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Synthesis, anti-microbial evaluation, and molecular modeling of new pyrazolo[3,4-*d*]pyrimidine derivatives

Ahmad F. Eweas · S. A. Swelam · O. A. Fathalla · N. M. Fawzy · Sh. I. Abdel-Moez

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Abstract Synthesis of some new pyrazolo[3,4-*d*]pyrimidine derivatives using readily available starting materials are described. A one-pot multi component cyclocondensation reaction was used to prepare the novel 3-methyl-4-aryl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidine-6-thiol which served as a new starting material for all new compounds in this research. The anti-microbial activities of the selective synthesized compounds have been evaluated. Some of the newly prepared compounds were found to have moderate to strong anti-microbial activity, e.g., compound **4a**, **6a**, and **8**, in comparison to the reference drugs. Molecular modeling of the most three biologically active new compounds **4a**, **6a**, and **8** compared to the reference drugs tobramycin and fluconazole was carried out using Field-align 2.0 software.

A. F. Eweas (✉) · O. A. Fathalla
Medicinal Chemistry Department, National Research Centre, Dokki, Cairo, Egypt
e-mail: a.eweas@tu.edu.sa

A. F. Eweas
Pharmaceutical Chemistry Department, College of Pharmacy, Taif University, Taif, Kingdom of Saudi Arabia

S. A. Swelam
Photochemistry Department, National Research Centre, Dokki, Cairo, Egypt

N. M. Fawzy
Department of Natural and Microbial Products, National Research Centre, Dokki, Cairo, Egypt

Sh. I. Abdel-Moez
Department of Microbiology and Immunology, National Research Centre, Dokki, Cairo, Egypt

Keywords Pyrazolopyrimidines · Hydrazino derivatives · Anti-microbial

Introduction

In the past few decades, the development of resistant microbes has been greatly accelerated by several concurrent trends which resulted in increasing the number of infections and which expands the need for new anti-microbial drugs.

In recent years pyrazolo[3,4-*d*]pyrimidines and related fused heterocyclic have attracted a great interest in medicinal chemistry and drug discovery in part this is attributed to their structure similarity to purines (Bakavoli *et al*., 2010). In this aspect a wide array of biological activity of pyrazolo[3,4-*d*]pyrimidines have been recently reported including CNS, metabolic diseases, oncology, selective cox-2 inhibitor, CCK1 receptor antagonist, non-steroidal anti-inflammatory, and anti-proliferative agents (Genin *et al*., 2000; Lyga *et al*., 1994; Jorda *et al*., 2011; Lee *et al*., 2003; Huang *et al*., 2000; Gomez *et al*., 2007) as well as blockbuster drugs, such as celecoxib (celebrex) and sildenafil (Viagra) (Penning *et al*., 1997; Terrett *et al*., 1996). Recently, extensive studies have been devoted to the synthesis of pyrazolo[3,4-*d*]pyrimidines (Liu *et al*., 2007; Schenone *et al*., 2008; Heravi *et al*., 2007; Quiroga *et al*., 2008). Multicomponent reactions (MCRs) have recently gained tremendous importance in organic and medicinal chemistry. The main contributing factors are the high atom economy, wide application in combinatorial chemistry and diversity–oriented synthesis (Ramón *et al*., 2005; Andreana *et al*., 2004; Denmark *et al*., 2003; Ramachary *et al*., 2004; Cozzi *et al*., 2005; Armstrong *et al*., 1996; Burke *et al*., 2004).

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Results and discussion

Chemistry

In continuation to our program directed to the synthesis and biological evaluation of fused heterocycles, we herein report the synthesis of new derivatives based on pyrazolopyrimidine moiety (Zaki *et al*., 1999; Swelam *et al*., 2008; Swelam *et al*., 2004; Swelam & Fawzy, 2004).

A one-pot cyclocondensation of pyrazolone **1**, aromatic aldehyde, and thiourea under acidic conditions afforded the corresponding pyrazolopyrimidine **4a–c** in good yields, compounds **4a–c** served as a new starting material throughout this study (Scheme 1).

The structures of 4-(4-aryl)-3-methyl-1-phenyl-1,7-dihydropyrazolo[3,4-*d*]pyrimidine-6-thione (**4a–c**) were confirmed via elemental analysis and spectral data. ^{1}H-NMR (DMSO-d_6) (δ, ppm) of **4a–c** showed signals at 3.00–3.20 (s, 1H, SH, exchangeable with D_2O) and δ 7.25–8.32 (m, Ar–H).

Cyclocondensation of **4a** with the appropriate aldehydes and chloroacetic acid in the presence of sodium acetate in acetic acid medium were carried out to produce the corresponding adducts **6a**, **b**. In addition to spectral prove, the structure assignments of the formed products were further confirmed chemically by alternative synthesis of **6a**, **b** via cyclocondensation of **4a** with chloroacetic acid then condensation of the products obtained with the appropriate aldehyde (Scheme 2).

The S-alkylation of **4a** with α- halo compounds namely chloroacetyl chloride and *P*-methoxy α-bromoacetophenone affording the corresponding ethanone derivatives **7**, **8**. The structures of the **7**, **8** were confirmed via micro analyzes and spectral data in addition to chemical confirmation reaction. ^{1}H-NMR (DMSO-d_6) (δ, ppm) showed peaks at 4.52 and 5.02 specific to (s, 2H, SCH_2).

