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Synthesis and biological evaluation of new pyrazolo[3,4-*d*]pyrimidine derivatives

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Abstract: Several new pyrazolopyrimidine compounds were achieved from aminocyanopyarazole **1**. The starting material **1** was initially coupled with orthoester at refluxed with various primary amines, ammonia, hydrazines and hydroxylamine to furnish a series of pyrazolo[3,4-*d*]pyrimidines. The reaction of imidate **2a-b** with hydrazide derivatives led to the formation of pyrazolo[3,4-*d*][1,2,4]triazolo[4,3-*c*]pyrimidines. Some of the synthesized compounds **3a** and **4c** were evaluated for their anti-inflammatory, antipyretic and nociceptive activities. We start by studing the toxicity of these two molecules by measuring the corresponding DL50. The DL50 of **3a** and **4c** are estimated to 1333.2mg / kg and 1593.5mg / kg respectively. Pharmacological evaluation showed that compounds **3a** and **4c** at doses (5.5-22.2 mg / Kg, i.p) exhibited anti-inflammatory activities compared to Ibuprofen (150 mg / Kg, i.p), used as a refer ence drug. Further, our study showed that the injection of derived pyrazolopyrimidines on hyperthermic animal leads to a decrease in temperature after 1 hours of treatment compared to paracetamol used as reference. In addition, the injection of derived pyrazolopyrimidines at different doses contains a potent nociceptive activity. This effect is dose-dependent compared to aspirin.

Keywords: pyrazolo[3,4-*d*]pyrimidines; anti-inflammatory; antipyretic; nociceptive activity; Dimroth rearrangement.

Introduction

In recent years, pyrazole and pyrimidine derivatives attracted organic chemists due to their widespread potential biological and chemotherapeutic activities. Pyrazolopyrimidines and related heterocyles are found to possess wide applications in the field of medicine and agriculture. They are biologically active isomeric purine analogues and have useful properties as antimetabolites in purine biochemical reactions[1-3]. They exhibit wide pharmacological activities like tuberculostatic[4] antimicrobial[5], neuroleptic[6], antitumor[7], antihypertensive[8] and antileishmanial activities[9].

Stimulated by the successful application of pyrazolo[3,4-*d*]pyrimidines, our objective was to synthesize a new class of pyrazolo[3,4-*d*]pyrimidine analogues **3a-c**, **4a-b** by introducing various groups at the pyrazolopyrimidine ring and to evaluate its anti-inflammatory, nociceptive and antipyretic activities.

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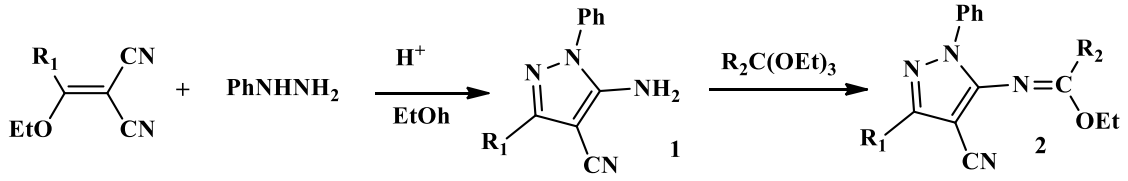
Results and Discussion

Chemistry

Several works mentioned the synthesis of 5-amino-4-cyano pyrazoles[10-12]. These products were prepared via a standard addition of hydrazine derivatives to ethoxymethylene compounds. To generalize the synthesis of 5-amino-4-cyano-1-substituted pyrazoles **1**, we have prepared a variety of unsaturated ethoxymethylene compounds in good yields and the corresponding pyrazoles. The 5-amino pyrazole-4-carbonitrile **1a-e** react with orthoester to give the corresponding imidate derivatives **2a-e** (Scheme 1), these later are used as precursors for the synthesis of various pyrazolo[3,4-*d*]pyrimidines.



