



Synthesis of novel Schiff base analogues of 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one and their evaluation for antioxidant and anti-inflammatory activity

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ABSTRACT

4-Aminoantipyridine (4-amino-1,5-dimethyl-2-phenylpyrazole-3-one) and its analogues have been found to be compounds of interest for their anti-inflammatory, analgesic, antiviral, antipyretic, antirheumatic and antimicrobial activities. In the present study, Schiff base analogues of 4-aminoantipyridine were synthesized by the condensation reaction with substituted benzaldehydes and then evaluated for their antioxidant and anti-inflammatory activities. From among the synthesized compounds (**3a–m**, **4** and **5**), **3k** and **3f** exhibited the highest antioxidant activity followed by **3g**, **3l**, **3c**, **3i**, **5**, **3m** and **3h**. The IC₅₀ values for compounds **3k** and **3f** were found to be 0.44 and 0.93 μM, respectively, comparable to that of ascorbic acid (IC₅₀ 0.41 μM), a standard antioxidant agent. From the comparisons between the hydroxylated and methoxylated compounds, the rank order of antioxidant activity for the products resulting from benzylidene phenyl ring substitution was 2,4,6-OH > 3,4-OH > 3-OMe-4-OH > 3,5-OMe-4-OH > 2,4-OH > 3-Me-4-OMe > 3,4-OMe > 4-OMe > 4-OH. The structure–activity relationship study revealed that the position and nature of the substituted group on the benzylidene phenyl ring of the Schiff base analogues of 4-aminoantipyridine play an important role in their antioxidant activity. The anti-inflammatory activity of **3f**, which also exhibited excellent antioxidant activity, was evaluated in terms of its inhibition of NO production, an inflammatory modulator, in LPS pretreated RAW 264.7 cells using the Griess method. We also examined whether or not this compound had effect on iNOS and COX-2 mRNA expression in RAW 264.7 cells. It was observed that compound **3f** significantly reduced NO production and inhibited LPS-stimulated iNOS and COX-2 mRNA levels in a dose-dependent manner. Overall, **3f** showed promising antioxidant and anti-inflammatory activities and may be used as the lead compound in a future study.

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1. Introduction

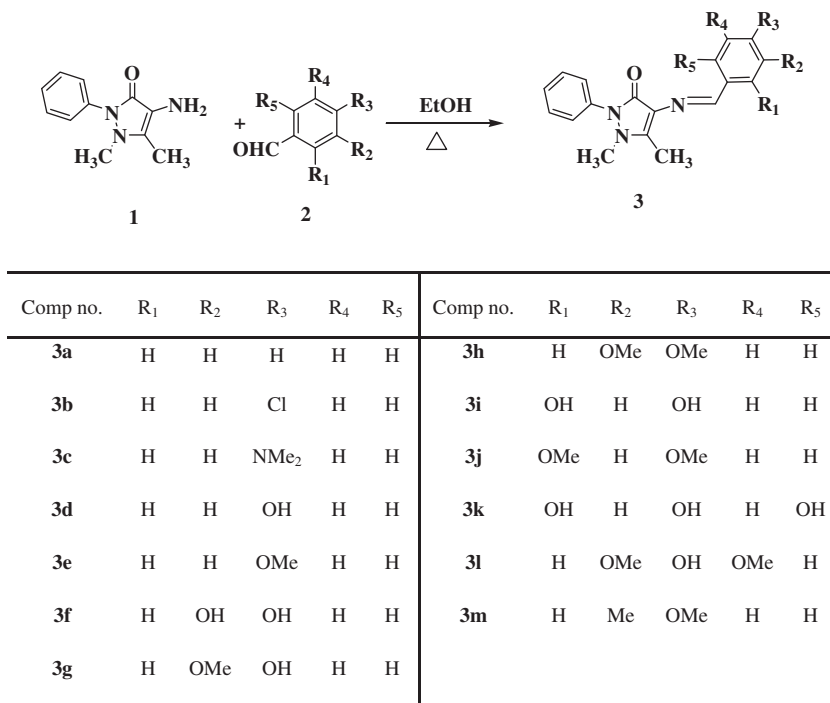
The pyrazolone derivative 4-aminoantipyridine (4-amino-1,5-dimethyl-2-phenylpyrazole-3-one) and its analogues have shown a wide range of biological activities such as anti-inflammatory,¹ analgesic,² antiviral,³ antipyretic, antirheumatic and antimicrobial activity.⁴ These classes of compounds also act as strong inhibitors of cyclooxygenase isoenzymes, platelet thromboxane synthesis and prostanoid synthesis.¹ Oxidative stress generates reactive oxygen species (ROS), including free radicals,⁵ which attack various biological macromolecules including proteins, enzymes, DNA, etc., and give rise to a number of inflammatory and metabolic disorders, cellular aging, reperfusion damage and cancer.^{6,7} Therefore, antioxidants have been shown to play an important role in protecting humans against many fatal diseases.

