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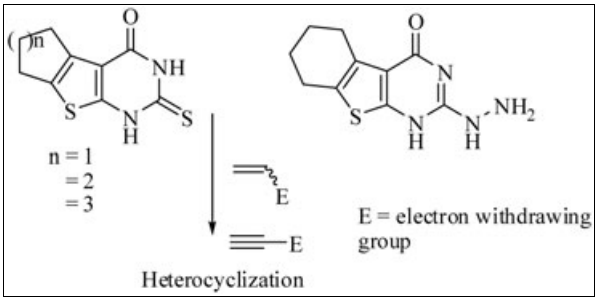

Diethyl azodicarboxylate and 3,4,5,6-tetrachloro-1,2-benzoquinone react with cyclopentano- and cycloheptano-fused thienopyrimidines to form the oxidative dimer of the starting material *via* S—S bond formation. Reaction of two equivalents of 2,2′-(cyclohexa-2′,5′-diene-1,4-diylidene)dimalononitrile with thienopyrimidines afforded 3-(4′,4′-dicyanomethylene-cycloalka[*a*]-2,5-dienyl)-4-oxo-6,7,8,9-tetrahydro-5*H*-cyclo-hepta[4,5]-[1,3]thiazolo[3,2-*a*]-thieno[2,3-*d*]pyrimidin-2-ylidene-2-dicarbonitriles. The thioenopyrimidines react with 2-[1,3-dioxo-1*H*-inden-2(3*H*)-ylidene]malononitrile to produce 1,3,5′- trioxo-1,3,3′,5′-tetrahydro*spiro*-(indene- 2,2′-thiazolo[2,3-*b*]-cycloalkyl[*b*]-thieno[2,3-*d*]pyrimidine)-3′-carbonitriles. However, the reaction of thienopyrimidines with 2,3-dicyano-1,4-naphthoquinone proceeded to afford the fused cycloalkyl-thieno form of naphtho[1,3]thiazolo[3,2-*a*]thieno[2,3-*d*]pyrimidine-6.7,12-triones. Reaction of 2-hydrazino-5,6,7,8-tetrahydrobenzo[*b*]thieno[2,3-*d*]pyrimidine-4(1*H*)-one with dimethyl acetylenedicarboxylate and ethyl propiolate, respectively, afforded cyclohexano-fused (*Z*)-dimethyl 2 [(*E*)-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidine-2(1*H*)-ylidene)hydrazono]succinate and thieno-pyrimidinotriazine. Both oxidative dimers of thienopyrimidines showed high inhibition of Hep-G2 cell growth compared with the growth of untreated control cells. Moreover, the cycloheptano-fused thiazinothienopyrimidine indicates a promising specific antitumor agent against Hep-G2 cells because its IC_{50} is < 20 μ*M*.

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INTRODUCTION

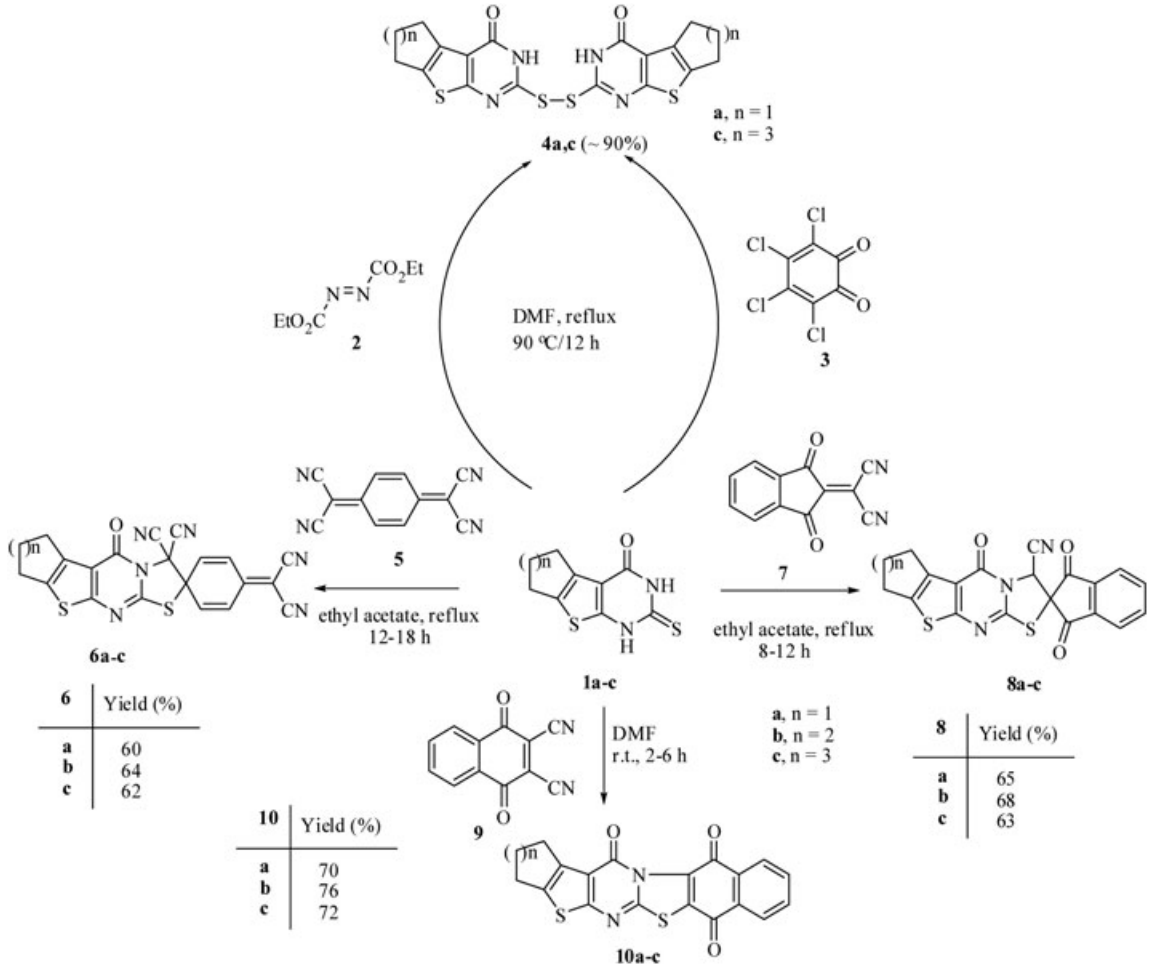
Fused pyrimidines are found in a variety of natural products (*e.g.*, purines, pyrrolopyrimidines, pyridopyrimidines, and pteridines), agrochemicals and veterinary products [1,2]. Pyrimidine derivatives and heteroannelated pyrimidines continue to attract great interest due to the wide variety of interesting biological activities observed for these compounds, such as antiviral [3], antitumor [4], anti-inflammatory [5], antimicrobial [6,7], antifungal [8], antihistaminic [9], and analgesic [10] activities. Thiazolopyrimidine derivatives are the bioisosteric analogs of purines and are potentially bioactive molecules. Aly and co-workers [11–13] have demonstrated that dimethyl acetylenedicarboxylate, ethyl propiolate, and *E*-dibenzoylethylene react with thienopyrimidines to form thiazolo [3,2-*a*]thieno-[2,3-*d*]-pyrimidin-2-ylidene)acetates, thieno [2,3-d]pyrimidin-2-ylthioacrylates, and thieno-[2′,3′:4,5] pyrimido[2,1-*b*][1,3]thiazin-6-ones, respectively. Many derivatives with different substitution patterns display interesting pharmacological activities. Thiazolopyrimidines possess antiviral activity against human cytomegalovirus [14]. Thiazolopyrimidines also display antipsychotic activity by antagonizing the activity of the corticotrophin releasing factor [14]. In addition, thiazolopyrimidines exhibit dual antimicrobial and anti-inflammatory activity comparable to ampicillin and indomethacin *in vivo* with no or minimal ulcerogenic effects [15]. Due to the aforesaid biological activities of thienopyrimidines, we reported on the synthesis of new thiazolopyrimidine derivatives [16]. In this article, we investigate the reactions of thieno[2,3-*d*]pyrimidines with diethyl azodicarboxylate, 3,4,5,6-tetrachloro-1, 2-benzoquinone, 2,2′-(cyclohexa-2′,5′-diene-1,4-diylidene)-dimalononitrile, 2-(1,3-dioxo-1*H*-inden-2(3*H*)-ylidene)-malononitrile, 2,3-dicyano-1,4-naphthoquinone, dimethyl acetylenedicarboxylate, and ethyl propiolate. The antitumor and antioxidant activities of some products were investigated.