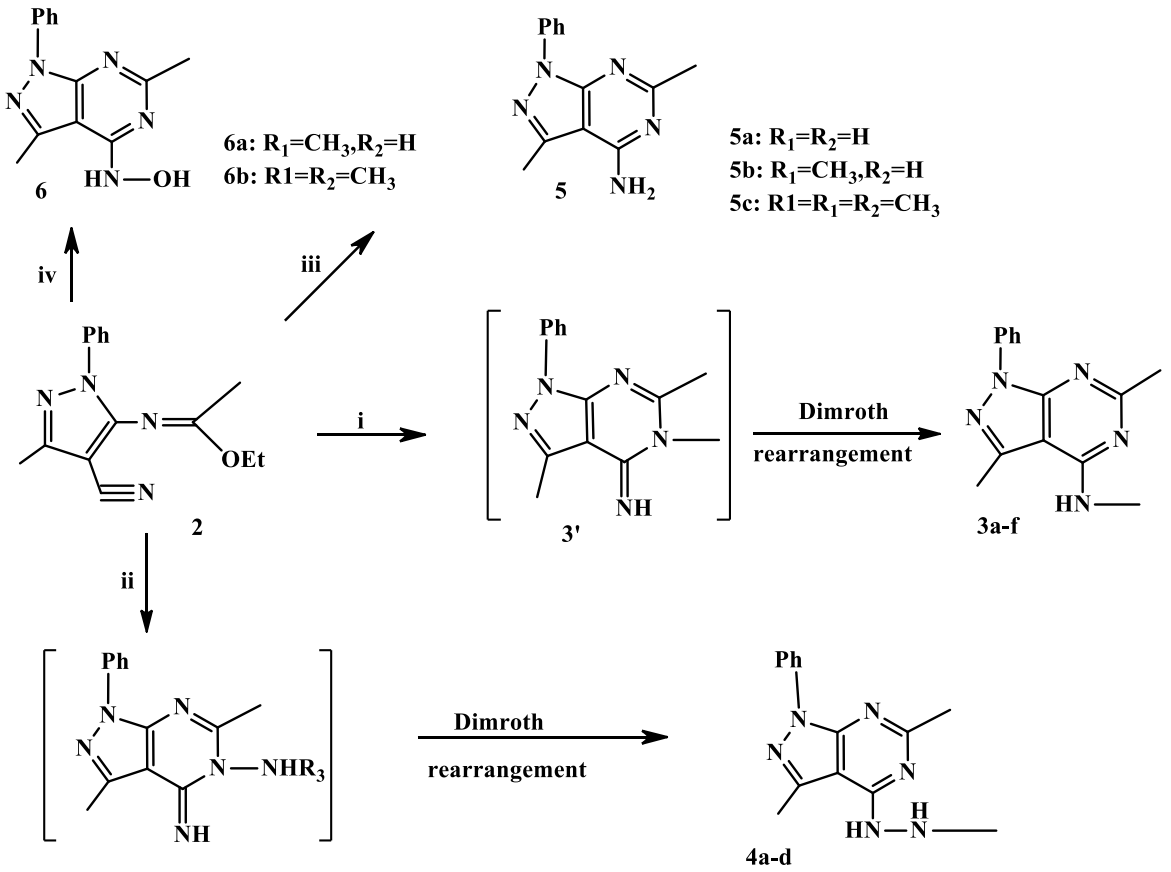
Scheme 1. Synthesis of 4-cyano-1-phenyl-1*H*-pyrazoloimidate **2**

Table 1. Synthesis of imidates derivatives

Entry	R_1	R_2	Yield %
2a	H	H	70
2b	H	Me	62
2c	Me	H	70
2d	Me	Me	40
2e	Me	Et	25

Reaction of imidates **2** with some primary aromatic amines, ammonia, hydrazines and hydroxylamine gave new pyrazolo[3,4-*d*]pyrimidine derivatives of significant biological interest since such compounds are substituted analogues of the well-known drug Allopurinol[13].

The imidate **2** reacted at their both electrophilic sites with aromatic amines to yield the pyrazolopyrimidines type **3a-f** in two steps. Firstly, the condensation of **2** with amines in toluene in the presence of a catalytic amount of acetic acid led to the intermediate **3'** by the nucleophilic attack of the NH_2 motif on imidic carbon. In the second step, the non isolable amidine **3'** was transformed into the novel pyrazolopyrimidines **3a-f** via Dimroth rearrangement. The isomerization of **3'** into the thermodynamically more stable pyrazolopyrimidines derivative **3a-f** (Scheme 2) seems to occur through acid / base-catalysed in tandem of ring opening followed by ring closure. This rearrangement is consistent with those reported in some earlier reports[14,15].



Scheme 2. Synthetic route of compounds **3-6.** Reagents and conditions: (i) R_3-NH_2,AcOH, Toluene. (ii) R_3-NH-NH_2, AcOH, Toluene. (iii) NH_3, EtOH. (iv) NH_2OH/HCl, EtOH, NEt_3.

Table 2. Synthesis of pyrazolo[3,4-*d*]pyrimidines derivatives

Entry	R_1	R_2	R_3
3a	CH_3	H	Ph
3b	CH_3	CH_3	Ph
3c	CH_3	H	CH_2-Ph
3d	CH_3	CH_3	CH_2-Ph
3e	CH_3	H	Naphtyl
3f	CH_3	CH_3	Naphtyl
4a	CH_3	H	H
4b	CH_3	CH_3	H
4c	CH_3	H	Ts
4d	CH_3	CH_3	Ts

Therefore, to confirm the structure of compounds **3**, an X-ray crystallographic study was carried out of compound **3d** obtained by condensation of **2d** with aniline (Figure 1). Crystals were obtained by slow evaporation from ethanol / DMSO-d_6 solution.

