

CHEMICAL REACTIVITY AND APPLICATION OF 4-ALKYLIDENE-3H-PYRAZOL-3-ONES: SYNTHESIS AND ANTIFUNGAL ACTIVITY OF POLYSUBSTITUTED PYRAZOLES

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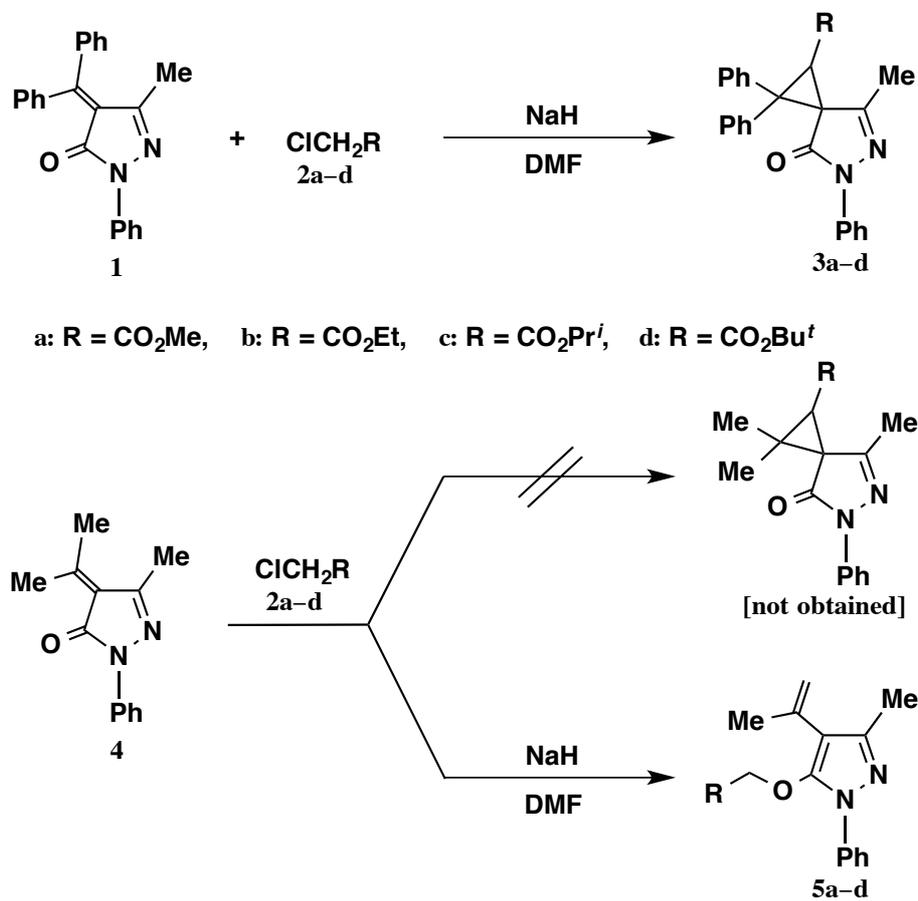
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Abstract – Chemical reactivity and application of 4-alkylidene-3H-pyrazol-3-ones are described. Furthermore, twelve of the newly synthesized *O*-substituted pyrazoles were evaluated for their antifungal activity *in vitro* against *Candida albicans* and *Saccharomyces cerevisiae*.

Pyrazoles are important class of heterocyclic compounds, which found a widespread use in various applications. Its derivatives have certainly been shown to exhibit various pharmaceuticals and biological activities.¹ The pyrazole ring is a constituent of a variety of natural products such as pyrazomycin² and L-β-(1-pyrazolyl)alanine.³ In this context, the synthesis of pyrazole derivatives continues to attract attention and provides an interesting challenge.⁴

In the course of our attempts to utilize the characteristic property of 3H-pyrazol-3-ones, we have discussed the synthesis of 1-acyl-1,2-dihydro-3H-pyrazol-3-ones through Lewis acid-mediated rearrangement of 3-acyloxy pyrazoles.⁵ More recently, as part of our study using 4-arylidene-3H-pyrazol-3-one as the starting material for the preparation of spiro-derivatives of pyrazole, we have reported a simple method for the synthesis of new spirocyclopropanepyrazoles **3a–d**, followed by the reaction of 4-arylidene-3H-pyrazol-3-one **1** with α-chloro esters **2a–d** (Scheme 1).⁶ The synthesis of 4-alkylidene- and 4-arylidene-3H-pyrazol-3-ones has been the subject of numerous publications over the past several decades.⁷ While a great number of compounds of this structural type have been prepared, there are relatively few reports describing the chemical reactivity of these compounds, particularly the 4-alkylidene derivatives.^{7d,7f,8} Thus, to expand the scope application of reactions of 4-alkylidene-3H-pyrazol-3-ones, we

focused our attention on the chemical reactivity of 4-alkylidene-3*H*-pyrazol-3-ones in the presence of a base and now present the results of our investigation, an efficient method for preparing new polysubstituted pyrazole derivatives with antifungal activity.



Scheme 1

The starting materials, 4-alkylidene derivatives **4**, **6**, and **8**, were prepared by treatment of 2,4-dihydro-5-methyl-2-phenyl-3*H*-pyrazol-3-one and ketones such as acetone, cyclopentanone, and cycloheptanone according to the method reported in literature.⁷ With the aim of extending the preparation of spiro-derivatives of pyrazole, we examined the reaction of **4** with α -chloro esters **2a-d**, but the reaction mode was completely changed (Scheme 1). Indeed, when a mixture of 4-isopropylidene-3*H*-pyrazol-3-one **4** with **2a-d** in the presence of NaH in DMF was stirred at 80 °C for 1 h, the corresponding *O*-substituted pyrazoles **5a-d** were isolated in 44, 37, 27, and 32% yields, respectively. In this case, the reaction was not clean and the expected spiro-compounds could not be detected at all.

The skeleton of **4** probably exists in three tautomeric forms as shown in Figure 1. However, our observation indicates that in the presence of a base, a deprotonation of **4** easily occurs and then *O*-substituted pyrazoles **5a-d** would be produced from OH form *via* *O*-attack nucleophilic substitution.

The reason for this chemical reactivity of **4** is not clear at present. Thus, it seems that the straightforward preparation of spiro-compounds⁹ by the reaction of **4** with **2a–d** is not easy.