RESULTS AND DISCUSSION

Reaction of thienopyrimidines 1a,c with diethyl azodicarboxylate (2) and 3,4,5,6-tetrachloro-1,2-benzoquinone (3). The oxidative dimers of thieno[2,3-*d*]pyrimidines **4a**,**c** were obtained in nearly 90% yield from the reaction of the target starting material **1a,c** with diethyl azodicarboxylate (**2**) and 3,4,5,6-tetrachloro-1,2-benzoquinone (**3**) in DMF (Scheme 1). Mechanistically, the initial addition of sulfur atom in **1a**,**c** of thieno[2,3-*d*]pyrimidines to the azo double bond of compound **2**, would generate salts **11**, which on neutralization produce molecules **12** (Scheme 2). Homolytic fission under heating process of two molecules of **12** would then reproduce the hydrazo form of **2** to and the radical **13**, which dimerizes to afford **4a**,**c** (Scheme 2). Previously, we reported that thioamido groups can be easily dimerized in presence of oxidizing π-deficient compounds [17]. TLC analysis of the solution indicated the presence of diethyl hydrazine-1,2-dicarboxylate (DEHD), as DEHD has been tested under the TLC visualization conditions. In the case of compound **3**, addition of the sulfur atom of **1a**,**c** to the carbonyl of **3** would form **14** (Scheme 3), which would react further similarly to **12**. Reduction of **3** would give tetrachloro-*o*-hydroquinone (*o*-TCHQ). Also TLC analysis of the solution indicated the presence of *o*-TCHQ. Compounds **4a**,**c** were too insoluble in DMSO for measurement of ^{13}C-NMR spectra. The mass spectrum indicated the molecular ion peak as 20–22%, with the monomer as the base peak. The NH-1 proton of **1a**,**c** disappeared in the ^{1}H-NMR of **4a**,**c**, whereas NH-3 remained at δ = 12.00–12.30 ppm.

Reaction of thienopyrimidines 1a–c with 2,2′-(cyclohexa-2′,5′-diene-1,4-diylidene)-dimalononitrile (5). Reaction of two moles of 2,2′-(cyclohexa-2′,5′-diene-1,4-diylidene) dimalononitrile (**5**) with one mole of thienopyrimidines **1a–c** in ethyl acetate afforded the fused pentyl-, hexyl- and heptyl 4-(dicyanomethylene)-5′-oxospiro[cyclohexa-(2,5)] diene-1,3′-thiazolo[3,2-*a*]thieno(2,3-*d*)pyrimidine-2′,2′-(5′*H*)-dicarbonitriles **6a–c** after chromatographic purification (Scheme 1). The IR spectroscopy of **6a**, as an example, indicated the absence of absorption of any NH groups. The nitrile groups appeared at ν = 2220–2206 and carbonyl of pyrimidinone moiety appeared at ν = 1686 cm^{-1}. The

Scheme 1. Dimerization and heterocyclization from the reactions of thienopyrimidines **1a–c** with π-deficient compounds.

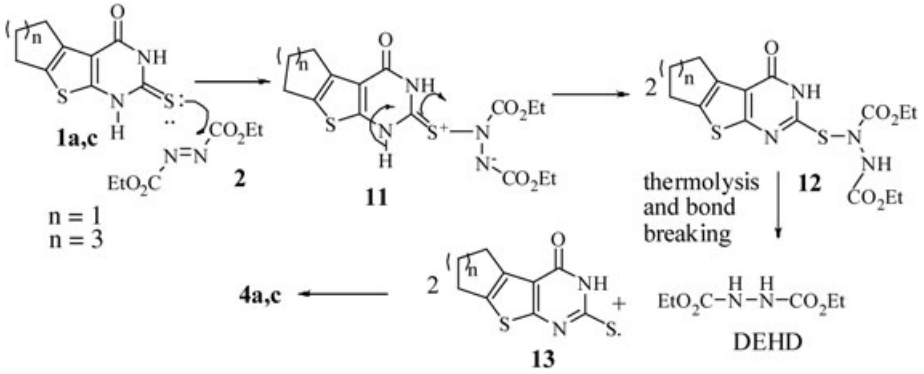


6	Yield (%)
a	60
b	64
c	62

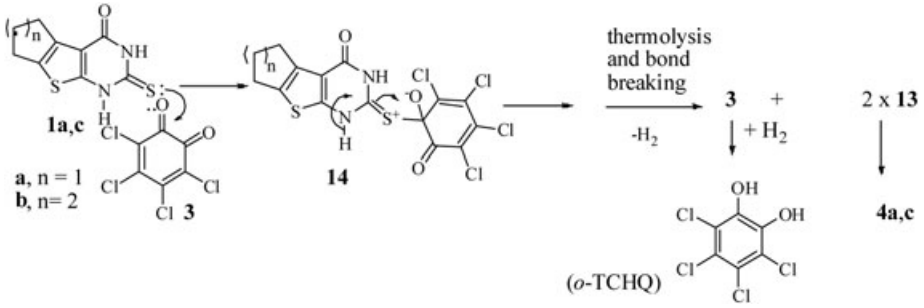
8	Yield (%)
a	65
b	68
c	63

10	Yield (%)
a	70
b	76
c	72

Scheme 2. Dimerization of thienopyrimidines **1a**,**c** with compound **2**.



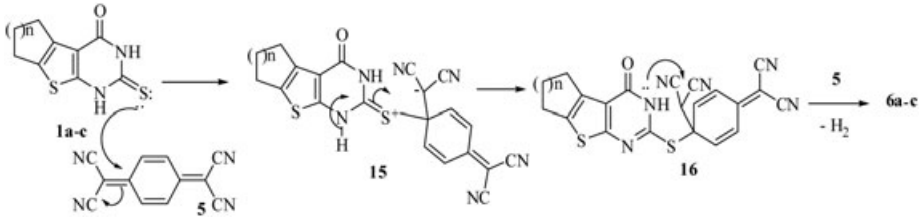
Scheme 3. Dimerization of thienopyrimidines **1a**,**c** with compound **3**.



^{1}H-NMR spectrum of **6a** showed two doublets of four methine groups at δ = 6.60 (*J* = 8.0 Hz) and 5.82 (*J* = 7.8 Hz), whereas the CH_2—CH_2 resonated as at δ = 3.07–2.88 (2m,4H, *o*-2CH_2) and 2.80–2.72 (m, 2H, m-CH_2). The ^{13}C-NMR spectrum of **6a** showed carbon signals at δ = 178.0 (C, cyclohexadiene), 163.0 (C=O), 159.5, 156.0, 118.0 (3C, pyrimidine), 138.6, 126.0 (2C, cyclopentene), 131.0, 120.0 (4CH, vinylic, cyclohexadiene), 113.0, 112.8, 112.6, 111.6 (4CN), 72.0 (C=C(CN)2), 52.8 (spiro, C), 50.2 (C, thiazole) and 32.0, 25.2, 23.1 ((3 CH2). The proton signal at δ = 5.82 ppm shows HMBC correlation with the carbonyl carbon. Reaction can be explained as due to initial nucleophilic addition of the sulfur lone pair to the vinylic carbon to give **15**, followed by neutralization to afford **16** (Scheme 4). Nucleophilic addition of the amido-NH to the substituted malononitrile-carbon followed by oxidation with another molecule of **5** would give **6** (Scheme 4). The mechanism was supported by the reported literature [18], which indicated that the nucleophilic sulfur lonepair of electrons would attack to the π-deficient bond and therefore initiate the reaction pathway.

