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New pyrazolo[3,4-*b*]pyrazines: synthesis and biological activity

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Some new pyrazolo[3,4-*b*]pyrazines and related heterocycles were synthesized and evaluated for their antifungal and antiparasitic activities. The key intermediate, 6-amino-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyrazine-5-carbonitrile (**3**) was obtained in a one-pot synthesis via the reaction of 5-amino-3-methyl-4-nitroso-1-phenylpyrazole **2** with malononitrile.

1. Introduction

The pyrazolo[3,4-*b*]pyrazine ring system has proved to be an interesting class of heterocycles. It has been reported that some of its derivatives are used as bone metabolism improvers [1], antiinflammatories [2], blood platelet aggregation inhibitors [2], anticancer agents with low toxicity [3, 4] and in controlling herbicides [5]. Also in dyes chemistry, pyrazolo[3,4-*b*]pyrazines are used as fluorescent [6] and disperse dyes [7]. Despite of all these interesting applications, pyrazolo[3,4-*b*]pyrazines have received little attention so far.

As a part of our program directed towards the synthesis of new fused pyrazine heterocycles [8–11], we report the synthesis of some unknown pyrazolo[3,4-*b*] pyrazines and their antifungal and antiparasitic activities.

2. Investigations, results and discussion

2.1. Chemistry

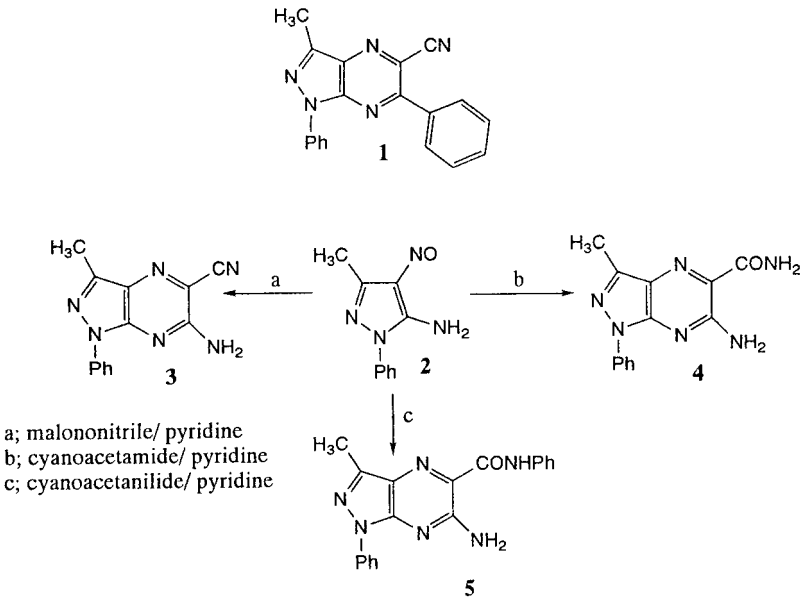
In a recent publication [12] we reported a facile synthesis of 1,6-diphenyl-3-methyl-1*H*-pyrazolo[3,4-*b*]pyrazine-5-carbonitrile (**1**) through the interaction of 5-amino-3-methyl-4-nitroso-1-phenylpyrazole (**2**) with benzoylacetonitrile in refluxing pyridine. The importance of compound **1** as an interesting intermediate in the synthesis of several new pyrazolo[3,4-*b*]pyrazines prompted us to explore the utility of the above reaction for the synthesis of other unknown derivatives of this class of heterocycles in an endeavor to study their antifungal and antiparasitic activities.

Thus, when the readily available aminonitrosopyrazole **2** reacted with malononitrile in boiling pyridine, the reaction product was 6-amino-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyrazine-5-carbonitrile (**3**). When malononitrile was replaced by 2-cyanoacetamide or 2-cyano-*N*-phenylacetamide the reaction products were identified as **4** and **5**, respectively (Scheme 1).