Coupling of compound **8** with appropriate to the aromatic aldehyde in basic medium afforded 3-(4-chlorophenyl)-2-((4-(4-chlorophenyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-6-yl)thio)-1-(4-methoxyphenyl)prop-2-en-1-one **9a** and 3-(4-bromophenyl)-2-((4-(4-chlorophenyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-6-yl) thio)-1-(4-methoxyphenyl)prop-2-en-1-one **9b** in good yields (Scheme 2).

The hydrazino derivative **10** was prepared by heating of **4a** with hydrazine hydrate in dioxane at refluxing temperature (Scheme 2). ^{1}HNMR (DMSO-d_6) of **10** showed signals at δ 2.15 (s, 3H, Me), δ 2.03 (bs, 1H, exchangeable with D_2O, NH), δ 4.02 (s, 2H, exchangeable with D_2O, NH_2). The resultant hydrazino product **10** underwent a series of cyclization reactions (Scheme 3).

First with triethylorthoformate, triethylorthoacetate to give the corresponding triazole derivatives **11a**, **b** (Scheme 3).

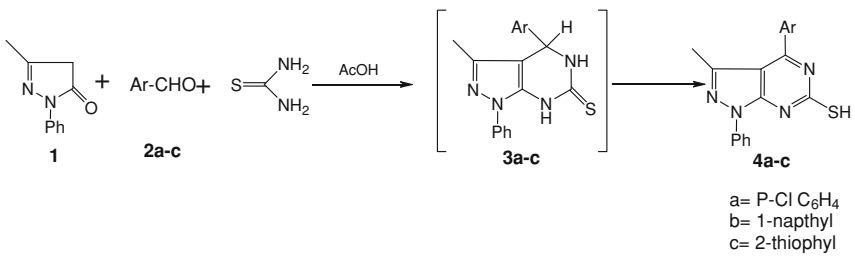
Pyrazolo[3,4-*d*]pyrimidin-6-yl]-5-methyl-1,2-dihydropyrazol-3-one derivative **12** was prepared via reaction of **10** with active methylene compound namely ethyl acetoacetate (Scheme 3). ^{1}HNMR spectrum of 12 showed signal at δ 6.54 (s, 1H) specific for pyrazolone ring.

The hydrazino derivative **10a** interact with α, β-unsaturated nitrite derivatives **13a**, **b** and **15a**, **b** afforded compounds **14a**, **b** and **16a**, **b**. The structures of the formed products were confirmed from their correct values in elemental analyzes and spectral data. IR (KBr, ν cm^{-1}) for compounds **14a**, **b** revealed peaks at 3,425–3,100 (NH_2), 2,210 (CN). IR (KBr, ν cm^{-1}) for compounds **16a**, **b** revealed peaks at 2,210 (CN), 1,653 (CO), respectively (Scheme 3).

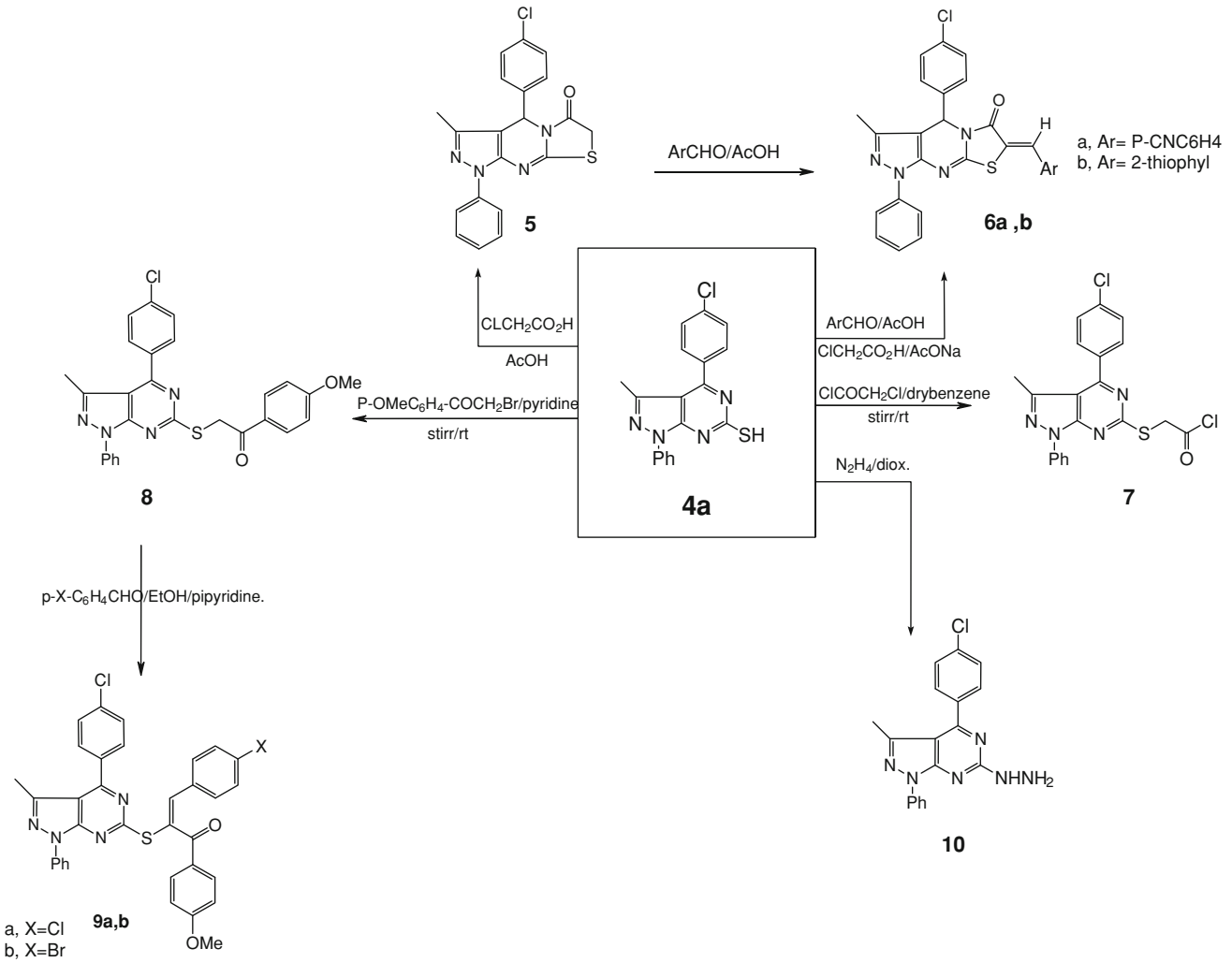
The synthesis of ketene dicyanoacetals (Tominaga *et al*., 1985), have attracted considerable attention as starting materials for synthesis of wide variety of heterocyclic compounds. Thus, compound **17** was formed upon heating of **10** with 2-((methylthio)methylene)malononitrile in basic medium. The structure of **17** was confirmed by its correct values in elemental analyzes and their agreeable spectral data. IR (KBr, ν cm^{-1}) of compound **17** revealed peaks at 3,401–3,010 (NH_2), 2,215 (CN) (Scheme 3). ^{1}H-NMR (DMSO-d_6) (δ, ppm) of compound showed signal at 4.25 (s, 3H, SCH_3) (Scheme 3, exp.).

