Dihydropyrazine Derivatives as a New Type of DNA Strand Breaking Agent

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The DNA strand-breaking activity of some dihydropyrazine derivatives, 2,3-dihydro-5,6-dimethylpyrazine (3), 2,3-dihydro-2,5,6-trimethylpyrazine (4), 2,3-dihydro-2,2,5,6-tetramethylpyrazine (5), *trans*-2,3-dimethyl-5,6,7,8,9,10-hexahydroquinoxaline (6), its *cis*-compound (7) and the mixture of 6 and 7 (8) was tested by agarose gel electrophoresis using plasmid pBR322 ccc-DNA as a substrate. The order of DNA strand-breaking activity in the presence of Cu²⁺ was (7) > (8) ≥ (5) > (2) > (6) > (4) ≥ (1) ≥ (3). 2,5-Bis(D-arabino-tetrahydroxybutyl)-2,5-dihydropyrazine (1) and 2,5-dihydro-3,6-dimethylpyrazine (2) have already been described in terms of DNA breaking activity in a previous paper.

The activity was suggested to be due to the dihydropyrazine skeleton, in addition to active oxygen radicals formed in an aqueous solution. The introduction of a methyl group to the dihydropyrazine skeleton enhanced the activities of dihydropyrazine derivatives. The possible chemical basis for DNA strand breakage by dihydropyrazine derivatives, especially in the presence of Cu²⁺, was discussed.

Key words dihydropyrazine; DNA strand-breaking activity; plasmid pBR322; radical ion; cupric ion

Many compounds, including drugs such as antitumor antibiotics and natural substances, have been shown to cause DNA strand breakage, especially in the presence of Cu²⁺. DNA strand breakage is closely related to many important biological phenomena such as mutation, canceration, aging and the like. In a previous paper,¹⁾ we found that a compound which was produced by the condensation of two molecules of D-glucosamine, and which was assigned on the basis of the positive-ion FAB-MS to 2,5-bis(D-arabino-tetrahydroxybutyl)-2,5-dihydropyrazine (1), caused single strand-breakage of the ccc-DNA of plasmid pBR322. Although the separation and purification of the compound thus produced was unsuccessful, because of its instability, 2,5-dihydro-3,6-dimethylpyrazine (2) and 2,3-dihydro-5,6-dimethylpyrazine (3) synthesized alternatively were shown to have similar DNA strand-breaking activity. Magas and Siger²⁾ in their review have listed 70 different pyrazines isolated from various foods. Since dihydropyrazine derivatives are supposed to be precursors to pyrazines in nature, the findings that some dihydropyrazines have DNA strand-breaking activity are worth noting, especially from a food hygienic point of view. However, so far there have been few reports on the physiological activities, such as DNA strand-breaking activity, of dihydropyrazine derivatives.

In this paper, therefore, to confirm the DNA strand-breaking activity of dihydropyrazines and to obtain more active compounds involved in breaking DNA, we have synthesized some dihydropyrazine derivatives (Fig. 1) and tested their activities in the presence or absence of Cu²⁺.

MATERIALS AND METHODS

Assay of DNA Strand-Breaking Activity The method of assaying the DNA strand-breaking activity of dihydropyrazines, using a covalently closed circular duplex

DNA of plasmid pBR322 (ccc-DNA) as a substrate, were described in a previous paper.¹⁾

Synthesis of Dihydropyrazine Derivatives The dihydropyrazines used were synthesized by the condensation of diketons and diamines. All synthesized products were too labile to exist at room temperature, and even at -30 °C some decomposition occurred.

(i) 2,3-Dihydro-5,6-dimethylpyrazine (3) was prepared by the procedure of Ishiguro *et al.*³⁾ To a solution of ethylenediamine (3.7 g) in ether (40 ml), which was mechanically stirred on an ice bath, a solution of diacetyl (4 g) in ether (32 ml) was slowly added dropwise. The reaction mixture was stirred overnight at room temperature until the mixture became clear. After that, the mixture was refluxed for 30 min. Potassium hydroxide was added to the mixture for dehydration. The product (3.4 g) was distilled off under reduced pressure in nitrogen, and was stored in a refrigerator. 66.7% yields, bp 43–48 °C (20 mmHg), colorless odorous oil: ¹H-NMR (CDCl₃) δ: 2.1 (6H, C₅- and C₆-CH₃, s), 3.3 (4H, CH₂, s); ¹³C-NMR δ: 23.1 (C₅- and C₆-CH₃), 44.8 (C₂, C₃), 159.1 (C₅, C₆). The data for protons were identical with the literature.⁴⁾ FAB-MS *m/z*: 221.1765 [(2M+H)⁺, C₁₂H₂₁N₄].

