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Phenyl-Substituted Dihydropyrazines with DNA Strand-Breakage Activity

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Monophenyl-substituted dihydropyrazines (Ph-DHP-1 to 4) of 2,3-dihydro-5,6-dimethylpyrazine (Me-DHP-1), which have the inductive effects of apoptosis and mutagenesis, were synthesized and their biological effect was investigated in terms of DNA strand-breakage. Differences between the phenyl- and methyl-substituted dihydropyrazines were examined.

Key words dihydropyrazine; radical generation; DNA breakage; biological effect; superoxide anion

In previous papers,[1,2] we have showed the relationship between the chemical structures of dihydropyrazine (DHP) derivatives and their DNA strand-breakage activity. For example, we have found that the DNA strand-breakage activity is remarkably enhanced upon addition of Cu^{2+}. Additionally, we observed an increase in the strand-breakage activity when electron-donating methyl groups were added to the DHP ring, and found that the ionization potential (IP) value was an effective measure for the estimated activity. The use of the IP value in this way offers the ability to actively design compounds with the potential for DNA strand-breakage activity. The phenyl-substituted dihydropyrazine, 2,3-dihydro-5-methyl-6-phenylpyrazine (**Ph-DHP-1**), should have a much higher DNA strand-breakage activity and produce a stronger intensity of radical species than the methyl-substituted dihydropyrazine, 2,3-dihydro-5,6-dimethylpyrazine (**Me-DHP-1**). Therefore, we aimed to synthesize phenyl-substituted dihydropyrazines (Ph-DHPs). It has already been shown[3] that Ph-DHPs cause cytotoxic and genotoxic damage that is different to that observed for Me-DHPs. In this report, the syntheses of four Ph-DHP derivatives and their DNA strand-breakage activities are compared.

Experimental

The dihydropyrazine derivatives (Chart 1) employed were synthesized by the condensation of diketones and diamines. 2,3-Dihydro-5,6-dimethylpyrazine (**Me-DHP-1**) was synthesized by the method of Yamaguchi *et al.*[1] Similar methods were also used to generate the phenyl derivative, 2,3-dihydro-5-methyl-6-phenylpyrazine. The starting materials, 1-phenylpropane-1,2-dione as the ketone and ethylenediamine, propane-1,2-diamine, 2-methylpropane-1,2-diamine and *trans*-cyclohexane-1,2-diamine as diamines, were purchased from Wako Pure Chemical Ind., Ltd.

Assay of DNA Strand-Breakage Activity The method used to assess the DNA strand-breakage activity of DHPs utilizes a covalently closed circular duplex DNA (ccc-DNA) of plasmid pBR322 and has been described previously.[4]

Semi-empirical MO Calculations The ionization potential (IP) based on Koopman's theorem, determined from PM3 molecular orbital (MO) calculations, was used as a measure of the DNA strand-breakage activity.[1] The calculations were performed using MacSpartan Pro software.

ESR Spectroscopy of Dihydropyrazines The ESR spectra were recorded on a JES-FA200 spectrometer (JEOL Co., Tokyo, Japan) using a Mn^{2+} marker as an external standard, and an ES-LC12 flat cell (JEOL Co.). The spectra were measured in a 50 mM Tris–HCl buffer (pH 7.1) using 5,5-dimethy-1-pyrroline *N*-oxide (DMPO) as a spin trapping agent, according to a previous paper.[4,5] The instrumental conditions used were: field center 335.9 mT, scan width ±5 mT, modulation frequency 100 kHz, modulation width 0.14 mT, time constant 0.3 s, amplitude 7×100, microwave power 10 mW, sweep time 2.0 min, and microwave frequency 9.427 GHz. The spectra were recorded 30 min after mixing.