Anti-microbial activities

Anti-microbial activity of the ten new compounds was evaluated against the following tested strains; bacterial strains *Escherichia coli* O157:H7, *S. typhimurium*, *Ps. areginosus*, *S. aureus*, *L. monocytogenes*, and *B. cereus*. Fungal strains *Candida albicans* and *Aspergillus flavus*. Tobramycin (10 μg/ml) was used as standard control positive anti-bacterial while



Scheme 1



Scheme 2

fluconazole (25 μg/ml) was used as standard anti-fungal, on the other hand dimethyl sulphoxide (DMSO) was used as control negative for both anti-bacterial and anti-fungal activity.

Results (Table 1) revealed that compound **6a** (100 μg/ml) gives the highest anti-bacterial activity against all tested strains with a mean zone of inhibition of 17.25 mm followed by **8** (100 μg/ml), 16.63 mm then **4a** (100 μg/ml), 16.13 mm then **10a** (100 μg/ml), 14.13 mm, and **7**, **4c** and **16a** (100 μg/ml), 14.00 mm finally compounds **16b**, **11a**, and **14a** showed the least zone of inhibition against all tested bacterial and fungal pathogenic strains 13.88, 13.60, and 8.63, respectively.

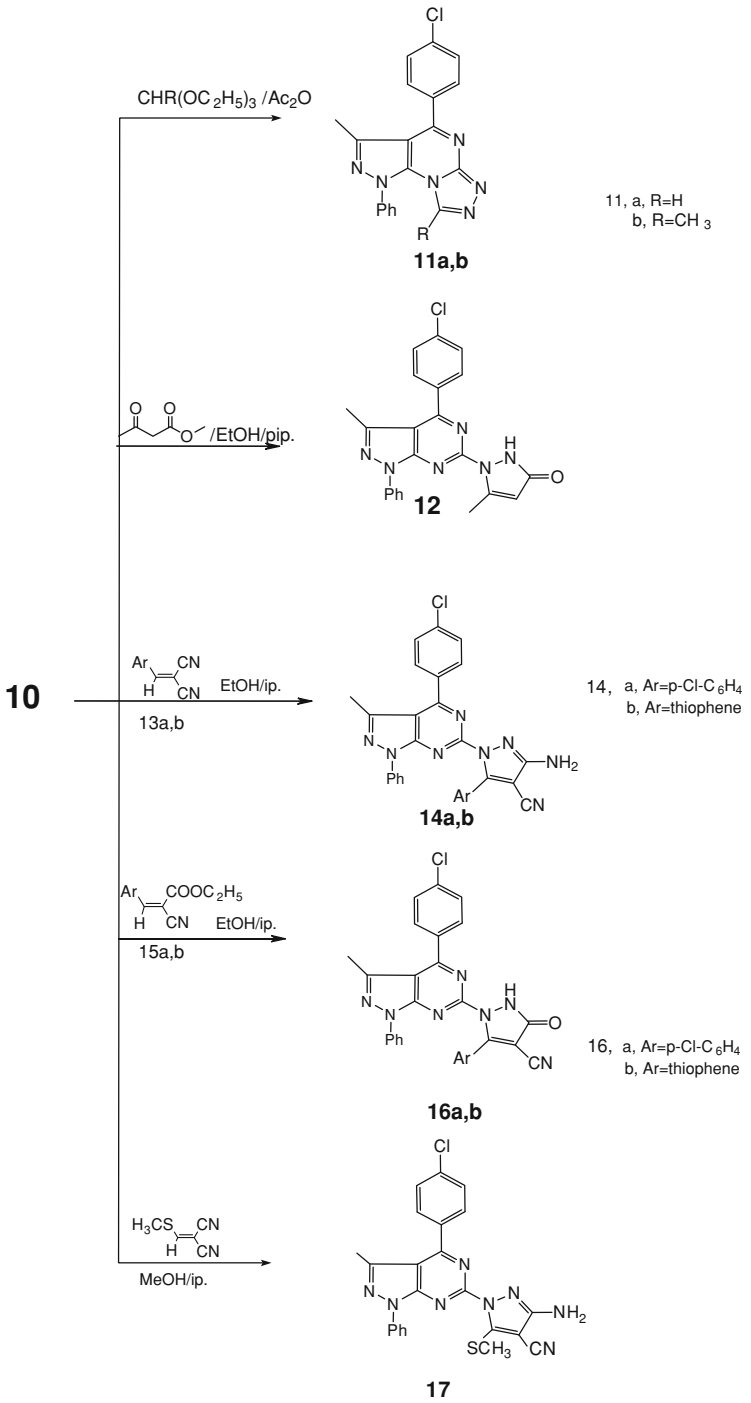
Molecular modeling study

Introduction

Assuming that the tested compound interact with the same targets as the reference drugs used for both anti-bacterial and anti-fungal activity, i.e., 16S rRNA on the bacterial 30S ribosome for tobramycin and the fungal cytochrome P450 14α-demethylase for fluconazole. Two molecules which both bind to a common active site tend to make similar interactions with the protein and hence have highly similar field properties like electrostatic interactions, H-bonding, and van der Waals interactions. Accordingly, aligning and scoring molecules based on the similarity in these properties compared to the reference drugs (tobramycin and fluconazole) is considered as a powerful tool as it concentrates on the aspects of the molecules that are important for biological activity. The alignments provided give ideas on how molecules with different structures could interact with the same protein, and the scores for those alignments provide insights into SAR and ideas for further synthesis.

Results

The three dimensional structures of both reference drugs tobramycin and fluconazole in their bioactive conformations