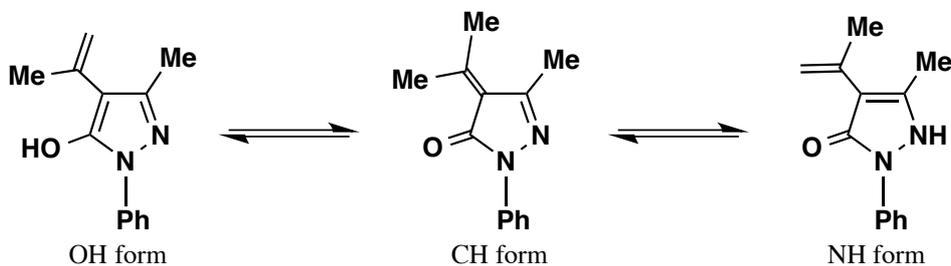
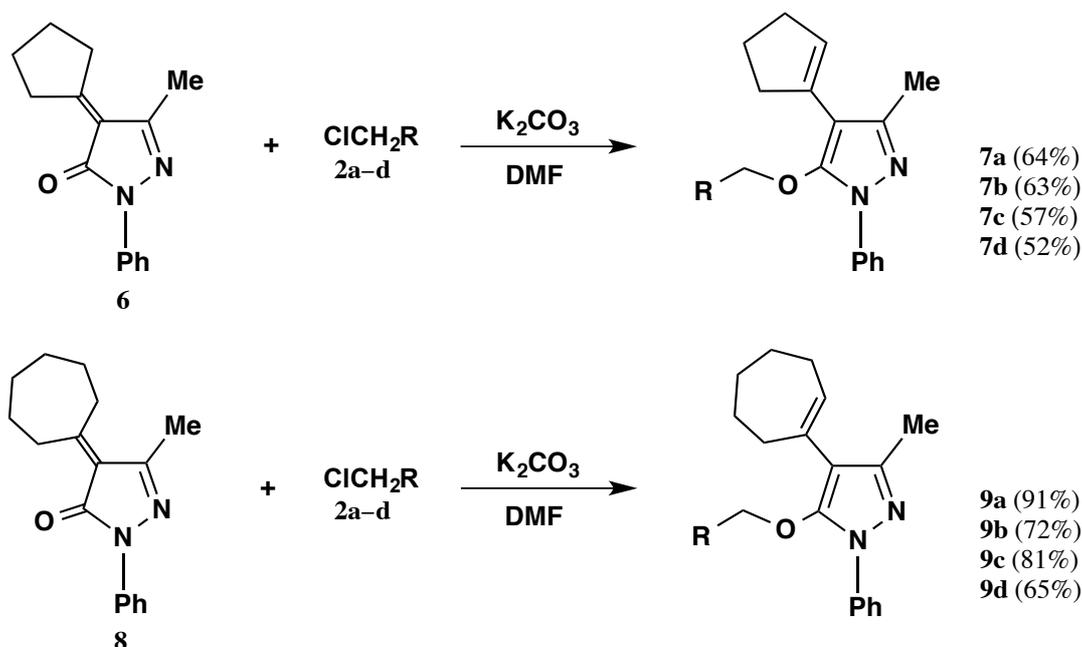


Figure 1. Tautomeric forms of compound **4**

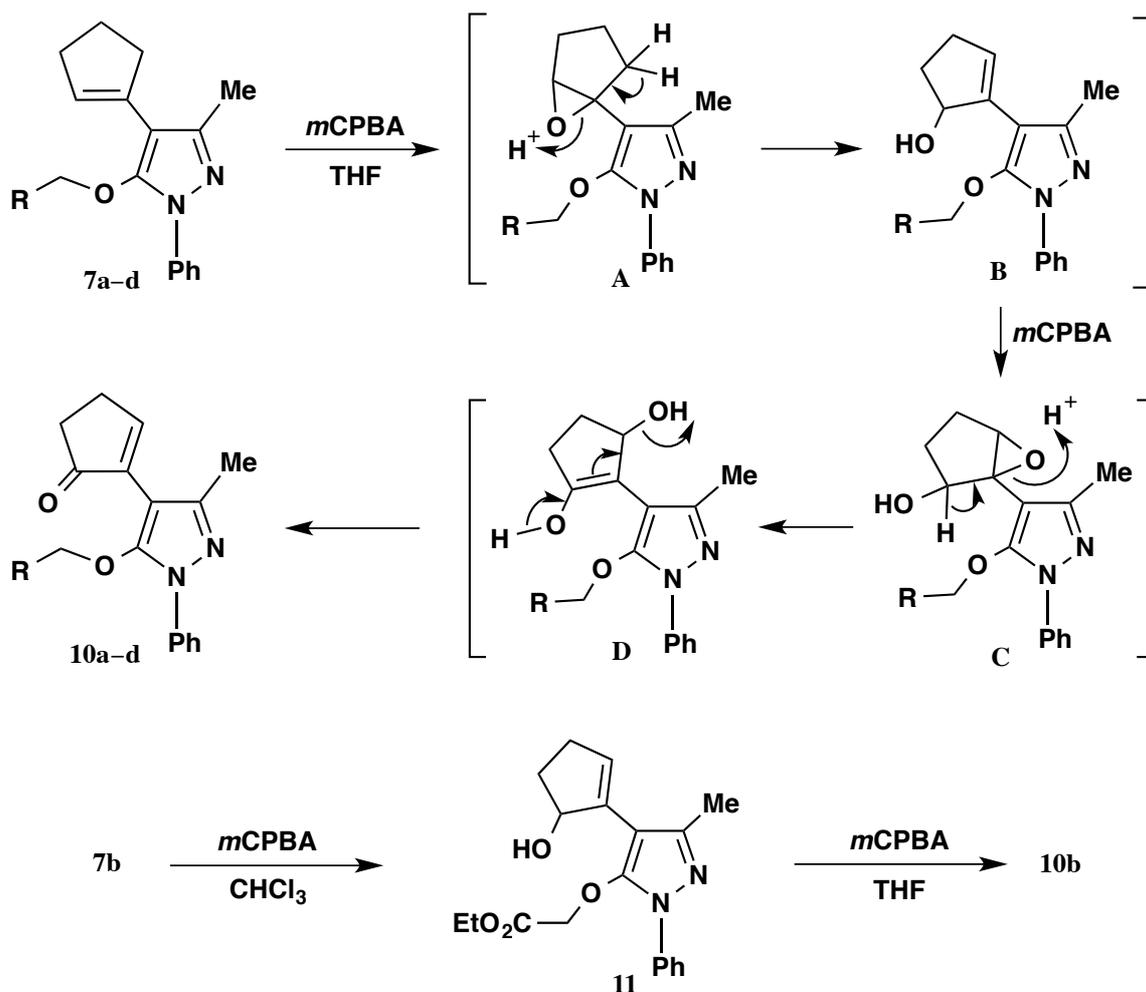


a: R = CO₂Me, b: R = CO₂Et, c: R = CO₂Prⁱ, d: R = CO₂Bu^t

Scheme 2

Subsequently, the reactions of 4-cyclopentylidene- and 4-cycloheptylidene-3*H*-pyrazol-3-ones **6** and **8** with **2a–d** in the presence of K₂CO₃ gave the corresponding *O*-substituted pyrazoles **7a–d** and **9a–d** in moderate to good yields (Scheme 2). These products **5a–d**, **7a–d**, and **9a–d** gave satisfactory elemental analyses and spectroscopic data consistent with their assigned structures. For examples, the IR spectra of **7a–d** display bands in the range of 1730–1770 cm⁻¹ due to the ester carbonyl group. The ¹H NMR spectra of **7a–d** exhibit a two-proton singlet near δ 4.4 attributable to the methylene protons between oxygen atom and carbonyl group and a one-proton singlet near δ 5.8 due to the olefin proton. The ¹³C

NMR spectra of **7a–d** show a signal near δ 70 due to the methylene carbon between oxygen atom and carbonyl group, a signal near δ 104 due to the pyrazole C-4 carbon, a signal near δ 128 due to the cyclopentene C-2 carbon, a signal near δ 133 due to the cyclopentene C-1 carbon, and a signal near δ 167 due to the ester carbonyl carbon (see experimental section).