Synthesis of Dihydropyrazine Derivatives 2,3-Dihydro-5-methyl-6-phenylpyrazine (**Ph-DHP-1**): To a solution of ethylenediamine (2.0 mmol) in ether (5.0 ml) mechanically stirred on an ice bath, a solution of 1-phenylpropane-1,2-dione (2.0 mmol) in ether (5.0 ml) was slowly added dropwise. The reaction mixture was stirred at room temperature until it became clear. The mixture was then refluxed for 30 min. Potassium hydroxide was added to the mixture to remove water and after filtration the filtrate was evaporated under reduced pressure in nitrogen to give a solid product, which was purified by recrystallization from *n*-hexane. (Yellowish crystals. Yield 71.0%. mp 36—37 °C. IR (Nujol) cm^{-1}: 1480 (C=N). ^{1}H-NMR (500 MHz; CDCl$_3$) δ: 2.10 (3H, t, J=1.46 Hz, CH$_3$), 3.45—3.50 (2H, m, CH$_2$), 3.54—3.60 (2H, m, CH$_2$), 7.40—7.60 (5H, m, aromatic-H). ^{13}C-NMR (125 MHz; CDCl$_3$) δ: 24.5 (CH$_3$), 45.0 (CH$_2$), 45.5 (CH$_2$), 127.5, 128.4, 129.6, 137.7 (aromatic-C), 158.9 (C=N), 162.0 (C=N). FAB-MS (*m/z*): 173 (M$^+$+1). HR-MS Calcd for $C_{11}H_{13}N_2$ (M$^+$+H): 173.1079. Found: 173.1062.)

A mixture of (2R*,2S*)-2,3-dihydro-2,5-dimethyl-6-phenylpyrazine (**Ph-DHP-2′**) and (3R*,3S*)-2,3-dihydro-3,5-dimethyl-6-phenylpyrazine (**Ph-DHP-2**) was prepared in a similar manner from 1-phenylpropane-1,2-dione and propane-1,2-diamine. (Yellowish oil. Yield 73.0%. IR (Nujol) cm^{-1}: (C=N). ^{1}H-NMR (500 MHz; CDCl$_3$) δ: 1.34 (6H, CH$_3$), 2.07 (6H, CH$_3$), 2.94—3.00 (2H, CH), 3.29—3.36 (2H, CH), 3.71—3.75 (1H, m, CH), 3.84—3.88 (1H, m, CH), 7.39—7.45 (10H, m, aromatic-H). ^{13}C-NMR (125 MHz; CDCl$_3$) δ: 19.05 (CH$_3$), 19.18 (CH$_3$), 24.44 (CH$_3$), 24.67 (CH$_3$), 49.79 (C$_2$-CH), 50.58 (CH), 51.46 (CH$_2$), 52.04 (CH$_2$), 127.55, 127.63, 128.52, 129.70, 137.60, 137.81 (aromatic-C), 158.07 (C=N), 158.91 (C=N), 161.10 (C=N), 162.03 (C=N). FAB-MS (*m/z*): 187.1 (M$^+$+1). HR-MS Calcd for $C_{12}H_{15}N_2$ (M$^+$+H): 187.1235. Found: 187.1220.)

A mixture of 2,3-dihydro-2,2,5-trimethyl-6-phenylpyrazine (**Ph-DHP-3′**) and 2,3-dihydro-3,3,5-trimethyl-6-phenylpyrazine (**Ph-DHP-3**) was prepared in a similar manner from 1-phenylpropane-1,2-dione and 2-methylpropane-1,2-diamine. (Yellowish oil. Yield 70.4%. IR (Nujol) cm^{-1}: 1480 (C=N). ^{1}H-NMR (500 MHz; CDCl$_3$) δ: 1.19 (6H, s, C2-CH$_3$), 2.04 (3H, s, C3-CH$_3$), 3.47 (0.24H, d, J=6.3 Hz, C3-methylene), 3.55 (2H, s, C2-methylene), 7.37—7.44 (5H, m, aromatic-H). ^{13}C-NMR (125 MHz; CDCl$_3$) δ: 24.8 (C3-CH$_3$), 25.6, 25.8 (C2-CH$_3$), 51.3 (C2), 56.8 (C1), 127.4, 127.6, 128.5, 129.4, 129.6, 137.9 (aromatic-C), 156.2 (C=N), 161.8 (C=N). FAB-MS (*m/z*): 201.1 (M$^+$+1). HR-MS Calcd for $C_{13}H_{17}N_2$ (M$^+$+H): 201.1392. Found: 201.1368. Signal assignments were confirmed by distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum correlation (HMQC), correlation spectroscopy (COSY) spectra.)

trans-2-Methyl-3-phenyl-,5,6,7,8,9,10-hexahydroquinoxaline (**Ph-DHP-4**) was prepared in a similar manner from 1-phenylpropane-1,2-dione and *trans*-cyclohexane-1,2-diamine. (Yellowish oil. Yield 84.1%. IR (Nujol) cm^{-1}: 1445 (C=N). ^{1}H-NMR (500 MHz; CDCl$_3$) δ: 1.39 (2H, m, C7H, C8H), 1.50 (2H, m, C7H, C8H), 1.86 (2H, s, C6H$_2$), 2.10 (3H, s, C2-CH$_3$), 2.39 (2H, s, C5H$_2$), 2.68 (2H, s, C4aH, C8aH), 7.39—7.45 (5H, m, aromatic-H). ^{13}C-NMR (125 MHz; CDCl$_3$) δ: 24.39 (C2-CH$_3$), 25.44, 25.61 (C6H$_2$, C7H$_2$), 33.42, 33.57 (C5H$_2$, C8H$_2$), 58.75, 59.57 (C4aH, C8aH),