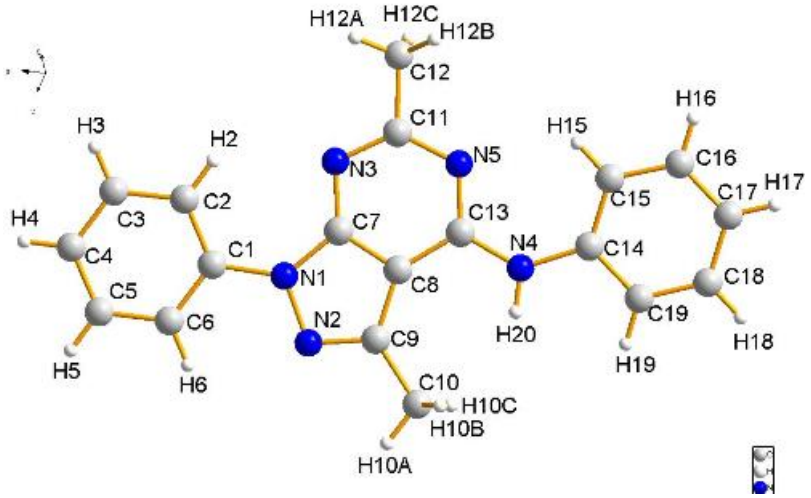
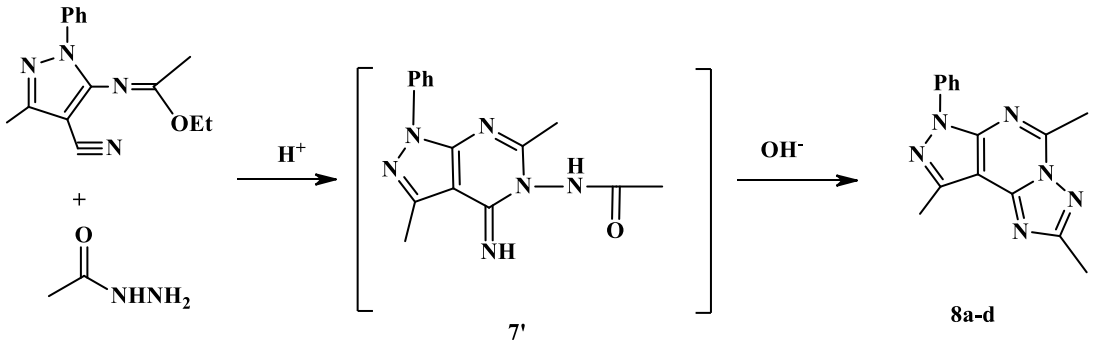


Figure 1. X-ray crystal analysis of compound **3d**

It seemed of interest to study the analogous reactions of imidate derivatives **2** with hydrazides. Treatment of imidates **2** with an equivalent of hydrazides in toluene for 24 h gave pyrazolo[3,4-*d*][1,2,4]triazolo[4,3-*c*]pyrimidines **8**.

Successive two nucleophilic additions of NH_2 group of hydrazide to the imidic carbon and to the cyano was observed to yield the intermediates amidopyrazolopyrimidines **7'**. The formation of **7'** was followed by an intracyclisation via elimination of water to give pyrazolotriazolopyrimidines **8**. The IR spectrum revealed the absence of the characteristic absorption bands corresponding to cyano, amino and CO groups.



Scheme 3. Synthesis of pyrazolo[3,4-*d*][1,2,4]triazolo[4,3-*c*]pyrimidines

Table 3. Synthesis of pyrazolo[3,4-*d*][1,2,4]triazolo[4,3-*c*]pyrimidines

compounds	R_1	R_2	R_4	Yield (%)	mp°C
8a	CH_3	H	Ph	79	232
8b	CH_3	CH_3	Ph	42	241
8c	CH_3	H	CH_3	48	201
8d	CH_3	CH_3	CH_3	52	130

Biological evaluation

Determination of LD50 of compounds 3a and 4c in adult mice

The acute toxicity of the two test compounds (3-methyl-*N*,1-diphenyl-1*H*-pyrazolo [3,4-*d*]pyrimidin-4-amine **3a** and 4-methyl-*N*-(3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidine-4-yl)benzenesulfonohydraine **4c**) in mice indicated their good safety profiles and their median lethal intraperitoneal doses (LD_{50}) values were found to be 1332.2 and 1593.5 mg / kg b.w., respectively.

Evaluation of Antipyretic activity

The effect of tested compounds (**3a** and **4c**) on normal body temperature in rats is presented in Table 4. The results showed that **3a** and **4c** at dose of 22.2 mg / kg (1/50 LD_{50}) caused significant lowering of the body temperature up to 4 hours. The normal mean temperature 38.37°C at 0 hour was reduced to 36.67 °C after 4 hours for the compound **3a**. Further, Lowering of body temperature was noticed for the compound 4c, in fact the mean temperature 38.97°C at 0 hour was reduced to 36.67°C within a 4 hour period. Time of peak effect was obtained from 2 to 4 h after oral administration of test drugs. Paracetamol (150mg / kg b.w.) also suppressed hyperthermia induced by yeast during all the observation times when compared with control values.

