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SYNTHESIS OF THIOSEMICARBAZONE DERIVATIVES OF 3-FORMYL-*N*-PYRAZOLYLMETHYLENEINDOLES AND THEIR ANTIFUNGAL ACTIVITIES

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Abstract – In order to discover natural-product-based antifungal candidates, a series of new thiosemicarbazone derivatives of 3-formyl-*N*-pyrazolylmethyleneindoles were prepared. Some derivatives showed good antifungal activities. Against *Curvularia lunata*, compound **I-2** showed 2.2 times antifungal activity compared to the precursor indole. Against *Valsa mali*, compound **I-1** exhibited 1.7 times antifungal activity compared to indole.

The indole (**1a**, Figure 1) scaffold is found in a wide range of bioactive heterocycles and natural products.[1] Moreover, some well-known commercial clinical drugs such as sumatriptan (**A**, Figure 1), fluvastatin (**B**, Figure 1), and rizatriptan (**C**, Figure 1) contain the indole fragment.[2-4] Indole and its derivatives also possess antifungal activities in agriculture.[5-7] The phytopathogenic fungi are considered to cause a direct loss in the economic value of agriculture.[8,9] Especially, apple *Valsa* canker is a threatening canker disease of the bark of apple trees caused by *Valsa mali* Miyabe et Yamada, and results in negative impacts on both yield and quality of apples.[10-12] Thus, the discovery of new agents for effective management of those congeneric phytopathogenic fungi is highly urgent. Pyraclostrobin (**D**, Figure 1) containing the pyrazole fragment is a commercial strobilurin fungicide against a variety of plant pathogenic fungi and inhibits their mitochondrial complex III.[13-16] Previously, we found a thiosemicarbazone derivative of *N*-benzylindole (**E**, Figure 1) showing potent antifungal activity against *V. mali*.[17] Consequently, to find natural-product-based pesticide candidates,[18-20] a series of new thiosemicarbazone derivatives of 3-formyl-*N*-pyrazolylmethyleneindoles (**I**, Figure 1) were designed and synthesized by introduction of two fragments such as pyrazole and thiosemicarbazide into indoles. Meanwhile, their antifungal activities were tested against two typical phytopathogenic fungi *Curvularia*

lunata (Walk.) and *V. mali*.

