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Scientific paper

Synthesis, Characterization and Cytotoxicity of Substituted [1]Benzothieno[3,2-*e*][1,2,4]triazolo [4,3-*a*]pyrimidines

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Abstract

A new series of 4-benzyl-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidines was synthesized motivated by the widely reported anticancer activity of thieno[2,3-*d*]pyrimidines and triazolothienopyrimidines. The *in vitro* cytotoxic activity of some selected compounds was evaluated against two human cell lines: prostate cancer (PC-3) and colon cancer (HCT-116). A preliminary study of the structure–activity relationship of the target compounds was discussed. Most of the synthesized compounds showed remarkable activity on the tested cell lines, while compound **16c** had the highest potency against the PC-3 cell line with an IC_{50} of 5.48 μM compared to Doxorubicin (IC_{50} = 7.7 μM), the reference standard used in this study. On the other hand, **6c** and **18c** were the most active against HCT-116 (IC_{50} = 6.12 and 6.56 μM, respectively) relative to IC_{50} = 15.82 μM of the standard. Thus, some of the synthesized thienopyrimidine derivatives, specially **6c**, **16c** and **18c**, have the potential to be developed into potent anticancer agents.

Keywords: Thienopyrimidines; 1,2,4-Triazoles; Anticancer activity; PC-3; HCT-116

1. Introduction

Despite decades of research that have resulted in an enormous leap in cancer therapy, cancer remains a major cause of death worldwide thus there is a continuous need for the discovery and development of new anticancer agents.[1,2] It is worth mentioning that 60% of world's total new annual cases occur in Africa, Asia and Central and South America.[3]

Thiophenes have been reported to possess interesting biological activities particularly as anticancer agents.[4,5] Many research groups reported the synthesis of biologically active thiophene derivatives through the well-known Gewald reaction.[6,7] As an example, Mohareb *et al.*[8] synthesized some thiophene derivatives and investigated their antitumor activity. The prepared compounds exhibited GI_{50} ranging from 0.02 to 0.08 μM against MCF-7, NCI-H450 and SF-268 cell lines compared to Doxorubicin.

Meanwhile, thieno[2,3-*d*]pyrimidines represent an important class of bioactive heterocycles attracting much attention due to their wide range of biological and pharmaceutical activities.[9,10]

The presence of pyrimidine ring in the basic building scaffolds of DNA and RNA modules (thymine, cytosine and uracil) is probably the reason of their diverse biological activities.[11] In addition, the tricyclic system, cycloalkylthieno[2,3-*d*]pyrimidine, which is considered to be a bioisostere of quinazoline, has been used as a core for the mechanism-based design and synthesis of a variety of compounds for anticancer therapy.[12–16]

On the other hand, the 1,2,4-triazole heterocycle is of great value as a building block in the structure of several anticancer drug candidates.[11,17,18] Letrozole, Anastrozole and Ribavirin are representative examples of commercially available anticancer drugs containing triazole scaffolds (Fig. 1).[19–21] Among these heterocycles, the mercapto substituted 1,2,4-triazole ring systems have been well studied and so far a variety of biological activities have reported for them.[17,22,23]

Recently, 4-amino-1,2,4-triazol-3-thione was used as an intermediate for the synthesis of several biologically active fused heterocyclic compounds where the amino and mercapto groups are appropriate nucleophile centers for many chemical modifications.[24] Further, many alkylated

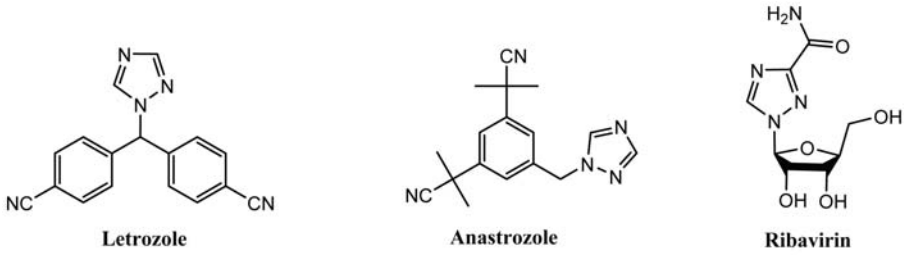


Figure 1. Chemical structures of anticancer drugs containing triazole moiety available on the market.

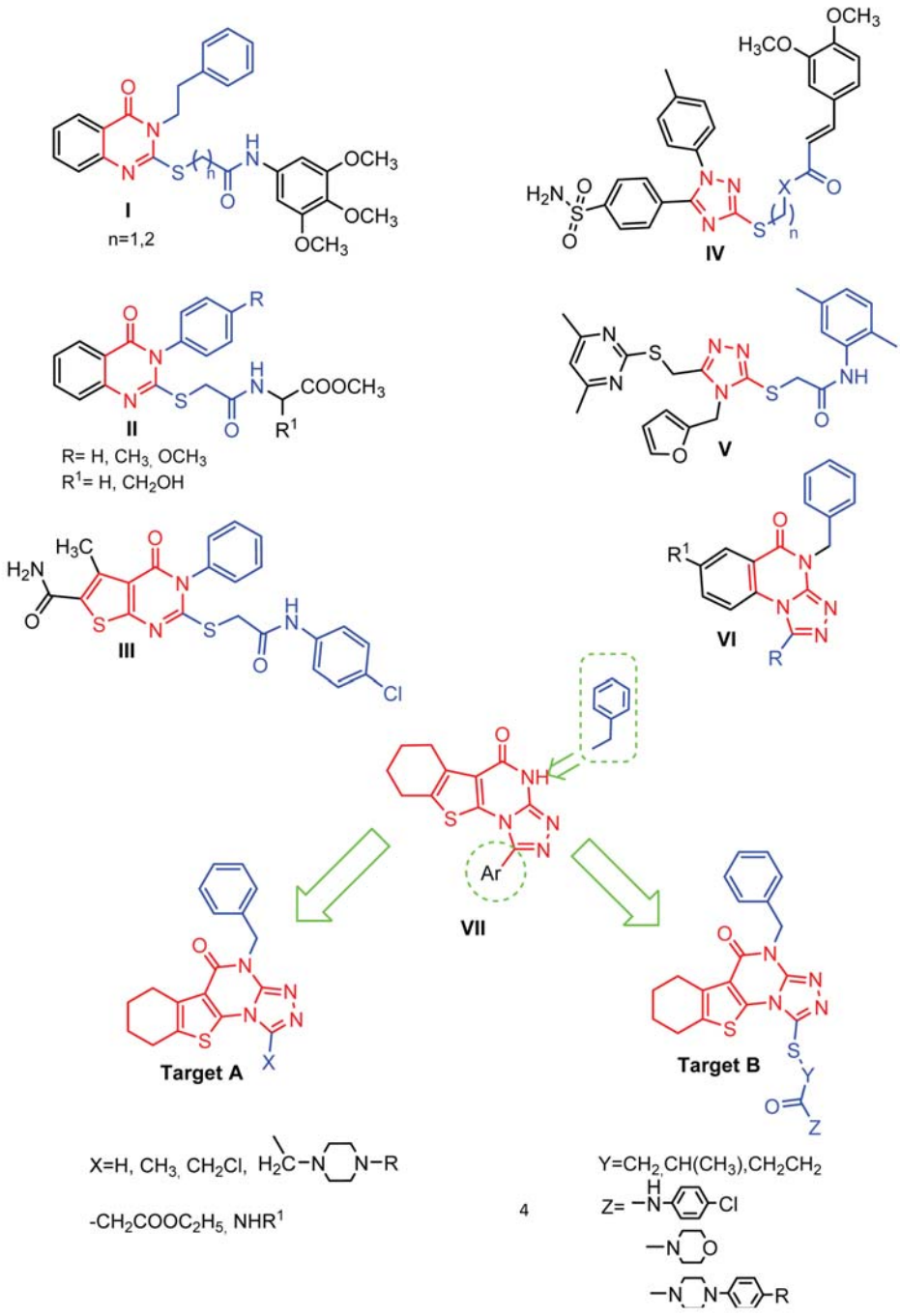


Figure 2. Structures of some reported pyrimidines, thieno[2,3-*d*]pyrimidines and triazole derivatives with cytotoxic activity showing the possible chemical optimization to obtain target compounds A and B

mercapto 1,2,4-triazoles linked to various aromatic ring systems either through amide or ester linkages have been reported to exhibit significant antitumor activities.[25–27]

In the last few years, many research groups investigated thienopyrimidine derivatives fused to 1,2,4-triazole moiety as potential cytotoxic agents.[28–30] For example, the fusion of a triazole ring to cycloalkylthieno[2,3-*d*]pyrimidine (**VII**) showed significant *in vitro* cytotoxic activity against human colorectal cancer cells (HCT-116) (IC_{50} = 2.8 μg/mL) compared to the reference drug Doxorubicin (Fig. 2).[31]

In our search for new classes of potential anticancer agents, the aforementioned findings prompted us to synthesize a series of 4-benzyl[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*] pyrimidines with varying the substitution at position 1 (Target compound A) in order to investigate the effect of combining these bioactive moieties on the anticancer activity. Moreover, we aimed in this work to prepare a series of 4-benzyl[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*] pyrimidines bearing various *S*-(substituted amino alkyl) moieties at position 1 (Target compound B) to act as cytotoxic agents. In this series, different alkyl linkers and different aliphatic and aromatic amines were used to study the effect of these variations on the cytotoxic activity. Some selected compounds were tested for possible anti-cancer activity against two cell lines (PC-3 and HCT-116).