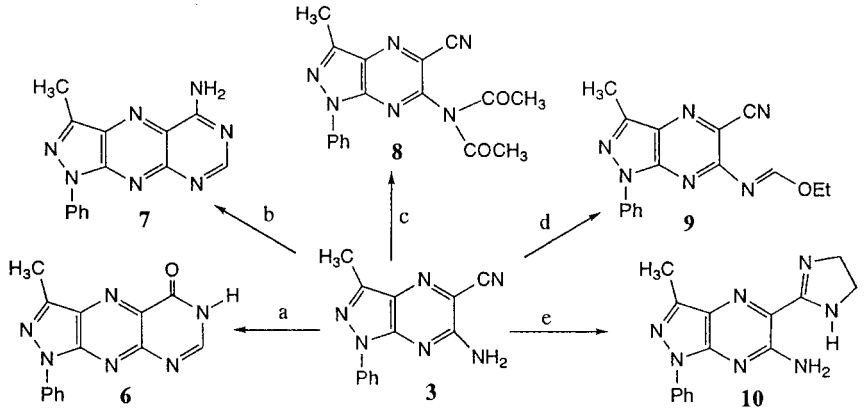
Compound **3** proved to be a useful key intermediate for the synthesis of several pyrazolo[3,4-*b*]pyrazines (Scheme 2). Thus, the interaction of **3** with formic acid and formamide gave the pyrazolopyrazinopyrimidines **6** and **7**, respectively. When **3** was boiled in acetic anhydride the reaction product was the diacetyl derivative **8**, while its heating under reflux in triethyl orthoformate resulted in the formation of the ethoxymethylenamino derivative **9**.

Earlier work has described the direct conversion of the cyano function of *o*-amino nitriles into the corresponding 4,5-dihydro-1*H*-imidazol-2-yl group via the reaction of the amino nitrile with ethylenediamine in the presence of carbon disulfide [13–15], *p*-toluenesulfonic acid [16] or phosphorus pentasulfide [17]. Accordingly, 6-amino-5-(2-imidazolin-2-yl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]-pyrazine (**10**) could be obtained via the interaction of **3**

Scheme 1



Scheme 2

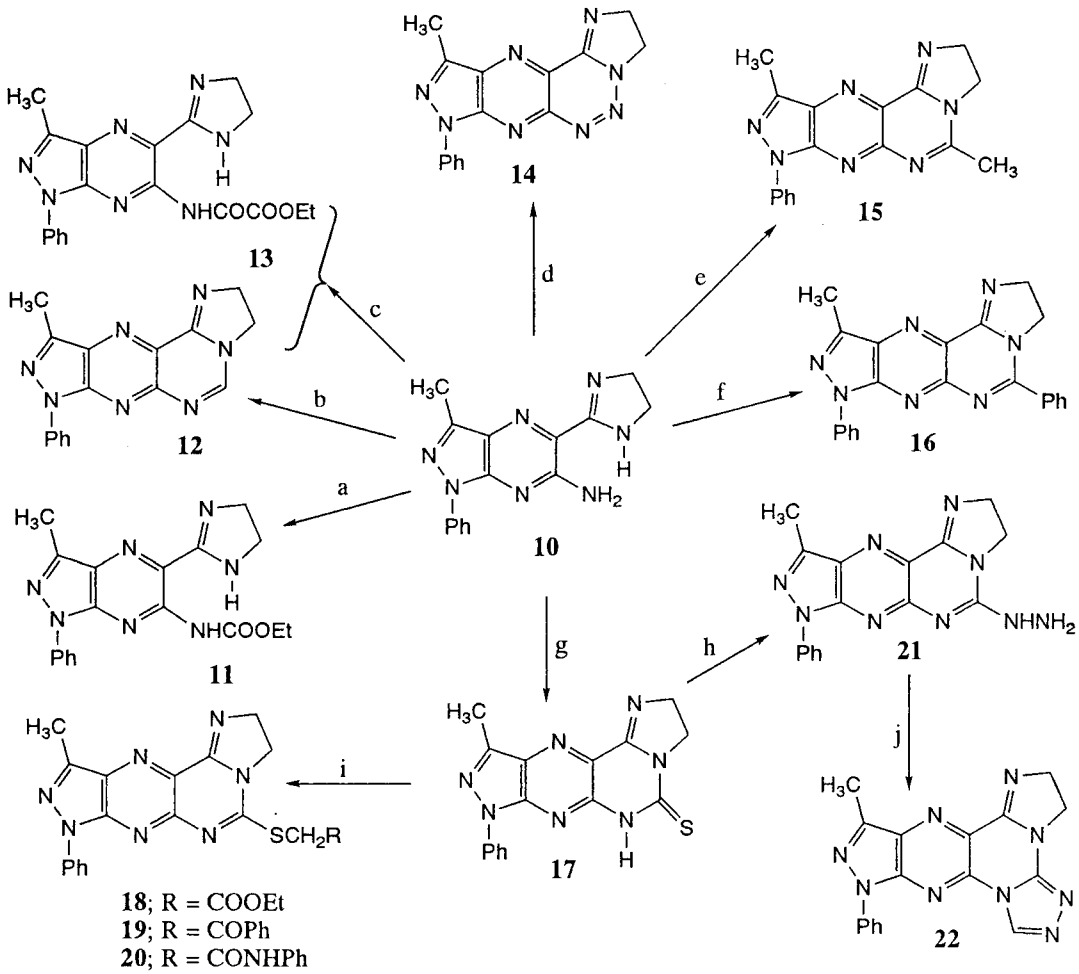


a; HCOOH, b; $HCONH_2$, c; Ac_2O, d; $(EtO)_3CH$, e; $H_2N(CH_2)_2NH_2/CS_2$

with ethylenediamine in the presence of carbon disulfide on a boiling water bath.

