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Research Article

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Synthesis and antitumor activity of novel pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one derivatives

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ABSTRACT

*Starting from pyrazolo[3,4-d]pyrimidine ethyl ester **4** and its corresponding acid hydrazide **5**, several new compounds were synthesized such as schiff bases **6a-e**, acetyl azide derivative **7**, phthalimido derivatives **8**, compounds containing oxadiazole ring **9**,**10a-c**, triazole ring system **11**, **14**,**15**, thiadiazole moiety **13** and phenyl thiosemicarbazide part **12**. Some of the synthesized compounds were screened for their antitumor activity against human breast adenocarcinoma cell line (MCF-7) using doxorubicin as a positive control. Compounds **6d**, **10b**, **12** were found to exhibit good cytotoxic activity with IC50 equal to (4.6, 4.6, 4.8 µg/mL), respectively.*

Key words: Pyrazolo[3,4-*d*]pyrimidine-4(5*H*)-ones, hydrazide, schiff bases, antitumor activity

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INTRODUCTION

Cancer still remains one of the most widespread and feared diseases in the world. Cancer cells are formed when the normal cells lose their normal regulatory mechanism which control their growth and multiplication. Gene mutation, including the activation of proto-oncogenes (e.g. Ras gene which is involved in the signaling pathway leading to cell division) [1] and inactivation of tumor suppressor genes (e.g. P53 which codes proteins that are involved in checking, repairing or suicide the damaged DNA) [2,3] is the most common cause leading to cancer.

Three approaches for the treatment of cancer are radiation, surgery and chemotherapy. Combination of chemotherapy alongside radiation and surgery is more effective and has less toxicity [1].

2,6,9-Trisubstituted purines (e.g. **CGP79883**) were reported to have potent cytotoxic activity [4,5]. Due to structural similarity between purines and pyrazolo[3,4-*d*]pyrimidines, great attention has been focused on pyrazolo[3,4-*d*]pyrimidines as antitumor agents [6,7].

Several mechanisms were reported for the cytotoxic activity of this class of compounds, including: adenosine receptor antagonists [8-10] and inhibitors of: CDKs [4], IGF-IR [11], Src [12], Lck [13], EGFR [14], mTOR [15] and $P^{38\alpha}$ [16,17].

Many pharmacophores were introduced to pyrazolo[3,4-*d*]pyrimidines to increase their cytotoxic activity. For example, introducing hydrazone moiety to N5 of pyrazolo[3,4-*d*]pyrimidine derivatives **1** increase their activity toward breast adenocarcinoma MCF-7 cell line [18].

Thiadiazoles [19,20], oxadiazoles [21,22], triazoles [21] and thiosemicarbazide [20] moieties are also examples for such pharmacophores with antitumor activities.

Based on the above findings, we thought to prepare new pyrazolo[3,4-*d*]pyrimidine derivatives substituted at C6 **2** with different groups of known reported cytotoxic activities. Most of the newly synthesized compounds were subjected to *in-vitro* antitumor evaluation against breast adenocarcinoma cell line MCF-7 using doxorubicine -the known anti breast cancer agent- [23] as a positive reference.

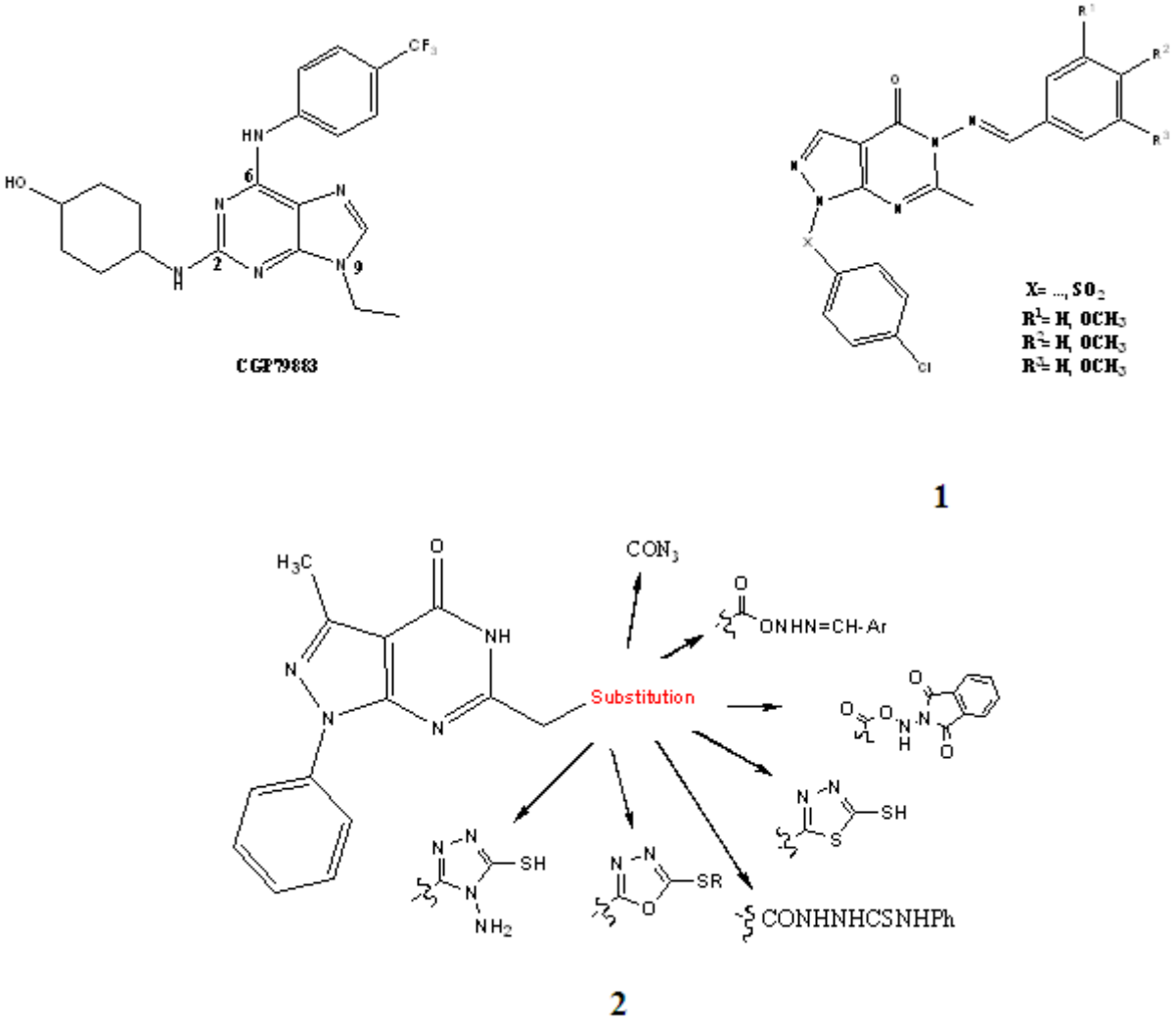


Fig. 1 Structure of CGP79883, compound 1 and general structure of test compounds 2.

