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Synthesis and antitumor activity of novel pyrazolo[1,5-*a*]pyrimidine derivatives

Mervat Mostafa El-Enany[a], Mona Monir Kamel[b], Omneya Mahmoud Khalil[b] and Hala Bakr El-Nassan[b,*]

[a] *Pharmacology Chemistry Department, Faculty of Pharmaceutical Science and Pharmaceutical Industries, Future University, Cairo, 11562, Egypt***
[b] *Organic Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, 11562, Egypt*

**Corresponding author at: Organic Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, 11562, Egypt. Tel.: +00202.23632245; fax: +00202.23635140. E-mail address: hala_bakr@hotmail.com (H.B. El-Nassan).*
*** Sabbatical leave from Cairo University.*

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ABSTRACT

A novel series of pyrazolo[1,5-*a*]pyrimidine-3-carbonitriles substituted with 7-amino, 7-substituted amino and 5-substituted amino groups was synthesized. Some of the newly synthesized compounds were tested *in vitro* on human colon tumor cell line (HCT116). Compound **14a** displayed the highest activity among the tested compounds with IC_{50} that equals to 0.0020 µM.

1. Introduction

Cancer is defined as malignant growth of cells. Most tumors arise from a combination of genetic mutations in the cell. These genetic changes lead to activation of oncogenes and suppression or deletion of tumor suppressor genes. As a result, there is unregulated cell proliferation and also a delay in programmed cell death, apoptosis [1]. Most of the clinically used antineoplastic drugs aim to suppress the proliferative process (e.g.: DNA replication or chromosome segregation) [1].

Pyrazolo[1,5-*a*]pyrimidines are of considerable chemical and pharmacological importance as purine analogs and many derivatives of pyrazolo[1,5-*a*]pyrimidines have been reported to exhibit cytotoxic activity [2-10]. Different mechanisms account for the cytotoxic effect of this class of compounds, where they have been reported to act as vascular endothelial growth factor receptor inhibitor [2] and cyclin dependent kinase inhibitors [3,6-8].

Several 7-substituted aminopyrazolo[1,5-*a*]pyrimidine derivatives were reported to have antiproliferative activity against HCT116 and other cell lines (e.g. compounds **1**-**3**) [3,6,8] (Figure 1).

In the present study, several pyrazolo[1,5-*a*]pyrimidine derivatives, bearing 2-methylsulphanyl group, 3-nitrile group and 7-amino (**6a**, **6b**, **7a**-**d**, **8** and **9**) or 7-substituted amino group (**14a**-**c**) were prepared. Besides, different substitutions were introduced at position 5 (aromatic ring, amino group or carbonyl group) (Scheme 1 and 2).

Meanwhile, the study aimed to synthesize 7-substituted amino-5-methylpyrazolo[1,5-*a*]pyrimidine derivatives. Nevertheless, the product obtained using two different reaction conditions was the 7-methyl derivative.

2. Experimental

2.1. Instrumentation

Melting points were determined using a Griffin apparatus and were uncorrected. IR spectra were recorded on Mattson Genesis II FT-IR and values were represented in cm^{-1}. ^1H NMR were carried out on Varian Gemini 200 MHz spectrophotometer, Microanalytical center, Cairo University, Cairo, Egypt, using TMS as an internal standard and chemical shifts were recorded in ppm on δ scale and coupling constants (*J*) are given in Hz. The electron impact (EI) mass spectra were recorded on Shimadzu QP-2010 plus, Microanalytical center, Cairo University, Cairo, Egypt. Analytical thin layer chromatography (TLC) on silica gel plates containing UV indicator was employed routinely to follow the course of reactions and to check the purity of products. All reagents and solvents were purified and dried by standard techniques. Elemental microanalyses were performed at Microanalytical Center, Cairo University, Cairo, Egypt, and were within ±0.4%.

2.2. Synthesis

2.2.1. General procedure for the synthesis of 7-amino-2-methyl sulphanyl-5-(substituted phenyl)-4,5-dihydropyrazolo [1,5-a] pyrimidine-3,6-dicarbonitriles 6a,b and 7-amino-2-methyl sulphanyl-5-(substituted phenyl)pyrazolo[1,5-a]pyrimidine-3,6-dicarbonitriles 7a-d

A mixture of 5-amino-3-methylsulphanyl-1*H*-pyrazole-4-carbonitrile (**4**) [11] (0.31 g, 0.002 mol), the appropriate substituted benzylidenemalononitrile **5a-f** [12-15] (0.002 mol) and triethylamine (2 mL) in absolute ethanol (20 mL) was heated under reflux for 7 h. The precipitate formed was filtered, dried and crystallized from acetic acid.