Table 4. Evaluation of Antipyretic activity

	dose	**t0**	**30min**	**1h**	**2h**	**3h**	**4h**
Compound 3a	1/50	38,37±0,5	38,03 ±0,3	36,43±0,4	36,77±0,9	37,53±0,5	36,67±0,3
	1/100	38,1±0,89	36,83±0,6	37.17±0,7	37,37±0,6	36.37±1,0	37,5±1,04
	1/200	37,57	36,8±1,22	36,85±0,7	35,95±1,3	36.55±0,9	36,95±1,4
Compound 4c	1/50	38,97	36,73±1,2	37,1±0,14	36,15±0,0	37,45±0,4	36,15±0,2
	1/100	38,07	37,4±0,4	37,47±0,0	36,57±1,1	37,53±0,3	37±0,79
	1/200	38,27	37,7±0,61	37,77±0,7	37,47±0,4	37,47±0,8	37,4±1,04
paracetamol		38,03	37,37±0,3	37,17±0,3	37,17±0,2	36,9±0,95	37,83±0,2

*$p \leq 0.05$: vs control

Evaluation of nociceptive activity

The effect of two newly synthesized pyrozolopyrimidine derivatives (**3a** and **4c**) on acetic induced writhing in mice was given in Figure 2. The present study revealed that all compounds showed a significant nociceptive effect ($P < 0.001$) at both doses (1/50 and 1/100); they were able to reduced pain induced by acetic acid writhing responses in dose dependant manner as compared to positive control group (untreated group).

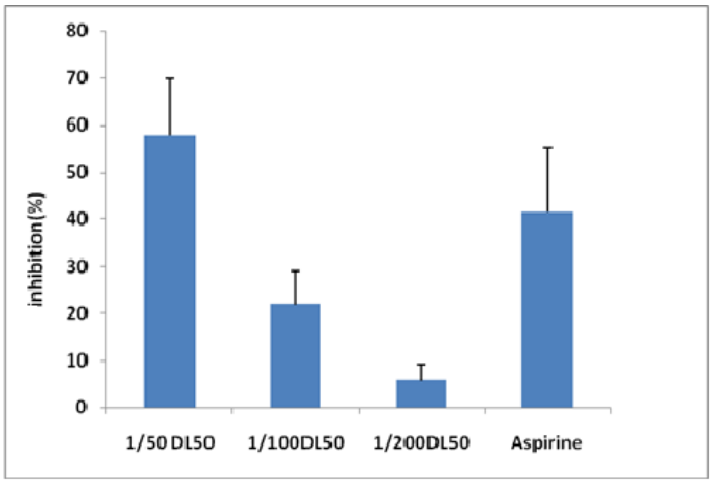


Figure 2. Nociceptive activity of certain pyrazolo[3,4-*d*]pyrimidines (3a)

Evaluation of anti-inflammatory activity

Inflammation is a complex reaction to injurious agents including microbes. It involves vascular responses such as activation and migration of leukocytes and systemic reactions. The newly synthesized pyrozolopyrimidine derivatives were screened for anti-inflammatory activity using carrageenin induced rat hind paw edema method. Anti-inflammatory activity of tested compounds and reference drug (ibuprofen) at different assessment times after injection are shown in Table 5.

The results revealed that the tested compound exhibited anti-inflammatory activity. Compound **3a** was effective in the inhibition of paw edema than ibuprofen during all experimental periods. After 2 hours, this activity was decreased in the order of compound **3a** > ibuprofen.

Table 5. Evaluation of anti-inflammatory activity

	paw edema **(mm)**				
	dose	**0h**	**2h**	**3h**	**4h**
Control	-----	2,15±0,21	7,41 ±0,28	7,41 ±0,28	8,01 ±0,18
Compound 3a	22,2mg/kg PC	2,65±0,49	5,55 ±0,63	5,25±0,63	4,2±0,1*
	11 ,1mg/kgPC	2,75±0,63	5,95±0,37	6,25±0,91	5,85±0,65
	5,55mg/kg PC	2,57 ±1,24	5,8±0,22	6,85±0,68	6,85±0,14
Ibuprofen	150mg/kg PC	2,05 ±0,76	6,25±1,3	5,14±0,9	4,19±0,72*

Conclusion

In conclusion, we have reported a simple and convenient approach to the synthesis of pyrazolo[3,4-*d*]pyrimidines by cyclization followed by Dimroth rearrangement of imidates derived from 5-amino-4-cyanopyrazoles in the presence of primary amines, hydrazines and hydroxylamine; while the condensation of imidates with some hydrazides derivatives gave the corresponding pyrazolo[4,3-*d*][1,2,4]triazolo[4,3-*c*]pyrimidines. Further, the results obtained in this study indicate that these compounds possess potent anti-inflammatory, nociceptive and antipyretic properties, which are mediated via peripheral and central inhibitory mechanisms.

Acknowledgement

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Experimental Section

Chemistry

General: Commercially available reagent grade chemicals were used as received without additional purification. All reactions were followed by TLC (E. Merck Kieselgel 60 F-$_{254}$), Melting point were measured on an Electrothermal apparatus. IR spectra were recorded on a Perkin-Elmer PARAGON FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on an AC Bruker spectrometer at 300 MHz (^{1}H) and 75 MHz (^{13}C) using $(CD_3)_2SO$ and $CDCl_3$ as solvents. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (0 ppm) as internal reference and the following multiplicity abbreviations were used: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet; J (hertz). The mass spectra were recorded on an ion trap mass spectrometer (Bruker Daltonics Data analysis 3.4). High resolution mass spectra (HRMS) were obtained on a Jeol GCmate spectrometer via direct introduction.

General experimental procedure for the preparation of imidates (**2a-e**).

The required pyrazole (1.0 mmol) was treated with triethyl orthoformate or triethyl orthoacetate (1.5 mmol) and few drops of acetic acid. The mixture was refluxed for 24h. After cooling, the product was filtered off and washed with ether.