(ii) 2,3-Dihydro-2,5,6-trimethylpyrazine (4) was prepared in the same manner from diacetyl and 1,2-diaminopropane. 84.9% yields, bp 37 °C (10 mmHg), colorless odorous oil. ¹H-NMR (CDCl₃) δ: 1.2 (3H, C₂-CH₃, d), 2.13 or 2.14 (3H, C₅- or C₆-CH₃, s), 2.8 (1H, C₃-CH_a, mult), 3.2 (1H, C₂-CH, mult), 3.6 (1H, C₃-CH_b, mult). ¹³C-NMR δ: 18.8 (C₂-CH₃), 22.7 or 22.9 (C₅- or C₆-CH₃), 49.2 (C₂), 51.1 (C₃), 157.9 or 158.7 (C₅ or C₆). FAB-MS *m/z*: 249.2078 [(2M+H)⁺, C₁₄H₂₅N₄].

(iii) 2,3-Dihydro-2,2,5,6-tetramethylpyrazine (5) was prepared in the same manner from diacetyl and 1,2-diamino-2-methylpropane. 59.3% yields, bp 38–39 °C (5 mmHg), colorless odorous oil. ¹H-NMR (CDCl₃) δ: 1.1 (6H, C₂-CH₃, s), 2.11 (3H, C₆-CH₃, s), 2.16 (3H,

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Fig. 1. Some Dihydropyrazine Derivatives

C_5-CH_3 , t), 3.3 (2H, CH_2 , q). ^{13}C -NMR δ : 22.8 (C_5-CH_3), 23.3 (C_6-CH_3), 25.8 (C_2-CH_3), 50.9 (C_2), 56.2 (C_3), 156.2 or 158.4 (C_5 or C_6). The data for the protons were identical with the literature.^{5,6} FAB-MS m/z : 277.2384 [$(2M+H)^+$, $C_{16}H_{29}N_4$].

(iv) *trans*-2,3-Dimethyl-5,6,7,8,9,10-hexahydroquinoxaline (**6**) was prepared in the same manner from diacetyl and *trans*-1,2-diaminocyclohexane. 51.7% yields, bp 80–81 °C (4 mmHg). The distilled product changed to viscous solid mass. 1H -NMR ($CDCl_3$) δ : 2.1 (6H, CH_3 , s), 2.5 (2H, C_5 - and $C_{10}-H_{axial}$, s), 1.13–1.98 (8H, mult). ^{13}C -NMR δ : 23.0 (CH_3), 25.6 (C_7 and C_8), 33.5 (C_6 and C_9), 58.9 (C_5 and C_{10}), 158.8 (C_2 and C_3). FAB-MS m/z : 165.1390 [$(M+H)^+$, $C_{10}H_{17}N_2$].

(v) *cis*-2,3-Dimethyl-5,6,7,8,9,10-hexahydroquinoxaline (**7**) was prepared in the same manner from diacetyl and *cis*-1,2-diaminocyclohexane. 49.9% yields, bp 74 °C (6 mmHg), pale yellow odorous oil. 1H -NMR ($CDCl_3$) δ : 2.1 (6H, CH_3 , s), 3.4 (1H, C_5 or $C_{10}-H$ equatorial, s), 1.13–1.68 (9H, mult). ^{13}C -NMR δ : 22.3 (CH_3), 23.0 (C_7 and C_8), 27.8 (C_6 and C_9), 54.0 (C_5 and C_{10}), 158.2 (C_2 and C_3). FAB-MS m/z : 165.1393 [$(M+H)^+$, $C_{10}H_{17}N_2$].