7-Amino-5-(2-chlorophenyl)-2-methylsulphanyl-4,5-dihydro pyrazolo[1,5-a]pyrimidine-3,6-dicarbonitrile (**6a**): Yield: 33%. M.p.: 246-247 °C. FT-IR (cm^{-1}): 3453, 3307, 3236 (NH/NH_2), 2228, 2192 (CN). ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 2.6 (s, 3H, SCH_3), 5.7 (d,

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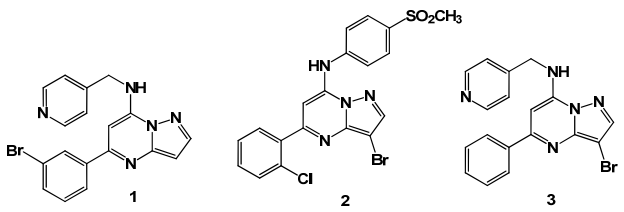
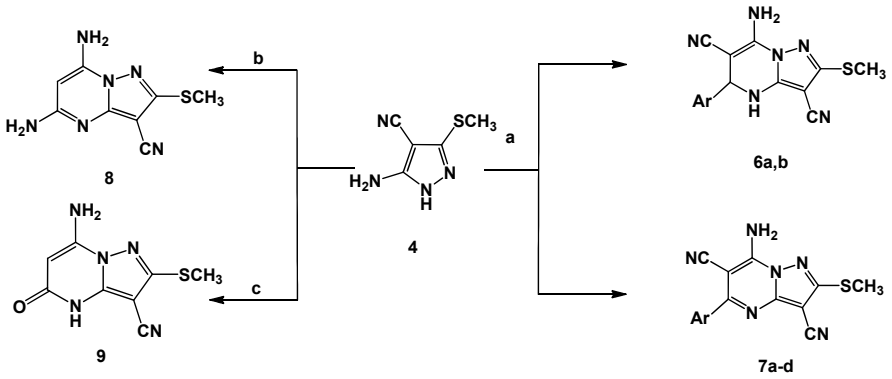


Figure 1. Examples of 7-substituted aminopyrazolo[1,5-a]pyrimidines with antiproliferative activity.



Compound	Ar	Compound	Ar
5a, 6a	2-ClC_6H_4	5d, 7b	3-$NO_2C_6H_4$
5b, 6b	2-$CH_3OC_6H_4$	5e, 7c	4-ClC_6H_4
5c, 7a	2-OHC_6H_4	5f, 7d	4-$(CH_3)_2NC_6H_4$

Reagents: a) $ArCH=CH(CN)_2$ 5a-f, triethylamine, ethanol;
b) $CH_2(CN)_2$, triethylamine, ethanol;
c) $NCCH_2COOC_2H_5$, Fusion at 160 °C.

Scheme 1

1H, *H*5, *J*=2 Hz), 7.3 (s, 1H, N*H*, D_2O exchangeable), 7.4-7.5 (m, 4H, Ar-*H*), 9.2 (s, 2H, NH_2, D_2O exchangeable). Anal. Calcd. for $C_{15}H_{11}ClN_6S$: C, 52.55; H, 3.23; N, 24.51. Found: C, 52.62; H, 3.44; N, 24.27%.

7-Amino-5-(2-methoxyphenyl)-2-methylsulphanyl-4,5-dihydro pyrazolo[1,5-a]pyrimidine-3,6-dicarbonitrile (**6b**): Yield: 48%. M.p.: 244-245 °C. FT-IR (cm^{-1}): 3425, 3277, 3219 (NH/NH_2), 2218, 2189 (CN). ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 2.6 (s, 3H, SCH_3), 3.7 (s, 3H, OCH_3), 5.4 (s, 1H, *H*5), 7.2 (s, 1H, N*H*, D_2O exchangeable), 6.9-7.3 (m, 4H, Ar-*H*), 9.0 (s, 2H, NH_2, D_2O exchangeable). MS (m/z (%)): 338 [M^+, 1.12%]. Anal. Calcd. for $C_{16}H_{14}N_6OS$: C, 56.79; H, 4.17; N, 24.83. Found: C, 56.50; H, 3.84; N, 24.97%.