The imidazolinyl derivative **10** is a versatile compound and could serve as a point of departure for the synthesis of some fused tetracyclic heterocycles related to the pyrazolo[3,4-*b*]pyrazine system. Thus, the ethyl carbamate derivative **11** was obtained by the reaction of **10** with ethyl chloroformate in ethanol in the presence of sodium acetate. Reaction of **10** with triethyl orthoformate gave the tetracyclic heterocycle **12**. Two compounds, however, were isolated when **10** was allowed to react on boiling with neat diethyl oxalate. A solid separated from the reaction mixture on cooling was identified as 6,7-dihydro-3-methyl-1-phenyl-1*H*-pyrazolo[4′,3′:5,6]pyrazino[2,3-*e*]-

Scheme 3



18; R = COOEt
19; R = COPh
20; R = CONHPh

a; ClCOOEt, b; $(EtO)_3CH$, c; $(COOEt)_2$, d; HNO_2, e; AcCl, f; PhCOCl, g; CS_2/pyridine, h; NH_2NH_2, i; $ClCH_2COOEt$ (19), $BrCH_2COPh$ (20), $ClCH_2CONHPh$ (21), j; $(EtO)_3CH$.

imidazo[1,2-*c*]pyrimidine (**12**), while the second product was recovered from the filtrate by dilution with petroleum ether and was identified as the oxalylamino derivative **13**. Under diazotization conditions **10** gave the triazine **14**. The pyrimidines **15** and **16** were obtained when **10** was reacted with acetyl chloride and benzoyl chloride respectively in pyridine.

The reaction of **10** with carbon disulfide in pyridine yielded the thione derivative **17**. The substituted mercapto derivatives **18**, **19** and **20** could be obtained via the condensation of **17** with ethyl chloroacetate, phenacyl bromide and 2-chloro-*N*-phenylacetamide, respectively. Nucleophilic displacement of the mercapto group of **17** by a hydrazino function was achieved by treatment with hydrazine hydrate to give **21**. The latter compound gave the pentacyclic heterocycle **22** upon treatment with triethyl orthoformate.

2.2. Antifungal activity

Nine of the newly synthesized compounds were screened for their antifungal activity against four species of fungi namely *Aspergillus flavus*, *Penicillium chrysogenum*, *Fusarium latertium* and *Rhizopus stolonifer* using the disc-diffusion method [19, 20]. The results are illustrated in Table 1. All the compounds under test were inactive against *Aspergillus flavus,* however, they exhibited a moderate activity against the other three strains of fungi using the antifungal lotion Trosyd^R 1% (tioconazole) as control.

Table 1: Antifungal activity of some pyrazolo[3,4-*b*]pyrazines[a]

Compd.	*Aspergillus flavus*	*Penicillium chrysogenum*	*Fusarium laterium*	*Rhizopus stolonifer*
Tioconazole	32	35	36	28
3	–	–	10	18
6	–	13	15	14
7	–	13	12	–
8	–	15	12	12
9	–	12	14	12
10	–	13	10	–
12	–	14	14	–
16	–	12	13	12
17	–	15	16	–

[a] The activity is expressed as the diameter of the inhibition zone (mm)

Table 2: Antiparasitic activity of some pyrazolo[3,4-*b*]pyrazines (MIC (mg/ml))

Compd.	*Trichomonas vaginalis*	*Leishmania tropica*	*Leishmania infantum*
Metronidazole	5	–	–
Pentamidine	–	5	5
2	10	25	25
3	100	>100	>100
5	50	100	100
6	>100	>100	>100
7	>100	>100	>100
8	>100	>100	>100
9	50	50	50
10	>100	>100	>100
11	>100	>100	>100
13	>100	>100	>100
15	>100	>100	>100
16	5	10	10
17	>100	>100	>100
18	100	100	100
20	10	25	25
21	>100	>100	>100

2.3. Antiparasitic activity

Sixteen of these new compounds were also tested against *Trichomonas vaginalis* and promastigotes of two *Leishmania* strains. I*n vitro* trichomonacidal activity was performed on a *Trichomonas vaginalis* wild strain grown in oxoid liquid medium (*Trichomonas medium* code CM 161). The MIC was determined after 48 h [21] using metronidazole as the reference drug. The results are illustrated in Table 2. Compound **16** displayed good *in vitro* activity against *T. vaginalis* and against promastigotes of two *Leishmania* strains. Compounds **2** and **20** showed greater trichomonacidal than leishmanicidal activity. The other derivatives were inactive.