Ethyl *N*-4-cyano-1-phenyl-1*H*-pyrazol-5-ylformimidate 2a.

White solid, yield: 70%, mp 56-57 °C. IR (cm^{-1}): 1596, 1625 (C=N), 2225 (CN). ^{1}H NMR: (300 MHz, DMSO-d$_6$): δ (ppm) 1.26 (t, 3H), δ = 4.27 (q, 2H), δ = 7.35-7.61 (m, 6), 8.51 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d$_6$): δ (ppm) C_7 12.7, C_6 63.9, C_1 150.5, C_2 81.7, C_3 150.5, C_4 114.2, C_5 162.1, C_{arom} 123.2-137.5.

Ethyl *N*-4-cyano-1-phenyl-1*H*-pyrazol-5-ylacetimidate 2b.

White solid, yield: 62%, mp 72-74°C. IR(cm^{-1}): 1596, 1651 (C=N), 2229 (CN). ^{1}H NMR: (300 MHz, DMSO-d$_6$): δ (ppm) 1.26 (t, J^3 = 7,2 Hz, 3H), 2.49 (s, 3H), 4.27 (q, J^3 = 7.2 Hz, 2H), 7.35-7.61 (m, 5H), 8.51 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d$_6$): δ (ppm) C_8 18.6, C_7 14.3, C_6 64.1, C_1 151.5, C_2 83.8, C_3 141.9, C_4 114.3, C_5 167.9, C_{arom} 123.9-138.5.

Ethyl *N*-4-cyano-3-methyl-1-phenyl-1*H*-pyrazol-5-ylfomimidate 2c.

White solid, yield: 70%, mp 105°C. IR(cm^{-1}): 1593, 1618 (C=N), 2214 (CN). ^{1}H NMR: (300 MHz, DMSO-d$_6$): δ (ppm) 1,47 (t, J^3 = 6.9 Hz, 3H), 2.30 (s, 3H), 4.27 (q, J^3 = 6.9 Hz, 2H), 7.35-7.69 (m, 5H), 8.51 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d$_6$): δ (ppm) C_7 13.2, C_8 14.3, C_6 64.4, C_2 82.3, C_4 114.7, C_3 151.01, C_1 151.0, C_5 162.6, C_{arom} 124.0-138.1. MS m/z: 254.

Ethyl *N*-4cyano-3-methyl-1-phenyl-1*H*-pyrazol-5-ylacetimidate 2d

Yield: 67%, IR (cm^{-1}): 1595, 1646 (C=N), 2220 (CN). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.57 (s, 3H), 7.14 (t, 1H), 7.28 (t, 1H), 7.38 (t, 2H), 7.50 (t, 2H), 7.59 (d, 2H), 8.17 (d, 2H), 8.41 (s, 1H), 8.75 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 15.2, C_2 101.9, C_1 142.8, C_3 154.5, C_5 156.0, C_4 156.3, C_{arom} 120.9-139.2.

General experimental procedure for synthesis of pyrazolo[3,4-*d*]pyrimidin-4-amines (3a-f).

A mixture of compound **2** (1 mmol) and primary amine or hydrazine (1 mmol) refluxed in toluene (10 ml) with acetic acid for 24h. The separated product was filtered, washed with ether, dried and crystallized from ethanol to give compounds **3**.

3-methyl-*N*,1-diphenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine 3a.

White solid, yield: 67%, mp 224°C. IR (cm^{-1}): 1563; 1580; 1609 (C=N), 3443 (NH). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.57 (s, 3H), 7.14 (t, 1H), 7.28 (t, 1H), 7.38 (t, 2H), 7.50 (t, 2H), 7.59 (d, 2H), 8.17 (d, 2H), 8.41 (s, 1H), 8.75 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 15.2, C_2 101.9, C_1 142.8, C_3 154.5, C_5 156.0, C_4 156.3, C_{arom} 120.9-139.2. HRMS calculated for $C_{18}H_{15}N_5$: 301.1327; found: 301.1328.

3,6-dimethyl-*N*,1-diphenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine 3b.

White solid, yield: 63%, mp 177°C. IR (cm^{-1}): 1560; 1581; 1616 (C=N), 3446 (NH). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.47 (s, 3H); 2.70 (s, 3H); 7.11 (t, 1H); 7.27 (t, 1H); 7.37-8.18 (m, 8H); 8.59 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 15.2, C_7 26.7, C_2 100.10, C_1 142.8, C_3 154.3, C_5 156.8, C_4 157.5, C_{arom} 120.9-140.1. HRMS calculated for $C_{19}H_{17}N_5$: 315.1484; found: 315.1482.

***N*-benzyl-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine 3c.**

White solid, yield: 77%, mp 125°C. IR (cm^{-1}): 1567; 1590 (C=N), 3440 (NH). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.68 (s, 3H), 4.23 (d, J^2 = 6 Hz, 1H), 4.78 (d, J^2 = 6 Hz, 1H), 7.22 (m, 1H), 7.27 (t, 2H), 7.35 (d, 2H), 7.49 (t, 2H), 7.92 (t, 2H), 8.15 (d, 2H), 8.31 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 15.2, C_7 43.8, C_2 101.1, C_1 142.8, C_3 154.3, C_5 156.4, C_4 157.5, C_{arom} 120.1-140.8. HRMS calculated for $C_{19}H_{17}N_5$: 315.1484; found: 315.1485.

