Full Paper

Synthesis and Biological Evaluation of Some Hydroxypyrazole Derivatives as Anti-inflammatory-Antimicrobial Agents

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Some hydroxypyrazole derivatives **2** – **7** were synthesized by cyclocondensation of the keto-ester **1** with hydrazines hydrate or substituted hydrazines followed by reduction and acylation with acetic anhydride or trifluoroacetic anhydride. The newly synthesized compounds were evaluated for their anti-inflammatory, antimicrobial activities. In addition, the ulcerogenic and acute toxicity profiles were determined. Compounds *N*-(4-(5-hydroxy-1-trifluoroacetyl-1*H*-pyrazol-3-yl)phenyl) trifluoroacetamide **4b**, 3-(4-nitrophenyl)-1-(4-methoxyphenyl)-1*H*-pyrazol-5-ol **5b**, and *N*-(4-(5-hydroxy-1-methyl-1*H*-pyrazol-3-yl)phenyl)trifluoroacetamide **7b** were proved to be the most active anti-inflammatory, antimicrobial agents in the present study with a good safety margin and minimal or no ulcerogenic effect.

Keywords: Hydroxypyrazole / Anti-inflammatory / Antimicrobial / Ulcerogenic effect / Acute Toxicity

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Introduction

Identification of novel compounds which treat both infectious and inflammatory states more effectively and which lack side effects associated with current therapies remains a major challenge in biomedical research. The use of several drugs to treat inflammatory conditions associated with infection is a problem, especially in case of patients with impaired liver or kidney functions, or to avoid drug-drug interaction. In addition, from the pharmacoeconomic cost-effective stand-point and seeking for a better patient compliance, a dual anti-inflammatory, antimicrobial agent with minimum adverse effects and a high safety margin is highly desirable. This promoted us searching for agents that have a dual effect as anti-inflammatory, antimicrobial agents. Compounds containing pyrazole functionality have been reported to exhibit anti-inflammatory activity [1 – 5]. Moreover, Tanitame et al. reported the antimicrobial activities of several pyrazole derivatives [6 – 8]. We have already reported the anti-inflammatory and the antimicrobial activities of some lead compounds containing the pyrazole moiety attached to different heterocyclic rings [9-17]. Some of our reported compounds **A** [11], **B** [12], **C** [13, 14], and **D** [15] showed pronounced dual anti-inflammatory, antimi-

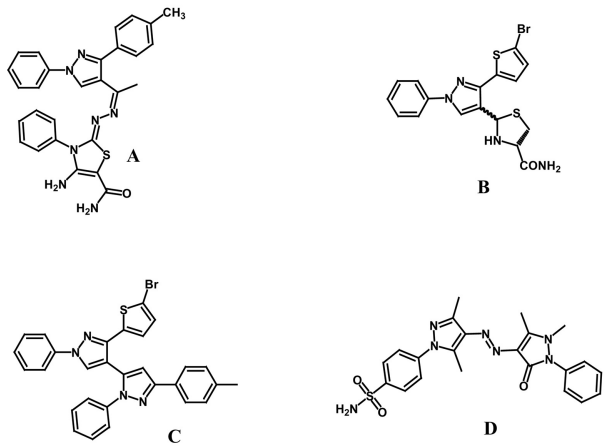

Figure 1. Structures of reported active pyrazole derivatives A, B, C, and D.

