Bioorganic & Medicinal Chemistry 19 (2011) 4506–4512

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of some new [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines and [1,2,4]triazolo[3,4-*b*][1,3,4] thiadiazoles starting from 5-nitro-2-furoic acid and evaluation of their antimicrobial activity

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ARTICLE INFO

Article history:
Received 26 April 2011
Revised 4 June 2011
Accepted 8 June 2011
Available online 16 June 2011

Keywords:
1,2,4-Triazole
Dihydrotriazolothiadiazoles
Triazolothiadiazoles
Triazolothiadiazines
Cyclization
Antimicrobial activity
Cytotoxic activity

ABSTRACT

New series of fused 1,2,4-triazoles such as, 6-(aryl)-3-(5-nitrofuran-2-yl)-5,6-dihydro-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazoles **4–8**, 6-(alkyl/aryl amino)-3-(5-nitrofuran-2-yl)-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazoles **9–13** and 6-(4-substituted phenyl)-3-(5-nitrofuran-2-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines *14*–**18** have been synthesized via the reaction of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** with various reagents such as hetero aromatic aldehydes, alkyl/aryl isothiocyanates and 4-substituted phenacyl bromides, respectively. The structures of the newly synthesized compounds have been confirmed on the basis of elemental analysis and spectral studies. The newly synthesized triazolo derivatives have been investigated for their in vitro antibacterial activity. Most of the tested compounds showed interesting antibacterial activity against *Staphylococcus aureus*. Furthermore, the most potent antibacterial compounds **11–13** were evaluated for their in vitro cytotoxic activity against human cancer cell lines. It was found that compounds **11** and **13** showed higher cytotoxicity against Hep-G2 cell line as compared to standard.

1. Introduction

Infectious microbial diseases remain pressing problems world wide, because resistance to a number of antimicrobial agents among variety of clinically significant species of microorganisms (like methicillin-resistant *Staphylococcus aureus* (MRSA)) has become an important global health problem.[1–3] Hence, there will always be a vital need to discover new antimicrobial agents. Literature survey has revealed the importance of 1,2,4-triazoles as antimicrobial agents[4–19] such as Fluconazole,[20,21] Ravuconazole,[21] Voriconazole,[21] Itraconazole and Posaconazole[20–22] (Fig. 1). In addition, furan derivatives have been widely used as antimicrobial agents[23–31] for example, Furazolidone,[32] Nifuroxazide,[33] Nitrofurantoin,[33] Furoxone and RBx7644[34] (Fig. 2). Prompted by these observations and as part of a synthetic effort directed toward the synthesis of antimicrobial agents, the key intermediate, 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** has been condensed with various reagents such as, hetero aromatic aldehydes, alkyl/aryl isothiocyanates and 4-substituted phenacyl bromides to afford 6-(aryl)-3-(5-nitrofuran-2-yl)-5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazoles **4–8**, 6-(alkyl/aryl amino)-3-(5-nitrofuran-2-yl)[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazoles **9–13** and 6-(4-substituted phenyl)-3-(5-nitrofuran-2-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines **14–18**, respectively.

2. Results and discussion

2.1. Chemistry

The target compounds were synthesized as outlined in Schemes 1–4. The title compound, 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** was prepared by a one-step synthesis via the combining of 5-nitro-2-furoic acid **1** with thiocarbohydrazide **2** at 170–180 °C. While the compound **3** may exist in thione–thiol tautomeric forms, in the present study, the thiol structure was dominated in the solid state. Usual spectroscopic methods IR and ^{1}H NMR distinguished between these constitutional isomers. The IR spectra, showed absorption band at about 2600 cm^{-1} indicative of SH group and no absorption band in the range 1300–1200 cm^{-1} attributable to the C=S group. The ^{1}H NMR spectrum of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** showed NH_2 protons as a singlet at 5.46 ppm (integrating two protons) and SH proton as a singlet at 13.19 ppm. Other peaks were observed at appropriate chemical shifts and integral values (Scheme 1).

Reaction of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** with hetero aromatic aldehydes in the presence of *p*-toluene

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doi:10.1016/j.bmc.2011.06.024

