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SYNTHESIS AND EVALUATION FOR BIOLOGICAL ACTIVITIES OF 2-THIO-ACYLATED THIAZOLES CONTAINING PYRAZOLE MOIETY

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Abstract – An approach to the synthesis of novel 2-thio-acylated thiazoles containing pyrazole moiety is described. The pyrazole-thiazolethione derivative as the key building block for bis-heterocycles was formed *via* a Knoevenagel-type condensation of rhodanine with pyrazol-3-one. Thermal treatment of the pyrazole-thiazolethione derivative with acid anhydride and/or chloride in refluxing toluene caused diacylation reaction to give the corresponding 2-thio-acylated thiazoles containing pyrazole moiety. All the synthesized compounds were characterized by spectroscopic analysis and were tested for their DNA cleavage activity *in vitro*. Furthermore, they were evaluated for their antifungal activity against *Candida albicans* and *Saccharomyces cerevisiae*.

Thiazole and related compounds belong to a class of heterocyclic compounds having both a nitrogen and sulfur atom as part of the aromatic five-member ring. Although they have been known from long ago to be biologically active, their varied biological features are still of great scientific interest. Thiazole moiety has been found as an integral part of the structure of therapeutic agents and is widely used like sulfathiazole as antimicrobial agent, ravuconazole as antifungal agent, ritonavir as antiretroviral agent, meloxicam as nonsteroidal anti-inflammatory drug, and antibiotics like penicillin.[1] Moreover, 2-thio-substituted 1,3-thiazoles can be found in a vast number of pharmaceutically active molecules (Figure 1).[2] Hence, their synthesis continues to attract attention and provides an interesting challenge.[3]

On the other hand, pyrazole and its derivatives are also an important skeleton found in many synthetic bioactive compounds.[4] Pyrazole derivatives have wide-ranging collection of conventional biological and pharmaceutical activities, such as antitumor,[5] anti-inflammatory,[6] antibacterial,[7] hypoglycemic,[8] and antihypertensive[9] activities. In this context, a large number of general methods for the preparation of pyrazole derivatives have recently been reported.[10]

