Tetraazaindenes Derived from Dihydropyrazines with DNA Strand-Breaking Activity

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Dihydropyrazines (DHPs), which exhibit DNA strand-breaking activity and other biological activities associated with the generation of radical species, reacted with thiourea to give tetraazaindene (TAI) derivatives, despite thiourea is a well known radical scavenger. The structural determination of the TAIs was carried out and formation mechanism of TAI was investigated.

Key words dihydropyrazine; tetraazaindene; thiourea; X-ray crystallography; molecular orbital calculation

We previously described the DNA strand-breaking activity[1—3] of dihydropyrazines (DHPs) *in vitro*, as well as other characteristics such as the generation of radical species[4,5] and high chemical reactivity.[6—8] We also investigated the biological effects of DHPs, such as induction of apoptosis[9] and mutagenesis.[10] Among the various phenomena, the effect of DHPs on the viability of *Escherichia coli* (*E. coli*) in the presence of Cu^{2+} was examined. It was predicted that the lethal effect of DHPs on *E. coli* would be accelerated in the presence of Cu^{2+}, probably due to radical species generated from the DHPs, as is the case of the DNA strand-cleaving reaction *in vitro*. Unexpectedly, however, the lethal effect was not hindered by the addition of thiourea, a well known radical scavenger, but was considerably enhanced by thiourea. This phenomenon was thought to be due to the generation of an unknown product from the interaction of DHP and thiourea. In an attempt to elucidate the interaction of DHPs and thiourea, tetraazaindene derivatives were newly identified as that enhanced the lethal effect of DHPs on *E. coli*. In this report, we present a new aspect of the chemical reactivity of DHPs toward diamino compounds.

Experimental

Instruments IR spectra were obtained using a JASCO FT/IR-4200 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained using a JEOL JNM-ECA500 spectrometer and *ca.* 10% solution (DMSO), with TMS as an internal standard. Chemical shifts are expressed as δ values. Signal assignments were confirmed by ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) spectra. Mass spectra were obtained using JEOL JMS-700 and JMS-BU20 instruments.

Synthesis of Tetraazaindene Derivatives The dihydropyrazine derivatives (**1a**, **1b**, **4**) were synthesized by the condensation of diketones and diamines and by the method of Yamaguchi *et al.*[1,11] Other chemicals were purchased from Wako Pure Chemical Ind. Ltd.

1) 3a*R**,7a*S**-Dimethylhexahydroimidazo[4,5-*b*]pyrazine-2-thione (**3a**). To a solution of thiourea (**2a**) (1.5 mmol) in water, which was mechanically stirred at room temperature, 2,3-dihydro-5,6-dimethylpyrazine (**1a**) (1 mmol) was added dropwise, and the reaction mixture was cooled to 2 °C for several hours. The material precipitated in the reaction mixture was collected by filtration, washed with EtOH, and dried under vacuum. Yield 80.2%, mp 164—166 °C (CH_3OH), colorless prisms. IR (KBr) cm^{-1}: 1534 (–N–C=S), 1144 (C=S). ^{1}H-NMR (500 MHz; DMSO) δ: 1.14 (6H, s, C3a–CH_3, C7a–CH_3), 2.20 (2H, br, N4H, N7H), 2.51—2.62 (4H, m, C5–CH_2, C6–CH_2), 7.98 (2H, br, N1H, N3H). ^{13}C-NMR (125 MHz; DMSO) δ: 22.9 (C3a–CH_3, C7a–CH_3), 38.6 (C5, C6), 75.7 (C3a, C7a), 180.9 (C=S). FAB-MS (*m*/*z*): 187.2 (M^++1). *Anal.* Calcd for $C_7H_{14}N_4S$: C, 45.13; H, 7.58; N, 30.08: Found: C, 45.00; H, 7.47; N, 30.34%.