7-Amino-5-(2-hydroxyphenyl)-2-methylsulphanylpyrazolo[1,5-a] pyrimidine-3,6-dicarbonitrile (**7a**): Yield: 22%. M.p.: 224-225 °C. FT-IR (cm^{-1}): 3436, 3344 (NH_2), 2200 (CN). ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 2.7 (s, 3H, SCH_3), 7.1-8.2 (m, 4H, Ar-*H*), 8.9 (s, 2H, NH_2, D_2O exchangeable), 9.3 (s, 1H, O*H*, D_2O exchangeable). Anal. Calcd. for $C_{15}H_{10}N_6OS$: C, 55.89; H, 3.12; N, 26.07. Found: C, 56.02; H, 3.59; N, 26.23%.

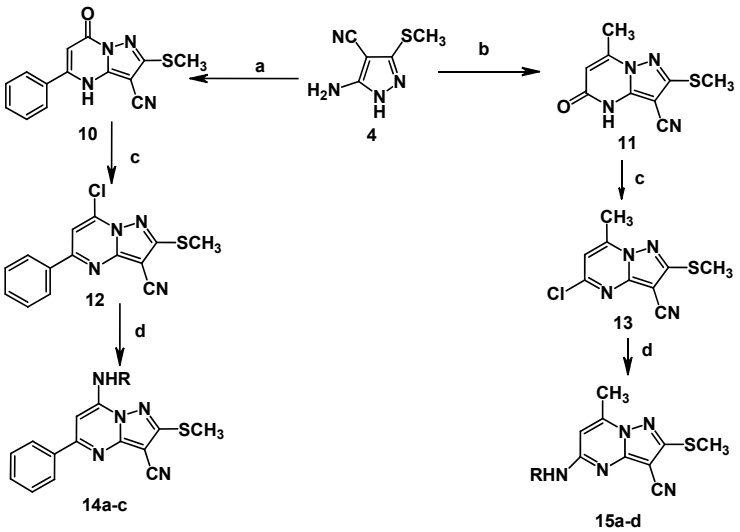
7-Amino-2-methylsulphanyl-5-(3-nitrophenyl)pyrazolo[1,5-a] pyrimidine-3,6-dicarbonitrile (**7b**): Yield: 44%. M.p.: 287-288 °C. FT-IR (cm^{-1}): 3308, 3273 (NH_2), 2214, 2131 (CN), 1528, 1345 (NO_2). ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 2.7 (s, 3H, SCH_3), 7.8 (t, 1H, *J*=8 Hz, Ar-*H*), 8.3 (d, 1H, *J*=7.8 Hz, Ar-*H*), 8.4 (d, 1H, *J*=8 Hz, Ar-*H*), 8.6 (s, 1H, Ar-*H*), 9.4 (br s, 2H, NH_2, D_2O exchangeable). Anal. Calcd. for $C_{15}H_9N_7O_2S$: C, 51.27; H, 2.58; N, 27.90. Found: C, 51.71; H, 2.30; N, 27.80%.

7-Amino-5-(4-chlorophenyl)-2-methylsulphanylpyrazolo[1,5-a] pyrimidine-3,6-dicarbonitrile (**7c**): Yield: 45%. M.p.: 273-274 °C. FT-IR (cm^{-1}): 3439, 3297 (NH_2), 2222 (CN). ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 2.7 (s, 3H, SCH_3), 7.6 (d, 2H, *J*=7.6 Hz, Ar-*H*), 7.8 (d, 2H, *J*=7.6 Hz, Ar-*H*), 9.3 (br s, 2H, NH_2, D_2O exchangeable). MS (m/z (%)): 342 [(M+2)$^+$, 38.96%], 340 [M^+, 100%]. Anal. Calcd. for $C_{15}H_9ClN_6S$: C, 52.86; H, 2.66; N, 24.65. Found: C, 52.70; H, 2.30; N, 24.86.

7-Amino-5-(4-dimethylaminophenyl)-2-methylsulphanylpyrazolo [1,5-a]pyrimidine-3,6-dicarbonitrile (**7d**): Yield: 32%. M.p.: 280-281 °C. FT-IR (cm^{-1}): 3449, 3370 (NH_2), 2209 (CN). ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 2.7 (s, 3H, SCH_3), 3.0 (s, 6H, -N(CH_3)$_2$), 6.7 (d, 2H, *J*=8.8 Hz, Ar-*H*), 7.8 (d, 2H, *J*=9.2 Hz, Ar-*H*), 9.0 (br s, 2H, NH_2, D_2O exchangeable). Anal. Calcd. for $C_{17}H_{15}N_7S$: C, 58.43; H, 4.32; N, 28.05. Found: C, 58.73; H, 4.64; N, 28.30%.