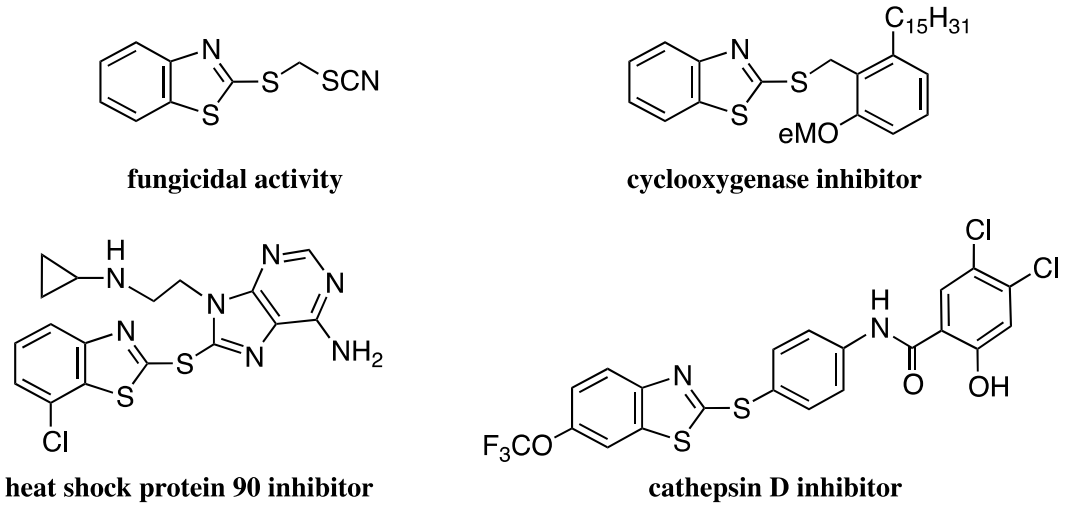
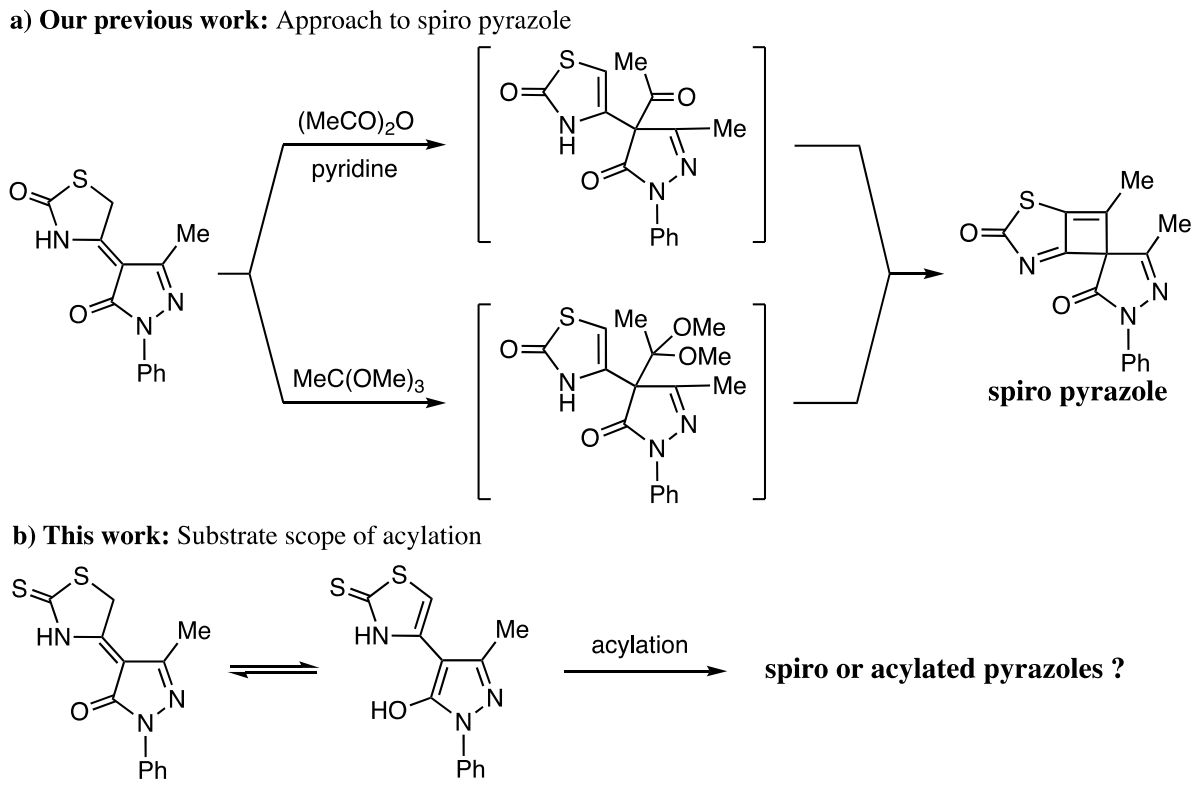


Figure 1. Selected bioactive 2-thio-substituted 1,3-thiazoles

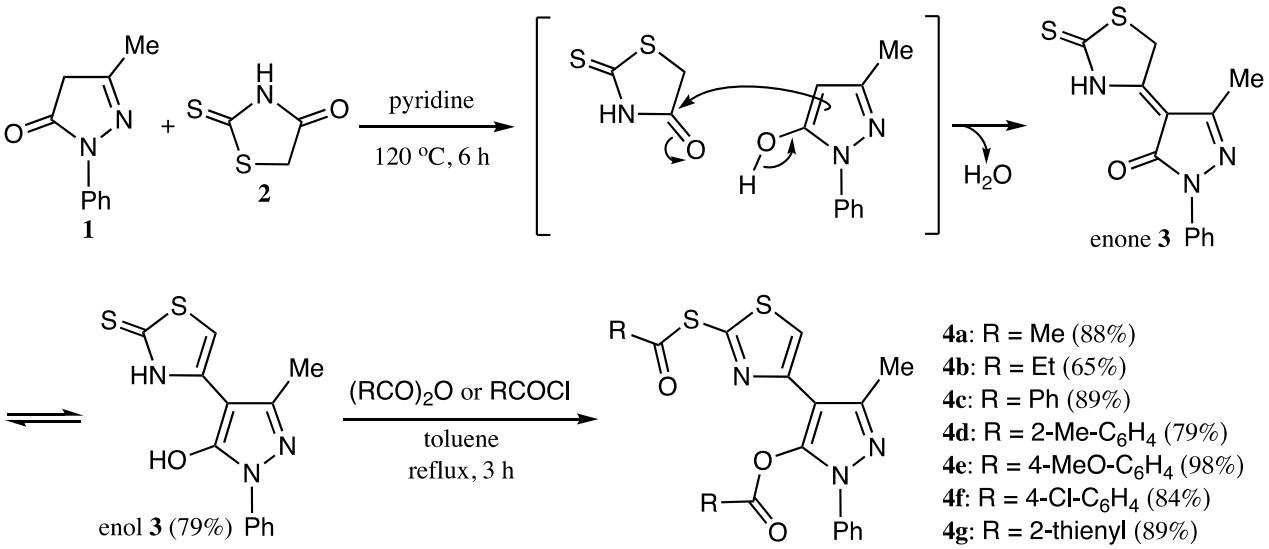
In connection with the synthesis of novel thiazoles containing pyrazole moiety,[11] we have reported the novel synthesis of spiro pyrazole derivatives containing cyclobutathiazole moiety starting from pyrazole-thiazolidinone derivative (Scheme 1a).[12] Owing to the importance of thiazoles containing pyrazole moiety, we decided to extend our previous studies to synthesize the pyrazole-thiazole derivatives from pyrazole-thiazolethione derivative as the key substrate, which might have useful biological and therapeutic activities, especially *in vitro* their DNA cleavage[13] and antifungal[14] activities (Scheme 1b).