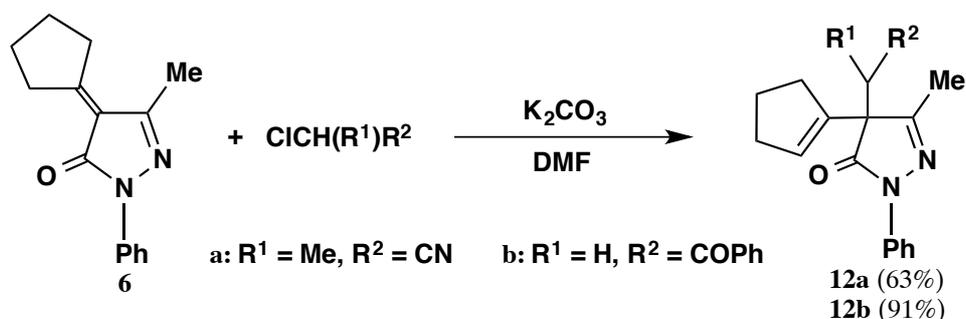
a: R = CO_2Me , b: R = CO_2Et , c: R = CO_2Pr^i , d: R = CO_2Bu^t

Scheme 3

During our study of the synthesis of polysubstituted pyrazoles, we found that *O*-substituted pyrazoles **7a–d** were reacted with *m*-chloroperbenzoic acid (*m*CPBA) to afford the corresponding 4-(2-cyclopenten-1-one)-substituted pyrazoles **10a–d**. After some optimization, the best results were obtained when **7a–d** were treated with an excess amount of *m*CPBA in THF, cyclopentenone-containing pyrazoles **10a–d** were isolated in 73, 79, 92, and 89% yields (Scheme 3). The IR spectra of **10a–d** display a carbonyl band of cyclopentenone at 1706 or 1708 cm^{-1} . The ^{13}C NMR spectra of **10a–d** show a signal at δ 207.8 due to

the carbonyl carbon of cyclopentenone. By comparison of NMR spectra, MS spectra, and elemental analyses of **10a–d** it seems that the structural assignments given to these compounds are correct.

A plausible reaction mechanism for the formation of **10a–d** is illustrated in Scheme 3. The reaction of **7a–d** with *m*CPBA probably causes an epoxidation to give the epoxides **A**, which would undergo ring opening *via* a protonation to afford the intermediate hydroxy cyclic alkenes **B**. An epoxidation of **B** again produces the epoxides **C**, which would undergo a protonation/ring-opening reaction to provide the dihydroxy cyclic alkenes **D**. A dehydration of **D** easily occurs and then 4-(2-cyclopenten-1-one)-substituted pyrazoles **10a–d** would be formed. To our knowledge, this type of tandem epoxidation reaction between a cyclic alkene and peroxides has not been described previously. Fortunately, we found the reaction condition under which the key intermediate cyclopentenol-containing pyrazole **11** could be isolated. Thus, when **7b** was treated with 1.0 equivalent of *m*CPBA in CHCl_3 at 0–5 °C overnight, **11** was obtained in 93% yield. Treatment of compound **11** with 1.0 equivalent of *m*CPBA in THF produced **10b** (84%), which was identical with an authentic sample prepared from **7b** and an excess amount of *m*CPBA in THF according to Scheme 3. Therefore, it appears that 4-(2-cyclopenten-1-one)-substituted pyrazoles **10a–d** may be formed directly from *O*-substituted pyrazoles **7a–d**, perhaps by a mechanism involving epoxidation/ring-opening to cyclopentenol-containing pyrazoles, followed by an epoxidation/dehydration sequence.



Scheme 4

In addition to the reactions described above, the behavior of the other reagents was also investigated. Interestingly, when a mixture of compound **6** with 2-chloropropionitrile or 2-chloroacetophenone in the presence of K_2CO_3 in DMF was stirred at 80 °C for 1 h or room temperature overnight, the corresponding *C*-substituted pyrazoles **12a,b** were isolated in 63 and 91% yields, respectively (Scheme 4). In this case, *O*-substituted pyrazoles such as **7a–d** could not be obtained at all. The reason for this change of behavior is not clear at present.

Table 1. *In vitro* antifungal activity of **5a–d**, **7a–d**, and **9a–d** against *C. albicans* and *S. cerevisiae*.

Entry	Compound	MIC ($\mu\text{g/mL}$)	
		<i>C. albicans</i> ^a	<i>S. cerevisiae</i> ^b
1	5a	13 ^c	13 ^c
2	5b	13 ^c	13 ^c
3	5c	13 ^c	13 ^c
4	5d	13 ^c	13 ^c
5	7a	13 ^c	13 ^c
6	7b	13 ^c	13 ^c
7	7c	13 ^c	13 ^c
8	7d	13 ^c	13 ^c
9	9a	13 ^c	13 ^c
10	9b	13 ^c	13 ^c
11	9c	13 ^c	13 ^c
12	9d	13 ^c	13 ^c
13	Miconazole	2 ^d	0.5 ^e
14	Itraconazole	2 ^d	4 ^f
15	DMSO	> 12.5%	> 6.25%
16	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.

Twelve of the newly synthesized *O*-substituted pyrazole derivatives **5a–d**, **7a–d**, and **9a–d** were tested for their antifungal activity *in vitro* 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-A2¹⁰ and our previous paper.⁶ Miconazole and Itraconazole were used as standard drugs for comparison of the antifungal activity. The results obtained are summarized in Table 1. All the synthesized compounds were active against both *Candida* and *Saccharomyces* with MIC \geq 13 $\mu\text{g/mL}$ (entries 1–12). It is worth noting that the entire tested compounds **5a–d**, **7a–d**, and **9a–d** showed more potent antifungal activity than those of the spirocyclopropanepyrazoles **3a–d**, which were active against *Candida* with MIC \geq 25 $\mu\text{g/mL}$ and *Saccharomyces* with MIC \geq 50 $\mu\text{g/mL}$.⁶

In conclusion, we have demonstrated the reactions of 4-alkylidene-3*H*-pyrazol-3-ones **4**, **6**, and **8** with α -chloro esters in the presence of a base. This methodology offers significant advantages with regard to the supply of polysubstituted pyrazole derivatives, 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 showed that polysubstituted pyrazoles **5a–d**, **7a–d**, and **9a–d** were moderately to fairly active against the tested fungi whereas the reference drug such as Miconazole and Itraconazole exhibited good to excellent results against both the fungi. Our results suggest that **5a–d**, **7a–d**, and **9a–d**

would play a role *in vivo*. At the present time, we continue to investigate the applications of 4-alkylidene-3*H*-pyrazol-3-ones.