(vi) The mixture of *trans*- and *cis*-2,3-dimethyl-5,6,7,8,9,10-hexahydroquinoxaline (**8**) was prepared in the same manner from diacetyl and the mixture of *trans*- and *cis*-1,2-diaminocyclohexane. bp 64–65 °C (4 mmHg). The distilled product, which was changed to a viscous solid mass, was shown to be a mixture of *trans* and *cis* compounds from the NMR data.

Spectrometry 1H - and ^{13}C -NMR spectra were recorded on a JEOL GX-400 spectrometer using tetramethylsilane (TMS) as an internal standard.

Enzymes and Chemicals Bovine erythrocyte superoxide dismutase (3050 unit/mg protein) and bovine liver catalase (3765 unit/mg protein) were purchased from Sigma Chemical Co. The other chemicals were purchased from Wako Jun-yaku Co., and used without further purification.

RESULTS AND DISCUSSION

Induction of DNA Strand Breakage by Dihydrodimethylpyrazine Figure 2 shows the time course of the single strand breakage of pBR322 ccc-DNA by 2,3-dihydro-5,6-dimethylpyrazine (**3**) with or without 1 mM $CuCl_2$. The amounts of ccc-DNA decreased gradually with time, and the reaction was remarkably accelerated by the addition of 1 mM Cu^{2+} . In the presence of Cu^{2+} , all the ccc-DNA was converted into linear-DNA via open-circular DNA, and the linear-DNA thus produced was further fragmented after a prolonged reaction (data not

Fig. 2. Time Course of pBR322 ccc-DNA Breakage by **3** with or without Cu^{2+}

Plasmid pBR322 ccc-DNA was incubated with 10 mM **3** at 37 °C in 50 mM Tris-HCl buffer (pH 7.2) with (●) or without (○) 1 mM $CuCl_2$. At intervals, the amounts of remaining ccc-DNA separated from the broken products by agarose gel electrophoresis were determined. ■, sample-free control DNA.

shown). Cupric chloride alone at the concentrations used here did not affect the DNA strand breakage. The activities of the other dihydropyrazine derivatives in causing the single-strand breakage of ccc-DNA, in the presence or absence of Cu^{2+} , are summarized in Table 1, where the relative amounts of ccc-DNA remaining after 1 or 3 h incubation are shown as an index of activity. A sample of dihydrofructosazine: 2,5-bis(D-*arabino*-tetrahydroxybutyl)-2,5-dihydropyrazine (**1**), which was unstable, consequently being changed to less reactive compounds during the storage, showed activity at almost the same level as that of the dehydrochlorinated product of D-glucosamine hydrochloride reported before.¹ 2,5-Dihydro-3,6-dimethylpyrazine (**2**),⁷ which has a dihydropyrazine skeleton similar to dihydrofructosazine (**1**), exhibited DNA strand-breaking activity higher than that of **1**. However, **1** was somewhat unstable and difficult to deal with, possibly because it has two double bonds apart from each other. Therefore, **3** with conjugated double bonds, which was shown to be the most stable among dihydropyrazine derivatives,⁸ was synthesized, and its activity on DNA was compared with the former. The DNA strand-breaking activity of **3** was slightly lower than that of **2**, but was comparable to that of **1**. These results supported the hypothesis that dihydrofructosazine would be a principle agent in the HCl-free D-glucosamine sample causing the DNA strand breakage, and they showed that the DNA strand-breaking activities of these compounds are due to the dihydropyrazine skeleton; also, for exhibiting their activities, the substituted groups on the dihydropyrazine skeleton are not necessarily sugar residues.

The DNA strand-breaking activities of all these dihydropyrazines were remarkably accelerated by the addition of Cu^{2+} . Since Cu^{2+} is known to accelerate the autoxidation of some reducing substances in their aqueous, oxygen-containing solutions to produce active oxygen and the other radicals,^{9,10} it was suggested that the autoxidation of dihydropyrazine was stimulated by Cu^{2+} to produce active radicals, which would consequently play the leading role in causing the DNA strand breakage.