Scheme 1

DNA is an important cellular receptor and many chemicals exert their antitumor effects through binding to DNA thereby changing the replication of DNA and inhibiting the growth of tumor cells.[15] Then discussing the mechanism of compounds cleaving and/or binding to DNA possesses significant meanings. For these reasons, we have been interested in the preparation of functionalized pyrazole-thiazole derivatives to evaluate their DNA cleavage and antifungal activities and now report the results of our investigation.

In the first step, we examined a Knoevenagel-type condensation of pyrazol-3-one **1** with rhodanine **2** (Scheme 2). To get the optimized reaction conditions, we carried out several experiments on pyrazole-thiazolethione derivative **3**, testing different reaction conditions, for example, substrate/base molar ratio, solvent, reaction temperature, and reaction time. Best result was obtained when a mixture of **1** and **2** in the presence of pyridine under the solvent-free was stirred at 120 ˚C for 6 h. Indeed, the expected pyrazole-thiazolethione **3** was isolated as a single isomer of enol form in 79% yield. This product **3** gave satisfactory elemental analysis and spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR, and MS) consistent with their assigned structures (see experimental section).



Scheme 2

In the next step, we tried an acylation of **3** in detail. Interestingly, the reaction mode was completely changed. Thus, **3** was reacted with acetic anhydride in refluxing toluene for 3 h to afford the diacetylated pyrazole-thiazole derivative **4a** as the only isolated product in 88% yield (Scheme 2). In this acetylation in the presence of pyridine as a base, the spiro pyrazole derivative as shown in Scheme 1a could not be detected at all. This result indicates that this type of straightforward preparation of spiro pyrazoles starting from pyrazole-thiazolethione **3** as the key substrate is not easy. The reason for this change of behavior is not clear at present.

The IR spectrum of **4a** displays two bands at 1783 and 1699 cm^{-1} because of two C=O groups. The ^{1}H NMR spectrum of **4a** in $CDCl_3$ exhibits two three-proton singlets at δ 2.31 and 2.52 assignable to two acetyl methyl protons and a three-proton singlet at δ 2.53 assignable to the methyl protons. The ^{13}C NMR spectrum of **4a** in $CDCl_3$ shows a signal at δ 14.9 because of the methyl carbon, two signals at δ□20.7 and 30.3 because of two acetyl methyl carbons, a signal at δ 104.7 because of the pyrazole C-4 carbon, and two signals at δ 167.6 and 191.1 because of two C=O carbons. By comparison of NMR, MS, and elemental analysis of **4a** it seems that the structural assignments given to this compound is correct. Subsequently, the reactions of compound **3** with acid anhydride and/or chloride in refluxing toluene gave the corresponding thiazoles **4b**–**g** containing pyrazole moiety with 65–98% isolated yields. These products **4b**–**g** were characterized by spectroscopic analyses (see experimental section).

Table 1. DNA cleavage by **3** and **4a**–**g** in the absence and/or presence of Cu^{2+}

Entry	Compound[a]	DNA type	Relative amounts of DNA (%)	
			Without $CuCl_2$[b]	With $CuCl_2$[c]
1	Control	ccc- oc-	100 0	100 0
2	**3**	ccc- oc-	100 0	33 67
3	**4a**	ccc- oc-	100 0	60 40
4	**4b**	ccc- oc-	100 0	85 15
5	**4c**	ccc- oc-	100 0	98 2
6	**4d**	ccc- oc-	100 0	97 3
7	**4e**	ccc- oc-	100 0	63 37
8	**4f**	ccc- oc-	100 0	93 7
9	**4g**	ccc- oc-	100 0	83 17

[a]Amount: Control (0 mM), **3** and **4a**–**g** (10 mM). [b]Amount: $CuCl_2$ (0 mM). [c]Amount: $CuCl_2$ (1 mM). As activity was accelerated upon addition of Cu^{2+}, the quantity of compounds and the incubation time were minimized until differences in activity could be observed.