Antipyridine has been found to be effective in scavenging peroxy radicals (ROO[•]), but not effective against other ROS and RNS (reactive nitrogen species) or to the oxidative burst from neutrophils.^{1,8,9} Whereas aminoantipyridine (**1**, Scheme 1) was found to be a highly efficient scavenger of ROSs, for example, hydroxyl radical (HO[•]), hypochlorous acid (HOCl), peroxy radical (ROO[•]), singlet oxygen (¹O₂), RNSs, [e.g. nitric oxide (NO) and peroxynitrite (ONOO[−])], as well as the oxidative burst from neutrophils.^{1,8–11} Of significance, in the progression of inflammatory diseases (cancer, coronary artery disease, etc.), macrophages produce large quantities of NO, an inflammatory mediator leading to inflammation.^{12,13}

The inducible NO synthase (iNOS) plays an important role in the production of inflammatory mediators. This enzyme oxidizes the guanidine moiety of L-arginine, leading to the production of NO which is associated with various carcinomas and inflammatory conditions including Type-1 diabetes and arthritis.¹⁴ Lipopolysaccharide (LPS), an endotoxin which is derived from the cell wall of gram-negative bacteria,¹⁵ can induce multiple signaling pathways

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Scheme 1. Synthesis of 4-aminoantipyrene analogues 3a–m.

to stimulate the production of inflammatory modulators involving NO, PGE₂, TNF- α and interleukins.¹⁶ Non-steroidal anti-inflammatory drugs (NSAIDs), which are known to inhibit COX enzymes, are currently used as important therapeutic agents for the treatment of pain and inflammation. Various side effects have been observed for many commercially available selective COX-2 inhibitors. For example, celecoxib has relatively few gastrointestinal side effects but its long-term use is related to cardiovascular injury, an effect which limits its clinical applications.¹⁷ Therefore, there is a critical need for the development of novel drugs with a broad spectrum of activities and improved safety profiles that could be used on a long-term basis for mitigating chronic inflammatory conditions.

In a previous study, we reported the synthesis, molecular structure and antioxidant activity of 4-[benzylideneamino]-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one, a Schiff base ligand of 4-aminoantipyrene.¹⁸ The hydroxyl radical scavenging activity¹⁹ of *N*-alkylated-4-aminoantipyrenes and antibacterial property²⁰ of a series of Schiff base analogues of 4-aminoantipyrene were also reported. Here, we describe the synthesis and biological evaluation of a series of novel Schiff base analogues of 4-aminoantipyrene (4-amino-1,5-dimethyl-2-phenylpyrazole-3-one) designed for use as antioxidant and anti-inflammatory agents. The syntheses of the Schiff base analogues of 4-aminoantipyrene were prepared by condensation reaction with various substituted benzaldehydes in ethanol. The structures of the new compounds were elucidated by IR, ¹H-NMR, and mass spectrometry. The *in vitro* DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity of the synthesized compounds was assayed according to the Blois method.²¹ The effect of the Schiff base analogue of 4-aminoantipyrene on NO production was assayed using the Griess method with LPS-stimulated RAW264.7 cells. The toxicity profile of the Schiff base analogues on the RAW264.7 cells was assessed by an MTT assay. We also investigated whether or not the Schiff base analogues had an effect on the expression of iNOS and COX-2 proteins using a reverse transcription-polymerase chain reaction (RT-PCR) method.