2. Experimental

2. 1. Chemistry

All melting points were determined with Stuart SMP10 apparatus and the values given are uncorrected. IR spectra (KBr, cm^{-1}) were determined on Shimadzu IR 8400s spectrophotometer (Faculty of Pharmacy, Cairo University, Egypt). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Mercury 300-BB 300 MHz (Microanalytical Center, Faculty of Science, Cairo University, Egypt) and Bruker 400-BB 400 MHz spectrometers (Microanalytical Unit, Faculty of Pharmacy, Cairo University, Egypt) using TMS as the internal standard. Chemical shift values are given in ppm on δ scale. Mass spectra were recorded on Hewlett Packard 5988 spectrophotometer (Microanalytical Center, Faculty of Science, Cairo University, Egypt). Elemental analyses were carried out at the Regional center for Mycology and Biotechnology, Faculty of Pharmacy, Al Azhar University, Egypt; values found were within ±0.35% of the theoretical ones. Progress of the reactions was monitored by TLC using aluminum sheets precoated with UV fluorescent silica gel (Merck 60F 254) and visualized using UV lamp. The solvent system used was chloroform : benzene : methanol [9:5:2].

The starting compounds, ethyl 2-amino-4,5,6,7-tetrahydro[1]benzothiophene-3-carboxylate (**1**),[32] 3-benzyl-2-sulphanyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4(*3H*)-one (**2**)[33] and the α- and β-chloroamides (**13a–d**, **14a–d**, **15a–d**)[34–40] were prepared according to reported procedures.

2. 1. 1. 3-Benzyl-2-hydrazino-5,6,7,8-tetrahydro [1]benzothieno[2,3-*d*]pyrimidin-4(3*H*)-one (3)

A mixture of 3-benzyl-2-sulphanyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4(*3H*)-one (**2**) (1.64 g, 50 mmol) and hydrazine hydrate 99–100% (7 mL, 140 mmol) in dry pyridine (25 mL) was heated under reflux for 25 h. The mixture was evaporated under reduced pressure and the residue was treated with ethanol. The solid product was collected by filtration, washed with ethanol, dried and crystallized from ethyl acetate.

Yield: 50%; mp: 226–228 °C; IR (KBr, cm^{-1}): 3248–3211 (NH, NH_2), 3061–3035 (CH aromatic), 2916, 2848 (CH aliphatic), 1666 (C=O), 1624, 1568, 1529 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.75–1.78 (m, 4H, 2 × CH_2 at C-6, C-7), 2.62–2.74 (m, 2H, CH_2 at C-5), 2.79–2.81(m, 2H, CH_2 at C-8), 5.21 (s, 2H, N$\underline{CH_2}C_6H_5$), 6.98 (s, 1H, NH, D_2O exchangeable), 7.15–7.34 (m, 5H, Ar-H); EI-MS *m/z* 326 (M^+, 26.29%); Anal. Calcd for $C_{17}H_{18}N_4OS$ (326.42): C, 62.55; H, 5.56; N, 17.16. Found: C, 62.74; H, 5.64; N, 17.38.

2. 1. 2. 4-Benzyl-6,7,8,9-tetrahydro[1]benzothieno [3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5 (4*H*)-one (4a)

A mixture of 3-benzyl-2-hydrazino-5,6,7,8-tetrahydro[1]benzothieno[2,3-*d*]pyrimidin-4(*3H*)-one (**3**) (0.32 g, 1 mmol) and formic acid (5 mL, 130 mmol) was heated under reflux for 4 h. The white precipitate formed upon cooling was collected by filtration, washed with water, dried and crystallized from ethyl acetate.

Yield: 78%; mp: 198–200 °C; IR (KBr, cm^{-1}): 3115 (CH aromatic), 2922, 2850 (CH aliphatic), 1670 (C=O), 1595, 1552, 1517 (C=C aromatic); ^{1}H-NMR ($CDCl_3$-d_6) δ: 1.81–1.92 (m, 4H, 2 × CH_2 at C-7, C-8), 2.76–2.79 (m, 2H, CH_2 at C-6), 3.02–3.06 (m, 2H, CH_2 at C-9), 5.48 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.26–7.67 (m, 5H, Ar-H), 8.37 (s, 1H, aromatic CH); EI-MS *m/z* 336 (M^+, 35.04), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{18}H_{16}N_4OS$ (336.4): C, 64.26; H, 4.79; N, 16.65. Found: C, 64.42; H, 4.86; N, 16.90.

2. 1. 3. 4-Benzyl-1-methyl-6,7,8,9-tetrahydro [1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*] pyrimidin-5(4*H*)-one (4b)

A mixture of **3** (0.32 g, 1 mmol) and acetic acid (10 mL, 70 mmol) was heated under reflux for 6 h. The reaction mixture was poured onto ice cold water (25 mL). The white precipitate formed was collected by filtration, washed with water, dried and crystallized from acetonitrile.

Yield: 85%; mp: 242–244 °C; IR (KBr, cm^{-1}): 3061, 3043 (CH aromatic), 2937, 2870 (CH aliphatic), 1664 (C=O), 1593, 1558, 1541 (C=C aromatic); ^{1}H-NMR ($CDCl_3$-d_6) δ: 1.83–1.91 (m, 4H, 2 × CH_2 at C-7, C-8), 2.75 (s, 3H, CH_3), 2.77–2.78 (m, 2H, CH_2 at C-6), 3.04–3.07 (m, 2H, CH_2 at C-9), 5.44 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.22–7.66 (m, 5H, Ar-H); ^{13}C-NMR ($CDCl_3$-d_6) δ: 12.07, 22.06, 22.97, 24.92, 25.63, 45.94, 118.29, 128.15, 128.63, 129.73, 130.91, 134.06, 136.12, 138.67, 144.07, 149.09, 156.29; EI-MS *m/z* 350 (M^+, 64.90), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{19}H_{18}N_4OS$ (350.42): C, 65.12; H, 5.18; N, 15.99. Found: C, 65.38; H, 5.29; N, 16.31.

2. 1. 4. 1-Chloromethyl-4-benzyl-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo [4,3-*a*] pyrimidin-5(4*H*)-one (5)

To a solution of **3** (1 g, 3 mmol) in dry DMF (10 mL), chloroacetyl chloride (1.5 mL, 20 mmol) was added dropwise with cooling. The solution was then heated under reflux in a boiling water bath for 9 h. After cooling, the reaction mixture was poured onto ice-cold water and the suspension formed was stirred at room temperature for 2 h. The separated solid was collected by filtration, washed with cold water, dried and crystallized from methanol.

Yield: 88%; mp: 188–190 °C; IR (KBr, cm^{-1}): 3080, 3040 (CH aromatic), 2939, 2852 (CH aliphatic), 1681 (C=O), 1622, 1591, 1550 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.74–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.76–2.80 (m, 2H, CH_2 at C-6), 2.82–2.87 (m, 2H, CH_2 at C-9), 5.11 (s, 2H, $\underline{CH_2}Cl$), 5.29 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.15–7.34 (m, 5H, Ar-H); EI-MS *m/z* 386 (M+2, 3.9); 384 (M^+, 16.95%); Anal. Calcd for $C_{19}H_{17}ClN_4OS$ (384.87): C, 59.29; H, 4.45; N, 14.56. Found: C, 59.41; H, 4.52; N, 14.71.

2. 1. 5. General procedure for the preparation of compounds 6a–d

A mixture of **5** (0.25 g, 0.6 mmol) and the appropriate *N*-substituted piperazine (4 mmol) in absolute ethanol (30 mL) was heated under reflux for 6 h. The product obtained was collected by filtration, washed with water and crystallized from the suitable solvent.

4-Benzyl-1-[[4-methylpiperazin-1-yl]methyl]-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (6a). Crystallized from aqueous ethanol; yield: 36%; mp: 172–174 °C; IR (KBr, cm^{-1}): 3040, 3020 (CH aromatic), 2922, 2850 (CH aliphatic), 1677 (C=O), 1591 (C=C aromatic); ^{1}H-NMR ($CDCl_3$-d_6) δ: 1.86–1.92 (m, 4H, 2 × CH_2 at C-7, C-8), 2.27 (s, 3H, CH_3), 2.43–2.50 (m, 4H, 2 × CH_2 piperazine), 2.63–2.70 (m, 2H, CH_2 at C-6), 2.78–2.81 (m, 2H, CH_2 at C-9), 3.06–3.10 (m, 4H, 2 × CH_2 piperazine), 3.88 (s, 2H, CH_2), 5.46 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.28–7.69 (m, 5H, Ar-H); EI-MS *m/z* 448 (M^+, 0.57), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{24}H_{28}N_6OS$ (448.56): C, 64.26; H, 6.29; N, 18.73. Found: C, 64.38; H, 6.37; N, 18.56.

4-Benzyl-1-[[4-phenylpiperazin-1-yl]methyl]-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (6b). Crystallized from ethyl acetate; yield: 48%; mp: 228–230 °C; IR (KBr, cm^{-1}): 3057, 3032 (CH aromatic), 2941, 2918, 2848, 2821 (CH aliphatic), 1672 (C=O), 1587, 1558, 1539 (C=C aromatic); ^{1}H-NMR ($CDCl_3$-d_6) δ: 1.85–1.87 (m, 4H, 2 × CH_2 at C-7, C-8), 2.77–2.80 (m, 6H, CH_2 at C-6 and 2 × CH_2 piperazine), 3.05–3.10 (m, 2H, CH_2 at C-9), 3.18–3.19 (m, 4H, 2 × CH_2 piperazine), 3.95 (s, 2H, CH_2), 5.47 (s, 2H, N-$\underline{CH_2}$-C_6H_5), 6.83–7.70 (m, 10H, Ar-H); EI-MS *m/z* 511 (M+1, 4.13), 510 (M^+, 6.27%); Anal. Calcd for $C_{29}H_{30}N_6OS$ (510.63): C, 68.21; H, 5.92; N, 16.46. Found: C, 68.44; H, 5.98; N, 16.82.