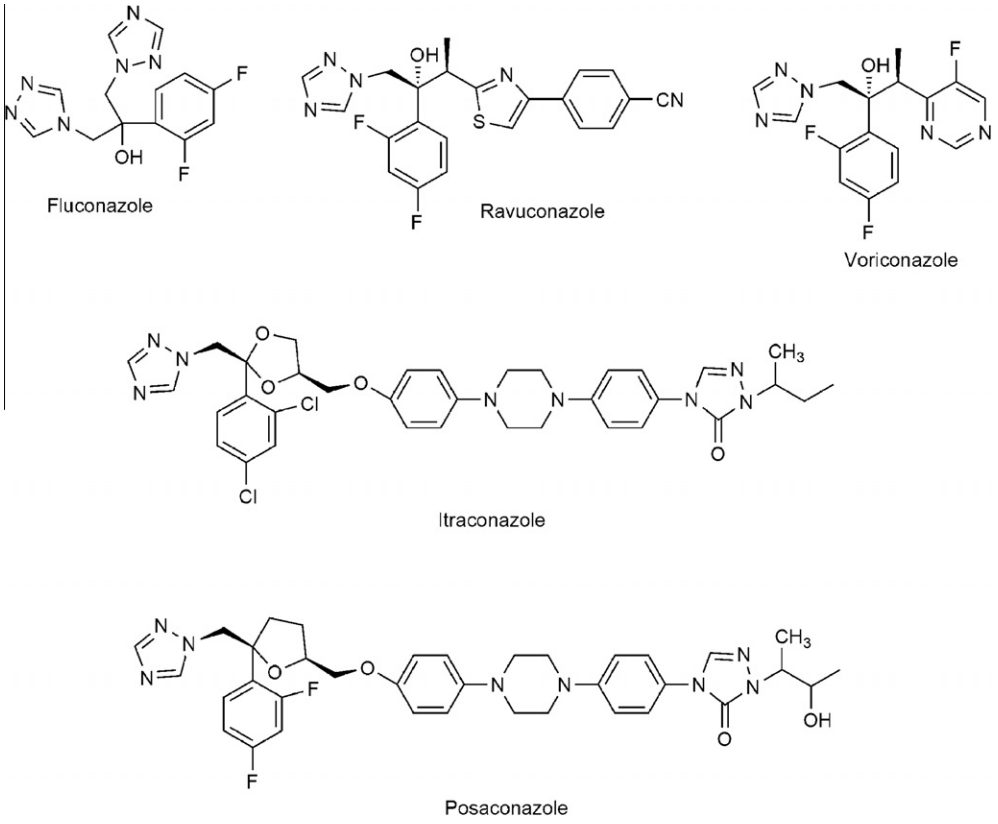


Figure 1. Structures of some antimicrobial drugs characterized by heterocyclic 1,2,4-triazole ring.

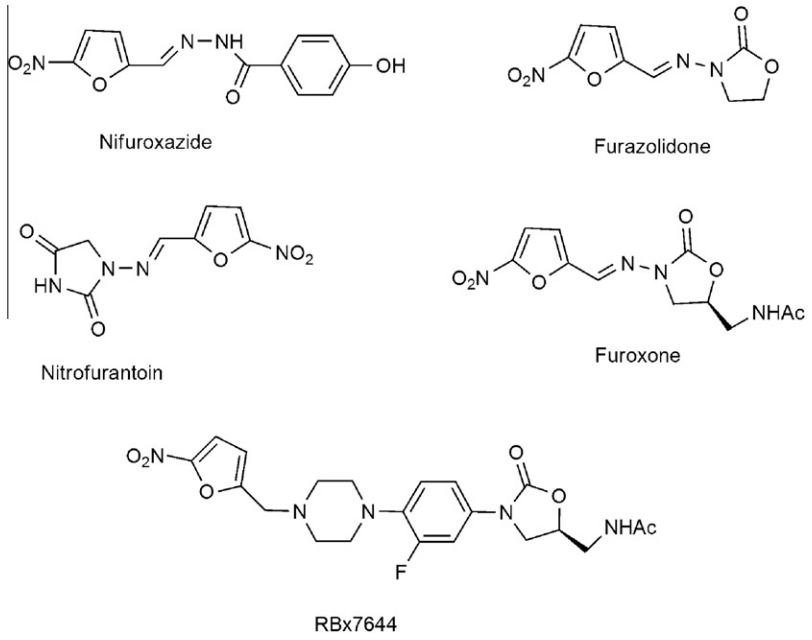
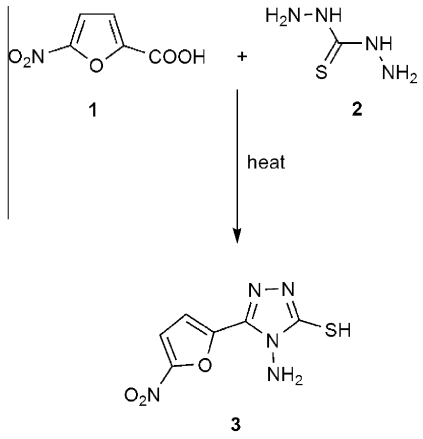