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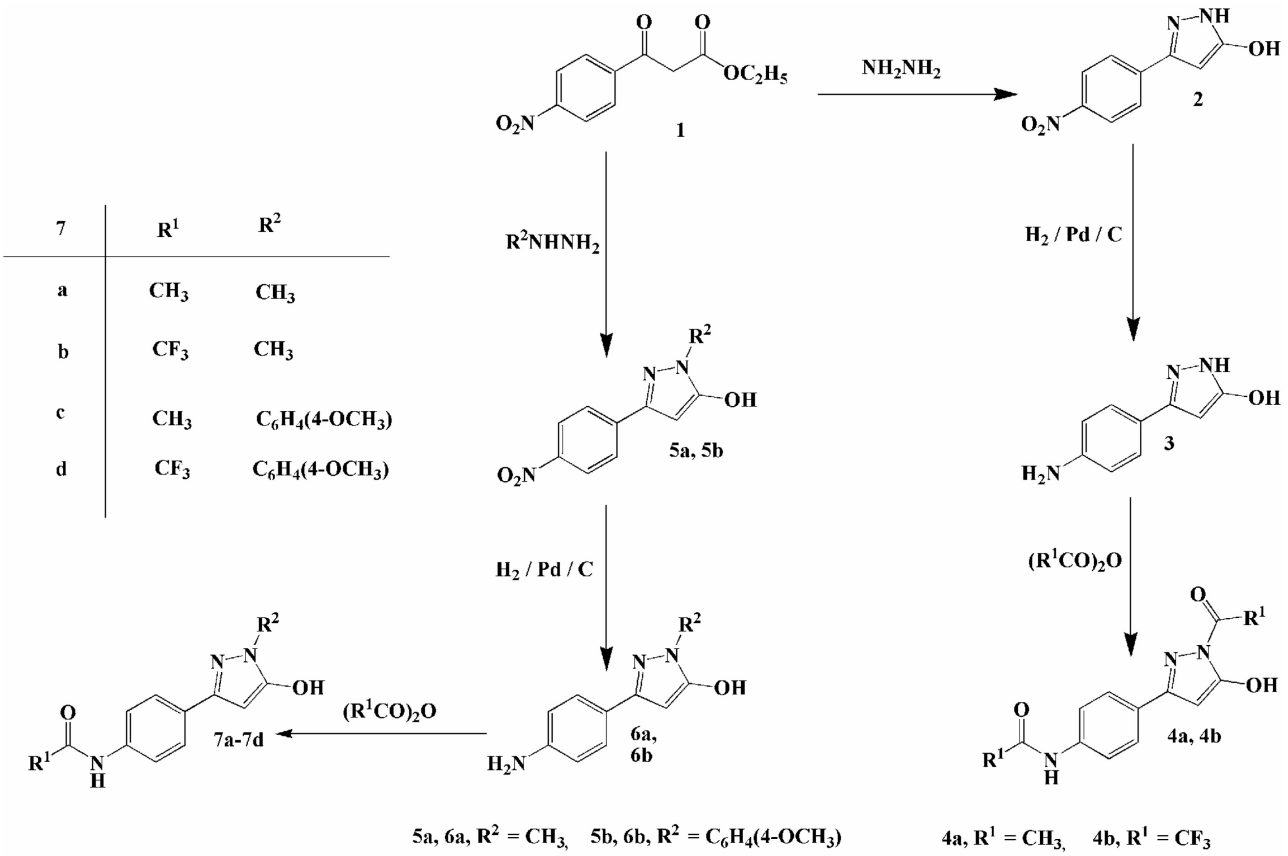

Scheme 1. Synthesis route of target compounds.

crobial activity (Figure 1). The present investigation deals with the synthesis of some hydroxypyrazole derivatives in order to investigate their anti-inflammatory and antimicrobial activities. It should be pointed out that, in addition to the targeted anti-inflammatory and antimicrobial activities, the ulcerogenic and acute toxicity profiles of the newly synthesized compounds were determined. The results revealed that some derivatives showed promising activities.