4-Benzyl-1-[[4-(4-chlorophenyl)piperazin-1-yl]methyl]-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (6c). Crystallized from ethyl acetate; yield: 51%; mp: 254–256 °C; IR (KBr, cm^{-1}): 3100, 3040 (CH aromatic), 2929, 2819 (CH aliphatic), 1670 (C=O), 1581, 1550, 1510 (C=C aromatic); ^{1}H-NMR ($CDCl_3$-d_6) δ: 1.86–1.88 (m, 4H, 2 × CH_2 at C-7, C-8), 2.77–2.80 (m, 6H, CH_2 at C-6 and 2 × CH_2 piperazine), 3.05–3.10 (m, 2H, CH_2 at C-9), 3.12–3.13 (m, 4H, 2 × CH_2 piperazine), 3.96 (s, 2H, CH_2) 5.47 (s, 2H, N-$\underline{CH_2}$-C_6H_5), 6.87–7.70 (m, 9H, Ar-H); EI-MS *m/z* 546 (M+2, 32.02), 544 (M^+, 37.08%); Anal. Calcd for $C_{29}H_{29}ClN_6OS$ (545.08): C, 63.90; H, 5.36; N, 15.42. Found: C, 64.07; H, 5.44; N, 15.67.

4-Benzyl-1-[[4-(4-methoxymphenyl)piperazin-1-yl]methyl]-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (6d). Crystallized from acetonitrile; yield: 37%; mp: 238–240 °C; IR (KBr, cm^{-1}): 3040, 3000 (CH aromatic), 2926, 2808 (CH aliphatic), 1681 (C=O), 1591, 1556, 1535 (C=C aromatic); ^{1}H-NMR ($CDCl_3$-d_6) δ: 1.85–1.87 (m, 4H, 2 × CH_2 at C-7, C-8), 2.75–2.78 (m, 6H, CH_2 at C-6 and 2 × CH_2 piperazine), 3.05–3.09 (m, 6H, CH_2 at C-9 and 2 × CH_2 piperazine), 3.75 (s, 3H, OCH_3), 3.94 (s, 2H, CH_2) 5.47 (s, 2H, N-$\underline{CH_2}$-C_6H_5), 6.83–7.70 (m, 9H, Ar-H); EI-MS *m/z* 541 (M+1, 5.84), 540 (M^+, 15.74), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{30}H_{32}N_6O_2S$ (540.68): C, 66.64; H, 5.97; N, 15.54. Found: C, 66.88; H, 6.05; N, 15.66.

2. 1. 6. 4-Benzyl-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-1,5(2H,4*H*)-dione (7)

A mixture of **3** (0.64 g, 2 mmol) and *N,N*-carbonyldiimidazole (CDI) (0.7 g, 4.3 mmol) in dry benzene (30 mL) was heated under reflux for 15 h. After cooling,

the solvent was evaporated under reduced pressure and the residue was triturated with cold water. The solid product was collected by filtration, dried and crystallized from acetonitrile.

Yield: 81%; mp: 306–308 °C; IR (KBr, cm^{-1}): 3170 (NH), 3055, 3034 (CH aromatic), 2933, 2852 (CH aliphatic), 1720, 1683 (2 × C=O), 1610, 1560, 1523 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.78–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.73–2.80 (m, 2H, CH_2 at C-6), 2.81–2.84 (m, 2H, CH_2 at C-9), 5.05 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.28–7.35 (m, 5H, Ar-H), 12.0 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6) δ: 22.03, 22.86, 24.47, 25.22, 43.86, 115.40, 127.84, 128.14, 128.80, 130.05, 131.47, 136.45, 138.97, 141.21, 149.17, 156.63; EI-MS *m/z* 352 (M^+, 32.62), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{18}H_{16}N_4O_2S$ (352.41): C, 61.35; H, 4.58; N, 15.90. Found: C, 61.54; H, 4.65; N, 15.88.

2. 1. 7. General procedure for the preparation of compounds 8a–e

A mixture of **3** (0.32 g, 1 mmol) and the appropriate isothiocyanate (2 mmol) in absolute ethanol (30 mL) was heated under reflux for 8 h. The precipitated product was collected by filtration, dried and crystallized from ethanol/$CHCl_3$ (2:1).

4-Benzyl-1-methylamino-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (8a). Yield: 43%; mp: 206–208 °C; IR (KBr, cm^{-1}): 3370, 3196 (NH), 2944, 2880 (CH aliphatic), 1681 (C=O), 1575, 1537, 1506 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.74–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.62–2.65 (m, 2H, CH_2 at C-6), 2.78–2.81 (m, 2H, CH_2 at C-9), 2.81 (s, 3H, CH_3), 5.22 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.19–7.38 (m, 5H, Ar-H), 9.29 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6) δ: 22.34, 23.10, 24.76, 25.67, 31.21, 43.31, 115.03, 127.09, 127.56, 127.66, 128.86, 130.82, 136.52, 138, 151.17, 158.15, 164.13; EI-MS *m/z* 365 (M^+, 68.95), 91 ($[C_7H_7]^+$, 97.00%); Anal. Calcd for $C_{19}H_{19}N_5OS$ (365.45): C, 62.44; H, 5.24; N, 19.16. Found: C, 62.61; H, 5.30; N, 19.34.

4-Benzyl-1-ethylamino-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (8b). Yield: 46%; mp: 200–202 °C; IR (KBr, cm^{-1}): 3358, 3257, 3169 (NH), 2972, 2848 (CH aliphatic), 1681 (C=O), 1571, 1535, 1506 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 0.96 (t, *J* = 7.2 Hz, 3H, CH_3), 1.76–1.79 (m, 4H, 2 × CH_2 at C-7, C-8), 2.65–2.70 (m, 2H, CH_2 at C-6), 2.80–2.85 (m, 2H, CH_2 at C-9), 3.39 (q, *J* = 7.2 Hz, 2H, $\underline{CH_2}$-CH_3), 5.26 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.19–7.36 (m, 5H, Ar-H), 9.30 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6) δ: 14.28, 21.80, 22.57, 24.24, 25.14, 38.20, 42.64, 126.56, 127.03, 128.35, 130, 130.32, 133.61, 136, 150.52, 157.64, 163.8; EI-MS *m/z* 379 (M^+, 100), 91 ($[C_7H_7]^+$, 97.00%).

4-Benzyl-1-butylamino-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (8c). Yield: 30%; mp: 172–174 °C; IR (KBr, cm^{-1}): 3360, 3178 (NH), 2924, 2850 (CH aliphatic), 1685 (C=O), 1535, 1454 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 0.76 (t, *J* = 14.7 Hz, 3H, CH_3), 1.18–1.23 (m, 2H, $\underline{CH_2}$-CH_3), 1.31–1.36 (m, 2H, CH_2-$\underline{CH_2}$-CH_2), 1.75–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.61–2.65 (m, 2H, CH_2 at C-6), 2.75–2.77 (m, 2H, CH_2 at C-9), 3.36 (t, *J* = 12.6 Hz, 2H, NH-$\underline{CH_2}$), 5.19 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.18–7.38 (m, 5H, Ar-H), 9.20 (s, 1H, NH, D_2O exchangeable); EI-MS *m/z* 407 (M^+, 1.32), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{22}H_{25}N_5OS$ (407.53): C, 64.84; H, 6.18; N, 17.18. Found: C, 65.01; H, 6.22; N, 17.39.

4-Benzyl-1-allylamino-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (8d). Yield: 38%; mp: 184–186 °C; IR (KBr, cm^{-1}): 3360, 3167 (NH), 2924, 2850 (CH aliphatic), 1685 (C=O), 1651 (C=N), 1531, 1454 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.75–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.64–2.70 (m, 2H, CH_2 at C-6), 2.79–2.82 (m, 2H, CH_2 at C-9), 4.00–4.08 (m, 2H, CH_2 allylic), 4.98–5.01 (m, 1H, $\underline{CH_2}$=CH), 5.11–5.14 (m, 1H, $\underline{CH_2}$=CH), 5.24 (s, 2H, N$\underline{CH_2}C_6H_5$), 5.69–5.74 (m,1H, CH_2=$\underline{CH}$), 7.18–7.33 (m, 5H, Ar-H), 9.40 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6) δ: 21.82, 22.59, 24.23, 25.16, 42.73, 45.50, 115, 117, 126.59, 127, 128.31, 130.32, 131, 134.54, 136.4, 138.6, 151, 158.2, 164.1; EI-MS *m/z* 391 (M^+, 2.27), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{21}H_{21}N_5OS$ (391.49): C, 64.43; H, 5.41; N, 17.89. Found: C, 64.67; H, 5.48; N, 18.04.

4-Benzyl-1-[(4-methoxyphenyl)amino]-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*] pyrim idin-5(4*H*)-one (8e). Yield: 65%; mp: 244–246 °C; IR (KBr, cm^{-1}): 3215 (NH), 3111, 3070 (CH aromatic), 2941, 2835 (CH aliphatic), 1683 (C=O), 1618 (C=N), 1543, 1512, 1487 (C= C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.77–1.79 (m, 4H, 2 × CH_2 at C-7, C-8), 2.72–2.79 (m, 2H, CH_2 at C-6), 2.80–2.86 (m, 2H, CH_2 at C-9), 3.74 (s, 3H, OCH_3), 5.14 (s, 2H, N$\underline{CH_2}C_6H_5$), 6.87–7.37 (m, 9H, Ar-H), 9.39 (s, 1H, NH, D_2O exchangeable); EI-MS *m/z* 457 (M^+, 0.75), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{25}H_{23}N_5O_2S$ (457.55): C, 65.63; H, 5.07; N, 15.31. Found: C, 65.79; H, 5.12; N, 15.47.

2. 1. 8. Ethyl(4-benzyl-5-oxo-4,5-dihydro-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4] triazolo[4,3-*a*] pyrimidin-1-yl) acetate (9)

A mixture of **3** (0.32 g, 1 mmol) and diethyl malonate (2 mL, 13 mmol) was refluxed for 9 h. The reaction was allowed to cool, the formed residue was triturated with ethanol, collected by filtration, dried and crystallized from isopropanol to yield the title compound **9**.