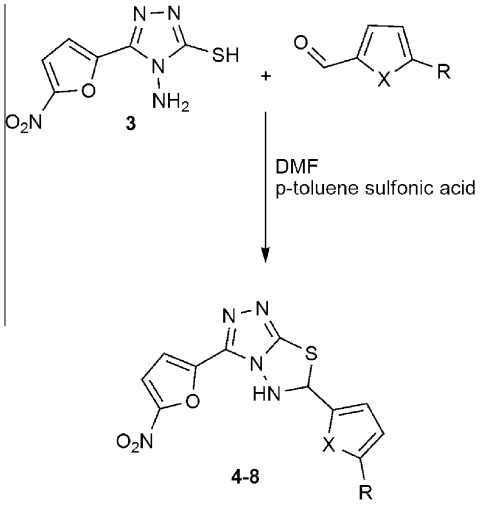
Figure 2. Structures of some antimicrobial drugs containing 5-nitrofuran moiety.

sulfonic acid as a catalyst yielded, 6-(aryl)-3-(5-nitrofuran-2-yl)-5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazoles **4**–**8**. Their proton NMR spectra lacked signals characteristic of SH and NH_2 protons, and showed two new peaks at 5.61–5.74 ppm (integration for one proton) and 6.33–6.64 ppm (integration for one proton) attributed to CH and NH protons, respectively, establishing that CHO group of the hetero aromatic aldehydes reacted with SH and NH_2 groups of the intermediate **3** to afford 5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives **4**–**8** (Scheme 2).

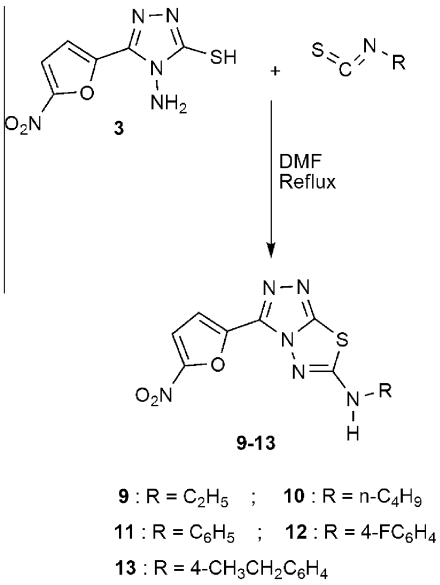
Furthermore, 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** was heated under reflux with alkyl/aryl isothiocyanates in dimethylformamide as a solvent to give 6-(alkyl/aryl amino)-3-(5-nitrofuran-2-yl)[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazoles **9–13**. In the proton NMR spectra, the disappearance of SH and NH_2 sig-



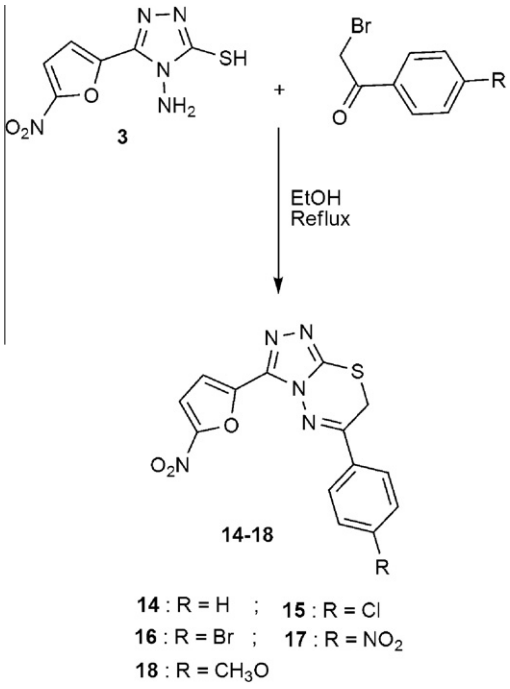
Scheme 1. Synthesis of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3**.



Scheme 2. Synthetic pathway of compounds **4**–**8**.



Scheme 3. Synthetic pathway of compounds **9**–**13**.



Scheme 4. Synthetic pathway of compounds **14**–**18**.

nals and appearance of new singlet characteristic for alkyl/aryl amino proton recorded in the range of 9.14–9.96 ppm (integration for one proton) confirmed the conversion of compound **3** into 1,2,4-triazolo[3,4-*b*][1,3,4]thiadiazole derivatives **9–13** (Scheme 3).

Moreover, 6-(4-substituted phenyl)-3-(5-nitrofuran-2-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines **14–18** were prepared through the reaction of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** with 4-substituted phenacyl bromides in absolute ethanol. Their proton NMR spectra showed that the signals due to SH and NH_2 protons disappeared, instead, a new signal observed at 4.16–4.32 ppm (integration for two protons) attributed to CH_2 group. This confirmed that the compound **3** underwent ring closure to give the 1,2,4-triazolo[3,4-*b*][1,3,4]thiadiazines **14–18** (Scheme 4).

All compounds were spectroscopically characterized and the spectral data agree with the proposed structures.