3. Experimental

All m.p.'s are uncorrected and were determined on a Mel-Temp II apparatus. IR spectra were recorded on a Pye-Unicam SP3-100 spectrophotometer using the KBr wafer technique. ^{1}H NMR spectra were recorded on a 90 MHz Varian EM-390 NMR spectrometer in suitable deuterated solvents using TMS as an internal standard. Elemental analyses were determined on a Perkin-Elmer 240 C microanalyser and the results for the indicated elements were within ±0.4% of the calculated values, unless otherwise indicated.

3.1. 5-Amino-3-methyl-4-nitroso-1-phenylpyrazole (2)

This compound was prepared according to the literature [18].

3.2. 6-Amino-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyrazine-5-carbonitrile (3)

A mixture of **2** (20.2 g, 0.1 mol) and malononitrile (6.6 g, 0.1 mol) in pyridine (60 ml) was heated under reflux for 3 h. The reaction mixture was left to cool and the precipitate formed was filtered, washed with water, and dried. Recrystallization from a dioxane/water mixture (1 : 1) gave yellow needles, m.p. 240 °C, yield 16.25 g (65%). IR: ν (cm^{-1}) 3400, 3200 (NH_2), 2220 (CN). ^{1}H NMR (DMSO-d_6): δ 2.56 (s, 3 H, CH_3), 6.43 (s, 2 H, NH_2), 7.43 (m, 3 H, Ar–H), 8.1 (m, 2 H, Ar–H).
$C_{13}H_{10}N_6$

3.3. 6-Amino-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyrazine-5-carboxamide (4)

A mixture of **2** (1 g, 0.005 mol) and 2-cyanoacetamide (0.42 g, 0.005 mol) in pyridine (15 ml) in the presence of few drops of piperidine was heated under reflux for 3 days. After cooling, the reaction mixture was poured into ice-cold water. The precipitate formed was filtered, washed with water, dried and recrystallized from a mixture of dioxane/water (1 : 1) to give brown crystals, m.p. 255 °C, yield 0.5 g. (37%). IR: ν (cm^{-1}) 3450, 3400, 3300, 3250, 3200 (NH_2 & $CONH_2$), 1690 (CO). ^{1}H NMR (DMSO-d_6): δ 2.49 (s, 3 H, CH_3), 7.37 (m, 3 H, Ar–H), 8.09 (m, 2 H, Ar–H), 7.60 (s, 2 H, NH_2), 8.20 (s, 2 H, NH_2).
$C_{13}H_{12}N_6O$

3.4. 6-Amino-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyrazine-5-N-phenylcarboxamide (5)

A mixture of 5-amino-3-methyl-4-nitroso-1-phenylpyrazole **2** (2.02 g, 0.01 mol) and 2-cyano-*N*-phenylacetamide (1.6 g, 0.01 mol) in pyridine (20 ml) was heated under reflux for 3 h. After cooling, the precipitate formed was filtered off, dried and recrystallized from a mixture of ethanol/dioxane (1 : 1) as yellow needles, m.p. 215 °C, yield 2 g (58%). IR: ν (cm^{-1}) 3425, 3310 (NH_2 & NH), 1660 (C=O). ^{1}H NMR (DMSO-d_6): δ 2.63 (s, 3 H, CH_3), 4.0 (s, 2 H, NH_2), 7.37 (m, 8 H, Ar–H), 7.77 (m, 2 H, Ar–H), 10.27 (s, 1 H, NH).
$C_{19}H_{16}N_6O$

3.5. 3-Methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino[2,3-d]pyrimidin-5(6H)-one (6)

A mixture of **3** (1.0 g, 0.004 mol) and formic acid (10 ml) was heated under reflux for 4 h. The reaction mixture was cooled and then poured into cold water. The precipitate formed was filtered, washed with water, dried and recrystallized from dioxane to give yellow crystals, m.p. >360 °C, yield 0.6 g (54%). IR: ν (cm^{-1}) 3200 (NH), 1640 (C=O). ^{1}H NMR: the sample was insoluble in all deuterated solvents.
$C_{14}H_{10}N_6O$

3.6. 5-Amino-3-methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino[2,3-d] pyrimidine (7)