Yield: 41%; mp: 220–222 °C; IR (KBr, cm^{-1}): 3035, 3055 (CH aromatic), 2935, 2854 (CH aliphatic), 1732, 1678 (2 × C=O), 1635 (C=N), 1589, 1539, 1504 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.18 (t, J = 7.2 Hz, 3H, CH_3), 1.78–1.82 (m, 4H, 2 × CH_2 at C-7, C-8), 2.63 (s, 2H, $\underline{CH_2}$-CO), 2.77–2.81 (m, 2H, CH_2 at C-6), 2.91–2.98 (m, 2H, CH_2 at C-9), 4.16 (q, J = 7.2 Hz, 2H, $\underline{CH_2}$-CH_3), 5.28 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.28–7.37 (m, 5H, Ar-H); ^{13}C-NMR (DMSO-d_6) δ: 14.45, 21.92, 22.77, 24.55, 25.52, 32.31, 45.51, 62.0, 117.61, 127.99, 128.39, 128.86, 131.32, 132.78, 136.59, 138.98, 142.06, 149.17, 155.17, 168.17; EI-MS *m/z* 422 (M^+, 60.13), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{22}H_{22}N_4O_3S$ (422.46): C, 62.54; H, 5.25; N, 13.26. Found: C, 62.71; H, 5.34; N, 13.48.

2. 1. 9. 4-Benzoyl-1-(3-benzyl-5,6,7,8-tetrahydro-4-oxo-3,4-dihydro[1]benzothieno[2,3-*d*]pyrimidin-2-yl)thiosemicarbazide (10)

To an ice cold solution of ammonium thiocyanate (0.17 g, 2 mmol) in dry acetone (5 mL), a solution of benzoyl chloride (0.3 mL, 2 mmol) in acetone (5 mL) was added dropwise. An ice-cold suspension of **3** (0.34 g, 1 mmol) in acetone (15 mL) was added to the previous mixture. The reaction mixture was heated on a water-bath for 15 h. The reaction mixture was cooled and filtered. The filtrate was evaporated and the obtained product was crystallized from ethanol/$CHCl_3$ (2:1).

Yield: 33%; mp: 114–116 °C; IR (KBr, cm^{-1}): 3346, 3159 (NH), 3057, 3030 (CH aromatic), 2927, 2856 (CH aliphatic), 1687, 1674 (2 × C=O), 1622 (C=N), 1598, 1581, 1539 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.76–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.65–2.73 (m, 2H, CH_2 at C-6), 2.81–2.84 (m, 2H, CH_2 at C-9), 5.31 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.27–7.91 (m, 10H, Ar-H), 9.79, 11.71, 12.49 (s, 3H, NH, D_2O exchangeable); EI-MS *m/z* 489 (M^+, 2.60), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{25}H_{23}N_5O_2S_2$ (489.61): C, 61.33; H, 4.73; N, 14.30. Found: C, 61.49; H, 4.79; N, 14.51.

2. 1. 10. 4-Benzyl-1-sulphanyl-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (11)

A mixture of **3** (1.4 g, 4.3 mmol), KOH (0.42 g, 7.5 mmol) and CS_2 (4.5 mL, 7.5 mmol) in absolute ethanol (70 mL) was heated under reflux for 25 h. The solvent was evaporated under reduced pressure. The obtained residue was dissolved in H_2O (20 mL) followed by acidification with dilute HCl (1 mL). The precipitated product was collected by filtration, dried and crystallized from methanol.

Yield: 50%; mp: 274–276 °C; IR (KBr, cm^{-1}): 3446 (NH), 3182, 3134 (CH aromatic), 2947, 2852 (CH aliphatic), 1662 (C=O), 1618 (C=N), 1585, 1516, 1489 (C=C aromatic), 1159 (C=S); ^{1}H-NMR (DMSO-d_6) δ: 1.77–1.82 (m, 4H, 2 × CH_2 at C-7, C-8), 2.77–2.80 (m, 2H, CH_2 at C-6), 2.89–2.92 (m, 2H, CH_2 at C-9), 5.14 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.26–7.38 (m, 5H, Ar-H), 14.06 (s, 1H, SH, D_2O exchangeable); EI-MS *m/z* 368 (M^+, 30.43), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{18}H_{16}N_4OS_2$ (368.48): C, 58.67; H, 4.38; N, 15.21. Found: C, 58.92; H, 4.41; N, 15.42.

2. 1. 11. General procedure for the alkylation of the thienotriazolopyrimidine 11 yielding 12a,b, 16a–d, 17a–d, 18a–d

A mixture of the triazolo derivative **11** (0.36 g, 1 mmol) and the appropriate alkyl iodide or α- and β-chloroamides (**13a–d, 14a–d, 15a–d**) (1.5 mmol) in the presence of anhydrous sodium acetate (5 mmol) in absolute ethanol (70 mL) was heated under reflux till TLC indicated completion of the reaction. The product precipitated was collected by filtration, dried and crystallized from the appropriate solvent.

4-Benzyl-1-methylsulphanyl-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (12a). Reaction time: 12 h, crystallized from ethanol, yield: 43%; mp: 246–248 °C; IR (KBr, cm^{-1}): 2916, 2846 (CH aliphatic), 1674 (C=O), 1585, 1546, 1508 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.76–1.83 (m, 4H, 2 × CH_2 at C-7, C-8), 2.62 (s, 3H, CH_3), 2.79–2.80 (m, 2H, CH_2 at C-6), 2.92–2.93 (m, 2H, CH_2 at C-9), 5.31 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.24–7.42 (m, 5H, Ar-H); EI-MS *m/z* 382 (M^+, 24.5), 91 ($[C_7H_7]^+$, 81.15%); Anal. Calcd for $C_{19}H_{18}N_4OS_2$ (382.5): C, 59.66; H, 4.74; N, 14.65. Found: C, 59.89; H, 4.79; N, 14.91.

4-Benzyl-1-ethylsulphanyl-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (12b). Reaction time: 15 h, crystallized from ethanol, yield: 46%; mp: 210–212 °C; IR (KBr, cm^{-1}): 2935, 2854 (CH aliphatic), 1670 (C=O), 1585, 1550, 1508 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.27 (t, 3H, CH_3), 1.75–1.82 (m, 4H, 2 × CH_2 at C-7, C-8), 2.77–2.81 (m, 2H, CH_2 at C-6), 2.90–2.94 (m, 2H, CH_2 at C-9), 3.05 (q, 2H, $\underline{CH_2}$-CH_3), 5.31 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.26–7.43 (m, 5H, Ar-H); EI-MS *m/z* 396 (M^+, 39.74), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{20}H_{20}N_4OS_2$ (396.53): C, 60.58; H, 5.08; N, 14.13. Found: C, 60.85; H, 5.14; N, 14.28.

***N*-(4-chlorophenyl)-2-[(4-benzyl-6,7,8,9-tetrahydro-5-oxo-4,5-dihydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-1-yl)sulfanyl]acetamide (16a).** Reaction time: 9.30 h, crystallized from ethyl acetate/ethanol, yield: 57%; mp: 238–240 °C; IR (KBr, cm^{-1}): 3259 (NH), 3190, 3064 (CH aromatic), 2941, 2858 (CH aliphatic), 1685 (br. 2 × C=O), 1591, 1548, 1506 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.76–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.65–2.70 (m, 2H, CH_2 at C-6), 2.88–2.90 (m, 2H, CH_2 at C-9), 3.84 (s, 2H, S$\underline{CH_2}$), 5.32 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.28–7.42 (m, 9H, Ar-H), 10.15 (s, 1H, NH,

D_2O exchangeable); EI-MS *m/z* 537 (M+2, 11.44), 535 (M^+, 25.2), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{26}H_{22}ClN_5O_2S_2$ (536.07): C, 58.25; H, 4.14; N, 13.06. Found: C, 58.44; H, 4.11; N, 13.21.

4-Benzyl-1-{[2-morpholino-2-oxoethyl]sulphanyl}-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (16b). Reaction time: 5.30 h, crystallized from acetonitrile, yield: 73%; mp: 256–258 °C; IR (KBr, cm^{-1}): 3020, 3000 (CH aromatic), 2966, 2870 (CH aliphatic), 1670, 1633 (2 × C=O), 1585, 1550, 1508 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.78–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.79–2.81 (m, 2H, CH_2 at C-6), 2.92–2.98 (m, 2H, CH_2 at C-9), 3.39 (t, *J* = 9.9 Hz, 4H, CH_2-N), 3.53 (t, *J* = 9.9 Hz, 4H, CH_2-O), 4.18 (s, 2H, S$\underline{CH_2}$), 5.32 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.26–7.42 (m, 5H, Ar-H); EI-MS *m/z* 495 (M^+, 5.25), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{24}H_{25}N_5O_3S_2$ (495.62): C, 58.16; H, 5.08; N, 14.13. Found: C, 58.42; H, 5.17; N, 14.29.

4-Benzyl-1-{[2-(4-phenylpiperazin-1-yl)-2-oxoethyl]sulphanyl}-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (16c). Reaction time: 5.30 h, crystallized from acetonitrile, yield: 65%; mp: 224–226 °C; IR (KBr, cm^{-1}): 3040, 3000 (CH aromatic), 2918, 2812 (CH aliphatic), 1672, 1635 (C=O), 1585, 1550, 1508 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.78–1.81 (m, 4H, 2 × CH_2 at C-7, C-8), 2.76–2.80 (m, 2H, CH_2 at C-6), 2.91–2.98 (m, 2H, CH_2 at C-9), 3.09–3.14 (m, 4H, 2 × CH_2 piperazine), 3.55–3.60 (m, 4H, 2 × CH_2 piperazine), 4.23 (s, 2H, S$\underline{CH_2}$), 5.32 (s, 2H, N$\underline{CH_2}C_6H_5$), 6.80–7.42 (m, 10H, Ar-H); EI-MS *m/z* 570 (M^+, 1.19), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{30}H_{30}N_6O_2S_2$ (570.73): C, 63.13; H, 5.30; N, 14.73. Found: C, 63.40; H, 5.36; N, 14.89.