2.2.2. 5,7-Diamino-2-methylsulphanylpyrazolo[1,5-a] pyrimidine-3-carbonitrile (8)

A mixture of 5-amino-3-methylsulphanyl-1*H*-pyrazole-4-carbonitrile (**4**) (0.77 g, 0.005 mol), malononitrile (0.33 g, 0.005 mol) and triethylamine (2 mL) in absolute ethanol (30 mL) was heated under reflux for 10 h. The precipitate formed was filtered, dried and crystallized from acetic acid. Yield: 26%. M.p.: 254-255 °C. FT-IR (cm^{-1}): 3479, 3431, 3373, 3317 (NH_2), 2203 (CN). ^1H NMR (200 MHz, DMSO-d_6, δ ppm): 2.6 (s, 3H, SCH_3), 5.3 (s, 1H, *H*6), 6.7 (s, 2H, NH_2, D_2O exchangeable), 7.3 (s, 2H, NH_2, D_2O exchangeable). Anal. Calcd. for $C_8H_8N_6S$: C, 43.62; H, 3.66; N, 38.15. Found: C, 43.99; H, 3.55; N, 38.60%.



Compound	R
14a, 15a	C_6H_{11}
14b, 15b	C_6H_5

Compound	R
14c, 15c	4-$CH_3OC_6H_4$
15d	2-$CH_3C_6H_4$

Reagents: a) $C_6H_5COCH_2COOC_2H_5$, Fusion at 160 °C;
b) $CH_3COCH_2COOC_2H_5$, Fusion at 170 °C (method A), acetic acid (method B);
c) $POCl_3$;
d) RNH_2, triethylamine, ethanol.

Scheme 2

2.2.3. 7-Amino-2-methylsulphanyl-5-oxo-4,5-dihydropyrazolo [1,5-a]pyrimidine-3-carbonitrile (9)

A mixture of 5-amino-3-methylsulphanyl-1*H*-pyrazole-4-carbonitrile (**4**) (2.31 g, 0.015 mol) and ethyl cyanoacetate (1.69 g, 1.70 mL, 0.015 mol) was heated at 160 °C in an oil bath for 2 h. The solid formed was triturated with ethanol (10 mL), filtered, dried and crystallized from DMF. Yield: 93%. M.p.: >300 °C. FT-IR (cm^{-1}): 3433, 3319 (NH/NH_2), 2220 (CN), 1650 (CO). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.6 (s, 3H, SCH_3), 5.2 (d, 1H, *H6*, *J*=2.2 Hz), 7.6 (s, 2H, NH_2, D_2O exchangeable), 11.9 (br s, 1H, N*H*, D_2O exchangeable). Anal. Calcd. for $C_8H_7N_5OS$: C, 43.43; H, 3.18; N, 31.65. Found: C, 43.01; H, 3.36; N, 31.22%.

2.2.4. 2-Methylsulphanyl-5-phenyl-7-oxo-4,7-dihydropyrazolo [1,5-a]pyrimidine-3-carbonitrile (10)

A mixture of 5-amino-3-methylsulphanyl-1*H*-pyrazole-4-carbonitrile (**4**) (2.31 g, 0.015 mol) and ethyl benzoylacetate (2.88 g, 2.60 mL, 0.015 mol) was heated at 160 °C in an oil bath for 2 h. The solid formed was triturated with ethanol (10 mL), filtered, dried and crystallized from acetic acid. Yield: 67%. M.p.: 268-269 °C. FT-IR (cm^{-1}): 3466 (NH), 2222 (CN), 1718 (CO). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.6 (s, 3H, SCH_3), 6.2 (s, 1H, N*H*, D_2O exchangeable), 7.5-7.8 (m, 6H, Ar-*H*+ *H6*). Anal. Calcd. for $C_{14}H_{10}N_4OS$: C, 59.56; H, 3.57; N, 19.84. Found: C, 59.97; H, 3.57; N, 20.16%.

2.2.5. 7-Methyl-2-methylsulphanyl-5-oxo-4,5-dihydropyrazolo [1,5-a]pyrimidine-3-carbonitrile (11)

Method A: A mixture of 5-amino-3-methylsulphanyl-1*H*-pyrazole-4-carbonitrile (**4**) (2.31 g, 0.015 mol) and ethyl acetoacetate (1.95 g, 1.90 mL, 0.015 mol) was heated at 170 °C in an oil bath for 2 h. The solid product was triturated with ethanol (10 mL), filtered, dried and crystallized from acetic acid.