Results and discussion

Chemistry

The target compounds were synthesized according to the presented Scheme 1. Cyclocondensation of the key intermediate keto-ester **1** with hydrazine hydrate resulted in pyrazole compounds **2**. The nitro group of the latter compound was reduced with hydrogen in the presence of palladium and carbon to give rise to amino compound **3**. Acylation of compound **3** with acetic anhydride or trifluoroacetic anhydride gave the diacylated compounds **4a**, **4b**. On the other hand, the key compound keto-ester **1** was allowed to react with substituted hydrazines to afford the pyrazole derivatives **5a**, **5b**. The amino derivatives **6a**, **6b** were obtained by treating **5a**, **5b** with hydrogen in the presence of palladium and carbon. Acylation of compound **6a**, **6b** with acetic anhydride or trifluoroacetic anhydride gave the acylated compounds **7a – 7d.** It is worth to mention that the newly synthesized pyrazole compounds are present in the hydroxypyrazole form rather than the pyrazolone tautomers as revealed from IR and ^{1}H-NMR spectra.

Biological screening

Anti-inflammatory activity

Cotton pellet-induced granuloma bioassay

The anti-inflammatory activity of the synthesized compounds **2 – 7** was evaluated applying the cotton-pellet granuloma bioassay in rats [18] using indomethacin as a reference standard. The ED_{50} values were determined for each compound. Data were expressed as the mean ± SEM. Significant difference between the control and the treated groups was evaluated using Student's t-test (Table 1).

Table 1. The anti-inflammatory (ED_{50} [μmol])* and ulcerogenic activities* of test compounds.

Test compound	ED_{50} [μmol]	% Ulceration
Indomethacin	9.68	100
2	11.44	0.0
3	9.84	0.0
4a	10.82	10
4b	9.96	0.0
5a	11.76	10
5b	9.94	10
6a	11.36	0.0
6b	11.34	0.0
7a	14.48	10
7b	8.34	0.0
7c	12.46	0.0
7d	13.28	10

* All data are significantly different from control ($P < 0.001$).

The difference in results was considered significant when $P < 0.001$ (Table 1). All test compounds showed significant anti-inflammatory activity. Compounds **3**, **4b**, **5b**, and **7b** (ED_{50} = 9.84, 9.96, 9.94, and 8.34 μmol, respectively) possessed anti-inflammatory activity comparable to that of indomethacin (ED_{50} = 9.68 μmol). The amino derivative **3** showed better anti-inflammatory activity than its nitro-compound precursor **2**. On other hand, acylation of the amino group of **3** did not affect the activity. Moreover, reduction of nitro compound **5b** greatly reduced the activity, whereas the activity of amino compound **6a** was greatly enhanced after introduction of triflouroacetyl group to form compound **7b**. In general, the trifluoroacetyl derivatives have higher activity than the acetyl derivatives.

Carrageenan-induced rat paw edema

Compounds **3**, **4b**, **5b**, and **7b** that showed anti-inflammatory activity comparable to that of indomethacin in the cotton pellet-induced granuloma bioassay, were further evaluated for their *in vivo* systemic effect using the carrageenan-induced paw edema bioassay in rats [19]. The results (Table 2) revealed that test compounds **3**, **4b**, **5b**, and **7b** exhibited systemic anti-inflammatory activity (% protection 68.3, 73.4, 71.4, and 77.5, respectively) comparable to that of indomethacin (% protection 74.4).

Ulcerogenic effects

All synthesized compounds were evaluated for their ulcerogenic potential in rats [20]. All the test compounds proved to have a superior GI safety profile (0 – 10% ulceration) in the population of the test animals at oral doses of 30 mmol/kg per day when compared with indomethacin. This reference drug was found to cause 100% ulceration under the same experimental conditions (Table 1). Gross observation of the isolated rat stomachs showed a normal stomach texture for compounds **2**, **3**, **4b**, **6a**, **6b**, **7b**, and **7c** (0% ulceration).

Table 2. Effect of compounds **3**, **4b**, **5b**, and **6b** on carrageenan-induced rat paw edema [mL], % protection, and activity relative to indomethacin.