Reaction of thienopyrimidines 1a–c with 2-(1,3-dioxo-1*H*-inden-2(3*H*)-ylidene)-malononitrile (7). Thienopyrimidines **1a–c** react with 2-(1,3-dioxo-1*H*-inden-2(3*H*)-ylidene) malononitrile to (**7**) give (toluene:ethyl acetate; 10:1), the corresponding 1,3,5′-trioxo-1,3,3′,5′-tetrahydro-*spiro* [indene-2,2′-thiazolo[2,3-*b*]cycloalkyl[*b*]thieno[2,3-*d*]-pyrimidine]-3′-carbonitriles **8a–c** after chromatographic purification (Scheme 1). The IR spectrum of **8a** showed the absorption of the cyano group as a sharp peak at ν = 2215 cm^{-1}. The ^{13}C-NMR spectrum indicated the cyano carbon atom at δ = 113.0 ppm. The *spiro* carbon at δ = 79.7 ppm and the two carbonyl carbon signals of the indanedione appeared at δ = 197.4, all show HMBC correlation with H-4 δ = 5.60 ppm). The remaining carbonyl signal of pyrimidinone appeared at δ = 161.2 ppm. The mechanism of the reaction pathway is similar to the reaction of **1a–c** with **5**. However, the addition of NH-3 to the $CH(CN)_2$ is accompanied by elimination of HCN.

Scheme 4. Suggested mechanism of the reaction between **1a-c** and **5**.



Reaction of thienopyrimidines 1a–c with 2,3-dicyano-1,4-naphthoquinone (9). Surprisingly, refluxing of thienopyrimidines **1a–c** with 2,3-dicyano-1,4-naphthoquinone (**9**) in ethyl acetate afforded naphtho[1,3]thiazolo[3,2-*a*] thieno[2,3-*d*]pyrimidine-6,7,12-triones **10a–c** in 70–76% yields (Scheme 1). The IR spectrum showed the disappearance of the absorption of the cyano and NH groups, which indicated that the reaction proceeded with elimination of two HCN molecules. The IR spectroscopy indicated the carbonyl groups in **10a** at ν = 1702–1685. The ^{1}H-NMR spectrum of **10a**, as an example, revealed the Ar protons as two multiplets at δ = 8.05–7.96 (4H) and 7.72–7.68 (2H). The ^{13}C-NMR spectrum revealed three carbonyl signals at δ = 178.3, 176.1 for the naphthoquinone ring, and 165.2 for the pyrimidine ring. The methylene carbons resonated at δ = 32.1, 25.5, and 21.3 ppm. The two vinylic carbons of the naphthoquinone appeared at δ = 147.9 and 118.0 ppm.

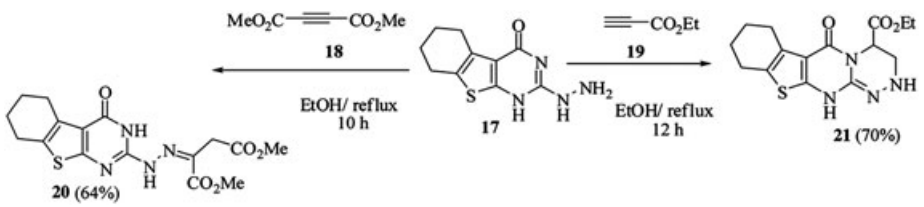
Reaction of hydrazinothienopyrimidine 17 with dimethyl acetylenedicarboxylate (18). Refluxing the cyclohexyl derivative of 2-hydrazinylthieno-[2,3-*d*]pyrimidinone **17** with dimethyl acetylenedicarboxylate (**18**) afforded (*Z*)-dimethyl 2[(*E*)-4-oxo-3,4-dihydrothieno-[2,3-*d*]pyrimidine-2(1*H*)-ylidene)hydrazono]-succinate in 64% yield (Scheme 5). The structure of **20** follows from its NMR spectra. In the DMAD-derived substructure, the ester carbonyl at δ = 168.47 ppm that gives HMBC correlation to both a methoxy group at δ = 3.74 ppm and the downfield methylene at δ = 3.82 ppm, is assigned as C-5′; these methoxy and methylene protons are assigned as H-6′ and H-4′, respectively, and their attached carbons at δ = 53.11 and 32.59 ppm must be C-6′ and C-4′. The imino carbon at δ = 133.95 ppm shows HMBC correlation to H-4′ and is assigned as C-3′. The remaining ester carbonyl at δ = 164.26 ppm gives HMBC correlation to the other methoxyl protons at δ = 3.83 ppm; they are assigned as C-2′ and H-1′, respectively, and the corresponding methoxyl carbon at δ = 53.06 ppm must be C-1′. The remaining carbonyl carbon at δ = 164.26 ppm is assigned as C-4. Proton and carbon assignments in the cyclohexanothiophene system are made by analogy with earlier work [19]. No carbon gives correlation with the NH protons, which would help differentiate structure **20a** from its tautomers **20b**; the lack of correlation is attributed to exchange (Fig. 1). The reaction mechanism can be described as due addition of hydrazine-NH_2 to the acetylenic triple bond to initially produce **22**, which was followed by tautomerism into conjugation, to give **20** (Scheme 6 and Fig. 1).

Reaction of hydrazinothienopyrimidine 17 with ethyl propiolate (19). When compound **17** reacted with ethyl propiolate (**19**) in refluxing ethanol, cyclohexano thienopyrimidinetriazine **19ab** was obtained in 70% yield (Scheme 7). The mechanism postulated initial nucleophilic attack on C_α of **18**, rather than C_β where nucleophiles would more typically attack. Attack at C_β would presumably lead to the regioisomeric product **21c** (Scheme 7). In the NMR spectra of compound **21**, the ethoxyl carbons and protons are assigned straightforwardly: C-5′, δ = 14.04; H-5′, δ = 1.21; C-4′, δ = 60.52; H-4′, δ = 4.12 ppm. One carbonyl carbon at δ = 169.58 ppm gives HMBC correlation with H-4′, and is assigned as C-3′. This carbonyl also gives HMBC correlation with the downfield triplet and doublet at δ = 4.41 and 3.42 ppm, which are assigned as H-2′ and H-1′, respectively; HSQC correlations then locate C-2′ and C-1′ at δ = 65.03 and 37.39 ppm. These two carbons give HMBC correlations to each other's attached protons; C-2′ also gives HMBC correlation to the less broad of the two NH protons δ = 11.42 ppm, which is assigned as the localized proton N_α-H. N_α-H also gives HMBC correlation with a signal at δ = 149.70 ppm, which is assigned as C-2. The observation of correlation between N_α-H and C-2′, not C-1′, leads us to support structure **21a,b** over **21c**. This assignment could be made absolutely unambiguous if C-2 or the remaining carbonyl C-4 (δ = 165.22 ppm) gave HMBC correlation with either H-1′ or H-2′, which unfortunately they do not. Proton and carbon assignments in the cyclohexanothiophene substructure are consistent with the observed correlations.

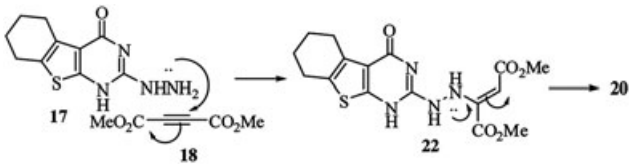
BIOLOGICAL SECTION

Material and methods. ***Cell culture.*** Four cell lines were used through this work: human hepatocellular carcinoma (HepG2), human colon carcinoma (HCT-116), human lymphoblastic leukemia (1301), and raw murine macrophage (RAW 264.7). All cell lines were purchased from ATCC, VA. HepG2 and 1301 cells were routinely cultured in Dulbeco's Modified Eagle's medium, while HCT-116 cells were cultured in Mc Coy's medium and RAW 264.7 cells

Scheme 5. Reactions of thienopyrimidinne **17** with acetylenic esters **18** and **19**.