Method B: A mixture of 5-amino-3-methylsulphanyl-1*H*-pyrazole-4-carbonitrile (**4**) (2.31 g, 0.015 mol) and ethyl acetoacetate (1.95 g, 1.90 mL, 0.015 mol) in glacial acetic acid (50 mL) was heated under reflux for 15 h. The solid formed was filtered, dried and crystallized from acetic acid.

Yield: 90% (*Method A*), 77% (*Method B*). M.p.: 300-301 °C [16]. FT-IR (cm^{-1}): 3470 (NH), 2225 (CN), 1661 (CO). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.2 (s, 3H, CH_3), 2.6 (s, 3H, SCH_3), 5.8 (s, 1H, *H6*), 13.2 (s, 1H, N*H*, D_2O exchangeable).

2.2.6. General procedure for the synthesis of pyrazolo[1,5-a] pyrimidines 12 and 13

A mixture of pyrazolo[1,5-*a*]pyrimidine derivatives **10** or **11** (0.004 mol) and phosphorus oxychloride (20 mL) was heated under reflux for 7 h. The reaction mixture was cooled and poured gradually onto crushed ice. The resulting product was filtered, dried, and crystallized from ethanol.

7-Chloro-2-methylsulphanyl-5-phenylpyrazolo[1,5-a] pyrimidine-3-carbonitrile (**12**): Yield: 73%. M.p.: 196-197 °C. FT-IR (cm^{-1}): 2220 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.7 (s, 3H, SCH_3), 7.5-8.2 (m, 6H, Ar-*H* + *H6*). Anal. Calcd. for $C_{14}H_9ClN_4S$: C, 55.90; H, 3.01; N, 18.62. Found: C, 56.00; H, 3.23; N, 18.20%.

5-Chloro-7-methyl-2-methylsulphanylpyrazolo[1,5-a] pyrimidine-3-carbonitrile (**13**): Yield: 71%. M.p.: 220-221 °C. FT-IR (cm^{-1}): 2223 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.6 (s, 3H, CH_3), 2.7 (s, 3H, SCH_3), 7.5 (s, 1H, *H6*). Anal. Calcd. for $C_9H_7ClN_4S$: C, 45.28; H, 2.95; N, 23.47. Found: C, 45.65; H, 2.96; N, 23.45%.

2.2.7. General procedure for the synthesis of 2-methylsulphanyl-5-phenyl-7-(substituted amino)pyrazolo[1,5-a]pyrimidine-3-carbonitriles 14a-c

A mixture of 7-chloropyrazolo[1,5-*a*]pyrimidine derivative **12** (0.60 g, 0.002 mol), the appropriate primary amine (0.002 mol) and triethylamine (0.50 mL) in absolute ethanol (20 mL) was heated under reflux for 9 h. The reaction mixture was concentrated under reduced pressure, and the solid formed upon cooling was filtered, dried and crystallized from the suitable solvent.

7-Cyclohexylamino-2-methylsulphanyl-5-phenylpyrazolo[1,5-a] pyrimidine-3-carbonitrile (**14a**): (Crystallized from ethanol). Yield: 60%. M.p.: 194-195 °C. FT-IR (cm^{-1}): 3383 (NH), 2213 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 1.2-1.9 (m, 11H, aliphatic-*H*), 2.7 (s, 3H, SC*H*$_3$), 7.0 (s, 1H, *H6*), 7.5-8.2 (m, 5H, Ar-*H*), 7.8 (s, 1H, N*H*, D_2O exchangeable). Anal. Calcd. for $C_{20}H_{21}N_5S$: C, 66.08; H, 5.82; N, 19.26. Found: C, 66.20; H, 5.60; N, 19.39%.

7-Anilino-2-methylsulphanyl-5-phenylpyrazolo[1,5-a]pyrimidine-3-carbonitrile (**14b**): (Crystallized from acetic acid). Yield: 44%. M.p.: 254-255 °C. FT-IR (cm^{-1}): 3349 (NH), 2211 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.8 (s, 3H, SC*H*$_3$), 6.7-8.3 (m, 11H, Ar-*H*), 10.3 (s, 1H, N*H*, D_2O exchangeable). Anal. Calcd. for $C_{20}H_{15}N_5S$: C, 67.20; H, 4.22; N, 19.59. Found: C, 67.05; H, 4.12; N, 19.84%.

