Antimicrobial and antiquorum-sensing studies. Part 2: Synthesis, antimicrobial, antiquorum-sensing and cytotoxic activities of new series of fused [1,3,4]thiadiazole and [1,3]benzo...

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ORIGINAL RESEARCH

Antimicrobial and antiquorum-sensing studies. Part 2: synthesis, antimicrobial, antiquorum-sensing and cytotoxic activities of new series of fused [1,3,4]thiadiazole and [1,3]benzothiazole derivatives

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Abstract New series of [1,3,4]thiadiazolo[3,2-*a*]pyrimidines, [1,3,4]thiadiazolo[2,3-*b*]quinazolines, and pyrimido[2,1-*b*][1,3]benzothiazoles have been synthesized and characterized by analytical and spectrometrical methods (IR, MS, ^{1}H, and ^{13}C NMR). Sixteen of the synthesized compounds; namely, **3a**, **b**, **5a–f**, **8a**, **b**, **10**, **11a–c**, and **13a**, **b** were screened for antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*. They were found to be either moderately active, slightly active or inactive against the selected microorganisms. The antifungal activity of these compounds were also tested against *Candida albicans*, *Aspergillus fumigatus* 293, and *Aspergillus flavus* 3375. Compound **11a** showed potent antifungal activity against the three selected fungi; the rest of the tested compounds displayed either weaker activity or were completely inactive. The same compounds were examined for antiquorum-sensing activity against *Chromobacterium violaceum* ATCC 12472, where compounds **3a**, **10**, **11a**, and **13a**, **b** exhibited promising activity. The in vitro cytotoxic activity of these compounds was also studied by brine shrimp lethality bioassay, and results indicated that compounds **3a**, **11a**, and **13a** have the highest cytotoxic activity.

Keywords Thiadiazolopyrimidines · Thiadiazoloquinazolines · Pyrimidobenzothiazoles · Antimicrobial activity · Antiquorum-sensing activity · Cytotoxic activity

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Introduction

The presence of multi-drug resistant pathogenic bacteria is increasing worldwide. This resistance resulted from growth of bacteria in specialized surface-attached communities, called biofilms (Costerton *et al*., 1999). Bacteria in biofilms were shown to be resistant to antibiotics (Olson *et al*., 2002). Consequently, the development of novel chemotherapeutics that inhibit bacterial pathogenesis is urgent (Hentzer and Givskov, 2003). A promising approach is to target cell-to-cell communications, commonly known as quorum-sensing (QS). Quorum-sensing produce signal molecules known as quorum-sensing autoinducers (QSAs) (Hastings and Greenberg, 1999), such as *N*-acyl-homoserine lactones (acyl HSLs) that allow intraspecies and interspecies communications. Once the threshold level of QSAs is reached, the autoinducers will bind to cognate receptors (Williams, 2007) to form a complex, which will in turn regulate the expression of target genes, particulary those responsible for virulence (Chan *et al*., 2010). Thus, antiquorum-sensing treatment may be a plausible way to attenuate bacterial virulence without killing the pathogens, and this may reduce bacterial drug resistance (Adonizio *et al*., 2006).

Literature survey revealed that [1,3,4]thiadiazolo[3,2-*a*]pyrimidine nucleus is associated with diverse pharmacodynamic and chemotherapeutic activities (Coburn and Glennon, 1973; Coburn *et al*., 1974; El-Ashmaway *et al*., 2010; El-Gohary and Shaaban, 2013; El-Sayed *et al*., 2011; Herrling, 1978; Maekawa and Mizumitsu, 1977; Mahran *et al*., 1998; Suiko and Maekawa, 1977; Taher *et al*., 2012), including antimicrobial (Coburn and Glennon, 1973; Coburn *et al*., 1974; El-Gohary and Shaaban, 2013; Mahran *et al*., 1998). In addition, [1,3,4]thiadiazolo[2,3-*b*]quinazolines are known to possess interesting pharmacological properties, including antitumor (El-Ashmaway *et al*., 2010)

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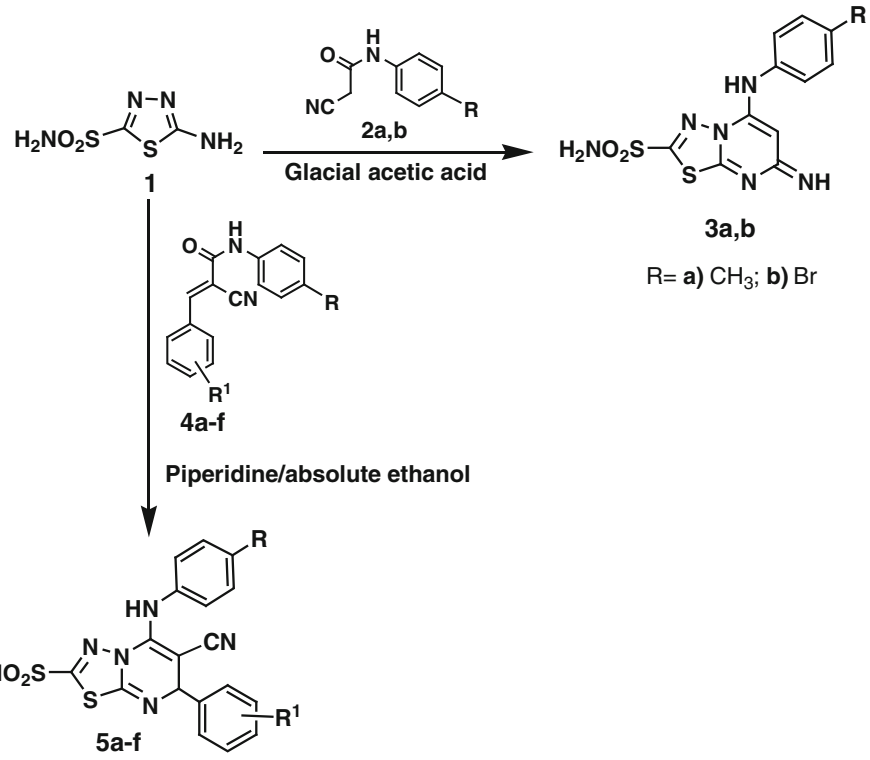
and CNS depressant activities (Alagarsamy *et al.*, 2006). Moreover, pyrimido[2,1-*b*][1,3]benzothiazoles have also been extensively investigated for their pharmacological uses (Chaitanya *et al.*, 2010; Deshmukh *et al.*, 2011; El-Sherbeny, 2000; Gupta *et al.*, 2009; Sahu *et al.*, 2012; Shendarkar *et al.*, 2011; Vartale *et al.*, 2011), some of these compounds showed antimicrobial activity (Sahu *et al.*, 2012; Shendarkar *et al.*, 2011; Vartale *et al.*, 2011). Considering these published data and in continuation to our previous study (El-Ashmaway *et al.*, 2010; El-Sayed *et al.*, 2011, El-Gohary and Shaaban, 2013) and in searching for new compounds of potent antimicrobial activity, we are reporting herein, the synthesis of new series of thiadiazolopyimidine, thiadiazoloquinazoline and pyrimidobenzothiazole derivatives and evaluation of their antibacterial, antifungal, antiquorum-sensing, and cytotoxic activities.