Table 1. DNA Single-Strand Breakage by Dihydropyrazines in the Absence or Presence of Cu^{2+}

Compound ^{a)}	Concn. (mM)	ccc-DNA remaining (%)	
		No Cu^{2+} , 3 h	with Cu^{2+} , 1 h
Control	—	100	100
1	0.1	100	92
	1	92	64
	10	72	0
	10	89	8
2^{b)}	1	58	0
	10	82	47
	10	60	21
	10	63	0
3	0.001	100	100
	0.01	100	100
	0.1	100	95
	1	82	47
4	10	60	21
	0.001	100	100
	0.01	100	99
	0.1	100	92
5	1	91	44
	10	71	0
	0.001	99	100
	0.01	99	98
6	0.1	97	64
	1	91	0
	10	71	0
	0.001	100	98
7	0.01	100	95
	0.1	100	84
	1	93	23
	10	65	0
8	0.001	100	100
	0.01	99	92
	0.1	99	45
	1	69	0
8	10	24	0
	0.1	96	80
	1	80	0
8	10	53	0

a) The numbers of compounds tested. **1**: dihydrofructosazine; this is in impure form containing some non-active compounds.¹⁾ **2**: 2,5-dihydro-3,6-dimethylpyrazine.⁶⁾ **3**: 2,3-dihydro-5,6-dimethylpyrazine, **4**: 2,3-dihydro-2,5,6-trimethylpyrazine, **5**: 2,3-dihydro-2,2,5,6-tetramethylpyrazine, **6**: *trans*-2,3-dimethyl-5,6,7,8,9,10-hexahydroquinoxaline, **7**: *cis*-2,3-dimethyl-5,6,7,8,9,10-hexahydroquinoxaline, **8**: mixture of *trans* and *cis*-2,3-dimethyl-5,6,7,8,9,10-hexahydroquinoxaline.
b) Data were taken from the reference 1.

Since dihydropyrazines are generally inclined to be oxidized to produce less active compounds by releasing protons (H^+), a methyl group and others which have been shown to donate electrons were introduced into the dihydropyrazine skeleton to enrich the electron density by preventing the release of H^+ , and their DNA strand-breaking activities were tested. All the compounds synthesized according to the conventional methods, 2,3-dihydro-2,5,6-trimethylpyrazine (**4**), 2,3-dihydro-2,2,5,6-tetramethylpyrazine (**5**), *trans*-2,3-dimethyl-5,6,7,8,9,10-hexahydroquinoxaline (**6**), its *cis*-compound (**7**), and the mixture of its *cis*- and *trans*-compound (**8**) exhibited higher DNA strand-breaking activities than **3**, and their activities were accelerated by the addition of 1 mM Cu^{2+} . The order of activity of these compounds was $7 > 8 \geq 5 > 2 > 6 > 4 \geq 1 \geq 3$. The result that the mixture of *cis*- and *trans*-compounds (**8**) was situated in a position between *cis*- (**7**) and *trans*- (**6**), indicated that the activities are influenced by the steric structure of dimethylhexahydroquinoxaline. Although **7** was the most active compound among the

Fig. 3. Change in Electron Density in Dihydropyrazine Ring by Cu^{2+} Fig. 4. Effect of Bathocuproine on the DNA Breakage by the Activity of **3**

Plasmid pBR322 ccc-DNA was incubated with 10 mM **3** at 37°C in 50 mM Tris-HCl buffer (pH 7.2) containing 1 mM CuCl_2 in the presence of the indicated concentrations of bathocuproine disulfonate. After 1 h, the amounts of remaining ccc-DNA (●) were determined. ■, control with neither bathocuproine nor **3**.

dihydropyrazine derivatives tested, it was unstable and colored pink during storage, even at -30°C in a freezer, while **6** was seemingly stable. Thus, the higher activity was suggested to be dependent on the extent of their lability, as in the case of **2** and **3**. That the order of activity was $8 > 5 > 4 > 3$ implies that the greater electron density supplied by alkyl groups to the dihydropyrazine skeleton, the more their activities will be enhanced.