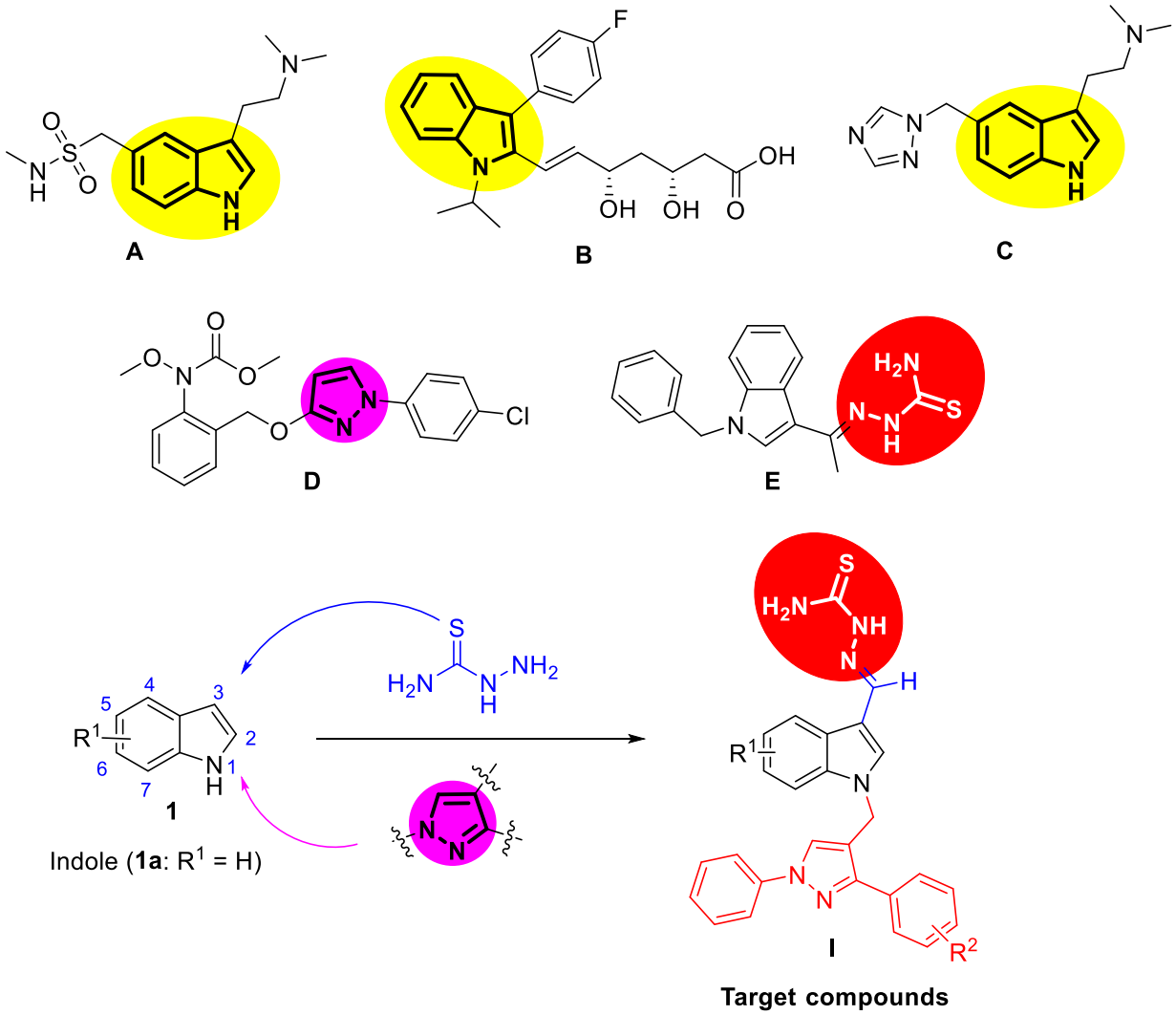
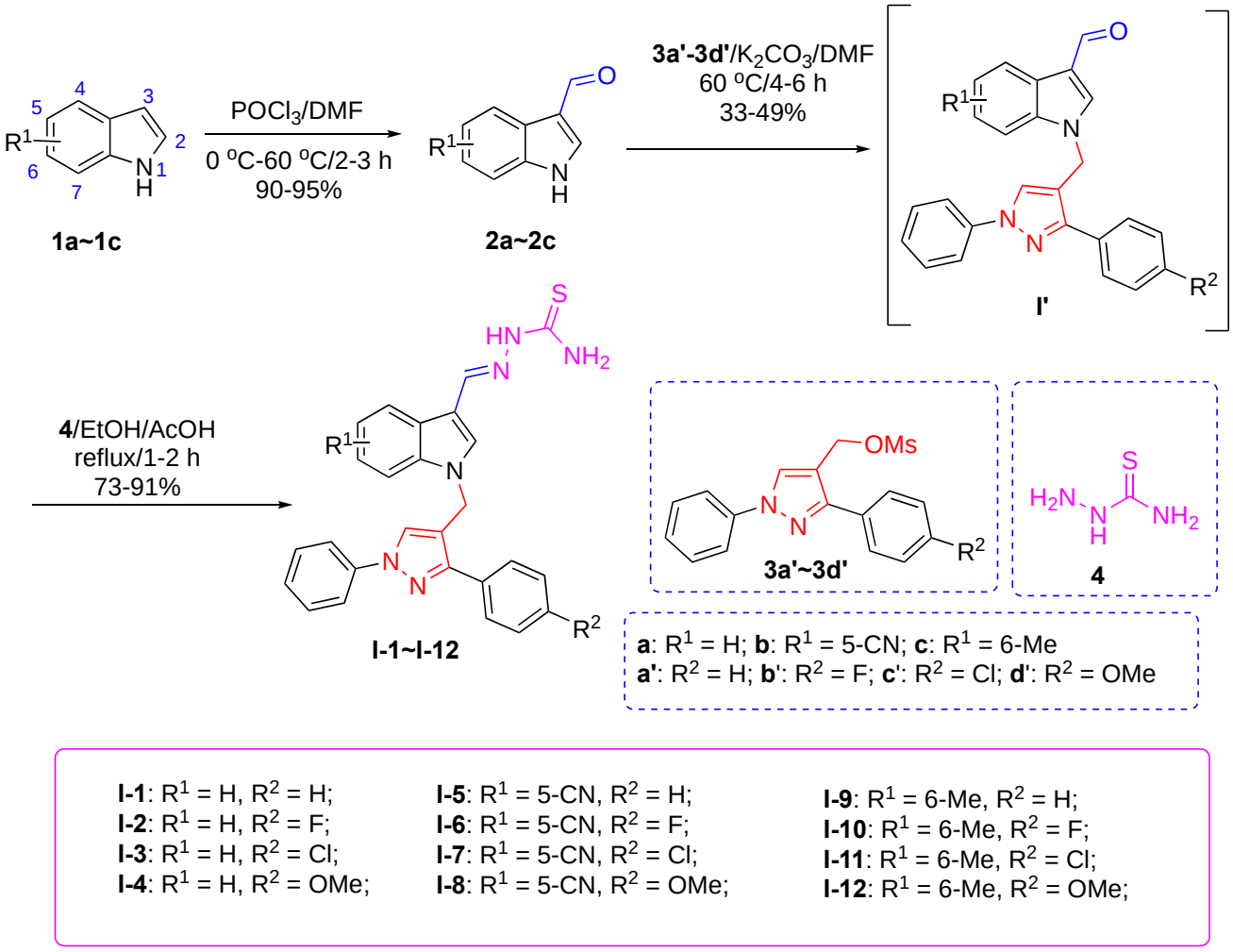