7-(4-Methoxyanilino)-2-methylsulphanyl-5-phenylpyrazolo[1,5-a]pyrimidine-3-carbonitrile (**14c**): (Crystallized from ethanol). Yield: 70%. M.p.: 192-193 °C. FT-IR (cm^{-1}): 3305 (NH), 2211 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.8 (s, 3H, SC*H*$_3$), 3.8 (s, 3H, OC*H*$_3$), 6.5-7.9 (m, 10H, Ar-*H*), 8.2 (s, 1H, N*H*, D_2O exchangeable). Anal. Calcd. for $C_{21}H_{17}N_5OS$: C, 65.09; H, 4.42; N, 18.07. Found: C, 65.00; H, 4.23; N, 17.77%.

2.2.8. General procedure for the synthesis of 7-methyl-2-methylsulphanyl-5-(substituted amino)pyrazolo[1,5-a] pyrimidine-3-carbonitriles 15a-d

A mixture of 5-chloropyrazolo[1,5-*a*]pyrimidine derivative **13** (0.48 g, 0.002 mol), the appropriate primary amine (0.002 mol) and triethylamine (0.50 mL) in absolute ethanol (20 mL) was heated under reflux for 11 h. The reaction mixture was concentrated under reduced pressure, and the product formed upon cooling was filtered, dried and crystallized from ethanol.

5-Cyclohexylamino-7-methyl-2-methylsulphanylpyrazolo[1,5-a] pyrimidine-3-carbonitrile (**15a**): Yield: 91%. M.p.: 180-181 °C. FT-IR (cm^{-1}): 3378 (NH), 2211 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 1.2-1.9 (m, 11H, aliphatic-*H*), 2.4 (s, 3H, C*H*$_3$), 2.7 (s, 3H, SC*H*$_3$), 6.4 (s, 1H, *H6*), 7.7 (br s, 1H, N*H*, D_2O exchangeable). Anal. Calcd. for $C_{15}H_{19}N_5S$: C, 59.77; H, 6.35; N, 23.23. Found: C, 59.55; H, 6.31; N, 22.92%.

5-Anilino-7-methyl-2-methylsulphanylpyrazolo[1,5-a]pyrimidine-3-carbonitrile (**15b**): Yield: 77%. M.p.: 209-210 °C. FT-IR (cm^{-1}): 3215 (NH), 2230 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.3 (s, 3H, C*H*$_3$), 2.7 (s, 3H, SC*H*$_3$), 6.2 (s, 1H, *H6*), 7.1-7.5 (m, 5H, Ar-*H*), 10.0 (s, 1H, N*H*, D_2O exchangeable). Anal. Calcd/ for $C_{15}H_{13}N_5S$: C, 60.99; H, 4.43; N, 23.71. Found: C, 60.86; H, 4.60; N, 23.45%.

5-(4-Methoxyanilino)-7-methyl-2-methylsulphanylpyrazolo[1,5-a]pyrimidine-3-carbonitrile (**15c**): Yield: 59%. M.p.: 168-169 °C. FT-IR (cm^{-1}): 3362 (NH), 2217 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.3 (s, 3H, C*H*$_3$), 2.7 (s, 3H, SC*H*$_3$), 3.8 (s, 3H, OC*H*$_3$), 6.0 (s, 1H, *H6*), 7.0 (d, 2H, *J*=8.7 Hz, Ar-*H*), 7.3 (d, 2H, *J*=8.7 Hz, Ar-*H*), 9.9 (s, 1H, N*H*, D_2O exchangeable). MS (m/z (%)): 325 [M$^+$, 100%]. Anal. Calcd. for $C_{16}H_{15}N_5OS$: C, 59.06; H, 4.64; N, 21.52. Found: C, 59.39; H, 5.10; N, 21.11%.

5-(2-Methylanilino)-7-methyl-2-methylsulphanylpyrazolo[1,5-a] pyrimidine-3-carbonitrile (**15d**): Yield: 53%. M.p.: 192-193 °C. FT-IR (cm^{-1}): 3368 (NH), 2214 (CN). ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.2 (s, 3H, C*H*$_3$C$_6$H$_4$), 2.3 (s, 3H, C*H*$_3$), 2.7 (s, 3H, SC*H*$_3$), 5.7 (s, 1H, *H6*), 7.3-7.4 (m, 4H, Ar-*H*), 9.9 (s, 1H, N*H*, D_2O exchangeable). Anal. Calcd. for $C_{16}H_{15}N_5S$: C, 62.11; H, 4.88; N, 22.63. Found: C, 62.40; H, 4.55; N, 22.29%.

