *para*-substitution. As a result, ΔT_m became smaller.

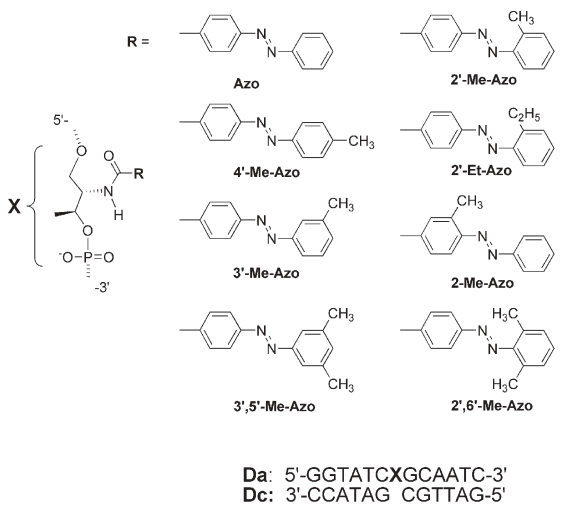

Fig. 1 Structures of modified azobenzenes and the DNA sequences used in this study.

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† Electronic supplementary information (ESI) available: experimental procedures for the synthesis of alkylated azobenzenes and modified oligodeoxyribonucleotides involving modified azobenzene, Energy-minimized structures of **Da/Dc** duplex involving **2′,6′-Me-Azo**, change of UV-Vis spectra of **2′,6′-Me-Azo** by the thermal *cis* → *trans* isomerization. See DOI: 10.1039/b708952j

Table 1 Effect of the insertion position of alkyl group on azobenzene on the T_m of **Da/Dc** duplex in the *trans*- and *cis*-forms

Azobenzene	T_m/°C[a]		ΔT_m[b]
	trans	*cis*	
Azo	48.9	43.2	5.7
4′-Me-Azo	46.8	45.4	1.4
3′-Me-Azo	49.7	44.8	4.9
2′-Me-Azo	50.7	40.1	10.6
2′-Et-Azo	49.6	39.8	9.8
2-Me-Azo	48.8	39.3	9.5
3′,5′-Me-Azo	49.0	44.4	4.6
2′,6′-Me-Azo	50.9	36.3	14.6

[a] Solution conditions: [**Da**] = [**Dc**] = 5 μM, [NaCl] = 0.1 M, pH 7.0 (10 mM phosphate buffer). [b] Change of T_m induced by the *cis–trans* isomerization.

The *meta*-substitution of an azobenzene group (**3′-Me-Azo**) did not significantly affect the stability of the duplex: ΔT_m was 4.9 °C, which was almost the same as that of **Azo**. However, in the case of 2′-methylazobenzene (**2′-Me-Azo**), in which a methyl group is attached at an *ortho* position of the benzene ring far from the DNA backbone, ΔT_m increased significantly compared with the value for **Azo**: T_m of the *trans*-form (50.7 °C) was 1.8 °C higher than that of non-substituted *trans*-azobenzene (*trans*-**Azo**), whereas the value of the *cis*-form (40.1 °C) was 3.1 °C lower. As a result, ΔT_m for **2′-Me-Azo** increased to 10.6 °C, which was about 5 °C higher than that of **Azo**. Both methylation and ethylation (**2′-Et-Azo**) raised the value of ΔT_m. Similarly, **2-Me-Azo** methylated at an *ortho*-position on the benzoyl side also showed an increase in ΔT_m. Interestingly, the di-substitution of azobenzene at the *ortho*-positions (**2′,6′-Me-Azo**) exhibited even larger ΔT_m: the T_m of the *trans*-form was 50.9 °C whereas that of the *cis*-form was as low as 36.3 °C. Consequently, this ΔT_m (14.6 °C) was the largest among all of the azobenzenes that were synthesized in this study. The melting curves of unmodified (**Azo**) and **2′,6′-Me-Azo** are shown in Fig. 2.[15] Large improvements were only observed at the *ortho*-position: azobenzene di-substituted at the *meta*-positions (**3′,5′-Me-Azo**) did not show a change in ΔT_m. Thus, the photoregulation ability was improved by modifying the *ortho*-positions of azobenzene.

