Promising Antimicrobial Agents: Synthetic Approaches to Novel Tricyclic and Tetracyclic Pyrimidinones with Antimicrobial Properties

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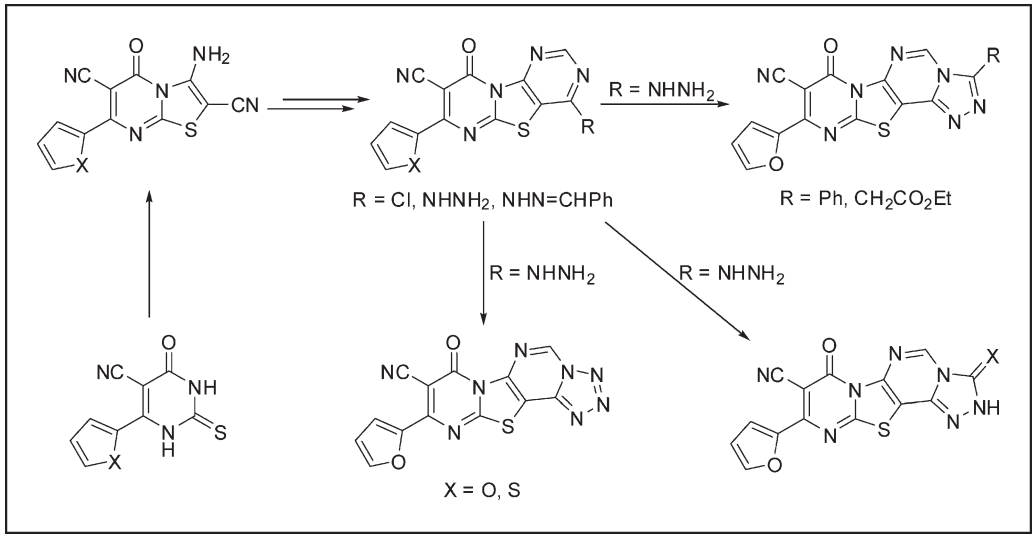

New tricyclic pyrimidinone derivatives were obtained from the corresponding thiazolopyrimidinone or hydrazino systems. The annelation of tricyclic hydrazino compound with 1,2,4-triazole and tetrazole moieties gave novel tetracyclic condensed pyrimidinones. The investigation of the antimicrobial properties of tricyclic and tetracyclic pyrimidinones, by agar-well diffusion assay, was carried out against six pathogenic bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella spp*, and *Salmonella typhyrium*) and four pathogenic fungi (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus*, and *Trichderma horozianum*). Most of the compounds tested exhibited some degree of antimicrobial activity against microorganisms. Among these compounds, 4-benzylidenhydrazino-8-cyano-7-(furan-2-yl)thiazolo[3,2-*a*:4,5-*d*']dipyrimidin-9-one (**12**) showed the most favorable antibacterial activity, while compound **17** showed the highest effect on fungi. Interestingly, tetrazole derivative **19** displayed a remarkable effect on fungi much more than the corresponding 3-substituted triazole derivatives on the one hand, whereas the lowest effect on bacteria on the other.

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INTRODUCTION

Pyrimidine and its derivatives are ubiquitous in nature. As such, the pyrimidine subunit has found widespread applications in therapeutically active compounds. Most importantly, pyrimidine bases are fundamental constituents of the building blocks of DNA and RNA and hence play a significant role in biochemical vital processes for human beings and animals [1,2]. Various analogs of thiopyrimidinones display antibacterial, antifungal, antiviral [3–5], and antileishmanial activities [3,6], whereas some derivatives of dihydropyrimidine (DHPM) have interesting biological properties such as antimicrobial [7], antiviral [8], and anticancer [9] activities and moreover are found to be useful in the treatment of benign prostatic hyperplasia [10]. More recently, these partly reduced DHPMs have emerged as anti-inflammatory agents [11]. Very recently, *S*-alkylpyrimidines possessing antifungal and antibacterial activities have been also reported in the literature [12]. Some time ago, a series of chloropyrimidines were identified as a new class of antimicrobial agents [13]. Also,

numerous nucleosides containing 1-substituted pyrimidines have found utility as anticancer and antiviral chemotherapeutic agents [8,14,15]. It should be kept in mind that thiazoles have occupied a unique place and have remarkably contributed to biological and medicinal chemistry [16–18]. Such medicines as sulfathiazole, phthalylsulfathiazole and related compounds are widely used in medical practice [19]. The thiazole ring unit is a useful structural component of natural compounds, *e.g*., Vitamin B1 (thiamine), penicillin, and carboxylase [19,20]. The 2-aminothiazole ring system has been employed in the preparation of a number of important drugs required for treatment of hypertension [21], inflammation [22], bacterial [23], and HIV infections [24]. Furthermore, aminothiazoles are well known for their antifungal [25], antimicrobial [26–28], antiviral [29], anti-inflammatory, and antioxidant [30] applications and also have been utilized for the treatment of both breast and prostate cancer [31,32], as a novel class of adenosine receptor antagonists [33,34] and in the development of cyclin-dependent kinase (CDK) inhibitors [35]. Moreover, some of the thiazole analogues are used as fungicides, inhibiting *in vivo* the growth of *Xanthomonas* and as an ingredient of herbicides or an schistosomicidal and anthelmintic drugs [36]. Literature survey reveals that triazole-containing substances are also well known for their diverse pharmaceutical activities including antimicrobial [37], insecticidal [38], antitumor [37,39], and anticonvulsant [40] effects, and moreover, triazolopyrimidines have recently been identified as adenosine A_3 receptor antagonists [41]. Interestingly, the fused tetrazoles have been found to exhibit similar biological properties to those of their corresponding triazole analogs [42].

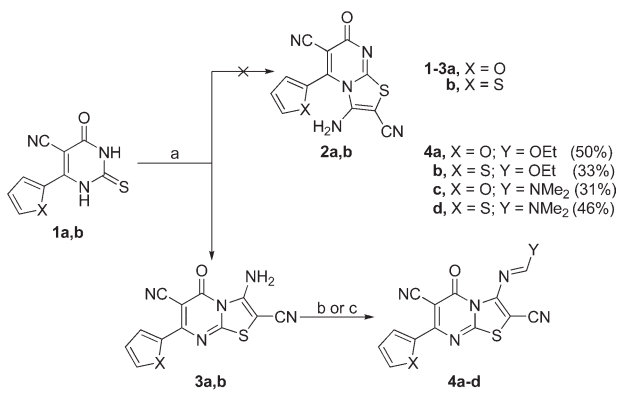
On the basis of the above data and continuing our studies on condensed heterocycles as a part of a chemotherapeutic research program [43–45], it was envisaged that the combined effect of all the above pharmacophores could result in interesting chemotherapeutic activity. Therefore, the goal of the present work was to synthesize substances containing a fused pyrimidine-thiazole scaffold as part of a tricyclic framework **8**, **10–12** and tetracyclic compounds of the same tricyclic structure with a heterocycle anellated to the pyrimidine ring **13**, **16–19** and to screen for their antibacterial and antifungal activities.