A mixture of **3** (1.0 g, 0.004 mol) and formamide (6 ml) was heated under reflux for 3 h. After cooling, the reaction mixture was poured into cold water. The solid precipitate was filtered, washed with water, dried and recrystallized from dioxane to give yellow crystals, m.p. 295 °C, yield 0.52 g (57%). IR: ν (cm^{-1}) 3300, 3100 (NH_2). ^1H NMR (DMSO-d_6): δ 2.73 (s, 3 H, CH_3), 7.4 (m, 3 H, Ar−H), 8.0 (s, 2 H, NH_2), 8,33 (m, 2 H, Ar−H), 8.57 (s, 1 H, pyrimidine).
$C_{14}H_{11}N_7$

3.7. 6-Diacetylamino-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]pyrazine-5-carbonitrile (8)

A mixture of **3** (1.0 g, 0.004 mol) and acetic anhydride (10 ml) was heated under reflux for 6 h. The reaction mixture was then cooled and poured into cold water. The precipitate formed was filtered, washed with water, dried and recrystallized from ethanol as brownish-yellow crystals, m.p. 190 °C, yield 1.0 g (75%). IR: ν (cm^{-1}) 2220 (CN), 1710, 1725 (CO). ^1H NMR ($CDCl_3$): δ 2.4 (s, 6 H, 2 $COCH_3$), 2.86 (s, 3 H, CH_3), 7.5 (m, 3 H, Ar−H), 8.10 (m, 2 H, Ar−H)
$C_{17}H_{14}N_6O_2$

3.8. 6-Ethoxymethyleneamino-3-methyl-1-phenyl-1H-pyrazolo[3,4-b]-pyrazine-5-carbonitrile (9)

A mixture of **3** (1.0 g, 0.004 mol) and triethyl orthoformate (10 ml) was heated under reflux for 3 h. The reaction mixture was then cooled and poured into cold water. The solid precipitate was filtered, washed with water, dried and recrystallized from ethanol to give yellow needles, m.p. 160 °C, yield 0.68 g (56%). IR: ν (cm^{-1}) 2230 (CN). ^1H NMR ($CDCl_3$): δ 1.50 (t, 3 H, $\underline{CH_3}CH_2$), 2.7 (s, 3 H, CH_3), 4.90 (q, 2 H, $CH_3\underline{CH_2}$), 7.43 (m, 3 H, Ar−H), 8.13 (m, 2 H, Ar−H), 8.60 (s, 1 H, $\underline{CH}$OEt).
$C_{16}H_{14}N_6O$

3.9. 6-Amino-5-(2-imidazolin-2-yl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-b] pyrazine (10)

To a mixture of **3** (1.0 g, 0.004 mol) and ethylenediamine (8 ml) was added dropwise carbon disulfide (0.8 ml). The reaction mixture was heated on a water bath for 6 h. After cooling, the reaction mixture was poured into ice-cold water with strong stirring. The solid precipitate was filtered off, washed with water, dried and recrystallized from ethanol as yellow needles, m.p. 232 °C, yield 0.9 g (77.5%). IR: ν (cm^{-1}) 3350, 3120 (NH_2 and NH). ^1H NMR ($CDCl_3$): δ 2.56 (s, 3 H, CH_3), 3.56 (m, 2 H, CH_2 imidazoline), 4.03 (m, 2 H, CH_2 imidazoline), 5.97 (s, 2 H, NH_2), 7.33 (m, 3 H, Ar−H), 7.80 (s, 1 H, NH), 8.20 (m, 2 H, Ar−H).
$C_{15}H_{15}N_7$

3.10. Ethyl N-[5-(2-imidazolin-2-yl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-b] pyrazin-6-yl]carbamate (11)

A mixture of compound **10** (1.17 g, 0.004 mol) and ethyl chloroformate (0.45 g, 0.005 mol) and sodium acetate (0.8 g, 0.01 mol) in ethanol (30 ml) was heated under reflux for 3 h. After cooling, the reaction mixture was poured into water. The solid precipitate was filtered, washed with water and dried. Recrystallization from a mixture of ethanol/dioxane (2 : 1) gave yellow crystals, m.p. 275 °C, yield 0.8 g (55%). IR: ν (cm^{-1}) 3400 (NH), 3250 (NH), 1700 (C=O). ^1H NMR (CF_3CO_2D): δ 1.13 (t, 3 H, $\underline{CH_3}CH_2$), 2.67 (s, 3 H, CH_3), 3.9 (s, 2 H, CH_2 imidazoline), 3.93 (q, 2 H, $\overline{CH_3}\underline{CH_2}$), 4.03 (s, 2 H, CH_2 imidazoline), 7.07 (s, 1 H, NH), 7.47 (m, 3 H, Ar−H), 8,37 (m, 2 H, Ar−H), 12.37 (s, 1 H, NH).
$C_{18}H_{19}N_7O_2$.