In our third studies, we have tested *in vitro* DNA cleavage activity of the synthesized compounds **3** and **4a**–**g**. The values obtained for activity were based on the remaining amounts of covalently closed circular duplex DNA, namely ccc-DNA, of plasmid pBR322.[16] The data of DNA cleavage activity is summarized in Table 1. Indeed, in the absence of Cu^{2+}, all the tested compounds showed no DNA cleavage activity. These activities of compounds **3**, **4a**, and **4e**, however, were obviously accelerated by the addition of 1

mM Cu^{2+} (entries 2, 3, and 7). Furthermore, it was found that compounds **4b** and **4g** have weak activity with Cu^{2+} (entries 4 and 9).

Finally, eight of the newly synthesized compounds **3** and **4a**–**g** were also tested for their antifungal activity against *Candida albicans* and *Saccharomyces cerevisiae*. *In vitro* susceptibility tests were performed to evaluate minimum inhibitory concentrations (MICs) using the method described in the guidelines of NCCLS Document M27-A2[17a] and our previous paper.[17b] Miconazole and Itraconazole were used as standard drugs for comparison of the antifungal activity. The results obtained are summarized in Table 2. All the synthesized compounds were no active against *Saccharomyces cerevisiae* with MIC ≥ 128 mg/mL. It is worth noting that the entire tested compounds **3** and **4c** showed moderate antifungal activity against *Candida albicans* with MIC ≥ 16 and 32 μg/mL (entries 1 and 4). Furthermore, compounds **4a**,**b** were weak active against *Candida albicans* with MIC ≥ 64 μg/mL (entries 2 and 3).

Table 2. *In vitro* antifungal activity of **3** and **4a**–**g** against *C. albicans* and *S. cerevisiae*

Entry	Compound	MIC (μg/mL)	
		C. albicans[a]	*S. cerevisiae*[b]
1	**3**	16[c]	>128[c]
2	**4a**	64[c]	>128[c]
3	**4b**	64[c]	>128[c]
4	**4c**	32[c]	>128[c]
5	**4d**	>128[c]	>128[c]
6	**4e**	>128[c]	>128[c]
7	**4f**	>128[c]	>128[c]
8	**4g**	>128[c]	>128[c]
9	Miconazole	1[d]	1[e]
10	Itraconazole	1[d]	1[f]
11	DMSO	> 12.5%	> 6.25%
12	EtOH	> 12.5%	> 6.25%

[a] RPMI1640 medium, 37 ˚C. [b] YM medium, 25 ˚C. [c] containing 1.0% DMSO.
[d] containing 0.015% DMSO. [e] containing 0.04% DMSO. [f] containing 0.03% DMSO.

In conclusion, we have demonstrated the reactions of the key substrate pyrazole-thiazolethione with acid anhydride and/or chloride. This methodology offers significant advantages with regard to the supply of 2-thio-acylated thiazoles containing pyrazole moiety, which may be important building blocks in organic synthesis and for the preparation of biologically active compounds with interest in medicinal chemistry. In this present work, we have found that compounds **3**, **4a**, and **4e** showed high DNA cleavage activity *in vitro* with Cu^{2+}. In addition, compounds **4b** and **4g** exhibited weak DNA cleavage activity. Furthermore, we showed that compounds **3** and **4c** were moderately to fairly active against *Candida albicans* whereas the reference drug such as Miconazole and Itraconazole exhibited good to excellent results against both the fungi. Further synthetic applications for novel thiazoles containing pyrazole moiety are in progress.

EXPERIMENTAL

All melting points are uncorrected. The IR spectra were recorded on a JASCO FT/IR-4100 spectrometer. The ^{1}H and ^{13}C NMR spectra were measured with a JEOL JNM-A500 spectrometer at 500.00 and 125.65 MHz or with a JEOL JNM-ECZ600R/S1 spectrometer at 600.17 and 150.91 MHz, respectively. The ^{1}H and ^{13}C chemical shifts (δ) are reported in parts per million (ppm) relative to TMS as internal standard. Positive FAB MS spectra were obtained on a JEOL JMS-700T spectrometer. Elemental analyses were performed on YANACO MT-6 CHN analyzer.