2) 1,3a*R**,7a*S**-Trimethylhexahydroimidazo[4,5-*b*]pyrazine-2-thione (**3b**) which was prepared similarly from **1a** and methylthiourea (**2b**). Yield 70.5%, mp 173—175 °C (dec.) (CH_3OH), colorless prisms. IR(KBr) cm^{-1}: 1509 (–N–C=S), 1146 (C=S). ^{1}H-NMR (500 MHz; DMSO) δ: 1.04 (3H, s, C7a–CH_3), 1.19 (3H, s, C3a–CH_3), 2.05 (1H, br, N4H), 2.34—2.55 (4H, m, C5–CH_2, C6–CH_2), 2.77 (3H, s, N1–CH_3), 2.94 (1H, br, N7H), 8.06 (1H, br, N3H). ^{13}C-NMR (125 MHz; DMSO) δ: 20.6 (C7a–CH_3), 21.7 (C3a–CH_3), 27.7 (N1–CH_3), 38.8 (C5–CH_2, C6–CH_2), 72.9 (C3a), 79.0 (C7a), 180.5 (C=S). FAB-MS (*m*/*z*): 201.2 (M^++1). *Anal.* Calcd for $C_8H_{16}N_4S$: C, 47.97; H, 8.05; N, 27.97: Found: C, 47.85; H, 7.82; N, 27.97.

3) 3a*R**,5,5,7a*S**-Tetramethylhexahydroimidazo[4,5-*b*]pyrazine-2-thione (**3c**) which was prepared similarly from 2,3-dihydro-2,2,5,6-tetramethylpyrazine (**1b**) and **2a**. Yield 76.4%, mp 158—160 °C (CH_3OH), colorless prisms. ^{1}H-NMR (500 MHz; DMSO) δ: 0.94 (3H, s, C5–CH_3), 0.99 (3H, s, C5–CH_3), 1.12 (3H, s, C3a–CH_3), 1.17 (3H, s, C7a–CH_3), 1.87 (1H, br, N7H), 2.20 (1H, br, N4H), 2.31 (1H, d, *J*=13.2, C6H), 2.43 (1H, d, *J*=13.2, C6H), 7.87 (1H, br, N3H), 8.02 (1H, br, N1H). ^{13}C-NMR (125 MHz; DMSO) δ: 22.5 (C7a–CH_3), 25.8 (C3a–CH_3), 30.6 (C5–CH_3), 48.6 (C5), 49.2 (C6–CH_2), 75.6 (C3a), 76.2 (C7a), 179.2 (C=S). EI-MS (*m*/*z*): 214.0 (M^+). *Anal.* Calcd for $C_9H_{18}N_4S$: C, 50.43; H, 8.46; N, 26.14: Found C, 50.35; H, 8.59; N, 26.05.

4) 1,3a*R**,6,6,7a*S**-Pentamethylhexahydroimidazo[4,5-*b*]pyrazine-2-thione (**3d**) which was prepared similarly from **1b** and **2b**. Yield 49.8%, mp 146—148 °C (CH_3OH), colorless prisms. IR (KBr) cm^{-1}: 1516 (–N–C=S), 1131 (C=S). ^{1}H-NMR (500 MHz; DMSO) δ: 0.85 (3H, s, C6–CH_3), 0.96 (3H, s, C6–CH_3), 1.02 (3H, s, C7a–CH_3), 1.21 (3H, s, C3a–CH_3), 2.17 (1H, br, N4H), 2.31 (2H, m, C5–CH_2), 2.56 (1H, br, N7H), 2.72 (3H, s, N1–CH_3), 8.00 (1H, br, N3H). ^{13}C-NMR (125 MHz; DMSO) δ: 22.4 (C6–CH_3), 22.5 (C6–CH_3), 27.8 (N1–CH_3), 28.1 (C7a–CH_3), 30.7 (C3a–CH_3), 48.1 (C6), 49.7 (C5–CH_2), 73.5 (C3a), 78.4 (C7a), 179.4 (C=S). EI-MS (*m*/*z*): 228.0 (M^+). *Anal.* Calcd for $C_{10}H_{20}N_4S\cdot0.6H_2O$: C, 50.22; H, 8.93; N, 23.43: Found C, 50.29; H, 8.85; N, 23.20. The structure was confirmed by X-ray analysis.

