DNA Mismatch-Specific Base Flipping by a Bisacridine Macrocycle

Arnaud David,[a] Nathalie Bleimling,[b] Christine Beuck,[c] Jean-Marie Lehn,[a] Elmar Weinhold,*[c] and Marie-Paule Teulade-Fichou*[a]

*Most, if not all, enzymes that chemically modify nucleobases in DNA flip their target base from the inside of the double helix into an extrahelical position. This energetically unfavorable conformation is partly stabilized by specific binding of the apparent abasic site being formed. Thus, DNA base-flipping enzymes, like DNA methyltransferases and DNA glycosylases, generally bind very strongly to DNA containing abasic sites or abasic-site analogues. The macrocyclic bisacridine **BisA** has previously been shown to bind abasic sites. Herein we demonstrate that it is able to specifically recognize DNA base mismatches and most likely induces base flipping. Specific binding of **BisA** to DNA mismatches was studied by thermal denaturation experiments by using short duplex oligodeoxynucleotides containing central TT, TC, or TG mismatches or a TA match. In the presence of the macrocycle a strong increase in the melting temperature of up to 7.1 °C was observed for the mismatch-containing duplexes, whereas the melting temperature of the fully matched duplex was unaffected. Furthermore, **BisA** binding induced an enhanced reactivity of the mispaired thymine residue in the DNA toward potassium permanganate oxidation. A comparable reactivity has previously been observed for a TT target base mismatch in the presence of DNA methyltransferase M·TaqI. This similarity to a known base-flipping enzyme suggests that insertion of **BisA** into the DNA helix displaces the mispaired thymine residue into an extrahelical position, where it should be more prone to chemical oxidation. Thus, DNA base flipping does not appear to be limited to DNA-modifying enzymes but it is likely to also be induced by a small synthetic molecule binding to a thermodynamically weakened site in DNA.*

Introduction

Native DNA assumes a double-helical structure, in which the nucleobases are buried in the interior of the DNA helix. In general, this structural arrangement leads to a reduced reactivity of the nucleobases toward various alkylating and oxidizing agents compared with that of the free nucleotides. Thus, it is not surprising that enzymes acting on nucleobases in DNA have evolved a special binding mode for getting steric access to their target base. They rotate their target base, including its sugar-phosphate backbone, from an innerhelical to an extrahelical position so as to place it in their active site for catalysis.[1] Base flipping, also called nucleotide flipping, was first observed by X-ray crystallography of protein–DNA cocrystals of the *C*5-cytosine DNA methyltransferase (MTase) M·*Hha*I.[2] Later, target base flipping was also observed in protein–DNA cocrystal structures of the *C*5-cytosine DNA MTase M·*Hae*III and the *N*6-adenine DNA MTase M·*Taq*I.[3] Furthermore, other biophysical and biochemical methods to detect DNA base flipping have been developed.[4] These methods make use of modified duplex oligodeoxynucleotides containing fluorescent or photoactivatable probes at the target base position.[5–7] In addition, duplexes with mismatched target bases or base analogues with reduced Watson–Crick hydrogen-bonding potential were found to bind to the enzymes with enhanced affinity; this can be attributed to the thermodynamics of base flipping.[8] By using these methods, evidence for base flipping by many more DNA MTases was obtained, and it is now believed that all of them flip their target base out of the DNA helix prior to methylation.

However, base flipping is not restricted to DNA MTases and has also been observed in protein–DNA cocrystal structures of a number of DNA base-excision repair glycosylases that recognize damaged nucleobases in DNA.[9]

Most interestingly, many DNA MTases and DNA glycosylases bind with very high affinity to duplexes containing a stable abasic-site analogue at their target position.[8, 10] Moreover, it has been shown by X-ray crystallography that these enzymes are able to move the sugar-phosphate backbone even without their target base in an extrahelical conformation.[9c–e, 9i, 11] This high-affinity binding to abasic sites can be rationalized by the thermodynamics of the base flipping process: The energetic