The preparation of pyrazole-thiazolethione 3 from 1 and 2 in the presence of pyridine. A mixture of 3*H*-pyrazol-3-one **1** (4.176 g, 24 mmol), rhodanine **2** (2.664 g, 20 mmol), and pyridine (1.582 g, 20 mmol) was stirred at 120 ˚C for 6 h. The resulting mixture was recrystallized from MeOH/petroleum ether to give 4-(5-hydroxy-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)-2(3*H*)-thiazolethione (**3**): this compound was obtained as pale brown needles (4.589 g, 79%), mp 147-149 ˚C (dec.); IR (KBr): ν 3418 (OH), 3163 cm^{-1} (NH); ^{1}H NMR ($CDCl_3$): δ 2.27 (s, 3H, Me), 3.51 (br, 1H, OH), 6.79 (s, 1H, olefinic H), 7.27-7.30 (m, 1H, Ph-H), 7.47-7.51 (m, 2H, Ph-H), 7.70-7.73 (m, 2H, Ph-H), 12.92 (br, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 12.4 (Me), 94.3 (pyrazole C-4), 108.1 (thiazole C-5), 120.3, 125.7, 129.0, 133.7 (Ph-C), 136.9 (thiazole C-4), 146.5 (pyrazole C-3), 187.7 (thiazole C-2); MS: *m/z* 290 $[M+H]^+$; high-resolution MS: Calcd for $C_{13}H_{12}N_3OS_2$ 290.0422, Found 290.0422. Anal. Calcd for $C_{13}H_{11}N_3OS_2 \cdot 0.4H_2O$: C, 52.65; H, 4.01; N, 14.17. Found: C, 52.67; H, 4.07; N, 14.3.

General procedure for the preparation of diacylated pyrazole-thiazoles 4a-g from 3 and acyl anhydride and/or chloride. A mixture of **3** (0.289 g, 1 mmol) and Ac_2O (0.510 g, 5 mmol), $(EtCO)_2O$ (0.651 g, 5 mmol), PhCOCl (0.703 g, 5 mmol), 2-Me-C_6H_4COCl (0.773 g, 5 mmol), 4-MeO-C_6H_4COCl (0.853 g, 5 mmol), 4-Cl-C_6H_4COCl (0.875 g, 5 mmol), or 2-thiophenecarbonyl chloride (0.733g, 5 mmol) in toluene (5 mL) was refluxed for 3 h. After removal of the solvent *in vacuo*, the residue was recrystallized from $CHCl_3$/petroleum ether to yield **4a-g**.

4-[2-(Acetylthio)thiazol-4-yl]-3-methyl-1-phenyl-1*H*-pyrazol-5-ol acetate (4a): Colorless needles (0.327 g, 88%), mp 114-115 ˚C; IR (KBr): ν 1783, 1699 cm^{-1} (CO); ^{1}H NMR ($CDCl_3$): δ 2.31 (s, 3H, OCOMe), 2.52 (s, 3H, SCOMe), 2.53 (s, 3H, Me), 7.33-7.36 (m, 1H, Ph-H), 7.39 (s, 1H, olefinic H), 7.43-7.46 (m, 2H, Ph-H), 7.55-7.57 (m, 2H, Ph-H); ^{13}C NMR ($CDCl_3$): δ 14.9 (Me), 20.7 (OCO*Me*), 30.3 (SCO*Me*), 104.7 (pyrazole C-4), 116.4 (thiazole C-5), 123.1, 127.6, 129.2, 137.6 (Ph-C), 141.9 (pyrazole C-5), 146.85 (thiazole C-4), 146.92 (pyrazole C-3), 154.2 (thiazole C-2), 167.6 (O*C*OMe), 191.1 (S*C*OMe); MS: *m/z* 374 $[M+H]^+$. Anal. Calcd for $C_{17}H_{15}N_3O_3S_2$: C, 54.67; H, 4.05; N, 11.25. Found: C, 54.61; H, 4.08; N, 11.18.