5) 3a,7a-Tricyclo[3.4.4]butanohexahydro-1*H*-imidazo[4,5-*b*]pyrazine-2(3*H*)-thione (**5a**) which was prepared similarly from the mixture (**4**) of 1,2,3,5,6,7-hexahydroquinoxaline and 2,3,5,6,7,8-hexahydroquinoxaline, and **2a**. Yield 68.0%, mp 163—165 °C (dec.) (CH_3OH), colorless prisms. IR (KBr) cm^{-1}: 1523 (–N–C=S), 1125 (C=S). ^{1}H-NMR (500 MHz; DMSO) δ: 1.29 (2H, C3a–CH_2–$\underline{CH_2}$), 1.38 (2H, C3a–$\underline{CH_2}$–), 1.40 (2H, C7a–CH_2–$\underline{CH_2}$), 1.61 (2H, C3a–$\underline{CH_2}$–), 2.05 (2H, br, N4H, N7H), 2.50 (2H, C5–CH_2), 2.65 (2H, C6–CH_2), 8.01 (2H, N1H, N3H). ^{13}C-NMR (125 MHz; DMSO) δ: 20.8 (C3a–CH_2–$\underline{CH_2}$, C7a–CH_2–$\underline{CH_2}$), 32.8 (C3a–$\underline{CH_2}$–, C7a–$\underline{CH_2}$–), 40.1 (C5–CH_2, C6–CH_2), 74.9 (C3a, C7a), 182.7 (C=S). FAB-MS (*m*/*z*): 213.2 (M^++1). *Anal.* Calcd for $C_9H_{16}N_4S\cdot0.5H_2O$: C, 48.84; H, 7.74; N, 25.31: Found: C, 48.56; H, 7.60; N, 25.46%.

6) 3a,7a-Tricyclo[3.4.4]-1-methylbutanohexahydro-1*H*-imidazo[4,5-*b*]pyrazine-2(3*H*)-thione (**5b**) which was prepared similarly from **4** and **2b**. Yield 52.5%, mp 161—163 °C (dec.) (CH_3OH), colorless prisms. IR (KBr) cm^{-1}: 1495 (–N–C=S), 1130 (C=S). ^{1}H-NMR (500 MHz; DMSO) δ: 1.12 (1H, N7H), 1.13 (1H, C7a–$\underline{CH}$), 1.25 (1H, N4H), 1.26 (2H, C3a–CH_2–$\underline{CH_2}$), 1.47 (1H, C3a–$\underline{CH}$), 1.49 (2H, C7a–CH_2–$\underline{CH_2}$), 1.59 (1H, C7a–$\underline{CH}$), 1.68 (1H, C3a–$\underline{CH}$), 2.42—2.58 (4H, C5–CH_2, C6–CH_2), 2.74 (3H, N1–CH_3), 8.00 (1H, N3H). ^{13}C-NMR (125 MHz; DMSO) δ: 20.0 (C3a–CH_2–$\underline{CH_2}$), 21.6 (C7a–CH_2–$\underline{CH_2}$), 27.1 (N1–CH_3), 29.6 (C7a–$\underline{CH_2}$–), 32.7 (C3a–$\underline{CH_2}$–),

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40.1 (C5–CH_2), 40.4 (C6–CH_2), 71.6 (C3a), 78.8 (C7a), 181.9 (C=S). FAB-MS (*m/z*): 227.1 (M^++1). *Anal.* Calcd for $C_{10}H_{18}N_4S$: C, 53.06; H, 8.02; N, 24.75: Found: C, 52.76; H, 8.02; N, 24.66%.

Single Crystal X-Ray Analysis of 3d Single crystals of **3d** were obtained by recrystallization from MeOH–DMSO. A colorless prismatic crystal with approximate dimensions of 0.90×0.50×0.20 mm was mounted in a glass fiber. All measurements were made on a Rigaku RAXIS RAPID imaging plate area detector with graphite monochromated Mo-$K\alpha$ radiation. The data were collected at a temperature of 23±1 °C to a maximum 2θ value of 55.0°. The structures were solved by direct method (SIR-92),[12] and the non-hydrogen atoms were refined anisotropically. Hydrogen atoms were refined using the riding model. All calculations were performed using the Crystal Structure[13,14] crystallographic software package.

3d: $C_{11}H_{24}N_4SO$, M=260.4, monoclinic, space group $P2_1/n$, a=7.8268(8), b=11.6274(9), c=16.909(1) Å, β=104.854(3)°, V=1487.4(2) Å^3, D_c=1.163 g cm^{-3}, Z=4, R=0.070 for 2180 observed reflections ($I>3.00\sigma(I)$), R_w=0.118. The crystallographic data are deposited at CCDC 734273.

Molecular Orbital Calculations Semi-empirical MO calculations were run through the CS Chem3D Pro interface and WINMOPAC3.5 using MOPAC2002[15] on an Intel personal computer. PM5-optimized structures were used as starting geometries for the DFT (density functional theory) calculations.

