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Original article

Synthesis and antitumor activity of novel pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*] pyrimidin-5-one derivatives

Hala B. El-Nassan*

Organic Chemistry Department, Faculty of Pharmacy, Cairo University, 33 Kasr El-Aini Street, Cairo 11562, Egypt

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ABSTRACT

A series of new pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidines with different substituents at position 3 were synthesized. The effect of the newly synthesized compounds was tested *in vitro* on human breast adenocarcinoma cell line (MCF7). Some of the synthesized compounds exploited potent antitumor activity, especially the 3-amino derivative **12** which displayed the highest activity among the test compounds with IC_{50} equal to 3.74 μg/mL.

1. Introduction

Cancer is the second leading cause of death in the world. In the last few years, a large number of fused pyrimidine derivatives were reported as anticancer agents used in clinics or in clinical trials [1–7]. Pyrido[2,3-*d*]pyrimidines were reported to exhibit antitumor activity which may be attributed to inhibition of cyclin dependent kinase [1,2], check point kinase [3] or mammalian target of rapamycin [4].

Another fused pyrimidine ring system was [1,2,4]triazolo[4,3-*a*] pyrimidine and many derivatives of this class were reported to exhibit antitumor activity [5–7].

The combination of these two classes to give the tricyclic pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidine ring system and the subsequent influence on the biological activities are of current interest [8–14]. None of these publication discussed the effect of pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidines as antitumor agents.

In this work, we aimed to synthesize new pyrido[2,3-*d*][1,2,4] triazolo[4,3-*a*]pyrimidin-5-one derivatives bearing different substituents at position 3 [H, alkyl, oxo, thioxo, alkylsulphanyl, aryl, amino and chloromethyl groups] in order to, examine the effect of substitution at position 3 on the antitumor activity.

2. Results and discussion

2.1. Chemistry

The synthesis of the target compounds is outlined in Schemes 1–3.

The starting compound, 5-(4-chlorophenyl)-2,3-dihydro-7-phenyl-2-thioxopyrido[2,3-*d*]pyrimidin-4(1*H*)-one **(3)** was prepared *via* reacting 6-amino-2-thiouracil **(1)** and 3-(4-chlorophenyl)-1-phenyl-2-propen-1-one (**2**) [15] in DMF according to the procedure reported by Quiroga *et al.* [16].

Reacting compound **3** with hydrazine hydrate in ethanol afforded 2-hydrazinopyrido[2,3-*d*]pyrimidin-4(3*H*)-one **4**. While conducting the same reaction in DMF resulted in 5-oxo-1*H*-pyrido[2,3-*d*][1,2,4] triazolo[4,3-*a*]pyrimidine **5**.

The formation of compound **5** was confirmed by ^{1}H NMR that showed only one exchangeable singlet signal at δ 13.6 ppm. Besides, the mass spectrum of compound **5** showed the molecular ion peaks at *m*/*z* 373 (M) and 375 (M+2) in the ratio of 3:1 [Cl pattern].

Besides, the same compound was obtained by reacting compound **4** with triethyl orthoformate or by its heating under reflux in DMF.

A possible mechanism for the formation of compound **5** in DMF was outlined in Fig. 1.

On the other hand, reacting compound **4** with acetic anhydride or propionic anhydride resulted in 3-methyl **6a** and 3-ethyl **6b**

* Tel.: +20 2 23632245; fax: +20 2 23635140.
E-mail address: hala_bakr@hotmail.com.

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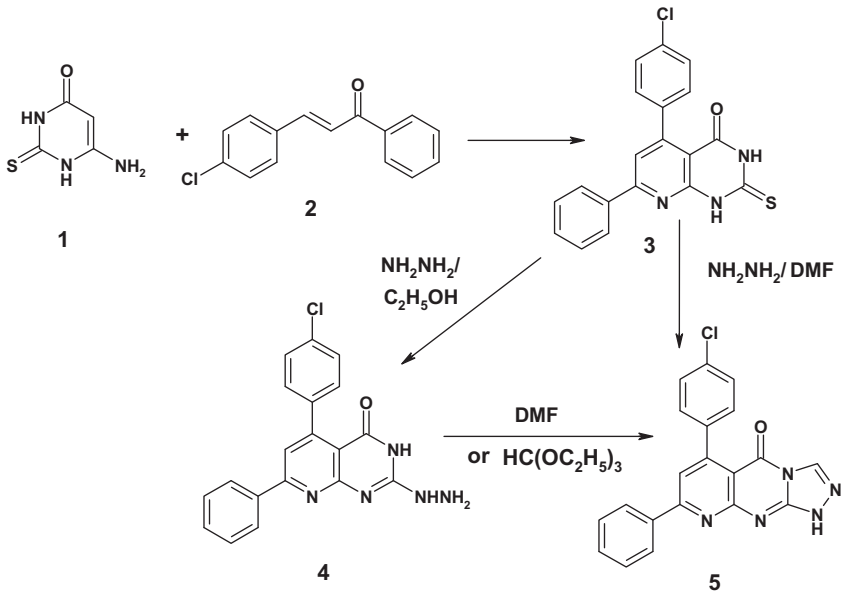


