Acceleration of DNA Cleavage by Benzidine Derivatives under Weakly Acidic Conditions

Rin-ichi YAMASHITA, Takahiro TOMIMOTO, and Yushin NAKAMURA*

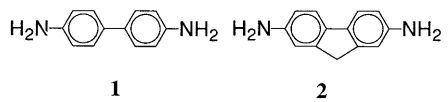
Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda-shi, Chiba 289, Japan. Received November 10, 1993; accepted March 1, 1994

Benzidine derivatives, especially 2,7-diaminofluorene and benzidine, accelerated the cleavage of a plasmid DNA (form I) under weakly acidic conditions at 37 °C. The acceleration was not inhibited by EDTA, superoxide dismutase, catalase, or hydroxyl radical scavengers. The rate of the cleavage was influenced by the pH value of the reaction buffer. The decrease in form I DNA in this reaction obeyed first-order kinetics. It was confirmed that the cleavage was enhanced by the amines themselves, and not by their oxidized derivatives. 2,7-Diaminofluorene also cleaved a 3′-end-labeled 40-mer DNA with A and G base specificity. These results indicate that the acceleration is caused by depurination followed by breakage of the DNA sugar backbone.

Keywords DNA cleavage; depurination; benzidine derivative; benzidine; 2,7-diaminofluorene; aromatic amine

Many aromatic amines, such as aminofluorene and aminobiphenyl, are carcinogenic and mutagenic. Their administration *in vivo* often yields nucleic acid- and protein-bound adducts. These amines should be metabolically converted into strongly electrophilic reactants which chemically bind to nucleophilic sites, particularly to nucleic acids and proteins, in the target cells. The hydroxylamine derivatives including *N*- and/or *O*-acyl hydroxylamine, are considered as proximate reactants.[1] For example, the administration of *N*-acetylaminofluorene (AAF) to rat gives DNA adducts, mainly *N*-(deoxyguanosin-8-yl)-AAF and a few other derivatives,[2] but *in vitro*, conversion to *N*-acetoxy derivatives is necessary for the reaction with DNA. In addition, some hydroxylamines cleave DNA *in vitro*. 3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P2) is a mutagenic product resulting from the pyrolysis of tryptophan in proteins. The hydroxylamino derivative of Trp-P2 cleaves plasmid DNA *in vitro*. However, Trp-P2 itself can not cleave DNA.[3]

Benzidine (4,4-diaminobiphenyl, **1**), a typical mutagen and carcinogen, binds covalently to DNA when administered to rats. This adduct was identified as *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine.[4] However, *in vitro*, no DNA adduct was observed in the absence of peroxidase and/or hydrogen peroxide.[5] The DNA damaged by **1** was easily cleaved to short fragments under alkaline conditions.[6] But it is not known whether **1** itself directly accelerates the DNA cleavage *in vitro* in the absence of the relevant oxidant. We report the acceleration of the *in vitro* DNA cleavage under weakly acidic conditions by some aromatic amines, typically **1** and 2,7-diaminofluorene (**2**) themselves, and the reaction mechanism. This property of benzidine derivatives might be responsible for their cytotoxicity.



1 **2**

Fig. 1. Structures of Benzidine (**1**) and 2,7-Diaminofluorene (**2**)

Experimental

Chemicals Compound **1**[7] was synthesized from hydrazobenzene by the method of Snyder *et al.*[8] 4-Aminobiphenyl (**3**) was obtained by the reduction of 4-nitrobiphenyl (purchased from Sigma) with iron powder in aqueous EtOH. 2,4′-Diaminobiphenyl (**4**) was synthesized and purified by the method of Lown *et al.*[9] The other amines were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan and purified by recrystallization. The purity of aromatic amines was assessed by using a Shimadzu LC-6A HPLC system. The purities of **1**, **2**, and **3** were more than 99%, those of **4**, 2-aminobiphenyl (**5**), 2-aminofluorene (**6**), 1-aminonaphthalene (**7**), 3,6-diaminoacridine (**8**), ethidium bromide (**9**), and aniline (**10**) were more than 98%, and those of the other five amines were more than 95%.