Scheme 3

were extracted from their X-ray crystallography PDB files while bound to their receptors 16S RNA PDB code 1LC4 for tobramycin and 14α-sterol demethylase (CYP51) for fluconazole PDB code 2WV2.

The most biologically active tested compounds **6a**, **8**, and **4a** were constructed using Chem3D ultra 12.0 software [chemical structure drawing standard; Cambridge soft corporation, USA (2010)], then they were energetically minimized by using MOPAC with 100 iterations and minimum RMS gradient of 0.10. The aforementioned compounds were then aligned to the reference drug molecules tobramycin and fluconazole, used as reference drugs in the anti-microbial testing based on their molecular fields using Fieldalign software (v 2.0.1). The interaction between a ligand and a protein involves electrostatic fields and surface properties (e.g., hydrogen bonding, hydrophobic surfaces… etc.).

Table 1 The anti-microbial activity of the tested compounds against bacterial and fungal strains isolated from food and milk of animal origin

Compound no.	*E. coli* O157	*S. typhimurium*	*L. monocytogenes*	*S .aureus*
4a	0	12	24	18
4c	14	14	16	14
6a	18	15	20	28
7	9	11	29	14
8	16	13	14	16
10a	10	13	16	16
11a	10	13	20	14
14a	0	9	18	12
16a	0	9	29	0
16b	0	10	32	14
Tobramycin (10 μg/ml)	20	18	20	19
Flucanazole (25 μg/ml)	-	-	-	-
DMSO	-	-	-	-

Compound no.	*Ps. Arginosus*	*B. cereus*	*C. albicans*	*A .flavus*	Mean zone of inhibition
4a	24	22	29	0	16.13
4c	16	18	10	10	14.00
6a	18	22	9	8	17.25
7	22	10	8	9	14.00
8	18	18	26	12	16.63
10a	16	26	16	0	14.13
11a	12	22	18	0	13.60
14a	9	12	9	0	8.63
16a	14	24	24	12	14.00
16b	14	9	20	12	13.88
Tobramycin (10 μg/ml)	18	19	-	-	19
Flucanazole (25 μg/ml)	-	-	17	16	16.5
DMSO	-	-	-	-	-

The result of alignments reveals that the tested compounds filed similarity compared to tobramycin ranged from 0.427 to 0.518 where maximum similarity value is 1.00 (Table 2). While their filed similarity compared to fluconazole is ranged from 0.478 to 0.517 (Table 3).

Compound **8** showed the highest filed similarity compared to tobramycin. On the other hand, compound **4a** showed the highest filed similarity compared to fluconazole. The alignment results of the tested compounds are shown in Tables 2, 3.