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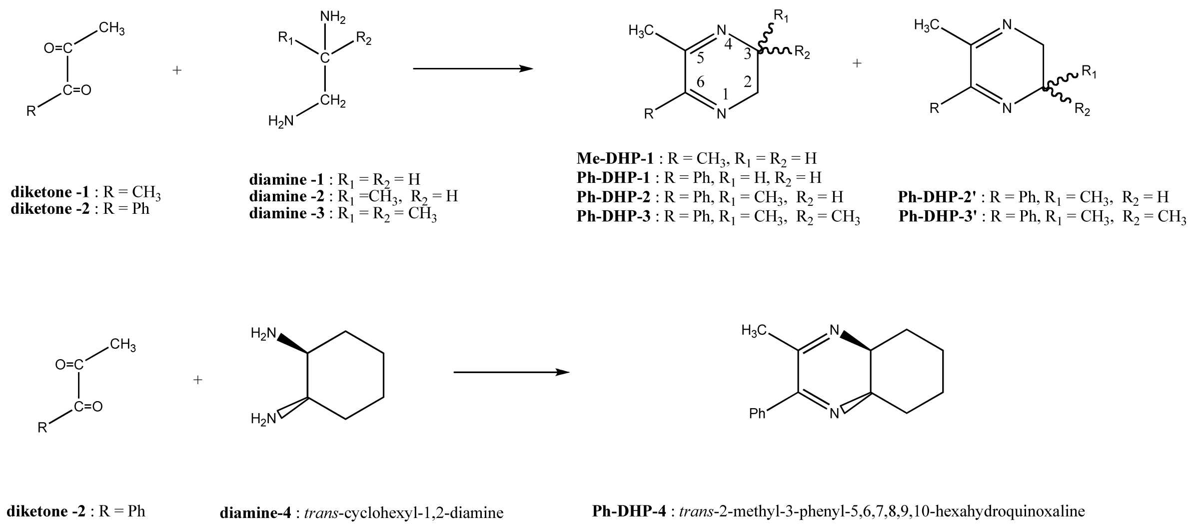


diketone -1 : R = CH_3
diketone -2 : R = Ph

diamine -1 : $R_1 = R_2 = H$
diamine -2 : $R_1 = CH_3$, $R_2 = H$
diamine -3 : $R_1 = R_2 = CH_3$

Me-DHP-1 : R = CH_3, $R_1 = R_2 = H$
Ph-DHP-1 : R = Ph, $R_1 = H$, $R_2 = H$
Ph-DHP-2 : R = Ph, $R_1 = CH_3$, $R_2 = H$
Ph-DHP-3 : R = Ph, $R_1 = CH_3$, $R_2 = CH_3$

Ph-DHP-2' : R = Ph, $R_1 = CH_3$, $R_2 = H$
Ph-DHP-3' : R = Ph, $R_1 = CH_3$, $R_2 = CH_3$

diketone -2 : R = Ph
diamine-4 : *trans*-cyclohexyl-1,2-diamine
Ph-DHP-4 : *trans*-2-methyl-3-phenyl-5,6,7,8,9,10-hexahydroquinoxaline

Chart 1. The Synthesis of Phenyl-Substituted Dihydropyrazines

127.67, 128.56, 129.61, 137.65 (aromatic-C), 158.48, 161.60 (C=N). FAB-MS (*m*/*z*): 227.1 ($M^+ + 1$). HR-MS Calcd for $C_{15}H_{19}N_2$ ($M^+ + H$): 227.1548. Found: 227.1526.)