EXPERIMENTAL

The IR spectra were recorded on a JASCO FT/IR-4100 spectrometer. The ¹H and ¹³C NMR spectra were measured with a JEOL JNM-A500 spectrometer at 500 and 125 MHz, respectively. The ¹H and ¹³C 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 starting compounds, 4-alkylidene-3*H*-pyrazol-3-ones **4**, **6**, and **8**, were prepared in this laboratory according to the procedure reported in literature.⁷

General procedure for the preparation of *O*-substituted pyrazoles **5a–d from 4-isopropylidene-3*H*-pyrazol-3-one **4** and α-chloro esters **2a–d**.** To an ice-cooled and stirred solution of **4** (0.428 g, 2 mmol) in DMF (10 mL), 60% NaH (0.160 g, 4 mmol) was added. The stirring was continued at rt until evolution of gas ceased. To the obtained solution, methyl chloroacetate (**2a**) (0.651 g, 6 mmol), ethyl chloroacetate (**2b**) (0.735 g, 6 mmol), isopropyl chloroacetate (**2c**) (0.819 g, 6 mmol), or *tert*-butyl chloroacetate (**2d**) (0.904 g, 6 mmol) was added with stirring and ice-cooling, and then the mixture was stirred at 80 °C for 1 h. After removal of the solvent *in vacuo*, cold H₂O was added to the residue. The resulting mixture was extracted with CHCl₃ (60 mL). The extract was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with CHCl₃ as the eluent to give **5a–d**.

Methyl {[3-methyl-1-phenyl-4-(propen-2-yl)-1*H*-pyrazol-5-yl]oxy}acetate (5a**):** Pale yellow oil (0.252 g, 44%); IR (neat): ν 1765, 1745 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 2.07 [dd, *J* = 0.9, 1.5 Hz, 3H, CH₂=C(*Me*)-], 2.27 (s, 3H, pyrazole 3-Me), 3.67 (s, 3H, OMe), 4.54 (s, 2H, OCH₂), 5.04–5.05 (m, 1H, olefin H), 5.20–5.21 (m, 1H, olefin H), 7.26–7.28 (m, 1H, Ph-H), 7.40–7.43 (m, 2H, Ph-H), 7.70–7.72 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.9 (pyrazole 3-Me), 23.5 [CH₂=C(*Me*)-], 52.1 (CO₂*Me*), 69.2 (OCH₂), 108.2 (pyrazole C-4), 116.2 (CH₂=), 122.5, 126.5, 128.9 (Ph-C), 135.6 [CH₂=C(*Me*)-], 138.5 (Ph-C), 146.9 (pyrazole C-3), 148.9 (pyrazole C-5), 168.1 (C=O); MS: *m/z* 287 [M+H]⁺. Anal. Calcd for C₁₆H₁₈N₂O₃: C, 67.12; H, 6.34; N, 9.78. Found: C, 67.01; H, 6.36; N, 9.76.

Ethyl {[3-methyl-1-phenyl-4-(propen-2-yl)-1*H*-pyrazol-5-yl]oxy}acetate (5b**):** Pale yellow oil (0.222 g, 37%); IR (neat): ν 1762, 1739 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.20 (t, *J* = 7.0 Hz, 3H, CO₂CH₂*Me*), 2.08 [dd, *J* = 0.9, 1.5 Hz, 3H, CH₂=C(*Me*)-], 2.27 (s, 3H, pyrazole 3-Me), 4.15 (q, *J* = 7.0 Hz, 2H, CO₂CH₂*Me*), 4.54 (s, 2H, OCH₂), 5.04–5.06 (m, 1H, olefin H), 5.21–5.22 (m, 1H, olefin H), 7.26–7.28 (m, 1H, Ph-H), 7.40–7.43 (m, 2H, Ph-H), 7.72–7.74 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.9 (pyrazole 3-Me), 14.0 (CO₂CH₂*Me*), 23.6 [CH₂=C(*Me*)-], 61.4 (CO₂CH₂*Me*), 69.3 (OCH₂), 108.1 (pyrazole C-4), 116.4 (CH₂=),

122.5, 126.5, 128.9 (Ph-C), 135.6 [CH₂=C(Me)-], 138.5 (Ph-C), 146.9 (pyrazole C-3), 149.0 (pyrazole C-5), 167.6 (C=O); MS: *m/z* 301 [M+H]⁺. Anal. Calcd for C₁₇H₂₀N₂O₃: C, 67.98; H, 6.71; N, 9.33. Found: C, 67.78; H, 6.71; N, 9.31.

Isopropyl {[3-methyl-1-phenyl-4-(propen-2-yl)-1*H*-pyrazol-5-yl]oxy}acetate (5c): Pale yellow oil (0.170 g, 27%); IR (neat): ν 1757, 1735 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.18 (d, *J* = 6.1 Hz, 6H, CO₂CHMe₂), 2.07 [dd, *J* = 0.9, 1.5 Hz, 3H, CH₂=C(Me)-], 2.27 (s, 3H, pyrazole 3-Me), 4.53 (s, 2H, OCH₂), 5.02–5.07 (m, 2H, olefin H and CO₂CHMe₂), 5.21–5.23 (m, 1H, olefin H), 7.25–7.28 (m, 1H, Ph-H), 7.39–7.42 (m, 2H, Ph-H), 7.73–7.75 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.8 (pyrazole 3-Me), 21.6 (CO₂CHMe₂), 23.6 [CH₂=C(Me)-], 69.2 (CO₂CHMe₂), 69.4 (OCH₂), 108.0 (pyrazole C-4), 116.5 (CH₂=), 122.4, 126.4, 128.9 (Ph-C), 135.6 [CH₂=C(Me)-], 138.5 (Ph-C), 146.9 (pyrazole C-3), 149.0 (pyrazole C-5), 167.2 (C=O); MS: *m/z* 315 [M+H]⁺. Anal. Calcd for C₁₈H₂₂N₂O₃: C, 68.77; H, 7.05; N, 8.91. Found: C, 68.64; H, 7.12; N, 8.93.

***tert*-Butyl {[3-methyl-1-phenyl-4-(propen-2-yl)-1*H*-pyrazol-5-yl]oxy}acetate (5d):** Pale yellow oil (0.210 g, 32%); IR (neat): ν 1756, 1731 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.41 (s, 9H, CO₂CMe₃), 2.07 (m, 3H, CH₂=C(Me)-), 2.27 (s, 3H, pyrazole 3-Me), 4.45 (s, 2H, OCH₂), 5.04–5.05 (m, 1H, olefin H), 5.22–5.23 (m, 1H, olefin H), 7.25–7.27 (m, 1H, Ph-H), 7.39–7.42 (m, 2H, Ph-H), 7.74–7.76 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.8 (pyrazole 3-Me), 23.7 [CH₂=C(Me)-], 27.8, 28.0, 28.1 (CO₂CMe₃), 69.7 (OCH₂), 82.5 (CO₂CMe₃), 107.9 (pyrazole C-4), 116.5 (CH₂=), 122.4, 126.4, 128.9 (Ph-C), 135.7 [CH₂=C(Me)-], 138.5 (Ph-C), 146.9 (pyrazole C-3), 149.1 (pyrazole C-5), 166.7 (C=O); MS: *m/z* 329 [M+H]⁺. Anal. Calcd for C₁₉H₂₄N₂O₃: C, 69.49; H, 7.37; N, 8.53. Found: C, 69.49; H, 7.31; N, 8.59.