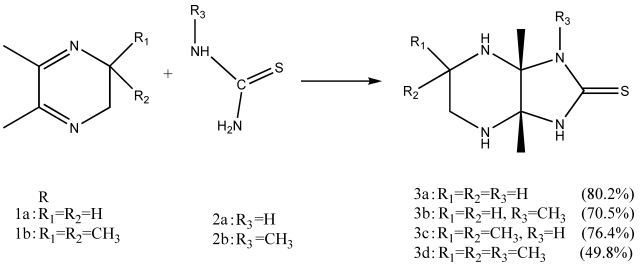


Chart 1

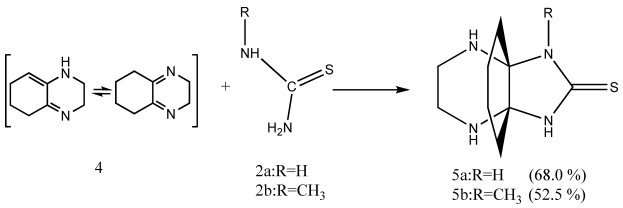


Chart 2

Results and Discussion

In the search for compounds with strong bactericidal effects on *E. coli*, the reaction products of DHPs with thiourea have been investigated. In previous studies,[6,7] we found that DHPs reacted with ethylenediamine to give tetraazadecalin derivatives. Similarly, it was thought that thiourea, might also react with DHPs as envisaged in Chart 1.

DHP was found to react with thiourea in water containing the bacterial culture medium for *E. coli*, under a similar condition as that caused a lethal effect on *E. coli*. A new spot appeared in the reaction mixture and was detected by TLC. It was also confirmed that the product was formed also in pure water or ethanol, and the subsequent isolation of the products was attempted.

DHPs such as 2,3-dihydro-5,6-dimethylpyrazine[1] (**1a**), 2,3-dihydro-2,2,5,6-tetramethylpyrazine[1] (**1b**) and hexahydroquinoxaline[11] (**4**) were reacted with thiourea/methylthiourea to give tetraazaindene derivatives (TAIs) **3a**, **3b**, **3c**, **3d**, **5a**, and **5b**. The stereochemical configuration of the TAIs were established by NMR spectrometry and X-ray crystallography. In terms of the stereochemistry of 3a*R**,7a*S**-dimethylhexahydroimidazo[4,5-*b*]pyrazine-2-thione (**3a**), it was not known whether the A and B rings of the TAI skeleton were fused in *cis*- or *trans*-configuration. From the reaction of **1a** with **2b**, 1,3a*R**,7a*S**-trimethylhexahydroimidazo[4,5-*b*]pyrazine-2-thione (**3b**) was obtained. The stereochemistry of the angularly substituted methyl groups at the C_{3a} and C_{7a} positions is shown in Fig. 1. An NOE observed between the methyl signals of N_1 and C_{3a}/C_{7a} indicates that **3b** has a *cis*-fused ring juncture. Single-crystal X-ray analysis of 1,3a*R**,6,6,7a*S**-pentamethylhexahydroimidazo[4,5-*b*]pyrazine-2-thione (**3d**), supported these structural assignments (see Fig. 2). Although the formation of four isomers is theoretically possible as shown in Fig. 3, the predominant formation of **3d** (*syn-cis* isomer) is clearly supported by comparing the heat of formation. It is also supported by the PM5-calculated heats of formation for **3b** that the superiority of *cis*-fused isomer over *trans*-fused one can be predicted (Fig. 4). Thereby, the formation of **5a** *via* a *cis*-fused addition of **2a** to DHP (**4**) is predictable. Also, we had previously reported the structurally similar propellane-type compound.[11]

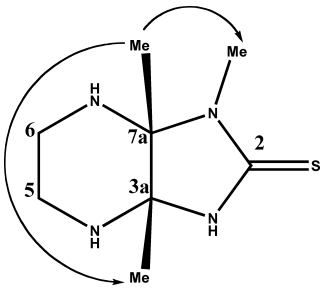


Fig. 1. NOE Correlation in **3b**

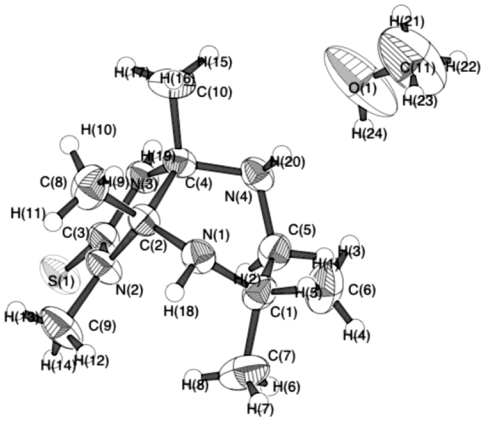


Fig. 2. ORTEP Drawing of (**3d**+CH_3OH)