EXPERIMENTAL SECTION

General

Melting points were determined on a Graffin apparatus and were uncorrected.

Element analyses (C, H, N) were carried out on Perkin-Elmer 2400 analyzer (Perkin-Elmer, Norwalk, CT, USA) at the Micro analytical unit of Cairo University, Egypt. All compounds were within ± 0.4% of the theoretical values. IR spectra were determined as KBr discs on Shimadzu IR 435 Spectrophotometer and values were represented in cm^{-1}. ^1H NMR spectra were carried out on a Bruker 300 MHz NMR Spectrophotometer in Cairo University, Egypt, using (Bruker, Munich, Germany) in DMSO-*d6* as a solvent, TMS as internal standard and chemical shifts were recorded in ppm on δ scale. Mass spectra were run on Hewlett Packard 5988 Spectrometer, Micro analytical center, Cairo University, Egypt. Progress of the reactions was monitored by TLC using TLC sheets precoated with UV fluorescent silica gel MERCK 60 F 254 that were visualized by UV lamp.

2.1.1. Procedure for the synthesis of Ethoxycarbonylmethyl-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one (**4**)
A mixture of the amino carboxamide **3** (2.16 g, 0.01 mol) and diethyl malonate (10 mL) was heated under reflux for 8 h. The separated solid which formed after cooling, was filtered, washed with ethanol 95%, dried and crystallized from ethanol 95%.

Yield: 54%; mp: 184-186^{0}C; IR (cm^{-1}): 3440 (NH), 3063 (CH arom.), 2976 (CH aliph.), 1744, 1680 (2C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 1.21 (t, 3H, *J*= 6.9Hz, $CH_2\underline{CH_3}$), 2.50 (s, 3H, CH_3), 3.80 (s, 2H, CH_2), 4.14 (q, 2H, *J*= 6.9Hz, $\underline{CH_2}CH_3$), 7.34, 7.35 (dd, 1H, J_1= 1.8Hz, J_2= 8.1Hz, Ar-H), 7.50, 7.52 (dd, 2H, J_1= 8.1Hz, J_2= 5.7Hz, Ar-H), 8.00 (d, 2H, *J*= 5.7Hz, Ar-H), 12.39 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{16}H_{16}N_4O_3$: C, 61.53; H, 5.16; N, 17.94. Found: C, 61.53; H, 5.39; N, 17.75; EIMS (*m/z*) (relative abundance %): 313 [M+1 $^{+\cdot}$, 21.38], 312 [M $^{+\cdot}$, 100], 240 [$C_{13}H_{12}N_4O^{+\cdot}$, 33.91], 238 [$C_{13}H_{10}N_4O^{+\cdot}$, 57.93] and 199 [$C_{11}H_9N_3O^{+\cdot}$, 31.83].

2.1.2. Procedure for the synthesis of 6-Hydrazinocarbonylmethyl-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(5**)
A mixture of **4** (3.12 g, 0.01 mol) and hydrazine hydrate 99% (0.055 g, 0.011 mol) in absolute ethanol (30 mL) was heated under reflux for 4 h. The solid that formed while hot was filtered, dried and crystallized from propanol.

Yield: 49%; mp: 282-284^{0}C; IR (cm^{-1}): 3289, 3146 (NH and NH_2), 3061 (CH arom.), 2992 (CH aliph.), 1708, 1667 (2C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.51 (s, 3H, CH_3), 3.53 (s, 2H, CH_2), 4.30 (s, 2H, NH_2 , D_2O exchangeable), 7.33, 7.35 (dd, 1H, J_1= 6.6Hz, J_2= 7.8Hz, Ar-H), 7.50, 7.53 (dd, 2H, J_1= 7.8Hz, J_2= 8.1Hz, Ar-H), 8.00 (d, 2H, *J*= 8.1Hz, Ar-H), 9.24 (s, 1H, CO$\underline{NH}$, D_2O exchangeable), 12.31 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{14}H_{14}N_6O_2$: C, 56.37; H, 4.73; N, 28.17. Found: C, 56.50; H, 4.85; N, 28.45; EIMS (*m/z*) (relative abundance %): 299 [M+1 $^{+\cdot}$, 12.77], 298 [M $^{+\cdot}$, 70.31], 267 [$C_{14}H_{11}N_4O_2^+$, 95.20], 240 [$C_{13}H_{12}N_4O^{+\cdot}$, 64.28], 77 [$C_6H_5^+$, 88.94], and 64 [$C_5H_4^+$, 100].

2.1.3. General procedure for the synthesis of compounds **6a-e**
To a suspension of compound **5** (2.98 g, 0.01 mol) in hot absolute ethanol (20 mL), the appropriate aromatic aldehyde (0.01 mol) and a catalytic amount of glacial acetic acid (0.5 mL) were added. The mixture was heated under reflux for 3-5 h (monitored by TLC), then filterd, washed with ethanol 95%, dried and crystallized from the appropriate solvent (given below).

2.1.3.1. *(Z* and *E*) 6-(N^1-benzylidenehydrazinocarbonylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(6a)**. (Crystallized from dioxane); Yield: 61%; mp: 281-283^{0}C; IR (cm^{-1}): 3430 (NH), 3072 (CH arom.), 2960 (CH aliph.), 1729, 1687 (2C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.52 (s, 3H, CH_3), 3.73, 4.08 (2s, 2H, CH_2, *Z* and *E* isomers), 7.31, 7.32 (dd, 1H, J_1= 1.5Hz, J_2= 7.8Hz, Ar-H), 7.44 (m, 5H, Ar-H), 7.65 (m, 2H, Ar-H), 7.99 (d, 2H, *J*= 2.4Hz, Ar-H), 8.02, 8.21 (2s, 1H, C=N$\underline{H}$, *Z* and *E* isomers), 11.69 (s, 1H, CON$\underline{H}$, D_2O exchangeable), 12.45 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-*d6*) δ ppm 13.25 (CH_3), 40.05, 41.08 (CH_2), 103.99 (C-3a), 120.65, 121.01 (C-3", C-5"), 126.33 (C-2', C-6'), 126.74, 127.02 (C-2", C-6"), 128.69, 128.98 (C-4"), 129.84, 130.06 (C-1"), 133.94 (C-4'), 138.31 (C-3', C-5'), 143.47 (C-1'), 145.73, 146.88 (C-6), 152.53 (C-3), 155.83, 156.51 (N=CH), 158.38, 163.02 (C-4=O), 168.90 (C=O); Anal.Calcd for $C_{21}H_{18}N_6O_2$: C, 65.27; H, 4.70; N, 21.75. Found: C, 65.49; H, 4.30; N, 22.01; EIMS (*m/z*) (relative abundance %): 387 [M+1 $^{+\cdot}$, 9.34], 386 [M $^{+\cdot}$, 13.34], 385 [M-H $^+$, 21.26], 384 [$C_{21}H_{16}N_6O_2^{+\cdot}$, 38.02], 328 [$C_{20}H_{16}N_4O^{+\cdot}$, 51.87], 327 [$C_{20}H_{15}N_4O^+$, 53.48], 240 [$C_{13}H_{12}N_4O^{+\cdot}$, 100], 198 [$C_{11}H_8N_3O^+$, 42.65] and 118 [$C_7H_6N_2^{+\cdot}$, 56.89].