Experimental

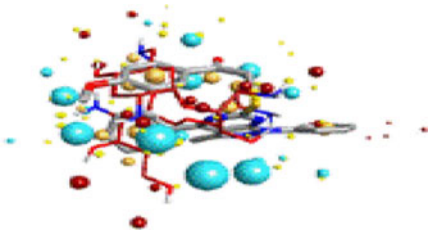
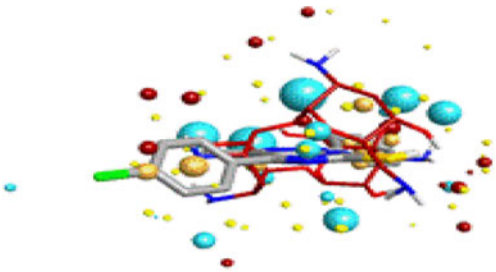
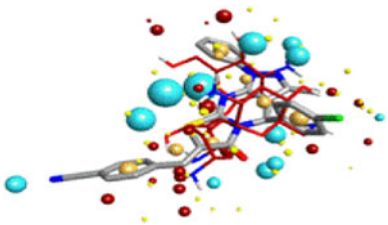
Chemistry

The purity of the newly synthesized compounds was evidenced by TLC. All solvents which were used for crystallization were of analytical grade. All melting points were determined on a Gallenkamp apparatus and uncorrected. Elemental analyzes were carried out at the Micro analytical Laboratory of the National Research Centre, Cairo, Egypt (satisfactory micro analyzes were obtained C ±0.40, H ±0.02, Cl ±0.21, N ±0.30 and S ±0.23). The IR spectra were recorded in KBr disks, on a Jasco Fourier Transform Infrared Spectrophotometer Model FT/IR3000E. The ^{1}H-NMR were recorded (DMSO-d_6), on JOEL-JNM-EX 270 FTNMR system (NRC) and chemical shifts (δ) are expressed in ppm using TMS as an internal standard. Splitting patterns are indicated as s singlet, d doublet, m multiple, b broad signal. The MS were performed at 70 eV on a Finnegan MAT SSQ7000 spectrometer.

*3-Methyl-1-phenyl-4-(aryl)-1H-pyrazolo[3,4-d]pyrimidine-6-thiol (**4a–c**)*

A mixture of equimolecular amounts of 3-methyl-1-phenyl-5-pyrazolone **1**, appropriate aromatic aldehyde (namely, *p*-chlorobenzaldehyde, 1-naphthalene aldehyde, and 2-thiophene aldehyde, respectively), and thiourea in acetic acid (20 ml) was heated to 80–85°C for 8 h with stirring, after reaction completion, the reaction mixture was cooled and

Table 2 Field alignment of compound **4a**, **6a**, and **8** against reference drug tobramycin

Compound no.	Filed align picture	Field align score
8		0.518
4a		0.499
6a		0.427

added to ice water (100 ml). The solid precipitated was filtered off, air dried, and crystallized from ethanol affording (**4a–c**).

4a: Yield 75%, m.p. 221–223°C, M.wt. $C_{18}H_{13}ClN_4S$ (352.84), molecular formula $C_{18}H_{13}ClN_4S$; ^{1}H NMR (DMSO-d_6) δ 2.16 (s, 3H, Me), 3.00 (s, 1H, SH, exchangeable with D_2O), δ 7.25–8.32 (m, 9H, Ar–H); MS *m*/*z* (353, 32%) (355, 10%) (77, 100%).

4b: Yield 77%, m.p. 185–187°C, M.wt. (368.47), molecular formula $C_{22}H_{16}N_4S$; ^{1}H NMR (DMSO-d_6) δ 2.19 (s, 3H, Me), 3.01 (s, 1H, SH, exchangeable by D_2O), δ 7.31–8.02 (m, 12H, Ar–H); MS *m*/*z* (368, 40%), (370, 14%), (77, 100%).

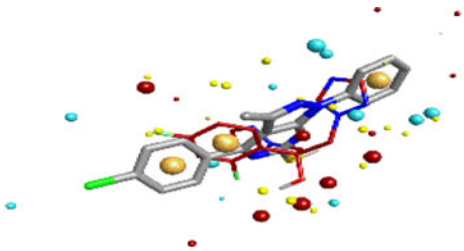
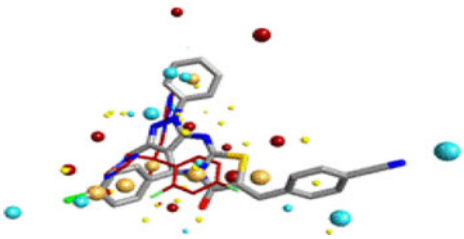
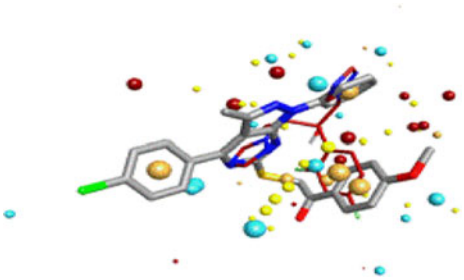
4c: Yield 74%, m.p. 148–150°C, M.wt. (324.43), molecular formula $C_{16}H_{15}N_4S_2$; ^{1}H NMR (DMSO-d_6) δ 2.21 (s, 3H, Me), 3.20 δ (s, 1H, SH, exchangeable by D_2O), δ 7.31–7.98 (m, 8H, Ar–H); MS *m*/*z* (324, 12%), (65, 100%).