[a] *Dr. M.-P. Teulade-Fichou, Dipl.-Chem. A. David, Prof. Dr. J.-M. Lehn*
Laboratoire de Chimie des Interactions Moléculaires
Collège de France, CNRS UPR 285
11 place Marcelin Berthelot, 75005 Paris (France)
Fax: (+33) 1-44-27-13-56
E-mail: mp.teulade-fichou@college-de-france.fr

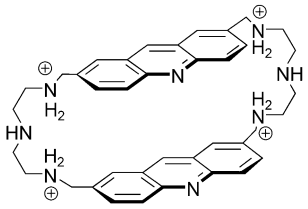
[b] *N. Bleimling*
Max-Planck-Institut für molekulare Physiologie
Abteilung III – Physikalische Biochemie
Otto-Hahn-Strasse 11, 44227 Dortmund (Germany)

[c] *Prof. Dr. E. Weinhold, Dipl.-Chem. C. Beuck*
Institut für Organische Chemie der RWTH Aachen
Professor-Pirlet-Strasse 1, 52056 Aachen (Germany)
Fax: (+49) 241-80-92528
E-mail: elmar.weinhold@oc.rwth-aachen.de

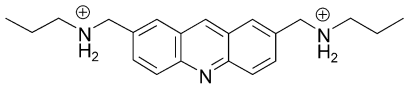
  DOI: 10.1002/cbic.200300693

costs for disrupting the Watson–Crick hydrogen bonds and base-stacking interactions of the target base are mainly paid by the binding of the target nucleotide in an extrahelical position and by stabilization of the apparent abasic site being formed. With an abasic site already present, the enzyme can directly bind without paying the energetic penalty for target base-pair disruption; this leads to high-affinity binding. Most interestingly, this argument can be reversed by stating that molecular structures with the ability to bind strongly to abasic sites in DNA should have the potential to flip out nucleobases.

In the course of our studies on structural recognition of DNA by synthetic ligands,[12] we demonstrated that the macrocyclic bisacridine **BisA** (Scheme 1) is able to bind specifically to DNA duplexes containing an apurinic (AP) site.[13] NMR and modeling



BisA



MonoA

Scheme 1. *Structural representation of the macrocycle **BisA** and the monomeric derivative **MonoA** at pH 6.0.*

studies have shown that a GC base pair next to a stable abasic site (a tetrahydrofuran analogue) is sandwiched by the two acridine units of the macrocycle.[14] The insertion of **BisA** into the abasic-site pocket proceeds through an unprecedented binding mode, namely threading bis-intercalation. Furthermore, binding of **BisA** leads to a distortion of the abasic site, in which the reduced sugar is pushed out, while the unpaired thymine is slightly shifted toward the major groove. In contrast to the abasic site duplex, fully paired control DNA is not recognized by the macrocycle. These observations led us to assume that **BisA** could also insert specifically into duplex DNA containing base mismatches and flip one of the mismatched bases out of the helix.

Here we present a study of the interaction of **BisA** with different duplex oligodeoxynucleotides containing TT, TC, or TG mismatches or a TA match at the target base pair position of the adenine-specific DNA MTase M·*Taq*I. We used thermal-denaturation experiments to demonstrate specific recognition of the mismatched site by **BisA**. Additionally, in analogy to M·*Taq*I, the enhanced reactivity of the mismatched thymine residue toward potassium permanganate oxidation is in accordance with a binding mode of **BisA** that involves base flipping.

Results and Discussion

Binding of the **BisA** macrocycle to duplexes containing various base mismatches was investigated by thermal-denaturation experiments. For these experiments, 17-mer duplex oligodeoxynucleotides containing a TT, TC, or TG mismatch or a TA match in the middle of the sequence were employed (Table 1). The control

Table 1. *Thermal stability of 17-mer duplexes in the absence and presence of **BisA** or **MonoA**.*[a]