2.1.3.2. *(Z* and *E*) 6-(N^1-3-hydroxybenzylidenehydrazinocarbonylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(6b)**. (Crystallized from dioxane); Yield: 52%; mp: 262-264^{0}C; IR (cm^{-1}): 3359, 3178 (OH and 2NH), 3074 (CH arom.), 2965 (CH aliph.), 1708, 1687 (2C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.52 (s, 3H, CH_3), 3.72, 4.07 (2s, 2H, CH_2, *Z* and *E* isomers), 6.80 (m, 2H, Ar-H), 7.06 (s, 1H, Ar-H), 7.26, 7.33 (dd, 1H, J_1= 3.9Hz, J_2= 6.9Hz, Ar-H), 7.34, 7.36 (dd, 1H, J_1= 7.8Hz, J_2= 3.6Hz, Ar-H), 7.46, 7.49 (dd, 2H, J_1= 7.8Hz, J_2= 7.8Hz, Ar-H), 7.91, 8.06 (2s, 1H, N=C$\underline{H}$, *Z* and *E* isomers), 8.02 (d, 2H, *J*= 7.2 Hz, Ar-H), 9.60, 9.63 (2s, 1H, OH, D_2O exchangeable, *Z* and *E* isomers), 11.63 (s, 1H, CON$\underline{H}$, D_2O exchangeable) and 12.42 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{21}H_{18}N_6O_3$: C, 62.68; H, 4.51; N, 20.88. Found: C, 62.39; H, 4.73; N, 21.19; EIMS (*m/z*) (relative abundance %): 403 [M+1 $^{+\cdot}$, 7.02], 402 [M $^{+\cdot}$, 19.68], 283 [$C_{14}H_{13}N_5O_2^{+\cdot}$, 24.32], 267 [$C_{14}H_{11}N_4O_2^+$, 83.38], 240 [$C_{13}H_{12}N_4O^{+\cdot}$, 100], 212 [$C_{11}H_8N_4O^{+\cdot}$, 29.52] and 200 [$C_{11}H_{10}N_3O^+$, 30.19].

2.1.3.3. *(Z* and *E*) 6-(N^1-4-chlorobenzylidenehydrazinocarbonylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(6c)**. (Crystallized from DMF/ethanol); Yield: 75%; mp: 346-348^{0}C; IR (cm^{-1}): 3431 (2NH),

3073 (CH arom.), 2967 (CH aliph.), 1708, 1667 (2C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.51 (s, 3H, CH_3), 3.73, 4.08 (2s, 2H, CH_2), 7.30, 7.32 (dd, 1H, J_1= 7.2Hz, J_2= 6.6Hz, Ar-H), 7.48 (m, 4H, Ar-H), 7.71 (m, 2H, Ar-H), 7.99, 8.21 (2s, 1H, N=CH, *Z* and *E* isomers), 8.02 (d, 2H, *J*= 1.5Hz), 11.70 (s, 1H, CONH, D_2O exchangeable) and 12.39 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{21}H_{17}ClN_6O_2$: C, 59.93; H, 4.07; N, 19.97. Found: C, 59.62; H, 4.36; N, 20.10; EIMS (*m/z*) (relative abundance %): 422 [M+2 $^{+\cdot}$, 6.16], 421 [M+1 $^{+\cdot}$, 5.86], 420 [M $^{+\cdot}$, 25.43], 283 [$C_{14}H_{13}N_5O_2$ $^{+\cdot}$, 34.78], 267 [$C_{14}H_{11}N_4O_2$ $^{+}$, 76.23], 240 [$C_{13}H_{12}N_4O$ $^{+\cdot}$, 100], 212 [$C_{11}H_8N_4O$ $^{+\cdot}$, 24.89] and 200 [$C_{11}H_{10}N_3O$ $^{+}$, 33.47].

2.1.3.4. *(Z* and *E*) 6-(*N*^{1}-4-dimethylaminobenzylidenehydrazinocarbonylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(6d)**. (Crystallized from dioxane); Yield: 69%; mp: 287-289^{0}C; IR (cm^{-1}): 3433 (2NH), 3060 (CH arom.), 2930 (CH aliph.), 1708, 1688 (2C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.50 (s, 3H, CH_3), 2.94 (s, 6H, -N(CH_3)$_2$), 3.68, 4.03 (2s, 2H, CH_2, *Z* and *E* isomers), 6.71 (m, 4H, Ar-H), 7.31, 7.33 (dd, 1H, J_1= 0.9Hz, J_2= 7.5Hz, Ar-H), 7.48 (m, 2H, Ar-H), 7.85 (s, 1H, N=CH), 8.02 (d, 2H, *J*= 7.8Hz, Ar-H), 11.38 (s,1H, CONH, D_2O exchangeable) and 12.40 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{23}H_{23}N_7O_2$: C, 64.32; H, 5.40; N, 22.83. Found: C, 64.12; H, 5.62; N, 23.06; EIMS (*m/z*) (relative abundance %): 430 [M+1 $^{+\cdot}$, 0.44], 429 [M $^{+\cdot}$, 1.16], 267 [$C_{14}H_{11}N_4O_2$ $^{+}$, 11.86], 240 [$C_{13}H_{12}N_4O$ $^{+\cdot}$, 19.69], 80 [$C_4H_4N_2$ $^{+\cdot}$, 46.34] and 64 [C_5H_4 $^{+}$, 100].