Scheme. 1. Synthesis of the starting compound 3 and its reaction with hydrazine hydrate.

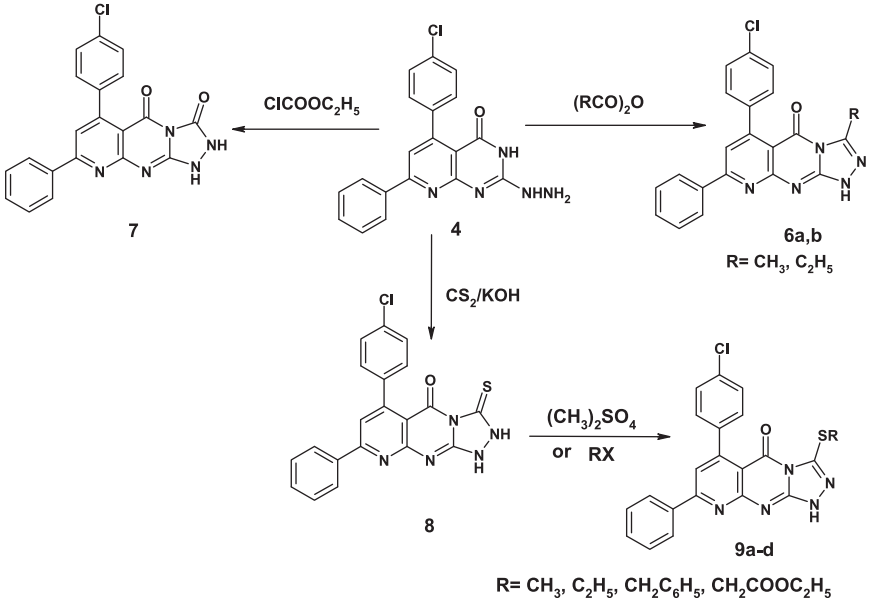
derivatives, respectively. The formation of compound **6a** was confirmed by ^{1}H NMR that revealed the presence of a singlet signal at δ 3.0 ppm corresponding to methyl protons and an exchangeable singlet signal at δ 12.8 ppm corresponding to NH proton. Also, the mass spectrum of compound **6a** showed the molecular ion peaks at *m*/*z* 387 (M) and 389 (M+2) in the ratio of 3:1 [Cl pattern]. While, the ^{1}H NMR spectrum of compound **6b** showed triplet and quartet signals at δ 1.0 and 2.2 ppm, respectively, corresponding to ethyl protons as well as an exchangeable singlet signal at δ 11.3 ppm corresponding to NH proton.

The reaction of compound **4** with ethyl chloroformate in dry pyridine or carbon disulphide in ethanolic KOH solution resulted in 3-oxo and 3-thioxopyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidines **7** and **8**, respectively. Both compounds were confirmed by ^{1}H NMR spectra that showed two exchangeable singlet signals at δ 9.2 and 11.2 ppm in the spectrum of **7** and at δ 8.2 and 13.6 ppm in the spectrum of **8**.