3-Methyl-1-phenyl-4-[2-(propionylthio)thiazol-4-yl]-1*H*-pyrazol-5-ol propionate (4b): Pale yellow prims (0.260 g, 65%), mp 79-80 ˚C; IR (KBr): ν 1788, 1717 cm^{-1} (CO); ^{1}H NMR ($CDCl_3$): δ 1.20 (t, *J* =

7.5 Hz, 3H, OCOCH$_2$*Me*), 1.26 (t, *J* = 7.5 Hz, 3H, SCOCH$_2$*Me*), 2.52 (s, 3H, Me), 2.60 (q, *J* = 7.5 Hz, 3H, OCOC*H*$_2$Me), 2.76 (q, *J* = 7.5 Hz, 3H, SCOC*H*$_2$Me), 7.31-7.34 (m, 1H, Ph-H), 7.37 (s, 1H, olefinic H), 7.41-7.44 (m, 2H, Ph-H), 7.53-7.55 (m, 2H, Ph-H); ^{13}C NMR ($CDCl_3$): δ 8.76 (OCOCH$_2$*Me*), 9.40 (SCOCH$_2$*Me*), 15.0 (Me), 27.6 (OCO*C*H$_2$Me), 37.5 (SCO*C*H$_2$Me), 104.9 (pyrazole C-4), 116.3 (thiazole C-5), 123.3, 127.6, 129.2, 137.7 (Ph-C), 142.1 (pyrazole C-5), 146.9 (thiazole C-4), 147.0 (pyrazole C-3), 154.4 (thiazole C-2), 171.1 (O*C*OCH$_2$Me), 195.5 (S*C*OCH$_2$Me); MS: *m/z* 402 $[M+H]^+$. Anal. Calcd for $C_{19}H_{19}N_3O_3S_2$: C, 56.84; H, 4.77; N, 10.47. Found: C, 57.03; H, 4.79; N, 10.51.

4-[2-(Benzoylthio)thiazol-4-yl]-3-methyl-1-phenyl-1*H*-pyrazol-5-ol benzoate (4c): Colorless needles (0.440 g, 89%), mp 157-158 ˚C; IR (KBr): ν 1752, 1663 cm^{-1} (CO); ^{1}H NMR ($CDCl_3$): δ 2.60 (s, 3H, Me), 7.24-7.29 (m, 1H, Ph-H), 7.37-7.39 (m, 2H, Ph-H), 7.40 (s, 1H, olefinic H), 7.43-7.49 (m, 4H, Ph-H), 7.57-7.63 (m, 4H, Ph-H), 7.86-7.88 (m, 2H, Ph-H), 8.14-8.15 (m, 2H, Ph-H); ^{13}C NMR ($CDCl_3$): δ 15.0 (Me), 105.2 (pyrazole C-4), 116.3 (thiazole C-5), 123.1, 127.56, 127.60, 128.3, 128.8, 129.1, 129.3, 130.8, 134.2, 134.5, 135.7, 137.8 (Ph-C), 142.2 (pyrazole C-5), 146.9 (thiazole C-4), 147.4 (pyrazole C-3), 154.4 (thiazole C-2), 163.7 (O*C*OPh), 187.4 (S*C*OPh); MS: *m/z* 498 $[M+H]^+$; high-resolution MS: Calcd for $C_{27}H_{20}N_3O_3S_2$ 498.0946, Found 498.0951. Anal. Calcd for $C_{27}H_{19}N_3O_3S_2 \cdot 0.25H_2O$: C, 64.59; H, 3.91; N, 8.37. Found: C, 64.58; H, 4.05; N, 8.37.

3-Methyl-4-[2-[(2-methylbenzoyl)thio]thiazol-4-yl]-1-phenyl-1*H*-pyrazol-5-ol 2-methylbenzoate (4d): Pale yellow prisms (0.413 g, 79%), mp 153-154 ˚C; IR (KBr): ν 1750, 1683 cm^{-1} (CO); ^{1}H NMR ($CDCl_3$): δ 2.48, 2.50 (s, 6H, 2xCOC$_6$H$_4$-2-*Me*), 2.59 (s, 3H, Me), 7.23-7.31 (m, 5H, Ph-H), 7.43 (s, 1H, olefinic H), 7.39-7.46 (m, 4H, Ph-H), 7.60-7.62 (m, 2H, Ph-H), 7.72-7.74 (m, 1H, Ph-H), 8.14-8.16 (m, 1H, Ph-H); ^{13}C NMR ($CDCl_3$): δ 15.0 (Me), 20.9, 21.7 (2xCOC$_6$H$_4$-2-*Me*), 105.2 (pyrazole C-4), 116.3 (thiazole C-5), 123.3, 126.0, 126.1, 127.3, 127.6, 128.9, 129.3, 131.7, 131.8, 132.1, 132.9, 133.2, 135.5, 137.9, 141.9 (Ph-C), 142.3 (pyrazole C-5), 147.1 (thiazole C-4), 147.3 (pyrazole C-3), 155.1 (thiazole C-2), 163.8 (O*C*OC$_6$H$_4$-2-Me), 189.1 (S*C*OC$_6$H$_4$-2-Me); MS: *m/z* 526 $[M+H]^+$. Anal. Calcd for $C_{29}H_{23}N_3O_3S_2$: C, 66.26; H, 4.41; N, 7.99. Found: C, 66.21; H, 4.49; N, 7.96.