2.1.3.5. *(Z* and *E*) 6-(*N*^{1}-4-nitrobenzylidenehydrazinocarbonylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(6e)**. (Crystallized from DMF/ethanol); Yield: 72%; mp: 260-262^{0}C; IR (cm^{-1}): 3430 (2NH), 3077 (CH arom.), 2931 (CH aliph.), 1708, 1687 (2C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.50 (s, 3H, CH_3), 3.78, 4.14 (s, 2H, CH_2, *Z* and *E* isomers), 7.31, 7.32 (dd, 1H, J_1= 1.2Hz, J_2= 8.1Hz, Ar-H), 7.46, 7.48 (dd, 2H, J_1= 8.1Hz, J_2= 7.5Hz, Ar-H), 7.72 (d, 2H, *J*= 6Hz, Ar-H), 8.02 (m, 4H, Ar-H), 8.27, 8.80 (2s, 1H, N=CH, *Z* and *E* isomers), 11.94 (s, 1H, CONH, D_2O exchangeable) and 12.44 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{21}H_{17}N_7O_4$: C, 58.47; H, 3.97; N, 22.73. Found: C, 58.60; H, 4.09; N, 22.41; EIMS (*m/z*) (relative abundance %): 431 [M $^{+\cdot}$, 37.49], 430 [M-H $^{+}$, 50.10], 369 [$C_{20}H_{13}N_6O_2$ $^{+}$, 54.61], 280 [$C_{13}H_8N_6O_2$ $^{+\cdot}$, 96.95] and 279 [$C_{13}H_7N_6O_2$ $^{+}$, 100].

2.1.4. procedure for the synthesis of (3-Methyl-4-oxo-1-phenyl-4,5-dihydro-1*H*-pyrazolo[3,4-*d*]pyrimidin-6-yl)-acetyl azide **(7**)

A solution of sodium nitrite (0.7 g, 0.002 mol) in water (7 mL) was added with stirring to a solution of the acid hydrazide **5** (0.59 g, 0.002 mol) in acetic acid (10 mL) during 5 minutes at room temperature. The solid product was filtered, washed with cold water, dried and crystallized from ethanol 95%.

Yield: 80%; mp: 357-359^{0}C; IR (cm^{-1}): 3433 (NH), 3040 (CH arom.), 2951 (CH aliph.), 2358 (N_3), 1798, 1683 (2C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.49 (s, 3H, CH_3), 3.26 (s, 2H, CH_2), 7.45 (m, 3H, Ar-H), 8.18 (d, 2H, *J*= 8.1Hz, Ar-H) and 13.16 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{14}H_{11}N_7O_2$: C, 54.37; H, 3.58; N, 31.70. Found: C, 58.60; H, 4.09; N, 22.41; EIMS (*m/z*) (relative abundance %): 310 [M+1 $^{+\cdot}$, 22.14], 309 [M $^{+\cdot}$, 4.60], 252 [$C_{13}H_{10}N_5O$ $^{+}$, 23.93], 198 [$C_{11}H_{10}N_4$ $^{+\cdot}$, 22.71] and 77 [C_6H_5 $^{+}$, 100].

2.1.5. Procedure for the synthesis of *6-(N*-phethalamidocarbamoylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(8)**

To a solution of the acid hydrazide **5** (1.49 g, 0.005 mol) in glacial acetic acid (10 mL), phthalic anhydride (0.74 g, 0.005 mol) was added and the mixture was heated under reflux for 4 h. After cooling, the reaction mixture was poured into crushed ice (≈30 g). The separated solid was filtered, washed with water, dried and crystallized from acetic acid.

Yield: 67%; mp: 311-313°C; IR (cm^{-1}): 3431, 3256 (2NH), 3001 (CH arom.), 2932 (CH aliph.), 1771-1667 (4C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.52 (s, 3H, CH_3), 3.87 (s, 2H, CH_2), 7.33, 7.35 (dd, 1H, J_1= 1.8Hz, J_2= 7.5Hz, Ar-H), 7.53, 7.56 (dd, 2H, J_1= 7.8Hz, J_2= 7.5Hz, Ar-H), 7.96 (m, 4H, Ar-H), 8.11 (d, 2H, *J*= 7.8Hz, Ar-H), 11.06 (s, 1H, CONH, D_2O exchangeable) and 12.51 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{22}H_{16}N_6O_4$: C, 61.68; H, 3.76; N, 19.62. Found: C, 61.39; H, 3.90; N, 19.55; EIMS (*m/z*) (relative abundance %): 429 [M+1 $^{+\cdot}$, 19.94], 428 [M $^{+\cdot}$, 53.65], 267 [$C_{14}H_{11}N_4O_2$ $^{+}$, 85.97], 266 [$C_{14}H_{10}N_4O_2$ $^{+\cdot}$, 80.80], 240 [$C_{13}H_{12}N_4O$ $^{+\cdot}$, 100], and 162 [$C_7H_6N_4O$ $^{+\cdot}$, 57.24].

2.1.6. procedure for the synthesis of 6-[(5-mercapto-3*H*-[1,3,4]-oxadiazol-5-yl)methyl]-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(9**)
To a suspension of **5** (2.98 g, 0.01 mol) in absolute ethanol (20 mL), potassium hydroxide (0.84 g, 0.015 mol) was added, stirred for a while then carbon disulphide (20 mL) was added. The reaction mixture was heated under reflux for 8 h, poured into cold water (≈ 30 ml) and acidified with conc. HCl till neutral to litmus paper. The formed solid was filtered, washed with water, dried and crystallized from ethanol 95%.

Yield: 71%; mp: 268-270°C; IR (cm^{-1}): 3430 (2NH), 3067 (CH arom.), 2875 (CH aliph.), 2751 (SH), 1685 (C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.50 (s, 3H, CH_3), 4.33 (s, 2H, CH_2), 7.34, 7.35 (dd, 1H, J_1= 7.8Hz, J_2= 1.2Hz, Ar-H), 7.47, 7.50 (dd, 2H, J_1= 8.1Hz, J_2= 7.5Hz, Ar-H), 7.98 (d, 2H, *J*= 7.8Hz, Ar-H), 12.56 (s, 1H, NH, D_2O exchangeable) and 14.50 (s, 1H, SH, D_2O exchangeable); Anal.Calcd for $C_{15}H_{12}N_6O_2S$: C, 52.93; H, 3.55; N, 24.69. Found: C, 52.63; H, 3.80; N, 24.53; EIMS (*m/z*) (relative abundance %): 341 [$M+1^{\urcorner +\cdot}$, 8.58], 340 [$M^{\urcorner +\cdot}$, 8.92], 240 [$C_{13}H_{12}N_4O^{\urcorner +\cdot}$, 15.96], 150 [$C_6H_6N_4O^{\urcorner +\cdot}$, 100].