Scheme 6. Suggested mechanism of the reaction of **17** and **18**.



were grown in RPMI-1640. Media was supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine, containing 100 units/mL penicillin G sodium, 100 units/mL streptomycin sulfate, and 250 ng/mL amphotericin B. Cells were maintained at subconfluency at 37°C in humidified air containing 5% CO_2. For subculturing, monolayer cells were harvested after trypsin/EDTA treatment at 37°C. Cells were used when confluence had reached 75%. Tested samples were dissolved in dimethyl sulfoxide (DMSO). All cell culture materials were obtained from Cambrex BioScience (Copenhagen, Denmark). All chemicals were from Sigma/Aldrich, except mentioned. All experiments were repeated three times, unless mentioned.

Cytotoxicty assay. Cytotoxicity of tested samples against different types of cells was measured using the (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) cell viability assay. MTT assay is based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form dark blue insoluble formazan crystals to which cell membranes are largely impermeable, resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at $\nu = 570$ nm [20].

Reagents preparation. MTT solution: 5 mg/mL of MTT in 0.9% NaCl. Acidified isopropanol: 0.04 N HCl in absolute isopropanol.

Procedure. Cells (0.5×10^5 cells/well) in serum-free media were plated in a flat bottom 96-well microplate and treated with 20 μL of different concentrations of each tested compound for 20 h at 37°C, in a humidified 5% CO_2 atmosphere. After incubation, medium was removed, and 40 μL of MTT solution/well was added and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 μL of acidified isopropanol/well and the plate was shaken at room temperature, followed by photometric determination of the absorbance at $\nu = 570$ nm using microplate ELISA reader. Triplicate repeats were performed for each concentration and the average was calculated. The results were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by <100% relative viability.

Calculations. Percentage of relative viability was calculated using the following equation: [absorbance of treated cells/absorbance of control cells)] × 100. Then the half maximal inhibitory concentration IC_{50} was calculated from the equation of the dose response curve.

Antioxidant activity (scavenging of DPPH). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable deep violet radical due to its unpaired electron. In the presence of an antioxidant radical scavenger, which can donate an electron to DPPH, the deep violet color decolorize to the pale yellow nonradical form [20]. The change in color and the subsequent fall in absorbance are monitored spectrophotometrically at $\nu = 520$ nm.

Reagents preparation. Ethanolic DPPH: 0.1 m*M* DPPH/absolute ethanol. Standard ascorbic acid solution: serial dilutions of ascorbic acid in concentrations ranging from 0 to 2.5 μ*M* in distilled water. A standard calibration curve was plotted using serial dilutions of ascorbic acid in concentrations ranging from 0–2.5 μ*M* in distilled water.

Procedure. In a flat bottom 96-well microplate, a total test volume of 200 μL was used. In each well, 20 μL of different concentrations (0–100 μg/mL final concentration) of tested compounds were mixed with 180 μL of ethanolic DPPH and incubated for 30 min at 37°C. Triplicate wells were prepared for each concentration and the average was calculated. Then photometric determination of absorbance at $\nu = 515$ nm was made, using a microplate ELISA reader [21].

Calculations. The half maximal scavenging capacity (SC_{50}) values for each tested compounds and ascorbic acid was estimated *via* two competitive dose curves. Abs_{50} of ascorbic acid = $(Abs_{100} - Abs_0)/2$. SC_{50} of ascorbic acid was calculated using the curve equation. SC_{50} of each compound was determined using the curve equation using Abs_{50} of ascorbic acid.

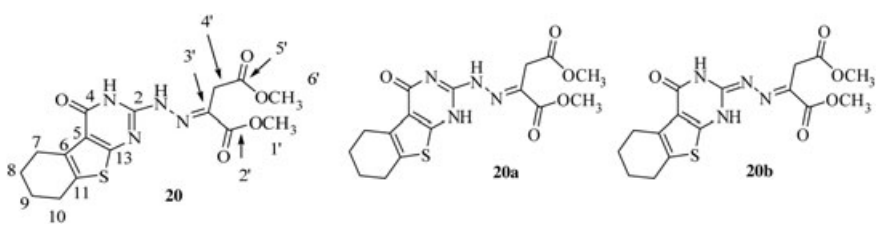
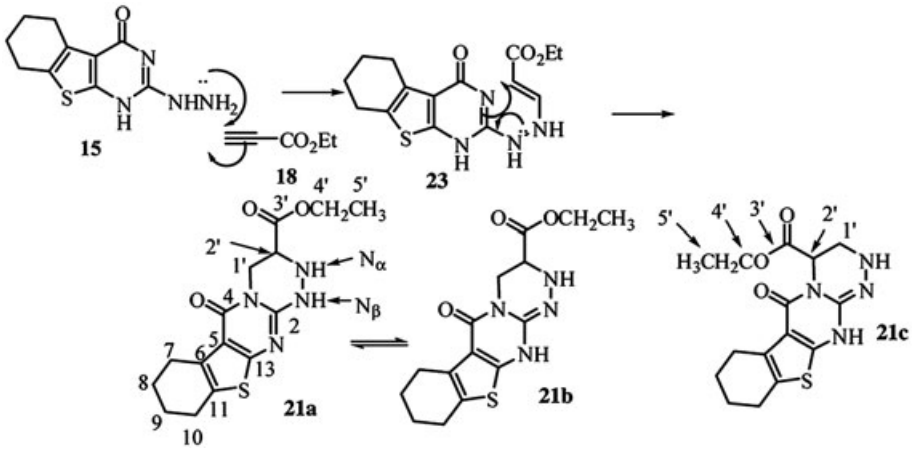


Figure 1. Proton exchange in compound **20**.

Scheme 7. Suggested mechanism of the reaction of thienopyrmidine **17** with ethyl propiolate (**19**).



Results. ***Cytotoxicity of the compounds against Hep-G2 cells.*** Using MTT assay, we studied the effect of the compounds on the proliferation of different cell lines after 48 h incubation. Incubation of Hep-G2 cell line with gradual doses of the compounds led to insignificant change in the growth of Hep-G2 cells as indicated from their IC_{50} values (>100 μ*M*), except compound **4c** which resulted in a high inhibition of the cell growth of Hep-G2 cells compared with the growth of untreated control cells, as concluded from its low IC_{50} value of 26.18 μ*M*.

Cytotoxicity of the compounds against HCT-116 cells. Incubation of colon carcinoma HCT-116 cell line with gradual doses of different tested compounds resulted in an unchanged level of growth of HCT-116 cells as indicated from their IC_{50} values (>100 μ*M*), except compound **4c** which resulted in a high inhibition of the cell growth of HCT-116 cells compared with the growth of untreated control cells, as concluded from its low IC_{50} value of 43.34 μ*M*.

Cytotoxicity of the compounds against T-lymphocytes and macrophages. Using MTT assay, we investigated the effect of the compounds on the proliferation of two types of immune cells, human lymphoblastic leukemia (1301, T-lymphocytes) and raw murine macrophage (RAW 264.7). Incubation of 1301 cells for 48 h incubation with gradual doses of the compounds **4c** and **8a** (Fig. 2) resulted in an insignificant inhibition in the 1301 cells, where their IC_{50} values were >100 μ*M*. On the other hand, compounds **4a**,**b** and **8b** (Fig. 2) led to significant induction in the growth of 1301 cells ranging from 1.22-fold of the control to 3.46-fold of the control, especially at high tested concentrations (50 and 100 μ*M*).