RESULTS AND DISCUSSION

Treatment of the 2-thioxopyrimidines **1a**,**b** [4,46] with bromomalononitrile, in ethanol containing potassium hydroxide, provided bicyclic products. Principally, there are three possible cyclization sites, *i.e*., either at *N*-3 or *N*-1 or partial cyclization at both, depending on the mode of cyclization. In practice, these reactions led to, in each case, the formation of only one isolable product as evidenced by TLC analysis. The structure of the isolated products was considered to be 5-one structure **3** rather than the related isomeric 7-one structure **2** based on the fact that the *N*-3 nitrogen atom of the pyrimidine ring in 2-thiouracil analogues has higher nucleophilic character when compared with *N*-1 atom, and hence, *N*-3 nitrogen is more reactive towards electrophiles than the *N*-1 position, which is part of a push-pull system with the cyano group in the 5-position of the pyrimidine ring. Therefore, the *N*-3 and not the *N*-1 always participates in the cyclization processes as clearly indicated from literature reports [4,47–52]. The IR and ^{13}C NMR spectra provide further evidence for the proposed structure by comparison of these spectra with those of similar annelated pyrimidinones. The IR spectra of the products isolated from the studied reactions showed among its peaks those for carbonyl carbon of the pyrimidinone ring at ν 1692 and 1690 cm^{-1}, respectively. This high frequency absorption is in favor of structure **3** [47,51,52]. Literature reports [47,53] have shown that the chemical shift for the carbonyl carbon in pyrimidin-4-one derivatives is markedly affected by the nature of the adjacent nitrogen (*N*-3) (pyrrole type in our structure **3** and pyridine type as in structure **2**). For instance, the ^{13}C NMR spectrum of compound **3a**, as a typical example, displayed the signal of the carbonyl carbon residue at δ 160.8 ppm. Such an upfield chemical shift value is in agreement with pyrimidin-5-one **3** rather than with pyrimidin-7-one **2**, for which carbonyl stretching frequencies would be expected to appear in the region ν 1640–1660 cm^{-1}, and the cyclic carbonyl groups would be expected to resonate in the lower field region ($\delta_C \sim 170$ ppm) as reported by Shawali *et al*. [47]. Consequently, it is reasonable to conclude that the studied reactions are completely regioselective and the structure of the isolated products is pyrimidin-5-one **3**; the alternative cyclization mode to the respective 7-one **2** is therefore discarded. Condensation of 3-amino-2-cyanothiazolopyrimidines **3a**,**b** with triethyl orthoformate gave the corresponding imino ethers **4a**,**b** while with dimethylformamide dimethylacetal (DMFDMA), the amidines **4c**,**d** were obtained (Scheme 1).

Closure of a second pyrimidine ring of the thiazolodipyrimidine ring system was carried out by heating *N*-ethoxymethylene derivative **4a** at reflux in an alcoholic solution of hydrazine hydrate (Scheme 2) to yield a reaction product of molecular formula $C_{13}H_7N_7O_2S$, which corresponded to the addition of the hydrazine to **4a** and the loss of one molecule of ethanol. The IR spectrum of the reaction product was characterized by the absence of one absorption for the cyano group and the presence of an absorption band in the region ν 3382–3200 cm^{-1} due to the hydrazino moiety in

Scheme 1. Synthetic pathway of thiazolopyrimidines **3**, **4**.

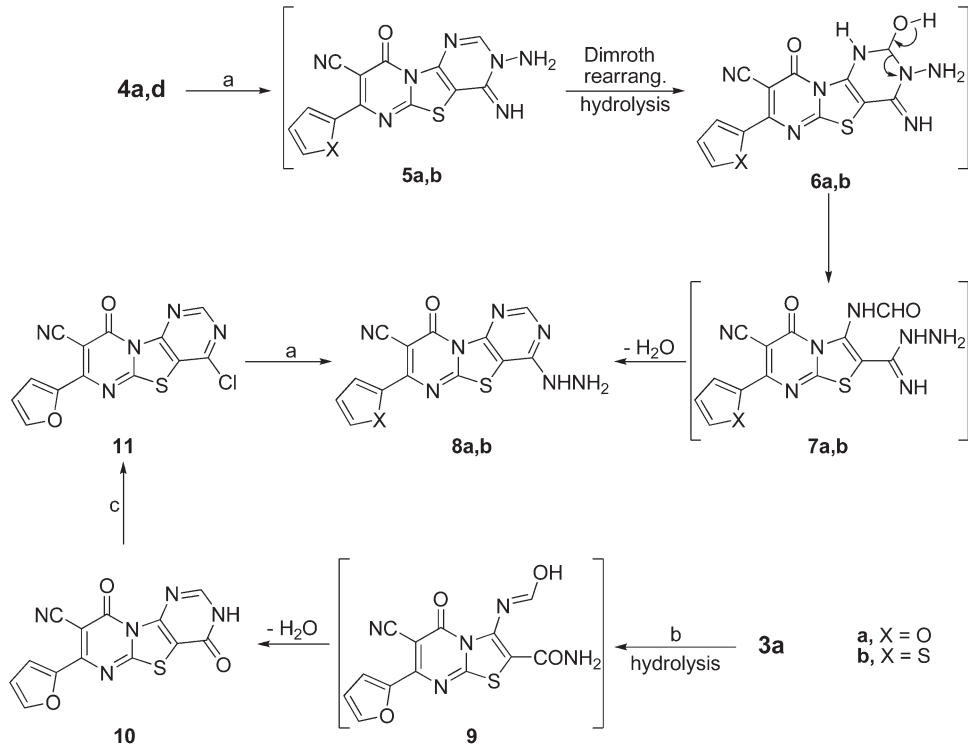


Reagents and conditions: (a) $BrCH(CN)_2$, KOH. EtOH, r.t. (41-45%); (b) $HC(OEt)_3$, reflux (33-50%); (c) $(MeO)_2CHNMe_2$, xylene, reflux (31-46%)
r.t.= room temperature