Figure 1. Design of target compounds **I**

As shown in Scheme 1,3-formylindoles (**2a**–**2c**) were obtained in 90–95% yields from indoles (**1a**–**1c**) by Vilsmeier-Haack-Arnold (VHA) reaction in the presence of $POCl_3$ and DMF.[21] Then, compounds **2a**–**2c** reacted with pyrazole derivatives (**3a′**–**3d′**) in the presence of K_2CO_3 and DMF to afford 3-formyl-*N*-pyrazolylmethyleneindoles (**I′**),[22] which directly reacted with thiosemicarbazide to give target compounds **I-1**–**I-12** in 73–91% yields.[17] Their structures were characterized by IR, melting points, HRMS, and ^{1}H NMR.

The 96 h antifungal results of compounds **I-1**–**I-12** at 50 μg/mL against two phytopathogenic fungi *V. mali* and *C. lunata* were described in Table 1. Against *V. mali*, the 96 h inhibition rates of compounds **I-1** and **I-2** were 50.7 and 34.9%, respectively, which were higher than that of their precursor indole (31.7%). Structure-activity relationships analysis showed that R^1 as the electron-withdrawing group (R^1 = 5-CN) was not beneficial for the antifungal activity against *V. mali.* For example, the 96 h inhibition rates of

I-5–**I-8** (R^1 = 5-CN) were only 0~1.6%. Additionally, the 96 h inhibition rates of **I-1**–**I-4** against *V. mali*



Scheme 1. Synthesis of target compounds **I-1**–**I-12**

were 50.7%, 34.9%, 12.8%, and 8.4%, respectively. It demonstrated that introduction of electron-withdrawing (R^2 = F, Cl) or electron-donating group (R^2 = OMe) on the phenyl of compound **I-1** cannot give the promising compound. Against *C. lunata*, the 96 h inhibition rates of compounds **I-2**, **I-4**, **I-9**, **I-10**, and **I-12** were 40.7%~50.3%, which were equal to or higher than the positive control carbendazim (40.6%; a commercial fungicide). Generally, like the antifungal activity against *V. mali*, the antifungal activity of 5-cyanoindole (**1b**) and its derivatives **I-5**–**I-8** was worse than that of indole (**1a**) and its derivatives **I-1**–**I-4**, and 6-methylindole (**1c**) and its derivatives **I-9**–**I-12**. It suggested that introduction of the electron-withdrawing group (R^1 = 5-CN) on the phenyl of compounds **1a** and **I-1**–**I-4** will lead to less potent compounds. Furthermore, when compared with R^2 as the chlorine atom or the methoxy group, introduction of R^2 as the fluorine atom on the phenyl of compounds **I-1**, **I-5**, and **I-9** was necessary for the antifungal activity against *C. lunata*. For instance, the 96 h inhibition rates of compounds **I-2**, **I-6**, and **I-10** against *C. lunata* were 50.3%, 34.9%, and 42.0%, respectively; however,