As the formation of TAIs from interaction of DHP and thiourea was unambiguously confirmed, the reaction of DHP with urea also was thought amenable and the reaction was attempted. However, contrary to expectations, any reaction products were not obtained. To clarify the marked difference in the reactivity between thiourea and urea, evaluation of the frontier molecular orbital (FMO) interaction energies was carried out, according to the perturbation equation[16]: $\Delta E = 2\beta^2(C_{nucl}\cdot C_{elec})^2/(E_{HOMO(nucl)}-E_{LUMO(elec)})$, in which the interactions between the nitrogen atom of urea (thiourea) and the carbon atom (C2) of DHP were considered as illustrated in

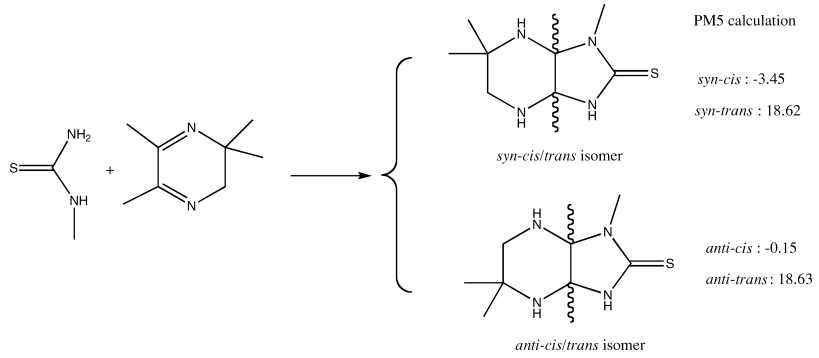


Fig. 3. The Comparison of the Heat of Formation (kcal/mol) among the Four Isomers

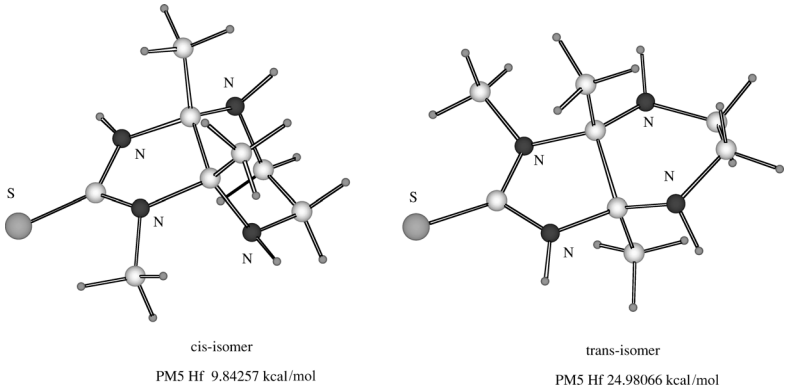


Fig. 4. PM5-Calculated Structures and Heats of Formation for *cis*- and *trans*-Fused Isomers of **3b**

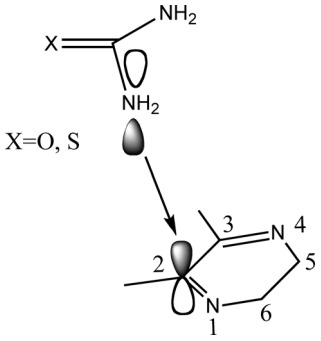


Fig. 5. The Interaction between the Nitrogen Atom of Urea (Thiourea) and the Carbon Atom (C2) of DHP

Fig. 5. As can be seen ΔE values obtained from the values in Tables 1 and 2, the FMO interaction energy for urea (-0.015 kcal/mol) is comparable to that (-0.016 kcal/mol) for thiourea. Similar results were obtained even in high-level calculations such as *ab initio* and DFT (B3LYP) methods at 6-31G(d) level. Therefore, the formation of the TAI-type DHP-urea adduct could not be rule out. The explanation by another mechanism is needed.