However, compound **4a** is considered as the highest inducer of T-lymphocytes (1301 cells), starting from the lowest tested concentration (12.5 μ*M*), which led to a growth induction of 1.47-fold of the control. Incubation of macrophages (RAW 264.7) for 48 h incubation with gradual doses of the compounds **4c** and **8a** resulted in an insignificant inhibition in the macrophages. Moreover, compound **8c** exhibited no effect on the growth of macrophages. On the other hand, compound **4a** led to significant induction in the growth of macrophages ranging from 1.18-fold of the control to 3.99-fold of the control, especially at high tested concentrations (50, 100 μ*M*). However, compound **4a** is considered as the highest inducer of macrophages (RAW 264.7), starting from the lowest tested concentration (12.5 μ*M*), which led to a growth induction of 1.63-fold of the control (Fig. 2).

Antioxidant activity was indicated *via* the inhibition of the cell growth of both of HCT-116 and Hep-G2 cells of the test compounds **4a,c** and **8a–c** (Fig. 3). Interestingly, compound **8c** showed a strong antioxidant activity (Fig. 3).

CONCLUSIONS

Compounds **4a** and **8b** enhanced macrophage growth. Additionally, **4a** and **8b** led to significant induction in the growth of 1301 cells. However, compound **4a** can be considered as a promising immunostimulant agent, as it led to the highest induction of both T-lymphocytes (1301 cells), macrophages (RAW 264.7). Compound **4c** resulted in a high inhibition of the cell growth of both of HCT-116 and Hep-G2 cells. Additionally, compound **8c** was a strong antioxidant.

EXPERIMENTAL

General procedure. ***Reaction of thienopyrimidines 1a, c with compounds 2 and 3.*** A mixture of compounds **1a**,**c** (1 mmol) and diethyl azodicarboxylate and/or 3,4,5,6-tetrachloro-1,2-benzoquinone (**2** and/or **3**, 1 mmol) in DMF (20 mL) was stirred for 4 h and then heated at 90°C for 12 h. TLC analysis indicated the presence of DEHD and/or *o*-TCHQ after consumption of the starting materials **1a** and/or **1c**. After cooling, the reaction mixture was poured into ice water. The solid product formed was collected by filtration, was washed by ethyl acetate (100 mL) and recrystallizated from DMF/EtOH.

Bis[(5,6,7-trihydrocyclopenta[b]thieno[2,3-d]pyrimidin-4-one)]-2-disulphide (4a). Orange crystals, 0.402 g (90%),

mp 270–272°C. IR (KBr): ν = 3332–3320 (w, NH), 3065–3050 (w, Ar—CH), 2980–2870 (w, aliph.-CH), 1685 (s, pyrimidine C=O), 1558 (s, C=C). ^{1}H-NMR (400.13 MHz, DMSO-d_6): δ = 12.00 (br, s, 2H, NH), 2.80–2.68 (m, 8H, CH_2—CH_2), 2.27–2.18 (m, 4H, CH_2—CH_2). MS (EI, 70 eV): *m*/*z* (%) = 446 ([M^+], (20), 224 (23), 223 (100), 151 (24), 150 (40), 124 (32), 94 (18), 68 (24). Anal. Calcd. for $C_{18}H_{14}N_4O_2S_4$: C, 48.41; H, 3.16; N, 12.55; S, 28.72. Found: C, 48.34; H, 3.08; N, 12.46; S, 28.92%.

Bis[(5,6,7,8,9-pentahydrocyclohepta[b]thieno[2,3-d]-pyrimidin-4-one)]-2-disulphide (4c). Orange crystals, 0.452 g (90%), mp 290–292°C. IR (KBr): ν = 3335–3326 (w, NH), 3075–3030 (w, vinylic-CH), 2966–2870 (w, aliph.-CH), 1687 (s, pyrimidine C=O), 1560 (s, C=C). ^{1}H-NMR (400.13 MHz, DMSO-d_6): δ = 12.30 (s, 2H, NH), 3.10–2.80 (m, 12H, CH_2—CH_2), 2.22–2.08 (m, 8H, CH_2—CH_2). MS (EI, 70 eV): *m*/*z* (%) = 502 ([M^+], 22), 298 (30), 254 (24), 251 (100), 250 (84), 220 (40), 180 (14), 152 (28), 122 (26), 96 (16), 70 (26). Anal. Calcd. for $C_{22}H_{22}N_4O_2S_4$: C, 52.56; H, 4.41; N, 11.15; S, 25.51. Found: C, 52.42; H, 4.30; N, 11.06; S, 25.40%.

Reaction of thienopyrimidines 1a–c with 2,2′-(cyclohexa-2′, 5′- diene- 1,4- diylidene)dimalononitrile (5). *General procedure.* Solutions of compounds **1a–c** (1 mmol) and **5** (0.408 g, 2 mmol) in ethyl acetate (30 mL) were refluxed for 12–18 h. The reaction was

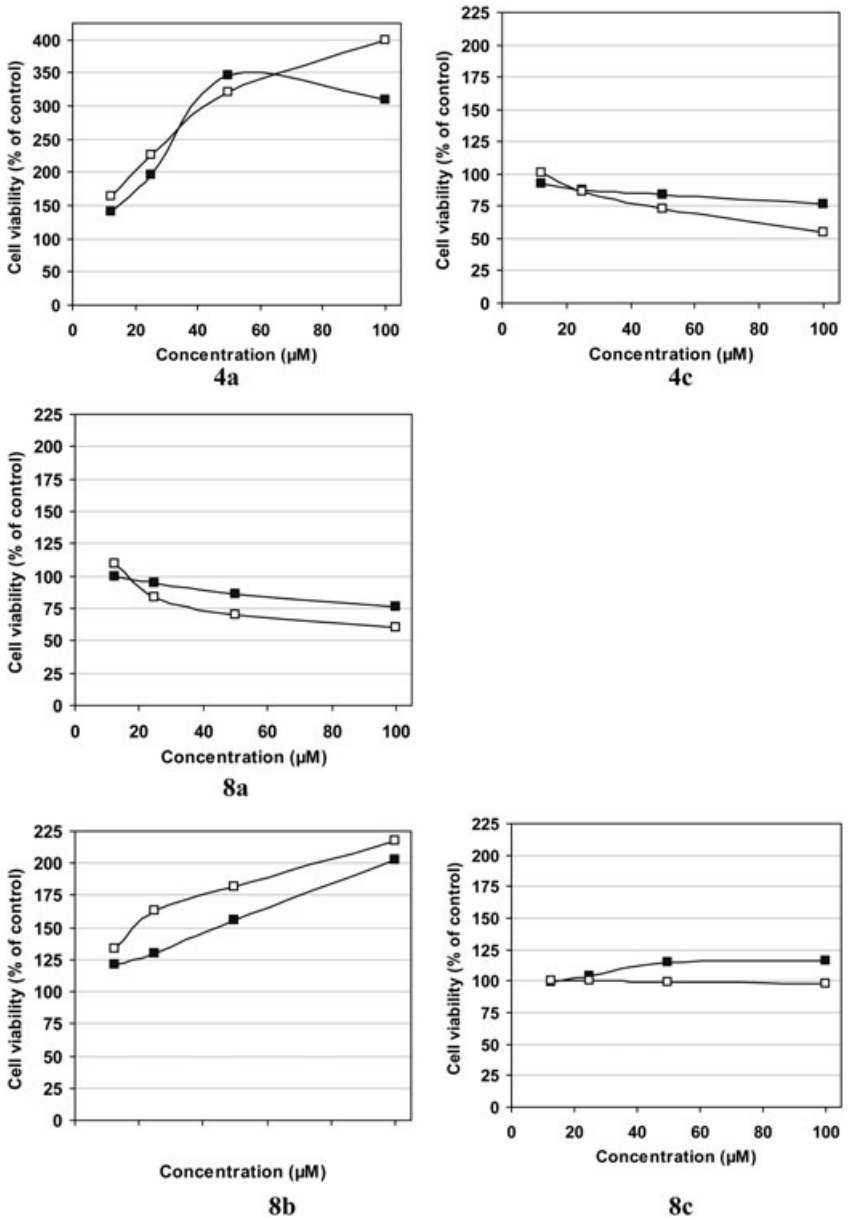


Figure 2. The effect of compounds **4a**,**c** and **8a**–**c** on the growth of two types of immune cells, human lymphoblastic leukemia (1301, T-lymphocytes, black squares line), and raw murine macrophage (RAW 264.7, white squares line), as measured by MTT assay. Results are represented as percentage of control untreated cells.