addition to a single cyano group at ν 2219 cm^{-1} and carbonyl function at ν 1697 cm^{-1}. The ^1H NMR spectrum of that product indicated the disappearance of the resonance signals from protons of the ethyl unit and the appearance of signals from the hydrazino moiety and a pyrimidine methine in their proper positions, besides the expected furyl resonances. Accordingly, this compound could be formulated as the tricyclic hydrazino derivative **8a**, formed most likely *via* a Dimroth-type rearrangement [54–56] of the initial cyclization product **5a**. The ^{13}C NMR spectrum of the isolated product was also in accordance with the proposed structure (see Experimental section). The pathway of this reaction, as illustrated in Scheme 2, may involve, first, the anticipated formation of imino compound **5a** followed by subsequent covalent hydration under the applied reaction conditions to afford the 2-hydroxy intermediate **6a**. Then, the pyrimidine ring opens and forms the formyl intermediate **7a**, which undergoes spontaneous heteroannelation with the more nucleophilic imino group to give the rearranged hydrazino compound **8a** (Scheme 2). A similar treatment of amidine **4d** with hydrazine hydrate resulted in the formation of the Dimroth rearrangement product **8b**. Intermediacy of **5b**, **6b**, and **7b** are most likely. It is worth mentioning that the latter products of the reaction of **4a**,**d** with hydrazine hydrate were recovered completely unchanged when subjected to conditions leading to hydrolysis of the imino group to carbonyl function, thus supporting the Dimroth rearrangement of **5a**,**b** to **8a**,**b** (Scheme 2).

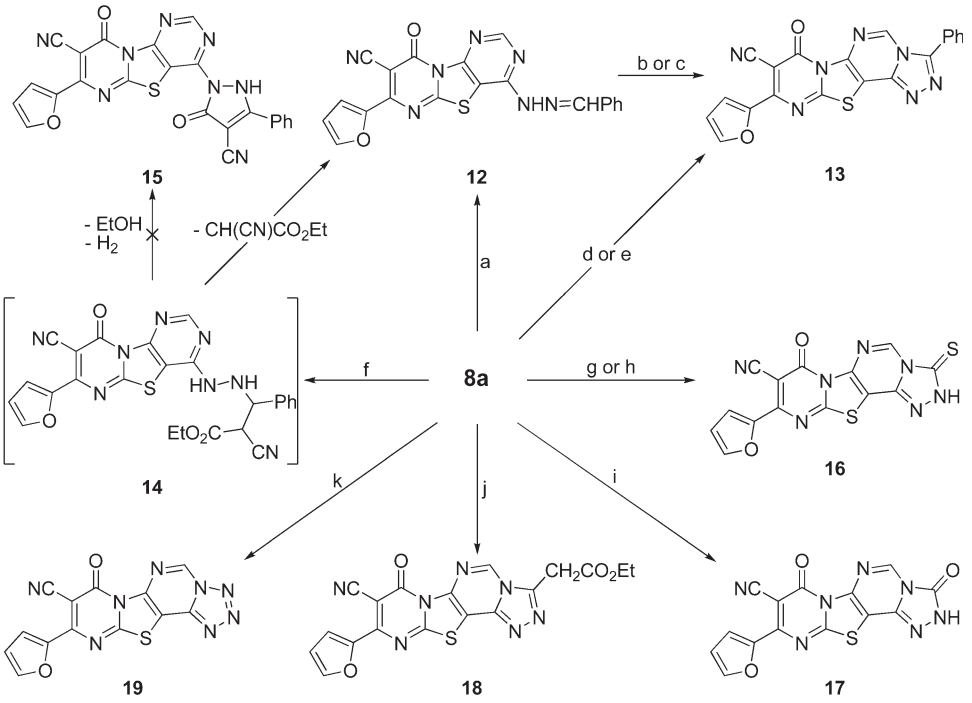
Nevertheless, a proof of structure **8** was accomplished by using an alternative synthetic route involving the cycliztion of aminonitrile **3a** with formic acid to give the dione **10** through the intermediate formation of carboxamide **9**. A further reaction of **10** with phosphorus

Scheme 2. Synthetic pathway of thiazolodipyrimidines **8**, **10**, **11**.



Reagents and conditions: (a) $N_2H_4.H_2O$, abs. EtOH, reflux (32-61%); (b) HCO_2H, reflux (56%); (c) $POCl_3$, reflux (77%)

Scheme 3. Synthetic pathway of tetracyclic pyrimidinones **13**, **16–19**.



Reagents and conditions: (a) PhCHO, abs. EtOH, pip., reflux (40%); (b) $PhNO_2$, reflux (72%); (c) $FeCl_3$, EtOH, reflux (63%); (d) $PhCO_2H$, $POCl_3$, reflux (59%); (e) PhCOCl, reflux (62%); (f) $PhCH{=}C(CN)CO_2Et$, EtOH, pip., reflux (53%); (g) PhNCS, NaOEt, reflux (75%); (h) CS_2, py., reflux (64%); (i) $ClCO_2Et$, py., reflux (89%); (j) $CH_2(CO_2Et)_2$, reflux (69%); (k) gl. AcOH, HCl, $NaNO_2$, 0-5 °C (60%)

oxychloride produced the respective chloro derivative **11**, hydrazinolysis of which led to the hydrazino compound **8a** (61% yield), whose spectral characteristics were completely coincident with the previously isolated sample (Scheme 2).

Treatment of **8a** with benzaldehyde, in boiling absolute ethanol in the presence of piperidine furnished the corresponding acyclic condensation product **12**. Oxidative cyclodehydrogenation of Schiff's base **12** by boiling in nitrobenzene or by treatment with ethanolic iron(III) chloride solution led to, in every case, a single product for which the tetracyclic-condensed structure **13** was established on the basis of its analytical and spectroscopic data. The absence of the methine proton of the hydrazone **12** in the ^{1}H NMR spectrum of **13** confirmed the structure. It is interesting to note that the same product **13** could be also obtained directly from cyclocondensation of the hydrazino derivative **8a** with benzoic acid in boiling phosphorus oxychloride. This fact was supported by heating compound **8a** at reflux in an excess of benzoyl chloride, wherein compound **13** was also isolated.

It is remarkable to report here that an unexpected reaction took place on reacting **8a** with ethyl benzylidenecyanoacetate in the presence of piperidine in an attempt to obtain the pyrazolyl derivative **15**. To our surprise, this reaction did not give the desired **15** and instead the Schiff's base **12** was isolated as indicated from TLC analysis, mp, mixed mp, and IR data of the reaction product. This result can be explained by assuming the formation of Michael adduct **14** as a first step. Subsequent ethyl cyanoacetate elimination leads eventually to the final benzylidene derivative **12**, what is in agreement with a previous literature report [37].