3.11. 6,7-Dihydro-3-methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino-[2,3-e]-imidazo[1,2-c]pyrimidine (12)

A mixture of **10** (1.17 g, 0.004 mol) and triethyl orthoformate (15 ml) was heated under reflux for 3 h. After cooling, the reaction mixture was poured into cold water. The solid precipitate was filtered, washed with water, dried and recrystallized from a mixture of *N,N*-dimethylformamide (DMF)/water (2 : 1) to give brownish-yellow crystals, m.p. >360 °C, yield 0.64 g (54%). IR: ν (cm^{-1}) 3050 (CH arom.), 2950 (CH aliph.). ^1H NMR (CF_3CO_2D): δ 3.00 (s, 3 H, CH_3), 4.67 (m, 2 H, CH_2), 5,1 (m, 2 H, CH_2), 7.53 (m, 3 H, Ar−H), 8.07 (m, 2 H, Ar−H), 9.10 (s, 1 H, CH pyrimidine).
$C_{16}H_{13}N_7$.

3.12. Reaction of 10 with diethyl oxalate

A mixture of compound **10** (1.17 g, 0.004 mol) and diethyl oxalate (15 ml) was heated under reflux for 5 h. After cooling, the solid precipitate was filtered, washed with a little cold ethanol, dried and recrystallized from dioxane to give **12** as brownish-yellow crystals, m.p. >360 °C, yield 0.52 g (43%). Spectroscopic data (IR and NMR) and melting point were identical to those of a sample obtained previously.

The filtrate was poured into petroleum ether and the precipitate formed was filtered and dried. Recrystallization from ethanol gave yellow crystals of 6-ethyloxalylamino-5-(2-imidazolin-2-yl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*] pyrazine (**13**), m.p. 150 °C, yield 0.50 g (32%). IR: ν cm^{-1}; 3400 (NH), 3300 (NH), 1682 (C=O), 1645 (C=O). ^1H NMR (DMSO-d_6): δ 1.30 (t, 3 H, $\underline{CH_3}CH_2$), 2.57 (s, 3 H, CH_3), 3.43 (s, 2 H, CH_2 imidazoline), 3.63 (s, 2 H, $\overline{CH_2}$ imidazoline), 4.20 (q, 2 H, $CH_3\underline{CH_2}$), 7.40 (m, 3 H, Ar−H), 8.17 (m, 2 H, Ar−H), 8.9 (s, 1 H, NH), 9.03 (s, 1 H, NH).
$C_{19}H_{19}N_7O_3$

3.13. 6,7-Dihydro-3-methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino[2,3-e] imidazo[1,2-c]-(1,2,3)-triazine (14)

To a cold solution (5 °C) of compound **10** (1.17 g, 0.004 mol) in a mixture of acetic acid (10 ml) and hydrochloric acid (10 ml, 20%), cold sodium nitrite solution (0.34 g, 0.005 mol) in water (10 ml) was added dropwise with stirring over 15 minutes. The reaction mixture was stirred for further 30 minutes at room temperature and then it was poured into water. The precipitate formed was filtered, washed with water, dried and recrystallized from a mixture of dioxane/DMF (1 : 1) to give yellow crystals, m.p. >360 °C, yield 0.63 g (52%). IR: ν (cm^{-1}) 3050 (CH arom.), 2950 (CH aliph.). ^1H NMR (CF_3CO_2D): δ 2.87 (s, 3 H, CH_3), 4.37 (m, 2 H, CH_2 imidazoline) 4.73 (m, 2 H, CH_2 imidazoline), 7.33 (m, 3 H, Ar−H), 8.13 (m, 2 H, Ar−H).
$C_{15}H_{12}N_8$

3.14. 6,7-Dihydro-3,8-dimethyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino-[2,3-e]imidazo[1,2-c]pyrimidine (15)

A mixture of compound **10** (0.58 g, 0.002 mol) and acetyl chloride (0.5 ml) in pyridine (20 ml) was heated under reflux for 6 h. The reaction mixture was then cooled and poured into water. The solid precipitate formed after standing for 1 h was filtered, washed with water, dried and recrystallized from acetic acid to give yellow crystals, m.p. 318 °C, yield 0.38 g (61%). IR: ν (cm^{-1}) 3060 (CH arom.), 2930 (CH aliph.). ^1H NMR (CF_3CO_2D): δ 2.93 (s, 3 H, CH_3), 3.27 (s, 3 H, CH_3), 4.87 (m, 2 H, CH_2 imidazoline), 5.12 (m, 2 H, CH_2 imidazoline), 7.63 (m, 3 H, Ar−H), 8.12 (m, 2 H, H−Ar).
$C_{17}H_{15}N_7$