Quantification of Cleavage of Plasmid DNA by Aromatic Amines Plasmid pUC119 DNA[10] was grown in *Escherichia coli* strain JM109 and isolated in supercoiled form (form I) by equilibrium centrifugation in CsCl–ethidium bromide gradients.[11] A 0.4 μg sample of the plasmid DNA (form I) was incubated with 1.5 μl of acetonitrile solution of an amine in 40 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer (total volume 15 μl) at 37 °C. Form I and open circular form (form II) DNAs were separated by electrophoresis on agarose gel, stained with ethidium bromide and quantified using a Shimadzu CS-9000 fluorescence densitometer. The correction factor for form I DNA was determined to be 1.49 by the method of Haidle *et al.*[12]

Quantification of Cleavage of 3′-Labeled 40-mer DNA Thirty-six-base-pair fragment of the exon 2 region of human c-*myc* prooncogene[13] and complementary 40-mer were synthesized using an Applied Biosystems Model 381A DNA Synthesizer and annealed. The double-stranded DNA fragment was purified by electrophoresis on polyacrylamide gel and labeled by enzymatic extension of the 3′-end with the Klenow fragment (Takara, Kyoto, Japan) of DNA polymerase I and [α-^{32}P]dGTP (370 MBq/ml). After purification by EtOH precipitation, $>10^4$ cpm of 3′-end-labeled 40-mer DNA was incubated with 1.5 μl of acetonitrile solution of **2** and 13.5 μl of 40 mM Mes buffer at 37 °C for 48 h, followed again by EtOH precipitation. The obtained DNA was electrophoresed on polyacrylamide gel and quantified using a Fujix, Bas 2000, Bio-imaging analyzer.

Results

The acceleration of the cleavage of the plasmid DNA by **1** and **2** is shown in Fig. 2. The introduction of a single strand break converts the supercoiled (form I) pUC 119 plasmid DNA to an open circular DNA (form II). At pH 5.0, the addition of 50 μM **1** and 5 μM **2** effectively converted form I to form II DNA (lanes 2 and 6).

The relationship between the pH values of the reaction buffers and the cleavage ratios are displayed in Fig. 3. The rate of the cleavage without amines was very slow at pH

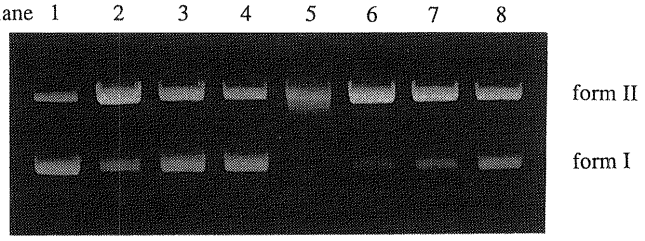


Fig. 2. Agarose Gel Electrophoresis of pUC119 DNA Incubated with **1** or **2**

The 0.4 μg of pUC119 form I DNA was incubated in 40 mM Mes buffer (pH 5.0) for 2 h at 37 °C with 0, 50, 10, or 5 μM of **1** (lanes 1—4), or 50, 5, 1, or 0.5 μM of **2** (lanes 5—8).

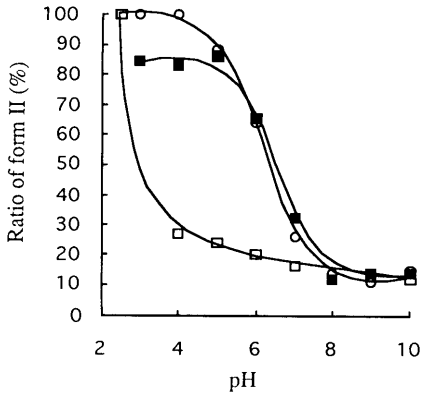


Fig. 3. Relationship between the pH Values of Reaction Buffers[a] and the Ratios of Nicked Form II DNA[b]

a) The reaction buffers were as follows: pH 2.5—4, 40 mM citric acid–Na_2HPO_4 buffer; pH 5—6, 40 mM Mes buffer; pH 7—8, 50 mM Tris–HCl buffer; pH 9—10, 40 mM sodium carbonate-sodium bicarbonate buffer. *b*) Other reaction conditions were the same as those of Fig. 2. □, without amines; ○, 5 μM **2**; ■, 50 μM **1**.