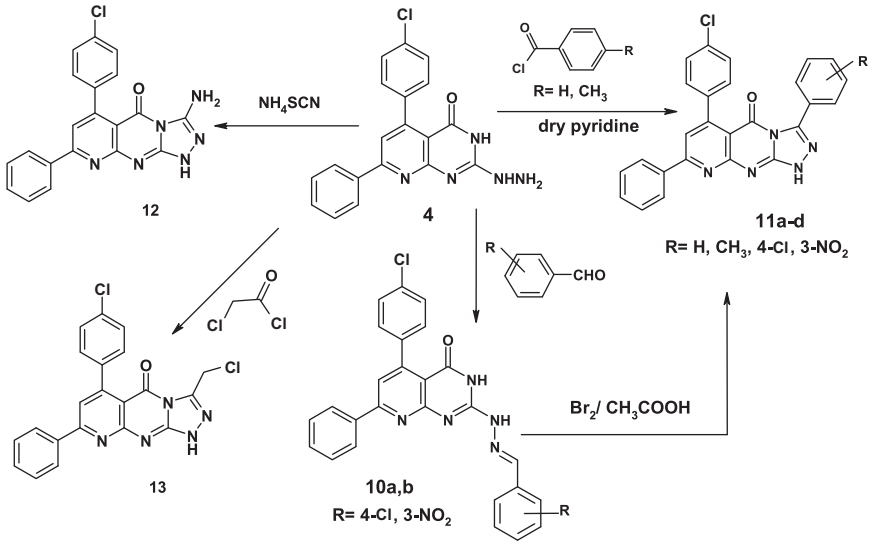
Alkylation of 3-thioxo derivative **8** with dimethyl sulfate, ethyl iodide, benzyl chloride or ethyl chloroacetate gave the corresponding 3-alkylsulphanyl derivatives **9a**–**d**. ^{1}H NMR spectra of compounds **9a**–**d** revealed the disappearance of the NH signals at δ 8.2 and 13.6 ppm and the appearance of only one exchangeable singlet signal at δ 12.5–12.9 ppm.

The 3-aryl derivatives **11c**–**d** could be obtained *via* two pathways; either by reacting compound **4** with benzoyl chloride or 4-methylbenzoyl chloride in dry pyridine, or by oxidative cyclization of the corresponding hydrazone **10a,b** in bromine/acetic acid mixture.

Reacting 2-hydrazino derivative **4** with ammonium isothiocyanate in acetic acid afforded the 3-amino derivative **12**. The



Scheme. 2. Synthesis of 3-alkyl, 3-oxo, 3-thioxo and 3-alkylsulphanyl derivatives.



Scheme. 3. Synthesis of 3-aryl, 3-amino and 3-chloromethyl derivatives.

^{1}H NMR spectrum of the latter compound showed an exchangeable singlet signal at δ 6.9 ppm corresponding to NH_2 protons as well as NH signal at δ 13.6 ppm.

Finally, reacting compound **4** with chloroacetyl chloride in dry pyridine afforded the 3-chloromethyl derivative **13**. Its ^{1}H NMR spectrum showed CH_2 protons as a singlet signal at δ 4.2 ppm and NH proton as an exchangeable broad singlet signal at δ 13.5 ppm.

2.2. In vitro anticancer screening

The newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against human breast cancer cell line, MCF7. Doxorubicin which is one of the most effective anticancer agents was used as the reference drug in this study.

The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of breast cancer cell line (MCF7). The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability.

The IC_{50} of the synthesized compounds compared to the reference drug are shown in Table 1 and the results are represented graphically in Fig. 2.

From the results in Table 1, it was found that the best results were obtained by compounds bearing thioxo group [compound **8**], alkylthio group [compounds **9a,b,d**] or amino group [compound **12**] at position 3. Regarding the alkylthio derivatives, better result was obtained by methylsulphanyl derivative **9a** than by ethylsulphanyl derivative **9b** or benzylsulphanyl derivative **9c**. Compound **12** was the most potent compound in this screening with IC_{50} equal to 3.74 μg/mL.

Derivatives substituted with aryl, ethyl or chloromethyl at position 3 showed moderate cytotoxic activity with IC_{50} between 9.07 and 14 μg/mL.

On the other hand, derivatives substituted with H, CH_3 or CO at position 3 showed poor cytotoxic activity with $IC_{50} > 20$ μg/mL.

It is worth noting that the starting compound **3** showed good cytotoxic activity against MCF7 with IC_{50} equal to 5.11 μg/mL.

Further studies needed to be done to explore the mechanism of action as well as the effect of substitution at other positions of the ring.