Test compound	Increase in paw edema [mL] ± SEM[a, b)]	% Protection	Activity relative to Indomethacin
Control	0.98 ± 0.027	0.0	0.0
Indomethacin	0.25 ± 0.024	74.4	100
3	0.31 ± 0.026	68.3	91.80
4b	0.26 ± 0.015	73.4	98.55
5b	0.28 ± 0.012	71.4	95.96
7b	0.22 ± 0.036	77.5	104.16

a) SEM denotes the standard error of the mean.
b) All data are significantly different from control ($P < 0.001$).

Acute toxicity

Compounds **3**, **4b**, **5b**, and **7b** were further evaluated for their oral acute toxicity in male mice using the method described in the literature [21, 22]. The results indicated that most of the tested compounds proved to be nontoxic and well tolerated by the experimental animals up to 120 mg/kg. Moreover, these compounds were tested for their toxicity through parenteral route [12]. The results revealed that all the test compounds were nontoxic up to 55 mg/kg.

Antimicrobial activity

The designed compounds **2**–**7** have been evaluated for their antimicrobial activity. The microdilution susceptibility test in Müller-Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid) were used for the determination of antibacterial and antifungal activity [23]. The minimal inhibitory concentration (MIC) listed in Table 3 showed that although all test compounds have antifungal activity lower than that of clotrimazole (Canesten®, Bayer). All test compounds possessed lower activity against *B. subtilis* than that of ampicillin but active against *E. coli* and *S. typhimurium*. Compounds **3**, **4a**, **4b**, **5a**, **6a**, **6b**, and **7b** possessed half-fold the activity of ampicillin against *E. coli*, while **5b** showed comparable activity to that of ampicillin against *E. coli*. On the other hand, compounds **4a**, **4b**, **5b**, and **6b** showed half-fold the activity of ampicillin against *S. typhimurium*. The amino derivative **3** and its acylated derivatives **4a** and **4b** showed better antibacterial activity than their nitro-compound precursor **2** against *E. coli*, while reduction of nitro compound **5b**

Table 3. Minimal inhibitory concentration (MIC) of test compounds [μg/mL].

Test compound	*E. coli*	*S. typhimurium*	*S. aureus*	*B. subtilis*	*C. albicans*
Ampicillin	25	12.5	12.5	12.5	
Clotrimazole					12.5
2	100	100	100	100	>200
3	50	50	>200	100	>200
4a	50	25	>200	>200	>200
4b	50	25	100	100	>200
5a	50	100	100	100	>200
5b	25	25	50	>200	>200
6a	50	50	100	>200	>200
6b	50	25	100	100	>200
7a	100	>200	>200	>200	>200
7b	50	50	100	>200	>200
7c	>200	>200	100	>200	>200
7d	100	50	100	>200	>200

reduced its activity against *E. coli*. Although reduction of nitro compound **2** resulted in active amino compound **3** against *S. typhimurium*, the activity was increased after acylation of **3**. Furthermore, reduction of nitro compound **5b** to amino compound **6b** did not affect the activity against *S. typhimurium*.

It can be safely concluded that compound **3**, **4b**, **5b**, and **7b** were proved to be the most active anti-inflammatory, antimicrobial agents in the present study with minimal or no ulcerogenic effect and a good safety margin. Compound **5b** showed anti-inflammatory activity comparable to that of indomethacin with minimal ulcerogenic effect and no toxicity up to 120 mg/kg orally. In addition, its antibacterial activity against *E. coli* is comparable to that of ampicillin, while its activity against *S. typhimurium* is about 50% that of ampicillin.