3.15. 6,7-Dihydro-1,8-diphenyl-3-methyl-1H-pyrazolo[4′,3′:5,6]pyrazino-[2,3-e]imidazo[1,2-c]pyrimidine (16)

A mixture of compound **10** (0.58 g, 0.002 mol) and benzoyl chloride (0.28 g, 0.002 mol) in pyridine (5 ml) was heated under reflux for 3 h. After cooling, the reaction mixture was poured into water and then neutralized with hydrochloric acid. The solid precipitate was filtered, washed with water, dried and recrystallized from DMF to give brownish-yellow crystals, m.p. >360 °C, yield 0.52 g (68%). IR: ν (cm^{-1}) 3050 (CH arom.), 2900, 2850 (CH aliph.). ^1H NMR (CF_3CO_2D): δ 2.95 (s, 3 H, CH_3), 4.70 (m, 2 H, CH_2 imidazoline), 4.67 (m, 2 H, CH_2 imidazoline), 7.60 (m, 4 H, Ar−H), 7.9 (m, 6 H, Ar−H).
$C_{22}H_{17}N_7$

3.16. 6,7-Dihydro-3-methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino-[2,3-e]imidazo[1,2-c]pyrimidine-9(10H)-thione (17)

A mixture of compound **10** (1.47 g, 0.005 mol) and carbon disulfide (15 ml) in pyridine (15 ml) was heated under reflux on a water bath for 30 h. The solid precipitate that was formed on hot was filtered, washed with water, dried and recrystallized from acetic acid as yellow needles, m.p. >360 °C, yield, 0.92 g (55%). IR: ν (cm^{-1}) 3250 (NH). ^1H NMR (DMSO-d_6): δ 2.60 (s, 3 H, CH_3), 4.07 (m, 2 H, CH_2 imidazoline), 4.23 (m, 2 H, CH_2 imidazoline), 7.40 (m, 3 H, Ar−H), 8.17 (m, 2 H, Ar−H), 10.5 (s, 1 H, NH).
$C_{16}H_{13}N_7S$

3.17. Alkylation of the thione 17

A mixture of compound **17** (1.68 g, 0.005 mol), the appropriate halo derivative (ethyl chloroacetate, phenacyl bromide or 2-chloro-N-phenylacetamide, 0.005 mol) and sodium acetate (0.8 g, 0.01 mol) in ethanol (40 ml) was heated under reflux for 2 h. The reaction mixture was then cooled and poured into water. The solid precipitate was filtered, washed with water, dried and recrystallized from the appropriate solvent.

3.17.1. Ethyl 6,7-dihydro-3-methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino-[2,3-e]imidazo[1,2-c]pyrimidin-9-ylthio)acetate (18)

Yellow crystals from a mixture of ethanol/dioxane (1 : 2), m.p. 240 °C, yield 1.22 g (58%.) IR: ν (cm^{-1}) 3060 (CH arom.), 2930 (CH aliph.), 1730 (C=O). ^1H NMR (CF_3CO_2D): δ 1.40 (t, 3 H, $\underline{CH_3}CH_2$), 2.97 (s, 3 H, CH_3), 4.40 (s, 4 H, 2CH_2 imidazoline), 4.87 (q, 2 H, $CH_3\underline{CH_2}$), 5.10 (s, 2 H, CH_2), 7.75 (s, 5 H, Ar−H).
$C_{20}H_{19}N_7O_2S$

3.17.2. 9-Benzoylmethylthio-6,7-dihydro-3-methyl-1-phenyl-1H-pyrazolo-[4′,3′:5,6]pyrazino[2,3-e]imidazo[1,2-c]pyrimidine **(19)**

Fine yellow crystals from a mixture of ethanol/dioxane (2 : 1), m.p. 248 °C, yield 1.2 g (53%). IR: ν (cm^{-1}) 3050 (CH arom.), 2910 (CH aliph.), 1680 (C=0). ^{1}H NMR (CF_3CO_2D): δ 2.93 (s, 3 H, CH_3), 4.73 (m, 2 H, CH_2 imidazoline), 4.93 (m, 2 H, CH_2 imidazoline), 5.23 (s, 2 H, $PhCO\underline{CH_2}$), 7.67 (s, 8 H, Ar−H), 8.17 (m, 2 H, Ar−H).
$C_{24}H_{19}N_7OS$.