4 or above, while the addition of amines apparently increased the cleavage rates in the range of pH 4—6. The cleavage was extremely fast even without amines at pH 2.5.

The DNA cleavage was examined using 15 aromatic and non aromatic amines. The results are shown in Table I. The DNA cleavage enhancements by the amines under the reaction conditions used were evaluated by comparison of the ratios of form I to the nicked form II DNA. Compound **2** was the most effective to cleave the DNA among the tested amines. The DNA cleavage enhancement by **2** was too high to allow comparison with other amines at the same concentration (50 μM). On the other hand, 2,3-diaminofluorene (**11**), a positional isomer of the amino substituents of **2**, did not exhibit enhancement. Compound **6** exhibited moderate enhancement. Compound **1**, which has a 4,4′-diaminobiphenyl skeleton similar to **2**, was quite effective, whereas, **3**, **5** and **4**, an amino positional isomer of **1**, only slightly enhanced the DNA cleavage. Other amines, including ethylenediamine (**12**), did not exhibit apparent enhancement.

The cleavages by **1** and **2** were followed by monitoring of the ratio of the retained form I DNA every hour (Fig. 4). The results indicate that the cleavages obey first-order kinetics. The values of the reaction rate constants K (h^{-1}) were calculated from the slopes of these linear rate plots. The K values of the cleavages with no amine, 5 μM **2**, and 50 μM **1** were 0.04, 0.38, and 0.77, respectively.

Table I. DNA Cleavage by the Aromatic Amines[a]

Compound	Concentration (μM)	Form I (%)	Form II (%)
—	50	83	17
Benzidine (**1**)	50	35	65
4-Aminobiphenyl (**3**)	50	66	34
2-Aminobiphenyl (**5**)	50	71	29
2,4′-Diaminobiphenyl (**4**)	50	67	33
2-Aminofluorene (**6**)	50	50	50
2,3-Diaminofluorene (**11**)	50	86	14
2,7-Diaminofluorene (**2**)	10	30	70
1-Aminonaphthalene (**7**)	50	80	19
2-Aminonaphthalene (**13**)	50	80	19
1,5-Diaminonaphthalene (**14**)	50	69	31
1,8-Diaminonaphthalene (**15**)	50	78	22
3,6-Diaminoacridine (**8**)	50	90	10
Ethidium bromide (**9**)	50	82	18
Aniline (**10**)	50	74	26
Ethylenediamine (**12**)	50	71	29

a) The reactions were carried out in 40 mM Mes buffer pH 6.0 at 37°C for 5 h in the dark.

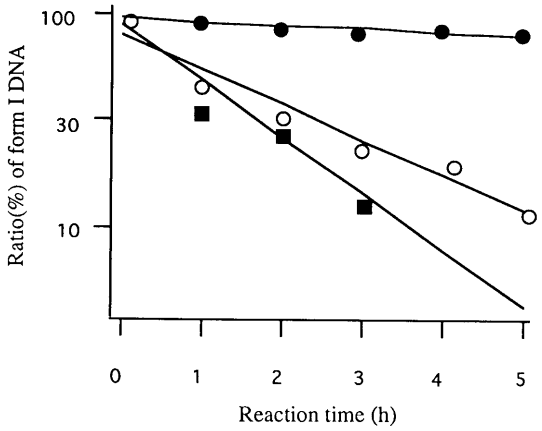


Fig. 4. Semilogarithmic Plot of Ratios of Form I DNA *vs.* Time

All reactions were run in 40 mM Mes pH 5.0 buffer at 37 °C. ●, control; ○, 5 μM **2**; ■, 50 μM **1**.