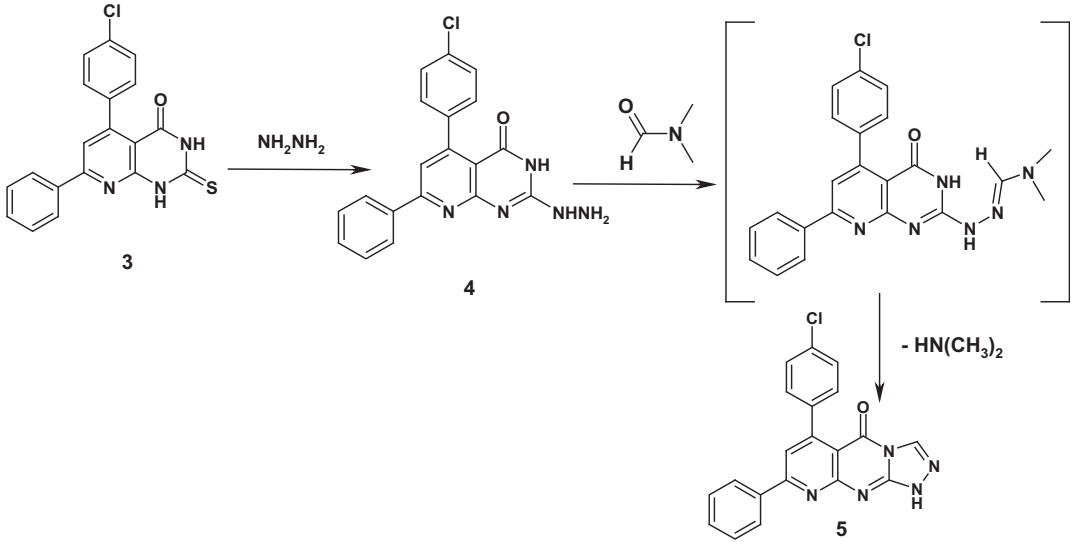


Fig. 1. A possible mechanism for the formation of compound **5** in DMF.

Table 1
Results of *in vitro* cytotoxic activity of the synthesized compounds on human breast adenocarcinoma cell line (MCF7).

Compound no.	IC_{50} in μg/mL
Doxorubicin	2.97
3	5.11
5	21.60
6a	>50
6b	14.00
7	24.30
8	6.78
9a	3.89
9b	5.20
9c	12.61
9d	4.50
11a	9.22
11b	9.07
12	3.74
13	11.70

3. Conclusion

The objective of the present study was to synthesize new pyrido[2,3-*d*][1,2,4]triazolo[4,3-*a*]pyrimidin-5-ones bearing different substituents at position 3 and to examine the effect of substitution at position 3 on the cytotoxic activity. Some of these new compounds exhibited good antitumor activity against MCF7 when compared to doxorubicin as a reference drug. The best results were obtained by derivatives bearing thioxo, alkylthio or amino groups at position 3. The 3-amino derivative **12** was the most potent compound in this screening with IC_{50} equal to 3.74 μg/mL.

4. Experimental part

4.1. General

Melting points were determined using a Griffin apparatus and were uncorrected. IR spectra were recorded on Shimadzu IR 435 spectrophotometer and values were represented in cm^{1}. ^{1}H NMR and ^{13}C NMR were carried out on Varian Gemini 200 MHz spectrophotometer at The Microanalytical center, Cairo University, Cairo, Egypt, using TMS as an internal standard and chemical shifts were recorded in ppm on δ scale. The electron impact (EI) mass spectra were recorded on Hewlett Packard 5988 spectrometer at The 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 The Microanalytical Center, Cairo University, Cairo, Egypt, and were within ±0.4%.

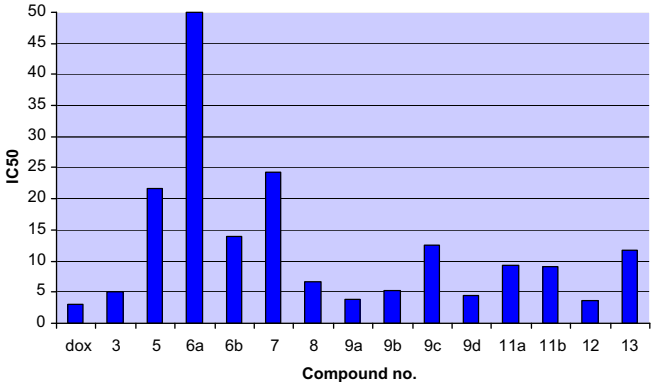


Fig. 2. IC_{50} in μg/mL of the synthesized compounds and doxorubicin against human breast adenocarcinoma cell line (MCF7).

4.1.1. 5-(4-Chlorophenyl)-2-hydrazino-7-phenylpyrido[2,3-d]pyrimidin-4(3H)-one (**4**)

A mixture of 2-thioxopyrido[2,3-*d*]pyrimidine **3** (1.46 g, 4 mmol) and hydrazine hydrate (99%, 3 mL, 60 mmol) in absolute ethanol (20 mL) was heated under reflux for 15 h. The reaction mixture was cooled and the solid formed was filtered, dried and crystallized from DMF. Yield: 70%; mp: 277–278 °C; IR (cm^{-1}): 3400, 3302 (NH/NH_2), 1685 (CO); ^{1}H NMR (DMSO-d_6) δ ppm 2.0 (br s, 2H, NH_2, D_2O exchangeable), 6.1 (br s, 1H, NH, D_2O exchangeable), 7.4–8.1 (m, 10H, Ar–H), 10.0 (br s, 1H, NH, D_2O exchangeable); MS *m/z*: 365 [$(M+2)^+$, 25.40%], 363 [M^+, 100%]; Anal. Calcd for $C_{19}H_{14}ClN_5O$: C, 62.73; H, 3.88; N, 19.25. Found: C, 62.95; H, 3.80; N, 19.22.