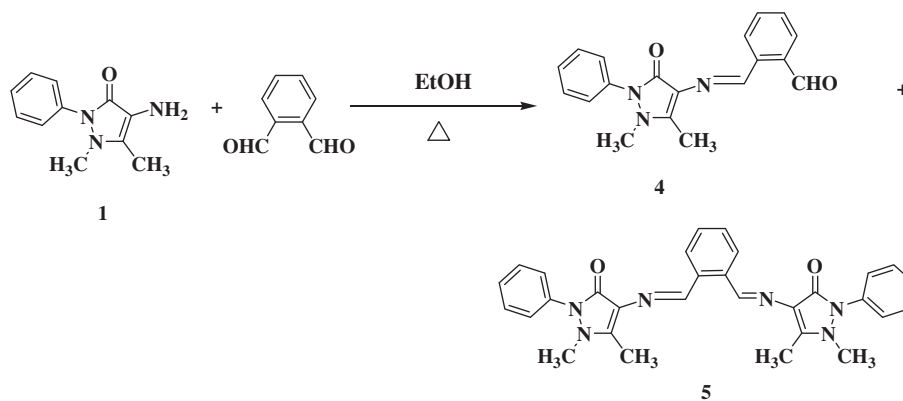
2. Results and discussion

2.1. Synthesis of the Schiff base analogues of 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one

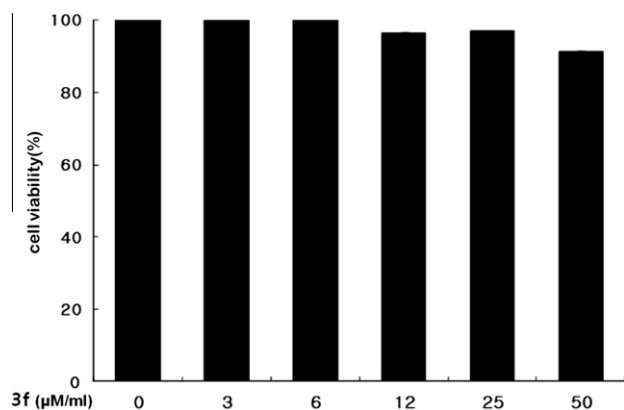
Synthesis of the Schiff base analogues was carried out according to a convenient one-step procedure, that is, by the condensation of commercially available 4-aminoantipyrene and different substituted benzaldehydes in ethanol, which provided excellent yields (80–94%). The synthetic routes of the desired Schiff base analogues (3a–m, 4 and 5) are outlined in Scheme 1 and 2. All compounds except 3b, 3d, 3e and 3g are new. The structures of the compounds were elucidated by IR, ¹H NMR and mass spectral data. In the IR spectra results for the compounds, the characteristic >C=O and –C=N– stretching absorption bands appeared around 1617–1724 cm^{–1} and 1579–1596 cm^{–1}, respectively. The ¹H NMR spectra showed a characteristic singlet for the imino proton (–CH=N–) at 9.41–9.83 ppm. The =C–CH₃ and –N–CH₃ protons were observed as singlets at 2.33–2.52 and 3.09–3.23 ppm, respectively, and equivalent to three protons each. The aromatic protons were assigned in the usual way, according to their substitution pattern. The methoxy protons of compounds 3e, 3g, 3h, 3j, 3l and 3m appeared as singlets at 3.80–3.96 ppm. The *N,N'*-dimethyl protons of compound 3c were observed as a singlet at 3.07 ppm, equivalent to six protons. Compound 4 exhibited a characteristic singlet for the –CH=O proton at 10.84 ppm, which was absent in compound 5, indicating the further condensation of the formyl group from compound 4 with the amino group of 4-aminoantipyrene. In addition, the EI-MS spectra of 3a–m, 4 and 5 showed a molecular ion peak with intensities of 88–100%.

2.2. Antioxidant activity

The newly synthesized Schiff base analogues (3a–m, 4 and 5) of 4-aminoantipyrene were evaluated for their free radical-scavenging activity using DPPH. The DPPH radical scavenging activities for the compounds are shown in Table 1. In order to study their

Scheme 2. Synthesis of 4-aminoantipyrene derivatives **4** and **5**.**Table 1**
DPPH radical scavenging activity of the Schiff base analogues of 4-aminoantipyrene

Compd no.	IC ₅₀ (μM)	Compd no.	IC ₅₀ (μM)
3a	31.26 ^a	3i	2.5
3b	31.68	3j	14.53
3c	2.43	3k	0.44
3d	18.8	3l	1.66
3e	15.27	3m	5.22
3f	0.93	4	34.46
3g	1.13	5	3.14
3h	7.27	Ascorbic acid	0.41

^a Taken from Ref. 18.Figure 1. Effect of **3f** on the viability of RAW264.7 cells.

structure–activity relationship, we synthesized and tested the antioxidant activity of hydroxylated, non-hydroxylated and methoxylated Schiff base analogues of 4-amino-1,5-dimethyl-2-phenylpyrazole-3-one. Comparisons of the hydroxylated and non-hydroxylated Schiff base analogues revealed that the DPPH radical scavenging activities of the former analogues were generally higher than those of the latter. Compounds **3a**, **3b** and **4**, bearing no OH group, exhibited much higher IC₅₀ values (>30 μM) than those having at least one OH group in the aromatic ring. Among this series of compounds (**3a–m**, **4** and **5**), **3k** and **3f** showed the highest DPPH free radical scavenging activity followed by **3g**, **3l**, **3c**, **3i**, **5**, **3m** and **3h**, respectively. The IC₅₀ values of compound **3k** and **3f** were 0.44 and 0.93 μM, respectively, which are comparable to that of the standard antioxidant agent, ascorbic acid (IC₅₀ 0.41 μM). We have previously reported the proposed antioxidant mechanism for compound **3a**, a non-hydroxylated Schiff base analogue of 4-