According to molecular modelling, methylation of the *para*-position inhibits hybridization due to the steric hindrance of the incorporated methyl group with a phosphodiester linkage and deoxyribose at the counter strand **Dc**. It should be noted that the size of the Watson–Crick base-pair in this case is around 11 Å,[16] whereas for unmodified azobenzene it is 11–12 Å.[17] **4′-Me-Azo**, in which the *para*-position of the azobenzene is methylated, was too long to intercalate effectively between the base-pairs, and thus the duplex was destabilized in the *trans*-form. In the case of *ortho*- (or *meta*-) substitution, such destabilization did not occur because the methyl group was located far from the counter strand in the duplex. Rather, such alkylation stabilized the duplex by hydrophobic interaction. However in the *cis*-form, methyl groups on the 2′-position (or 2-position) protruded towards the base-pair and inhibited base-pairing due to steric hindrance (see Supplemental Fig. 1†). Such inhibition of base-pairing would be enhanced by the double-methylation of the 2′-(*ortho*-)positions. As a result, a much larger ΔT_m was induced by *cis–trans* isomerization of **2′,6′-Me-Azo**.

Not only visible-light irradiation, but also heat isomerizes *cis*-azobenzene to the *trans*-form. One of the problems caused by the modification of azobenzene is degradation of the thermal stability of *cis*-azobenzene.[18] In particular, donor–acceptor ("push–pull") modification at the *para*- (or *ortho*-position) of azobenzene usually lowers the thermal stability of *cis*-azobenzene. In our cases, carboxyl groups in the vicinity of the threoninol pull and alkyl groups push the electron, so thermal isomerization should be accelerated. In fact, the mono-methylation of azobenzene (**4′-**, **3′-**, and **2′-Me-Azo**) accelerated the thermal isomerization: in a single-stranded **Da**, the half-life of *cis*-**4′-Me-Azo** was one-third of that of unmodified *cis*-**Azo** at 60 °C (Fig. 3). Similarly, *ortho*-substitution (*cis*-**2′-Me-Azo**), which enhanced ΔT_m, also accelerated the thermal isomerization, although the acceleration effect in this case was smaller than that of *para*-substitution. Unexpectedly, **2′,6′-Me-Azo**, which contains two methyl groups at both *ortho* potions and which displays the largest ΔT_m, showed very slow thermal isomerization. Its half-life was as long as 25 h (rate constant was 0.028 h^{-1}) at 60 °C, which is as much as 8-times slower than unmodified *cis*-**Azo**.[19] The half-life of *cis*-**2′,6′-Me-Azo** at 37 °C was estimated by extrapolation of the Arrhenius plots to be 200–400 h, indicating that its thermal *cis*-to-*trans* isomerization could be practically suppressed under physiological conditions. Thus, methylation of the two *ortho*-positions exhibited both

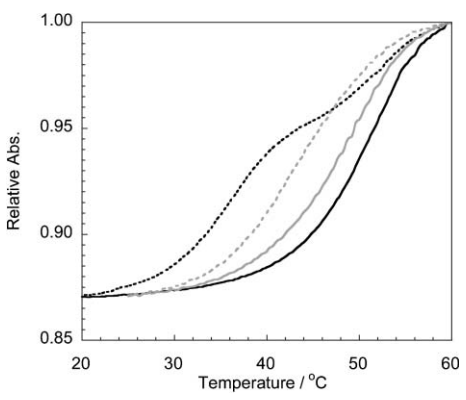

Fig. 2 Melting curves of a **Da/Dc** duplex involving non-substituted (**Azo**: gray line) and 2′,6′-dimethylazobenzene (**2′,6′-Me-Azo**) either in the *trans*-(solid line) or the *cis*-forms (black line).

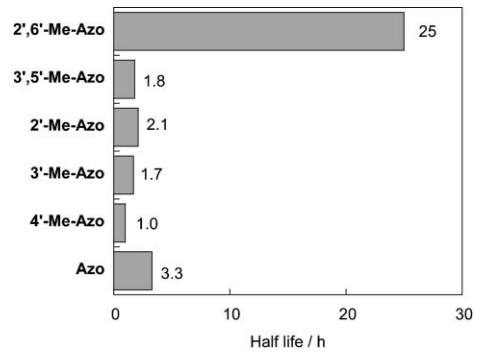

Fig. 3 Half-lives of *cis*-azobenzene to *trans*-form in the single-stranded **Da** at 60 °C in the presence of 0.1 M NaCl at pH 7.0 (10 mM phosphate buffer). [**Da**] = 20 μM. All the thermal isomerizations are first order (See Supplemental Fig. 2† for the change of UV-Vis spectra of *cis*-**2′,6′-Me-Azo**).

effective photo-regulation of hybridization and thermal durability of the *cis*-form.