***N*-benzyl-3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine 3d.**

White solid, yield: 68%, mp 119°C. IR (cm^{-1}): 1509, 1570, 1586 (C=N), 3450 (NH). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.68 (s, 3H), 4.23 (d, *J* = 6 Hz, 1H), 4.78 (d, *J* = 6 Hz, 1H), 7.22 (m, 1H), 7.27 (t, 2H), 7.35 (d, 2H), 7.49 (t, 2H), 7.92 (t, 2H), 8.15 (d, 2H), 8.31 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 15.2, C_7 43.8, C_2 101.1, C_1 142.8, C_3 154.3, C_5 156.4, C_4 157.5, C_{arom} 120.1-140.8. MS m/z: 316.

3-methyl-*N*-(naphtalen-2-yl)-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine 3e.

Broun solid, yield: 80%, mp 209°C. IR (cm^{-1}): 1560, 1589 (C=N), 3386 (NH). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.77 (s, 3H), 7.28-8.21(m, 13H), 9.28 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 15.4, C_2 101.6, C_1 143.1, C_3 154.7, C_5 156.6, C_4 157.8, C_{arom} 121.0-139.3. HRMS calculated for $C_{22}H_{17}N_5$: 351.1484; found: 351.1486.

3,6-dimethyl-*N*-(naphtalen-2-yl)-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine 3f.

Broun solid, yield: 48%, mp 208°C. IR (cm^{-1}): 1562, 1588 (C=N), 3415 (NH). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.28 (s, 3H), 2.62 (s, 3H), 7.28-8.20 (m, 11H), 9.15 (s, 1H).

^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 15.4, C_7 26.6, C_1 132.9, C_2 99.8, C_3 155.9, C_4 165.8, C_5 157.4, C_{arom} 120.9-139.4.

General experimental procedure for synthesis of pyrazolo[3,4-*d*]pyrimidin-4-hydrazines (4a-d).

A mixture of hydrazine (1 mmol) and imidate **2** (1 mmol) was heated at reflux for 24 h in toluene (10mL) with acetic acid. The product, which precipitates, was filtered and recrystallized from ethanol.

1-(3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)hydrazine 4a.

Yellow solid, yield: 37%, mp 231°C. IR (cm^{-1}): 1488, 1560, 1581 (C=N), 3268, 3312 (NH). ^{1}H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.60 (s, 3H), 4.75 (s, 2H), 7.62 (t, 1H), 7.48 (t, 2H), 8.13 (d, 2H), 8.34 (s, 1H), 8.80 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 14.9, C_1 142.7, C_2 99.3, C_3 153.5, C_4 158.0, C_5 157.5, C_{arom} 120.3-142.2. MS m/z: 241.

1-(3,6-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl)hydrazine 4b.

Yellow solid, yield: 52%, IR (cm^{-1}): 1599, 1650, 1698 (C=N), 3036, 3194 (NH). ^{1}H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.44 (s, 3H), 2.59 (s, 3H), 5.67 (s, 2H), 7.30 (t, 1H), 7.49 (t, 2H), 7.16 (d, 2H), 8.99 (s, 1H).

4-methyl-*N*'-(3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl) benzene-sulfonohydrazide 4c.

White solid, yield: 84%, mp 268°C. IR (cm^{-1}): 1488, 1560, 1581 (C=N), 3268, 3312 (NH). ^{1}H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.60 (s, 3H), 4.75 (s, 2H), 7.62 (t, 1H), 7.48 (t, 2H), 8.13 (d, 2H), 8.34 (s, 1H), 8.80 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 14.9, C_1 142.7, C_2 99.3, C_3 153.5, C_4 158.0, C_5 157.5, C_{arom} 120.3-142.2. MS m/z: 241.

4-methyl-*N*'-(3,4-dimethyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yl) benzene-sulfonohydrazide 4d.

White solid, yield: 36%, mp 212°C. IR (cm^{-1}): 1559, 1593, 1655 (C=N), 3217, 3367 (NH). ^{1}H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.39 (s, 3H), 2.45 (s, 3H), 2.69 (s, 3H), 7.23-7.51 (m, 9H), 7.72 (s, 1H), 8.00 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 14.4, C_7 23.0, C_8 21.5, C_1 142.4, C_2 99.9, C_3 144.4, C_4 161.8, C_5 154.2, C_{arom} 121.9-140.3.

General experimental procedure for synthesis of 4-amino-pyrazolo[3,4-*d*] pyrimidine (5a-c).

A solution of imidate **2** (1.0 mmol) in ethanol (5mL) was treated with ammoniac (2.0 mmol) and a catalytic amount of acetic acid. The reaction mixture was refluxed for 6h, and the formed solid was collected by filtration, dried and recrystallized from ethanol to give compound **5**.

1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine 5a.