2.3. Biological testing

2.3.1. Materials and methods

The human colon tumor cell line (HCT116) was obtained as a gift from National Cancer Institute NCI, Maryland MD, USA. All chemicals and solvents were purchased from Sigma–Aldrich.

2.3.2. Measurement of potential cytotoxicity

The cytotoxic activity of some of the newly synthesized compounds was tested in vitro on human colon tumor cell line (HCT116) using Sulforhodamine-B stain (SRB) assay according to the method of Skehan *et al.* [17]. Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the tested compounds to allow attachment of the cells to the wall of the plate. The tested compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the tested compound (0, 1, 2.5, 5 and 10 µg/mL) were added to the cell monolayer. Triplicate wells prepared for each individual dose. Monolayer cells were incubated with the tested compound for 48 h at 37 °C in atmosphere of 5% CO_2. After 48 h, cells were fixed with trichloroacetic acid, washed with water and stained for 30 min with 0.4% (w:v) Sulforhodamine-B stain dissolved with 1% acetic acid. Excess stain was removed by four washes with 1% acetic acid and attached stain was recovered with Tris EDTA buffer. Colour intensity was measured in ELISA reader. The relation between surviving fraction and compound concentration was plotted and IC_{50} (the concentration required for 50% inhibition of cell viability) was calculated for each compound and results are given in Table 1. Figure 2 represents IC_{50} in µM of the synthesized compounds and doxorubicin against human colon tumor cell line (HCT116)

Table 1. Results of *in vitro* cytotoxic activity of some of the synthesized compounds on human colon tumor cell line (HCT116).

Compound no.	IC_{50} in *µM**
Doxorubicin	0.0069
7b	0.0062
7c	0.0375
8	0.0433
9	0.0814
14a	0.0020
14b	0.0113
14c	0.0238
15c	0.0077
15d	0.0598

*The values given are means of three experiments.

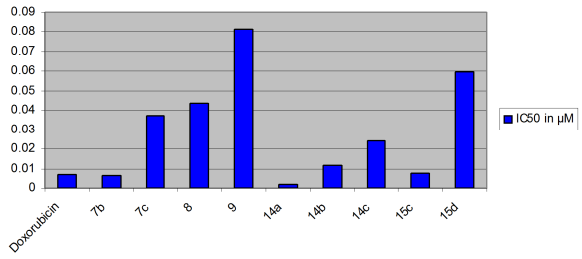


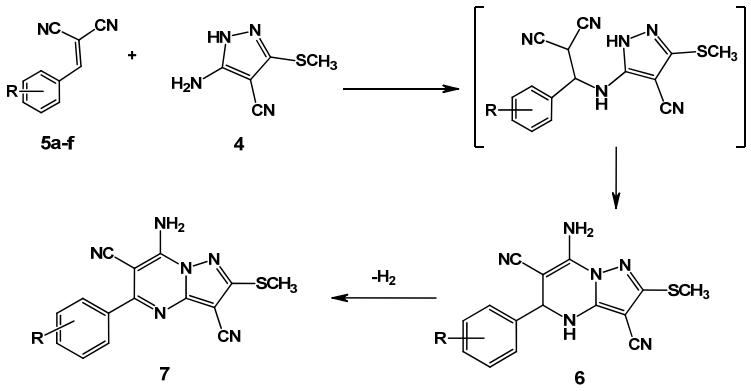
Figure 2. IC_{50} in µM of the synthesized compounds and doxorubicin against human colon tumor cell line (HCT116).

3. Results and discussion

3.1. Chemistry

The synthesis of the target compounds is outlined in Schemes 1 and 2. The starting compound, 5-amino-3-methylsulphanyl-1*H*-pyrazole-4-carbonitrile (**4**) [11] was reacted with the appropriate substituted benzylidenemalononitrile **5a-f** [12-15] to afford 7-amino-2-methylsulphanyl-5-(substituted phenyl)-4,5-dihydropyrazolo[1,5-*a*]pyrimidine-3,6-dicarbonitriles **6a**,**b** or 7-amino-2-methylsulphanyl-5-(substituted phenyl)pyrazolo[1,5-*a*]pyrimidine-3,6-dicarbonitriles **7a-d**.