According to Asano, the thermal isomerization of the *cis* to the *trans*-form has two routes.[20] The first of these is inversion, which proceeds through a transition state in which one of the nitrogen atoms is sp hybridized. The other is a rotation mechanism which involves the rupturing of a nitrogen–nitrogen π-bond and rotation around the remaining σ-bond. Presumably, the introduction of methyl groups on the two *ortho*-positions would restrict either the rotation around the nitrogen–nitrogen bond or the inversion process due to the close proximity of the benzene ring to the two methyl groups, and thus suppress the *cis* → *trans* isomerization.[21]

In conclusion, the introduction of two methyl groups into the *ortho* positions of the same benzene ring greatly raised its photoregulation ability and concurrently suppressed the thermal isomerization of the *cis*-form. A robust photoregulator, **2′,6′-Me-Azo**, was developed, and the clear-cut photoregulation of various DNA functions now becomes promising.

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Notes and references

‡ See Supplemental Information† for syntheses of alkylated azobenzenes[22,23] and their phosphoramidite monomers. All of the modified oligonucleotides were synthesized on an ABI 3400 DNA/RNA Synthesizer by using the corresponding phosphoramidite monomer and other conventional precursors. The purification was achieved by using Poly-Pak cartridges and then by a reversed-phase HPLC (Merck LiChrospher 100 RP-18(e) column, with a linear gradient of a mixture of acetonitrile and H_2O containing 50 mM ammonium formate, 0.5 mL min^{-1}, detection at 260 nm).[13] The purified DNAs were then characterized by MALDI-TOFMS. MALDI-TOFMS for **Da** with **Azo**: obsd. 4021 (calcd. for protonated form: 4020), **4′-Me-Azo**: obsd. 4035 (calcd. 4034), **3′-Me-Azo**: obsd. 4035 (calcd.: 4034), **2′-Me-Azo**: obsd. 4035 (calcd. 4034), **2′-Et-Azo**: obsd. 4048 (calcd. 4048), **2-Me-Azo**: obsd. 4032 (calcd. 4034), **3′,5′-Me-Azo**: obsd. 4049 (calcd. 4048), **2′,6′-Me-Azo**: obsd. 4049 (calcd. 4048).

§ The T_m values were determined from the maxima in the first derivatives of the melting curves, which were obtained by measuring the absorbance at 260 nm as a function of temperature. A JASCO model V-530 or V-550 spectrophotometer equipped with a programmable temperature-controller was used. Both the heating and cooling curves were measured, and the values of T_m that were obtained coincided with each other to 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}. The conditions of the sample solutions were as follows: [NaCl] = 0.1 M, pH 7.0 (10 mM phosphate buffer), [**Da**] = [**Dc**] = 5 μM.

Photo-isomerization of azobenzene: The light source for the photo-irradiation was a 150 W Xenon lamp. For the *trans* → *cis* isomerization, a UV-D36C filter (Asahi Tech. Co.) was used, and UV light (λ = 300 ∼ 400 nm: 5.3 mW cm^{-2}) was irradiated to **Da/Dc** solution at 60 °C for 5 min. The *cis* → *trans* isomerization was carried out by irradiating with visible light (λ > 400 nm) through an L-42 filter (Asahi Tech. Co.) at 60 °C for 5 min. In both cases, a water filter was used to cut off the infrared light.

Half-life of thermal isomerization of cis-azobenzene to the trans-form: UV light (λ = 300 ∼ 400 nm: 5.3 mW cm^{-2}) was irradiated to a solution of **Da** involving azobenzene ([NaCl] = 0.1 M, pH 7.0 (10 mM phosphate buffer), [**Da**] = 20 μM) at 60 °C for 5 min to isomerize *trans*-azobenzene to the *cis*-form. Then the solution was inserted into a UV-Vis spectrometer equipped with a temperature controller (JASCO model V-530 or V-550), and spectra were monitored at 60 °C at a predetermined interval. The half-lives were obtained from the changes in the absorbance at the absorption maximum of *trans*-azobenzene (around 340 nm). It should be noted that all of the thermal *cis* → *trans* isomerizations were first-order (See Supplemental Fig. 2†).

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15 In the case of **2′,6′-Me-Azo**, about 65% of the total azobenzene was isomerized to the *cis*-form under the conditions employed (See Supplemental Fig. 2† for the UV-Vis spectra). The melting curve of a **Da/Dc** duplex involving **2′,6′-Me-Azo** showed two sigmoids because not only the *cis*-form, but also 35% of the *trans*-form was involved in the solution. The upper sigmoid (minor one) in Fig. 2 corresponds to *trans*-**2′,6′-Me-Azo**, whereas the lower sigmoid (major one) corresponds to the *cis*-form. The T_m of the *cis*-form was determined from this lower sigmoid.
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