General procedure for the preparation of *O*-substituted pyrazoles 7a–d and 9a–d from 4-cyclopentylidene- or 4-cycloheptylidene-3*H*-pyrazol-3-one 6 or 8 and α -chloro esters 2a–d. A mixture of **6** (0.480 g, 2 mmol) or **8** (0.536 g, 2 mmol), methyl chloroacetate (**2a**) (0.651 g, 6 mmol), ethyl chloroacetate (**2b**) (0.735 g, 6 mmol), isopropyl chloroacetate (**2c**) (0.819 g, 6 mmol), or *tert*-butyl chloroacetate (**2d**) (0.904 g, 6 mmol), and K₂CO₃ (0.553 g, 4 mmol) in DMF (10 mL) was stirred at rt overnight. After removal of the solvent *in vacuo*, cold H₂O was added to the residue. The resulting mixture was extracted with CHCl₃ (60 mL). The extract was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with CHCl₃ as the eluent to afford **7a–d** and **9a–d**.

Methyl {[4-(cyclopenten-1-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-yl]oxy}acetate (7a): Pale yellow oil (0.401 g, 64%); IR (neat): ν 1766, 1745 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.94–1.99 (m, 2H, cyclopentene 4-H), 2.31 (s, 3H, pyrazole 3-Me), 2.46–2.51 (m, 2H, cyclopentene 3- or 5-H), 2.65–2.69 (m, 2H, cyclopentene 3- or 5-H), 3.68 (s, 3H, OMe), 4.44 (s, 2H, OCH₂), 5.70–5.86 (m, 1H, olefin H), 7.25–7.29

(m, 1H, Ph-H), 7.37–7.43 (m, 2H, Ph-H), 7.69–7.71 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 14.6 (pyrazole 3-Me), 23.4 (cyclopentene C-4), 32.9, 35.3 (cyclopentene C-3 and -5), 52.1 (CO₂Me), 69.5 (OCH₂), 104.4 (pyrazole C-4), 122.4, 126.6 (Ph-C), 128.2 (cyclopentene C-2), 129.0 (Ph-C), 133.3 (cyclopentene C-1), 138.4 (Ph-C), 147.4 (pyrazole C-3), 149.1 (pyrazole C-5), 167.9 (C=O); MS: *m/z* 313 [M+H]⁺. Anal. Calcd for C₁₈H₂₀N₂O₃: C, 69.21; H, 6.45; N, 8.97. Found: C, 69.09; H, 6.53; N, 8.93.

Ethyl {[4-(cyclopenten-1-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-yl]oxy}acetate (7b): Pale yellow oil (0.410 g, 63%); IR (neat): ν 1762, 1739 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.20 (t, *J* = 7.0 Hz, 3H, CO₂CH₂Me), 1.95–2.00 (m, 2H, cyclopentene 4-H), 2.31 (s, 3H, pyrazole 3-Me), 2.47–2.50 (m, 2H, cyclopentene 3- or 5-H), 2.66–2.69 (m, 2H, cyclopentene 3- or 5-H), 4.16 (q, *J* = 7.0 Hz, 2H, CO₂CH₂Me), 4.43 (s, 2H, OCH₂), 5.84–5.85 (m, 1H, olefin H), 7.25–7.28 (m, 1H, Ph-H), 7.40–7.43 (m, 2H, Ph-H), 7.71–7.72 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 14.0 (CO₂CH₂Me), 14.6 (pyrazole 3-Me), 23.4 (cyclopentene C-4), 32.9, 35.4 (cyclopentene C-3 and -5), 61.3 (CO₂CH₂Me), 69.6 (OCH₂), 104.3 (pyrazole C-4), 122.4, 126.5 (Ph-C), 128.2 (cyclopentene C-2), 129.0 (Ph-C), 133.3 (cyclopentene C-1), 138.4 (Ph-C), 147.4 (pyrazole C-3), 149.2 (pyrazole C-5), 167.5 (C=O); MS: *m/z* 327 [M+H]⁺. Anal. Calcd for C₁₉H₂₂N₂O₃: C, 69.92; H, 6.79; N, 8.58. Found: C, 69.74; H, 6.85; N, 8.54.

Isopropyl {[4-(cyclopenten-1-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-yl]oxy}acetate (7c): Pale yellow oil (0.388 g, 57%); IR (neat): ν 1757, 1734 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.19 (d, *J* = 6.1 Hz, 6H, CO₂CHMe₂), 1.94–2.04 (m, 2H, cyclopentene 4-H), 2.30 (s, 3H, pyrazole 3-Me), 2.47–2.50 (m, 2H, cyclopentene 3- or 5-H), 2.65–2.69 (m, 2H, cyclopentene 3- or 5-H), 4.41 (s, 2H, OCH₂), 5.05 (sep, *J* = 6.1 Hz, 1H, CO₂CHMe₂), 5.84–5.85 (m, 1H, olefin H), 7.24–7.28 (m, 1H, Ph-H), 7.39–7.42 (m, 2H, Ph-H), 7.71–7.74 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 14.6 (pyrazole 3-Me), 21.6 (CO₂CHMe₂), 23.4 (cyclopentene C-4), 32.9, 35.4 (cyclopentene C-3 and -5), 69.2 (CO₂CHMe₂), 69.8 (OCH₂), 104.3 (pyrazole C-4), 122.3, 126.5 (Ph-C), 128.2 (cyclopentene C-2), 129.0 (Ph-C), 133.4 (cyclopentene C-1), 138.5 (Ph-C), 147.4 (pyrazole C-3), 149.3 (pyrazole C-5), 167.1 (C=O); MS: *m/z* 341 [M+H]⁺. Anal. Calcd for C₂₀H₂₄N₂O₃: C, 70.56; H, 7.11; N, 8.23. Found: C, 70.46; H, 7.08; N, 8.24.

***tert*-Butyl {[4-(cyclopenten-1-yl)-3-methyl-1-phenyl-1*H*-pyrazol-5-yl]oxy}acetate (7d):** Pale yellow oil (0.368 g, 52%); IR (neat): ν 1757, 1731 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.42 (s, 9H, CO₂CMe₃), 1.94–1.99 (m, 2H, cyclopentene 4-H), 2.31 (s, 3H, pyrazole 3-Me), 2.47–2.51 (m, 2H, cyclopentene 3- or 5-H), 2.66–2.69 (m, 2H, cyclopentene 3- or 5-H), 4.33 (s, 2H, OCH₂), 5.85–5.86 (m, 1H, olefin H), 7.24–7.28 (m, 1H, Ph-H), 7.39–7.42 (m, 2H, Ph-H), 7.72–7.74 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 14.6 (pyrazole 3-Me), 23.4 (cyclopentene C-4), 28.0 (CO₂CMe₃), 32.9, 35.4 (cyclopentene C-3 and -5), 70.1 (OCH₂), 82.5 (CO₂CMe₃), 104.2 (pyrazole C-4), 122.3, 126.5 (Ph-C), 128.2 (cyclopentene C-2), 129.0 (Ph-C), 133.4 (cyclopentene C-1), 138.4 (Ph-C), 147.4 (pyrazole C-3), 149.3 (pyrazole C-5), 166.5

(C=O); MS: m/z 355 [M+H]⁺. Anal. Calcd for C₂₁H₂₆N₂O₃: C, 71.16; H, 7.39; N, 7.90. Found: C, 71.03; H, 7.44; N, 7.90.