2.1.7 General procedure for the synthesis of compounds **10a-c**
A solution of **9** (3.40 g, 0.01 mol) and the appropriate halo derivative (0.01 mol) in ethanolic potassium hydroxide (0.08 g KOH in 20 mL absolute ethanol) was heated under reflux for 3 h. On cooling, the reaction mixture was poured into ice-cold water (≈30 mL). The separated solid was filtered, dried and crystallized from acetone/chloroform mixture (7:3)**.**

2.1.7.1 6-[(3-Methyl sulfanyl-[1,3,4]-oxadiazol-2-yl)methyl]-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(10a**)
Yield: 41%; mp: 251-253°C; IR (cm^{-1}): 3429 (NH), 3068 (CH arom.), 2930 (CH aliph.), 1683 (C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.52 (s, 3H, CH_3), 2.72 (s, 3H, SCH_3), 4.44 (s, 2H, CH_2), 7.45 (m, 3H, Ar-H), 7.93 (d, 2H, *J*= 8.4Hz, Ar-H) and 12.56 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{16}H_{14}N_6O_2S$: C, 54.23; H, 3.98; N, 23.71. Found: C, 54.30; H, 3.94; N, 23.93; EIMS (*m/z*) (relative abundance %): 355 [$M+1^{\urcorner +\cdot}$, 21.29], 354 [$M^{\urcorner +\cdot}$, 100], 239 [$C_{13}H_{11}N_4O^{\urcorner +}$, 37.79], 198 [$C_{11}H_{10}N_4^{\urcorner +\cdot}$, 17.20] and 77 [$C_6H_5^{\urcorner +}$, 63.16].

2.1.7.2 6-[(3-Ethyl sulfanyl-[1,3,4]-oxadiazol-2-yl)methyl]-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(10b)**
Yield: 48%; mp: 164-166°C; IR (cm^{-1}): 3428 (NH), 3073 (CH arom.), 2929 (CH aliph.), 1685 (C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 1.36 (t, 3H, *J*= 7.2Hz, $CH_2\underline{CH_3}$), 2.51 (s, 3H, CH_3), 3.27 (q, 2H, *J*= 7.2Hz, $\underline{CH_2}CH_3$), 4.45 (s, 2H, CH_2), 7.30, 7.31 (dd, 1H, J_1= 1.8Hz, J_2= 7.2Hz, Ar-H), 7.45, 7.47 (dd, 2H, J_1= 8.4Hz, J_2= 7.2Hz, Ar-H), 7.92 (d, 2H, *J*= 8.1Hz, Ar-H) and 12.61 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{17}H_{16}N_6O_2S$: C, 55.42; H, 4.38; N, 22.81. Found: C, 55.37; H, 4.46; N, 22.82; EIMS (*m/z*) (relative abundance %): 369 [$M+1^{\urcorner +\cdot}$, 11.64], 368 [$M^{\urcorner +\cdot}$, 26.22], 271 [$C_{13}H_{11}N_4OS^{\urcorner +}$, 29.16], 257 [$C_{12}H_9N_4OS^{\urcorner +}$, 35.98], 243 [$C_{11}H_7N_4OS^{\urcorner +}$, 46.21], 236 [$C_{13}H_8N_4O^{\urcorner +\cdot}$, 50.32] and 111 [$C_4H_3N_2S^{\urcorner +}$, 100].

2.1.7.3 6-[(3-Ethoxycarbonylmethyl sulfanyl-[1,3,4]-oxadiazol-2-yl)methyl]-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(10c)**
Yield: 59%; mp: 247-249°C; IR (cm^{-1}): 3414 (NH), 3074 (CH arom.), 2976 (CH aliph.), 1677 (C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.51 (s, 3H, CH_3), 4.43 (s, 2H, CH_2), 4.51 (s, 2H, SCH_2), 7.35 (m, 8H, Ar-H), 7.88 (d, 2H, *J*= 8.4Hz, Ar-H) and 12.61 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{22}H_{18}N_6O_2S$: C, 61.38; H, 4.21; N, 19.52. Found: C, 61.09; H, 4.17; N, 19.27; EIMS (*m/z*) (relative abundance %): 431 [$M+1^{\urcorner +\cdot}$, 0.76], 430 [$M^{\urcorner +\cdot}$, 0.82], 362 [$C_{20}H_{18}N_4OS^{\urcorner +\cdot}$, 2.08], 312 [$C_{19}H_{12}N_4O^{\urcorner +\cdot}$, 19.52], 278 [$C_{14}H_{10}N_6O^{\urcorner +\cdot}$, 19.70], 181 [$C_{11}H_7N_3^{\urcorner +\cdot}$, 32.19], 165 [$C_{10}H_3N_3^{\urcorner +\cdot}$, 25.99], and 121 [$C_6H_7N_3^{\urcorner +\cdot}$, 100].

2.1.8 Procedure for the synthesis of 6-(4-amino-5-mercapto-4*H*-[1,2,4]triazol-3-ylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(11)**
A mixture of compound **9** (3.40 g, 0.01 mol) and hydrazine hydrate 99% (0.50 g, 0.01 mol) in absolute ethanol (30 mL) was heated under reflux for 8 h. The solid that formed while hot was filtered, dried and crystallized from ethanol 95%.

Yield: 48%; mp: 313-315°C; IR (cm^{-1}): 3425-3157 (2NH and NH_2), 3074 (CH arom.), 2950 (CH aliph.), 2646 (SH), 1680 (C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.49 (s, 3H, CH_3), 4.16 (s, 2H, CH_2), 5.51 (s, 2H, NH_2, D_2O exchangeable), 7.29, 7.31 (dd, 1H, J_1= 1.2Hz, J_2= 7.2Hz, Ar-H), 7.43, 7.45 (dd, 2H, J_1= 8.4Hz, J_2= 7.2Hz, Ar-H), 7.88 (d, 2H, *J*= 8.7Hz, Ar-H), 12.54 (s, 1H, NH, D_2O exchangeable) and 13.63 (s, 1H, SH, D_2O exchangeable);

Anal.Calcd for $C_{15}H_{14}N_8OS$: C, 50.84; H, 3.98; N, 31.62. Found: C, 50.63; H, 4.32; N, 31.48; EIMS (*m/z*) (relative abundance %): 354 [$M^{+\cdot}$, 31.18], 240 [$C_{13}H_{12}N_4O^{+\cdot}$, 17.95], 221 [$C_7H_7N_7S^{+\cdot}$, 19.77], 184 [$C_{10}H_6N_3O^{+}$,21.59], 161 [$C_7H_7N_5^{+}$, 20.91], 80 [$C_4H_4N_2^{+\cdot}$, 36.36] and 64 [$C_5H_4^{+}$, 100].