	5′-CCAGTTCG **T** AGTAACCC-3′ 3′-GGTCAAGC **X** TCATTGGG-5′			
Additive	**X** = T TT mismatch	**X** = C TC mismatch	**X** = G TG mismatch	**X** = A TA match
None	37.7	37.1	40.4	45.0
BisA				
1 equiv	43.5 (+5.8)	42.9 (+5.8)	43.0 (+2.6)	45.0 (0)
2 equiv	44.5 (+6.8)	44.2 (+7.1)	43.5 (+3.1)	45.0 (0)
MonoA				
2 equiv	39.8 (+2.1)	38.5 (+1.4)	n.d.[b]	45.6 (+0.6)

[a] T_m values are given in °C (±0.2), and ΔT_m values relative to the duplexes without additive are provided in parentheses. [b] not determined.

TA 17-mer duplex melted at $T_m = 45.0\,^{\circ}C$ under our experimental conditions. As expected, lower T_m values were observed for the corresponding mismatched TT, TC, or TG 17-mer duplexes. Interestingly, a significant increase in the melting temperature of the TT-mismatch-containing duplex (Figure 1A) was observed in the presence of one equivalent of **BisA** ($\Delta T_m = +5.8\,^{\circ}C$). The effect leveled off at higher **BisA** concentrations. With two equivalents of **BisA**, the TT 17-mer duplex is stabilized by $\Delta T_m = +6.8\,^{\circ}C$. A very similar effect was observed with the duplex containing a TC mismatch (Figure 1B). The melting temperature in the presence of two equivalents of **BisA** is increased by 7.1 °C compared to the melting temperature of the TC 17-mer duplex alone. In the case of the more stable duplex containing a TG mismatch (Figure 1C) the stabilization by two equivalents of **BisA** is more modest ($\Delta T_m = +3.1\,^{\circ}C$). However, in all three cases, the stabilizing effect of **BisA** almost completely compensates for the drop in melting temperature resulting from the introduction of the mismatches. Thus, the increase in melting temperature, which should reflect the binding affinity of **BisA** to the mismatched site, is inversely correlated to the thermodynamic stability of the mismatch as indicated by the corresponding T_m values. Such behavior is expected for mismatch-specific binding and has also been observed with other synthetic ligands that recognize mismatches.[15]

Most importantly, no shift of the melting curve toward higher temperatures was observed with the fully paired TA 17-mer duplex in the presence of one or two equivalents of **BisA** (Figure 1D). This result indicates that **BisA** does not significantly bind to fully paired DNA and that binding of **BisA** is mismatch-specific.

In contrast to the results with the macrocycle **BisA**, the monomeric derivative **MonoA** (Scheme 1) leads only to a weak stabilization of the TT and TC 17-mer duplexes (Table 1). It also