Methyl {[4-(cyclohepten-1-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (9a): Pale yellow oil (0.619 g, 91%); IR (neat): ν 1765, 1745 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.58–1.65 (m, 4H, cycloheptene 2CH₂), 1.80–1.84 (m, 2H, cycloheptene CH₂), 2.22 (s, 3H, pyrazole 3-Me), 2.24–2.28 (m, 2H, cycloheptene CH₂), 2.43–2.45 (m, 2H, cycloheptene CH₂), 3.67 (s, 3H, OMe), 4.62 (s, 2H, OCH₂), 5.91 (t, J = 6.7 Hz, 1H, olefin H), 7.22–7.26 (m, 1H, Ph-H), 7.38–7.41 (m, 2H, Ph-H), 7.70–7.72 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.6 (pyrazole 3-Me), 26.8, 26.9, 28.9, 32.3, 34.3 (cycloheptene 5CH₂), 52.0 (CO₂Me), 68.9 (OCH₂), 110.1 (pyrazole C-4), 122.4, 126.1, 128.7 (Ph-C), 134.0 (cycloheptene C-2), 134.8 (cycloheptene C-1), 138.7 (Ph-C), 147.1 (pyrazole C-3), 148.2 (pyrazole C-5), 168.2 (C=O); MS: m/z 341 [M+H]⁺. Anal. Calcd for C₂₀H₂₄N₂O₃ 0.3H₂O: C, 69.46; H, 7.00; N, 8.10. Found: C, 69.45; H, 7.05; N, 8.10.

Ethyl {[4-(cyclohepten-1-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (9b): Pale yellow oil (0.510 g, 72%); IR (neat): ν 1763, 1739 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.19 (t, J = 7.0 Hz, 3H, CO₂CH₂Me), 1.57–1.66 (m, 4H, cycloheptene 2CH₂), 1.80–1.84 (m, 2H, cycloheptene CH₂), 2.22 (s, 3H, pyrazole 3-Me), 2.22–2.28 (m, 2H, cycloheptene CH₂), 2.43–2.45 (m, 2H, cycloheptene CH₂), 4.15 (q, J = 7.0 Hz, 2H, CO₂CH₂Me), 4.62 (s, 2H, OCH₂), 5.91 (t, J = 6.4 Hz, 1H, olefin H), 7.22–7.26 (m, 1H, Ph-H), 7.38–7.41 (m, 2H, Ph-H), 7.72–7.74 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.6 (pyrazole 3-Me), 14.0 (CO₂CH₂Me), 26.8, 27.0, 28.9, 32.4, 34.4 (cycloheptene 5CH₂), 61.3 (CO₂CH₂Me), 69.1 (OCH₂), 110.1 (pyrazole C-4), 122.4, 126.1, 128.8 (Ph-C), 134.0 (cycloheptene C-2), 134.8 (cycloheptene C-1), 138.7 (Ph-C), 147.1 (pyrazole C-3), 148.3 (pyrazole C-5), 167.8 (C=O); MS: m/z 355 [M+H]⁺. Anal. Calcd for C₂₁H₂₆N₂O₃ 0.3H₂O: C, 70.09; H, 7.28; N, 7.78. Found: C, 70.08; H, 7.44; N, 7.71.

Isopropyl {[4-(cyclohepten-1-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (9c): Pale yellow oil (0.596 g, 81%); IR (neat): ν 1758, 1735 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.17 (d, J = 6.4 Hz, 6H, CO₂CHMe₂), 1.58–1.66 (m, 4H, cycloheptene 2CH₂), 1.80–1.84 (m, 2H, cycloheptene CH₂), 2.22 (s, 3H, pyrazole 3-Me), 2.25–2.29 (m, 2H, cycloheptene CH₂), 2.43–2.45 (m, 2H, cycloheptene CH₂), 4.61 (s, 2H, OCH₂), 5.05 (sep, J = 6.4 Hz, 1H, CO₂CHMe₂), 5.91 (t, J = 6.4 Hz, 1H, olefin H), 7.22–7.26 (m, 1H, Ph-H), 7.38–7.41 (m, 2H, Ph-H), 7.74–7.76 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.6 (pyrazole 3-Me), 21.6 (CO₂CHMe₂), 26.8, 27.0, 28.9, 32.4, 34.5 (cycloheptene 5CH₂), 69.1 (OCH₂ and CO₂CHMe₂), 110.0 (pyrazole C-4), 122.3, 126.0, 128.7 (Ph-C), 134.0 (cycloheptene C-2), 134.9 (cycloheptene C-1), 138.7 (Ph-C), 147.1 (pyrazole C-3), 148.3 (pyrazole C-5), 167.4 (C=O); MS: m/z 369 [M+H]⁺. Anal. Calcd for C₂₂H₂₈N₂O₃ 0.2H₂O: C, 71.02; H, 7.59; N, 7.53. Found: C, 71.02; H, 7.72; N, 7.49.

tert-Butyl {[4-(cyclohepten-1-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (9d): Pale yellow oil (0.500 g, 65%); IR (neat): ν 1756, 1732 cm^{-1} (C=O); ^1H NMR (CDCl_3): δ 1.41 (s, 9H, CO_2CMe_3), 1.58–1.66 (m, 4H, cycloheptene 2CH_2), 1.81–1.85 (m, 2H, cycloheptene CH_2), 2.22 (s, 3H, pyrazole 3-Me), 2.25–2.29 (m, 2H, cycloheptene CH_2), 2.43–2.45 (m, 2H, cycloheptene CH_2), 4.54 (s, 2H, OCH_2), 5.91 (t, $J = 6.4$ Hz, 1H, olefin H), 7.21–7.26 (m, 1H, Ph-H), 7.37–7.40 (m, 2H, Ph-H), 7.74–7.76 (m, 2H, Ph-H); ^{13}C NMR (CDCl_3): δ 13.6 (pyrazole 3-Me), 26.8, 27.0 (cycloheptene 2CH_2), 28.0 (CO_2CMe_3), 28.9, 32.4, 34.6 (cycloheptene 3CH_2), 69.3 (OCH_2), 82.4 (CO_2CMe_3), 109.9 (pyrazole C-4), 122.2, 126.0, 128.7 (Ph-C), 134.0 (cycloheptene C-2), 134.9 (cycloheptene C-1), 138.8 (Ph-C), 147.1 (pyrazole C-3), 148.3 (pyrazole C-5), 166.9 (C=O); MS: m/z 383 $[\text{M}+\text{H}]^+$. Anal. Calcd for $\text{C}_{23}\text{H}_{30}\text{N}_2\text{O}_3 \cdot 0.2\text{H}_2\text{O}$: C, 71.55; H, 7.83; N, 7.26. Found: C, 71.55; H, 7.92; N, 7.22.