2.1.9 Procedure for the synthesis of 6-[(*N*-phenylthiosemicarbazido)carbonylmethyl]-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(12)**
To a solution of the acid hydrazide **5** (2.98 g, 0.01 mol) in absolute ethanol (30 mL), the phenylisothiocyanate (0.01 mol) was added. The reaction mixture was heated under reflux for 6 h, the solid that formed while hot was filtered, dried and crystallized from dioxane.

Yield: 82%; mp: 265-267°C; IR (cm^{-1}): 3437-3333 (4NH), 3125 (CH arom.), 2956 (CH aliph.), 1708, 1685 (2C=O), 1260 (C=S); ^{1}H NMR (DMSO-*d6*) δ ppm 2.52 (s, 3H, CH_3), 3.73 (s, 2H, CH_2), 7.18 (m, 1H, Ar-H), 7.34 (m, 5H, Ar-H), 7.49, 7.52 (dd, 2H, J_1= 7.5Hz, J_2= 7.8Hz, Ar-H), 8.02 (d, 2H, *J*= 8.4Hz, Ar-H), 9.60 (s, 1H, NH, D_2O exchangeable), 9.77 (s, 1H, NH, D_2O exchangeable), 9.81(s, 1H, NH, D_2O exchangeable), 10.33(s, 1H, NH, D_2O exchangeable) and 12.36 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{21}H_{19}N_7O_2S$: C, 58.19; H, 4.52; N, 22.62. Found: C, 58.34; H, 4.52; N, 22.24; EIMS (*m/z*) (relative abundance %): 435 [M+2$^{+\cdot}$, 7.05], 434 [M+1$^{+\cdot}$, 5.90], 433 [$M^{+\cdot}$, 7.22], 368 [$C_{20}H_{14}N_7O^{+}$, 40.08] 147 [$C_7H_5N_3O^{+\cdot}$, 24.38], 135 [$C_6H_5N_3O^{+\cdot}$, 92.13] and 80 [$C_4H_4N_2^{+\cdot}$, 100].

2.1.10 Procedure for the synthesis of 6-[2-(phenylamino-1,3,4-thiadiazol-5-yl)methyl]-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(13)**
A solution of the phenylthiosemicarbazide **12** (4.33g, 0.01 mol) in conc. sulphuric acid (10 mL) was kept at room temperature for 4 h, while stirring. Thereafter, it was poured into crushed ice, and neutralized with 10% NaOH. The separated solid was filtered, dried and crystallized from ethanol 95%

Yield: 78%; mp: 265-267°C; IR (cm^{-1}): 3431 (2NH), 3083 (CH arom.), 2937 (CH aliph.), 1698 (C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.52 (s, 3H, CH_3), 4.45 (s, 2H, CH_2), 7.32, 7.35 (dd, 1H, J_1= 7.5Hz, J_2= 6.3Hz, Ar-H), 7.55 (m, 7H, Ar-H), 8.02 (d, 2H, *J*= 8.4Hz, Ar-H), 10.48 (s, 1H, NH, D_2O exchangeable) and 12.61 (s, 1H, NH, D_2O exchangeable); Anal.Calcd for $C_{21}H_{17}N_7OS$: C, 60.71; H, 4.12; N, 23.60. Found: C, 60.52; H, 4.42; N, 23.49; EIMS (*m/z*) (relative abundance %): 416 [M+1$^{+\cdot}$, 8.44], 415 [$M^{+\cdot}$, 19.82], 265 [$C_{14}H_{11}N_5O^{+\cdot}$, 25.02], 135 [$C_6H_7N_4^{+}$, 80.57] and 64 [$C_5H_4^{+}$, 100].

2.1.11 Procedure for the synthesis of 6-(5-mercapto-4-phenyl-4-*H*-[1,2,4]-triazol-3-ylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(14)**
A solution of the Phenylthiosemicarbazide **12** (4.33g, 0.01 mol) in 2*N* NaOH (10 mL) was heated under reflux for 8 h. After cooling, the solution was filtered, and the filterate was acidified with dil. HCl. The separated solid was filtered, dried and crystallized from dioxane.

Yield: 75%; mp: 311-313°C; IR (cm^{-1}): 3431 (NH), 3041 (CH arom.), 2924 (CH aliph.), 2750 (SH), 1679 (C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 2.51 (s, 3H, CH_3), 4.01 (s, 2H, CH_2), 7.36 (m, 3H, Ar-H), 7.49 (m, 5H, Ar-H), 7.95 (d, 2H, *J*= 7.5Hz), 12.33 (s, 1H, NH, D_2O exchangeable) and 13.91 (s, 1H, SH, D_2O exchangeable); Anal.Calcd for $C_{21}H_{17}N_7OS$: C, 60.71; H, 4.12; N, 23.60. Found: C, 60.61; H, 4.32; N, 23.50; EIMS (*m/z*) (relative abundance %): 415 [$M^{+\cdot}$, 0.04], 412 [$C_{21}H_{14}N_7OS^{+\cdot}$, 0.45], 80 [$C_4H_4N_2^{+\cdot}$, 40.51] and 64 [$C_5H_4^{+}$, 100].

2.1.12 Procedure for the synthesis of 6-[4-(ethyl-5-mercapto-4*H*-[1,2,4]-triazol-3-ylmethyl)-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one **(15)**

Method A:
A solution of the acid hydrazide **5** (2.98 g, 0.01 mol) and ethyl isothiocyanate (0.87 g, 0.01 mol) in absolute ethanol (30 mL), was heated under reflux for 1.5 h. The separated solid that formed on hot, was filtered, dried and crystallized from chloroform /acetone mixture (8:2) to yield 2.64 g, (72%) of compound **15**.