the 96 h inhibition rates of compounds **I-3**/**I-4**, **I-7**/**I-8**, and **I-11**/**I-12** were 37.8%/40.8%, 30.1%/10.9%, and 32.3%/40.7%, respectively.

Table 1. Antifungal activities of **I-1**−**I-12** against two phytopathogenic fungi at 50 μg/mL for 96 h

Compound	Inhibition rate (%) [a]	
	V. mali	*C. lunata*
1a	31.7 ± 0.6	24.2 ± 3.1
1b	28.1 ± 5.0	7.0 ± 1.0
1c	48.4 ± 2.4	25.8 ± 1.5
I-1	50.7 ± 0.4	27.4 ± 0.3
I-2	34.9 ± 0.5	50.3 ± 0.3
I-3	12.8 ± 1.7	37.8 ± 0.2
I-4	8.4 ± 0.2	40.8 ± 0.2
I-5	0	23.9 ± 0.6
I-6	0	34.9 ± 0.1
I-7	1.6 ± 0.3	30.1 ± 0.9
I-8	1.5 ± 0.2	10.9 ± 0.3
I-9	23.3 ± 0.5	42.7 ± 0.3
I-10	8.7 ± 0.1	42.0 ± 0.3
I-11	2.2 ± 0.1	32.3 ± 0.3
I-12	23.9 ± 0.3	40.7 ± 0.1
carbendazim	100 ± 0	40.6 ± 0.1

[a]Values are mean ± SE of three replicates.

Table 2. Toxicity regression analysis of compound **I-1** at 96 h against *V. mali*

Compound	EC_{50} (μg/mL)	95% Confidence interval (μg/mL)	Toxic regression equation [a]	*r*
1a	78.97	68.58 – 91.92	Y = -6.853 + 3.612X	0.977
I-1	46.44	35.14 – 61.12	Y = -7.882 + 4.728X	0.992

[a] Regression analysis by IBM SPSS Statistics 20.0 ($P < 0.05$).

The toxicity regression analysis of compound **I-1** was conducted against *V. mali*. As shown in Table 2,

the EC_{50} value at 96 h of compound **I-1** against *V. mali* was 46.44 μg/mL, which was 1.7-fold higher antifungal activity of that of compound **Ia** (EC_{50}: 78.97 μg/mL).

In addition, the toxicity regression analysis of compound **I-2** was further done against *C. lunata*. As shown in Table 3, the EC_{50} value at 96 h of compound **I-2** against *C. lunata* was 46.10 μg/mL, which was 2.2-fold higher antifungal activity of that of compound **Ia** (EC_{50}: 100.74 μg/mL). Notably, the antifungal activity of compound **I-2** was more pronounced than that of the commercial fungicidal agent carbendazim (EC_{50} = 73.49 μg/mL). It suggested that compound **I-2** can be used as a lead for further optimization as a fungicidal agent against *C. lunata*.

Table 3. Toxicity regression analysis of **I-2** at 96 h against *C. lunata*

Compound	EC_{50} (μg/mL)	95% Confidence interval (μg/mL)	Toxic regression equation [a]	*r*
carbendazim	73.49	63.24 – 86.35	Y = -6.116 + 3.277X	0.985
1a	100.74	84.00 – 125.06	Y = -5.450 + 2.721X	0.975
I-2	46.10	40.51 – 52.38	Y = -6.911 + 4.154X	0.994

[a] Regression analysis by IBM SPSS Statistics 20.0 ($P < 0.05$).