Another new tetracyclic pyrimidinone derivative **16** was synthesized from the hydrazino compound **8a** by reaction with one carbon inserting agents. Thus, interaction of **8a** with phenyl isothiocyanate in ethanolic sodium ethoxide solution gave the target **16** (Scheme 3). This reaction is assumed to proceed most likely with *in situ* evolution of aniline. In support of this hypothesis, the desired **16** was also obtained by an independent route involving the reaction of **8a** with carbon disulfide at reflux in pyridine, leading to a reaction product that was identical to **16** obtained by the prescribed method according to TLC analysis, mp, mixed mp, and IR data.

The hydrazino derivative **8a** proved to be a useful precursor for the synthesis of other tetracyclic pyrimidinones. Thus, reaction of **8a** with an excess of ethyl chloroformate, at reflux in pyridine, led to the triazolone

Table 1

Mean diameter of inhibition zone (mm) as a criterion of antibacterial activity for selected tricyclic and tetracyclic derivatives.

Compd.	Test bacterial isolate					
	S. aureus	*Klep. spp*	*S. typhy.*	*B. cereus*	*E. coli*	*P. aeruginosa*
8a	2.9 ± 0.2	1.7 ± 0.2	1.4 ± 0.2	1.7 ± 0.2	0.0	0.0
10	1.9 ± 0.2	1.7 ± 0.2	0.0	1.4 ± 0.2	1.7 ± 0.2	0.0
11	1.7 ± 0.1	1.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.2	1.4 ± 0.2
12	1.4 ± 0.2	1.6 ± 0.2	1.3 ± 0.2	1.9 ± 0.2	1.5 ± 0.2	1.7 ± 0.2
13	1.7 ± 0.2	1.5 ± 0.2	0.0	1.8 ± 0.2	0.0	0.0
16	1.6 ± 0.2	1.6 ± 0.2	0.0	1.8 ± 0.2	0.0	0.0
17	1.4 ± 0.1	1.8 ± 0.2	0.0	2.1 ± 0.2	1.7 ± 0.2	2
18	1.5 ± 0.2	1.6 ± 0.2	0.0	1.8 ± 0.2	0.0	0.0
19	1.7 ± 0.2	1.5 ± 0.2	0.0	1.5 ± 0.2	0.0	0.0
Standard[a]	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.2	1.2 ± 0.1	1.2 ± 0.2	1.3 ± 0.2

[a] Standard for bacteria: Oxacillin 1 mg/mL.

analogue **17**, while with diethyl malonate, the ethyl ester **18** was isolated in acceptable yield. Elucidation of structure for the latter products was established on the basis of elemental and spectroscopic analyses in each case (see Experimental section).

As an extension to this synthetic route, treatment of compound **8a** with sodium nitrite resulted in the formation of a similar product with annelated tetrazole ring **19**, formed most likely *via* diazotization, by the action of *in situ* generated nitrous acid on **8a**, followed by self-condensation. A similar diazotization of hydrazinopyrimidines with sodium nitrite has been reported previously [42,49,54].

Antimicrobial evaluation. As shown by the results in Tables 1 and 2, the majority of the new tricyclic and tetracyclic compounds tested displayed *in vitro* antibacterial and antifungal activities. In general, the chemical structure of the whole molecule, comprising the nature of the heterocyclic system as well as the type of the substituted function present in the heterocyclic ring structure, has a pronounced effect on antimicrobial activity. In particular, it has been found that antimicrobial activity was highly dependent on the type of substituent at the 4-position of pyrimidine ring in the tricyclic core (compounds **8a** and **10–12**, respectively). The most toxic compound to the test bacterial isolates was that containing a benzylidenhydrazino moiety at position 4 of pyrimidine ring in the tricyclic ring system (compound **12**) as compared with the other 4-substituted analogues (compounds **8a**, **10**, and **11**). Replacement of the benzylidenhydrazino moiety by a chlorine atom diminished slightly the activity of **11**; however, 4-chloro analog **11** exhibited more pronounced antibacterial activity than the corresponding 4-hydrazino **8a** or 4-oxo **10** derivatives. Fusion of 3-substituted 1,2,4-triazoles with the parent thiazolodipyrimidine structure (compounds **13** and **16–18**) led in general to a decrease in antibacterial activity relative to the corresponding tricyclic products. However, the antifungal activity of tetracyclic triazole derivative **17** was found to be the highest. This was followed by the tetrazole derivative **19**, which displayed a remarkable effect on fungi much more than the other 3-substituted triazole derivatives (compounds **13**, **16**, and **18**). Despite promising *in vitro* antifungal activity of **19**, only poor activity was found against test bacterial isolates (*Staphylococcus aureus*, *Klebsiella spp*, and *Bacillus cereus*).

CONCLUSION

New heterocyclic motifs were obtained from the reaction of aminonitriles **3a**,**b** and hydrazino compound **8a** with several commercially available reactants. All the tricyclic and tetracyclic compounds described in this work retain a thiazolodipyrimidinone core, while a fourth fused heterocyclic nucleus (triazole or tetrazole)

Table 2

Mean diameter of inhibition zone (mm) as a criterion of antifungal activity for selected tricyclic and tetracyclic derivatives.

Compd.	Test fungal isolate			
	A. niger	*A. flavus*	*A. fumigatus*	*T. horozianum*
8a	2.6 ± 0.2	2.4 ± 0.2	1.7 ± 0.2	2
10	2.5 ± 0.2	2.8 ± 0.4	0.0	2.4 ± 0.4
11	2.4 ± 0.2	2.2 ± 0.2	1.6 ± 0.2	2.4 ± 0.4
12	2	3 ± 0.4	0.0	2.2 ± 0.2
13	2.1 ± 0.2	2.6 ± 0.4	0.0	2.5 ± 0.2
16	2.5 ± 0.2	2.5 ± 0.2	1.6 ± 0.2	2.5 ± 0.2
17	3.1 ± 0.2	3.2 ± 0.2	2.8 ± 0.4	2.9 ± 0.4
18	2.5 ± 0.2	2.6 ± 0.2	0.0	1.8 ± 0.2
19	2.4 ± 0.2	2.5 ± 0.2	1.8 ± 0.2	2.5 ± 0.2
Standard[a]	2.6 ± 0.2	2.8 ± 0.1	2.8 ± 0.1	2.4 ± 0.2

[a] Standard for fungi: Mycostatine 1 mg/mL.

was constructed by cyclocondensation reactions of tricyclic hydrazino compound **8a** with various chemical reagents. The biological potential of all the fused pyrimidinone derivatives was further investigated by screening for their antimicrobial activity. The test compounds displayed different levels of antibacterial and antifungal activities, with the assays carried out on six pathogenic bacteria and four pathogenic fungi. The most potent compounds against test bacterial and fungal isolates were 4-benzylidenhydrazino compound **12** and dione derivative **17**, respectively. Further studies on structure–activity relationships (SAR) combined with molecular modeling approaches of these condensed heterocyclic derivatives are currently underway, and the results of this research will be reported in due course. We believe that research in this direction should be encouraged in order to broaden the applicability of these new heterocyclic frameworks to serving as leads for designing novel chemotherapeutic agents.