Results and discussion

Chemistry

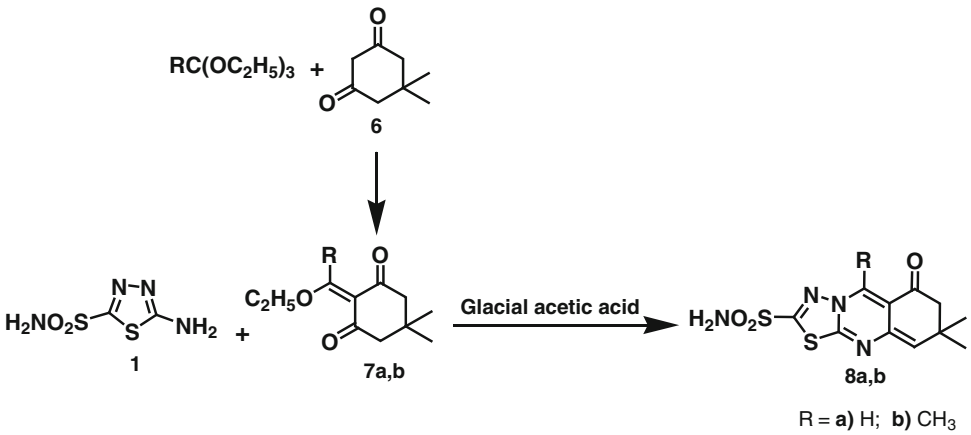
A general approach to synthesize the designed compounds is outlined in Schemes 1, 2, and 3. 2-Amino-[1,3,4]thiadiazole-5-sulfonamide (**1**) and 2-amino-6-methyl-[1,3]benzothiazole (**9**) were used as key starting materials in this study for syntheses of new fused heterocycles. Amine **1** was synthesized via acid hydrolysis of 2-acetamido-[1,3,4]thiadiazole-5-sulfonamide (Petrow *et al.*, 1958). Cyclocondensation of **1** with cyanoacetanilide derivatives **2a**, **b** (Deka *et al.*, 2012) in refluxing glacial acetic acid afforded 7-imino-5-(substituted phenyl)amino-7*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidine-2-sulfonamide derivatives **3a**, **b** (Scheme 1). On the other hand, refluxing **1** with benzylidenecyanoacetanilide derivatives **4a–f** (Meskini *et al.*, 2010) in absolute ethanol and in the presence of catalytic amount of piperidine gave 6-cyano-7-(substituted phenyl)-5-(substituted phenyl)amino-7*H*-[1,3,4]thiadiazolo[3,2-*a*]pyrimidine-2-sulfonamide derivatives **5a–f** (Scheme 1). The ^{1}H NMR spectrum of compound **3b** showed four significant singlets at δ 5.20, 8.28, 10.03, and 11.65 ppm for (C_6–H), (NH_2), and (2NH), respectively. The IR spectrum of compound **5b** showed absorption bands at 3,309, 3,193 cm^{-1} for (NH_2 and NH), in addition to the absorption band characteristic for cyano group that appeared at 2,200 cm^{-1}. ^{1}H NMR spectrum of the same compound displayed three singlets at δ 2.08, 3.00, and 5.05 ppm assignable to (CH_3), (NH), and (C_7–H), respectively.

Reaction of dimedone **6** with the triethyl orthoformate or triethyl orthoacetate in refluxing absolute ethanol, and in the presence of catalytic amount of piperidine/glacial acetic acid

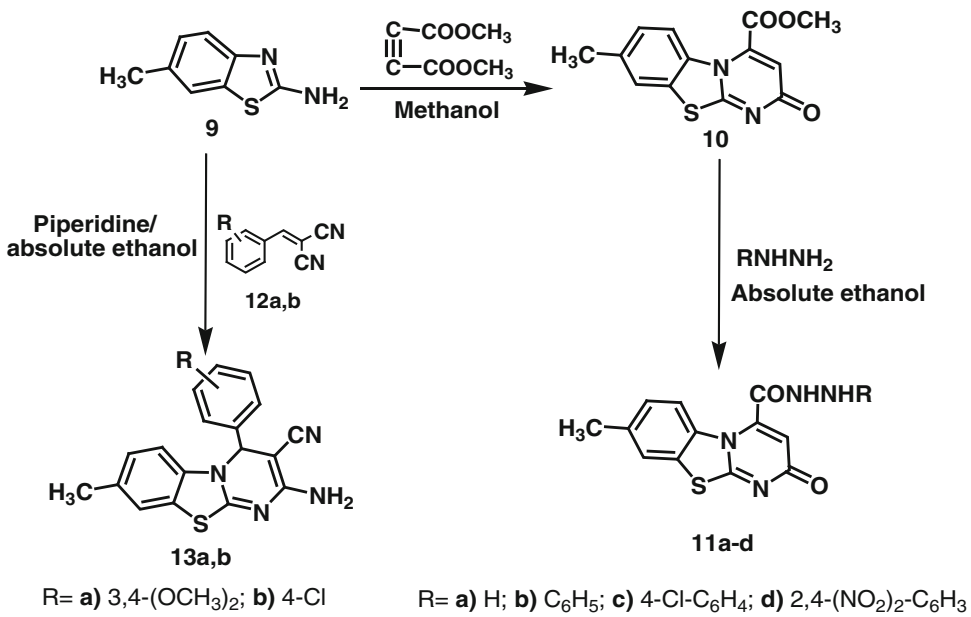


Scheme 1 Synthesis of compounds **3a**, **b** and **5a–f**

Scheme 2 Synthesis of compounds **8a**, **b**



Scheme 3 Synthesis of compounds **10**, **11a–d**, and **13a**, **b**



(3:2) afforded 2-(ethoxymethylene)-5,5-dimethylcyclohexane-1,3-dione (**7a**) and 2-(1-ethoxyethylidene)-5,5-dimethylcyclohexane-1,3-dione (**7b**), respectively. ^{1}H NMR spectrum of **7b** displayed the triplet–quartet pattern characteristic for the ethyl protons at the expected regions, in addition to three singlets at δ 1.03, 2.23, and 2.56 ppm for ($2CH_3$), (C=C–CH_3), and ($2CH_2$), respectively. Cyclocondensation of aminothiadiazole **1** with **7a**, **b** in refluxing glacial acetic acid afforded the thiadiazolo[2,3-*b*]quinazolines **8a**, **b** in acceptable yields (Scheme 2). Mass spectrum of **8a** exhibited a molecular ion peak at *m*/*z* 312 which is in agreement with its molecular formula $C_{11}H_{12}N_4O_3S_2$. ^{1}H NMR spectrum of the same compound showed three significant singlets at δ 6.73, 7.16, and 8.56 ppm for (C_9-H), (C_5-H), and (NH_2), respectively.