3.17.3. (6,7-Dihydro-3-methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino-[2,3-e] imidazo[1,2-c]pyrimidin-9-ylthio)acetanilide **(20)**

Yellow crystals from ethanol, m.p. 175 °C, yield 1.4 g (62%). IR: ν (cm^{-1}) 3250 (NH), 3050 (CH arom.) 2900 (CH aliph.), 1695 (CO). ^{1}H NMR (CF_3CO_2D): δ 2.93 (s, 3 H, CH_3), 4.07 (s, 2 H, CH_2), 4.5 (m, 2 H, CH_2 imidazoline), 4.8 (m, 2 H, CH_2 imidazoline), 7.57 (m, 6 H, Ar−H), 8.2 (m, 4 H, Ar−H), 10.57 (s, 1 H, NH).
$C_{24}H_{20}N_8OS$.

3.18. (6,7-Dihydro-3-methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino-[2,3-e] imidazo[1,2-c]pyrimidin-9-yl)hydrazine **21**

A mixture of the thione **17** (3.35 g, 0.01 mol) and excess hydrazine hydrate (5 ml, 80%) in pyridine (15 ml) was heated under reflux for 6 h. After cooling, the solid precipitate was filtered, washed with water, dried and purified by heating in boiling dioxane and filtered to give yellowish-brown crystals, m.p. >360 °C, yield 1.7 g (52%). IR: ν (cm^{-1}) 3350, 3150 ($NHNH_2$). ^{1}H NMR: the compound is insoluble in all deuterated solvents.
$C_{16}H_{15}N_9$.

3.19. 6,7-Dihydro-3-methyl-1-phenyl-1H-pyrazolo[4′,3′:5,6]pyrazino-[2,3-e]imidazo[1,2-c]-(1,2,4)-triazolo[4,3-a]pyrimidine **22**

A mixture of compound **21** (0.83 g, 0.0025 mol) and triethyl orthoformate (15 ml) was heated under reflux for 6 h. The reaction mixture was then cooled and poured into water. The solid precipitate was filtered, washed with water, and recrystallized from a mixture of DMF/water (1 : 2) to give orange-yellow crystals, m.p. 335 °C, yield 0.44g (51%). IR: ν (cm^{-1}) 3060 (CH arom.), 2920 (CH aliph.). ^{1}H NMR (CF_3CO_2D): δ 2.97 (s, 3 H, CH_3), 4.13 (m, 2 H, CH_2 imidazoline), 4.8 (m, 2 H, CH_2 imidazoline), 7.66 (m, 3 H, Ar−H), 7.9 (m, 2 H, Ar−H), 8.4 (s, 1 H, CH triazole).
$C_{17}H_{13}N_9$.

3.20. Antifungal testing

The compounds tested were dissolved in DMF to give a solution of 1% concentration. Filter paper discs (Whatman No. 3 filter paper, 5 mm diameter) were saturated with this solution. The saturated filter paper discs were placed on the surface of solidified Czapek's Dox agar dishes seeded by the test fungi. The inhibition zones were measured in mm at the end of an incubation period of 48 h at 28 °C.

3.21. Antiparasitic testing

Leishmanicidal activity was evaluated on *Leishmania infantum* strain (MCAN/FR/74 LPMA 57; WHO) and *L. tropica* (MHOM/FR/65 LPMA 59; WHO). *Leishmania infantum* was originally isolated from the ganglia of dogs in Marseilles and *Leishmania tropica* from a human case of cutaneous leishmaniasis with numerous typical ulcerations. These isolates contained numerous *Leishmania amastigotes* and were cultivated in NNN (Novy, Mac Neal, Nicolle) [22] and Tobie [23] media where they were transformed into promastigote forms. These strains were maintained in continuous culture in RPMI 1640 (Gibco) containing 10% heat-inactivated fetal calf serum. Streptomycin (50 mg/l) and penicillin G (50 units/ml) were also added (these concentrations did not affect *Leishmania* growth).
Promastigotes (10^6 *Leishmania*/ml) were inoculated in tubes containing 5 ml of the above-described medium and incubated at 24 °C [24]. Subcultures were made once a week and each subculture was checked for abundance and motility of promastigote forms. They were counted with a Malassez cell and the volume of inoculum was adjusted to distribute 10^6 *Leishmania*/ml. The test compounds were first dissolved in dimethylformamide (10 mg/ml) then distributed to the culture tubes to obtain final concentrations of 100; 50; 25; 10; 5; 1 and 0.5 mg/l. Dimethylformamide was completely inactive on the parasites at these concentrations. Each strain and each concentration was tested in triplicate. The minimal inhibitory concentrations (MIC) of the compounds were determined after the parasites had been in culture for 7 days by checking for the presence or absence of promastigotes microscopically (x 400). The absence of promastigotes in the tubes was confirmed by retroculture. If the parasites did not recover, that concentration of a compound was considered leishmanicidal. The MIC for each compound was then compared with that of pentamidine determined under the same conditions.

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