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Experimental

Chemistry

All chemicals were purchased from E. Merck (Whitehouse Station, NJ, USA), Fluka AG (Buchs SG, Switzerland), TCI (Tokyo, Japan), and Aldrich (St. Louis, MO, USA). Melting points were determined in open glass capillaries using a Thomas capillary melting point apparatus (Philadelphia, PA, USA) and are uncorrected. Infrared (IR) spectra were recorded on 470-Shimadzu infrared spectrophotometer using the KBr disc technique (Shimadzu Corp., Kyoto, Japan). ^{1}H-NMR spectra were recorded on JEOL JNM-AL 300 FT NMR spectrometer (DMSO-d_6) (JEOL, Tokyo, Japan), and the chemical shifts are given in δ (ppm) downfield from tetramethylsilane (TMS) as an internal standard. Splitting patterns were designated as follows: s: singlet; d: doublet; t: triplet; m: multiplet. Elemental analyses were performed on Perkin-Elmer 2400 elemental analyzer (Perkin-Elmer, Hitachi, Tokyo, Japan) and were found within ±0.4% of the theoretical values. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel-precoated aluminium sheets (Type 60 GF254, Merck) and the spots were detected by exposure to UV lamp at λ 254 nm for few seconds.

*3-(4-Nitrophenyl)-1H-pyrazol-5-ol (**2**), 1-methyl-3-(4-nitrophenyl)-1H-pyrazol-5-ol **5a** and 1-(4-methoxyphenyl)- 3-(4-nitrophenyl)-1H-pyrazol-5-ol **5b***

A mixture of ethyl 3-(4-nitrophenyl)-3-oxopropanoate **1** (4.74 g, 20 mmol) and hydrazine hydrate, methyl hydrazine, or 4-methoxyphenylhydrazine (24 mmol) in ethanol (40 mL) was heated under reflux for 4 h. The reaction mixture was allowed to attain room temperature; the precipitated solid product was filtered, washed with water and crystallized from ethanol (Table 4).

*3-(4-Aminophenyl)-1H-pyrazol-5-ol (**3**), 3-(4-aminophenyl)-1-methyl-1H-pyrazol-5-ol **6a** and 3-(4-aminophenyl)-1-(4-methoxyphenyl)-1H-pyrazol-5-ol **6b***

To a solution of the appropriate nitro compound **2**, **5a**, or **5b** (10 mmol) in methanol (100 mL), was added 0.5 g of 10% Pd/C. The mixture was stirred overnight under a balloon of hydrogen gas. The reaction mixture was filtered over celite, followed by evaporation of the filtrate under reduced pressure. The obtained solid product was crystallized from ethanol (Table 4).

*N-(4-(5-Hydroxy-1-substituted-1H-pyrazol-3-yl)phenyl)acetamides **4a**, **7a**, **7c** and N-(4-(5-hydroxy-1-substituted-1H-pyrazol-3-yl)phenyl)trifluoroacetamides **4b**, **7b**, **7d***

Acetic anhydride or trifluoroacetic anhydride (25 mmol) was added slowly at room temperature to the selected amino compound **3**, **6a**, or **6b** (10 mmol), followed by warming the mixture till dissolution of all solids. The reaction mixture was stirred at room temperature for 15 min, then water was added and allowed to stand for overnight. The separated white solid product was filtered, washed with water, and crystallized from ethanol (Table 4).

In case of compounds **4a**, **4b** the mixture was stirred at room temperature with 2N NaOH (5 mL) for 5 h followed by neutralization with diluted HCl before the crystallization step.

Biological screening

Anti-inflammatory testing

Adult male Sprague-Dawley rats (120 – 140 g) were used (Medical Research Institute, Alexandria University). They were acclimated one week prior to use and allowed unlimited access to standard rat chow and water. Prior to the start of experiment, the animals were randomly divided into groups (6 rats each). Cotton pellet (35 ± 1 mg) cut from dental rolls were impregnated with 0.2 mL (containing 0.01 mmol) of a solution of the test compound in chloroform or acetone and the solvent was allowed to evaporate. Each cotton pellet was subsequently injected with 0.2 mL of an aqueous solution of antibiotics (1 mg penicillin G and 1.3 mg dihydrostreptomycin/mL). Two pellets were implanted subcutaneously, one in each axilla of

Table 4. Physical and analytical data of compounds **2** – **7**.