3-Methyl-4-[2-[(4-methoxylbenzoyl)thio]thiazol-4-yl]-1-phenyl-1*H*-pyrazol-5-ol 4-methoxylbenzoate (4e): Colorless needles (0.548 g, 98%), mp 158-160 ˚C; IR (KBr): ν 1685, 1604 cm^{-1} (CO); ^{1}H NMR ($CDCl_3$): δ 2.58 (s, 3H, Me), 3.82, 3.87 (2xOMe), 6.91-7.00 (m, 4H, Ph-H), 7.24-7.28 (m, 1H, Ph-H), 7.36 (s, 1H, olefinic H), 7.36-7.39 (m, 2H, Ph-H), 7.60-7.62 (m, 2H, Ph-H), 7.85-7.88 (m, 2H, Ph-H), 8.06-8.10 (m, 2H, Ph-H); ^{13}C NMR ($CDCl_3$): δ 15.0 (Me), 55.6, 55.7 (2xOMe), 105.3 (pyrazole C-4), 114.1, 114.3 (Ph-C), 116.3 (thiazole C-5), 120.4, 123.0, 127.4, 129.3, 129.9, 133.1, 137.9 (Ph-C), 142.4 (pyrazole C-5), 146.8 (thiazole C-4), 147.5 (pyrazole C-3), 154.7 (thiazole C-2), 163.2 (O*C*OC$_6$H$_4$-4-OMe), 164.5, 164.6 (Ph-C), 185.8 (S*C*OC$_6$H$_4$-4-OMe); MS: *m/z* 558 $[M+H]^+$; high-resolution MS: Calcd for $C_{29}H_{24}N_3O_5S_2$ 558.1157, Found 558.1156. Anal. Calcd for

$C_{29}H_{23}N_3O_5S_2 \cdot 0.5H_2O$: C, 61.47; H, 4.27; N, 7.42. Found: C, 61.39; H, 4.17; N, 7.60.

4-[2-[(4-Chlorobenzoyl)thio]thiazol-4-yl]-3-methyl-1-phenyl-1*H*-pyrazol-5-ol 4-chlorobenzoate (4f): Colorless needles (0.477 g, 84%), mp 188-189 ˚C; IR (KBr): ν 1750, 1665 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ 2.58 (s, 3H, Me), 7.28-7.31 (m, 1H, Ph-H), 7.38-7.42 (m, 4H, Ph-H), 7.41 (s, 1H, olefinic H), 7.46-7.48 (m, 2H, Ph-H), 7.59-7.62 (m, 2H, Ph-H), 7.76-7.82 (m, 2H, Ph-H), 8.06-8.10 (m, 2H, Ph-H); ^{13}C NMR ($CDCl_3$): δ 15.1 (Me), 104.9 (pyrazole C-4), 116.4 (thiazole C-5), 123.1, 127.0, 127.7, 128.9, 129.1, 129.3, 129.6, 132.3, 133.9, 137.7, 140.8, 141.1 (Ph-C), 142.2 (pyrazole C-5), 147.05 (thiazole C-4), 147.15 (pyrazole C-3), 153.6 (thiazole C-2), 163.1 (O*C*OC_6H_4-4-Cl), 186.2 (S*C*OC_6H_4-4-Cl); MS: *m/z* 366 M^+. Anal. Calcd for $C_{27}H_{17}N_3Cl_2O_3S_2$: C, 57.25; H, 3.02; N, 7.42. Found: C, 57.02; H, 3.29; N, 7.37.