In summary, to develop new natural-product-based antifungal candidates, a series of new thiosemicarbazone derivatives of 3-formyl-*N*-pyrazolylmethyleneindoles were prepared. Against *C. lunata*, compound **I-2** showed 2.2 folds antifungal activity compared to the precursor indole. Against *V. mali*, compound **I-1** exhibited 1.7 times antifungal activity compared to indole. Their structure-activity relationships suggested that R^1 as the electron-withdrawing group (R^1 = 5-CN) was not beneficial for the antifungal activities against *C. lunata* and *V. mali*; introduction of R^2 as the electron-withdrawing or electron-donating group on the phenyl of compound **I-1** cannot give the potent compounds against *V. mali*; introduction of R^2 as the fluorine atom on the phenyl of compounds **I-1**, **I-5**, and **I-9** was beneficial for the antifungal activity against *C. lunata*. These will lay the foundation for guiding the synthesis and application of indole derivatives as fungicidal agents in the future.

EXPERIMENTAL

3-Formylindoles (**2a**−**2c**) were prepared according to our previous method.[21] *Data for **2a***: ACS: 487-89-8. Yield: 92%, brown solid, mp 189−190 °C [lit., 190−192 °C];[21] *Data for **2b***: ACS: 17380-18-6. Yield: 90%, yellow solid, mp 249−250 °C [lit., 241−243 °C];[21] *Data for **2c***: ACS: 4771-49-7. Yield: 95%, yellow solid, mp 191−192 °C [lit., 187−189 °C].[21]

General procedure for synthesis of target compounds I-1−**I-12**

A mixture of **2a**−**2c** (1 mmol), **3a′**−**3d′** (1.2 mmol), and K_2CO_3 (2 mmol) in DMF (5 mL) was allowed to stir at 60 °C for 4~6 h. Then the mixture was poured into ice water (50 mL), and the precipitated product was collected and washed with ice water to give compounds **I′** in 33~49% yields, which were used directly for the next step without further purification. A mixture of **I′** (1 mmol), **4** (1 mmol), and acetic acid (2 drops) in absolute EtOH (5 mL) was refluxed for 1~2 h. When the reaction was complete checked by TLC analysis, the mixture was allowed to cool and filtered to give the solid, which was further recrystallized from absolute EtOH to afford target compounds **I-1**−**I-12** in 73~91% yields.

Data for **I-1**: Yield: 82%, white solid, mp 239−240 °C; IR cm^{-1} (KBr): 3488, 3421, 3058, 2861, 2356, 1611, 1569, 1448, 1383, 1339, 1274, 1229, 1169, 831, 747, 686; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.18 (s, 1H), 8.50 (s, 1H), 8.27 (d, J = 8.0 Hz, 1H), 8.23 (s, 1H), 8.04 (s, 1H), 7.86 (d, J = 8.0 Hz, 2H), 7.72 (s, 1H), 7.66 (d, J = 7.0 Hz, 2H), 7.47-7.52 (m, 3H), 7.38-7.44 (m, 4H), 7.33 (t, J = 7.5 Hz, 1H), 7.23 (t, J = 7.5 Hz, 1H), 7.18 (t, J = 7.5 Hz, 1H), 5.51 (s, 2H). HRMS [ESI]: calcd for $C_{26}H_{22}N_6NaS$ ($[M + Na]^+$), 473.1519; found, 473.1524.

Data for **I-2**: Yield: 87%, white solid, mp 237−238 °C; IR cm^{-1} (KBr): 3486, 3419, 3054, 2828, 2356, 1612, 1446, 1377, 1335, 1276, 1228, 1169, 832, 741, 675; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.13 (s, 1H), 8.50 (s, 1H), 8.24 (d, J = 8.0 Hz, 1H), 8.20 (s, 1H), 7.99 (s, 1H), 7.85 (d, J = 8.0 Hz, 2H), 7.70 (s, 1H), 7.67-7.69 (m, 2H), 7.47-7.51 (m, 3H), 7.38 (s, 1H), 7.34 (t, J = 7.5 Hz, 1H), 7.23-7.27 (m, 2H), 7.23 (t, J = 7.5 Hz, 1H), 7.17 (t, J = 7.5 Hz, 1H), 5.50 (s, 2H). HRMS [ESI]: calcd for $C_{26}H_{21}FN_6NaS$ ($[M + Na]^+$), 491.1425; found, 491.1435.