White solid, yield 83 % ; mp 228 °C; IR(cm^{-1}): 3283 (NH_2), 1480, 1500, 1590(C=N). ^{1}H NMR: (300 MHz, DMSO-d_6): δ (ppm) 4.69 (s,2H), 7.36 (t, *J* = 7.3Hz, 1H), 7.48 (t, *J* = 7.3Hz, 2H), 7.52 (d, *J* = 7.3Hz, 2H), 7.60 (s,1H), 7.72 (s,1H). ^{13}C RMN (75 MHz, DMSO-d_6): δ (ppm)114.1, 124.2, 129.0, 129.5, 130.0, 136.9, 141.3, 149.8, 156.8; HRMS calculated for $C_{11}H_9N_5$: 211.0858, found: 211.0859.

3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine 5b.
Yield 68 %; mp 192 °C; IR(cm^{-1}) 3317(NH_2), 1626, 1647, 1665(C=N). RMN ^1H (δ ppm, DMSO-d_6): 2.76 (s, 3H), 5.97 (s, 2H), 7.33(t, *J* = 7.1Hz, 1H) , 7.57(t, *J* = *7*.1Hz, 2H), 8.16 (d, *J* = *7*.1Hz, 2H), 8.46 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 14.8, 101.2, 121.4, 126.3, 129.1, 138.8, 141.8, 154.4, 156.4, 158.4. HRMS calculated for $C_{12}H_{11}N_5$: 225.1014, found: 225.1018.

6-methyl-1-phenyl-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine 5c.
Yield 70 % ; mp 160 °C; IR(cm^{-1}) 3320(NH_2),1597, 1638, 1663(C=N). RMN ^1H (δ ppm, DMSO-d_6): 2.65 (s, 3H), 4.28 (s,2H), 7.28(t, *J* = 7.3Hz, 1H), 7.56 (t, *J* = 7.3Hz, 2H), 8.19(d, *J* = 7.3Hz, 2H), 8.29 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 14.4, 100.2, C_{arom} 120.2, 124.6, 129.1, 138.8, 142.7, C_3 154.1, 156.5, 158.5. HRMS calculated for $C_{12}H_{11}N_5$: 225.1014, found: 225.1016.

General experimental procedure for synthesis of *N*-hydroxy-pyrazolo [3,4-*d*]pyrimidin-4-amines (**6a-b**).

A mixture of imidate **2** (1 mmol) and hydroxylamine hydrochloride (1 mmol) in ethanol was heated for 24 h with triethylamine (1 mmol). The solvent was then removed and the residue was extracted with dichloromethane (3×50 ml). Collected organic layers were dried over $MgSO_4$, and then the solvent was evaporated.

***N*-hydroxy-3-methyl-1-phenyl-1*H*- pyrazolo[3,4-*d*]pyrimidin-4-amine 6a.**
White solid, yield: 36%, mp 265°C. IR (cm^{-1}): 1561, 1593, 1650 (C=N), 3286 (OH), 3405 (NH). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.65 (s, 3H), 7.30 (t, 1H), 7.50 (t, 2H), 8.04 (d, 2H), 8.49 (s, 2H), 8.72 (s, 1H).^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 14.6, C_1 142.8, C_2 100.7, C_3 146.4, C_4 149.3, C_5 146.3, C_{arom} 120.7-138.6. HRMS calculated for $C_{12}H_{11}N_5O$: 241.0964; found: 241.0956.

***N*-hydroxy-3,6-dimethyl-1-phenyl-1*H*- pyrazolo[3,4-*d*]pyrimidin-4-amine 6b.**
White solid, yield: 32%, mp 240°C. IR (cm^{-1}): 1573, 1594, 1654 (C=N), 2971 (OH), 3262 (NH).^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.74 (s, 3H), 2.83 (s, 3H), 7.30-7.56 (m, 5H), 7.98 (s, 1H), 8.04 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_6 15.3, C_7 21.2, C_1 142.8, C_2 101.6, C_3 148.8, C_4 156.4, C_5 157.1, C_{arom} 121.1-138.2. HRMS calculated for $C_{13}H_{13}N_5O$: 255.1120; found: 255.1112.

General experimental procedure for synthesis of pyrazolo[3,4-*d*][1,2,4]triazolo [4,3-*c*]pyrimidines (**7a-d**).

A mixture of hydrazide (1 mmol) and imidate **2** (1 mmol) was heated at reflux for 24 h in toluene (10mL) with acetic acid. The product, which precipitates, was filtered and recrystallized from ethanol.

7a: White solid, yield: 79%, mp 232°C. IR (cm^{-1}): 1538, 1590, 1652 (C=N). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.81 (s, 3H), 7.39-8.28 (m, 10H), 9.61 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_7 15.1, C_2 101.6, C_1 138.8, C_3 142.4, C_4 145.5, C_5 149.6, C_6 164.2, C_{arom} 121.7-136.7. HRMS calculated for $C_{19}H_{14}N_6$: 326.1280; found:326.1280.