EXPERIMENTAL

Melting points are uncorrected. IR spectra were recorded (KBr) on a Pye Unicam SP-1000 spectrophotometer. NMR spectra were obtained on a Varian Gemini 300 MHz spectrometer in DMSO-d_6 as solvent and TMS as internal reference. Chemical shifts are expressed in δ ppm. EI mass spectra were recorded on a Shimadzu GC MS-QP 1000 EI mass spectrometer at 70 eV. Compounds **1a**,**b** were prepared according to known methods [4,46].

General procedure for the preparation of 7-substituted-3-amino-5-oxo-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitriles (3a,b). To a warm ethanolic potassium hydroxide solution [prepared by dissolving potassium hydroxide (0.005 mol) in ethanol (30 mL)] of either pyrimidinethione **1a** or **1b** (0.005 mol), bromomalononitrile (0.005 mol) was added portionwise with stirring. The reaction content was then left overnight at room temperature, whereby the solid product so precipitated upon dilution with water was filtered off, dried, and recrystallized from the appropriate solvents to give the title compounds **3a** (0.64 g; 45%) and **3b** (0.61 g; 41%), respectively.

3-Amino-7-(2-furyl)-5-oxo-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitrile (3a). This compound was obtained as a yellow solid (DMF/H_2O), mp 267–268°C; IR (ν/cm^{-1}): 3380, 3272 (NH_2), 2221, 2202 (2CN), 1692 (CO); ^{1}H NMR (δ ppm): 6.74 (dd, 1H, J = 1.6, 3.7 Hz, furan H-4), 7.31 (d, 1H, J = 3.7 Hz, furan H-3), 7.90 (d, 1H, J = 1.6 Hz, furan H-5), 8.48 (s, 2H, NH_2, D_2O-exchangeable); ^{13}C NMR (δ ppm): 79.9, 98.5 (C-2, C-6), 111.2, 112.1 (furan C-3,4), 116.8 (CN), 118.0 (CN), 144.2 (furan C-5), 155.0 (furan C-2), 158.5, 159.0 (C-8a, C-3), 160.8 (CO), 167.9 (C-7); *Anal*. Calcd. for $C_{12}H_5N_5O_2S$ (283.270): C, 50.88; H, 1.78; N, 24.72; S, 11.32. Found: C, 50.59; H, 1.60; N, 24.51; S, 11.09.

3-Amino-5-oxo-7-(2-thienyl)-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitrile (3b). This compound was obtained as a yellowish white solid (DMF), mp 251-252 °C; IR (ν/cm^{-1}): 3374, 3260 (NH_2), 2218, 2202 (2CN), 1690 (CO); ^{1}H NMR (δ ppm): 7.05 (dd, 1H, J = 3.5, 4.9 Hz, thiophene H-4), 7.89 (d, 1H, J = 3.5 Hz, thiophene H-3), 8.04 (d, 1H, J = 4.9 Hz, thiophene H-5), 8.72 (s, 2H, NH_2, D_2O-exchangeable); Anal. Calcd. for $C_{12}H_5N_5OS_2$ (299.337): C, 48.15; H, 1.68; N, 23.40; S, 21.42. Found: C, 47.87; H, 1.53; N, 23.31; S, 21.15.

General procedure for the preparation of 7-substituted-3-(ethoxymethylene)amino-5-oxo-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitriles (4a,b). A mixture of either **3a** or **3b** (0.005 mol) and triethyl orthoformate (10 mL) was heated at reflux for 10 h. After distillation of the ortho ester, the viscous mass was treated with ether or petroleum ether (3 mL). The precipitated crystals of products **4a**,**b** were collected by filtration and recrystallized from the proper solvents to give the imino ethers **4a** (0.85 g; 50%) and **4b** (0.59 g; 33%), respectively.

3-(Ethoxymethylene)amino-7-(2-furyl)-5-oxo-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitrile (4a). This compound was obtained as a pale brown solid (EtOH/H_2O), mp 214°C; IR (ν/cm^{-1}): 2225, 2216 (2CN), 1700 (CO), 1621 (C=N); ^{1}H NMR (δ ppm): 1.41 (t, 3H, J = 7.3 Hz, CH_3 ethoxy), 4.43 (q, 2H, J = 7.3 Hz, OCH_2), 6.68 (dd, 1H, J = 1.6, 3.8 Hz, furan H-4), 7.35 (d, 1H, J = 3.8 Hz, furan H-3), 7.85 (d, 1H, J = 1.6 Hz, furan H-5), 8.36 (s, 1H, methylenic CH); MS: m/z (%) = 339 (M^+, 18%); Anal. Calcd. for $C_{15}H_9N_5O_3S$ (339.334): C, 53.09; H, 2.67; N, 20.64; S, 9.45. Found: C, 52.93; H, 2.51; N, 20.37; S, 9.30.

3-(Ethoxymethylene)amino-5-oxo-7-(2-thienyl)-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitrile (4b). This compound was obtained as a golden yellow solid (EtOH), mp 201–202°C; IR (ν/cm^{-1}): 2221, 2215 (2CN), 1704 (CO), 1621 (C=N); ^{1}H NMR (δ ppm): 1.39 (t, 3H, J = 7.4 Hz, ethoxy CH_3), 4.45 (q, 2H, J = 7.4 Hz, OCH_2), 7.24 (dd, 1H, J = 3.7, 4.8 Hz, thiophene H-4), 7.95 (d, 1H, J = 3.7 Hz, thiophene H-3), 8.10 (d, 1H, J = 4.8 Hz, thiophene H-5), 8.36 (s, 1H, methylenic CH); Anal. Calcd. for $C_{15}H_9N_5O_2S_2$ (355.390): C, 50.69; H, 2.55; N, 19.71; S, 18.04. Found: C, 50.47; H, 2.43; N, 19.49; S, 17.86.

General procedure for the preparation of 7-substituted-3-(dimethylaminomethylenamino)-5-oxo-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitriles (4c,d). To a solution of either **3a** or **3b** (0.005 mol), in dry xylene (30 mL), DMFDMA (0.006 mol) was added and the reaction content was then heated under reflux for 6 h. The reaction mixture was cooled and triturated with petroleum ether (40–60). The solid product obtained was filtered off, dried and recrystallized from the appropriate solvents to give the amidines **4c** (0.52 g; 31%) and **4d** (0.82 g; 46%), respectively.