Results and Discussion

The dihydropyrazine derivatives (Fig. 1) employed were synthesized by the condensation of a diketone as butane-2,3-dione or 1-phenylpropane-1,2-dione, and a diamine as an ethylenediamine derivative or cyclohexyldiamine. Diketone-1 (butane-2,3-dione) reacted with diamine-1 (ethylenediamine) to give **Me-DHP-1** (2,3-dihydro-5,6-dimethylpyrazine). In this case, no isomers were expected. However, when the asymmetric species **diketone-2** and **diamine-2** or **diamine-3** were used as starting materials, several isomers were produced. In the reaction of **diketone-2** with **diamine-2**, four isomers of **Ph-DHP-2** [mixture of (*R*) and (*S*)] and **Ph-DHP-2′** [mixture of (*R*) and (*S*)] were expected due to the asymmetric carbon atom. In the reaction of **diketone-2** with **diamine-3**, the formation of two isomers of **Ph-DHP-3** and **Ph-DHP-3′** was predicted. The product ratios of these isomers were estimated using the peak intensities from NMR spectra of the products. For example, the product ratio of **Ph-DHP-2** and **Ph-DHP-2′** is 1 : 1 based on the signal intensities at δ: 3.71—3.75 (1H, m, CH_2-H) and δ: 3.84—3.88 (1H, m, CH_2-H), which can be unambiguously assigned to one proton of the methylene signal of **Ph-DHP-2** and **Ph-DHP-2′**, respectively. Similarly, the product ratio of **Ph-DHP-3** and **Ph-DHP-3′** is 8 : 1 based on the signal intensities at δ: 3.55 (2H, s, C2-methylene) and δ: 3.47 (0.24H, d, $J=6.3$ Hz, C3-methylene); long-range coupling is observed between the C5-methyl and the C3-methylene for **Ph-DHP-3′**.

As shown in a previous paper,[1] the PM3 method is the most suitable for estimating the ionization potential (IP) when using this value as a measure of the relative activity for DNA scission. The IP values obtained on the basis of the PM3 calculation are summarized in Table 1. The order of DNA strand-breakage activity of the Me-DHPs observed experimentally is R-DHP-3>R-DHP-4>R-DHP-2>R-DHP-1, which agrees closely with the order expected based on the IP values.[1]

Ph-DHP-2 and **Ph-DHP-2′** as well as **Ph-DHP-3** and **Ph-DHP-3′** were obtained as product mixtures. The IP values of the mixture of **Ph-DHP-2** and **Ph-DHP-2′** (**Ph-DHP2**, **-2′**), and the mixture of **Ph-DHP-3** and **Ph-DHP-3′** (**Ph-DHP-3**, **-3′**) can be roughly estimated to be ≤ 9.62 and ≤ 9.59, respectively, based on the product ratio of the isomers. Thus, the order of IP values for the Ph-DHPs follows: R-DHP-3 (**Ph-DHP-3**, **-3′**)>R-DHP-4>R-DHP-2 (**Ph-DHP-2**, **-2′**) >R-DHP-1, which is similar to that observed for the Me-DHPs. However, the order suggested by the IP values was later found to disagree with the actual order of DNA strand-breakage activity as described below, the reason for which is not known at this stage.

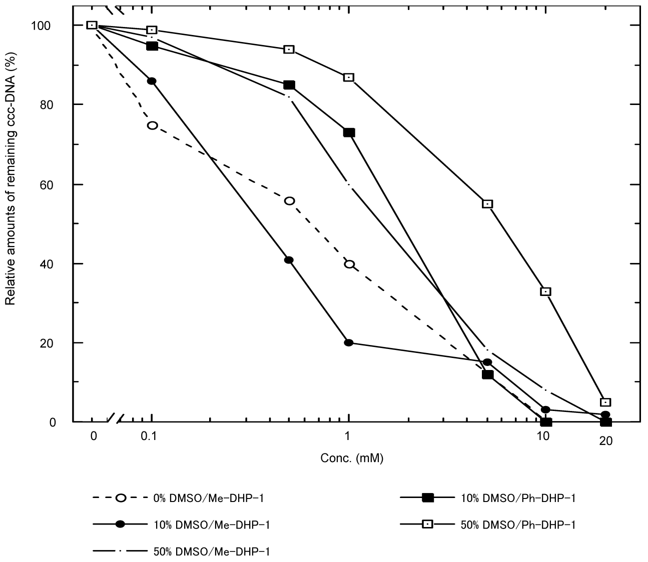
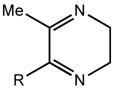
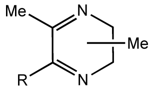
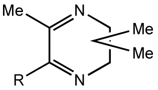
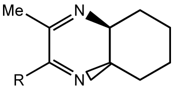