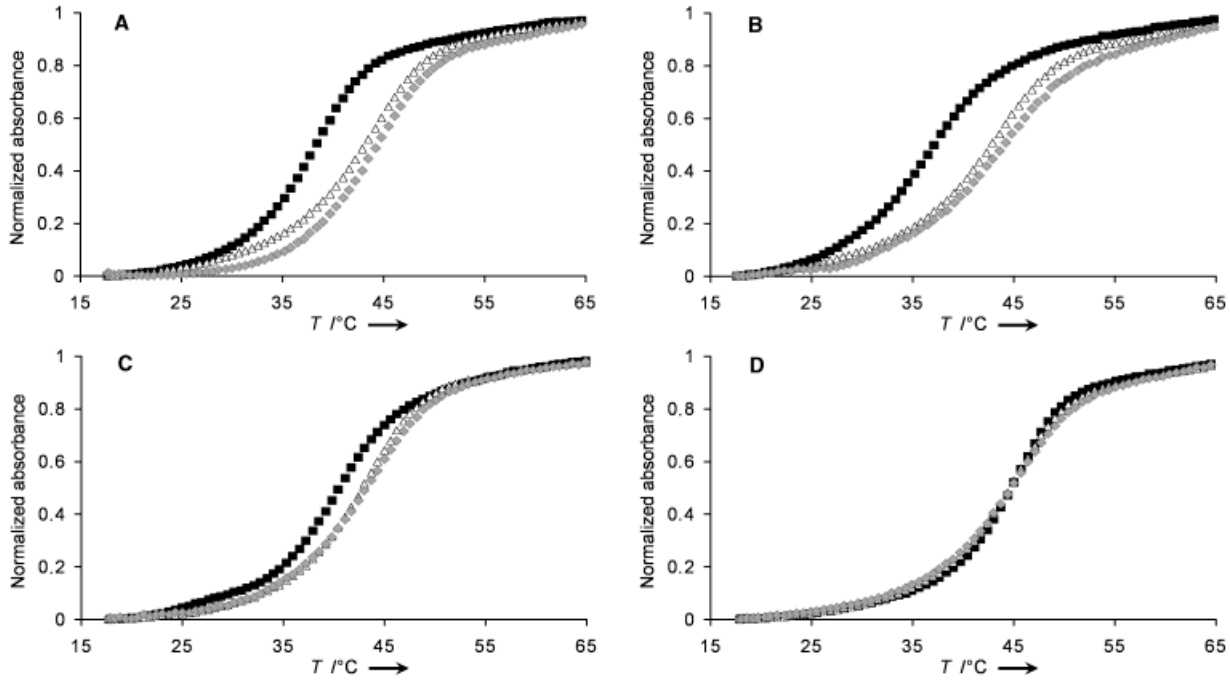


Figure 1. *Effect of* ***BisA*** *on the melting profile of 17-mer duplexes containing A) a TT mismatch, B) a TC mismatch, C) a TG mismatch, and D) a TA match at the corresponding position. Thermal denaturation studies were performed with duplexes (6 μM) in the absence of* ***BisA*** *(squares) or in the presence of 1 (triangles) or 2 (diamonds) equiv of* ***BisA*** *in cacodylate buffer (10 mM, pH 6.0) containing sodium chloride (10 mM).*

leads to an increased melting temperature of the fully base-paired TA 17-mer duplex. These results demonstrate that the control compound **MonoA**, which binds duplex DNA through classical intercalation,[16] is not able to discriminate between mismatched and matched DNA. In addition, these results establish that the macrocylic and the monomeric compound exhibit very different binding modes and that cyclization of two acridine units is needed for specific mismatch recognition.

Having demonstrated mismatch-specific binding of **BisA**, it was now interesting to investigate whether binding of **BisA** indeed induces flipping of one of the mismatched nucleobases, as proposed in the introduction. For this purpose we made use of the sensitivity of thymine residues toward potassium permanganate oxidation.[17] Since potassium permanganate attacks the C5=C6 double bond of the pyrimidine ring, thymine residues in double-stranded DNA are less reactive than in single-stranded DNA. Therefore, this oxidation reaction offers a convenient method of probing solvent accessibility of thymine residues in DNA. After oxidative conversion to 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) the glycosidic bond can be selectively cleaved with piperidine leading to DNA strand breakage that can easily be detected by denaturing polyacrylamide gel electrophoresis (PAGE).

In fact, this assay for accessible thymine residues has already been used to probe base flipping by DNA MTases M·*Taq*I and M·*Hha*I.[6] In the case of M·*Taq*I, the target adenine within the 5′-TCGA-3′ recognition sequence was replaced by thymine, resulting in a TT mismatch. Treatment of such a duplex (36-mer) with potassium permanganate followed by piperidine cleavage and analysis by denaturing PAGE is shown in Figure 2. In the presence of M·*Taq*I (lane 1), a strong band appears while the duplex alone (lane 2) does not show a prominent band. This result clearly indicates that enzymatic base flipping leads to an enhanced reactivity of the targeted thymine residue resulting in strand cleavage. Most remarkably, a concentration of only 10 nM of the **BisA** macrocycle also induces an enhanced reactivity of the mismatched thymine residue (lane 3) while no matched residue is susceptible to chemical oxidation. This result is almost identical to that observed in the presence of M·*Taq*I, and not only demonstrates that binding of **BisA** is mismatch-specific but also implies that it could lead to base flipping. In contrast to **BisA**, the classical intercalator **MonoA** does not lead to an enhanced reactivity of the mismatched thymine or any other residue (lane 4).