The reaction of the pyrazole derivative **4** with substituted benzylidenemalononitriles **5a-f** may proceed *via* initial nucleophilic attack by the exocyclic amino group of compound **4** on the activated double bond in **5a-f** to form a Micheal adduct. Intramolecular cyclization of the latter may result in the formation of the dihydro derivative **6** which may then aromatize into **7** (Scheme 3).

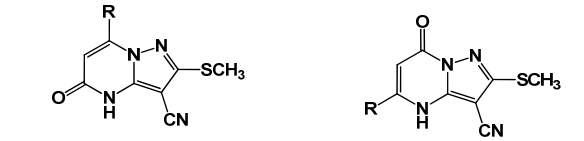


Scheme 3

Trials to oxidize **6a**,**b** into **7** (R=2-Cl, 2-CH_3O) *via* the use of two molar equivalents of benzylidenemalononitriles **5a**,**b** or by increasing the time of the reaction (up to 15 h) were unsuccessful.

Compound **4** was also reacted with malononitrile to give 5,7-diaminopyrazolo[1,5-*a*]pyrimidine **8**. ^{1}H NMR spectrum of compound **8** revealed the presence of a singlet signal at δ 5.3 ppm corresponding to *H*6 and two exchangeable singlet signals at δ 6.7 and δ 7.3 ppm corresponding to protons of the two NH_2 groups at positions 5 and 7, respectively.

Furthermore, 1*H*-pyrazole derivative **4** was reacted with ethyl cyanoacetate, ethyl benzoylacetate and ethyl acetoacetate to give one of two possible products; the 5-oxopyrazolo[1,5-*a*]pyrimidine or the 7-oxo isomer (Scheme 4). The assignment of the product as 5-oxo or 7-oxopyrazolo[1,5-*a*]pyrimidine depends on ^{1}H NMR study.



5-Oxopyrazolo[1,5-*a*]pyrimidine **7-Oxopyrazolo[1,5-*a*]pyrimidine**

R= NH_2, C_6H_5, CH_3

Scheme 4

The reaction of compound **4** with ethyl cyanoacetate and ethyl acetoacetate afforded 5-oxopyrazolo[1,5-*a*]pyrimidine derivatives **9** and **11**, respectively. Both compounds showed an exchangeable singlet signal at δ 11.9 ppm and δ 13.2 ppm, respectively, corresponding to NH proton due to tautomerism with the adjacent carbonyl group. The 7-oxo isomer, lacking such tautomerism, should give that signal at δ 5-7 ppm.

On the other hand, the reaction of compound **4** with ethyl benzoylacetate afforded 7-oxopyrazolo[1,5-*a*]pyrimidine **10**. Herein, ^{1}H NMR of compound **10** revealed the presence of an exchangeable singlet signal at δ 6.2 ppm corresponding to NH proton.

Chlorination of compounds **10** and **11** with $POCl_3$ followed by nucleophilic substitution with primary amines afforded 7-(substituted amino) and 5-(substituted amino)pyrazolo[1,5-*a*]pyrimidine derivatives **14a**-**c** and **15a**-**d**, respectively.

3.2. In vitro anticancer screening

Some of the newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against human colon tumor cell line, HCT116. Doxorubicin which is one of the most effective anticancer agents was used as the reference drug in this study.

The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability. The IC_{50} in μM of the tested compounds compared to the reference drug are shown in Table 1 and represented graphically in Figure 2.

From the results in Table 1, it was found that all the tested compounds exhibited good antitumor activity against HCT116 with IC_{50} between 0.0020 and 0.0814 μM.

Two of the tested compounds (**7b** and **14a**) showed antitumor activity superior to doxorubicin with IC_{50} that equals to 0.0069 and 0.0020 μM, respectively.

The best results were obtained by 5-phenyl-7-(substituted amino)pyrazolo[1,5-*a*]pyrimidine derivatives **14a**-**c**, and the highest activity was obtained by 7-cyclohexylamino-2-methylsulphanyl-5-phenylpyrazolo[1,5-*a*]pyrimidine-3-carbonitrile (**14a**).

4. Conclusion

In summary, a novel series of pyrazolo[1,5-*a*]pyrimidine-3-carbonitriles substituted with 7-amino (**6**-**9**), 7-substituted amino (**14a**-**c**) and 5-substituted amino (**15a**-**d**) was synthesized. Some of the newly synthesized compounds were tested *in vitro* on human colon tumor cell line (HCT116). Most of the tested compounds exhibited good antitumor activity, especially compound **14a** which displayed the highest activity among the tested compounds with IC_{50} that equals to 0.0020 μM.

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