Fig. 1. Influence of Added DMSO on Assay of DNA Strand-Breakage Activity

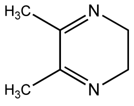
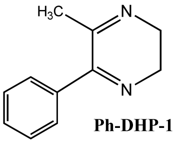
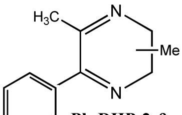
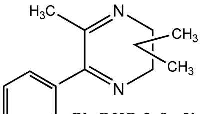
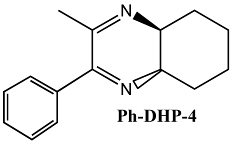
The solubility of the Ph-DHPs in water decreases from **Ph-DHP-4** to **Ph-DHP-1**, with **Ph-DHP-1** being barely soluble. As such, dimethyl sulfoxide (DMSO) may be added to increase solubility and so its influence must be considered in any bioassay. For the assay of DNA strand-breakage activity as shown in Fig. 1, the activity of **Me-DHP-1** employed as a

Table 1. Ionization Potential (IP) Values Determined on the Basis of the PM3 Calculation

	R-DHP-1	R-DHP-2		R-DHP-3		R-DHP-4
						
R=Ph	9.6233	9.602	9.6237	9.580	9.599	9.596
		(1 : 1)[a]		(8 : 1)[a]		
		DHP-2	**DHP-2′**	**DHP-3**	**DHP-3′**	
R=Me[b]	9.802	9.776		9.721		9.770

a) Product ratio of isomers. *b*) Obtained from a previous paper.[1)]

Table 2. DNA Strand-Breakage Activity of Ph-DHPs in the Absence or Presence of Cu^{2+}

Test compound	Conc. (mM)	Remaining ccc-DNA (%) Without Cu^{2+} for 3 h	Remaining ccc-DNA (%) With Cu^{2+} for 1 h
 **Me-DHP-1**	0.1	100	95
	1.0	99	47
	10.0	89	21
 **Ph-DHP-1**	0.1	100	95
	1.0	99	67
	10.0	89	5
 **Ph-DHP-2 & -2'**	0.1	98	90
	1.0	100	53
	10.0	94	1
 **Ph-DHP-3 & -3'**	0.1	100	91
	1.0	100	59
	10.0	100	15
 **Ph-DHP-4**	0.1	100	94
	1.0	100	78
	10.0	100	32

Plasmid pBR322 ccc-DNA was incubated with various concentrations of DHP in 50 mM Tris–HCl bufer (pH 7.1) containing 10% DMSO at 37 °C for 1 h with 1 mM $CuCl_2$ or for 3 h without $CuCl_2$. A small amount of remaining ccc-DNA indicates strong breakage activity.

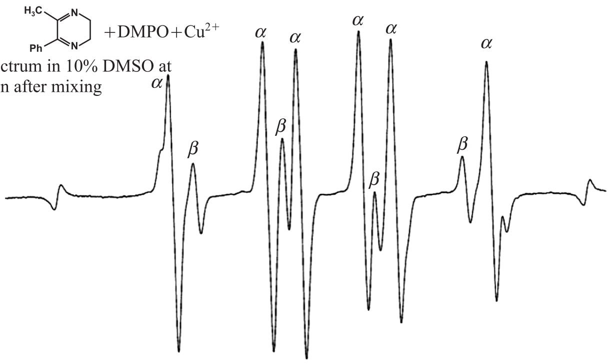


Fig. 2. ESR Spectrum Obtained from the Reaction of Ph-DHP-1 (1.5 mM) with $CuCl_2$ (0.01 mM) in the Presence of DMPO (100 mM) at pH 7.1 (50 mM Tris–HCl Buffer Containing 10% DMSO)

control is hardly affected by the amount of DMSO added (0—10%). However, for both **Me-DHP-1** and **Ph-DHP-1**, the activity was remarkably affected by the amount added (*ca.* 50%), with the phenyl-substituted DHP the more strongly influenced. This result suggests that DMSO exerts an influence as a scavenger of ·OH, indicating the activity of **Ph-DHP-1** is mainly due to the generation of hydroxyl radicals.[2)] Therefore, it is essential to reduce the amount of DMSO added, while maintaining the **Ph-DHP** solubility, in order to limit its influence on the assay. Thus, the strand-breakage activities of **Ph-DHPs** were observed in 50 mM Tris–HCl buffer (pH 7.1) containing 10% DMSO, as summarized in Table 2.