3-Methyl-1-phenyl-4-[2-[(2-thiophenecarbonyl)thio]thiazol-4-yl]-1*H*-pyrazol-5-ol 2-thiophenecarboxylate (4g): Colorless needles (0.451 g, 89%), mp 172-174 ˚C; IR (KBr): ν 1729, 1653 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ 2.59 (s, 3H, Me), 7.14-7.18 (m, 2H, Ph-H), 7.28-7.31 (m, 1H, Ph-H), 7.42 (s, 1H, olefinic H), 7.39-7.43 (m, 2H, Ph-H), 7.62-7.64 (m, 2H, 2xthiophene C-4), 7.68-7.69 (m, 1H, thiophene C-3), 7.72-7.74 (m, 1H, thiophene C-3), 7.78-7.80, (m, 1H, thiophene C-5), 7.97-7.98, (m, 1H, thiophene C-5); ^{13}C NMR ($CDCl_3$): δ 14.9 (Me), 105.2 (pyrazole C-4), 116.5 (thiazole C-5), 123.0 (2xthiophene C-3), 127.5, 128.2, 128.3, 129.2, 131.0 (Ph-C), 132.3 (thiophene C-5), 134.5, 134.7 (2xthiophene C-3), 136.0 (thiophene C-5), 137.7, 140.1 (2xthiophene C-2), 141.6 (pyrazole C-5), 146.7 (thiazole C-4), 147.3 (pyrazole C-3), 153.7 (thiazole C-2), 158.6 (O*C*O), 179.1 (S*C*O); MS: *m/z* 510 $[M+H]^+$. Anal. Calcd for $C_{23}H_{15}N_3O_3S_4$: C, 54.20; H, 2.97; N, 8.24. Found: C, 54.02; H, 3.09; N, 8.22.

Reaction of plasmid pBR322 with compounds 3 and 4a–g. The method of assaying the DNA cleavage activity, using a covalently closed circular duplex DNA (ccc-DNA) of plasmid pBR322 as a substrate, was described in our previous investigation.[16d] The results are listed in Table 1. The reaction mixture (100 μL) containing 1 μg of ccc-DNA of plasmid pBR322, 10 mM of **3** and **4a**–**g**, and 50 mM Tris-HCl buffer (pH7.4), was incubated at 37 ˚C. At interval, 20 μL of the reaction was mixed with 2 μL of 10 × Loading Buffer (TAKARA BIO INC. Shiga, Japan). The resulting mixture was directly by 1.0% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/mL) for 20 min. Under these conditions the order of anodal migration for the tree topological forms of the DNA was ccc-DNA, full-length linear duplex DNA (linear-DNA), and nicked open circular duplex DNA (oc-DNA). The ccc-DNA produced oc-DNA after single strand scission and linear-DNA after double-strand scission. They were all detected as clearly separated bands in agarose gels. The stained DNA bands were made visible using BioDoc-It™ Imaging Systems (UVP, Upland, CA) and then took the JPEG image file. For quantitative analysis of DNA on the gels, densitometric analyses of the images file were carried out using QuantiScan densitometry software (BIOSOFT, Cambridge, U.K.). The area under the ccc-DNA was multiplied by a factor of 1.42 to correct for its reduced binding of ethidium bromide as indicated by Lloyd

and coworkers.[16a]

Antifungal activity testing. *In vitro* susceptibility tests were performed to evaluate minimum inhibitory concentrations (MICs) using the method described in the guidelines of NCCLS Document M27-A2[17a] and our previous paper.[17b] The results are listed in Table 2. The susceptibility assays were determined by the broth dilution method performed in sterile flat-bottom 96-well microplates (Thermo Scientific, Waltham, USA) as described previously in NCCLS guidelines, M-27 A document (NCCLS, 2002). Briefly, *C. albicans* was inoculated at 35 ˚C and observed at 24 and 48 h. Five colonies greater than 1 mm in diameter were selected, suspended in saline solution and adjusted to a final concentration of 0.5 x 10^3 to 2.5 x 10^3 in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) buffered to pH 7.0 with 0.165M 3-morpholinepropanesulfonic acid (MOPS; DOJINDO Laboratories, Kumamoto Japan). *C. albicans* and *S. cerevisiae* were inoculated at 28 ˚C and observed at 24 and 48 h. Five colonies greater than 1 mm in diameter were selected, suspended in saline solution and adjusted to a final concentration of 0.5 x 10^3 to 2.5 x 10^3 in YM medium (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). The antifungal agents itraconazole and miconazole (Wako Pure Chemical Industries, Osaka, Japan), were used as positive control in the susceptibility tests. Itraconazole and miconazole were dissolved in DMSO. The drugs were prepared at the 1280 μg/mL concentration in DMSO. The drug solutions were diluted in RPMI medium or YM medium and final drugs concentrations ranged from 128 to 0.02 μg/mL After 48 h of incubation at 35 ˚C, MIC was determined visually by comparing its turbidity with the drug-free growth control well. The MIC values were defined as the lower drug concentration, which resulted in reduction of 80% in the turbidity in comparison with the drug-free growth control well.

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