With the fully paired TA duplex, the presence of **BisA** only leads to minor background activities (lane 6), and the monomeric control compound **MonoA** does not induce any enhanced reactivity (lane 7).

A clearly enhanced reactivity of the thymine residue is also observed when it is mispaired with a guanine residue (lane 12) but the resulting band has a weaker intensity than that obtained with a duplex containing a TT mismatch (lane 3). This difference could be explained by a weaker affinity of **BisA** to the thermodynamically more stable TG mismatch, which is known to form a wobble base pair.[18] In addition, this difference parallels the smaller **BisA**-induced stabilization (ΔT_m) of the 17-mer TG duplex in comparison with the 17-mer TT duplex (Table 1).

However, in the TC mismatch (lane 9), the mispaired thymine residue is much less reactive than in the TT or TG mismatch. Since the binding affinities of **BisA** to the TC and the TT mismatch

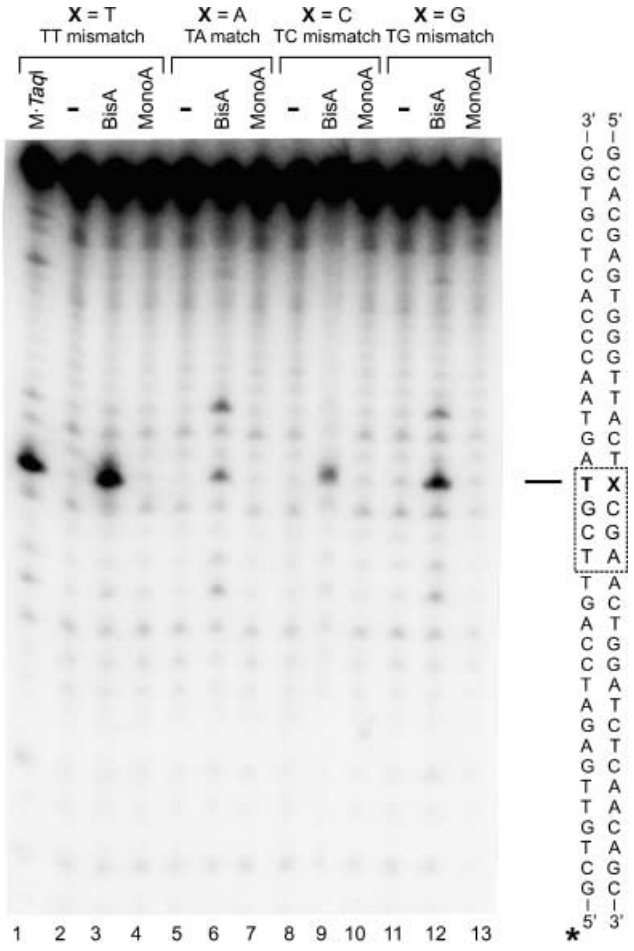
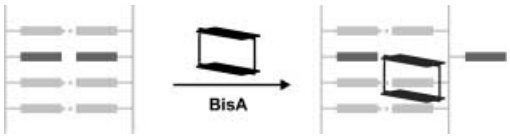


Figure 2. ***BisA****-enhanced chemical reactivity of mismatched thymine residues. 36-mer duplexes (5 nM) containing a TT, TC, or TG mismatch or a TA match at the corresponding position were treated with potassium permanganate and then with piperidine. Strand cleavage was analyzed by denaturing PAGE and autoradiography. The strand containing the probed thymine residue was ^{32}P-labeled at the 5′-end (indicated by a star). Prior to chemical treatment it was hybridized to four different DNA strands. The sequences of the resulting duplexes (X = T, A, C, or G) are given on the right-hand side. The sequence recognized by the DNA MTase M·TaqI (X = T) is boxed. The position of bands resulting from oxidation of the probed thymine residue is indicated by a line. Lanes 1–4: TT mismatch duplex in the presence of M·TaqI (50 nM), without addition, addition of* ***BisA*** *(10 nM), addition of* ***MonoA*** *(10 nM); lanes 5–7: TA match duplex alone, addition of* ***BisA*** *(10 nM), addition of* ***MonoA*** *(10 nM); lanes 8–10: TC mismatch duplex alone, addition of* ***BisA*** *(10 nM), addition of* ***MonoA*** *(10 nM); lanes 11–13: TG mismatch duplex alone, addition of* ***BisA*** *(10 nM), addition of* ***MonoA*** *(10 nM).*