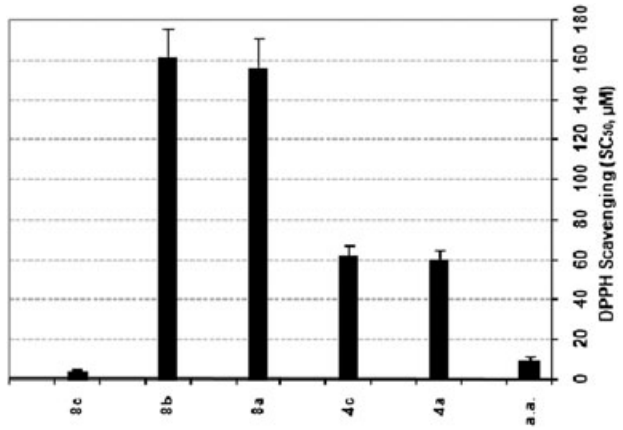


Figure 3. The antioxidant activity of the compounds **4a**,**c** and **8a-c** was investigated using DPPH assay. The results are represented as SC_{50} values (μM) as (Mean ± S.E., $n = 4$).

followed by TLC analysis. The reaction mixture was then cooled. The solid product formed was collected by filtration and recrystallized from the proper solvents.

3-(4′,4′-Dicyanomethylene-cyclohexa-[a]-2,5-dienyl)-4-oxo-6,7-dihydro-5H-cyclopenta-[4,5]-[1,3]thiazolo[3,2-a]thieno[2,3-d] pyrimidin-2-ylidene-2-dicarbonitrile (6a). Yellow crystals (benzene), 255 mg (60%), mp 222–224°C. IR (KBr): ν = 3090–3060 (w, vinylic-CH), 2982–2860 (w, aliph.-CH), 2220–2206 (s, CN), 1686 (s, pyrimidine C=O), 1557 (s, C=C). ^{1}H-NMR (400.13 MHz, DMSO-d_6): δ = 6.62 (d, 2H; vinylic-H, J = 8.0 Hz), 5.82 (d, 2H; vinylic-H, J = 7.8 Hz), 3.09–2.90 (m, 2H, CH_2—CH_2), 2.70–2.62 (m, 4H, CH_2). ^{13}C-NMR (100.6 MHz, DMSO-d_6): δ = 178.0 (C=C $(CN)_2$) 163.0 (C=O), 156.0, 154.8 (S—C—N), 138.6 (cyclopentenyl-C—S), 131.0 (vinylic-2CH), 126.0 (cyclopentenyl-C=), 120.2 (vinylic-2CH), 118.0 (C—CO), 113.0, 112.8, 112.6, 111.6 (CN), 72.0 (=$C(CN)_2$), 52.8 (q-$C(CN)_2$), 50.2 (q-chiral carbon), 32.0, 25.2, 23.1 (CH_2). MS (EI, 70 eV, %): m/z = 426 ([M^+], 100), 376 (22), 350 (24), 326 (30), 312 (28), 276 (26), 250 (18), 220 (14), 192 (40), 152 (18), 128 (32), 96 (16), 83 (24), 42 (12). Anal. Calcd. for $C_{21}H_{10}N_6OS_2$: C, 59.14; H, 2.36; N, 19.71; S, 15.04%. Found: C, 59.00; H, 2.28; N, 19.62; S, 14.98%.

3-(4′,4′-Dicyanomethylene-cyclohexa-2,5-dienyl)-4-oxo-5,6,7,8-tetrahydrobenzo[4,5]-[1,3]thiazolo[3,2-a]thieno[2,3-d]pyrimidin-2-ylidene-2-dicarbonitrile (6b). Yellow crystals (benzene), 282 mg (64%), mp 234–236°C. IR (KBr): ν = 3080–3050 (w, vinylic-CH), 2980–2840 (w, aliph.-CH), 2220–2210 (s, CN), 1682 (s, pyrimidinone C=O), 1555 (s, C=C). ^{1}H-NMR (400.13 MHz, DMSO-d_6): δ = 6.38 (d, 2H; vinylic-H, J = 8.1 Hz), 5.90 (d, 2H; vinylic-H, J = 7.9 Hz), 2.80–2.72 (b, s, 4H, $2CH_2$), 1.80–1.72 (m, 4H, $2CH_2$). ^{13}C-NMR (100.6 MHz, DMSO-d_6): δ = 178.2 (C=C $(CN)_2$) 162.0 (C=O), 159.2, 155.0 (S—C—N), 139.2 (cyclohexyl—C—S), 130.0 (vinylic-2CH), 125.2 (cyclohexyl—C=), 120.0 (vinylic-2CH), 116.4 (C—CO), 113.8, 113.2, 113.0, 112.0 (CN), 70.8 [=$C(CN)_2$], 55.8 (q-$C(CN)_2$), 48.2 (*spiro* carbon), 25.0, 24.1, 23.3, 20.0 (CH_2). MS (EI, 70 eV, %): m/z = 440 ([M^+], 100), 415 (28), 390 (20), 365 (18), 340 (24), 328 (34), 316 (24), 264 (34), 238 (40), 206 (24), 166 (20), 152 (18), 128 (14), 110 (16), 70 (16). Anal. Calcd. for $C_{22}H_{12}N_6OS_2$: C, 59.98; H, 2.75; N, 19.08; S, 14.56%. Found: C, 59.90; H, 2.74; N, 19.00; S, 14.50%.

3-(4′,4′-Dicyanomethylene-cyclohexa[a]-2,5-dienyl)-4-oxo-6,7,8,9-tetradihydro-5H-cyclohepta[4,5][1,3]thiazolo[3,2-a]thieno [2,3-d]pyrimidin-2-ylidene-2-dicarbonitrile (6c). Orange crystals (benzene: pet. Ether; 2:1), 281 mg (62%), mp 200–202°C. IR (KBr): ν = 3090–3040 (w, vinylic-CH), 2990–2820 (w, aliph.-CH), 2220–2212 (s, CN), 1680 (s, pyrimidine C=O), 1555 (s, C=C). ^{1}H-NMR (400.13 MHz, $CDCl_3$): δ = 6.32 (d, 2H; vinylic-H, J = 8.0 Hz), 5.90 (d, 2H; vinylic-H, J = 7.8 Hz), 2.80–2.75 (m, 4H, $2CH_2$), 2.30–2.25 (m, 2H, CH_2), 1.80–1.74 (m, 2H, CH_2), 1.20–1.14 (m, 2H, CH_2). ^{13}C-NMR (100.6 MHz, DMSO-d_6): δ = 179.0 [C=C $(CN)_2$], 161.0 (C=O), 159.0, 155.2 (S—C—N), 139.2 (cyclohexyl—C—S), 130.0 (vinylic-2CH), 125.6 (cyclohexyl—C=), 120.2 (vinylic-2CH), 117.8 (C—CO), 113.0, 112.6, 111.4, 111.2 (CN), 70.2 (=$C(CN)_2$), 55.6 (q-$C(CN)_2$), 48.8 (*spiro* carbon), 28.2, 25.4, 24.5, 22.8, 19.0 (CH_2—CH_2). MS (EI, 70 eV, %): m/z = ([M^+], 100), 428 (20), 404 (23), 380 (18), 342 (16), 330 (16), 278 (24), 252 (20), 220 (14), 194 (12), 166 (24), 152 (34), 128 (38), 96 (14), 70 (24). Anal. Calcd. for $C_{23}H_{14}N_6OS_2$: C, 60.78; H, 3.10; N, 18.49; S, 14.11%. Found: C, 60.86; H, 3.00; N, 18.30; S, 14.12%.