4.1.2. 6-(4-Chlorophenyl)-8-phenyl-1H-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**5**)

4.1.2.1. Method A. A mixture of 2-thioxopyrido[2,3-*d*]pyrimidine **3** (0.73 g, 2 mmol) and hydrazine hydrate (99%, 0.5 mL, 10 mmol) in DMF (7 mL) was heated under reflux for 15 h. The reaction mixture was cooled and the solid formed was filtered, dried and crystallized from DMF. Yield: 64%.

4.1.2.2. Method B. A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.37 g, 1 mmol) and DMF (5 mL) was heated under reflux for 3 h. The reaction mixture was cooled and the solid formed was filtered, dried and crystallized from DMF. Yield: 74%.

4.1.2.3. Method C. A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.37 g, 1 mmol) and triethyl orthoformate (8 mL) was heated under reflux for 3 h. The solid formed was filtered, dried and crystallized from DMF. Yield: 88%. mp: > 330 °C; IR (cm^{-1}): 3400 (NH), 1693 (CO); ^{1}H NMR (DMSO-d_6) δ ppm 7.4–8.4 (m, 10H, Ar–H), 9.4 (s, 1H, CH=N), 13.6 (br s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm 105.0, 117.9, 127.6, 128.8, 130.3, 130.8, 132.9, 136.4, 137.7, 150.3, 151.3, 152.8, 153.4, 154.3, 158.0, 159.4; MS *m/z*: 375 [$(M+2)^+$, 36.96%], 373 [M^+, 100%]; Anal. Calcd for $C_{20}H_{12}ClN_5O$: C, 64.26; H, 3.24; N, 18.74. Found: C, 64.49; H, 3.50; N, 18.40.

4.1.3. 6-(4-Chlorophenyl)-3-methyl-8-phenyl-1H-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**6a**)

A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.37 g, 1 mmol) and acetic anhydride (4 mL) was heated under reflux for 5 h. The reaction mixture was cooled and the solid formed was filtered, dried and crystallized from DMF. Yield: 74%; mp: > 330 °C; IR (cm^{-1}): 3410 (NH), 1693 (CO); ^{1}H NMR (DMSO-d_6) δ ppm 3.0 (s, 3H, CH_3), 7.4–8.3 (m, 10 H, Ar–H), 12.8 (br s, 1H, NH, D_2O exchangeable); MS *m/z*: 389 [$(M+2)^+$, 37.73%], 387 [M^+, 100%]; Anal. Calcd for $C_{21}H_{14}ClN_5O$: C, 65.04; H, 3.64; N, 18.06. Found: C, 64.70; H, 4.10; N, 18.39.

4.1.4. 6-(4-Chlorophenyl)-3-ethyl-8-phenyl-1H-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**6b**)

A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.37 g, 1 mmol) and propionic anhydride (0.26 g, 2 mmol) in pyridine (10 mL) was heated under reflux for 15 h. The reaction mixture was cooled and the solid formed was filtered, washed with ethanol, dried and crystallized from DMF. Yield: 57%; mp: > 330 °C; IR (cm^{-1}): 3421 (NH), 1693 (CO); ^{1}H NMR (DMSO-d_6) δ ppm 1.0 (t, 3H, CH_3, $J = 7.5$ Hz), 2.2 (q, 2H, CH_2, $J = 7.5$ Hz), 7.4–8.1 (m, 10 H, Ar–H), 11.3 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{22}H_{16}ClN_5O$: C, 65.76; H, 4.01; N, 17.43. Found: C, 65.71; H, 4.10; N, 17.77.

*4.1.5. 6-(4-Chlorophenyl)-8-phenyl-1H-pyrido[2,3-d] [1,2,4] triazolo[4,3-a]pyrimidin-3,5(2H)-dione (**7**)*

A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.37 g, 1 mmol) and ethyl chloroformate (0.22 g, 2 mmol) in pyridine (10 mL) was heated under reflux for 9 h. The reaction mixture was cooled and the solid was filtered, washed with ethanol, dried and crystallized from DMF. Yield: 78%; mp: > 330 °C; IR (cm^{-1}): 3387, 3340 (NH), 1690, 1654 (CO); ^1H NMR (DMSO-d_6) δ ppm 7.4–8.1 (m, 10H, Ar–H), 9.2 (br s, 1H, NH, D_2O exchangeable), 11.2 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{20}H_{12}ClN_5O_2$: C, 61.63; H, 3.10; N, 17.97. Found: C, 61.98; H, 3.16; N, 17.77.