Method B:
To a solution of the acid hydrazide **5** (2.98 g, 0.01 mol) in dioxane (30 mL), the ethylisothiocyanate (0.87 g, 0.01 mol) was added. The reaction mixture was stirred at room temperature for 7 h; the formed solid was filtered, dried and crystallized from chloroform /acetone mixture (8:2) to yield 2.75 g, (75%) of compound **(15**)

mp: 312-314°C; IR (cm^{-1}): 3437 (2NH), 3092 (CH arom.), 2928 (CH aliph.), 2358 (SH), 1686 (C=O); ^{1}H NMR (DMSO-*d6*) δ ppm 1.19 (t, 3H, *J*= 8.7Hz, $CH_2\underline{CH_3}$), 2.51 (s, 3H, CH_3), 3.99 (q, 2H, *J*= 8.7Hz, $\underline{CH_2}CH_3$), 4.25 (s, 2H, CH_2), 7.30, 7.31 (dd, 1H, J_1= 1.8Hz, J_2= 7.5Hz, Ar-H), 7.46, 7.48 (dd, 2H, J_1= 7.5Hz, J_2= 7.2Hz, Ar-H), 7.91 (d, 2H, *J*= 7.2Hz, Ar-H), 12.58 (s, 1H, NH, D_2O exchangeable) and 13.62 (s, 1H, SH, D_2O exchangeable); Anal.Calcd for $C_{17}H_{17}N_7OS$: C, 55.57; H, 4.66; N, 26.68. Found: C, 55.77; H, 4.74; N, 26.71; EIMS (*m/z*) (relative abundance %): 369 [M+2 $^{+\cdot}$, 31.51], 368 [M+1 $^{+\cdot}$, 44.12], 367 [M $^{+\cdot}$, 47.06], 325 [$C_{15}H_{13}N_6OS^+$, 66.39], 171 [$C_{10}H_9N_3^{+\cdot}$, 73.95], 75 [$C_6H_3^+$, 97.48] and 59 [$CHNS^{+\cdot}$, 100].

3. Biological testing

3.1Materials and Methods

The human breast tumor cell line (MCF-7) was obtained from NCI, Cairo, Egypt.

3.2. Measurement of potential cytotoxicity

The effects of compounds on the growth of tumor cell lines (MCF-7), were evaluated according to the procedure adopted by the National Cancer Institute, Cairo, Egypt for the *in- vitro* anticancer drug screening that use the protein-binding dye sulforhodamine B (SRB) to assess growth inhibition [28]. Cell were routinely maintained as adherent cell cultures in RPMI- 1640 medium supplemented with 10% heat-inactived fetal bovine serum (FBS) and 1% penicillin/ streptomycin at 37^{o}C in humidified atmosphere containing 5% CO_2.

The cell line was regularly subcultured to be maintained in the expotential growth phase. Cells were exposed for 48 h to five concentrations of compounds (0, 5, 12.5, 25and 50 ug/ml). Compounds were prepared in dimethylsulphoxide (DMSO), were freshly diluted with cell culture medium just prior the assays. Doxorubicin was used as positive control. For each test compound and the cell line a dose-response curve was generated and the growth inhibition of 50% (IC50), corresponding to the concentration of compound that inhibits 50% of the net cell growth was determined. The results of *in-vitro* cytotoxic activity experiments are presented in (Table 1).

Table 1. IC_{50} of some of the newly synthesized compounds against doxorubicine as positive control.

Compound no.	IC_{50} (μg/mL)[a]
5	13
6b	16
6d	4.6
8	13.4
9	17.6
10b	4.6
12	4.8
13	13.6
14	19.6
15	11.2
Doxorubicin	2.97

[a] *The values given are means of three experiments.*

Results of *in-vitro* cytotoxic activity of some of the newly synthesized compounds on human breast adenocarcinoma cell line (MCF-7).

RESULTS AND DISCUSSION

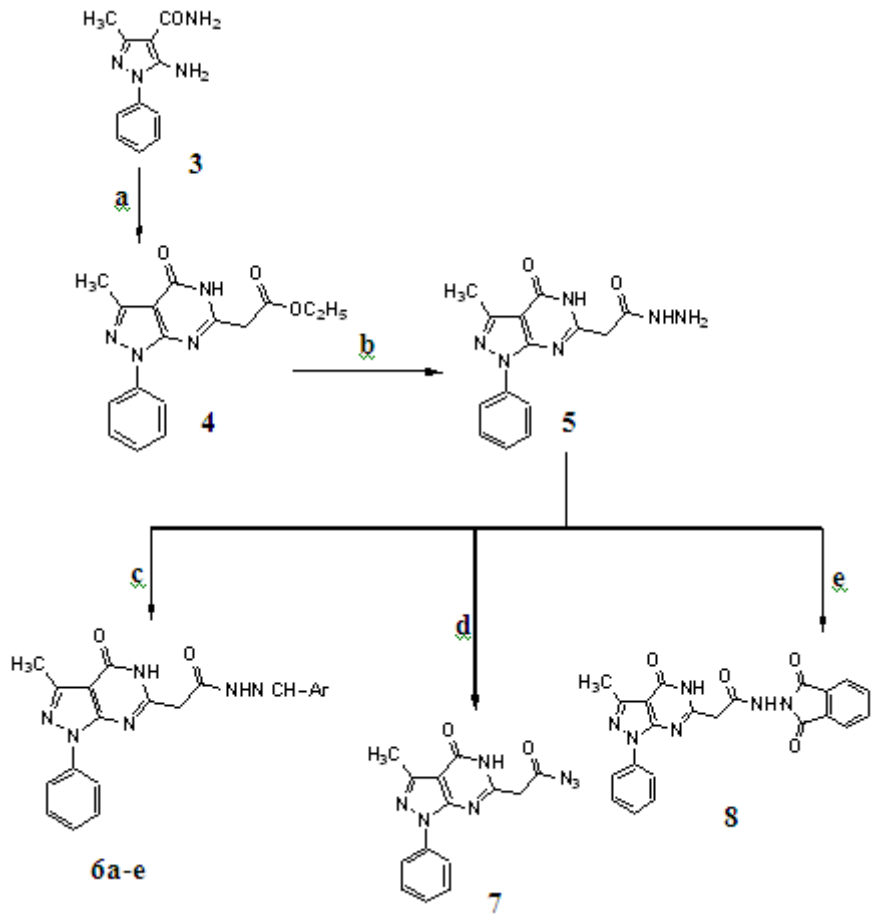
3.1. Chemistry

Synthesis of the target compounds was achieved by reacting 5-amino-3-methyl-1-phenyl-4-carboxamide (**3**) [24] with diethyl malonate to give 6-ethoxycarbonylmethyl derivative **4**. Hydrazinolysis of **4** with excess hydrazine hydrate in refluxing ethanol afforded the corresponding acid hydrazide **5** which was the key compound for synthesizing different new compounds.

Reaction of **5** with different aromatic aldehydes, namely, benzaldehyde, 3-hydroxybenzaldehyde, 4-chlorobenzaldehyde, 4-dimethylaminobenzaldehyde, 4-nitrobenzaldehyde in ethanol containing catalytic amount of glacial acetic acid gave compounds **6a-e** in their two geometrical isomers *Z* and *E*. As arylidene-hydrazide structure was reported to exist in two geometrical isomers (*E* and *Z*) about –C=N double bond [25, 26] and according to the literature [27], compounds containing imine bond are present in higher percentage in dimethyl-d_6 sulfoxide solution in the form of geometrical *E* isomer about –C=N double bond.