aminoantipyrene, based on its electrochemical properties.¹⁸ Introduction of a hydroxyl group into the 4-position of the benzylidene phenyl ring of **3a**, yielding **3d**, led to a ~2 fold increase in activity, whereas the 4-methoxy analogue, **3e**, showed slightly improved activity compared to that of compound **3d**. Substitution of two hydroxyl groups into the 3,4- and 2,4-positions of compound **3a**, produced **3f** and **3i**, respectively. These substitutions caused a rise in their antioxidant activity as compared to that of **3a**, of 34- (**3f**) and 12-fold (**3i**). Replacement of the hydroxyl groups of **3f** with a methoxy or methyl group, producing **3g**, **3h**, or **3m**, resulted in a significant decrease in activity. Similar results were also observed in the case of compound **3i**. Introduction of two methoxy groups into the 3- and 5-position of compound **3d**, led to **3l**, and an increased antioxidant activity >11-fold greater than that observed for **3d**. Introduction of a chlorine atom or an aldehyde group, a deactivating substituent, onto the benzylidene phenyl ring of **3a** resulted in compounds **3b** or **4**, respectively, and neither showed any marked change in antioxidant activity. The placement of a *N,N*-dimethylamino group, an activating substituent, on the phenyl ring of **3a**, produced **3c** and resulted in a ~13-fold increase in antioxidant activity. When the aldehyde group of **4** underwent a condensation reaction with another 4-aminoantipyrene molecule to furnish compound **5**, it exhibited ~11-fold higher antioxidant activity than observed for compound **4**. The above structure–activity relationship study led us to speculate that the structure and position of the substituted group on the benzylidene phenyl ring of the Schiff base analogues of 4-aminoantipyrene play important roles in their antioxidant activity.

2.3. Anti-inflammatory activity

To examine the anti-inflammatory activity of Schiff base analogues of 4-aminoantipyrene, we arbitrarily selected compounds **3k** and **3f**, which demonstrated a potent antioxidant activity of <1.0 μM at IC₅₀. However, only compound **3f** could be evaluated in terms of inhibition of the production of inflammatory modulators associated with NO because the most active antioxidant compound, **3k** was difficult to solubilize, even in DMSO. The cytotoxic effect of compound **3f** on RAW 264.7 cells was determined by MTT assay. At concentrations of 6, 12, 25 and 50 μg/mL, the viability of RAW 264.7 cells was not significantly inhibited by compound **3f** (Fig. 1).

In order to investigate the effects of **3f** on NO production by RAW 264.7 macrophage cells in the presence or absence of LPS, we measured the concentration of nitrate and the oxidative metabolite of NO in their cell culture medium using the Griess method. The RAW 264.7 cells were pretreated with **3f** for 30 min prior to stimulation with LPS (200 ng/mL). After 24 h of stimulation by

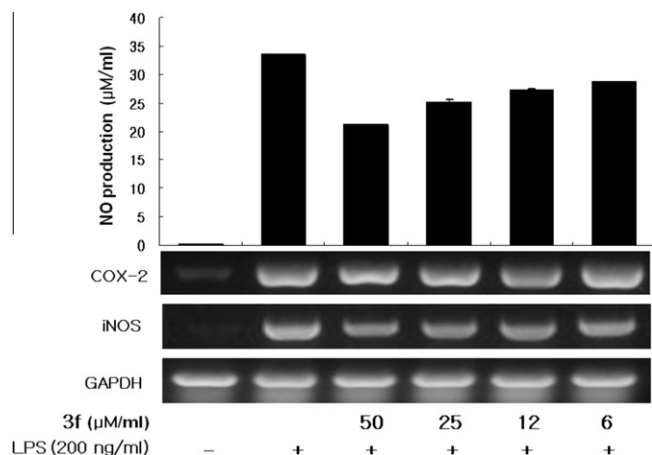


Figure 2. Effect of **3f** on NO production and iNOS and COX-2 mRNA expression in LPS-treated RAW264.7 cells. Cells were pretreated with different concentrations of **3f** for 30 min and then stimulated with LPS (200 ng/mL) for 24 h. After incubation, the NO production levels in the cultured media were measured by Griess reagent. Cells were harvested, total RNA was isolated, and iNOS and COX-2 mRNA expression was examined by RT-PCR analysis.