Treating of 2-aminobenzothiazole **9** with dimethyl acetylenedicarboxylate in refluxing methanol yielded methyl 8-methyl-2-oxopyrimido[2,1-*b*][1,3]benzothiazole-4-carboxylate (**10**) in 65 % yield (Scheme 3). The IR spectrum of **10** demonstrated the characteristic absorption bands for the two carbonyl groups at 1,745 and 1,664 cm^{-1}. In addition, ^{1}H NMR spectrum showed three characteristic singlets at δ 2.23, 3.96, and 7.88 ppm for (CH_3), (OCH_3), and (C_3-H), respectively.

New series of pyrimido[2,1-*b*][1,3]benzothiazole-4-carbohydrazide derivatives **11a–d** were prepared through reaction of compound **10** with hydrazine hydrate and phenylhydrazine derivatives in refluxing absolute ethanol (Scheme 3). The IR spectrum of **11b** revealed the disappearance of the carbonyl ester absorption band with concomitant appearance of the characteristic absorption bands for (2NH) groups at 3,363 and 3,321 cm^{-1}. The mass spectrum of the same compound showed a molecular ion peak at *m*/*z* 350, which is in agreement with its molecular

weight along with a base peak at *m/z* 210. ^{1}H NMR spectrum of **11d** displayed two characteristic singlets δ 9.98 and 11.34 ppm for (2NH).

Cyclocondensation of **9** with the appropriate benzylidenemalononitrile **12a**, **b** (Bigi *et al.*, 2000) in absolute ethanol, and in the presence of catalytic amount of piperidine afforded the corresponding pyrimido[2,1-*b*][1,3]benzothiazole-3-carbonitrile derivatives **13a**, **b** (Scheme 3). The IR spectrum of **13a** revealed the presence of two characteristic absorption bands at 3,425 and 2,191 cm^{-1} for (NH_2) and (C≡N) groups, respectively. ^{1}H NMR spectrum of the same compound showed two singlets at δ 3.00 and 5.18 ppm assignable to (NH_2) and (C_4–H), respectively. The mass spectrum of **13b** showed a molecular ion peak at *m/z* 353, which is in agreement with its molecular formula $C_{18}H_{13}ClN_4S$.

Antimicrobial and antiquorum-sensing screening

Sixteen of the synthesized compounds; namely, **3a**, **b**, **5a–f**, **8a**, **b**, **10**, **11a–c**, and **13a**, **b** were screened for in vitro antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus*, and *Escherichia coli* (Pearson *et al.*, 1980). Also, they were tested for antifungal activity against *Candida albicans*, *Aspergillus fumigatus* 293, and *Aspergillus flavus* 3372 (Holt, 1975). The antimicrobial screening results were determined by measuring the average diameter of the inhibition zones, expressed in millimeters (mm). Based on the results (Table 1), it could be inferred that compounds **5a**, **d**, **10**, **11a**, and **13a** have moderate activity against *S. aureus*. The rest of the tested compounds showed either weaker activity or were completely inactive against the same microorganism. Compounds **10**, **11a**, **b**, and **13a** displayed interesting activity against *B. cereus* compared to the reference drug, ampicillin.

Regarding the antifungal activity, compound **11a** demonstrated promising antifungal activity against the three selected fungi and it is proved to be the most active fungicide in this study. The rest of the tested compounds were found to be less active or completely inactive against the selected fungi.

The same compounds were tested for antiquorum-sensing activity against *Chromobacterium violaceum* ATCC 12472 (McClean *et al.*, 1997). The QS system of *C. violaceum* was used for this assay. QS in this wild type strain of bacteria produces violacein (a purple pigment) in response to autoinducer molecules known as acyl HSLs (McClean *et al.*, 2004; Cha *et al.*, 2011). Thus, drugs that inhibit acyl HSL-mediated QS activity in *C. violaceum* will prevent production of this purple pigment. Screening results for their ability to inhibit QS regulated violacein

Table 1 Antimicrobial and antiquorum-sensing activities of compounds **3–13**

Comp. no.	Inhibition zone diameter (mm)						
	E. coli	*B. cereus*	*S. aureus*	*C. albicans*	*A. fumigatus*	*A. flavus*	*Ch. violaceum*
3a	6	2	2	6	5	10	12
3b	–	–	–	4	–	3	–
5a	–	2	11	4	–	–	–
5b	–	2	4	4	–	–	–
5c	–	2	5	5	–	–	–
5d	–	2	9	3	–	–	–
5e	–	2	2	–	–	–	–
5f	–	2	7	–	–	–	–
8a	–	–	–	3	–	–	–
8b	–	–	–	4	–	–	–
10	–	5	9	6	7	–	9
11a	–	5	9	32	18	18	10
11b	4	5	4	–	–	–	–
11c	–	–	–	4	–	–	–
13a	–	5	11	15	9	9	12
13b	–	–	4	–	–	–	6
Ampicillin	23	3.5	14				
Fluconazole				22	–	–	

Sample concentration: 5 mg/mL, sample volume: 0.1 mL/well. Results are calculated after subtraction of DMSO activity. Not active (– inhibition zone < 2 mm); weak activity (2–8 mm); moderate activity (9–15 mm); strong activity (>15 mm)

E. coli Escherichia coli, *B. cereus Bacillus cereus*, *S. aureus Staphylococcus aureus*, *C. albicans Candida albicans*, *A. fumigatus Aspergillus fumigatus*, *A. flavus Aspergillus flavus*, *Ch. violaceum Chromobacterium violaceum*

production against *C. violaceum* (based on measuring the radius of pigment inhibition in mm) are presented in Table 1, and showed that compounds **3a**, **10**, **11a**, and **13a**, **b** have antiquorum-sensing activity.

Structure–activity relationship (SAR) studies

- For compounds **3a**, **b**, the presence of 5-phenylamino moiety substituted with electron-donating group enhanced the antibacterial, antifungal and antiquorum-sensing activities (compound **3a**).
- In the series of thiadiazolopyrimidines **5a–f**, the presence of 5-phenylamino moiety substituted with electron-donating group (compounds **5a–c**) improved the antifungal activity against *C. albicans*, while those substituted with electron-withdrawing group exhibited either weaker activity (compound **5d**) or were completely inactive (compounds **5e**, **f**) against the same microorganism. Regarding their antibacterial activity, the presence of unsubstituted phenyl moiety at 7-position increased the activity against *S. aureus* (compounds **5a**, **d**).
- Concerning the thiadiazoloquinazolines **8a**, **b**, the presence of methyl substituent at 5-position increased the antifungal activity against *C. albicans* (compound **8b**).
- Taking into account the structure of pyrimido[2,1-*b*][1,3]benzothiazole-4-carbohydrazides **11a–d**, it is assumed that the presence of free hydrazinocarbonyl moiety at 4-position enhanced the activity against *S. aureus*. In addition, the antifungal and antiquorum-sensing activities were also increased (compound **11a**). However, the presence of unsubstituted phenylhydrazinocarbonyl moiety at 4-position resulted in improvement of activity against *E. coli*, but decreased activity against *S. aureus*. On the other hand, antifungal and antiquorum-sensing activities were completely diminished (compound **11b**). Furthermore, the presence of electron-withdrawing substituent on the phenyl of the 4-phenylhydrazinocarbonyl moiety abolished the activity against all the tested microorganisms except *C. albicans* (compound **11c**).
- Regarding compounds **13a**, **b**, the presence of 4-phenyl moiety substituted with electron-donating group improved the antibacterial, antifungal and antiquorum-sensing activities (compound **13a**).
- Cytotoxicity testing using brine shrimp lethality bioassay.