*4-(4-Chlorophenyl)-3-methyl-1-phenyl-4,7-dihydropyrazolo[3,4-d]thiazolo[3,2-a]pyrimidin-6(1H)-one (**5**)*

To a warm solution of compound **4a** (3.52 g, 10 mmol) in (30 ml) acetic acid, chloroacetic acid (0.95 g, 10 mmol), anhydrous sodium acetate (1.64 g, 20 mmol) was added. The reaction mixture was heated gently with stirring on water bath at 60°C under reflux for 12 h. The reaction mixture was allowed to cool to room temperature, poured into ice water (100 ml), the precipitated solid filtered off, air dried, and crystallized from ethanol affording compound **5**.

5: Yield 70%, m.p. 164–166°C, M.wt. (494.890), molecular formula $C_{20}H_{15}ClN_4OS$; ^{1}H NMR (DMSO-d_6) δ 2.21 (s, 3H, Me), 3.82 (s, 2H, thiazolo), δ 7.02 (s, 1H, pyrimidine), δ 7.31–8.03 (m, 9H, Ar–H); MS *m*/*z* (495 HNCS, 65%), (497 HNCS, 22%), (77, 100%).

Table 3 Field alignment of compound **4a**, **6a**, and **8** against reference drug fluconazole

Compound no.	Filed align picture	Field align score
4a		0.517
6a		0.500
8		0.478

Color key

Reference compounds tobramycin and fluconazole are shown in *red color* while tested compounds shown in *gray color*

Filed points color key

Blue negative field points

Red positive field points

Yellow van der Waals surface field points

Gold/Orange hydrophobic field points

*(Z)-4-(4-Chlorophenyl)-3-methyl-1-phenyl-7-(arylmethylene)-4,7-dihydropyrazolo[3,4-d]thiazolo[3,2-a]pyrimidin-6(1H)-one (**6a**, **b**)*

General procedure *Method A* A mixture of compound **4a** (3.52 g, 10 mmol), chloroacetic acid (0.95 g, 10 mmol), appropriate aldehydes (10 mmol) (4-cyanobenzaldehyde, 2-thiophene aldehyde), and anhydrous sodium acetate (1.64 g, 20 mmol) in a mixture of acetic acid and acetic anhydride (30 ml, 1:1) was stirred under refluxing temperature for 8 h. The formed solid was collected by filtration, air dried, and crystallized from appropriate solvent affording **6a**, **b**.

Method B A mixture of compound **5** (4.94 g, 10 mmol) appropriate aldehyde (10 mmol) (4-cyanobenzaldehyde, 2-thiophene aldehyde) and anhydrous sodium acetate (1.64 g, 20 mmol) in a mixture of acetic acid and acetic anhydride (30 ml, 1:1) was stirred under refluxing temperature for 8 h. The deposited solid after cooling was filtered off, air dried, and crystallized from appropriate solvent affording **6a**, **b**.

6a: Yield 55%, m.p. 148–150°C, crystallized from benzene, M.wt. (508.01) molecular formula $C_{28}H_{18}ClN_5OS$, IR (KBr, ν cm^{-1}) 2,216 (CN); ^{1}H NMR (DMSO-d_6) δ 2.16 (s, 3H, Me), δ 7.42 (s, 1H, pyrimidine), δ 7.31–8.03 (m, 13H, Ar–H), δ 8.54 (s, 1H, Ar–CH); MS *m/z* (508, 65%), (510, 21%), (96, 100%).

6b: Yield 51%, m.p. 196–198°C, crystallized from methanol, M.wt. (488.02), molecular formula $C_{25}H_{17}ClN_4OS_2$; ^{1}H NMR (DMSO-d_6) δ 2.25 (s, 3H, Me), δ 6.42 (s, 1H, pyrimidine), δ 7.31–8.03 (m, 12H, Ar–H), δ 9.02 (s, 1H, Ar–CH); MS *m/z* (488, 25%), (490, 8%), (65, 100%).

*2-((4-(4-Chlorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)acetyl chloride (**7**)*

An equimolecular amounts of compound **4a** (3.52 g, 10 mmol) and chloroacetylchloride (1.12 g, 10 mmol) in dry benzene (20 ml) was stirred at room temperature for 2 h. The solid formed was filtered off and crystallized from benzene.

7: Yield 56%, m.p. 180–182°C, M.wt. (393.88), molecular formula $C_{20}H_{14}ClN_4OS$; ^{1}H NMR (DMSO-d_6) δ 2.18 (s, 3H, Me), δ 4.52 (s, 2H, SCH_2), δ 7.31–8.03 (m, 9H, Ar–H), MS *m/z* (393, SCHCOCl, 55%), (395, SCHCOCl, 18%), (65, 100%).

*2-((4-(4-Chlorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-1-(4-methoxyphenyl)ethanone (**8**)*

An equimolecular amounts of compound **4a** (3.52 g, 10 mmol) and *P*-methoxy β-acetophenone (10 mmol) in dry pyridine (20 ml) was stirred at room temperature for 12 h. The precipitated solid was filtered off and crystallized from ethanol.

8: Yield 63%, m.p. 160–162°C, M.wt. (500.97), molecular formula $C_{27}H_{21}ClN_4O_2S$; ^{1}H NMR (DMSO-d_6) δ 2.23 (s, 3H, Me), δ 5.02 (s, 2H, SCH_2), δ 4.12 (s, 3H, OCH_3), δ 7.35–8.23 (m, 13H, Ar–H), MS *m/z* (500 SCHCOCl, 15%), (502, SCHCOCl, 5%), (75, 100%).