Table II. Influence of Inhibitors on the Cleavage by **2**[a]

Entry	Reagent	Concentration	Ratio of cleavage[b]
1	EDTA	1 mM	1.00
2	SOD	0.1 mg/ml	0.98
3	Catalase	0.01 mg/ml	1.05
4	EtOH	0.2 M	1.01
5	iso-PrOH	0.2 M	0.97
6	Thiourea	0.2 M	0.99
7	Mannitol	0.2 M	0.94
8	DTT	0.2 M	0.98
9	O_2 bubbling		1.01
10	Degassed[c]		1.00

a) Form I plasmid with 5 μM **2** and inhibitors were incubated in 40 mM Mes buffer pH 5.0 at 37 °C for 5 h in the dark. *b*) The ratio of the % value of form II DNA produced by the incubation in the presence of each inhibitor and that in the absence of inhibitor. *c*) The reaction buffer was degassed by ultrasonication under reduced pressure followed by argon gas bubbling and then the reaction vessel was sealed.

The cleavage by **2** in the presence of various inhibitors and enhancers was studied to elucidate the mechanism involved. The results are shown in Table II. EDTA,

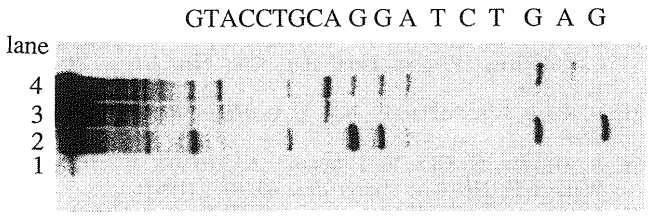


Fig. 5. Gel Electrophoresis of 3'-end Labeled 40-mer DNA

Lane 1, untreated; lane 2, Maxam–Gilbert G[14]; lane 3, incubated in 40 mM Mes and 1 mM EDTA buffer pH 5.0 for 48 h without amines; lane 4, incubated with 100 μM **2** in the same buffer as lane 3.

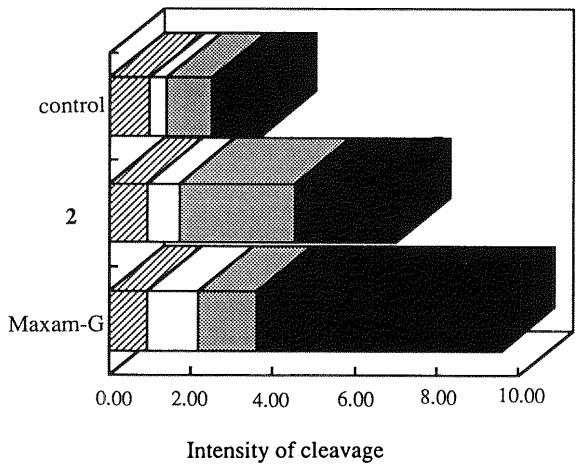


Fig. 6. Quantification of Gel Electrophoresis Results (Fig. 5)

The quantities of the cleaved DNA fragments were measured using a Bio-imaging analyzer and averaged per base (G, A, T, C). The averaged quantity of the cleaved fragment at C base on the control lane (Fig. 5, lane 3) was defined as the standard. The relative intensity of the cleavage in comparison with the standard was displayed as a bar chart. ■, G; ▩, A; □, T; ▨, C.

dithiothreitol (DTT), superoxide dismutase (SOD), catalase and four hydroxyl radical scavengers did not inhibit the cleavage (entries 1—8). O_2 bubbling also did not enhance the cleavage (entry 9). The cleavage proceeded even under degassed conditions (entry 10).

The base specificity of the enhancement of the DNA cleavage by **2** was investigated using polyacrylamide gel electrophoresis of the 3'-end-labeled 40-mer double-stranded DNA (Fig. 5). The relative intensity of the cleavage is displayed in Fig. 6. The 40-mer DNA was effectively cleaved with A and G base specificity by the addition of **2**.