Comp. N°	Yield [%]	Mp. [°C]	Mol. Formula (Mol. wt.)	IR (KBr, [cm^{-1}])	^1H NMR (DMSO-d_6)
2	76	248-9	$C_9H_7N_3O_3$ (205.17)	3530 (OH), 3390 (NH), 1627(C=N), 1562, 1343 (NO_2)	5.63 (s, 1H, pyr-C_4-H), 6.54 (d, 2H, J = 9 Hz, phenyl $C_{2,6}$-H), 6.62 (s, 1H, NH, D_2O exchangeable), 7.35 (d, 2H, J = 9 Hz, phenyl $C_{3,5}$-H), 10.59 (brs, 1H, OH, D_2O exchangeable)
3	65	274-5	$C_9H_9N_3O$ (175.18)	3525 (OH), 3385, 3210 (NH_2), 1625 (C=N).	5.21 (brs, 2H, NH_2, D_2O exchangeable), 5.61 (s, 1H, pyr-C_4-H), 6.55 (d, 2H, J = 9 Hz, phenyl $C_{3,5}$-H),), 6.68 (s, 1H, NH, D_2O exchangeable), 7.32 (d, 2H, J = 9 Hz, phenyl $C_{2,6}$-H), 10.14 (brs, 1H, OH, D_2O exchangeable)
4a	84	268-9	$C_{13}H_{13}N_3O_3$ (259.26)	3515 (OH), 3395 (NH), 1693 (C=O), 1660 (C=O), 1628 (C=N)	2.42 (s, 3H, CH_3), 2.48 (s, 3H, CH_3), 5.78 (s, 1H, pyr-C_4-H), 6.87 (s, 1H, NH, D_2O exchangeable), 7.65 (d, 2H, J = 9 Hz, phenyl $C_{3,5}$-H), 7.75 (d, 2H, J = 9 Hz, phenyl $C_{2,6}$-H), 10.57 (brs, 1H, OH, D_2O exchangeable)
4b	86	262-4	$C_{13}H_7F_6N_3O_3$ (367.20)	3515 (OH), 3390 (NH), 1720 (C=O), 1698 (C=O), 1624 (C=N)	5.94 (s, 1H, pyr-C_4-H), 6.97 – 7.85 (m, 5H, phenyl –H, NH), 11.26 (brs, 1H, OH, D_2O exchangeable)
5a	81	253-4	$C_{10}H_9N_3O_3$ (219.19)	3525 (OH), 1624 (C=N), 1573, 1347 (NO_2)	3.59 (s, 3H, pyr-CH_3), 5.96 (s, 1H, pyr-C_4-H), 7.94 (d, 2H, J = 9 Hz, phenyl $C_{2,6}$-H), 8.19 (d, 2H, J = 9 Hz, phenyl $C_{3,5}$-H), 10.57 (brs, 1H, OH, D_2O exchangeable)
5b	83	220-2	$C_{16}H_{13}N_3O_4$ (311.292)	3530 (OH), 1623 (C=N), 1567, 1350 (NO_2)	3.84 (s, 3H, CH_3), 5.98 (s, 1H, pyr-C_4-H), 6.97 (d, 2H, J = 9 Hz, methoxyphenyl $C_{3,5}$-H), 7.73 (d, 2H, J = 9 Hz, methoxyphenyl $C_{2,6}$-H), 7.96 (d, 2H, J = 9 Hz, nitrophenyl $C_{2,6}$-H), 8.22 (d, 2H, J = 9 Hz, nitrophenyl $C_{3,5}$-H), 10.96 (brs, 1H, OH, D_2O exchangeable)