*4.1.6. 6-(4-Chlorophenyl)-5-oxo-8-phenyl-1H-pyrido[2,3-d] [1,2,4] triazolo[4,3-a]pyrimidin-3(2H)-thione (**8**)*

A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.74 g, 2 mmol), KOH (0.23 g, 4 mmol) and carbon disulphide (4 mL) in absolute ethanol (40 mL) was heated under reflux for 5 h. The reaction mixture was evaporated to dryness and water (200 mL) was added then, the alkaline solution was filtered. The filtrate was acidified with conc. HCl (10 mL) and the separated solid was filtered, dried and crystallized from DMF. Yield: 90%; mp: > 330 °C; IR (cm^{-1}): 3450 (NH), 1705 (CO); ^1H NMR (DMSO-d_6) δ ppm 7.4–8.6 (m, 10H, Ar–H), 8.2 (br s, 1H, NH, D_2O exchangeable), 13.6 (s, 1H, NH, D_2O exchangeable); MS *m*/*z*: 407 [$(M+2)^+$, 3.28%], 405 [M^+, 7.61%], 190 [100%]; Anal. Calcd for $C_{20}H_{12}ClN_5OS$: C, 59.19; H, 2.98; N, 17.26. Found: C, 59.10; H, 2.90; N, 17.50.

4.1.7. 3-Alkylsulphanyl-6-(4-chlorophenyl)-8-phenyl-1Hpyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-ones

Compound **8** (0.40 g, 1 mmol) was dissolved in 10% ethanolic KOH (2 g in 20 mL ethanol). The reaction mixture was cooled and dimethyl sulfate, ethyl iodide, benzyl chloride or ethyl chloroacetate (1 mmol) was added and the reaction was stirred at room temperature for 2 h then left overnight. The reaction was diluted with water (30 mL) and acidified with acetic acid (5 mL). The separated solid was filtered, dried and crystallized from the appropriate solvent.

*4.1.7.1. 6-(4-Chlorophenyl)-3-methylsulphanyl-8-phenyl-1H-pyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**9a**).* Crystallized from ethanol. Yield: 51%; mp: 233–234 °C; IR (cm^{-1}): 3387 (NH), 2927, 2850 (CH aliphatic),1705 (CO); ^1H NMR (DMSO-d_6) δ ppm 2.6 (s, 3H, SCH_3), 7.2–8.2 (m, 10 H, Ar–H), 12.8 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{21}H_{14}ClN_5OS$: C, 60.07; H, 3.36; N, 16.68. Found: C, 60.28; H, 3.46; N, 16.90.

*4.1.7.2. 6-(4-Chlorophenyl)-3-ethylsulphanyl-8-phenyl-1H-pyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**9b**).* Crystallized from acetic acid. Yield: 94%; mp: 273–274 °C; IR (cm^{-1}): 3394 (NH), 2927, 2870 (CH aliphatic), 1697 (CO); ^1H NMR (DMSO-d_6) δ ppm 1.4 (t, 3H, CH_3, $J = 7.2$ Hz), 3.3 (q, 2H, CH_2, $J = 7.2$ Hz), 7.4–8.4 (m, 10 H, Ar–H), 12.5 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{22}H_{16}ClN_5OS$: C, 60.90; H, 3.72; N, 16.14. Found: C, 60.77; H, 3.48; N, 16.25.

*4.1.7.3. 3-Benzylsulphanyl-6-(4-chlorophenyl)-l-8-phenyl-1H-pyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**9c**).* Crystallized from ethanol. Yield: 60%; mp: 198–199 °C; IR (cm^{-1}): 3398 (NH), 2927, 2854 (CH aliphatic), 1701 (CO); ^1H NMR (DMSO-d_6) δ ppm 4.3 (s, 2H, SCH_2), 7.2–8.2 (m, 15 H, Ar–H), 12.9 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{27}H_{18}ClN_5OS$: C, 65.38; H, 3.66; N, 14.12. Found: C, 65.70; H, 3.24; N, 14.39.