Cytotoxicity via brine shrimp lethality test was studied in order to reveal new antimicrobial compounds (Harborne, 1998). Toxicity to brine shrimps has a good correlation with antitumor, pesticidal (Mclaughlin, 1991) and antitrypanosomal activities (Zani *et al.*, 1995) in man. The brine shrimp larvae (*Artemia salina*) responds similarly to the corresponding mammalian systems (Solis *et al.*, 1993) since the DNA-dependent RNA polymerases of *A. salina* have been shown to be similar to the mammalian type (Birndorf *et al.*, 1975). This test is not only used for predicting cytotoxicity, but also it is used to predict antitumor, antibacterial and pesticidal activities (Sanchez *et al.*, 1993). Thus, it is possible to evaluate the cytotoxicity of compounds using brine shrimp lethality bioassay, rather than the more tedious in vitro and in vivo antitumor assays.

Sixteen of the synthesized compounds; namely, **3a**, **b**, **5a–f**, **8a**, **b**, **10**, **11a–c**, and **13a**, **b** were screened for cytotoxic activity against brine shrimp larvae (nauplii) adopting the microplate assay method (Mayer *et al.,* 1982) and using 5-flurouracil as a reference drug.

Cytotoxic activity of the tested compounds was determined by measuring the median lethal concentration; LC_{50} (concentration that kills 50 % of brine shrimp nauplii) expressed in μg/mL. LC_{90} (concentration that kills 90 % of brine shrimp nauplii) values were also determined to establish the therapeutic index. The results were shown in Table 2 and indicated that compounds **11a**, **13a**, and **3a** have good cytotoxic activity against brine shrimp nauplii in comparison with 5-flurouracil with LC_{50} values of 404.85, 420.16, and 423.72 μg/mL, respectively, which represent high cytotoxicity and hence may predict antimicrobial potential of those compounds. The rest of the tested compounds showed weaker activity, whereas compounds **3b**, **5e**, **5d**, and **5f**, are the least cytotoxic analogs in this study with LC_{50} values of 714.28, 724.63, 781.25, and 806.45 μg/mL, respectively.

Structure–activity relationship (SAR) studies

- Concerning compounds **3a**, **b**, the presence of 5-phenylamino moiety substituted with electron-donating group enhanced the cytotoxic activity (compound **3a**).
- Regarding series **5a–f**, the presence of 5-phenylamino moiety substituted with electron-donating group (compounds **5a–c**) improved cytotoxic activity compared with those substituted with electron-withdrawing group (compounds **5d–f**).
- For thiadiazoloquinazolines **8a**, **b**, the absence of substituent at 5-position increased the cytotoxic activity (compound **8a**).
- Taking into account the structure of pyrimido[2,1-*b*][1,3]benzothiazole-4-carbohydrazides **11a–d**, the presence of free hydrazinocarbonyl moiety at 4-position enhanced the cytotoxic activity (compound **11a**).
- For compounds **13a**, **b**, it is clear that the presence of 4-phenyl moiety substituted with electron-donating group improved the cytotoxic activity (compound **13a**).

Table 2 Cytotoxic activity of compounds **3–13** using brine shrimp lethality bioassay

Comp. no.	Conc. (μg/mL)	LogC	Number of dead nauplii	% Mortality	LC_{50} (μg/mL)	LC_{90} (μg/mL)
3a	1,000	3	25	100.0	423.72	762.71
	500	2.698	21	84.0		
	100	2	16	64.0		
	10	1	10	40.0		
3b	1,000	3	16	64.0	714.28	1,285.71
	500	2.698	11	44.0		
	100	2	8	32.0		
	10	1	4	16.0		
5a	1,000	3	23	92.0	462.96	833.33
	500	2.698	19	76.0		
	100	2	16	64.0		
	10	1	9	36.0		
5b	1,000	3	22	88.0	476.19	857.14
	500	2.698	19	76.0		
	100	2	16	64.0		
	10	1	10	40.0		
5c	1,000	3	16	64.0	694.44	1,250.00
	500	2.698	12	48.0		
	100	2	7	28.0		
	10	1	4	16.0		
5d	1,000	3	14	56.0	781.25	1,406.25
	500	2.698	11	44.0		
	100	2	9	36.0		
	10	1	5	20.0		
5e	1,000	3	16	64.0	724.63	1,304.34
	500	2.698	10	40.0		
	100	2	7	28.0		
	10	1	3	12.0		
5f	1,000	3	14	56.0	806.45	1,451.61
	500	2.698	10	40.0		
	100	2	7	28.0		
	10	1	4	16.0		
8a	1,000	3	17	68.0	657.89	1,184.21
	500	2.698	12	48.0		
	100	2	9	36.0		
	10	1	6	24.0		
8b	1,000	3	16	64.0	694.44	1,250.00
	500	2.698	12	48.0		
	100	2	7	28.0		
	10	1	4	16.0		
10	1,000	3	23	92.0	462.96	833.33
	500	2.698	19	76.0		
	100	2	16	64.0		
	10	1	9	36.0		
11a	1,000	3	25	100.0	404.85	728.74
	500	2.698	24	96.0		
	100	2	20	80.0		
	10	1	15	60.0		

Table 2 continued

Comp. no.	Conc. (μg/mL)	LogC	Number of dead nauplii	% Mortality	LC_{50} (μg/mL)	LC_{90} (μg/mL)
11b	1,000	3	19	76.0	574.71	1,034.48
	500	2.698	15	60.0		
	100	2	11	44.0		
	10	1	6	24.0		
11c	1,000	3	18	72.0	625.00	1,125.00
	500	2.698	13	52.0		
	100	2	9	36.0		
	10	1	5	20.0		
13a	1,000	3	25	100.0	420.16	756.30
	500	2.698	22	88.0		
	100	2	16	64.0		
	10	1	11	44.0		
13b	1,000	3	19	76.0	588.23	1,058.82
	500	2.698	14	56.0		
	100	2	10	40.0		
	10	1	5	20.0		
5-Fu	1,000	3	25	100.0	409.83	737.70
	500	2.698	23	92.0		
	100	2	20	80.0		
	10	1	14	56.0		
Control	–	–	0	0.0	–	–

LC_{50} compound concentration required to kill 50 % of brine shrimp nauplii, *LC_{90}* compound concentration required to kill 90 % of brine shrimp nauplii

Conclusion

Concerning the antibacterial activity, all tested compounds exhibited lower activity than ampicillin against *E. coli*. On the other hand, compounds **10**, **11a**, **b**, and **13a** were found to be more active than ampicillin against *B. cereus*. Regarding the antifungal activity, compound **11a** was the most active analog compared to fluconazole against the three selected fungi. The rest of the tested compounds displayed either weaker activity or no activity toward the selected microorganisms. Compounds **3a**, **10**, **11a**, and **13a**, **b** demonstrated antiquorum-sensing activity. In addition, compounds **3a**, **11a**, and **13a** are the most cytotoxic analogs.