3-(Dimethylaminomethylenamino)-7-(2-furyl)-5-oxo-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitrile (4c). This compound was obtained as an orange solid (EtOH), mp 239–241°C; IR (ν/cm^{-1}): 2225, 2214 (2CN), 1700 (CO), 1618 (C=N); ^{1}H NMR (δ ppm): 3.05, 3.14 (2s, 6H, NMe_2), 6.85–7.17 (m, 2H, furan H-3,4), 7.83 (d, 1H, J = 1.9 Hz, furan H-5), 8.23 (s, 1H, methylenic CH); Anal. Calcd. for $C_{15}H_{10}N_6O_2S$ (338.340): C, 53.25; H, 2.98; N, 24.84; S, 9.48. Found: C, 52.99; H, 2.81; N, 24.57; S, 9.20.

3-(Dimethylaminomethylenamino)-5-oxo-7-(2-thienyl)-5*H*-thiazolo[3,2-*a*]pyrimidine-2,6-dicarbonitrile (4d). This compound was obtained as a brown solid (1,4-dioxane), mp 232–233°C; IR (ν/cm^{-1}): 2220, 2212 (2CN), 1695 (CO), 1617 (C=N); ^{1}H NMR (δ ppm): 2.89, 2.99 (2s, 6H, NMe_2), 7.30 (dd, 1H, J = 3.9, 5.2 Hz, thiophene H-4), 7.81 (d, 1H, J = 3.9

Hz, thiophene H-3), 7.97 (d, 1H, J = 5.2 Hz, thiophene H-5), 8.12 (s, 1H, methylenic CH); Anal. Calcd. for $C_{15}H_{10}N_6OS_2$ (354.417): C, 50.83; H, 2.84; N, 23.71; S, 18.09; Found: C, 50.55; H, 2.72; N, 23.46; S, 17.91.

General procedure for the preparation of 7-substituted-8-cyano-4-hydrazinothiazolo[3,2-*a*:4,5-*d*′]dipyrimidin-9-ones (8a,b). ***Method A for compounds 8a,b.*** A mixture of either **4a** or **4d** (0.005 mol), hydrazine hydrate (0.05 mol, 2.5 mL) and absolute ethanol (12 mL) was refluxed for 5 h. The precipitates formed after cooling overnight were collected by filtration, washed with cold alcohol, and recrystallized from the proper solvents to give the hydrazino compounds **8a** (0.89 g; 55%) and **8b** (0.55 g; 32%), respectively.

8-Cyano-7-(2-furyl)-4-hydrazinothiazolo[3,2-a:4,5-d′]dipyrimidin-9-one (8a). This compound was obtained as a light brown solid (EtOH), mp 246–247°C; IR (ν/cm^{-1}): 3382–3200 (NH, NH_2), 2219 (CN), 1697 (CO); ^{1}H NMR (δ ppm): 4.76 (s, br, 2H, NH_2, D_2O-exchangeable), 6.69 (dd, 1H, J = 1.6, 3.7 Hz, furan H-4), 7.25 (d, 1H, J = 3.7 Hz, furan H-3), 7.81 (d, 1H, J = 1.6 Hz, furan H-5), 7.95 (s, 1H, pyrimidine H-2), 10.15 (s, br, 1H, NH, D_2O-exchangeable); ^{13}C NMR (δ ppm): 95.3, 99.0, 111.5, 112.2 (furan C-3,4), 117.1 (CN), 143.5 (furan C-5), 155.2 (furan C-2), 157.4, 158.0, 158.5, 159.6, 161.4 (CO), 167.8 (C-7); *Anal*. Calcd. for $C_{13}H_7N_7O_2S$ (325.311): C, 48.00; H, 2.17; N, 30.14; S, 9.86. Found: C, 47.73; H, 1.99; N, 29.88; S, 9.72.

8-Cyano-4-hydrazino-7-(2-thienyl)thiazolo[3,2-a:4,5-d′]dipyrimidin-9-one (8b). This compound was obtained as canary yellow crystals (MeOH), mp 261°C; IR (ν/cm^{-1}): 3370–3208 (NH, NH_2), 2219 (CN), 1702 (CO); ^{1}H NMR (δ ppm): 4.95 (s, br, 2H, NH_2, D_2O-exchangeable), 7.16 (dd, 1H, J = 4.3, 4.8 Hz, thiophene H-4), 7.88–7.92 (m, 2H, thiophene H-3, pyrimidine H-2), 8.06 (d, 1H, J = 4.8 Hz, thiophene H-5), 10.54 (s, br, 1H, NH, D_2O-exchangeable); MS: m/z (%) = 341 (M^+, 26%); Anal. Calcd. for $C_{13}H_7N_7OS_2$ (341.378): C, 45.74; H, 2.07; N, 28.72; S, 18.79. Found: C, 45.60; H, 1.92; N, 28.46; S, 18.51.

Method B for compound 8a. Chloro compound **11** (0.002 mol) was mixed with hydrazine hydrate (0.006 mol), in absolute ethanol (20 mL). The mixture was stirred under reflux for 3 h. The precipitate formed during reflux was collected by filtration and found, after recrystallization from EtOH, identical in all respects to that obtained from method A (61% yield).

8-Cyano-7-(2-furyl)-4,9-dioxo-3,4-dihydro-9*H*-thiazolo[3,2-*a*:4,5-*d*′]dipyrimidine (10). Compound **3a** (0.003 mol) was heated under reflux in formic acid (10 mL) for 7 h. The reaction mixture was then diluted with cold water and allowed to stand overnight. The resulting precipitate was filtered off, washed with ethanol (20 mL), dried, and recrystallized from DMF/EtOH (2:1) as reddish brown crystals (0.52 g; 56%), mp 256–257°C; IR (ν/cm^{-1}): 3157 (NH), 2221 (CN), 1697, 1670 (2CO); ^{1}H NMR (δ ppm): 6.64 (dd, 1H, J = 1.7, 4.0 Hz, furan H-4), 7.33 (d, 1H, J = 4.0 Hz, furan H-3), 7.76 (d, 1H, J = 1.7 Hz, furan H-5), 8.01 (s, 1H, pyrimidine H-2), 12.52 (s, br, 1H, NH, D_2O-exchangeable); Anal. Calcd. for $C_{13}H_5N_5O_3S$ (311.280): C, 50.16; H, 1.62; N, 22.50; S, 10.30. Found: C, 49.87; H, 1.54; N, 22.42; S, 10.13.