Reaction between thieno[2,3-d]pyrimidines 1a–c and 2-(1,3-dioxo-1H-inden-2(3H)-ylidene)-malononitrile (7). *General procedure.* A mixture of **1a–c** (1 mmol) and 2-(1,3-dioxo-1*H*-inden-2(3*H*)-ylidene)malononitrile (**7**, 142 mg, 1 mmol) was heated at reflux in ethyl acetate (30 mL) for 12–17 h. The reaction was followed by TLC. The solvent was removed under vacuum, and the residue was applied on plate chromatography using silica; toluene:ethyl acetate; 10:1. The products were obtained and recrystallized from the stated solvents.

1,3,5′-Trioxo-1,3,3′,5′-tetrahydrospiro[indene-2,2′-thiazolo [2,3-b]-cyclopentyl[b]thieno-[2,3-d]pyrimidine]-3′-carbonitrile (8a). Orange crystals (ethanol), 263 mg (65%), mp 222–224°C. IR (KBr): ν = 3090–2998 (w, Ar—CH), 2975–2880 (m, aliph.-CH), 2215 (s, CN), 1705, 1685 (s, C=O), 1557 (s, C=C). ^{1}H-NMR (400.13 MHz, $CDCl_3$): δ = 7.95–7.90 (m, 2H; Ar—H), 7.40–7.35 (m, 2H; Ar—H), 5.80 (s, 1H; CH—CN), 2.70–2.62 (m, 4H; $2CH_2$—C_7 + C_8), 2.15–1.95 (m, 2H; CH_2). ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 195.9, 194.2 (C=O, indanedione), 163.2 (C=O, pyrimidinone), 161.0 (C=N), 159.2, 140.8 (2 C=C; Ar—C), 139.2 (C—S, thieno ring), 133.3, 128.5 (4CH, Ar—CH), 125.3, 117.5 (C=C; thieno ring), 113.0 (CN), 79.7 (*spiro* carbon), 41.7 (CH, thiazole ring), 32.1, 25.8, 21.2 (CH_2). MS (EI, 70 eV, %): m/z = 405 ([M^+], 100), 380 (22), 368 (16), 342 (14), 328 (24), 304 (24), 272 (16), 178 (40), 96 (20). Anal. Calcd. for $C_{20}H_{11}N_3O_3S_2$: C, 59.25; H, 2.73; N, 10.36; S, 15.82%. Found: C, 59.12; H, 2.62; N, 10.20; S, 15.78%.

1,3,5′-Trioxo-1,3,3′,5′-tetrahydrospiro[indene-2,2′-thiazolo [2,3-b]cyclohexyl[b]-thieno[2,3-d]pyrimidine]-3′-carbonitrile (8b). Orange crystals (EtOAc), 285 mg (68%), mp 242–244°C. IR (KBr): ν = 3096–3008 (w, Ar—CH), 2980–2820 (w, aliph.-CH), 1700, 1685 (s, C=O), 1560 (s, C=C). ^{1}H-NMR (400.13 MHz, $CDCl_3$): δ = 7.95–7.91 (m, 2H; Ar—H), 7.46–7.41 (m, 2H; Ar—H), 5.63 (s, 1H; CH—CN), 3.10–2.94 (m, 4H; $2CH_2$—C_6 + C_9), 2.20–1.91 (m, 4H; CH_2—C_7 + C_8). ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 197.0 (2 C=O, indandione), 161.4 (C=O, pyrimidinone), 159.2 (C=N), 159.0, 140.8 (2 C=C; Ar carbons), 139.2 (C—S, thieno ring), 132.4, 128.0 (4CH, Ar carbons), 126.0, 117.8 (C=C; thieno ring), 112.6 (CN), 79.4 (*spiro* carbon), 41.2 (CH, thiazol ring), 28.6, 25.6, 22.8, 20.0 (CH_2). MS (EI, 70 eV, %): m/z = 420 ([M+1], 38), 419 ([M^+, 100), 394 (28), 382 (18), 342 (22), 328 (16), 304 (16), 272 (20), 98 (24). Anal. Calcd. for $C_{21}H_{13}N_3O_3S_2$: C, 60.13; H, 3.12; N, 10.02; S, 15.29%. Found: C, 60.00; H, 3.08; N, 9.93; S, 15.20%.

1,3,5′-Trioxo-1,3,3′,5′-tetrahydrospiro[indene-2,2′-thiazolo[2,3-b]cycloheptyl[b]thieno-[2,3-d]pyrimidine]-3′-carbonitrile (8c). Orange crystals (acetone), 273 mg (63%), mp 198–200°C. IR (KBr): ν = 3087–3004 (w, Ar—CH), 2990–2820 (w, aliph.-CH), 1702, 1685 (s, C=O), 1554 (s, C=C). ^{1}H-NMR (400.13 MHz, $CDCl_3$): δ = 7.97–7.92 (m, 2H; 2 CH Ar—H), 7.52–7.47 (m, 2H; 2CH Ar—H), 5.92 (s, 1H; CH—CN), 2.65–2.60 (m, 4H; $2CH_2$—C_7 + C_{11}), 1.72–1.65 (m, 4H; $2CH_2$—C_8 + C_{10}), 1.29–1.24 (m, 2H; CH_2—C_9). ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 197.4, 197.0 (C=O, indandione), 160.8 (C=O, pyrimidinone), 160.0 (C=N), 154.6, 140.8 (2 C=C; Ar carbons), 138.2 (C—S, thieno ring), 132.0, 128.8 (4CH, Ar carbons), 124.2, 116.2 (C=C; thieno ring), 113.0 (CN), 79.7 (*spiro* carbon), 42.4 (CH, thiazolo ring), 31.8, 28.2, 26.2, 24.8, 16.3 (CH_2, aliphatic carbons). FAB MS: *m/z* = 433. Anal. Calcd. for $C_{22}H_{15}N_3O_3S_2$: C, 60.95; H, 3.49; N, 9.69; S, 14.79%. Found: C, 60.80; H, 3.38; N, 9.54; S, 14.62%.

Reaction of thienopyrimidines 1a–c with 2,3-dicyano-1,4-naphthoquinone (9). *General procedure.* A mixture of compounds **1a–c** (0.01 mol) and 2,3-dicyano-1,4-naphthoquinone (0.208 g, 0.01 mol) (**9**) in DMF (30 mL) was refluxed for 2–6 h. After cooling, the solid product was collected. The obtained precipitates were filtered, dried, and recrystallized.

6,7-Dihydro-5H-cyclopenta[4,5]naphtha-[2′,3′,4,5]-[1,3]-thiazolo[3,2-a]thieno[2,3-d]pyrimidine-6,7,12-trione (10a). Pale orange crystals (ethanol), 264 mg (70%), mp 272–274°C. IR (KBr): ν = 3060–3045 (w, Ar—CH), 2960–2880 (w, aliph.-CH), 1702–1685 (s, C=O), 1557 (s, C=C). ^{1}H-NMR (400.13 MHz, $CDCl_3$): δ = 8.05–7.96 (m, 4H; Ar—H), 7.72–7.68 (m, 2H; Ar—H), 2.53–2.44 (m, 4H, 2H-6 + 2H-7), 1.95–1.78 (m, 2H, H-5). ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 178.3, 176.1 (C=O, naphthoquinone ring), 165.2 (C=O, pyrimidine ring), 163.2 (C= N), 155.1, 149.0 (2C=C, thiazolo ring), 139.4 (C—S), 135.2 (2CH Ar—CH), 131.8 (2C=C), 130.3 (2CH, Ar—CH), 125.2, 117.3 (C=C, thieno ring), 116.0 (2C=C, thiazolo ring), 32.1, 25.5, 21.3 (CH_2). FAB MS: *m/z* = 378. Anal. Calcd. for $C_{19}H_{10}N_2O_3S_2$: C, 60.30; H, 2.66; N, 12.68; S, 16.95%. Found: C, 60.20; H, 2.60; N, 12.60; S, 16.90%.