General procedure for the preparation of 4-(2-cyclopenten-1-one)-substituted pyrazoles 10a–d from 7a–d and *m*-chloroperbenzoic acid. A mixture of 7a–d (1 mmol) and *m*CPBA (0.345 g, 2 mmol) in THF (10 mL) was stirred at rt overnight. To the reaction mixture, a saturated aqueous NaHCO_3 solution (30 mL) was added with stirring and ice-cooling. The resulting mixture was extracted with AcOEt (60 mL). The extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel with CHCl_3 as the eluent to provide 10a–d.

Methyl {[4-(2-cyclopenten-1-on-2-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (10a): Pale yellow oil (0.239 g, 73%); IR (neat): ν 1763, 1743, 1706 cm^{-1} (C=O); ^1H NMR (CDCl_3): δ 2.20 (s, 3H, pyrazole 3-Me), 2.54–2.56 (m, 2H, cyclopentenone 5-H), 2.76–2.79 (m, 2H, cyclopentenone 4-H), 3.67 (s, 3H, OMe), 4.46 (s, 2H, OCH_2), 7.29–7.30 (m, 1H, Ph-H), 7.40–7.44 (m, 2H, Ph-H), 7.67 (s, 1H, olefin H), 7.67–7.71 (m, 2H, Ph-H); ^{13}C NMR (CDCl_3): δ 13.6 (pyrazole 3-Me), 27.2 (cyclopentenone C-4), 34.4 (cyclopentenone C-5), 52.0 (CO_2Me), 69.4 (OCH_2), 97.9 (pyrazole C-4), 122.6, 126.9, 129.0 (Ph-C), 137.2 (cyclopentenone C-2), 138.3 (Ph-C), 147.8 (pyrazole C-3), 150.2 (pyrazole C-5), 161.6 (cyclopentenone C-3), 168.0, 207.8 ($2\text{C}=\text{O}$); MS: m/z 327 $[\text{M}+\text{H}]^+$. Anal. Calcd for $\text{C}_{18}\text{H}_{18}\text{N}_2\text{O}_4$: C, 66.25; H, 5.56; N, 8.58. Found: C, 66.21; H, 5.64; N, 8.32.

Ethyl {[4-(2-cyclopenten-1-on-2-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (10b): Pale yellow oil (0.269 g, 79%); IR (neat): ν 1759, 1739, 1706 cm^{-1} (C=O); ^1H NMR (CDCl_3): δ 1.20 (t, $J = 7.0$ Hz, 3H, $\text{CO}_2\text{CH}_2\text{Me}$), 2.20 (s, 3H, pyrazole 3-Me), 2.54–2.56 (m, 2H, cyclopentenone 5-H), 2.77–2.79 (m, 2H, cyclopentenone 4-H), 4.14 (q, $J = 7.0$ Hz, 2H, $\text{CO}_2\text{CH}_2\text{Me}$), 4.45 (s, 2H, OCH_2), 7.26–7.30 (m, 1H, Ph-H), 7.40–7.43 (m, 2H, Ph-H), 7.67 (s, 1H, olefin H), 7.66–7.73 (m, 2H, Ph-H); ^{13}C NMR (CDCl_3): δ 13.6 (pyrazole 3-Me), 14.0 ($\text{CO}_2\text{CH}_2\text{Me}$), 27.2 (cyclopentenone C-4), 34.4 (cyclopentenone C-5), 61.3 ($\text{CO}_2\text{CH}_2\text{Me}$), 69.6 (OCH_2), 97.8 (pyrazole C-4), 122.6, 126.8, 129.0 (Ph-C), 137.4 (cyclopentenone C-2), 138.4 (Ph-C), 147.8 (pyrazole C-3), 150.3 (pyrazole C-5), 161.6 (cyclopentenone C-3), 167.6, 207.8

(2C=O); MS: m/z 341 [M+H]⁺. Anal. Calcd for C₁₉H₂₀N₂O₄ 0.2H₂O: C, 66.34; H, 5.86; N, 8.14. Found: C, 66.41; H, 6.06; N, 7.98.

Isopropyl {[4-(2-cyclopenten-1-on-2-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (10c): Pale yellow oil (0.326 g, 92%); IR (neat): ν 1753, 1736, 1708 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.17 (d, J = 6.4 Hz, 6H, CO₂CHMe₂), 2.20 (s, 3H, pyrazole 3-Me), 2.55–2.56 (m, 2H, cyclopentenone 5-H), 2.77–2.80 (m, 2H, cyclopentenone 4-H), 4.42 (s, 2H, OCH₂), 5.02 (sep, J = 6.4 Hz, 1H, CO₂CHMe₂), 7.26–7.29 (m, 1H, Ph-H), 7.40–7.43 (m, 2H, Ph-H), 7.72 (s, 1H, olefin H), 7.67–7.74 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.7 (pyrazole 3-Me), 21.6 (CO₂CHMe₂), 27.2 (cyclopentenone C-4), 34.4 (cyclopentenone C-5), 69.2 (CO₂CHMe₂), 69.8 (OCH₂), 97.8 (pyrazole C-4), 122.6, 126.7, 129.0 (Ph-C), 137.4 (cyclopentenone C-2), 138.4 (Ph-C), 147.8 (pyrazole C-3), 150.4 (pyrazole C-5), 161.5 (cyclopentenone C-3), 167.2, 207.8 (2C=O); MS: m/z 355 [M+H]⁺. Anal. Calcd for C₂₀H₂₂N₂O₄ 0.6H₂O: C, 65.77; H, 6.07; N, 7.67. Found: C, 65.75; H, 6.42; N, 7.53.

tert-Butyl {[4-(2-cyclopenten-1-on-2-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (10d): Pale yellow oil (0.328 g, 89%); IR (neat): ν 1753, 1733, 1708 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.40 (s, 9H, CO₂CMe₃), 2.20 (s, 3H, pyrazole 3-Me), 2.54–2.56 (m, 2H, cyclopentenone 5-H), 2.77–2.79 (m, 2H, cyclopentenone 4-H), 4.34 (s, 2H, OCH₂), 7.25–7.29 (m, 1H, Ph-H), 7.40–7.43 (m, 2H, Ph-H), 7.67 (t, J = 3.0 Hz, 1H, olefin H), 7.72–7.74 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.6 (pyrazole 3-Me), 27.2 (cyclopentenone C-4), 28.0 (CO₂CMe₃), 34.4 (cyclopentenone C-5), 70.0 (OCH₂), 82.5 (CO₂CMe₃), 97.6 (pyrazole C-4), 122.5, 126.6, 129.0 (Ph-C), 137.4 (cyclopentenone C-2), 138.4 (Ph-C), 147.8 (pyrazole C-3), 150.5 (pyrazole C-5), 161.6 (cyclopentenone C-3), 166.7, 207.8 (2C=O); MS: m/z 369 [M+H]⁺. Anal. Calcd for C₂₁H₂₄N₂O₄ 1H₂O: C, 65.27; H, 6.28; N, 7.25. Found: C, 65.20; H, 6.43; N, 7.01.