Effect of the Cu^+ -Chelator on the DNA Strand-Breaking Activity of Dihydropyrazine We assumed a mechanism regarding the stimulating effect of Cu^{2+} on DNA strand-breakage by dihydropyrazine, as shown in Fig. 3, where a dihydropyrazine structure (A) with increased electron densities by the introduction of electron-donating group such as methyl one would be oxidized by Cu^{2+} to give a cation radical (B) together with Cu^+ , and the cation radical thus produced or/and Cu^+ -OOH complex¹¹⁾ formed in Cu^+ -containing aqueous solution would directly attack the DNA. To confirm this assumption, the effect of bathocuproine, a specific Cu^+ -chelator unreactive to Cu^{2+} , on the DNA strand breakage by **3**, was examined. As Fig. 4 shows, the DNA strand breakage by **3** was inhibited by bathocuproine in a concentration-dependent manner. The concentration of bathocuproine required to inhibit the reaction by half was about 0.3 mM. Next, to further confirm the generation of Cu^+ , the optical density (OD) at 480 nm originating from the Cu^+ -bathocuproine complex in the mixture of Cu^{2+} and **3** was measured. As Fig. 5 shows, the value of OD increased with increasing concentrations of **3**, clearly indicating the generation of Cu^+ from the mixture of Cu^{2+} and **3**.

Effects of Enzymes and Radical Scavengers on the

Fig. 5. Effect of the Concentration of 3 on the Formation of a Cu(I)-Bathocuproine Complex

One mM CuCl_2 was incubated with 100 μM bathocuproine disulfonate at 37 °C in 50 mM Tris-HCl buffer (pH 7.2) for 1 h in the presence of various concentrations of 3, and the OD at 480 nm, showing the presence of Cu(I)-bathocuproine complex, was measured.

Dihydropyrazine-Induced DNA Breakage To see the possible involvement of radicals in the DNA strand breakage by dihydropyrazine, the effects of active oxygen-related enzymes and some radical scavengers on the DNA strand breakage by 3 and *cis*-2,3-dimethyl-5,6,7,8,9,10-hexahydroquinoxaline (7) in the presence of 1 mM CuCl_2 were examined. As Table 2 shows, in both the cases, the reaction was strongly inhibited by Tiron, a superoxide radical (O_2^-)-scavenger, and only slightly by superoxide dismutase (SOD), which causes disproportionation of O_2^- to yield H_2O_2 . The inhibition by Tiron in this case, however, may have been due to a possible chelating action of Tiron on metal ions which promote the generation of a hydroxyl radical ($\cdot\text{OH}$) in the reaction mixture.¹²⁾ Catalase inhibited the reaction more strongly than SOD, suggesting that H_2O_2 , an intermediate in the reduction pathway from O_2^- to $\cdot\text{OH}$, may be concerned in some way in the reaction. Scavengers of $\cdot\text{OH}$, such as thiourea and KI, most effectively inhibited the reaction. The other $\cdot\text{OH}$ -scavengers, dimethyl sulfoxide (DMSO), D-mannitol and HCOONa , and a singlet oxygen ($^1\text{O}_2$)-quencher, 1,4-diazabicyclo[2.2.2]octane (DABCO), however, did not affect the reaction so much. These results led us to suggest that O_2^- , which was generated at the first stage of the autoxidation of dihydropyrazine in the O_2 -containing aqueous solution, would be changed into $\cdot\text{OH}$ via H_2O_2 , and that the $\cdot\text{OH}$ or/and $\text{Cu}^+-\text{OOH}^{11)}$ formed in the presence of Cu^{2+} may be finally responsible for the DNA strand breakage. In addition to undergoing disproportionation, O_2^- is known to react with H_2O_2 to give $\cdot\text{OH}$.¹³⁾

DNA strand breakage by dihydropyrazine was also inhibited effectively by 2-mercapto-ethylamine hydrochloride (MEA) and *S*-(2-aminoethyl)isothiuronium bromide hydrobromide (AET), which are well known to scavenge not only active oxygen radicals, but also general radical structures ($\cdot\text{R}$). Therefore, the cation radical (B), possibly generated from dihydropyrazine (A) in the presence of Cu^{2+} (Fig. 3), was suggested to be a possible principal species in the DNA strand-breaking activity.