Data for **I-3**: Yield: 73%, yellow solid, mp 246−247 °C; IR cm^{-1} (KBr): 3514, 3476, 3391, 3072, 2356, 1604, 1545, 1449, 1320, 1272, 1170, 1089, 834, 802, 738, 682, 624; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.18 (s, 1H), 8.50 (s, 1H), 8.26 (d, J = 7.5 Hz, 1H), 8.22 (s, 1H), 8.04 (s, 1H), 7.86 (d, J = 8.0 Hz, 2H), 7.73 (s, 1H), 7.69 (d, J = 8.0 Hz, 2H), 7.47-7.52 (m, 5H), 7.42 (s, 1H), 7.34 (t, J = 7.5 Hz, 1H), 7.21-7.24 (m, 1H), 7.18 (t, J = 8.0 Hz, 1H), 5.52 (s, 2H). HRMS [ESI]: calcd for $C_{26}H_{21}ClN_6NaS$ ($[M + Na]^+$), 507.1129; found, 507.1136.

Data for **I-4**: Yield: 88%, white solid, mp 225−226 °C; IR cm^{-1} (KBr): 3464, 3062, 2356, 1612, 1444, 1372, 1336, 1257, 1171, 828, 799, 740, 671; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.17 (s, 1H), 8.47 (s, 1H), 8.27 (d, J = 8.0 Hz, 1H), 8.22 (s, 1H), 8.03 (s, 1H), 7.85 (d, J = 8.0 Hz, 2H), 7.70 (s, 1H), 7.57-7.58 (m, 2H), 7.47-7.51 (m, 3H), 7.42 (s, 1H), 7.32 (t, J = 7.5 Hz, 1H), 7.24 (t, J = 7.5 Hz, 1H), 7.19 (t, J = 7.5 Hz, 1H), 6.96-6.98 (m, 2H), 5.47 (s, 2H), 3.78 (s, 3H). HRMS [ESI]: calcd for $C_{27}H_{24}N_6NaOS$ ($[M + Na]^+$), 503.1625; found, 503.1633.

Data for **I-5**: Yield: 86%, white solid, mp > 300 °C; IR cm^{-1} (KBr): 3494, 3061, 2356, 1608, 1444, 1372, 1335, 1257, 1171, 829, 803, 740, 672; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.19 (s, 1H), 8.81 (s, 1H), 8.49 (s, 1H), 8.19 (s, 1H), 8.08 (s, 1H), 7.90 (s, 1H), 7.85-7.86 (m, 3H), 7.70 (d, J = 8.8 Hz, 1H), 7.62-7.64 (m,

2H), 7.58 (d, *J* = 8.8 Hz, 1H), 7.48-7.52 (m, 2H), 7.37-7.44 (m, 3H), 7.34 (d, *J* = 7.2 Hz, 1H), 5.59 (s, 2H). HRMS [ESI]: calcd for $C_{27}H_{21}N_7NaS$ ([M + Na]$^+$), 498.1471; found, 498.1480.

Data for **I-6**: Yield: 85%, yellow solid, mp 257−258 ºC; IR cm^{-1} (KBr): 3461, 3307, 2922, 2356, 2229, 1602, 1542, 1453, 1340, 1276, 1221, 1169, 834, 741, 680, 644; ^{1}H NMR (500 MHz, DMSO-*d*$_6$) *δ*: 11.15 (s, 1H), 8.79 (s, 1H), 8.47 (s, 1H), 8.20 (s, 1H), 8.03 (s, 1H), 7.89 (s, 1H), 7.82-7.85 (m, 3H), 7.70 (s, 1H), 7.65-7.68 (m, 2H), 7.58 (d, *J* = 8.5 Hz, 1H), 7.48-7.51 (m, 2H), 7.34 (t, *J* = 7.5 Hz, 1H), 7.23-7.26 (m, 2H), 5.58 (s, 2H). HRMS [ESI]: calcd for $C_{27}H_{20}FN_7NaS$ ([M + Na]$^+$), 516.1377; found, 516.1372.