Discussion

It is well known that purine nucleotides are cleaved under strongly acidic conditions. This reaction is based on the depurination of the purine nucleotides, and is utilized in the Maxam–Gilbert method to cleave selectively the G and/or A nucleotides.[14] The pUC119 form I DNA was effectively cleaved by incubation in the Mes buffer at pH 3 or below. In a higher pH range, however, the cleavage proceeded only slightly; the rate constant of the cleavage at pH 5 was only 0.04, as shown in Fig. 4. On the other hand, the rate of cleavage was increased about 20 times by the addition of 50 μM **1**. In the neutral and basic pH ranges (pH 6 up to 10), the cleavages were not enhanced by the addition of the amines (Fig. 3). The cleavage by **2** selectively proceeded at A and G bases (Figs. 5 and 6). These results indicate that amines under weakly acidic conditions enhance the DNA backbone breakage by depurination similar to that observed under strongly acidic conditions without amines. This depurination mechanism is supported by the following results.

In vivo, aromatic amines are often oxidized metabolically into strongly electrophilic reactants. *In vitro*, however, the appropriate oxidant is necessary for the conversion of amines to the reactants. Compound **1** is oxidized to a highly reactive derivative called meriquinoid by a variety of oxidants, such as molecular oxygen, chromic acid and sodium chlorite.[15] Therefore the influence of oxidants on the cleavage by **1** was examined. The rate of the cleavage did not increase with sodium chlorite (data not shown) or under oxygen gas bubbling, and also did not decrease under degassed conditions (Table II, entries 9 and 10). The addition of DTT, a typical oxidation inhibitor, did not interfere with the reaction (Table II, entry 8). In addition, the decrease in form I DNA with reaction time obeyed first-order kinetics (Fig. 4). If the oxidized amines cleave the plasmid, the rate of the cleavage must increase with increase in the concentration of the oxidized amines. In that case, the initial velocity of the cleavage must be nearly zero because no oxidized amine should exist at the beginning of the cleavage. But in our experiment, the initial velocity was maximal and the rate of the cleavage decreased with time (Fig. 4). The results indicate that the oxidized aromatic amine is not involved in the cleavage. The activating reactant of the cleavages must be the amines themselves.

Numerous aromatic compounds and dyes, such as adriamycin, mitomycin C and acridine, generate hydroxyl radicals, which cleave DNA.[16] If the enhancement of the cleavage was caused by hydroxyl radicals, it should be inhibited by the addition of scavengers. As shown in Table II entries 2—7, catalase, SOD and four hydroxyl radical scavengers did not inhibit the cleavages by **2**. These results are in marked contrast to those of a similar experiment with Trp-P2(OH), where the DNA cleavage by Trp-P2(OH) was strongly inhibited by those inhibitors.[17] It was confirmed that the DNA cleavage by **2** did not proceed *via* active oxygen species such as superoxide, H_2O_2 and hydroxyl radical. Metal ions, which can cause DNA scission, were not involved since EDTA did not inhibit the cleavage (Table II, entry 1).

The mechanism of the depurination by benzidine derivatives can be explained as follows. In general, the binding of amines to DNA is caused by the electrostatic interaction between protonated amino substituent and phosphate anion. So, in the case of the benzidine derivatives, which should be intercalative, at least one of the amino substituents must be protonated and form an ion pair with a phosphate anion in order to stabilize the non-covalent binding to DNA. The pK_a values of the ammonium cations of **1** and **2** determined by titration were 5.0 and 4.0, respectively, so that the cations act as acids. If a purine base on the neighboring nucleotide were protonated by an acidic proton of this ammonium ion, this purine base would be liable to be eliminated from the sugar backbone following the DNA cleavage.