4-Benzyl-1-{[2-[4-(4-methoxyphenyl)piperazin-1-yl]-2-oxoethyl]sulphanyl}-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (16d). Reaction time: 6.30 h, crystallized from ethyl acetate, yield: 83%; mp: 230–232 °C; IR (KBr, cm^{-1}): 3040, 3000 (CH aromatic), 2941, 2818 (CH aliphatic), 1672, 1635 (2 × C=O), 1585, 1548, 1510 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.77–1.79 (m, 4H, 2 × CH_2 at C-7, C-8), 2.76–2.80 (m, 2H, CH_2 at C-6), 2.91–2.99 (m, 6H, CH_2 at C-9 and 2 × CH_2 piperazine), 3.53–3.59 (m, 4H, 2 × CH_2 piperazine), 3.68 (s, 3H, OCH_3), 4.22 (s, 2H, S$\underline{CH_2}$), 5.32 (s, 2H, N$\underline{CH_2}C_6H_5$), 6.80–7.42 (m, 9H, Ar-H); EI-MS *m/z* 600 (M^+, 2.79), 232 (M–$C_{18}H_{16}N_4OS_2$, 100%); Anal. Calcd for $C_{31}H_{32}N_6O_3S_2$ (600.76): C, 61.98; H, 5.37; N, 13.99. Found: C, 62.17; H, 5.46; N, 14.12.

***N*-(4-chlorophenyl)-2-methyl-2-[(4-benzyl-6,7,8,9-tetrahydro-5-oxo-4,5-dihydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-1-yl)sulphanyl]acetamide (17a).** Reaction time: 9.30 h, crystallized from chloroform, yield: 60%; mp: 264–266 °C; IR (KBr, cm^{-1}): 3305, 3261, 3194 (NH), 3066 (CH aromatic), 2945, 2858 (CH aliphatic), 1681 (br. 2 × C=O), 1610, 1589, 1548 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.48 (d, *J* = 6.6 Hz, 3H, CH_3), 1.71–1.79 (m, 4H, 2 × CH_2 at C-7, C-8), 2.60–2.62 (m, 2H, CH_2 at C-6), 2.83–2.85 (m, 2H, CH_2 at C-9), 4.16 (q, *J* = 6.6 Hz, 1H, CH), 5.34 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.23–7.42 (m, 9H, Ar-H), 10.03 (s, 1H, NH, D_2O exchangeable); ^{13}C-NMR (DMSO-d_6) δ: 17.09, 21.43, 22.14, 23.8, 24.68, 44.89, 47.15, 117.43, 120.46, 126.83, 127.44, 127.84, 128.23, 128.31, 131.1, 131.97, 135.97, 137.67, 137.98, 138.53, 149.62, 155.47, 168.49; EI-MS *m/z* 551(M+2, 14.86), 549 (M^+, 19.21), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{27}H_{24}ClN_5O_2S_2$ (550.10): C, 58.95; H, 4.40; N, 12.73. Found: C, 59.17; H, 4.48; N, 12.85.

4-Benzyl-1-{[2-morpholino-1-methyl-2-oxoethyl]sulphanyl}-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (17b). Reaction time: 5 h, crystallized from ethyl acetate, yield: 65%; mp: 260–262 °C; IR (KBr, cm^{-1}): 2943, 2860 (CH aliphatic), 1670, 1635 (2 × C=O), 1587, 1550, 1508 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.45 (d, *J* = 6 Hz, 3H, CH_3), 1.80–1.85 (m, 4H, 2 × CH_2 at C-7, C-8), 2.78–2.79 (m, 2H, CH_2 at C-6), 2.91–2.95 (m, 2H, CH_2 at C-9), 3.46 (t, *J* = 11 Hz, 4H, CH_2-N), 3.52 (t, *J* = 11 Hz, 4H, CH_2-O), 4.56 (q, *J* = 6 Hz, 1H, CH), 5.33 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.26–7.43 (m, 5H, Ar-H); EI-MS *m/z* 509 (M^+, 2.41), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{25}H_{27}N_5O_3S_2$ (509.65): C, 58.92; H, 5.34; N, 13.74. Found: C, 59.13; H, 5.41; N, 13.87.

4-Benzyl-1-{[2-(4-phenylpiperazin-1-yl)-1-methyl-2-oxoethyl]sulphanyl}-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (17c). Reaction time: 7 h, crystallized from acetonitrile, yield: 63%; mp: 240–242 °C; IR (KBr, cm^{-1}): 3020, 3000 (CH aromatic), 2931, 2820 (CH aliphatic), 1670, 1629 (2 × C=O), 1598, 1583, 1548 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.48 (d, *J* = 6.6 Hz, 3H, CH_3), 1.73–1.78 (m, 4H, 2 × CH_2 at C-7, C-8), 2.89–2.92 (m, 2H, CH_2 at C-6), 3.02–3.08 (m, 2H, CH_2 at C-9), 3.14–3.20 (m, 4H, 2 × CH_2 piperazine), 3.59–3.70 (m, 4H, 2 × CH_2 piperazine), 4.61 (q, *J* = 6.6 Hz, 1H, CH), 5.33 (s, 2H, N$\underline{CH_2}C_6H_5$), 6.78–7.43 (m, 10H, Ar-H); EI-MS *m/z* 584 (M^+, 2.10), 216 (M–$C_{18}H_{16}N_4OS_2$, 100), 91 ($[C_7H_7]^+$, 72.31%); Anal. Calcd for $C_{31}H_{32}N_6O_2S_2$ (584.76): C, 63.67; H, 5.52; N, 14.37. Found: C, 63.81; H, 5.58; N, 14.59.

4-Benzyl-1-{[2-[4-(4-methoxyphenyl)piperazin-1-yl]-1-methyl-2-oxoethyl]sulphanyl}6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (17d). Reaction time: 7 h, crystallized from acetonitrile, yield: 83%; mp: 236–238 °C; IR (KBr, cm^{-1}):

2933, 2816 (CH aliphatic), 1670, 1629 (2 × C=O), 1585, 1548, 1510 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.47 (d, J = 7.2 Hz, 3H, CH_3), 1.74–1.79 (m, 4H, 2 × CH_2 at C-7, C-8), 2.65–2.67 (m, 4H, 2 × CH_2 piperazine), 2.89–2.91 (m, 2H, CH_2 at C-6), 2.97–3.01 (m, 2H, CH_2 at C-9), 3.49–3.58 (m, 4H, 2 × CH_2 piperazine), 3.68 (s, 3H, OCH_3), 4.60 (q, J = 7.2 Hz, 1H, CH), 5.33 (s, 2H, N$\underline{CH_2}C_6H_5$), 6.80–7.43 (m, 9H, Ar-H); ^{13}C-NMR (DMSO-d_6) δ: 19.35, 21.45, 22.18, 23.96, 24.78, 38.66, 45.27, 49.60, 50.04, 55.14, 114.22, 117.0, 120.50, 127.45, 127.95, 128.31, 131.50, 131.88, 135.97, 138.0, 144.86, 149.0, 153.28, 155.56, 168.54; EI-MS *m/z* 614 (M^+, 2.22), 246 (M–$C_{18}H_{16}N_4OS_2$, 100), 91 ($[C_7H_7]^+$, 48.71%); Anal. Calcd for $C_{32}H_{34}N_6O_3S_2$ (614.78): C, 62.52; H, 5.57; N, 13.67. Found: C, 62.74; H, 5.66; N, 13.89.

***N*-(4-chlorophenyl)-3-[(4-benzyl-6,7,8,9-tetrahydro-5-oxo-4,5-dihydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-1-yl)sulphanyl]propanamide (18a).** Reaction time: 34 h, crystallized from chloroform, yield: 68%; mp: 228–230 °C; IR (KBr, cm^{-1}): 3309, 3275 (NH), 3100, 3000 (CH aromatic), 2931, 2840 (CH aliphatic), 1681 (br. 2 × C=O), 1589, 1546 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.74–1.79 (m, 4H, 2 × CH_2 at C-7, C-8), 2.62–2.65 (m, 2H, CH_2 at C-6), 2.72 (t, J = 15 Hz, 2H, $\underline{CH_2}$-CH_2S), 2.83–2.85 (m, 2H, CH_2 at C-9), 3.31(t, J = 15 Hz, 2H, CH_2-$\underline{CH_2}$S), 5.30 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.22–7.45 (m, 9H, Ar-H), 9.99 (s, 1H, NH, D_2O exchangeable); EI-MS *m/z* 551.8 (M+2, 0.65), 549.8 (M^+, 1.27), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{27}H_{24}ClN_5O_2S_2$ (550.10): C, 58.95; H, 4.40; N, 12.73. Found: C, 59.12; H, 4.47; N, 12.91.

4-Benzyl-1-{[2-morpholino-3-oxopropyl]sulphanyl}-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (18b). Reaction time: 30 h, crystallized from chloroform, yield: 51%; mp: 198–200 °C; IR (KBr, cm^{-1}): 2947, 2862 (CH aliphatic), 1674, 1639 (2 × C=O), 1589, 1554, 1508 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.74–1.80 (m, 4H, 2 × CH_2 at C-7, C-8), 2.75–2.77 (m, 2H, CH_2 at C-6), 2.77 (t, J = 12.6 Hz, 2H, $\underline{CH_2}$-CH_2S), 2.91–2.95 (m, 2H, CH_2 at C-9), 3.22 (t, J = 12.6 Hz, 2H, CH_2-$\underline{CH_2}$S), 3.43–3.44 (t, 4H, CH_2-N), 3.49–3.51 (t, 4H, CH_2-O), 5.30 (s, 2H, N$\underline{CH_2}C_6H_5$), 7.25–7.41 (m, 5H, Ar-H); EI-MS *m/z* 509 (M^+, 0.30), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{25}H_{27}N_5O_3S_2$ (509.65): C, 58.92; H, 5.34; N, 13.74. Found: C, 59.21; H, 5.36; N, 13.89.