*3-(4-Halophenyl)-2-((4-(4-chlorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl)thio)-1-(4-methoxyphenyl)prop-2-en-1-one **9a, b***

A mixture of equimolecular amounts of compound **8** (5.01 g, 10 mmol) and appropriate aromatic aldehyde (*p*-chlorobenzaldehyde and *p*-bromobenzaldehyde, respectively) (10 mmol) in ethanol (20 ml) in the presence of catalytic amount of pyrimidine (1 ml) was heated under refluxing temperature for 12 h. The solid formed on cooling was filtered off, air dried, and crystallized from ethanol.

9a: Yield 52%, m.p. 294–296°C, M.wt. (623.57), molecular formula $C_{34}H_{24}Cl_2N_4O_2S$; ^{1}H NMR (DMSO-d_6) δ 2.23 (s, 3H, Me), δ 4.12 (s, 3H, OCH_3), δ 7.35–8.23 (m, 17H, Ar–H), δ 8.01 (s, 1H, Ar–CH).

9b: Yield 48%, m.p. 310–312°C, M.wt. (668.02), molecular formula $C_{34}H_{24}BrClN_4O_2S$; ^{1}H NMR (DMSO-d_6) δ 2.23 (s, 3H, Me), δ 4.12 (s, 3H, OCH_3), δ 7.35–8.23 (m, 17H, Ar–H), δ 8.01 (s, 1H, CH).

*2-[4-(4-Chlorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl]-hydrazine (**10**)*

A solution of compound **4a** (3.52 g, 10 mmol) in dioxane (25 ml) and hydrazine hydrate (0.05 g, 10 mmol) was heated at refluxing temperature for 10 h. Then, the white crystals formed was collected by filtration, washed with cold water then dried under suction and crystallized from ethanol affording **10**.

10a: Yield 80%, m.p. 208–210°C, M.wt. (350.81), molecular formula $C_{18}H_{15}ClN_6$, IR (KBr, ν cm^{-1}) 3,310, 3,102 (NH_2 NH); ^{1}HNMR (DMSO-d_6) δ 2.15 (s, 3H, Me), δ 2.03 (bs, 1H, exchangeable with D_2O, NH), δ 4.02 (s, 2H, exchangeable with D_2O, NH_2), δ 7.35–8.23 (m, 9H, Ar–H); MS *m/z* (351, 71%), (353, 23%), (65, 100%).

*Preparation of **11a, b*** An equimolecular amounts of compound **10a** (3.50 g, 10 mmol) and triethylorthoformate or triethylorthoacetate (10 mmol) in AcOH (20 ml) was stirred at refluxing temperature for 8 h. The solid formed on cooling was filtered off and crystallized from ethanol.

*4-(4-Chlorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a]pyrimidine (**11a**)*

11a: Yield 60%, m.p. 180–182°C, M.wt. (362.82), molecular formula $C_{19}H_{13}ClN_6$; ^{1}H-NMR (DMSO-d_6), δ 2.23 (s, 3H, Me), δ 6.41 (s, 1H, triazolo), δ 7.42–7.99 (m, 9H, Ar–H); MS *m/z* (363, 25%), (365, 8%), (75, 100%).

*4-(4-Chlorophenyl)-3,8-dimethyl-1-phenyl-1H-pyrazolo[4,3-e][1,2,4]triazolo[4,3-a]pyrimidine (**11b**)*

11b: Yield 51%, m.p. 130–133°C, M.wt. (374.83), molecular formula $C_{20}H_{15}ClN_6$; ^{1}H NMR (DMSO-d_6) δ 1.19, 2.23 (2s, 6H, 2Me), δ 7.42–7.99 (m, 9H, Ar–H); MS *m/z* (375, 27%), (377, 9%), (75, 100%).

*1-(4-(4-Chlorophenyl)-3-methyl-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-6-yl)-5-methyl-1H-pyrazol-3(2H)-one (**12**)*

An equimolecular amounts of compound **10a** (3.50 g, 10 mmol), ethylacetoacetate (1.31 g, 10 mmol), and catalytic amount pyrimidine (1 ml) in ethanol (20 ml) was stirred at refluxing temperature for 8 h. After cooling to

room temperature the solid formed was filtered off, air dried, and crystallized from ethanol.

12: Yield 49%, m.p. 295–297°C, M.wt. (416.87), molecular formula $C_{22}H_{17}ClN_6O$; ^{1}H NMR (DMSO-d_6), δ 2.20, 2.09 (2s, 6H, 2Me), δ 6.54 (s, 1H, pyrazolo), δ 7.42–7.99 (m, 9H, Ar–H), δ 8.02 (bs, 1H, exchangeable with D_2O, NH); MS *m*/*z* (417, 16%), (419, 5%), (77, 100%).

*General procedure for preparation of **14a**, **b**, **16a**, **b** and **17***

A mixture of compound **10a** (10 mmol) and ethyl(*Z*)-2-cyano-3-substituted phenylacrylate derivatives **13a**, **b**, **15a**, **b** or 2-((methylthio)methylene) malononitrile (10 mmol) in ethanol (20 ml) was heated under reflux for 12–14 h. The reaction completion was followed by TLC. The reaction mixture was cooled, poured into ice water and the solid formed was filtered off, dried under air suction, and crystallized from methanol.