Data for **I-7**: Yield: 91%, white solid, mp 264−265 ºC; IR cm^{-1} (KBr): 3519, 3468, 3382, 3118, 3025, 2978, 2356, 2221, 1604, 1544, 1497, 1451, 1342, 1275, 1173, 817, 754, 690, 637; ^{1}H NMR (600 MHz, DMSO-*d*$_6$) *δ*: 11.19 (s, 1H), 8.82 (d, *J* = 1.8 Hz, 1H), 8.47 (s, 1H), 8.20 (s, 1H), 8.07 (s, 1H), 7.93 (s, 1H), 7.84-7.86 (m, 3H), 7.71-7.73 (m, 2H), 7.66-7.67 (m, 2H), 7.47-7.51 (m, 4H), 7.31-7.34 (m, 1H), 5.60 (s, 2H). HRMS [ESI]: calcd for $C_{27}H_{20}ClN_7NaS$ ([M + Na]$^+$), 532.1082; found, 532.1090.

Data for **I-8**: Yield: 87%, white solid, mp 248−249 ºC; IR cm^{-1} (KBr): 3642, 3421, 3306, 2970, 2356, 2229, 1600, 1541, 1494, 1452, 1340, 1275, 1221, 1169, 834, 740, 676, 646; ^{1}H NMR (600 MHz, DMSO-*d*$_6$) *δ*: 11.19 (s, 1H), 8.82 (d, *J* = 1.8 Hz, 1H), 8.47 (s, 1H), 8.21 (s, 1H), 8.08 (s, 1H), 7.90 (s, 1H), 7.87 (s, 1H), 7.83-7.85 (m, 2H), 7.71 (d, *J* = 8.4 Hz, 1H), 7.58-7.59 (m, 1H), 7.56 (d, *J* = 9.0 Hz, 2H), 7.84-7.51 (m, 2H), 7.30-7.32 (m, 1H), 6.98 (d, *J* = 9.0 Hz, 2H), 5.55 (s, 2H), 3.78 (s, 3H). HRMS [ESI]: calcd for $C_{28}H_{23}N_7NaOS$ ([M + Na]$^+$),528.1577; found, 528.1593.

Data for **I-9**: Yield: 77%, white solid, mp 229−230 ºC; IR cm^{-1} (KBr): 3489, 3420, 3358, 3057, 2356, 2228, 1610, 1566, 1491, 1444, 1367, 1337, 1274, 1230, 1168, 815, 757, 682; ^{1}H NMR (500 MHz, DMSO-*d*$_6$) *δ*: 11.12 (s, 1H), 8.49 (s, 1H), 8.18 (s, 1H), 8.09 (d, *J* = 8.0 Hz, 1H), 7.97 (s, 1H), 7.85 (d, *J* = 8.0 Hz, 2H), 7.66 (s, 1H), 7.65 (d, *J* = 4.0 Hz, 2H), 7.48-7.51 (m, 2H), 7.42-7.47 (m, 3H), 7.36 (s, 1H), 7.33 (t, *J* = 7.5 Hz, 1H), 7.17 (s, 1H), 6.98 (d, *J* = 8.0 Hz, 1H), 5.45 (s, 2H), 2.35 (s, 3H). HRMS [ESI]: calcd for $C_{27}H_{25}N_6S$ ([M + H]$^+$), 465.1856; found, 465.1865.