4-Benzyl-1-{[3-(4-phenylpiperazin-1-yl)-3-oxopropyl]sulphanyl}-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (18c). Reaction time: 26 h, crystallized from acetonitrile, yield: 40%; mp: 214–216 °C; IR (KBr, cm^{-1}): 2937, 2852 (CH aliphatic), 1676, 1643 (2 × C=O), 1585, 1552, 1508 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.70–1.71 (m, 4H, 2 × CH_2 at C-7, C-8), 2.70–2.81 (m, 4H, CH_2 piperazine), 2.84 (t, J = 6 Hz, 2H, $\underline{CH_2}$-CH_2S), 2.97–2.99 (m, 2H, CH_2 at C-6), 3.04–3.12 (m, 2H, CH_2 at C-9), 3.23 (t, J = 6 Hz, 2H, CH_2-$\underline{CH_2}$S), 3.46–3.50 (m, 4H, CH_2 piperazine), 5.30 (s, 2H, N$\underline{CH_2}C_6H_5$), 6.79–7.42 (m, 10H, Ar-H); ^{13}C-NMR (DMSO-d_6) δ: 21.39, 22.16, 23.98, 24.79, 31.18, 32.72, 44.83, 47.98, 48.35, 115.63, 119.17, 120.50, 127.37, 127.78, 128.26, 128.88, 131.60, 131.71, 136.02, 138.0, 140.66, 148.0, 150.60, 155.58, 168.22; EI-MS *m/z* 584 (M^+, 4.13), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{31}H_{32}N_6O_2S_2$ (584.76): C, 63.67; H, 5.52; N, 14.37. Found: C, 63.84; H, 5.63; N, 14.61.

4-Benzyl-1-{[3-[4-(4-methoxyphenyl)piperazin-1-yl]-3-oxopropyl]sulphanyl}-6,7,8,9-tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidin-5(4*H*)-one (18d). Reaction time: 29 h, crystallized from chloroform, yield: 42%; mp: 222–224 °C; IR (KBr, cm^{-1}): 3055, 3001 (CH aromatic), 2949, 2833 (CH aliphatic), 1674, 1641 (2 × C=O), 1587, 1554, 1510 (C=C aromatic); ^{1}H-NMR (DMSO-d_6) δ: 1.71–1.73 (m, 4H, 2 × CH_2 at C-7, C-8), 2.71–2.78 (m, 4H, 2 × CH_2 piperazine), 2.80 (t, J = 6.6 Hz, 2H, $\underline{CH_2}$-CH_2S), 2.83–2.92 (m, 4H, 2 × CH_2 at C-6 and C-9), 3.25 (t, J = 6.6 Hz, 2H, CH_2-$\underline{CH_2}$S), 3.45–3.55 (m, 4H, 2 × CH_2 piperazine), 3.68 (s, 3H, OCH_3), 5.31 (s, 2H, N$\underline{CH_2}C_6H_5$), 6.79–7.42 (m, 9H, Ar-H); EI-MS *m/z* 616 (M+2, 0.95), 614 (M^+, 4.66), 91 ($[C_7H_7]^+$, 100%); Anal. Calcd for $C_{32}H_{34}N_6O_3S_2$ (614.78): C, 62.52; H, 5.57; N, 13.67. Found: C, 62.70; H, 5.54; N, 13.84.

2. 2. In vitro Anticancer Screening

2. 2. 1. Materials and Methods

The prostate tumor cell line (PC-3) and the colon tumor cell line (HCT-116) were obtained frozen in liquid nitrogen (–180 °C) from the American Type Culture Collection (ATCC) and were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. All chemicals used in this study were of high analytical grade. They were obtained from either Sigma-Aldrich or Bio-Rad.

2. 2. 2. Measurement of Potential Cytotoxicity

The cytotoxic activity of some selected compounds was measured *in vitro* against human prostate cancer cell line (PC-3) and colon cancer cell line (HCT-116) at five different doses (0, 5.0, 12.5, 25.0 and 50.0 μg/mL). The screening was carried out at the Pharmacology Unit, Cancer Biology Department, National Cancer Institute, Cairo University using Sulforhodamine-B (SRB) assay, applying the method of Skehan *et al.*[41] as follows.

Cells were plated in 96 multi-well plate (104 cells/well) for 24 h before treatment with the tested compound to allow attachment to the wall of the plate. Different concentrations of the compounds (0, 5.0, 12.5, 25.0 and 50.0 μg/mL) were added to the cell monolayer in tri-

plicate and wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37 °C in atmosphere of 5% CO_2. After 48 h, cells were fixed, washed and stained with Sulforhodamine-B stain. Excess stain was washed with acetic acid and the attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration was plotted to get the survival curve of each tumor cell line. IC_{50} values (the concentration required for 50% inhibition of cell viability) were calculated using sigmodial dose response curve-fitting models (GraphPad, Prizm software incorporated), each concentration was repeated three times. The results are given in Table 1 and represented graphically in Fig. 3.

3. Results and Discussion

3. 1. Chemistry

The synthetic strategies adopted for the synthesis of the intermediate and final compounds are illustrated in Schemes 1 and 2. In Scheme 1, the starting compound ethyl 2-amino-4,5,6,7-tetrahydro[1]benzothiophene-3-carboxylate (**1**) was prepared according to the well-known Gewald procedure.[32] Reacting **1** with benzyl isothiocyanate in acetonitrile afforded the corresponding 3-benzyl-2-sulfanylthienopyrimidine derivative **2**. The **2** formed was treated with 99% hydrazine hydrate in dry pyridine to give the 2-hydrazino derivative **3**. Structural elucidation of **3** was based on IR and ^{1}H-NMR spectroscopy. Reacting the key intermediate **3** with formic acid or acetic acid induced cyclization to the corresponding triazolo derivatives **4a** and **4b**. IR and ^{1}H-NMR spectra confirmed the cyclization through the disappearance of NH and NH_2 signals. The presence of a signal at δ 12.07 ppm in ^{13}C-NMR verified the presence of the CH_3 group in **4b**. Compound **5** was obtained upon treatment of **3** with chloroacetyl chloride in dry DMF. The notable feature in the ^{1}H-NMR spectrum was the appearance of a singlet peak at δ 5.18 ppm indicating CH_2Cl group. The successful formation of the intermediate **5** prompted us to investigate the nucleophilic replacement of the active chlorine atom with different amines. Compound **5** underwent nucleophilic substitution with various substituted piperazines to afford **6a–d**. The ^{1}H-NMR spectra of the products **6a–d** showed the appearance of the protons of the piperazine moiety in the range of δ 2.43–3.19 ppm. Moreover, a singlet signal at δ 3.94–3.96 ppm characteristic to the CH_2 linking the triazole ring and the piperazine ring confirmed the successful incorporation of piperazine moieties.

Compound **7** was obtained in good yield by heating the key intermediate **3** with *N,N*-carbonyldiimidazole in dry benzene. IR spectrum of **7** showed absorption bands at ν 1720 and 1632 cm^{-1} indicating the presence of two C=O groups of the triazole ring and the pyrimidinone ring, respectively. Furthermore, the ^{1}H-NMR spectrum displayed an exchangeable singlet signal at δ 12.0 ppm corresponding to the NH proton of the triazole ring. ^{13}C-NMR spectrum of **7** showed two signals at δ 149.17 and 156.63 ppm confirming the presence of two carbonyl moieties. Furthermore, the reaction of **3** with various isothiocyanates yielded the corresponding 1-substituted aminotriazolo derivatives **8a–e**. ^{1}H-NMR spectra of **8a–e** showed D_2O exchangeable signals in the range of δ 9.29–9.39 ppm assignable to the NH.