To this end, the mechanisms shown in Fig. 6 were examined. Based on the fact that $_{SH}$thiourea exists in tautomeric equilibrium as evidenced by the existence of $-N_4=C_2<$ type bond length in the X-ray crystallographic data of **3d**, we considered that the initial stage of the cyclization may be triggered by pericyclic ene reaction between the HN=C–S–H moiety of $_{SH}$thiourea and the C=N moiety of DHP. The transition state (TS) for the $[2\pi+2\pi+2\sigma]$ interaction was successfully located at PM3 level. The reaction barrier for the intermolecular ene reaction was calculated to be *ca.* 15 kcal/mol. The second-step N–C bond formation for cyclization (route A) may be achieved by intramolecular nucleophilic attack of the nitrogen to the carbon atom ($-HC_3=N-$), in which proximity effect becomes operative. However, the possibility of the ene reaction (route B) can not be ruled out although the increase of sizable molecular strain would be expected in TS.

Thus the formation of TAIs is brought about by favorable FMO interaction between the π electron systems. As described in a previous paper, the formation of dimeric compounds of DHP[8] is explicable in the same way *via* ene reaction. The regiochemistry observed in **3d** can be explained in terms of low steric hindrance of the initial attack. In the formation reaction of tetraazadecalin derivatives obtained from DHP and ethylenediamine,[6] the coulomb interaction due to the nitrogen lone pair of the primary amine plays a leading role.

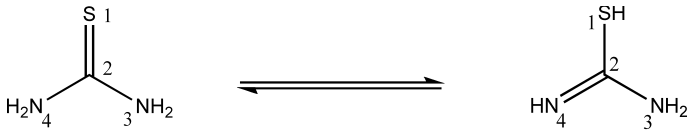
Upon addition of thiourea, the lethal effect of DHPs seemed to be reinforce. As thiourea served not as a radical scavenger but as a reactant with DHPs, the reaction afforded TAIs as products. According to the similar methods in our previous papers,[17—19] the growth inhibition and lethal effect of TAIs on *E. coli* were confirmed based on experiments using certain combinations (TAI only, TAI+Cu^{2+}, TAI+Cu^{2+}+thiourea, *etc.*); however, an effect as strong as that observed when using DHP+Cu^{2+}+thiourea occurred only with a combination of TAI+Cu^{1+}+thiourea. TAIs exhibited effects almost the same as those of DHPs, the lethal effect being somewhat stronger than that of DHP at 20 mM. However, the DNA strand-breaking activities of TAIs were very

Table 1. FMO Interaction Energies for the Initial Stage of the Reaction

	Pz(C2)	H		Pz(N)	L	H-L	$\Delta E/\beta^2$
DHP	−0.0656	−9.3420	$_{C=S}$Thiourea	−0.1092	−0.1370	−9.2050	0.000
	−0.0656	−9.3420	$_{SH}$Thiourea	−0.1231	−0.5920	−8.7500	0.000
	−0.0656	−9.3420	$_{C=O}$Urea	−0.1833	0.3090	−9.6510	0.000
	−0.0656	−9.3420	$_{OH}$Urea	0.2042	0.3570	−9.6990	0.000
	Pz(C2)	H		Pz(N)	nL	H-nL	
DHP	−0.0656	−9.3420	$_{C=S}$Thiourea	0.3000	−0.1080	−9.2340	0.000
	−0.0656	−9.3420	$_{SH}$Thiourea	0.5539	0.6370	−9.9790	0.000
	−0.0656	−9.3420	$_{C=O}$Urea	0.1846	0.5180	−9.8600	0.000
	−0.0656	−9.3420	$_{OH}$Urea	−0.5543	0.6950	−10.0370	0.000
	Pz(N)	H		Pz(C2)	L	H-L	
$_{C=S}$Thiourea	−0.1734	−8.9490	DHP	−0.4256	−0.2030	−8.7460	−0.001
$_{SH}$Thiourea	0.5119	−9.6350		−0.4256	−0.2030	−9.4320	−0.010
$_{C=O}$Urea	−0.4334	−10.5820		−0.4256	−0.2030	−10.3790	−0.007
$_{OH}$Urea	0.6467	−10.1750		−0.4256	−0.2030	−9.9720	**−0.015**
	Pz(N)	nH		Pz(C2)	L	nH-L	
$_{C=S}$Thiourea	−0.2473	−9.0590		−0.4256	−0.2030	−8.8560	−0.003
$_{SH}$Thiourea	0.6583	−10.0970		−0.4256	−0.2030	−9.8940	**−0.016**
$_{C=O}$Urea	0.6539	−10.6720		−0.4256	−0.2030	−10.4690	**−0.015**
$_{OH}$Urea	0.0766	−11.0160		−0.4256	−0.2030	−10.8130	0.000
	Pz(N)	nnH		Pz(C2)	L	nnH-L	
$_{C=S}$Thiourea	0.6821	−10.7610		−0.4256	−0.2030	−10.5580	**−0.016**
$_{SH}$Thiourea	−0.2651	−10.7500		−0.4256	−0.2030	−10.5470	−0.002

H: HOMO, nH: next HOMO, nnH: next next HOMO, L: LUMO, nL: next LUMO, β: resonance energy.