4-Chloro-8-cyano7-(2-furyl)thiazolo[3,2-*a*:4,5-*d*′]dipyrimidin-9-one (11). A suspension of compound **10** (0.002 mol) in phosphorus oxychloride (30 mL) was refluxed with stirring for 5 h and then left aside to cool to room temperature overnight under stirring. Excess reagent was removed under reduced pressure. The residue was poured to ice/water with stirring and the precipitate was filtered off, dried, and recrystallized from acetone to give the chloro compound **11** as a dark brown solid (0.51 g; 77%), mp > 300°C; IR (ν/cm^{-1}): 2223 (CN), 1699 (CO); ^{1}H NMR (δ ppm): 6.72 (dd, 1H, J = 1.8, 3.9 Hz, furan H-4), 7.31 (d, 1H, J = 3.9 Hz, furan H-3), 7.84 (d, 1H, J = 1.8 Hz, furan H-5), 8.45 (s, 1H, pyrimidine H-2); Anal. Calcd. for $C_{13}H_4ClN_5O_2S$ (329.726): C, 47.35; H, 1.22; Cl, 10.75; N, 21.24; S, 9.72. Found: C, 47.09; H, 1.10; Cl, 10.62; N, 20.98; S, 9.56.

4-Benzylidenhydrazino-8-cyano-7-(2-furyl)thiazolo[3,2-*a*:4,5-*d*′]dipyrimidin-9-one (12). ***Method A.*** Compound **8a** (0.005 mol) was dissolved in absolute ethanol (20 mL), then benzaldehyde (0.006 mol) and piperidine (0.5 mL) were added. The reaction mixture was heated at reflux for 1.5 h. On cooling, the deposited solid product was filtered off and dried. Recrystallization from EtOH gave yellow crystals of the title compound **12** (0.83 g; 40%), mp 261–262°C; IR (ν/cm^{-1}): 3330 (NH), 3080 (arom. CH), 2223 (CN), 1698 (CO), 1626 (C=N); ^{1}H NMR (δ ppm): 6.71–6.76 (m, 1H, furan H-4), 7.42–7.76 (m, 6H, furan H-3, Ph), 7.86 (d, 1H, J = 2.0 Hz, furan H-5), 7.95 (s, 1H, pyrimidine H-2), 8.49 (s, 1H, CH=N), 11.92 (s, 1H, NH, D_2O-exchangeable); *Anal*. Calcd. for $C_{20}H_{11}N_7O_2S$ (413.419): C, 58.11; H, 2.68; N, 23.72; S, 7.76. Found: C, 57.86; H, 2.52; N, 23.50; S, 7.49.

Method B. To a solution of **8a** (0.002 mol) and few drops of piperidine (0.5 mL) in ethanol (10 mL), ethyl benzylidenecyanoacetate (0.002 mol) was added. The reaction content was heated under reflux for 3 h. After cooling, the obtained crystalline product was collected by filtration, washed several times with water, and dried. Recrystallization from EtOH gave, upon air drying, a yellow product (0.44 g; 53%) identical in all aspects (mp, mixed mp, and IR data) to that described in method A.

9-Cyano-10-(2-furyl)-3-phenylpyrimido[2′,1′:2,3]thiazolo[5,4-*e*][1,2,4]triazolo[4,3-*c*]pyrimidin-8-one (13). ***Method A.*** Compound **12** (0.002 mol) was heated at reflux in nitrobenzene (10 mL) for 1 h. The final mixture was concentrated and the product deposited after cooling was recrystallized from dilute acetic acid to give the 3-phenyl derivative **13** as a brown solid (0.59 g; 72%), mp > 300°C; IR (ν/cm^{-1}): 3064 (arom. CH), 2220 (CN), 1697 (CO); ^{1}H NMR (δ ppm): 6.75 (dd, 1H, J = 1.7, 3.8 Hz, furan H-4), 7.34–7.79 (m, 6H, furan H-3, Ph), 7.89 (d, 1H, J = 1.7 Hz, furan H-5), 8.30 (s, 1H, pyrimidine H-5); Anal. Calcd. for $C_{20}H_9N_7O_2S$ (411.404): C, 58.39; H, 2.21; N, 23.83; S, 7.79. Found: C, 58.21; H, 2.08; N, 23.56; S, 7.80.

Method B. To a solution of **12** (0.0025 mol) in ethanol (50 mL), ethanolic iron(III) chloride solution [prepared by dissolving iron(III) chloride (0.005 mol) in ethanol (10 mL)] was added portionwise while stirring. The reaction content was then boiled for 15 min and left at room temperature overnight. The solid product that separated out was collected by filtration and recrystallized from dilute acetic acid to give a tetracyclic product (0.65 g; 63%) that was found to be identical in all aspects (mp, mixed mp, and IR data) to the product prepared by method A.

Method C. A mixture of **8a** (0.002 mol) and benzoic acid (0.004 mol) was refluxed with phosphorus oxychloride (10 mL) for 30 min. Excess phosphorus oxychloride was distilled off under reduced pressure. The residue was triturated with dilute sodium hydroxide solution to remove the unreacted material. The solid residue was recrystallized from dilute acetic acid to give a solid product (0.49 g; 59%). Again, this product was identified as **13**.

Method D. A mixture of **8a** (0.002 mol) and benzoyl chloride (10 mL) was refluxed for 4 h. The excess of benzoyl chloride was extracted with benzene and the residue was

recrystallized from dilute acetic acid to give a solid product (0.51 g; 62%). The material proved to be **13**.

9-Cyano-10-(2-furyl)-3-thioxo-2,3-dihydropyrimido[2′,1′:2,3]thiazolo[5,4-*e*][1,2,4]triazolo[4,3-*c*]pyrimidin-8-one (16). ***Method A.*** To a solution of **8a** (0.002 mol) in ethanolic sodium ethoxide [prepared by dissolving sodium metal (0.002 mol) in absolute ethanol (25 mL)], phenyl isothiocyanate (0.002 mol) was added dropwise. The mixture was refluxed with stirring for 10 h and then left to cool to room temperature overnight under stirring. The reaction mixture was then poured onto iced water and neutralized with dilute hydrochloric acid. The resulting precipitate was collected by filtration, washed several times with water and recrystallized from DMF to give pale brown crystals of the thione derivative **16** (0.55 g; 75%), mp 285–288°C; IR (ν/cm^{-1}): 3305–3110 (NH), 2225 (CN), 1700 (CO), 1395 (C=S); ^{1}H NMR (δ ppm): 6.64 (dd, 1H, J = 1.6, 3.7 Hz, furan H-4), 7.26 (d, 1H, J = 3.7 Hz, furan H-3), 7.80 (d, 1H, J = 1.6 Hz, furan H-5), 8.40 (s, 1H, pyrimidine H-5), 9.55 (s, 1H, NH, D_2O-exchangeable); Anal. Calcd. for $C_{14}H_5N_7O_2S_2$ (367.372): C, 45.77; H, 1.37; N, 26.69; S, 17.46. Found: C, 45.60; H, 1.27; N, 26.39; S, 17.36.