Experimental

Chemistry

All melting points (°C) were recorded on Fisher-Johns melting point apparatus and are uncorrected. The infrared spectra were recorded in KBr disk using a Unicam SP 1000 IR spectrometer (ν in cm^{-1}) at Faculty of Science, Mansoura University. Nuclear magnetic resonance (^1H and ^{13}C NMR) spectra were obtained on 300 MHz FT-NMR spectrometer at Faculty of Science, Cairo University. The chemical shifts are expressed in δ ppm using tetramethylsilane (TMS) as internal reference and DMSO-d_6 or $CDCl_3$ as solvents. Mass spectra were recorded on JEOL JMS-600H spectrometer using electron impact technique at 70 eV at Microanalytical Unit, Cairo University. Microanalyses (C, H, N) were performed at Microanalytical Unit, Cairo University, and were in agreement with the proposed structures. Reaction times were monitored using TLC plates, Silica gel 60 F_{254} precoated (E. Merck), and the spots were visualized by UV. Chloroform:methanol (9:1) was adopted as elution solvent. Compound **9** was obtained from Aldrich Chemical Co. (Milwaukee, WI). Compounds **2a**, **b** were prepared adopting the method of (Deka *et al.*, 2012). Compounds **4a–f** were prepared according to the reported procedure (Meskini *et al.*, 2010), while compounds **12a**, **b** were prepared following the literature method (Bigi *et al.*, 2000).

*General procedure for the preparation of 7-imino-5-(substituted phenyl)amino-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamides (**3a, b**)*

A mixture of amine **1** (0.45 g, 0.0025 mol) and the appropriate cyanoacetanilide **2a**, **b** (0.0025 mol) in glacial

acetic acid (10 mL) was heated under reflux for 12 h. The reaction mixture was cooled and the precipitated solid was collected by filtration, dried, and crystallized from ethanol/water (2:1).

*7-Imino-5-(4-methylphenyl)amino-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**3a**)* Yield 65 %, m.p. 187–188 °C. ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.23 (s, 3H, CH_3), 5.31 (s, 1H, C_6–H), 7.07 (d, 2H, Ar–H), 7.43 (d, 2H, Ar–H), 9.79 (s, 2H, NH_2), 11.74 (s, 1H, NH), 12.14 (s, 1H, NH). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 21.3, 89.4, 118.6 (2C), 127.3, 128.9 (2C), 139.7, 157.2, 159.6, 161.8, 163.9. MS *m*/*z* (%): 336 (9.47, M^+), 59 (100.00). Anal. Calcd. for $C_{12}H_{12}N_6O_2S_2$ (336.39): C 42.85, H 3.60, N 24.98. Found: C 43.10, H 3.76, N 24.72.

*5-(4-Bromophenyl)amino-7-imino-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**3b**)* Yield 70 %, m.p. 268–270 °C. ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 5.20 (s, 1H, C_6–H), 7.45 (d, 2H, Ar–H), 7.52 (d, 2H, Ar–H), 8.28 (s, 2H, NH_2), 10.03 (s, 1H, NH), 11.65 (s, 1H, NH). MS *m*/*z* (%): 400 (0.03, M^+-1), 171 (100.00). Anal. Calcd. for $C_{11}H_9BrN_6O_2S_2$ (401.26): C 32.93, H 2.26, N 20.94. Found: C 32.77, H 2.45, N 21.23.

*General procedure for the preparation of 6-cyano-7-(substituted phenyl)-5-(substituted phenyl)amino-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamides (**5a–f**)*

A mixture of amine **1** (0.45 g, 0.0025 mol), the appropriate benzylidenecyanoacetanilide **4a–f** (0.0025 mol) and a catalytic amount of piperidine (0.3 mL) in absolute ethanol (15 mL) was heated under reflux for 12–16 h. The solvent was concentrated, then poured onto cold water (50 mL). The precipitated solid was collected by filtration, dried, and crystallized from ethanol/water (3:1).

*6-Cyano-5-(4-methylphenyl)amino-7-phenyl-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**5a**)* Yield 60 %, m.p. 164–165 °C. IR spectrum (KBr, ν, cm^{-1}): 3429, 3303 (NH_2, NH), 2209 (C≡N). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.17 (s, 3H, CH_3), 2.99 (s, 1H, NH), 5.30 (s, 1H, C_7–H), 6.94–7.69 (m, 11H, Ar–H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 21.5, 40.9, 69.5, 117.1, 119.0 (2C), 125.8, 126.2, 127.6 (2C), 128.9 (2C), 129.5 (2C), 140.5, 141.9, 158.3, 161.9, 166.3. MS *m*/*z* (%): 424 (0.03, M^+), 84 (100.00). Anal. Calcd. for $C_{19}H_{16}N_6O_2S_2$ (424.50): C 53.76, H 3.80, N 19.80. Found: C 53.47, H 3.62, N 20.05.

*7-(4-Bromophenyl)-6-cyano-5-(4-methylphenyl)amino-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**5b**)* Yield 65 %, m.p. 162–163 °C. IR spectrum (KBr, ν, cm^{-1}): 3308, 3193 (NH_2, NH), 2200 (C≡N). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.08 (s, 3H, CH_3), 3.00 (s, 1H, NH), 5.05 (s, 1H, C_7–H), 6.99–7.79 (m, 10H, Ar–H, NH_2). MS *m*/*z* (%): 505 (0.63, M^++2), 504 (0.4, M^++1), 503 (0.09, M^+), 107 (100.00). Anal. Calcd. for $C_{19}H_{15}BrN_6O_2S_2$ (503.40): C 45.33, H 3.00, N 16.69. Found: C 45.07, H 2.85, N 16.43.

*6-Cyano-7-(3,4-dimethoxyphenyl)-5-(4-methylphenyl)amino-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**5c**)* Yield 60 %, m.p. 172–173 °C. IR spectrum (KBr, ν, cm^{-1}): 3338, 3192 (NH_2, NH), 2200 (C≡N). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.09 (s, 3H, CH_3), 3.04 (s, 1H, NH), 3.80 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 5.35 (s, 1H, C_7–H), 6.78–7.77 (m, 9H, Ar–H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 21.7, 41.3, 55.6, 55.7, 69.7, 112.5, 113.6, 116.8, 119.1 (2C), 121.7, 127.5, 128.2 (2C), 131.1, 140.1, 145.7, 148.2, 157.6, 162.8, 165.3. MS *m*/*z* (%): 483 (3.61, M^+-1), 84 (100.00). Anal. Calcd. for $C_{21}H_{20}N_6O_4S_2$ (484.55): C 52.05, H 4.16, N 17.34. Found: C 52.31, H 3.95, N 17.05.

*5-(4-Bromophenyl)amino-6-cyano-7-phenyl-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**5d**)* Yield 65 %, m.p. 176–177 °C. IR spectrum (KBr, ν, cm^{-1}): 3334, 3189 (NH_2, NH), 2201 (C≡N). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.98 (s, 1H, NH), 5.34 (s, 1H, C_7–H), 7.11–7.71 (m, 11H, Ar–H, NH_2). Anal. Calcd for $C_{18}H_{13}BrN_6O_2S_2$ (489.37): C 44.18, H 2.68, N 17.17. Found: C 44.39, H 2.48, N 16.93.