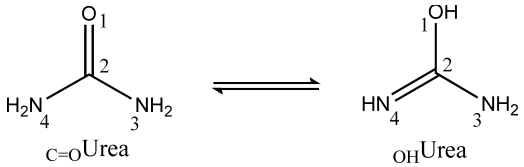
Table 2. FMO Energies and Coefficients of Urea, Thiourea and Their Tautomers



$_{C=S}$Thiourea $_{SH}$Thiourea

Hf 0.927 kcal/mol 12.939 kcal/mol

	nnHOMO	nHOMO	HOMO	LUMO	nLUMO	nnHOMO	nHOMO	HOMO	LUMO	nLUMO
Eigenvalues	−10.761	−9.059	−8.949	−0.137	−0.108	−10.750	−10.097	−9.635	−0.592	0.637
Pz(S1)	0.0000	0.7330	0.4997	−0.0003	0.3611	0.2012	−0.4418	−0.7960	0.0604	0.1628
Pz(C2)	0.0000	0.1355	0.0426	0.0007	−0.7431	0.0974	−0.0688	0.1317	−0.0078	−0.6968
Pz(N3)	0.6821	−0.2473	−0.1734	−0.1092	0.2999	−0.2651	0.6583	−0.2268	−0.1231	0.1474
Pz(N4)	−0.6821	−0.2473	−0.1734	0.1086	0.3000	0.1741	−0.3203	0.5119	0.0069	0.5539



$_{C=O}$Urea $_{OH}$Urea

Hf −56.180 kcal/mol −39.492 kcal/mol

	nHOMO	HOMO	LUMO	nLUMO	nHOMO	HOMO	LUMO	nLUMO
Eigenvalues	−10.672	−10.582	0.309	0.518	−11.016	−10.175	0.357	0.695
Pz(O1)	−0.0009	0.5988	−0.0008	0.4183	−0.0729	−0.2626	−0.0700	−0.2142
Pz(C2)	0.0000	−0.0307	0.0014	−0.7244	0.0653	0.2092	0.0521	0.7243
Pz(N3)	0.6539	−0.4311	−0.1833	0.1837	0.0186	−0.5181	0.2042	−0.0974
Pz(N4)	−0.6526	−0.4334	0.1824	0.1846	0.0766	0.6467	−0.0419	−0.5543

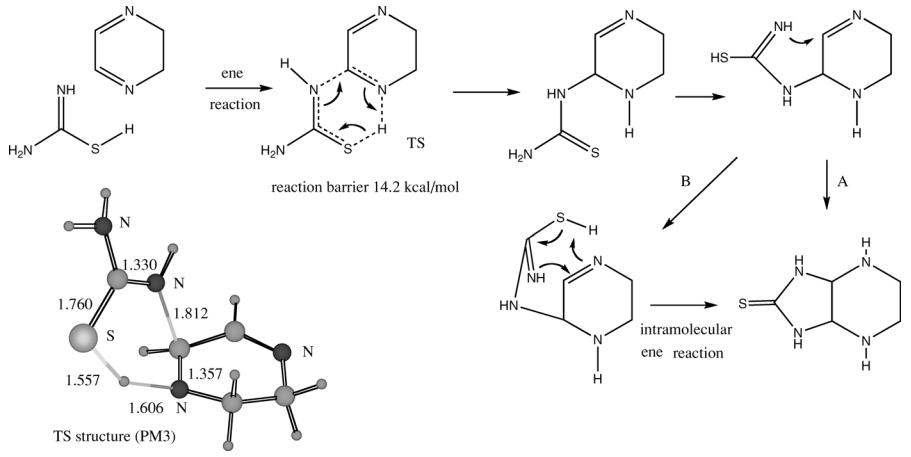


Fig. 6. Possible Reaction Mechanism *via* Transition State (TS) for the $[2\pi+2\pi+2\sigma]$ Interaction