should be similar, as indicated by a very similar increase in melting temperature (Table 1), the low reactivity may result from a binding mode in which the opposing cytosine residue rather than the thymine residue of the labeled strand is predominantly pushed out of the DNA helix.

In summary, the capacity of **BisA** to strongly stabilize the TT mismatch duplex, its inability to increase the melting temperature of the fully paired TA duplex, and its stimulation of enhanced reactivity of thymine in a mismatched TT duplex are clearly indicative of mismatch-specific binding of **BisA**. In addition, the oxidative display of the mismatched thymine suggests that the macrocycle **BisA** inserts at the TT site and concomitantly displaces one of the thymine residues from the helical stack. In analogy to the structure of **BisA** bound to a duplex with an abasic site[14] we propose that **BisA** sandwiches a base pair flanking the mismatch site thereby flipping one of the mismatched bases out of the DNA helix (Scheme 2). Although more functional and structural data are needed to definitively



Scheme 2. *Proposed binding mode of* ***BisA*** *to DNA containing a mismatched site. A neighboring base pair is sandwiched by the macrocycle so that one of the mismatched nucleobases is flipped out of the DNA helix.*

prove the presence of an extrahelical base in the complex of mismatch-containing DNA and **BisA**, this work clearly suggests that base flipping in DNA may not be restricted to enzymes but could also be induced by the binding of small synthetic ligands. In this respect it is interesting to note that β-cyclodextrin was recently employed to trap spontaneously flipped-out bases.[19] However, in contrast to our studies, which were performed with nanomolar or micromolar concentrations of **BisA**, β-cyclodextrin was employed at millimolar concentration in order to elicit a small *decrease* in melting temperature. This is in contrast to **BisA**, which leads to an *increase* in melting temperature of DNA containing a mismatch. Thus, different binding modes can be assumed, such as innerhelical binding for **BisA** and extrahelical binding for β-cyclodextrin.

Conclusion

We have demonstrated that the macrocycle **BisA**, which strongly binds to abasic sites, can also specifically recognize DNA base mismatches. The binding is accompanied by an increase in the melting temperature of mismatched DNA almost to the level of fully matched DNA. This property makes **BisA** interesting for hybridization experiments, for which degenerate pools of oligodeoxynucleotides need to be used. Here **BisA** could act as a mismatch rectifier and counteract the dilution of the degenerate probe due to nonpairing components.

In addition, **BisA** represents an attractive probe for recognizing DNA mismatches. Unfortunately, the macrocycle is poorly fluorescent due to interchromophoric quenching (excimer effect) of the two acridine units,[20] so that its direct use for optical detection of structural heterogeneity of DNA can be ruled out. However, the attachment of a small-sized fluorophore to the macrocyclic scaffold could provide a valuable sensor for single-base alteration, assuming that the functionalization will not affect the binding specificity.

Finally, the combination of **BisA** with potassium permanganate leads to specific cleavage of thymine from a GT mismatch, so that it can be regarded as a chemical analogue of a GT mismatch-specific DNA glycosylase.[9d] It is also conceivable that **BisA** binds to other DNA mismatches resulting from chemical damage of nucleobases. Thus, it could interfere with a variety of

different DNA repair glycosylases. The presence of **BisA** could sterically block binding of DNA glycosylases to their target site thus act as a DNA-glycosylase inhibitor. Such inhibitors are of pharmacological interest because most clinically used antitumor agents exert their cytotoxicity by damaging DNA, and their action is often counteracted by DNA repair enzymes.[21]