The order of the activity in the presence of Cu^{2+} is **Ph-DHP-2**, **2′**>**Ph-DHP-1**>**Ph-DHP-3**, **3′**>**Me-DHP-1**>**Ph-DHP-4**. Although this order disagrees with that expected based on the IP values, it is consistent with the activity based on the radical signal peak heights for radical species in ESR spectra, which were measured under reaction conditions of DNA strand breakage. As shown in Fig. 2, all the compounds (**Me-DHP-1** and **Ph-DHPs**) revealed approximately similar signal patterns for the DMPO-adducts,[4)] although with varying levels of intensity.

The spectrum was assigned to DMPO adducts of hydroxyl (β: quartet of lines) and carbon-centered (α: doublet of sixtet lines) radicals. The order of signal intensity for the carbon-centered radical and the hydroxyl radical do not necessarily parallel the order of DNA strand-breakage activity in some cases (not described here). Full details of the ESR data for all DHPs containing Me-DHPs and other compounds will be published in another report, currently in preparation. Furthermore, as described in the our previous paper,[3)] the relative lethal effect of Ph-DHPs on five strains (wild type, *recA*, *uvrB*, *katE katG*, *sodA sodB*) of *Escherichia coli* does not correspond with their radical signal intensity. Overall, the lethal effect of the Ph-DHPs increased in the presence of Cu^{2+}. Interestingly, the Ph-DHPs, in contrast to the Me-DHPs, were toxic to the *sodA sodB* strain regardless of the presence of Cu^{2+}. We have previously predicted[3)] that Ph-DHPs would affect cells differently than Me-DHPs because of their greater solubility in fats, which should improve their ability to penetrate the cell membrane, as shown in Fig. 3. The toxicity in the absence of Cu^{2+} was **Ph-DHP-4**>**Ph-**

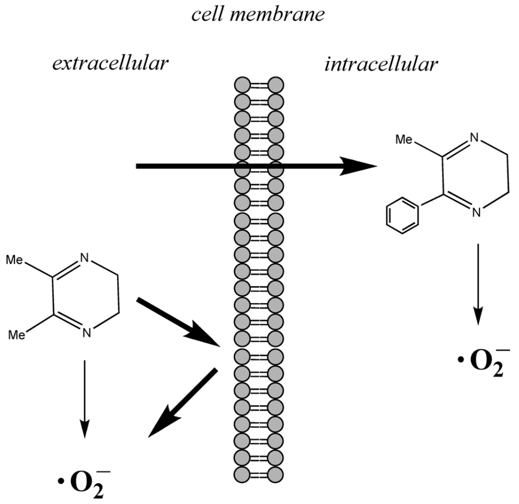


Fig. 3. The Action of DHPs in Bacterial Toxicity towards *E. coli*

DHP-1$\geq$**Ph-DHP-2**, **2′**>**Ph-DHP-3**, **3′**, whereas in the presence of Cu^{2+}, it was **Ph-DHP-3**, **3′**>**Ph-DHP-2**, **2′**>**Ph-DHP-1**>**Ph-DHP-4**. In both the presence or absence of Cu^{2+}, since the *sodA sodB* strain is sensitive to Ph-DHPs,[3] the superoxide anion might be a reason for the bacterial toxicity. As with the DNA-strand breakage results, the order of the ESR signal intensity for the hydroxyl and carbon-centered radicals did not appear to agree with the order of toxicity for the Ph-DHPs for *E. coli*. The existence of the superoxide anion was recognized by the water-soluble tetrazolium salt (WST)-1 assay.[3] As described in our previous paper,[2] **Ph-DHP-1** generated greater amounts of 8-OHdG than **Me-DHP-1** upon addition of Cu^{2+}. At the present time (the details will be published in another report), the order of forming 8-OHdG upon addition of Cu^{2+} is **Ph-DHP-3**, **3′**>Me-DHP-3 (2,3-dihydro-2,2,5,6-tetramethylpyrazine)>**Ph-DHP-2**, **2′**>Me-DHP-2 (2,3-dihydro-2,5,6-trimethylpyrazine)> **Ph-DHP-4**>**Ph-DHP-1**>**Me-DHP-1**. This order agrees with the order based on ESR signal intensity for the hydroxyl and carbon-centered radicals.

In summary, the biological effect of DHPs was found to depend on the amount of radical species generated from DHPs and the sensitivity of bacterial cells to these species. The chemical reactivity of DHPs with the component *in vivo* was considered to also be a factor in their bacterial toxicity, as shown with reactions of DHPs with ethylenediamine,[6—8] ketene[9] or thiourea.[10]

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