*4.1.7.4. Ethyl [6-(4-chlorophenyl)-5-oxo-8-phenyl-1H-pyrido[2,3-d] [1,2,4]triazolo[4,3-a]pyrimidin-3-ylsulphanyl] acetate (**9d**).* Crystallized from DMF. Yield: 83%; mp: 292–293 °C; IR (cm^{-1}): 3398 (NH), 2927, 2850 (CH aliphatic), 1701 (CO); ^1H NMR (DMSO-d_6) δ ppm 2.0 (m, 3H, CH_3), 3.8 (m, 2H, CH_2), 4.0 (s, 2H, SCH_2CO), 7.3–8.4 (m, 10 H, Ar–H), 12.5 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{24}H_{18}ClN_5O_3S$: C, 58.60; H, 3.69; N, 14.24. Found: C, 58.88; H, 3.36; N, 14.43.

*4.1.8. 2-Arylmethylenehydrazone-5-(4-chlorophenyl)-7-phenylpyrido[2,3-d]pyrimidin-4(3H)-ones **10a,b***

A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.37 g, 1 mmol) and 4-chlorobenzaldehyde or 3-nitrobenzaldehyde (2 mmol) in glacial acetic acid (15 mL) was heated under reflux for 4 h. The reaction mixture was cooled and the solid formed was filtered, dried and crystallized from DMF.

*4.1.8.1. 2-(4-Chlorophenyl)methylenehydrazone-5-(4-chlorophenyl)-7-phenylpyrido[2,3-d]pyrimidin-4(3H)-one (**10a**).* Yield: 91%; mp: 315–316 °C; IR (cm^{-1}): 3500, 3300 (NH), 1680 (CO); ^1H NMR (DMSO-d_6) δ ppm 7.4–8.4 (m, 14 H, Ar–H), 8.8 (s, 1H, CH=N), 11.2 (s, 1H, NH, D_2O exchangeable), 11.6 (s, 1H, NH, D_2O exchangeable); MS *m*/*z*: 487 [$(M+2)^+$, 30.21%], 485 [M^+, 45.19%], 374 [$M^+-C_6H_4Cl$, 100%]; Anal. Calcd for $C_{26}H_{17}Cl_2N_5O$: C, 64.21; H, 3.52; N, 14.40. Found: C, 64.62; H, 3.23; N, 14.52.

*4.1.8.2. 5-(4-Chlorophenyl)-2-(3-nitrophenyl)methylenehydrazone-7-phenylpyrido[2,3-d]pyrimidin-4(3H)-one (**10b**).* Yield: 92%; mp: 278–279 °C; IR (cm^{-1}): 3400, 3383 (NH), 1693 (CO), 1539, 1350 (NO_2); ^1H NMR (DMSO-d_6) δ ppm 7.4–8.5 (m, 14 H, Ar–H), 8.8 (s, 1H, CH=N), 11.2 (s, 1H, NH, D_2O exchangeable), 11.6 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{26}H_{17}ClN_6O_3$: C, 62.85; H, 3.45; N, 16.91. Found: C, 63.21; H, 3.88; N, 16.46.

*4.1.9. 6-(4-Chlorophenyl)-3-(substituted phenyl)-8-phenyl-1H-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-ones **11a–d***

*4.1.9.1. Method A (for the synthesis of compounds **11a,b**).* A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.37 g, 1 mmol) and benzoyl chloride or 4-methylbenzoyl chloride (1.5 mmol) in dry pyridine (8 mL) was heated under reflux for 20 h. The reaction mixture was cooled and the solid formed was filtered, washed with ethanol, dried and crystallized from DMF.

*4.1.9.2. Method B (for the synthesis of compounds **11c,d**).* A mixture of compounds **10a,b** (1 mmol) and anhydrous sodium acetate (0.21 g, 2.5 mmol) bromine (0.16 g, 1 mmol) and glacial acetic acid (10 mL) was heated in a water bath at 80 °C for 8 h. The reaction mixture was poured onto water (50 mL) and the solid formed was filtered, dried and crystallized from DMF.

*4.1.9.3. 6-(4-Chlorophenyl)-3,8-diphenyl-1H-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**11a**).* Yield: 52%; mp: 323–324 °C; IR (cm^{-1}): 3350 (NH), 1680 (CO); ^1H NMR (DMSO-d_6) δ ppm 7.3–8.3 (m, 15 H, Ar–H), 12.7 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{26}H_{16}ClN_5O$: C, 69.41; H, 3.58; N, 15.57. Found: C, 69.62; H, 3.48; N, 15.90.