*7-(4-Bromophenyl)-5-(4-bromophenyl)amino-6-cyano-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**5e**)* Yield 65 %, m.p. 166–167 °C. IR spectrum (KBr, ν, cm^{-1}): 3420 (broad band, NH_2, NH), 2201 (C≡N). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.91 (s, 1H, NH), 5.32 (s, 1H, C_7-H), 7.14–7.84 (m, 10H, Ar–H, NH_2). MS *m*/*z* (%): 570 (0.05, M^++2), 569 (0.11, M^++1), 568 (0.29, M^+), 64 (100.00). Anal. Calcd. for $C_{18}H_{12}Br_2N_6O_2S_2$ (568.26): C 38.04, H 2.13, N 14.79. Found: C 37.87, H 2.35, N 14.63.

*5-(4-Bromophenyl)amino-6-cyano-7-(3,4-dimethoxyphenyl)-7H-[1,3,4]thiadiazolo[3,2-a]pyrimidine-2-sulfonamide (**5f**)* Yield 72 %, m.p. 146–147 °C. ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 3.01 (s, 1H, NH), 3.83 (s, 3H, OCH_3),

3.85 (s, 3H, OCH_3), 5.19 (s, 1H, C_7–H), 6.49–7.78 (m, 9H, Ar–H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 41.6, 55.7, 55.9, 70.6, 114.6 (2C), 115.1, 116.4, 118.9, 120.7 (2C), 131.6 (2C), 133.2, 140.8, 146.2, 148.6, 157.3, 161.8, 165.5. MS *m/z* (%): 551 (0.01, M^++2), 549 (0.01, M^+), 112 (100.00). Anal. Calcd. for $C_{20}H_{17}BrN_6O_4S_2$ (549.42): C 43.72, H 3.12, N 15.30. Found: C 43.95, H 3.41, N 15.12.

*Preparation of 2-substituted 5,5-dimethylcyclohexane-1,3-diones (**7a, b**)*

Piperidine (0.9 mL) and glacial acetic acid (0.6 mL) were added to a mixture of dimedone **6** (1.4 g, 0.01 mol), and triethyl orthoformate or triethyl orthoacetate (0.015 mol) in absolute ethanol (20 mL). The mixture was heated under reflux for 48 h, then evaporated under reduced pressure. The residue obtained (**7a**) was triturated with crushed ice, filtered, dried, and crystallized from ethanol/water (2:1). For compound **7b**, evaporation of reaction mixture leaves yellow oily product.

*2-(Ethoxymethylene)-5,5-dimethylcyclohexane-1,3-dione (**7a**)* Yield 60 %, m.p. 140–142 °C. ^1H NMR spectrum: (DMSO-d_6, δ ppm): 1.03–2.43 (m, 13H, $2CH_3$, CH_2CH_3, $2CH_2$), 4.06–4.12 (q, 2H, CH_2CH_3), 5.06 (s, 1H, CH=C). MS *m/z* (%): 198 (2.55, M^++2), 197 (1.65, M^++1), 196 (1.97, M^+), 55 (100.00). Anal. Calcd. for $C_{11}H_{16}O_3$ (196.24): C 67.32, H 8.22. Found: C 67.54, H 8.41.

*2-(1-Ethoxyethylidene)-5,5-dimethylcyclohexane-1,3-dione (**7b**)* Yield 75 %, yellow oil. ^1H NMR spectrum: ($CDCl_3$, δ ppm): 1.03 (s, 6H, $2CH_3$), 1.32 (t, 3H, $CH_2\underline{CH_3}$), 2.23 (s, 3H, CH_3), 2.56 (s, 4H, $2CH_2$), 3.92–3.97 (q, 2H, $C\underline{H_2}CH_3$). MS *m/z* (%): 211 (1.92, M^++1), 210 (1.61, M^+), 83 (100.00). Anal. Calcd. for $C_{12}H_{18}O_3$ (210.27): C 68.54, H 8.63. Found: C 68.41, H 8.76.

*Preparation of 7,8-dihydro-8,8-dimethyl-6-oxo-5-unsubstituted/methyl-[1,3,4]thiadiazolo[2,3-b] quinazoline2-sulfonamides (**8a, b**)*

A mixture of amine **1** (0.45 g, 0.0025 mol) and the appropriate cyclohexane-1,3-dione derivative **7a**, **b** (0.0025 mol) in glacial acetic acid (10 mL) was heated under reflux for 24 h. The reaction mixture was evaporated under reduced pressure and the remaining products were crystallized from ethanol/water (3:1).

*7,8-Dihydro-8,8-dimethyl-6-oxo-[1,3,4]thiadiazolo[2,3-b] quinazoline-2-sulfonamide (**8a**)* Yield 68 %, m.p. 274–275 °C. IR spectrum (KBr, *v*, cm^{-1}): 3320 (broad band, NH_2), 1674 (C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 2.05 (s, 6H, $2CH_3$), 2.62 (s, 2H, CH_2), 6.73 (s, 1H, C_9–H), 7.16 (s, 1H, C_5–H), 8.56 (s, 2H, NH_2). MS *m/z* (%): 313 (0.08, M^++1), 312 (0.07, M^+), 64 (100.00). Anal. Calcd. for $C_{11}H_{12}N_4O_3S_2$ (312.37): C 42.30, H 3.87, N 17.94. Found: C 42.12, H 3.98, N 18.27.

*7,8-Dihydro-5,8,8-trimethyl-6-oxo-[1,3,4]thiadiazolo[2,3-b] quinazoline-2-sulfonamide (**8b**)* Yield 72 %, m.p. 268–269 °C. IR spectrum (KBr, *v*, cm^{-1}): 3302, 3182 (NH_2), 1679 (C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 1.75 (s, 6H, C_8–$2CH_3$), 2.05 (s, 3H, C_5–CH_3), 2.48 (s, 2H, CH_2), 6.50 (s, 1H, C_9–H), 7.89 (s, 2H, NH_2). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 14.6, 28.9 (2C), 31.8, 51.5, 112.8, 131.7, 133.4, 144.6, 157.4, 161.3, 169.9. MS *m/z* (%): 328 (0.01, M^++2), 327 (0.02, M^++1), 326 (0.02, M^+), 180 (100.00). Anal. Calcd. for $C_{12}H_{14}N_4O_3S_2$ (326.39): C 44.16, H 4.32, N 17.17. Found: C 44.37, H 4.57, N 16.99.

*Preparation of methyl 8-methyl-2-oxopyrimido [2,1-b][1,3]benzothiazole-4-carboxylate (**10**)*

Dimethyl acetylenedicarboxylate (DMAD) (1.42 g, 0.01 mol) was added dropwise to a stirred solution of 2-amino-6-methyl-[1,3]benzothiazole (**9**) (1.64 g, 0.01 mol) in methanol (20 mL). The reaction mixture was heated under reflux for 12 h. On cooling, the precipitated solid was collected by filtration, dried, and crystallized from ethanol/water (2:1).