LPS, the levels of NO in the culture media were determined. As shown in Figure 2, LPS stimulation resulted in a marked induction of NO production as compared to untreated cells. However, pretreatment with **3f**, 30 min prior to LPS stimulation significantly reduced NO production in a dose-dependent manner.

The effects of **3f** on iNOS and COX-2 mRNA expression in RAW 264.7 cells was examined by RT-PCR. In order to measure iNOS and COX-2 mRNA expression levels, the RAW 264.7 cells were pretreated with **3f** for 30 min prior to stimulation with LPS (200 ng/mL). After 24 h of stimulation by LPS, the cells were evaluated for iNOS and COX-2 mRNA expression. The iNOS and COX-2 mRNA levels were not detectable in the absence of LPS treatment, but these levels were significantly induced after LPS exposure. Pretreatment of the cells with **3f** inhibited LPS-stimulated iNOS levels in a dose-dependent manner. However, the results indicated that merely 50 µg/mL of **3f** inhibited the LPS-stimulated COX-2 mRNA levels (Fig. 2).

3. Conclusion

In summary, we prepared and evaluated a series of novel Schiff base analogues of 4-aminoantipyrene (4-amino-1,5-dimethyl-2-phenylpyrazole-3-one) for their antioxidant and anti-inflammatory activities. Most of the compounds showed significant antioxidant activity while compound **3k** and **3f** showed a potent antioxidant activity comparable to that of the standard antioxidant agent, ascorbic acid. Moreover, compound **3f** showed promising anti-inflammatory activity which could be beneficial for use in the treatment of inflammatory diseases. The results of this study may lead to the development of a new therapeutic agent useful in fighting diseases caused by oxidative stress and inflammation.

4. Materials and methods

4.1. General

The melting point of the synthesized compounds was determined using a Stuart SMP3 apparatus, and the results were uncorrected. The ^1H NMR spectra were recorded on a Varian GEMINI 200 spectrophotometer (200 MHz) using TMS as a reference standard. FT-IR spectra, using KBr pellets, was obtained with a Bruker Tensor 37 spectrophotometer. EI- and FAB-MS spectra were acquired using a Jeol JMS-700 mass spectrometer.

4.2. General method for the preparation of Schiff base analogues of 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one (**3a–m**)

An anhydrous ethanol solution (10 mL) of 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one (203 mg, 1 mmol) was added to an anhydrous ethanol solution (10 mL) of substituted benzaldehyde (1 mmol), and the mixture was refluxed at 80 °C for 4–6 h under atmospheric conditions (Scheme 1). The progress of the reaction was monitored by TLC. The precipitates formed were collected by filtration and purified by recrystallization with ethanol, and then dried in vacuo to produce the pure compound with a high yield (80–94%).

4.2.1. 4-Benzylideneamino-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**3a**)¹⁸

Yield 85%¹⁸; mp 178.3 °C (yellow crystal); IR (KBr) 1651 ($>\text{C}=\text{O}$), 1597 ($\text{C}=\text{N}$) cm^{-1} ; ^1H NMR (DMSO- d_6) δ ppm: 2.49 (s, 3H, $=\text{C}-\text{CH}_3$), 3.14 (s, 3H, $-\text{N}-\text{CH}_3$), 7.31–7.52 (m, 8H, ArH), 7.84–7.89 (m, 2H, ArH), 9.77 (s, 1H, $-\text{N}=\text{CH}$); HR-FAB-MS (m/z): 293 [$\text{M}+\text{H}$]⁺, calculated for $\text{C}_{18}\text{H}_{17}\text{N}_3\text{O}$, 292.1338; found 292.1339 (error: +0.4 ppm).

4.2.2. 4-(4-Chlorobenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**3b**)²⁰

Yield 90%; mp 252.1 °C (yellow solid); IR (KBr) 1649 ($>\text{C}=\text{O}$), 1593 ($\text{C}=\text{N}$) cm^{-1} ; ^1H NMR (DMSO- d_6) δ ppm: 2.49 (s, 3H, $=\text{C}-\text{CH}_3$), 3.16 (s, 3H, $-\text{N}-\text{CH}_3$), 7.35–7.48 (m, 7H, Ar-H), 7.77–7.82 (m, 2H, Ar-H), 9.72 (s, 1H, $-\text{N}=\text{CH}$); EI-MS m/z (%): 327 ($\text{M}+2$, 34), 326 ($\text{M}+1$, 21), 325 (M^+ , 100), 233 (20), 188 (38), 121 (36), 56 (96).