6a	67	240-2	$C_{10}H_{11}N_3O$ (189.21)	3525 (OH), 3392, 3228 (NH_2), 1631 (C=N)	3.59 (s, 3H, pyr-CH_3), 5.12 (brs, 2H, NH_2, D_2O exchangeable), 5.53 (s, 1H, pyr-C_4-H), 6.52 (d, 2H, J = 9 Hz, phenyl $C_{3,5}$-H), 7.33 (d, 2H, J = 9 Hz, phenyl $C_{2,6}$-H), 10.81 (brs, 1H, OH, D_2O exchangeable)
6b	61	245-6	$C_{16}H_{15}N_3O_2$ (281.30)	3525 (OH), 3395, 3220 (NH_2), 1624 (C=N)	3.82 (s, 3H, CH_3), 5.14 (s, 2H, NH_2, D_2O exchangeable), 5.94 (s, 1H, pyr-C_4-H), 6.69 (d, 2H, J = 9 Hz, aminophenyl $C_{3,5}$-H), 6.93 (d, 2H, J = 9 Hz, aminophenyl $C_{2,6}$-H), 7.55 (d, 2H, J = 9 Hz, methoxyphenyl $C_{3,5}$-H), 7.83 (d, 2H, J = 9 Hz, methoxyphenyl $C_{2,6}$-H), 10.89 (brs, 1H, OH, D_2O exchangeable)
7a	74	260-1	$C_{12}H_{13}N_3O_2$ (231.251)	3520 (OH), 3385 (NH), 1663 (C=O), 1630 (C=N)	2.36 (s, 3H, CH_3), 3.56 (s, 3H, pyr-CH_3), 5.89 (s, 1H, pyr-C_4-H), 7.61 – 7.78 (m, 5H, phenyl-H, NH), 11.32 (s, 1H, OH, D_2O exchangeable)
7b	69	246-8	$C_{12}H_{10}F_3N_3O_2$ (285.22)	3525 (OH), 3385 (NH), 1708 (C=O), 1628 (C=N)	3.53 (s, 3H, pyr-CH_3), 5.82 (s, 1H, pyr-C_4-H), 7.65 – 7.74 (m, 5H, phenyl-H, NH), 11.25 (s, 1H, OH, D_2O exchangeable)
7c	76	254-6	$C_{18}H_{17}N_3O_3$ (323.34)	3520 (OH), 3380 (NH), 1669 (C=O), 1632 (C=N)	2.29 (s, 3H, CH_3), 3.85 (s, 3H, CH_3), 5.96 (s, 1H, pyr-C_4-H), 6.86 – 7.79 (m, 9H, phenyl-H, NH), 10.98 (brs, 1H, OH, D_2O exchangeable)
7d	72	242-4	$C_{18}H_{14}F_3N_3O_3$ (377.31)	3525 (OH), 3383 (NH), 1696 (C=O), 1626 (C=N)	3.83 (s, 3H, CH_3), 5.94 (s, 1H, pyr-C_4-H), 6.78 – 7.93 (m, 9H, phenyl-H, NH), 11.05 (s, 1H, OH, D_2O exchangeable)