Table 2. Effect of Enzymes and Radical Scavengers on the DNA Strand-Breaking Activity of Dihydropyrazine Derivatives, 3 and 7

Sample	Enzyme or scavenger concn.	Rel. Amnts. of remaining ccc-DNA (%)	Inhibition (%)
3 None (C_0)	—	100.0	—
3 Control (C)	—	20.7	—
SOD ^{a)} (O_2^-)	100 $\mu\text{g}/\text{ml}$	17.4	-4.0
Catalase (H_2O_2)	100 $\mu\text{g}/\text{ml}$	66.1	57.3
Tiron ^{b)} (O_2^-)	100 mM	101.1	101.3
KI ($\cdot\text{OH}$)	100 mM	96.4	95.7
Thiourea ($\cdot\text{OH}$)	100 mM	107.6	108.8
DMSO ^{c)} ($\cdot\text{OH}$)	100 mM	19.2	-2.0
D-mannitol ($\cdot\text{OH}$)	100 mM	16.5	-5.7
HCOONa ($\cdot\text{OH}$)	100 mM	20.6	-0.1
DABCO ^{d)} ($^1\text{O}_2$)	100 mM	33.0	15.9
MEA ^{e)} ($\cdot\text{R}$)	100 mM	91.3	88.2
AET ^{f)} ($\cdot\text{R}$)	100 mM	81.5	76.7
7 Control (C)	—	26.3	—
SOD ^{a)} (O_2^-)	100 $\mu\text{g}/\text{ml}$	21.5	-8.1
Catalase (H_2O_2)	100 $\mu\text{g}/\text{ml}$	77.1	70.0
Tiron ^{b)} (O_2^-)	100 mM	67.9	58.2
KI ($\cdot\text{OH}$)	100 mM	93.7	94.3
Thiourea ($\cdot\text{OH}$)	100 mM	98.6	99.7
DMSO ^{c)} ($\cdot\text{OH}$)	100 mM	25.5	-1.2
D-mannitol ($\cdot\text{OH}$)	100 mM	21.4	-7.0
HCOONa ($\cdot\text{OH}$)	100 mM	23.3	-4.3
DABCO ^{d)} ($^1\text{O}_2$)	100 mM	33.3	6.7
MEA ^{e)} ($\cdot\text{R}$)	100 mM	62.6	50.3
AET ^{f)} ($\cdot\text{R}$)	100 mM	64.6	52.9

Plasmid pBR322 ccc-DNA was incubated either with 10 mM 3 or with 1 mM 7 at 37 °C in 50 mM Tris-HCl buffer (pH 7.2) containing 1 mM CuCl_2 in the presence of either enzymes or radical scavengers for 1 h. Percent inhibition of the DNA strand breakage was calculated as follows: inhibition (%) = $[(I - C)/(C_0 - C)] \times 100$, where I is the amount of remaining ccc-DNA of the system with the inhibitor, C that of a control without an inhibitor, and C_0 that of another control without either inhibitor or 3. Corresponding active oxygens and radicals are shown in parentheses. a) Superoxide dismutase. b) 4,5-Dihydroxy-1,3-benzenedisulfonic acid. c) Dimethylsulfoxide. d) 1,4-Diazabicyclo-[2.2.2]octane. e) 2-Mercapto-ethylamine hydrochloride. f) *S*-(2-Aminoethyl)isothiuronium bromide hydrobromide.

Actually, we have now succeeded in the detection of a certain carbon-centered radical, together with a hydroxyl radical, by an ESR measurement of dihydropyrazine derivatives in an aqueous solution (data not shown). Thornalley *et al.*^{14,15)} have shown the formation of monosaccharide radicals during the autoxidation of monosaccharide using an ESR technique. The fact shown in our previous paper,¹⁶⁾ that D-glucosamine and its 6-phosphate in the presence of Cu^{2+} cut the nucleotide sequence at specific (pyrimidine-purine ($5' \rightarrow 3'$)) sites, but not uniformly at all nucleotide sites, indirectly supports this assumption that a cation radical other than active oxygen may cause the DNA strand breakage. Another possibility, that methyl radicals having DNA strand-breaking activity may be generated from dihydromethylpyrazines, was predicted, since the activities of dihydropyrazine were accelerated by introducing methyl groups into the dihydropyrazine ring. However, the alkylating agent-detection reaction,¹⁷⁾ using γ -(4-nitrobenzyl)-pyridine, was negative, indicating the absence of methyl radicals in the aqueous solution of methyl derivatives of dihydropyrazine.

The elucidation of a chemical structure corresponding to the carbon-centered radical (B) proposed in Fig. 3 is

now being carried out.

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