The relationship between structure and DNA cleavage enhancement by amines was investigated (Table I). (i) The mono- and di-amino substituted biphenyls, **3**—**5**, and naphthalene derivatives, **7**, 2-aminonaphthalene (**13**), 1,5-diaminonaphthalene (**14**) and 1,8-diaminonaphthalene (**15**), only slightly affected the DNA cleavage compared with **1** and **2**. The basicity and DNA binding affinity are not much different among them. However, the dipole moment of these compounds, the distance between the two amino groups, and their direction vary appreciably. These differences would cause an alteration in the binding position of the amines to DNA, which appears to influence strongly the cleavage enhacement. Similarly the reason why **11**, an amino-positional isomer of **2**, did not enhance the DNA cleavage can be explained by the difference of dipole moment. (ii) The reason why the enhancement of the DNA cleavage by **2** is higher than that by **1** (Figs. 2 and 3) may be the higher DNA binding ability of **2** and the higher acidity of the ammonium ion of **2** in comparison with those of **1** (data not shown). (iii) Compounds **8** and **9**, which are strong intercalators, have extremely high DNA-binding ability compared with other amines. Neither of them, however, cleaved the plasmid (Table I). Under weakly acidic conditions, **8** is protonated at the position **10** nitrogen atom, but not at the amino groups. So it is assumed that a proton of the ammonium cation of **8** can not effectively form a hydrogen bond with the purine base. Compound **9** already has a positive charge itself, so further protonation of the amino-nitrogens under weakly acidic conditions would be unlikely. Therefore, **9** can not enhance the protonation of the purine base in the proposed cleaving mechanism.

References and Notes

1) B. Singer, D. Grunberger, "Molecular Biology of Mutagens and Carcinogens," Plenum Publishing, Co., New York, 1983, pp. 129—140.
2) E. Kriek, J. A. Miller, U. Juhl, E. C. Miller, *Biochemistry*, **6**, 177 (1967).
3) A. Wakata, N. Oka, K. Hiramoto, A. Yosioka, K. Negishi, Y. Wataya, H. Hayatsu, *Cancer Res.*, **45**, 5867 (1985).
4) B. Singer, D. Grunberger, "Molecular Biology of Mutagens and Carcinogens," Plenum Publishing, Co., New York, 1983, pp. 169—172; C. N. Martin, F. A. Beland, R. W. Roth, F. F. Kadlubar, *Cancer Res.*, **42**, 2678 (1982).
5) Y. Tsuruta, P. D. Josephy, A. D. Rahimtula, P. J. O'Brien, *Chem.-Biol. Interact.*, **54**, 143 (1985).
6) C. Bolognesi, C. F. Cesarone, L. Santi, *Carcinogenesis*, **4**, 265 (1981).
7) A. I. Vogel, "Practical Organic Chemistry," 3rd ed., Longmans, London, 1959, p. 633.
8) H. R. Snyder, C. Weaver, C. D. Marshall, *J. Am. Chem. Soc.*, **71**, 289 (1949).
9) J. W. Lown, B. C. Gunn, K. C. Majumdar, E. McGoran, *Can. J. Chem.*, **57**, 2305 (1979).
10) J. Vieira, J. Messing, *Methods Enzymol.*, **153**, 3 (1987).
11) J. Sambrook, E. F. Fritsch, T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, New York, 1989, p. 1.42.
12) R. S. Lloyd, C. W. Haidle, D. L. Robberson, *Biochemistry*, **17**, 1890 (1978).
13) J. Battey, C. Moulding, R. Taub, W. Murphy, T. Stewart, H. Potter, G. Lenoir, P. Leder, *Cell*, **34**, 779 (1983).
14) A. M. Maxam, W. Gilbert, *Methods Enzymol.*, **65**, 499 (1980).
15) M. R. Rifi, *Tetrahedron Lett.*, **58**, 5089 (1969).
16) B. Kalanaraman, E. Perez-Reyes, *Biochim. Biophys. Acta*, **630**, 119 (1980); H. Nakano, K. Sugioka, M. Nakano, M. Mizukami, H. Kimura, S. Tero-Kubota, Y. Ikegani, *ibid.*, **796**, 285 (1984); J. G. Peak, M. J. Peak, and C. S. Foote, *Photochem. Photobiol.*, **44**, 111 (1986).
17) K. Hiramoto, K. Negishi, Y. Wataya, H. Hayatsu, *Protein, Nucleic Acid and Enzyme*, **16**, 2869 (1988).