weak. A total of 40% inhibition of activity was observed when a combination of 20 mM DHP+1 mM Cu^{2+}+20 mM thiourea was used, indicating the role of thiourea as a radical scavenger. In our previous data,[1,5] the DNA strand-breaking activity and radical species generating ability of DHPs were appreciably accelerated in the presence of Cu^{2+}. Copper(II) converted into Cu^{1+} by the reducing ability of DHPs, simultaneously, the resulting cation radical of DHPs generated further to radical species such as $\cdot OH$, $\cdot O^{2-}$ and carbon-centered radicals, which attacked to DNA strand. On the other hand, it is claimed that Cu^{1+}–OOH complex[20] formed in reaction mixture also attacked the DNA. It was concluded that the lethal effect on *E. coli* and DNA strand-breaking activity for cccDNA of pBR322 were controlled by different mechanisms. These results indicate that TAI shows moderate bactericidal activity; however, the rationale for the bactericidal effect is still unknown. Details of the biological effects of TAIs will be published in another report, which is currently in preparation.

References and Notes

1) Yamaguchi T., Kashige N., Mishiro N., Miake F., Watanabe K., *Biol. Pharm. Bull.*, **19**, 1261—1265 (1996).
2) Kashige N., Yamaguchi T., Miake F., Watanabe K., *Biol. Pharm. Bull.*, **23**, 1281—1286 (2000).
3) Yamaguchi T., Ito S., Kashige N., Nakahara K., Harano K., *Chem. Pharm. Bull.*, **55**, 532—536 (2007).
4) Yamaguchi T., Matsumoto S., Watanabe K., *Tetrahedron Lett.*, **39**, 8311—8312 (1998).
5) Kashige N., Takeuchi T., Matsumoto S., Takechi S., Miake F., Yamaguchi T., *Biol. Pharm. Bull.*, **28**, 419—423 (2005).
6) Yamaguchi T., Ito S., Iwase Y., Watanabe K., Harano K., *Heterocycles*, **51**, 2305—2309 (1999).
7) Yamaguchi T., Ito S., Iwase Y., Watanabe K., Harano K., *Heterocycles*, **53**, 1677—1680 (2000).
8) Yamaguchi T., Eto M., Harano K., Kashige N., Watanabe K., Ito S., *Teterahedron*, **55**, 675—686 (1999).
9) Yamaguchi T., Nomura H., Matsunaga K., Ito S., Takata J., Karube Y., *Biol. Pharm. Bull.*, **26**, 1523—1527 (2003).
10) Takechi S., Yamaguchi T., Nomura H., Minematsu T., Nakayama T., *Mutat. Res.*, **560**, 49—55 (2004).
11) Maruoka H., Kashige N., Miake F., Yamaguchi T., *Chem. Pharm. Bull.*, **53**, 1359—1361 (2005).
12) *SIR92*: Altomare A., Cascarano G., Giacovazzo C., Guagliardi A., Burla M., Polidori G., Camalli M., *J. Appl. Cryst.*, **27**, 435 (1994).
13) *CrystalStructure* 3.6.0: Crystal Structure Analysis Package, Rigaku and Rigaku/MSC, 9009 New Trails Dr. The Woodlands TX 77381 USA, (2000—2004).
14) *CRYSTALS* Issue 10: Watkin D. J., Prout C. K., Carruthers J. R., Betteridge P. W., Chemical Crystallography Laboratory, Oxford, U.K. (1996).
15) PM3 and PM5 calculations were performed using MOPAC2002 Version 1.5, Stewart J. J. P., Fujitsu Limited, Tokyo, Japan (2003).
16) Fleming I., "Frontier Orbitals and Organic Chemical Reactions," Wiley, London, 1976.
17) Takeda O., Takechi S., Kathoh T., Yamaguchi T., *Biol. Pharm. Bull.*, **28**, 1161—1164 (2005).
18) Takeda O., Takechi S., Ito S., Omori H., Kathoh T., Yamaguchi T., *Biol. Pharm. Bull.*, **30**, 1663—1667 (2007).
19) Takechi S., Nakahara k., Adachi M., Yamaguchi T., *Biol. Pharm. Bull.*, **32**, 186—189 (2009).
20) Kawanishi S., Hiraku Y., *Jpn. J. Toxicol. Environ. Health*, **41**, 399—410 (1995).