*4.1.9.4. 6-(4-Chlorophenyl)-3-(4-methylphenyl)-8-phenyl-1H-pyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**11b**).* Yield: 72%; mp: 342–343 °C; IR (cm^{-1}): 3400 (NH), 1680 (CO); ^1H NMR (DMSO-d_6) δ ppm 2.3 (s, 3H, CH_3), 7.2–8.2 (m, 14 H, Ar–H), 12.8 (br s, 1H, NH, D_2O exchangeable); MS *m*/*z*: 465 [$(M+2)^+$, 2.68%], 463 [M^+, 6.11%], 450 [100%], 77 [69.64%]; Anal. Calcd for $C_{27}H_{18}ClN_5O$: C, 69.90; H, 3.91; N, 15.10. Found: C, 70.28; H, 3.91; N, 15.42.

*4.1.9.5. 3,6-Di(4-chlorophenyl)-8-phenyl-1H-pyrido[2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**11c**).* Yield: 66%; mp: > 330 °C; IR

(cm^{-1}): 3350 (NH), 1680 (CO); ^1H NMR (DMSO-d_6) δ ppm 7.3–8.3 (m, 14 H, Ar–H), 12.7 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{26}H_{15}Cl_2N_5O$: C, 64.48; H, 3.12; N, 14.46. Found: C, 64.50; H, 3.23; N, 14.67.

*4.1.9.6. 6-(4-Chlorophenyl)-3-(3-nitrophenyl)-8-phenyl-1H-pyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**11d**).* Yield: 64%; mp: > 330 °C; IR (cm^{-1}): 3390 (NH), 1708 (CO), 1550, 1339 (NO_2); ^1H NMR (DMSO-d_6) δ ppm 7.2–8.3 (m, 14 H, Ar–H), 12.6 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{26}H_{15}ClN_6O_3$: C, 63.10; H, 3.06; N, 16.98. Found: C, 63.44; H, 3.22; N, 16.90.

*4.1.10. 3-Amino-6-(4-chlorophenyl)-8-phenyl-1H-pyrido[2,3-d] [1,2,4]triazolo[4,3-a]pyrimidin-5-one (**12**)*

A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.74 g, 2 mmol) and ammonium thiocyanate (2.38 g, 30 mmol) in glacial acetic acid (20 mL) was heated under reflux for 10 h. The reaction mixture was cooled, poured into water (50 mL) and the solid formed was filtered, dried and crystallized from acetic acid. Yield: 77%; mp: > 330 °C; IR (cm^{-1}): 3417, 3394 (NH/NH_2), 1712 (CO); ^1H NMR (DMSO-d_6) δ ppm 6.9 (s, 2H, NH_2, D_2O exchangeable), 7.4–8.6 (m, 10 H, Ar–H), 13.6 (s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{20}H_{13}ClN_6O$: C, 61.78; H, 3.37; N, 21.61. Found: C, 61.70; H, 3.12; N, 21.38.

*4.1.11. 3-Chloromethyl-6-(4-chlorophenyl)-8-phenyl-1H-pyrido [2,3-d][1,2,4]triazolo[4,3-a]pyrimidin-5-one (**13**)*

A mixture of 2-hydrazinopyrido[2,3-*d*]pyrimidine **4** (0.74 g, 2 mmol) and chloroacetyl chloride (0.23 g, 2 mmol) in pyridine (15 mL) was heated under reflux for 15 h. The reaction mixture was cooled, poured into water (50 mL) and the solid was filtered, dried and crystallized from acetic acid. Yield: 72%; mp: > 330 °C; IR (cm^{-1}): 3390 (NH), 1690 (CO); ^1H NMR (DMSO-d_6) δ ppm 4.2 (s, 2H, CH_2Cl), 7.4–8.5 (m, 10H, Ar–H), 13.5 (br s, 1H, NH, D_2O exchangeable); Anal. Calcd for $C_{21}H_{13}Cl_2N_5O$: C, 59.73; H, 3.10; N, 16.79. Found: C, 60.07; H, 3.15; N, 16.77.

4.2. Biological testing

4.2.1. Materials and methods

The human breast adenocarcinoma cell line (MCF7) was obtained as a gift from NCI, MD, USA.

All chemicals and solvents were purchased from Sigma–Aldrich.

4.2.1.1. Measurement of potential cytotoxicity. The cytotoxic activity of the newly synthesized compounds was measured *in vitro* on human breast adenocarcinoma cell line (MCF7) using Sulforhodamine-B stain (SRB) assay applying 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 test compounds to allow attachment of the cells to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the test compound (0, 1, 2.5, 5 and 10 μg/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the test 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% (wt./vol) 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.

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