Yield 65 %, m.p. 193–194 °C. IR spectrum (KBr, *v*, cm^{-1}): 1745 ($COOCH_3$), 1664 (C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm): 2.23 (s, 3H, CH_3), 3.96 (s, 3H, OCH_3), 6.65–7.43 (m, 3H, Ar–H), 7.88 (s, 1H, C_3–H). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 23.9, 52.6, 103.3, 121.6, 124.1, 125.3, 127.8, 128.7, 138.5, 141.1, 157.5, 166.8, 169.7. MS *m/z* (%): 275 (12.03, M^++1), 274 (72.00, M^+), 216 (100.00). Anal. Calcd. for $C_{13}H_{10}N_2O_3S$ (274.30): C 56.92, H 3.67, N 10.21. Found: C 56.74, H 3.49, N 9.98.

*Preparation of 8-methyl-2-oxo-N′-(un)substituted pyrimido[2,1-b][1,3]benzothiazole-4-carbohydrazides (**11a–d**)*

A mixture of compound **10** (0.274 g, 0.001 mol) and hydrazine hydrate 98 % (0.5 g, 0.01 mol, excess) or aromatic hydrazine (0.001 mol) in absolute ethanol (15 mL) was heated under reflux for 16–18 h. The reaction mixture was concentrated and the precipitated solid was collected by filtration, washed with water, dried, and crystallized from dioxane.

*8-Methyl-2-oxopyrimido[2,1-b][1,3]benzothiazole-4-carbohydrazide (**11a**)* Yield 50 %, m.p. decomp. 210 °C. IR spectrum (KBr, *v*, cm^{-1}): 3319 (broad band, NH_2, NH), 1640 (2C=O). ^1H NMR spectrum: (DMSO-d_6, δ ppm):

2.29 (s, 3H, CH_3), 4.40 (s, 2H, NH_2), 6.64–7.38 (m, 3H, Ar–H), 7.47 (s, 1H, C_3–H), 10.67 (s, 1H, NH). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 23.9, 105.1, 121.3, 124.7 (2C), 127.5, 128.8, 138.7, 153.2, 157.6, 166.1, 170.4. Anal. Calcd. for $C_{12}H_{10}N_4O_2S$ (274.30): C 52.54, H 3.67, N 20.43. Found: C 52.83, H 3.92, N 20.21.

8-Methyl-2-oxo-N′-phenylpyrimido[2,1-b][1,3]benzothiazole-4-carbohydrazide (***11b***) Yield 60 %, m.p. 169–170 °C. IR spectrum (KBr, ν, cm^{-1}): 3363, 3321 (2NH), 1647 (2C=O). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.31 (s, 3H, CH_3), 6.56-7.93 (m, 9H, Ar–H, C_3–H), 9.82 (s, 1H, NH), 10.35 (s, 1H, NH). MS *m/z* (%): 351 (0.04, M^++1), 350 (0.01, M^+), 210 (100.00). Anal. Calcd. for $C_{18}H_{14}N_4O_2S$ (350.39): C 61.70, H 4.03, N 15.99. Found: C 61.93, H 3.86, N 16.27.

N′-(4-Chlorophenyl)-8-methyl-2-oxopyrimido[2,1-b][1,3]benzothiazole-4-carbohydrazide (***11c***) Yield 65 %, m.p. 156–157 °C. IR spectrum (KBr, ν, cm^{-1}): 3354, 3320 (2NH), 1654 (2C=O). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.23 (s, 3H, CH_3), 6.63–7.88 (m, 8H, Ar–H, C_3–H), 9.98 (s, 1H, NH), 11.34 (s, 1H, NH). MS *m/z* (%): 387 (0.09, M^++2), 385 (0.22, M^+), 58 (100.00). Anal. Calcd. for $C_{18}H_{13}ClN_4O_2S$ (384.84): C 56.18, H 3.40, N 14.56. Found: C 55.96, H 3.67, N 14.72.

8-Methyl-N′-(2,4-dinitrophenyl)-2-oxopyrimido[2,1-b][1,3]benzothiazole-4-carbohydrazide (***11d***) Yield 50 %, m.p. 172–174 °C. IR spectrum (KBr, ν, cm^{-1}): 3367, 3325 (2NH), 1656 (2C=O). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.34 (s, 3H, CH_3), 6.93–8.84 (m, 7H, Ar–H, C_3–H), 9.98 (s, 1H, NH), 11.34 (s, 1H, NH). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 23.7, 106.5, 114.8 (2C), 121.7, 123.1, 124.3, 125.2, 127.6, 128.7, 129.1 (2C), 138.9, 146.9, 151.8, 157.3, 166.7, 169.9. MS *m/z* (%): 441 (1.36, M^++1), 440 (1.38, M^+), 164 (100.00). Anal. Calcd. for $C_{18}H_{12}N_6O_6S$ (440.39): C 49.09, H 2.75, N 19.08. Found: C 49.31, H 2.97, N 19.37.

Preparation of 2-amino-8-methyl-4-(substituted)phenyl-4H-pyrimido[2,1-b][1,3]benzothiazole-3-carbonitriles (***13a, b***)

A mixture of amine **9** (0.41 g, 0.0025 mol), the appropriate benzylidenemalononitrile **12a**, **b** (0.0025 mol) and a catalytic amount of piperidine (0.3 mL) in absolute ethanol (15 mL) was heated under reflux for 12–16 h. The solvent was concentrated, then poured onto cold water (50 mL). The precipitated solid was collected by filtration, dried, and crystallized from ethanol/water (2:1).

2-Amino-4-(3,4-dimethoxyphenyl)-8-methyl-4H-pyrimido[2,1-b][1,3]benzothiazole-3-carbonitrile (***13a***) Yield 60 %, m.p. 164–165 °C. IR spectrum (KBr, ν, cm^{-1}): 3425 (broad band, NH_2), 2191 (C≡N). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.30 (s, 3H, CH_3), 3.00 (s, 2H, NH_2), 3.83 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 5.18 (s, 1H, C_4-H), 6.81-7.65 (m, 6H, Ar–H). ^{13}C NMR spectrum: (DMSO-d_6, δ ppm): 23.9, 38.8, 55.6, 55.7, 68.7, 112.6, 114.8 (2C), 116.3, 121.2, 126.5, 127.3 (2C), 127.9, 133.1, 137.9, 145.9, 148.1, 158.3, 163.1. MS *m/z* (%): 380 (0.14, M^++2), 379 (0.51, M^++1), 378 (0.22, M^+), 164 (100.00). Anal. Calcd. for $C_{20}H_{18}N_4O_2S$ (378.45): C 63.47, H 4.79, N 14.80. Found: C 63.68, H 4.91, N 14.63.