14a: Yield 49%, m.p. 275–277°C, M.wt. (537.42), molecular formula $C_{28}H_{18}Cl_2N_8$; IR (KBr, ν cm^{-1}), 3,425–3,100 (NH_2), 2,210 (CN); ^{1}H-NMR (DMSO-d_6), δ 2.29 (s, 3H, Me), δ 4.54 (s, 2H, exchangeable with D_2O, NH_2), δ 6.98–8.01 (m, 13H, Ar–H); MS *m*/*z* (538 HCN, 16%), (540 HCN, 16%), (542 HCN, 5%), (77, 100%).

14b: Yield 42%, m.p. 295–297°C, M.wt. (509.0), molecular formula $C_{26}H_{17}ClN_8S$; IR (KBr, ν cm^{-1}), 3,421–3,210 (NH_2), 2,210 (CN); ^{1}H-NMR (DMSO-d_6), δ 2.29 (s, 3H, Me), δ 4.34 (s, 2H, exchangeable with D_2O, NH_2), δ 6.98–7.94 (m, 12H, Ar–H); MS *m*/*z* (510 HCN, 16%), (512 HCN, 5), (77, 100%).

16a: Yield 54%, m.p. 268–270°C, M.wt. (584.47); molecular formula $C_{30}H_{23}Cl_2N_7O_2$; IR (KBr, ν cm^{-1}), 2,210 (CN), 1,653 (CO); ^{1}H-NMR (DMSO-d_6), δ 1.05, 2.29 (2s, 6H, 2Me), δ 2.54 (s, 2H, exchangeable with D_2O, NH_2), δ 7.34–7.84 (m, 13H, Ar–H); MS *m*/*z* (584 HCN, 25%), (586 HCN, 25%), (588 HCN, 8%), (77, 100%).

16b: Yield 34%, m.p. $>$ 310°C, M.wt. (558.05), molecular formula $C_{28}H_{22}ClN_7O_2S$; IR (KBr, ν cm^{-1}), 2,210 (CN), 1,653 (CO); ^{1}H-NMR (DMSO-d_6), δ 1.35, 2.19 (2s, 6H, 2Me), 4.54 (s, 2H, exchangeable with D_2O, NH_2), δ 7.34–7.84 (m, 12H, Ar–H); MS *m*/*z* (559 HCN, 25%), (561 HCN, 8%), (77, 100%).

17: Yield 39%, m.p. 245–247°C, M.wt. (472.96), molecular formula $C_{23}H_{17}ClN_8S$; IR (KBr, ν cm^{-1}), 3,401–3,010 (NH_2), 2,215 (CN); ^{1}H-NMR (DMSO-d_6), δ 2.20 (s, 3H, Me), δ 3.74,3.9 (2s, 2H, exchangeable with D_2O, NH_2), δ 4.25 (s, 3H, SCH_3), δ 6.56–7.94 (m, 9H, Ar–H).

Anti-microbial activity

Anti-bacterial as well as anti-fungal activity of seven tested compounds were in vitro evaluated using agar well diffusion test (Sgouras *et al*., 2004; Wiart, 2007) using two different concentration of the compounds (100 μg/ml) dissolved in (1 ml) DMSO as a qualitative method for studying the anti-microbial activity of the tested compounds against the following tested stains; bacterial strains *E. coli* O157, *S. typhimurium*, *Ps. areginosus*, *S. aureus*, *L. monocytogenes*, *B. cereus*. Fungal strains are *C. albicans* and *A. flavus*. As control positive tobramycin (10 μg/ml) was used as standard anti-bacterial while fluconazole (25 μg/ml) was used as standard anti-fungal while DMSO was used as control negative.

Strains selected for being tested are isolated, and feed by products of animal origin including poultry (*E. coli* O157 and *S. typhimuruim*) as well as from mastitic cow milk, (*L. monocytogenes*, *S.* and *B. cereus*) and from minced cow meet (*S. aureus*, *Ps. aerigenosus*). These strains are commonly accused of being a cause of food intoxication.

Muller–Hinton agar plates were inoculated with bacterial strains while sabouraud dextrose agar plates were inoculated with fungal strains prepared in conc. equivalent with 0.5 MacFerland for bacterial strains and 2×10^5 and streaked onto the agar plates using sterile swabs, and then 50 μg of the dissolved compound in DMSO were placed into the wells under sterile conditions. All plates were incubated at 37°C/24 h for bacterial growth and at 28°C/48–72 h for fungal growth. Zone of inhibition were measured in mm using a ruler. The experiment was carried out in duplicate and the mean of the zone of inhibition was tabulated in (Table 1).

Conclusion

In conclusion, we have developed a simple and efficient method for the synthesis of substituted pyrazolopyrimidines. We also believe that the procedural simplicity, the efficiency and the easy accessibility of the reaction partners gives access to a wide array of heterocyclic frameworks containing a pyrazolopyrimidine unit. The anti-microbial activity of the tested compounds against bacterial and fungal strains isolated from food and milk of animal origin was evaluated. The molecular modeling of the most biologically active compounds among the newly synthesized compounds was carried out using Fieldalign software revealed that these compounds showed moderate field similarity compared to the reference drugs used in the anti-microbial testing.

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