Method B. A mixture of compound **8a** (0.002 mol) and carbon disulfide (0.02 mol) in dry pyridine (10 mL) was heated under reflux for 6 h. The precipitated crystals were filtered off, dried, and recrystallized from DMF to produce the thione derivative **16** (0.47 g; 64%). Mixed melting points with a sample of **16** prepared from phenyl isothiocyanate according to method A showed no depression. The spectral data of compound **16** obtained from both sources were superimposable.

9-Cyano-10-(2-furyl)-3,8-dioxo-2,3-dihydro-8*H*-pyrimido[2′,1′:2,3]thiazolo[5,4-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine (17). To a mixture of dry pyridine (10 mL) and **8a** (0.0012 mol) was carefully added ethyl chloroformate (1 mL, 0.01 mol) and the mixture was refluxed for 48 h. After cooling, the reaction mixture was poured onto iced water containing a few drops of hydrochloric acid. The solid that separated out was filtered off, washed with water several times, dried, and then recrystallized from EtOH to give the dione derivative **17** as a yellow solid (0.38 g; 89%), mp > 300°C; IR (ν/cm^{-1}): 3300 (NH), 2221 (CN), 1702, 1680 (2CO); ^{1}H NMR (δ ppm): 6.88–7.15 (m, 2H, furan H-3,4), 7.78 (d, 1H, J = 2.0 Hz, furan H-5), 8.11 (s, 1H, pyrimidine H-5), 10.92 (s, br, 1H, NH, D_2O-exchangeable); MS: m/z (%) = 351 (M^+, 16%); Anal. Calcd. for $C_{14}H_5N_7O_3S$ (351.305): C, 47.87; H, 1.43; N, 27.91; S, 9.13. Found: C, 47.58; H, 1.33; N, 27.74; S, 8.96.

Ethyl {9-cyano-10-(2-furyl)-8-oxo-8*H*-pyrimido[2′,1′:2,3]thiazolo[5,4-*e*][1,2,4]triazolo[4,3-*c*]pyrimidin-3-yl}acetate (18). A suspension of compound **8a** (0.002 mol) in diethyl malonate (10 mL) was gently heated under reflux for 10 h. The reaction mixture was triturated with ethanol (15 mL) and then allowed to cool. The formed precipitate was collected by filtration and purified by recrystallization from 1,4-dioxane to give light brown crystals of the ethyl ester **18** (0.58 g; 69%), mp 209–210°C; IR (ν/cm^{-1}): 2960, 2835 (aliph. CH), 2223 (CN), 1730, 1698 (2CO); ^{1}H NMR (δ ppm): 1.25 (t, 3H, J = 7.2 Hz, ester Me), 4.20 (q, 2H, J = 7.2 Hz, ester CH_2), 5.11 (s, 2H, CH_2CO), 6.90–7.19 (m, 2H, furan H-3,4), 7.75 (d, 1H, J = 1.9 Hz, furan H-5), 8.70 (s, 1H, pyrimidine H-5); ^{13}C NMR (δ ppm): 14.0 (ester Me), 34.5 (CH_2), 60.7 (ester CH_2), 98.7 (C-9), 111.4, 112.5 (furan C-3,4), 118.3 (CN), 133.1, 138.5, 143.8 (furan C-5), 148.0, 155.6 (furan C-2), 156.3, 158.4, 160.1, 161.6 (ring CO), 166.9, 168.0 (ester CO, C-10); Anal. Calcd. for $C_{18}H_{11}N_7O_4S$ (421.395): C, 51.30; H, 2.63; N, 23.27; S, 7.61. Found: C, 51.06; H, 2.49; N, 22.99; S, 7.50.

9-Cyano-10-(2-furyl)pyrimido[2′,1′:2,3]thiazolo[5,4-*e*]tetrazolo[1,5-*c*]pyrimidin-8-one (19). Compound **8a** (0.003 mol) was dissolved in glacial acetic acid (15 mL) containing concentrated hydrochloric acid (1.5 mL), a small amount of insoluble material was filtered off, then the liquid was cooled in ice bath at 0–5°C. The mixture was stirred at this temperature and treated gradually with a cold saturated solution of sodium nitrite [1g of sodium nitrite (0.015 mol) in water (10 mL)] over a period of 15 min. The mixture was kept in ice bath at 0–5°C with continuous stirring for further 2 h, then it was left to stand overnight at room temperature and diluted with water, whereon precipitation took place. The solid thus formed was isolated by filtration, washed abundantly with cold water, recrystallized from aqueous DMF, and air dried to give the fused tetrazole derivative **19** as a yellow solid (0.58 g; 60%), mp 221–222°C; IR (ν/cm^{-1}): 2218 (CN), 1701 (CO); ^{1}H NMR (δ ppm): 6.75 (dd, 1H, J = 1.8, 4.0 Hz, furan H-4), 7.30 (d, 1H, J = 4.0 Hz, furan H-3), 7.84 (d, 1H, J = 1.8 Hz, furan H-5), 9.80 (s, 1H, pyrimidine H-5); MS: m/z (%) = 336 (M^+, 22%); Anal. Calcd. for $C_{13}H_4N_8O_2S$ (336.288): C, 46.43; H, 1.20; N, 33.32; S, 9.53. Found: C, 46.25; H, 1.12; N, 33.07; S, 9.41.

Antimicrobial activity. The preliminary antimicrobial activity of the synthesized tricyclic and tetracyclic derivatives was evaluated *in vitro* by means of the agar-well diffusion assay. The assay was carried out according to the method of Hufford *et al*. [57] with some modifications. A total of 10 test microorganisms were used for the current antimicrobial activity studies: two gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*), four gram negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhyrium*, and *Klebsiella spp*), and four fungi (*Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Trichoderma horozianum*). The culture media used were Nutrient agar for bacteria and Czapek's agar (Difco) for fungi. Twenty-five milliliters of the specified molten agar (45°C) was aseptically mixed with either 100 μL of a bacterial suspension or 1 mL of a fungal suspension and poured into 15 mm sterile Petri dishes. For the preparation of the inocula colonies of bacteria were suspended in nutrient broth incubated overnight and fungi were suspended in sterile saline solution (NaCl, 0.85%), respectively. Once the agar was hardened, 9-mm wells were bored using a sterile cork borer. One hundred milliliters of the DMF extract (2 μm) were placed into the wells and the plates were incubated for 24 h at 37°C for the bacteria and 24–72 h at 28°C for the fungi. The antimicrobial activity was measured as the diameter (mm) of clear zone of growth inhibition. Solvent controls (DMF) were included in every experiment as negative controls. DMF was used for dissolving the crude extracts and gave negative results, confirming that it did not influence on antimicrobial activity observed for the compounds tested.

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