On the other hand, the acid hydrazide **5** was subjected to diazotization to give the corresponding azido derivative **7**.

Moreover, reaction of **5** with phthalic anhydride in glacial acetic acid afforded the phthalimido derivative **8** (Scheme 1).

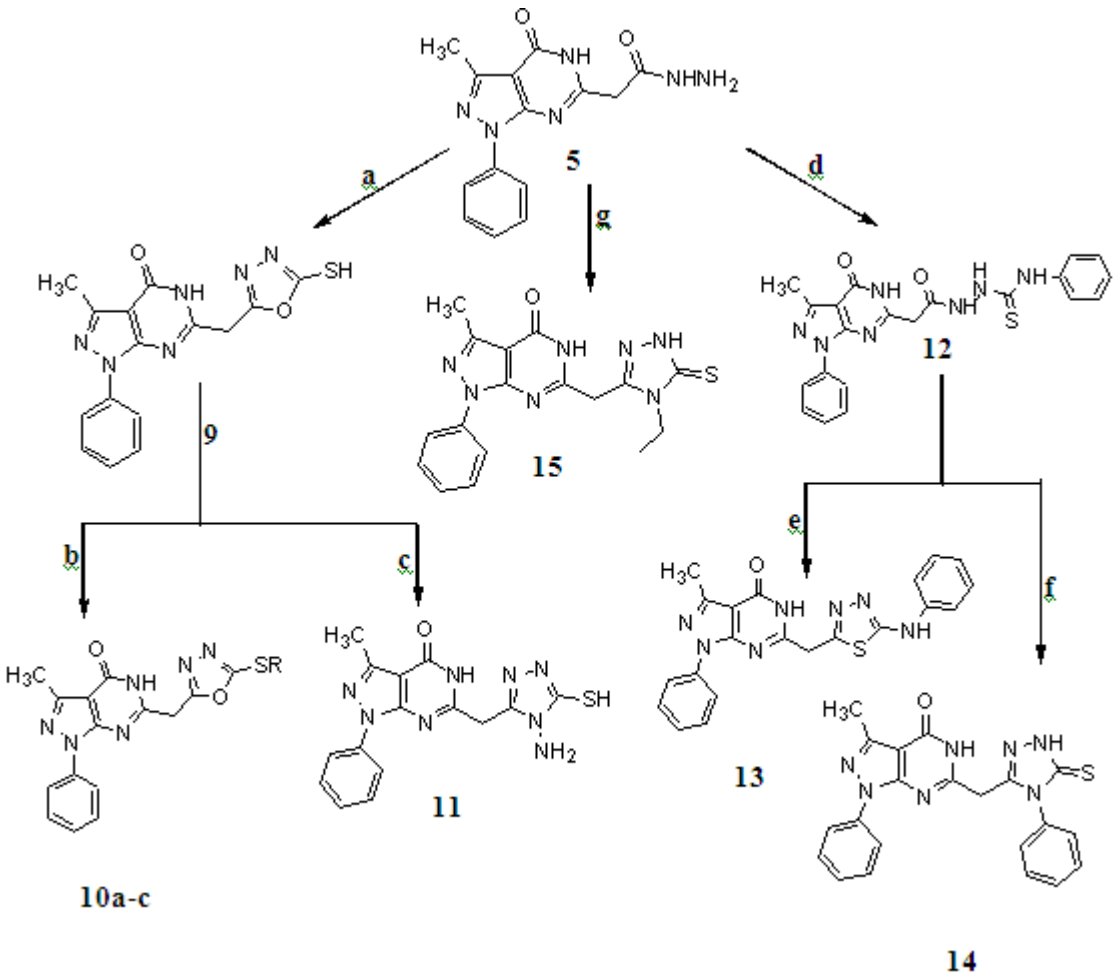


Ar = a, C_6H_5; b, 3-OH-C_6H_4; c, 4-Cl-C_6H_4; d, 4-$N(CH_3)_2$-C_6H_4; e, 4-NO_2-C_6H_4

Scheme 1. Reagents: a) diethyl malonate; b) N_2H_4, ethanol; c) ArCHO, gl. acetic acid, ethanol; d) $NaNO_2$ 20%, gl. acetic acid; e) phthalic anhydride, gl. acetic acid.

Oxadiazole derivative **9** was obtained from the reaction of acid hydrazide **5** and CS_2 in ethanolic potassium hydroxide. Compound **9** was used as a precursor for the formation of S-alkylated compounds **10a-c** using alkyl or aryl halides such as methyl iodide, ethyl iodide and benzyl chloride. In addition, reaction of **9** with hydrazine hydrate in ethanol yielded triazole ring with N4 amino group **11**.

Reacting the acid hydrazide **5** with ethyl or phenyl isothiocyanate afforded two different products. Phenyl thiosemicarbazide **12** was obtained and cyclized under acidic conditions using conc. sulfuric acid or in sodium hydroxide as a base, giving compounds **13** and **14**, respectively. While, ethyl thiosemicarbazide not formed from the reaction of **5** and ethylisothiocyanate either in refluxing ethanol or in less polar solvent such as dioxane and at room temperature, instead the cyclized triazole containing compound **15** was obtained. This occurred due to the –I effect of ethyl group of ethyl isothiocyanate which facilitate the cyclization process (Scheme 2).



R= a, CH_3; b, C_2H_5; c, CH_2-C_6H_5

Scheme 2. Reagents: a) CS_2, KOH, ethanol; b) RX, K_2CO_3, ethanol**;** c) N_2H_4, ethanol; d) PhNCS, ethanol; e) Conc. H_2SO_4; f) 2N NaOH; g) EtNCS, ethanol, reflux or EtNCS, dioxane, r.t.

Some of the newly synthesized pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-ones were tested *in-vitro* on human breast adenocarcinoma cell line (MCF-7). Three compounds **6d**, **10b** and **12** exploited good antitumor activity. These results suggested that 4-substituted benzylidene derivative with its hydrophobic character, substituted oxadiazole and phenyl thiosemicarbazide at C6 of pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-one resulted in good cytotoxic activity.

Compounds **6d**, **10b** and **12** showed significant antitumor activity against MCF-7 with IC50 equql to 4.6, 4.6 and 4.8 µg/mL. According to the data obtained, we found that 4-dimethylbenzylidene derivative **6d** was better than 3-hydroxybenzylidene derivative **6b** this was thought to be due to hydrophobicity in **6d** rather than **6b**. Moreover, S-ethyl substituted oxadiazole **10b** showed higher activity than the unsubstituted oxadiazole **9**. In addition, phenylthiosemicarbazide **12** showed better antitumor activity than the two cyclized forms **13**, **14**.

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