Experimental Section

BisA and **MonoA** were synthesized as described before.[20] The DNA MTase M·*Taq*I was isolated as previously reported.[3b] Oligodeoxynucleotides were purchased either from Eurogentec or from MWG-Biotech. The following 17-mer and 36-mer duplexes were used in this study:

5′-CCAGTTCG **T** AGTAACCC-3′
3′-GGTCAAGC **X** TCATTGGG-5′

X = T (TT 17-mer duplex), **X** = C (TC 17-mer duplex), **X** = G (TG 17-mer duplex), **X** = A (TA 17-mer duplex) and

5′-GCTGTTGAGATCCAGTTCG **T** AGTAACCCACTCGTGC-3′
3′-CGACAACTCTAGGTCAAGC **X** TCATTGGGTGAGCACG-5′

X = T (TT 36-mer duplex), **X** = C (TC 36-mer duplex), **X** = G (TG 36-mer duplex), **X** = A (TA 36-mer duplex).

Thermal-denaturation experiments: Thermal denaturation measurements were performed with a Uvikon XL spectrophotometer. The temperature of the six-cell holder was regulated by an electrical thermosystem and controlled by a temperature sensor immersed in a reference cell containing appropriate buffer. The rate of temperature change was usually 0.4 °C min^{-1}. T_m values were obtained by calculating the first derivative of the melting curves. The melting profiles were monitored at 270 nm and subtracted from the absorbance at 500 nm, which was used as internal base line. All measurements were performed with the 17-mer duplexes. To form the duplexes, we incubated equimolar amounts of complementary (TA duplex) or partly complementary oligodeoxynucleotides (TT, TC, and TG duplex) in cacodylate buffer (10 mM, pH 6.0) containing sodium chloride (10 mM) at 90 °C for 5 min, the duplexes were then slowly cooled to room temperature and stored at 4 °C for 12 h. **BisA** (6 μM or 12 μM) or **MonoA** (6 μM or 12 μM) was added to the duplexes (6 μM), and the solutions were allowed to equilibrate at 4 °C for 3 h before measurements were made.

Potassium permanganate oxidation assay: The 36-mer strand containing the target thymine residue was 5′-labeled by treatment with [γ-^{32}P]ATP and T4 polynucleotide kinase following standard procedures.[22] 36-mer duplexes were formed by mixing equimolar amounts of ^{32}P-labeled strand and the different (partly) complementary strands in Tris-HCl buffer (10 mM, pH 8.0) containing EDTA (1 mM). The mixtures were incubated at 95 °C for 2 min and then slowly cooled to 30 °C. Permanganate oxidations of labeled 36-mer duplexes (5 nM) were performed in sodium cacodylate buffer (100 μL, 10 mM, pH 6.0) containing potassium permanganate (0.8 mM) and either **BisA** (10 nM), **MonoA** (10 nM), or no additive. Oxidative modification in the presence of M·*Taq*I was carried out as described before[6] except that poly(dG-dC) was omitted. The solutions were allowed to equilibrate at room temperature for 1 h before the reactions were started by potassium permanganate addition. After incubation at room temperature for 1 min, the reactions were quenched by adding a solution of sodium acetate (100 μL, 1.5 M, pH 7.0) containing β-mercaptoethanol (1 M). Oligodeoxynucleotides were precipitated by addition of a solution (4 μL) of glycogen (20 mg mL^{-1}) and ethanol (600 μL) followed by incubation at −20 °C. For oxidation-specific strand cleavage, the pellets were dissolved in piperidine (100 μL, 1 M), and the solution was incubated at 90 °C for 1 h. After a second ethanol precipitation, samples were analyzed by denaturing PAGE (15% DNA-sequencing gel containing urea).[22] Radioactive bands were visualized by using a phosphorimager (Molecular Imager System GS525, Bio-Rad).

Keywords: bioorganic chemistry · DNA base flipping · DNA recognition · enzymes · macrocycles · nucleobases

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Received: June 23, 2003 [F693]