2-Amino-4-(4-chlorophenyl)-8-methyl-4H-pyrimido[2,1-b][1,3]benzothiazole-3-carbonitrile (***13b***) Yield 65 %, m.p. 176–177 °C. IR spectrum (KBr, ν, cm^{-1}): 3421, 3385 (NH_2), 2189 (C≡N). ^{1}H NMR spectrum: (DMSO-d_6, δ ppm): 2.29 (s, 3H, CH_3), 3.00 (s, 2H, NH_2), 4.45 (s, 1H, C_4–H), 6.98–7.65 (m, 7H, Ar–H). MS *m/z* (%): 355 (0.01, M^++2), 354 (0.09, M^++1), 353 (0.11, M^+), 164 (100.00). Anal. Calcd. for $C_{18}H_{13}ClN_4S$ (352.84): C 61.27, H 3.71, N 15.88. Found: C 61.14, H 3.54, N 16.15.

Biological testing

Antimicrobial and antiquorum-sensing activities

Sixteen of the synthesized compounds; namely, **3a**, **b**, **5a**–**f**, **8a**, **b**, **10**, **11a**–**c**, and **13a**, **b** were screened for antibacterial activity against *E. coli*, *S. aureus*, and *B. cereus* in (Luria–Bertani agar media) (Pearson *et al.*, 1980) and antifungal activity against *C. albicans* in (Saboured's agar), *A. fumigatus* 293, and *A. flavus* 3375 in (glucose minimal media) (Holt, 1975). In addition, their antipathogenic potential was checked by examining the antiquorum-sensing activity against *C. violaceum* ATCC 12472 in (Luria–Bertani agar media) (McClean *et al.*, 1997).

Escherichia coli, *B. cereus*, *S. aureus*, and *C. albicans* were obtained from the culture collection of the Department of Microbiology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt. Filamentous fungi; *A. fumigatus* 293 and *A. flavus* 3375 were kindly provided from Keller Lab, UW, USA. *Chromobacterium violaceum* ATCC 12472 was kindly provided from Prof. Bob Mclean, Department of Biology, Texas State University, USA. All bacterial strains were propagated in Luria–Bertani (LB) agar media (Merck, Germany). *Candida albicans* was grown in Saboured's media (Merck, Germany) and filamentous fungi were cultivated in glucose minimal media (Merck, Germany). Ampicillin was obtained from EPICO company; Fluconazole was obtained from Pfizer company.

Method

Antibacterial screening

All the bacterial strains were propagated in Luria–Bertani (LB) broth (1 % peptone, 0.5 % yeast extract, 0.5 % NaCl), and solidified with 1.5 % agar. Melted Muller Hinton agar (50 mL) at 50 °C were seeded with 50 µL of 1×10^6 CFU/mL of 18 h culture of tested microorganisms. The inoculated agar was mixed and poured into 15-cm-diameter plates to solidify. Wells were made in agar using cork borer. Tested compounds were dissolved in DMSO in eppendorff tubes for final concentration 5 mg/mL. Aliquots each of 100 µL of each compound were applied into the wells, DMSO was also included as a negative control, and ampicillin in a concentration of 5 mg/mL was used as a reference antibacterial agent. The compounds were allowed to diffuse for 2 h at 4 °C and incubated at 37 °C for 24 h (Pearson *et al.,* 1980). Inhibition zones were measured using Vernier caliper and the activity of the tested compounds was estimated in comparison to ampicillin (Table 1). The inhibition zone diameter of DMSO was subtracted from the antibacterial activity of tested compounds.

Antifungal screening

Saboured's media (50 mL) was inoculated with 50 µL of 1×10^6 CFU/mL of 24 h culture of *C. albicans*. For filamentous fungi, glucose minimal media (50 mL) was inoculated with 50 µL of 1×10^6 CFU/mL of Aspergilli. Wells were made in agar using cork borer. The tested compounds were dissolved in DMSO in eppendorff tubes for final concentration 5 mg/mL, and 100 µL of test solution was applied into the wells. The standard antifungal drug (fluconazole) was also added at the same concentration to each plate. In addition, DMSO (control solvent) was added to each plate. Plates were incubated at 37 °C for 48 h for *C. albicans* and *A. fumigatus* and at 30 °C for 48 h for *A. flavus* (Holt, 1975). Antifungal activity of the tested compounds was determined by measuring the diameter of the inhibition zone (Table 1). The inhibition zone diameter of DMSO was subtracted from the antifungal activity of tested compounds.

Antiquorum-sensing screening

Cultures were prepared by growing *C. violaceum* ATCC 12472 in Luria–Bertani (LB) broth and incubated for 16–18 h in an orbital incubator running at 28 °C and 150 rpm. Cultures were then adjusted to 0.5 McFarland standard (Ca. 1×10^6 CFU/mL). *Chromobacterium violaceum* was inoculated (100 µL/plate) in 50 mL LB agar and solidified. Wells were made in LB agar media using cork borer. The tested compounds were dissolved in DMSO in eppendorff tubes for final concentration 5 mg/mL and 100 µL of test solution was applied into the wells. DMSO (control solvent) was added to each plate. Plates were incubated at 30 °C for 48 h to check the inhibition of pigment production around the wells. Bacterial growth inhibition would result in a clear halo around the disk, while a positive quorum sensing inhibition is exhibited by a turbid halo harboring pigmentless bacterial cells of *C. violaceum* ATCC 12472 monitor strain (McClean *et al.*, 1997). Bacterial growth inhibition by the tested compounds was measured as radius (r_1) in mm, while both growth and pigment inhibition was measured as radius (r_2) in mm. The pigment inhibition (QS inhibition) was determined by subtracting bacterial growth inhibition (r_1) from the total radius (r_2); thus, QS inhibition $= (r_2 - r_1)$ in mm (Table 1).

Cytotoxicity testing using brine shrimp lethality bioassay

Artemia salina Leach (brine shrimp eggs) are readily available as fish food in pet shops. Artificial "sea water" is prepared by dissolving sea salt in distilled water (40 g/L) supplemented with dried yeast (6 mg/L). Brine shrimp eggs are hatched in artificial sea water during 48 h incubation in a warm room (22–29 °C), providing large numbers of larvae (nauplii). Brine shrimp larvae (nauplii) were collected with a Pasteur pipette after attracting the organisms to one side of the vessel with a light source. Nauplii were separated from the eggs by pipetting them 2–3 times in small beakers containing sea water. The test compounds were made up to 1 mg/mL in artificial sea water (water insoluble compounds were dissolved in 50 µL DMSO prior to adding sea water). Serial dilutions (1000, 500, 100, 10 µg/mL) were made in the wells in 96-multiwell microtiter plate in triplicate in 100 µL sea water. A suspension of nauplii containing 25 brine shrimp larvae in 100 µL sea water was added to each well with the help of a Pasteur pipette and the covered microwell plate was incubated at 22–29 °C for 24 h. The plate was then examined under a binocular microscope and the number of dead (non-mobile) nauplii in each well was counted. Methanol (100 µL) was then added to each well, and after 15 min the total number of shrimps in each well was counted (Mayer *et al.,* 1982). Plotting of log concentration (logC) versus % mortality for all tested compounds showed an approximate linear correlation, and the values of LC_{50} and LC_{90} were calculated by using Microsoft Excel XP (Table 2). All values were compared with the standard cytotoxic agent, 5-flurouracil with LC_{50} and LC_{90} values of 409.83 and 737.70 µg/mL, respectively.

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