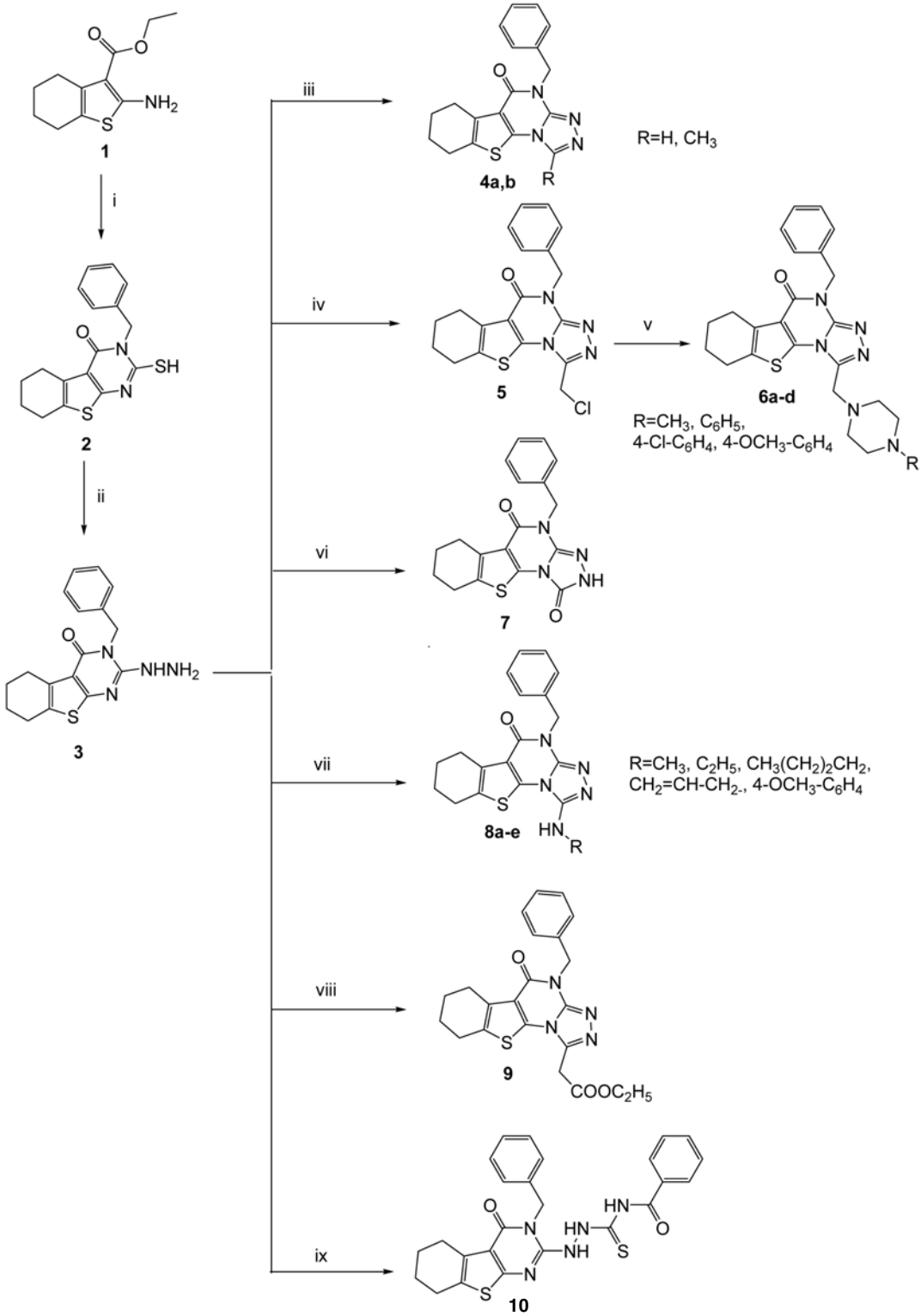
On the other hand, reacting **3** with diethyl malonate in acetic acid afforded the unexpected product 1-methyltriazolo derivative **4b**. The formation of **4b** may be explained by the hydrolysis and decarboxylation of the ester group in the intermediate compound **9** in acidic medium. However, the direct interaction of **3** with excess diethyl malonate in the absence of solvent at the refluxing temperature afforded the expected product **9**. The IR spectrum showed the presence of two C=O moieties at ν 1740 and 1666 cm^{-1} while the ^{1}H-NMR spectrum confirmed the presence of the ethyl ester group. Further evidence was obtained from the ^{13}C-NMR spectrum of **9** which confirmed the presence of ethyl ester group through signals at δ 14.45 and 62.0 ppm in addition to a signal at δ 32.31 ppm corresponding to the $–CH_2–$ flanked between the thienopyrimidin-2-ylsulphanyl group and carbonyl function. Furthermore, the reaction of **3** with benzoyl chloride and ammonium thiocyanate in dry acetone afforded the benzoyl thiourea derivative **10**. ^{1}H-NMR spectrum of compound **10** showed the presence of three D_2O exchangeable signals assignable to three NH moieties at δ 9.79, 11.71 and 12.49 ppm.

In Scheme 2, the reaction of **3** with carbon disulfide in ethanolic potassium hydroxide followed by acidification with hydrochloric acid yielded the thiol (**11**) / thione (**11a**) tautomers. One of the objectives of this work was to prepare a series of *S*-alkylated triazolopyrimidine derivatives with varying the linker skeleton as well as varying the bioactive amine to test their cytotoxicity.

Herein, a series of alkylated mercapto 1,2,4-triazoles was synthesized via the reaction of the key intermediate **11** with various alkyl halides or α- and β-chloroamides (**13a–d**, **14a–d**, **15a–d)** in absolute ethanol in the presence of anhydrous sodium acetate to afford the corresponding *S*-alkyl derivatives (**12a**,**b**, **16a–d**, **17a–d**, **18a–d**). The success of alkylation was confirmed by the absence of SH or NH signals in ^{1}H-NMR spectra of **12a** and **12b** together with the appearance of peaks characteristic to methyl and ethyl moieties in each compound, respectively. Moreover, the mass spectrum of **12a**,**b** showed their corresponding molecular ion peaks at *m/z* 382 and 396, respectively.

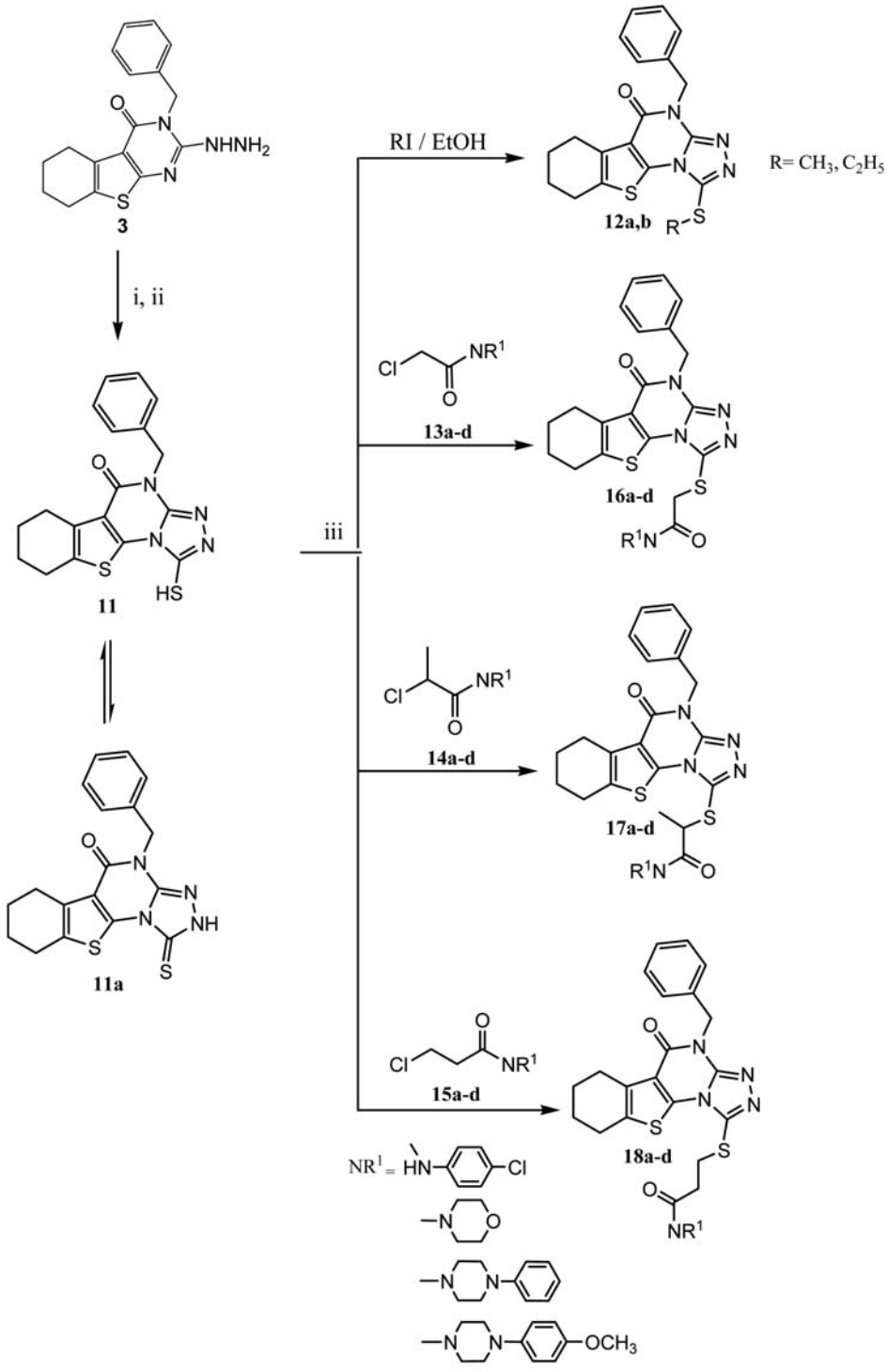
The structure of the mercapto alkylated derivatives **16a–d**, **17a–d** and **18a–d** linked to different secondary amines with different linkages was supported by elemental analyses and spectral data. IR spectra of all target

compounds indicated the appearance of new amide C=O absorption band at ν 1629–1643 cm^{-1}. Moreover, ^{1}H-NMR spectra of compounds **16a–d**, **17a–d** and **18a–d** showed the disappearance of the SH signal at δ 14.06 ppm. Besides, the alkyl protons in the linker between the triazolopyrimidine ring and the amine appeared as follows;



Scheme 1. Synthesis of triazolo derivatives **4a**,**b**, **5**, **6a–d**, **7**, **8a–d**, **9** and **10**: (i) $PhCH_2NCS$ / K_2CO_3 / acetonitrile, followed by acidification; (ii) NH_2–NH_2·H_2O / pyridine, reflux; (iii) RCOOH, reflux; (iv) $ClCH_2COCl$ / DMF, 100 °C; (v) piperazines / EtOH, reflux; (vi) CDI / benzene, reflux; (vii) RNCS / EtOH, reflux; (viii) diethyl malonate, reflux; (ix) benzoyl chloride / NH_4SCN / acetone, reflux.

^{1}H-NMR spectra of **16a–d** showed a characteristic singlet in the range of δ 3.84–4.23 ppm assigned for SCH_2 protons, while the spectra of **17a–d** revealed doublet signals in the range of δ 1.44–1.51 ppm assignable to CH_3 moiety and quartet signals assignable to CH protons in the range of δ 3.93–4.61 ppm. The presence of ethylene fragment (CH_2–CH_2) in compounds **18a–d** was revealed by two triplet signals in the range of δ 2.72–2.87 ppm and δ 3.20–3.40 ppm in ^{1}H-NMR spectra. Further proof for these compounds was obtained using ^{13}C-NMR spectroscopy where the spectrum of compound **18c** showed signals at δ 31.18 and 32.72 ppm assignable to the SCH_2 and



Scheme 2. Synthesis of triazolo derivatives **11**, **12a**,**b**, **16a–d**, **17a–d**, **18a–d**: (i) CS_2 / KOH / EtOH, reflux; (ii) diluted HCl; (iii) anhydrous sodium acetate / EtOH, reflux.