The preparation of 11 from 7b and *m*-chloroperbenzoic acid. A mixture of **7b** (0.326 g, 1 mmol) and *m*CPBA (0.173 g, 1 mmol) in CHCl₃ (10 mL) was stirred at 0–5 °C overnight. To the reaction mixture, a saturated aqueous NaHCO₃ solution (30 mL) was added with stirring and ice-cooling. The resulting mixture was extracted with CHCl₃ (60 mL). After the same work-up as described above for the preparation of **10a–d**, ethyl {[4-(2-cyclopenten-1-ol-2-yl)-3-methyl-1-phenyl-1H-pyrazol-5-yl]oxy}acetate (**11**) was obtained as pale yellow oil (0.319 g, 93%); IR (neat): ν 3386 (OH), 1759, 1740 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.23 (t, J = 7.0 Hz, 3H, CO₂CH₂Me), 1.89–1.92 (m, 1H, cyclopentenol 5-H), 2.32 (s, 3H, pyrazole 3-Me), 2.20–2.42 (m, 2H, cyclopentenol 4- and 5-H), 2.62–2.67 (m, 1H, cyclopentenol 4-H), 3.39 (br, 1H, OH), 4.19 (q, J = 7.0 Hz, 2H, CO₂CH₂Me), 4.45 (AB q, J = 15.3 Hz, 2H, OCH₂), 5.12–5.14 (m, 1H, cyclopentenol 1-H), 6.00–6.01 (m, 1H, olefin H), 7.28–7.31 (m, 1H, Ph-H), 7.41–7.45 (m, 2H, Ph-H), 7.67–7.69 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 14.0 (CO₂CH₂Me), 14.7 (pyrazole 3-Me), 30.5 (cyclopentenol C-4), 33.1 (cyclopentenol C-5), 61.5 (CO₂CH₂Me), 69.9 (OCH₂),

77.9 (cyclopentenol C-1), 102.5 (pyrazole C-4), 122.6, 126.9, 129.2 (Ph-C), 130.9 (cyclopentenol C-3), 136.9 (cyclopentenol C-2), 138.4 (Ph-C), 148.0 (pyrazole C-3), 149.1 (pyrazole C-5), 167.8 (C=O); MS: m/z 343 [M+H]⁺. Anal. Calcd for C₁₉H₂₂N₂O₄ 0.4H₂O: C, 65.28; H, 6.34; N, 8.01. Found: C, 65.32; H, 6.42; N, 7.77.

The preparation of 10b from 11 and *m*-chloroperbenzoic acid. A mixture of **11** (0.342 g, 1 mmol) and *m*CPBA (0.173 g, 1 mmol) in THF (10 mL) was stirred at rt overnight. After the same work-up as described above for the preparation of **10a–d**, **10b** was obtained in 84% yield (0.286 g).

General procedure for the preparation of C-substituted pyrazoles 12a,b from 4-cyclopentylidene-3H-pyrazol-3-one 6. A mixture of **6** (0.480 g, 2 mmol), 2-chloropropionitrile (0.537 g, 6 mmol) or 2-chloroacetophenone (0.928 g, 6 mmol), and K₂CO₃ (0.553 g, 4 mmol) in DMF (5 mL) was stirred at 80 °C for 1 h (in the case of the preparation of **12a**) or rt overnight (in the case of the preparation of **12b**). After the same work-up as described above for the preparation of **7a–d** and **9a–d**, C-substituted pyrazoles **12a,b** were obtained.

4-(Cyclopenten-1-yl)-4,5-dihydro- α ,3-dimethyl-5-oxo-1-phenyl-1H-pyrazole-4-acetonitrile (12a):

Pale yellow oil (0.370 g, 63%); IR (neat): ν 2242 (CN), 1714 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.21 (d, J = 7.0 Hz, 3H, CHMeCN), 1.91–1.96 (m, 2H, cyclopentene 4-H), 2.32 (s, 3H, pyrazole 3-Me), 2.34–2.48 (m, 4H, cyclopentene 3- and 5-H), 3.44 (q, J = 7.0 Hz, 1H, CHMeCN), 5.85–5.86 (m, 1H, olefin H), 7.19–7.22 (m, 1H, Ph-H), 7.38–7.42 (m, 2H, Ph-H), 7.85–7.87 (m, 2H, Ph-H); ¹³C NMR (CDCl₃): δ 13.8 (CHMeCN), 15.7 (pyrazole 3-Me), 22.7 (cyclopentene C-4), 27.9 (CHMeCN), 31.4, 32.7 (cyclopentene C-3 and -5), 62.1 (pyrazole C-4), 118.8 (Ph-C), 119.8 (CN), 125.6, 129.0 (Ph-C), 131.4 (cyclopentene C-2), 135.6 (cyclopentene C-1), 137.6 (Ph-C), 159.1 (pyrazole C-3), 170.4 (C=O); MS: m/z 294 [M+H]⁺. Anal. Calcd for C₁₈H₁₉N₃O 0.1H₂O: C, 73.24; H, 6.49; N, 14.24. Found: C, 73.26; H, 6.58; N, 14.11.

4-(Cyclopenten-1-yl)-2,4-dihydro-5-methyl-4-(2-oxo-2-phenylethyl)-1-phenyl-3H-pyrazol-3-one (12b):

Pale yellow oil (0.652 g, 91%); IR (neat): ν 1704, 1685 cm⁻¹ (C=O); ¹H NMR (CDCl₃): δ 1.87–1.94 (m, 2H, cyclopentene 4-H), 2.00 (s, 3H, pyrazole 3-Me), 2.27–2.31 (m, 2H, cyclopentene 3- or 5-H), 2.39–2.44 (m, 2H, cyclopentene 3- or 5-H), 3.80 (AB q, J = 18.1 Hz, 2H, CH₂COPh), 5.81–5.82 (m, 1H, olefin H), 7.15–7.17 (m, 1H, Ph-H), 7.37–7.46 (m, 4H, Ph-H), 7.55–7.58 (m, 1H, Ph-H), 7.92–7.95 (m, 4H, Ph-H); ¹³C NMR (CDCl₃): δ 14.2 (pyrazole 3-Me), 22.8 (cyclopentene C-4), 31.3, 32.5 (cyclopentene C-3 and -5), 41.1 (CH₂COPh), 58.1 (pyrazole C-4), 119.2, 124.9, 128.2, 128.7, 128.8 (Ph-C), 129.1 (cyclopentene C-2), 133.6 (Ph-C), 135.9 (cyclopentene C-1), 138.0, 138.5 (Ph-C), 160.4 (pyrazole C-3), 173.7 (pyrazole C-5), 195.4 (C=O); MS: m/z 359 [M+H]⁺. Anal. Calcd for C₂₃H₂₂N₂O₂: C, 77.07; H, 6.19; N, 7.82. Found: C, 77.12; H, 6.29; N, 7.80.

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-A2¹⁰ and our previous paper.⁶ The results are listed in Table 1. 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 0.5×10^3 to 2.5×10^3 in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) buffered to pH 7.0 with 0.165M 3-morpholinepropanesulfonic acid (MOPS; DOJINDO Laboratories, Kumamoto Japan). *S. cerevisiae* was 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 0.5×10^3 to 2.5×10^3 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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