the rat, under mild general anaesthesia. One group of animals received the standard reference indomethacin and the antibiotics at the same level. Pellets containing only the antibiotics were similarly implanted in control rats. Seven days later, the animals were sacrificed and the two cotton pellets with adhering granulomas were removed, dried for 48 h at 60°C, and weighed. The increment in dry weight (difference between the initial and final weights) was taken as a measure of granuloma ± S.E. This was calculated for each group and the percentage reduction in dry weight of granuloma from control value was

also calculated. The ED_{50} values were determined through dose-response curves, using doses of 4, 7, 10, and 15 mmol for each compound.

Carrageenan-induced rat paw edema

Male albino rats weighing 120 – 150 g (Medical Research Institute, Alexandria University) were used throughout the work. They were kept in the animal house under standard conditions of light and temperature with free access to food and water. The animals were randomly divided into groups of six rats each. The paw edema was induced by subplantar injection of 50 μL of 2% carrageenan solution in saline (0.9%). Indomethacin and the test compounds were dissolved in DMSO and were injected subcutaneously in a dose of 10 mmol/kg body weight, 1 h prior to carrageenan injection. The controls were injected with DMSO. The volume of paw edema (mL) was determined by means of water plethysmometer immediately after injection of carrageenan and 4 h later. The increase in paw volume between time 0 and + 4 h was measured [19]. The percentage protection against inflammation was calculated as follows:

$$V_c - V_d/V_c \times 100$$

where V_c is the increase in paw volume in the absence of test compound (control) and V_d is the increase of paw volume after injection of the test compound. Data were expressed as the mean ± SEM. Significant difference between the control and the treated groups was performed using Student's *t*-test and *P* values. The differences in results were considered significant when $P < 0.001$. The anti-inflammatory activity of the test compounds relative to that of indomethacin was also calculated.

Ulcerogenic effects

All synthesized compounds were evaluated for their ulcerogenic potential in rats [20]. Indomethacin was used as reference standard. Male albino rats (100 – 120 g) were fasted for 12 h prior to the administration of the compounds. Water was given *ad libitum*. The animals were divided into groups, each consisting of six animals. The control group received 1% gum acacia orally. The other groups received indomethacin or the test compounds orally in two equal doses at 0 and 12 h for three successive days at a dose of 30 mM/kg per day. Animals were sacrificed by diethyl ether 6 h after the last dose and the stomach was removed. An opening at the greater curvature was made and the stomach was cleaned by washing with cold saline and inspected with a 3X magnifying lens for any evidence of hyperemia, hemorrhage, definite hemorrhagic erosion, or ulcer. An arbitrary scale was used to calculate the ulcer index which indicates the severity of the stomach lesions [20]. The percentage ulceration for each group was calculated as follows:

$$\% \text{ Ulceration} = \frac{\text{Number of animals bearing ulcer in a group}}{\text{Total number of animals in the same group}} \times 100$$

Acute toxicity

The same biologically significant compounds were further investigated for their oral acute toxicity in male mice [21, 22] (each 20 g, supplied by Medical Research Institute, Alexandria University). Groups of mice each consisting of six animals were used. The compounds were given orally suspended in 1% gum acacia, in doses of 1, 10, 100, 200, 250, and 300 mg/kg, respectively. 24 h later, the % mortality in each group and for each compound was recorded. Moreover, these compounds were tested for their parenteral acute toxicity, groups of mice each consisting of six animals were used. The compounds or their vehicle, propylene glycol (control) were given by intraperitoneal injection in doses of 10, 25, 50, 75, and 100 mg/kg, respectively. Survival was followed up to 7 days [12].

Antimicrobial activity

The microdilution susceptibility test in Müller-Hinton Broth (Oxoid) and Sabouraud Liquid Medium (Oxoid) were used for the determination of antibacterial and antifungal activity [23]. Test organisms: *Escherichia coli* (*E. coli*) ATCC 25922 and *Salmonella typhimurium* (*S. typhimurium*) *ATCC* 3311 as Gram-negative bacteria, *Staphylococcus aureus* (*S. aureus*) ATCC 19433 and *Bacillus subtilis* (*B. subtilis*) *ATCC* 1042 as Gram-positive bacteria and *Candida albicans* (*C. albicans*) as a yeast fungus. Ampicillin trihydrate and clotrimazole were used as standard antibacterial and antifungal agents, respectively. Solutions of the test compounds, ampicillin trihydrate and clotrimazole were prepared in DMSO at concentration of 1600 mg/mL. From this stock different dilutions of the compounds (800, 400, down to 6.25 mg/mL) were prepared. The microorganism suspensions at 10^6 colony forming unit/mL/1c concentration were inoculated to the corresponding wells. Plates were incubated at 36°C for 24 to 48 h. The incubation chamber was kept sufficiently humid. At the end of the incubation period, the minimal inhibitory concentrations (MIC) were determined. Controls with DMSO and uninoculated media were also maintained.

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