4.2.3. 4-[4-(Dimethylamino)benzylideneamino]-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**3c**)

Yield 80%; mp 219.4 °C (yellow solid); IR (KBr) 1650 ($>\text{C}=\text{O}$), 1606, 1575 ($\text{C}=\text{N}$) cm^{-1} ; ^1H NMR (CDCl_3) δ ppm: 2.52 (s, 3H, $=\text{C}-\text{CH}_3$), 3.07 (s, 6H, $-\text{N}(\text{CH}_3)_2$), 3.15 (s, 3H, $-\text{N}-\text{CH}_3$), 6.77 (d, 2H, $J = 8.2$ Hz, Ar-H), 7.33–7.53 (m, 5H, Ar-H), 7.82 (d, 2H, $J = 8.2$ Hz, Ar-H), 9.74 (s, 1H, $-\text{N}=\text{CH}$); EI-MS m/z (%): 334 (M^+ , 88), 167 (12), 56 (100).

4.2.4. 4-(4-Hydroxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**3d**)²⁰

Yield 84%; mp 232.3 °C (yellow powder); IR (KBr) 3591(OH), 1617 ($>\text{C}=\text{O}$), 1579 ($\text{C}=\text{N}$) cm^{-1} ; ^1H NMR (CDCl_3) δ ppm: 2.42 (s, 3H, $=\text{C}-\text{CH}_3$), 3.13 (s, 3H, $-\text{N}-\text{CH}_3$), 6.84 (d, 2H, $J = 8.2$ Hz, Ar-H), 7.35–7.52 (m, 5H, Ar-H), 7.65 (d, 2H, $J = 8.2$ Hz, Ar-H), 9.46 (s, 1H, $-\text{N}=\text{CH}$); EI-MS m/z (%): 307 (M^+ , 100), 215 (33), 121 (20), 56 (78).

4.2.5. 4-(4-Methoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (**3e**)²⁰

Yield 91%; mp 168.1 °C (yellow crystal); IR (KBr) 1643 ($>\text{C}=\text{O}$), 1586 ($\text{C}=\text{N}$) cm^{-1} ; ^1H NMR (CDCl_3) δ ppm: 2.45 (s, 3H, $=\text{C}-\text{CH}_3$), 3.19 (s, 3H, $-\text{N}-\text{CH}_3$), 3.83 (s, 3H OCH_3), 6.81 (d, 2H, $J = 8.3$ Hz, Ar-H), 7.29–7.46 (m, 5H, Ar-H), 7.57 (d, 2H, $J = 8.3$ Hz, Ar-H), 9.41 (s, 1H, $-\text{N}=\text{CH}$); EI-MS m/z (%): 321 (M^+ , 100), 229 (23), 212 (18), 121 (22), 56 (93).

4.2.6. 4-(3,4-Dihydroxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one(**3f**)

Yield 90%; mp 287.2 °C (yellow crystal); IR (KBr) 3491 (OH), 1618 ($>\text{C}=\text{O}$), 1586 ($\text{C}=\text{N}$) cm^{-1} ; ^1H NMR (DMSO- d_6) δ ppm: 2.46 (s, 3H, $=\text{C}-\text{CH}_3$), 3.18 (s, 3H, $-\text{N}-\text{CH}_3$), 6.84 (d, 1H, $J = 2.4$ Hz, Ar-H), 7.05 (d, 1H, $J = 2.4$ Hz, Ar-H), 7.35–7.61 (m, 6H, Ar-H), 9.42 (s, 1H, $-\text{N}=\text{CH}$); EI-MS m/z (%): 323 (M^+ , 100), 231 (37), 121 (15), 56 (76).

4.2.7. 4-(4-Hydroxy-3-methoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (3g)

Yield 95%; mp 207.5 °C (yellow crystal) (mp. 205 °C)²²; IR (KBr) 3542 (OH) 1624 (>C=O), 1581 (C=N) cm⁻¹; ¹H NMR (DMSO-*d*₆) δ ppm: 2.43 (s, 3H, =C-CH₃), 3.17 (s, 3H, -N-CH₃), 3.80 (s, 3H, OCH₃), 6.88 (d, 1H, *J* = 2.5 Hz, Ar-H), 7.11 (d, 1H, *J* = 2.5 Hz, Ar-H), 7.36–7.63 (m, 6H, Ar-H), 9.41 (s, 1H, -N=CH); EI-MS *m/z* (%): 337 (M⁺, 100), 245 (34), 228 (17), 121 (16), 56 (84).