Data for **I-10**: Yield: 79%, yellow solid, mp 231−232 ºC; IR cm^{-1} (KBr): 3493, 3352, 3172, 2977, 2915, 2356, 1609, 1560, 1506, 1447, 1382, 1335, 1276, 1227, 1164, 830, 758, 685, 604; ^{1}H NMR (600 MHz, DMSO-*d*$_6$) *δ*: 11.16 (s, 1H), 8.50 (s, 1H), 8.18 (s, 1H), 8.11 (d, *J* = 8.4 Hz, 1H), 8.03 (s, 1H), 7.83-7.85 (m, 2H), 7.69-7.71 (m, 2H), 7.65 (s, 1H), 7.49-7.51 (m, 2H), 7.39 (d, *J* = 3.0 Hz, 1H), 7.31-7.34 (m, 1H), 7.27-7.30 (m, 2H), 7.17 (s, 1H), 6.97-6.99 (m, 1H), 5.44 (s, 2H), 2.36 (s, 3H). HRMS [ESI]: calcd for $C_{27}H_{24}FN_6S$ ([M + H]$^+$), 483.1762; found, 483.1774.

Data for **I-11**: Yield: 80%, white solid, mp 235−236 ºC; IR cm^{-1} (KBr): 3514, 3477, 3423, 3393, 2356, 1607, 1549, 1501, 1447, 1395, 1349, 1274, 1167, 832, 806, 749, 682, 625; ^{1}H NMR (600 MHz, DMSO-*d*$_6$) *δ*: 11.15 (s, 1H), 8.49 (s, 1H), 8.18 (s, 1H), 8.11 (d, *J* = 8.4 Hz, 1H), 8.02 (s, 1H), 7.83-7.85 (m, 2H), 7.69-7.70 (m, 2H), 7.67 (s, 1H), 7.50-7.52 (m, 4H), 7.38 (d, *J* = 3.0 Hz, 1H), 7.31-7.34 (m, 1H), 7.17

(s, 1H), 6.97-6.98 (m, 1H), 5.46 (s, 2H), 2.36 (s, 3H). HRMS [ESI]: calcd for $C_{27}H_{23}ClN_6NaS$ ([M + Na]$^+$), 521.1286; found, 521.1302.

Data for **I-12**: Yield: 83%, white solid, mp 219−220 ºC; IR cm^{-1} (KBr): 3473, 3419, 2356, 1611, 1574, 1493, 1444, 1371, 1337, 1255, 1171, 827, 753, 672; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.14 (s, 1H), 8.48 (s, 1H), 8.17 (s, 1H), 8.11 (d, J = 8.0 Hz, 1H), 8.01 (s, 1H), 7.82-7.84 (m, 2H), 7.64 (s, 1H), 7.56-7.59 (m, 2H), 7.47-7.51 (m, 2H), 7.37 (s, 1H), 7.32 (d, J = 7.2 Hz, 1H), 7.17 (s, 1H), 7.01 (d, J = 8.8 Hz, 2H), 6.98 (d, J = 8.8 Hz, 1H), 5.41 (s, 2H), 3.79 (s, 3H), 2.35 (s, 3H). HRMS [ESI]: calcd for $C_{28}H_{26}N_6NaOS$ ([M + Na]$^+$), 517.1781; found, 517.1793.

Biological assay

In vitro antifungal activities of compounds **1a**−**1c** *and* **I-1**−**I-12** *against C. lunata and V. mali*. The antifungal activities were conducted according to the mycelial growth method.[17,23] Firstly, 4.5 mg of tested compounds (carbendazim as a positive control) was dissolved in 2 mL of acetone (containing 10% DMSO), and their concentrations were at 50 μg/mL in the 90 mL of potato dextrose agar (PDA) medium. Whereafter, 15 mL of medium was poured into each Petri dish, and 2 mL of acetone (containing 10% DMSO) in 90 mL of PDA medium was set as the blank control group (CK). Subsequently, the mycelial disks of *C. lunata* or *V. mali* of 4 mm of diameter were inoculated in the center of the Petri dishes (three replicates for each treatment). Finally, all Petri dishes were cultured in an incubator at 26 ± 1 °C for 96 h and their mycelial colony diameters were measured. Their inhibition rate values were calculated as follows:

$$\text{Inhibition rate (\%)} = \frac{(C - 4) - (T - 4)}{C - 4} \times 100\%$$

where C and T represent the average colony diameters of the blank control and the treated groups, respectively. Finally, the linear regressions of inhibition rates (%) at 96 h against *C. lunata* and *V. mali* versus five concentrations of some potent compounds and carbendazim were conducted, and their corresponding EC_{50} values were obtained.

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