CH_2–CO moieties, respectively. In addition, ^{1}H-NMR spectra of all the target products **16a–d**, **17a–d** and **18a–d** displayed the expected signals of the morpholino, 4-chloroanilino and substituted piperazine moieties.

3. 2. In vitro Cytotoxicity

The *in vitro* cytotoxic activity of 24 selected compounds was evaluated against two human cancer cell lines including cells derived from human prostate cancer (PC-3) and human colon cancer (HCT-116) according to the standard protocol for IC_{50} determination. Doxorubicin (DOX), being one of the most effective anticancer agents, was chosen as the reference standard anticancer drug.[42] The IC_{50} values in μM are listed in Table 1 and the results are represented graphically in Fig. 3.

From the results in Table 1 it is evident that most of the tested compounds displayed moderate to potent cancer cell growth inhibition. Generally, all the tested compounds tended to be more active against HCT-116 than against PC-3. Examining the IC_{50} of the tested compounds against PC-3 cell line revealed that compounds **10**, **12b**, **17b** and **18c** exhibited significant anticancer activities with lower IC_{50} values compared to DOX, with compound **16c** being the most potent with an IC_{50} of 5.48 μM. Meanwhile, compound **12a** showed equipotent activity to DOX, while compounds **6a**, **6c**, **8a**, **17c** and **18a** exhibited IC_{50} values (ranging from 8.25–8.97 μM) very close to DOX (IC_{50} = 7.7 μM) against PC-3. As for the HCT-116 cell line, compounds **6c** and **18c** were the most active (IC_{50} = 6.56 and 6.12 μM, respectively) in contrast to 15.82 μM for the standard on the same cell line. In addition, compounds **4a**, **5**, **6a**, **8a**, **8b**, **8d**, **10**, **12b**, **17b**, **17c**, and **18a** displayed more potent cytotoxic activity compared to the standard with IC_{50} values ranging from 7.4 to 14.77 μM.

Table 1. Results of *in vitro* cytotoxic activity of some selected compounds against human prostate cancer cell line (PC-3) and colon cancer cell line (HCT-116). (Results in bold represent compounds with better activity than DOX.)

Compound no.	IC_{50} in μM*	
	PC-3	HCT-116
4a	13.58	**10.64**
4b	11.41	>100
5	10.13	**12.47**
6a	8.91	**14.77**
6c	8.25	**6.56**
7	11.35	29.51
8a	8.97	**12**
8b	15	**11**
8d	10.7	**12**
8e	10.05	17.15
10	**6.53**	**8.86**
11	>100	>100
12a	**7.8**	20.1
12b	**7**	**7.57**
16a	>100	>100
16c	**5.48**	17.52
17a	>100	60.53
17b	**6.4**	**7.63**
17c	8.5	**7.4**
17d	>100	20.33
18a	8.5	**8.18**
18b	16.01	>100
18c	**7.5**	**6.12**
18d	36	27.32
Doxorubicin	7.7	15.82

* The values given are means of three experiments.

Referring to the IC_{50} values listed in Table 1, the following SAR can be deduced: among the triazolo derivatives **4a** and **4b**, the unsubstituted derivative **4a** showed

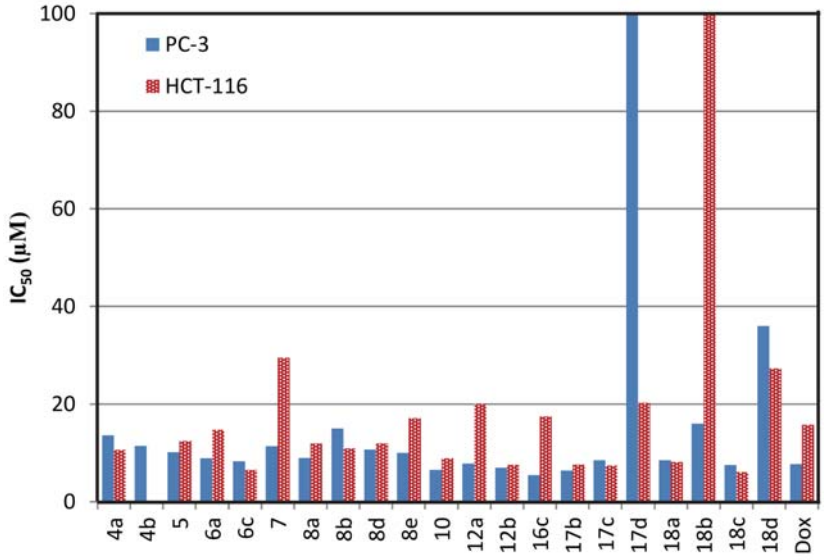


Figure 3. Cytotoxicity of some selected compounds against human prostate cancer cell line (PC-3) and colon cancer cell line (HCT-116)

good activity against HCT-116. Concerning the piperazine derivatives **6a–d**, compounds **6a** and **6c** displayed good activity against both cell lines whereas the 4-chlorophenyl piperazine derivative **6c** showed 2.4 fold higher activity than DOX against HCT-116 cell line in agreement with the reported anticancer activity of derivatives incorporating piperazine scaffolds and halogen atoms.[43,44] Upon analyzing the results of the substituted amino triazoles **8a–e**, compounds **8a**, **8b** and **8d** exhibited higher activity than DOX against HCT-116 but it was difficult to reach conclusions regarding the effect of varying the substituent since the cytotoxicity of **8a–e** was almost the same. The *N*-methyl derivative **8a** was the only potent analogue against PC-3 cell line. Interestingly, compound **10** displayed potent cytotoxic activity against both cell lines in accordance with the reported antitumor activity of thiosemicarbazide derivatives.[45]

Among the 1,2,4-triazole derivatives, the mercapto substituted 1,2,4-triazole ring systems have been studied and so far a variety of antitumor properties have been reported for a large number of these compounds.[25–27] Based on the above findings, we investigated herein in Scheme 2, the structure–activity relationship of *S*-alkylated series of compounds **12a**,**b**, **16a–d**, **17a–d** and **18a–d**, focusing in particular on the effect of the linker skeleton as well as varying the bioactive amine on the cytotoxic activity, the following was observed:

– The incorporation of ethyl substituent in **12b** resulted in a more potent derivative than **12a** against both cell lines.
– Among compounds **16a–d** with CH_2 linker, the 4-phenyl piperazine analogue **16c** showed selective high activity against PC-3.
– The cytotoxic activity of compounds **17a–d** with branched alkyl linker (–$CHCH_3$) showed that the incorporation of morpholine ring (**17b**) and phenyl piperazine moiety (**17c**) resulted in compounds with potent activity against both cell lines.
– The phenyl piperazine derivatives (**16c** and **18c**) afforded better cytotoxic activity compared to other amines against PC-3 and HCT-116, respectively.
–Extending the side chain caused pronounced change in the activity of the 4-chloroaniline derivative **18a** against both cell lines compared to **16a** with acetamide linkage and **17a** with branched linker which were devoid of activity.

4. Conclusion

A series of substituted 4-benzyl[1]benzothieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pyrimidines was designed, synthesized, and screened for their anticancer activity against PC-3 and HCT-116 cell lines. Many of the newly synthesized compounds showed remarkable activity on the tested cell lines with higher sensitivity towards the HCT-116 cell line. Compounds **10**, **12b**, **17b** and **18c** showed higher cytotoxic activity against both PC-3 and HCT-116 cell lines compared to DOX. Incorporation of a 4-phenylpiperazine moiety resulted in higher activity against both cell lines where compound **16c** was the most active against PC-3 with 1.4 fold higher activity than DOX, while **18c** showed 2.5 fold higher anticancer activity against HCT-116. The obtained results suggest that thienopyrimidines containing 1,2,4-triazole scaffold might be suitable candidates for further chemical modifications in order to obtain more potent and selective anticancer agents.

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Povzetek

Številna poročila o proti rakastem delovanju različnih tieno[2,3-*d*]pirimidinov in triazolotienopirimidinov so nas spodbudila k pripravi nove serije 4-benzil-6,7,8,9-tetrahidro[1]benzotieno[3,2-*e*][1,2,4]triazolo[4,3-*a*]pirimidinov. Raziskali smo *in vitro* citotoksično aktivnost izbranih spojin proti dvema človeškima celičnima linijama: raka prostate (PC-3) ter raka debelega črevesa in danke (HCT-116). Izvedli smo tudi začetno študijo odvisnosti med aktivnostjo tarčnih spojin in njihovo strukturo. Večina pripravljenih spojin je izkazala precejšnjo aktivnost proti testiranima celičnima linijama, zlasti obetavna je bila aktivnost spojine **16c** proti celični liniji PC-3 z IC_{50} vrednostjo 5.48 μM, kar je zelo ugodno v primerjavi z vrednostjo za doksorubicin (IC_{50} = 7.7 μM), referenčnim standardom uporabljenim v tej raziskavi. Po drugi strani pa sta se spojini **6c** in **18c** izkazali kot najbolj aktivni proti celični liniji HCT-116 (IC_{50} = 6.12 in 6.56 μM), kar je tudi ugodno v primerjavi z vrednostjo za standard (IC_{50} = 15.82 μM). Zato lahko zaključimo, da bi nekateri izmed sintetiziranih tienopirimidinskih derivatov, zlasti **6c**, **16c** in **18c**, lahko predstavljali potencialno zanimive spojine za nadaljnji razvoj v učinkovita zdravila proti raku.