4.2.8. 4-(3,4-Dimethoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (3h)

Yield 94%; mp 191.7 °C (yellow crystal); IR (KBr) 1649 (>C=O), 1598, 1579 (C=N) cm⁻¹; ¹H NMR (CDCl₃) δ ppm: 2.47 (s, 3H, =C-CH₃), 3.10 (s, 3H, -N-CH₃), 3.90 (s, 3H, OCH₃), 3.96 (s, 3H, OCH₃), 6.89 (d, 1H, *J* = 2.4 Hz, Ar-H), 7.29–7.55 (m, 7H, Ar-H), 9.71 (s, 1H, -N=CH); EI-MS *m/z* (%): 351 (M⁺, 100), 259 (21), 242 (21), 121 (15), 56 (91).

4.2.9. 4-(2,4-Dihydroxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (3i)

Yield 91%; mp 231.5 °C (yellow needles); IR (KBr) 3466 (OH), 1624 (>C=O), 1580 (C=N) cm⁻¹; ¹H NMR (DMSO-*d*₆) δ ppm: 2.41 (s, 3H, =C-CH₃), 3.21 (s, 3H, -N-CH₃), 6.42 (m, 2H, Ar-H), 7.28–7.59 (m, 6H, Ar-H), 9.59 (s, 1H, -N=CH); EI-MS *m/z* (%): 323 (M⁺, 100), 231 (40), 203 (18), 121 (14), 56 (64).

4.2.10. 4-(2, 4-Dimethoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (3j)

Yield 83%; mp 183.4 °C (yellow solid); IR (KBr) 1643 (>C=O), 1586 (C=N) cm⁻¹; ¹H NMR (DMSO-*d*₆) δ ppm: 2.47 (s, 3H, =C-CH₃), 3.23 (s, 3H, -N-CH₃), 3.81 (s, 3H, OCH₃), 3.84 (s, 3H, OCH₃), 6.62 (m, 2H, Ar-H), 7.18–7.49 (m, 6H, Ar-H), 9.49 (s, 1H, -N=CH); EI-MS *m/z* (%): 351 (M⁺, 100), 259 (56), 121 (20), 56 (97).

4.2.11. 4-(2, 4, 6-Trihydroxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (3k)

Yield 82%; mp 243.8 °C (reddish powder); IR (KBr) 3552 (OH), 3490 (OH), 1629 (>C=O), 1579 (C=N) cm⁻¹; ¹H NMR (CDCl₃) δ ppm: 2.33 (s, 3H, =C-CH₃), 3.13 (s, 3H, -N-CH₃), 6.80 (s, 2H, Ar-H), 7.35–7.60 (m, 5H, Ar-H), 9.83 (s, 1H, -N=CH); EI-MS *m/z* (%): 339 (M⁺, 100), 247 (43), 203(24), 121 (14), 84 (15), 56 (68).

4.2.12. 4-(3,5-Dimethoxy-4-hydroxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (3l)

Yield 92%; mp 258.9 °C (yellow powder); IR (KBr) 1632 (>C=O), 1588 (C=N) cm⁻¹; ¹H NMR (DMSO-*d*₆) δ ppm: 2.44 (s, 3H, =C-CH₃), 3.13 (s, 3H, -N-CH₃), 3.82 (s, 6H, 2×-OCH₃), 7.01(s, 2H, Ar-H), 7.36–7.53 (m, 5H, Ar-H), 9.46 (s, 1H, -N=CH); EI-MS *m/z* (%): 367 (M⁺, 100), 345 (30), 344 (47), 275 (45), 56 (70).

4.2.13. 4-(3-Methyl-4-methoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1H-pyrazol-3(2H)-one (3m)

Yield 89%; mp 269.8 °C (yellow crystal); IR (KBr) 1624 (>C=O), 1579 (C=N) cm⁻¹; ¹H NMR (DMSO-*d*₆) δ ppm: 2.21 (s, 3H, -CH₃), 2.42 (s, 3H, =C-CH₃), 3.12 (s, 3H, -N-CH₃), 3.81 (s, 3H, -OCH₃), 7.36–7.57 (m, 7H, Ar-H), 9.43 (s, 1H, -N=CH); EI-MS *m/z* (%): 335 (M⁺, 100), 259 (21), 201 (21), 135 (15), 56 (79).

4.3. Synthesis of Schiff base analogues of 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one with phthalaldehyde (4 and 5)

Phthalaldehyde (268 mg, 2 mmol) was added to a solution of 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one (203 mg, 1 mmol) in anhydrous ethanol (20 mL) at room temperature. The reaction mixture was refluxed for 6 h under atmospheric conditions (Scheme 2) and then cooled to room temperature. The progress

of the reaction was monitored by TLC. The solvent was evaporated and then the mixture was subjected to column chromatography on a silica gel using *n*-hexane-dichloromethane (2:1) to yield compounds 4 and 5.