7b: White solid, yield: 52%, mp 241°C. IR (cm^{-1}): 1504, 1540, 1591, 1658 (C=N). ^1H NMR: (300 MHz, DMSO-d_6): δ (ppm) 1.90 (s, 3H), 2.36 (s, 3H), 7.49-7.92 (m, 10H),

10.52 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_7 13.3, C_8 15.6, C_2 100.8, C_1 138.9, C_3 142.6, C_4 145.3, C_5 149.5, C_6 164.2, C_{arom}122.0-137.6. HRMS calculated for $C_{20}H_{16}N_6$: 340.1436; found: 340.1434.

7c: White solid, yield: 48%, mp 201°C. IR (cm^{-1}): 1497, 1544, 1595, 1650 (C=N). ^{1}H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.48 (s, 3H), 2.72 (s,3H), 7.39 (t, 1H), 7.57-8.10(m, 4H), 9.45 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_7 13.4, C_8 14.1, C_1 139.9, C_2 100.5, C_3 142.4, C_4 164.7, C_5 146.1, C_6 167.8, C_{arom}126.9-138.0. HRMS calculated for $C_{14}H_{12}N_6$: 264.1123; found: 264.1124.

7d: White solid, yield: 52%, mp 130 °C. IR (cm^{-1}): 1544, 1599, 1650, 1698 (C=N). ^{1}H NMR: (300 MHz, DMSO-d_6): δ (ppm) 2.58 (s, 3H), 2.80 (s, 3H), 3.34 (s, 3H), 7.36 (t, 1H), 7.54 (t, 2H), 8.07 (d, 2H).^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) C_7 13.8, C_8 14.7, C_9 20.7, C_2 101.6, C_1 138.8, C_3 142.6, C_4 145.3, C_5 149.6, C_6 164.2, C_{arom} 121.7-136.8.

Biological evaluation

Laboratory bred Wister rats (120-220 g) and Swiss mice (20-35 g) of either sex, purchased from Central Pharmacy (SIPHAT, Tunisia), were used for the in-vivo pharmacological testing. All experiments were conducted in accordance with the internationally accepted principals for laboratory animal use. All animals were maintained under standard laboratory conditions at 22 ±2°C, with relative humidity 50 ± 15% and photoperiod (12h light and dark cycle). Commercial pellet diet and water were provided ad libitum. The animals were fasted overnight prior to each experiment.

Acute toxicity study

Acute toxicity studies were performed on Swiss mice either sex selected randomly. Six single doses (2366, 1183, 473, 236, 47, 23 mg/kg) of 3-methyl-*N*,1-diphenyl-1*H*-pyrazolo [3,4-*d*]pyrimidin-4-amine **3a** and 4-methyl-*N'*-(3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidin-4-yl)benzenesulfonohydrazide **4c** were administrated intraperitoneally (i.p.) to different groups containing ten mice each. Mice were kept under regular observation for 48h for any adverse effect, including mortality. Other behavioural changes and parameters, such as body weight, food intake, urination, water intake, locomotor activity, changes in skin, respiration, tremors, temperature, etc., were also observed. None of the treated groups displayed any significant change of behaviour as compared with the untreated controls.

Acetic acid induced writhing test

The peripheral anti-nociceptive activity of pyrazolo[3,4-*d*]pyrimidines was evaluated by acetic acid induced writhing test[16]. Adult Wister rats of either sex were randomized into five groups of six mice each. Pyrazolo[3,4-*d*]pyrimidines (50 mg/Kg, i.p.) and aspirine (150 mg / kg, i.p.) were administered to respective group of rats. After 30 min, 1% aqueous solution of acetic acid (10 ml / kg) was administrated i.p. to induce pain sensation. Writhing movement was recognized as contraction of abdominal muscle together with stretching of hind limbs. The number of writhing movements for each mouse was counted for 20 min after acetic acid injection.

Carrageenin-induced paw oedema in rats

Pedal inflammation was produced according to the method described by Winter et al[17]. In this study 0.1 mL of 1% carrageenin was injected into the right hind foot paw of each rat under the subplanter aponeurosis. Animal grouping and dosage administration were as in the plate test. Animals were administrated intraperitoneally the product 1h before carrageenan injection. Measurement of paw size was carried out as in previous studies[18] by wrapping a piece of cotton thread round the paw and the length of the thread corresponding to the paw circumference was determined using a meter ruler.

Paw size were measured immediately before and 1-5h following injection. The inhibitor activity was determined as follows. [18]

$$\text{Percentage inhibition} = \frac{(Ct-C0)\,\text{control} - (Ct-C0)\,\text{treated}}{(Ct-C0)\,\text{control}} \times 100$$

Where Ct = paw circumference at time t, C0 = paw circumference before carrageenan injection and Ct-C0 = oedema.

Brewer's yeast induced pyrexia

The antipyretic activity of pyrazolo[3,4-*d*]pyrimidines was assisted by the yeast induces pyrexia method as described previously[19] with little modification. Adult rats were randomized into five different groups of six rats each.

The rectal temperature of each rat was measured 17h (normal control) after brewer's yeast injection using a clinical thermometer. Only rats that showed an increase in temperature of at least 0.7°C were used for this study. Animals were administrated (orally) the pyrazolo [3,4-*d*]pyrimidine 18h after the brewer's yeast injection and rectal temperature were measured at 60, 90 and 120 min post administration. The mean of the rectal temperature of each group were determined and compared with pre-drug (hyperpyretic state) temperature.

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