6,7-Dihydro-5H-cyclohexa[4,5]naphtho[2′,3′,4,5]-[1,3]thiazolo[3,2-a]thieno[2,3-d]pyrimidine-6,7,12-trione (10b). Orange crystals (ethanol), 297 mg (76%), mp 292–294°C. IR (KBr): ν = 3080–3030 (w, Ar—CH), 2980–2810 (aliph.-CH), 1702–1682 (s, C=O), 1555 (s, C=C). ^{1}H-NMR (400.13 MHz, $CDCl_3$): δ = 8.10–7.97 (m, 4H; Ar—H), 7.74–7.68 (m, 2H; Ar—H), 2.53–2.48 (m, 4H, 2H-6 + 2H-9), 1.61–1.49 (m, 4H, 2H-7 + 2H-8). ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 178.2, 176.0 (C=O, naphthoquinone ring), 165.8 (C=O, pyrimidine ring), 162.6 (C=N), 155.0, 148.6 (2C=C C=C, thiazolo ring), 139.0 (C—S), 135.0 (2CH, Ar—CH), 130.8 (2C=C), 130.1 (2CH, Ar—CH), 125.0, 117.0 (C=C, thieno ring), 116.2 (2C=C, thiazolo ring), 32.1, 28.2, 25.5, 21.3 (CH_2). FAB MS: *m/z* = 392. Anal. Calcd. for $C_{20}H_{12}N_2O_3S_2$: C, 61.21; H, 3.08; N, 7.14; S, 16.34%. Found: C, 61.14; H, 3.00; N, 7.00; S, 16.24%.

6,7-Dihydro-5H-cyclohepta[4,5]naphtho[2′,3′,4,5][1,3]-thiazolo[3,2-a]thieno[2,3-d]pyrimidine-6,7,12-trione (10c). Orange crystals (acetonitrile), 292 mg (72%), mp 302–304°C. IR (KBr): ν = 3090–3010 (w, Ar—CH), 2960–2810 (aliph.-CH), 1705–1687 (s, C=O), 1556 (s, C=C). ^{1}H-NMR (400.13 MHz, $CDCl_3$): δ = 7.89–7.84 (m, 2H; 2CH Ar—H), 7.76–7.72 (m, 2H; 2CH Ar—H), 2.85–2.83 (m, 2H), 2.65–2.58 (m, 2H), 1.50–1.44 (m, 4H), 1.20–1.16 (m, 2H), ^{13}C-NMR (100.6 MHz, $CDCl_3$): δ = 178.0, 176.2 (C=O, naphthoquinone ring), 166.2 (C=O, pyrimidine ring), 162.2 (C=N), 154.8, 148.6 (2C=C, thiazolo ring), 138.4 (C—S), 135.0 (2CH, Ar—CH), 130.8 (2C=C), 130.1 (2CH, Ar—CH), 125.2, 117.4 (C=C, thieno ring), 116.0 (2C=C, thiazolo ring), 32.1, 30.0, 28.0, 23.5, 19.2 (CH_2). FAB MS: *m/z* = 406. Anal. Calcd. for $C_{21}H_{14}N_2O_3S_2$: C, 62.05; H, 3.47; N, 6.89; S, 5.78%. Found: C, 61.90; H, 3.37; N, 6.80; S, 15.68 %.

Reaction of 2-hydrazino-5,6,7,8-tetrahydrobenzo-[b]thieno[2,3-d]pyrimidine-4(1H)-one (17) with dimethyl acetylenedicarboxylate (18). A mixture of **17** (0.236 g, 1 mmol) and dimethyl acetylenedicarboxylate (**18**, 0.142 g, 1 mmol) were heated at reflux in ethanol (25 mL) for 10 h. The formed yellow precipitate was collected by filtration and recrystallized.

(Z)-Dimethyl 2[(E)-4-oxo-3,4-dihydro[2,3-d]pyrimidine-2(1H) ylidene)hydrazono]succinate (20). Yellow crystals (ethyl acetate), 242 mg (64%), mp 230°C. IR (KBr): ν = 3330–3265 (NH), 2980–2820 (aliph.-CH), 1720–1710, 1685 (ester and pyrimidine—C=O), 1557 (C=C). ^{1}H-NMR (400 MHz, $CDCl_3$): δ =10.11 (b, 2H; NH), 3.83 (s, 3H; H-1′), 3.82 (s, 2H; H-4′), 3.74 (s, 3H; H-6′), 2.96 (bt, 2H; H-10), 2.70 (bt, 2H; H-7), 1.85 (m, 4H; H-8,9). ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 168.47 ("sextet", *J* = 4.3; C-5′), 164.26 (s; C-4), 163.77 (q; C-2′), 157.96 (s; C-13), 147.75 (s; C-2), 133.95 (t, *J* = 8.7; C-3′), 131.32 (m; C-6), 130.19 (m; C-11), 118.29 (s; C-5), 53.11 (q, *J* = 148.1; C-6′), 53.06 (q, *J* = 148.0; C-1′), 32.59 (t, *J* = 132.3; C-4′), 25.49 (tm, J_t = 133.2; C-10), 24.98 (tm, J_t = 131.2; C-7), 23.05 (tt, *J* = 122.3, 4.1; C-9), 22.24 (tt, *J* = 128.2, 4.2; C-8). FAB MS: *m/z* = 378. Anal. Calcd. for $C_{16}H_{18}N_4O_5S$: C, 50.78; H, 4.79; N, 14.81; S, 8.47%. Found: C, 50.60; H, 4.70; N, 14.74; S, 8.44%.

Reaction of ethyl propiolate (19) with 2-hydrazino-5,6,7,8-tetrahydrobenzo-[b]thieno[2,3-d]-pyrimidine-4(1H)-one (17). A mixture of **17** (0.236 g, 1 mmol) and ethyl propiolate (**19**, 0.098 g, 1 mmol) was heated at reflux in ethanol (25 mL) for 12 h. The formed pale orange precipitate was collected by filtration.

Ethyl 6-oxo-3,4,6,9-2H-5,6,7,8-tetrahydrobenzo[b]thieno pyrimido[2,1-c][1,2,4]triazine-4-carboxylate (19). Pale orange (EtOAc), 234 mg (70%), mp 260°C. IR (KBr): ν = 3420–3330 (NH), 2960–2830 (aliph.-CH), 1720, 1685 (ester and pyrimidine—C=O), 1558 (C=C). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 11.42 (s, 1H; N_α—H), 10.57 (b, 1H; N_β—H), 4.41 (t, *J* = 5.8, 1H; H-2′), 4.12 (q, *J* = 7.1, 2H; H-4′), 3.42 (d, *J* = 5.8, 2H; H-1′), 2.79 (m, 2H; H-10), 2.62 (t, *J* = 5.7, 2H; H-7), 1.77 (m, 2H; H-8), 1.73 (m, 2H; H-9), 1.21 (t, *J* = 7.1, 3H; H-5′). ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 169.58 (C-3′), 165.22 (C-4), 157.69 (C-13), 149.70 (C-2), 130.29 (C-6), 126.04 (C-11), 115.29 (C-5), 65.03 (C-2′), 60.52 (C-4′), 37.39 (C-1′), 25.27 (C-10), 24.21 (C-7), 22.66 (C-8), 21.85 (C-9), 14.04 (C-5′). FAB MS: *m/z* = 334.110. Anal. Calcd. for $C_{15}H_{18}N_4O_3S$: C, 53.88; H, 5.43; N, 16.75; S, 9.59%. Found: C, 53.70; H, 5.40; N, 16.70; S, 9.55%.

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