4.3.1. 2-[(2,3-Dihydro-1,5-dimethyl-3-oxo-2-phenyl-1H-pyrazol-4-ylimino)methyl]benzaldehyde (4)

Brown oily liquid. Yield 42%; IR (KBr) 1724 (>C=O), 1664 (>C=O), 1596 (C=N) cm⁻¹; ¹H NMR (CDCl₃) δ ppm: 2.42 (s, 3H, =C-CH₃), 3.09 (s, 3H, -N-CH₃), 6.69–6.78 (m, 3H, Ar-H), 7.38–7.69 (m, 6H, Ar-H), 9.49 (s, 1H, -N=CH), 10.84 (s, 1H, CHO); EI-MS *m/z* (%): 319 (M⁺, 100), 214 (32), 187 (23), 172 (19), 104 (16), 84 (10), 56 (86).

4.3.2. 4-[2-(2,3-Dihydro-1,5-dimethyl-3-oxo-2-phenyl-1H-pyrazol-4-ylimino-methyl)benzylideneamino]-1,2-dihydro-1,5-dimethyl-2-phenylpyrazol-3-one (5)

Brown oily liquid. Yield 47%; IR (KBr) 1658(>C=O), 1579(C=N) cm⁻¹; ¹H NMR (CDCl₃) δ ppm: 2.45 (s, 3H, =C-CH₃), 3.10 (s, 3H, -N-CH₃), 6.71–6.85 (m, 6H, Ar-H), 7.49–7.86 (m, 8H, Ar-H), 9.56 (s, 1H, -N=CH); EI-MS *m/z* (%): 504 (M⁺, 33), 334 (22), 333 (100), 245 (27), 203 (19), 172 (19), 104 (36), 84 (21), 56 (91).

4.4. DPPH radical scavenging activity

The free radical-scavenging activity of the synthesized compounds was assayed according to the Blois method with some modification,²¹ and using DPPH. To 0.1 ml samples of different concentrations (2.5–20 mg/mL) in ethanol, 4 ml of 1.5 × 10⁻⁵ M DPPH solution was added, thoroughly mixed, and then left to stand at room temperature in a dark place. After 30 min of incubation, the absorbance of the solution was measured at 520 nm, the result of which was used to calculate DPPH radical scavenging activity (%).

4.5. Cell culture

The RAW264.7 cells were obtained from Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 g/mL streptomycin and 100 M MEM non-essential amino acid solution. In all experiments, the cells were grown to 80–90% confluence and subjected to not more than 20 cell passages.

4.6. Measurement of cell viability

Cell viability was assessed by MTT assay. RAW264.7 cells seeded onto 96-well plates (5 × 10⁴ cells/well) were treated with various concentrations of compound 3f. After 24 h incubation, 20 μl of MTT (5 mg/ml) was added and then incubated for 4 h. The culture medium was removed, and then the cells were dissolved in 0.04 N HCl/isopropyl alcohol. The optical densities (OD) of the resulting cell cultures were measured at 570 nm and 630 nm using a microplate reader.

4.7. Measurement of nitric oxide

The RAW264.7 cells (5 × 10⁵ cells/well) were seeded onto a 24-well culture plate at 37 °C for O/N in medium. The cells were pre-incubated with various concentrations of 3f for 24 h. NO production was monitored by measuring the nitrite levels in the culture media using Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid). After incubating for 10 min, the absorbance was measured at 570 nm. Nitrite levels in the samples were calculated from a standard curve with a known concentration of sodium nitrite.

4.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the RAW264.7 cells was prepared using a TRIzol reagent (Gibco, Grand Island, NY). The concentration and integrity of RNA was determined by measuring absorbance at 260/280 nm. The forward and reverse primers for iNOS were 5'-TCTTTGACGCTCGGAAGTGTAGCA-3' and 5'-CGTGAAGCCATGACCTTTCGCATT-3', respectively. The forward and reverse primers for COX-2 were 5'-TTGCTGT ACAAGCAGTGGCAAAGG-3' and 5'-AGGACAAACACCGGAGGGAATC TT-3', respectively. The forward and reverse primers for the mouse GAPDH mRNA expression (used as a control for total RNA content for each sample) were 5'-AACTTTGGCA TTGTGGAAGGGCTC-3' and 5'-TGGAAGAGTGGGAGTTGCTGTGA-3', respectively. RT-PCR was performed using a ONE-STEP RT-PCR PriMix kit (Invitrogen Co., USA), according to the manufacturer's instructions.

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