2.2. Antimicrobial evaluation

The newly synthesized compounds **3–18** were tested for their in vitro antibacterial activity against *S. aureus* (e.g., for Gram-positive bacteria) and *Escherichia coli* (e.g., for Gram-negative bacteria). The minimum inhibitory concentration (MIC, μg/ml, i.e., the lowest concentration required to inhibit the growth of bacteria) and the minimum bactericidal concentration (MBC, μg/ml, i.e., the lowest concentration of the compound required to kill bacteria) of all the compounds were determined by the broth dilution method.[35–38] For comparison, ampicillin was used as reference drug (Table 1). Moderate antibacterial activity was observed for the tested compounds against *E. coli* in comparison to reference

Table 1
Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) in μg/ml of compounds **3**–**18**

Compound	*Staphylococcus aureus*		*Escherichia coli*	
	MIC	MBC	MIC	MBC
3	50	200	25	200
4	25[a]	200	25	200
5	25[a]	200	25	200
6	25[a]	200	25	200
7	25[a]	200	50	200
8	25[a]	200	50	200
9	25[a]	100	25	200
10	25[a]	200	25	100
11	25[a]	50[b]	50	100
12	25[a]	50[b]	25	100
13	25[a]	50[b]	25	50
14	100	200	50	200
15	25[a]	200	25	200
16	25[a]	200	25	200
17	50	100	25	200
18	50	200	25	200
Ampicillin	25	50	12.5	25

MIC, minimum inhibitory concentration (the lowest concentration that inhibited the bacterial growth). MBC, minimum bactericidal concentration (the lowest concentration at which no bacterial growth was observed).
[a] Equal MIC value to reference.
[b] Equal MBC value to reference

ampicillin (Table 1). In contrast, when compared with the reference drug ampicillin, most of the compounds exhibited considerable antibacterial activity against *S. aureus* (Table 1). The MIC value (25 μg/ml) of the compounds, **4–13**, **15** and **16** was equal to reference. The other compounds in the series **3**, **17** and **18** showed moderate activity. Only the compound **14** exhibited weak activity against *S. aureus* (Table 1). The obtained results revealed that antibacterial activity of the newly synthesized heterocyclic compounds, **4–8** containing 1,2,4-triazole moiety fused with 1,3,4-dihydrothiadiazole ring and compounds, **9–13** containing 1,2,4-triazole moiety fused with 1,3,4-thiadiazole ring depend on the basic skeleton of the molecule rather the substituents and all were found to be as effective as reference drug ampicillin (MIC = 25 μg/ml). Meanwhile, among triazolothiadiazine derivatives **14–18** it was noticed that the activity depend on the substituents rather the basic skeleton of the molecule. The compounds, **15** and **16** bearing *p*-chlorophenyl moiety and *p*-bromophenyl moiety respectively at position 6 of the thiadiazine ring were found to be as effective as reference drug (MIC = 25 μg/ml) against *S. aureus*. However, compounds **17** and **18** bearing *p*-nitrophenyl moiety and *p*-methoxyphenyl moiety respectively at position 6 of the thiadiazine ring exhibited moderate activity while, compound **14**, bearing unsubstituted phenyl moiety at position 6 of the thiadiazine ring showed weak activity.

In general, compounds **11–13** exhibited antibacterial activity (MIC and MBC) against *S. aureus* equivalent to that of standard ampicillin.

2.3. Cytotoxicity evaluation

The most potent antibacterial compounds **11–13** were evaluated for their in vitro cytotoxic activity against Hep-G2 (hepatocarcinoma) and HCT-116 (human colon tumor) cell lines using Sulforhodamine B assay.[39,40] Inhibitory activities (IC_{50}) were presented as microgram concentrations of the compounds as shown in Table 2. It was found that compounds **11** and **13** showed the most significant cytotoxicity against Hep-G2 cell line compared to standard (Doxorubicin). The structure–activity relationship study among the tested compounds revealed that the cytotoxic activity was dependent on the substituents. Incorporation of unsubstituted phenyl ring or 4-methylbenzyl moiety into the triazolothiadiazole ring at 6-position via amino bridge developed more effective cytotoxic analogues (compounds **11** and **13**) than the compared standard drug (Doxorubicin) against Hep-G2 cell line. While incorporation of *p*-fluorophenyl moiety into the triazolothiadiazole ring at 6-position via amino bridge providing lower potency analogue (compound **12**) than the compared standard drug (Doxorubicin) against Hep-G2 cell line.

Table 2
IC_{50} values (in μg/ml) for compounds **11**–**13** against HCT-116 (human colon tumor cell line) and Hep-G2 (hepatocarcinoma cell line)

Compound	Hep-G2	HCT-116
11	4.23	4.38
12	11.3	8.91
13	3.78	6.65
Doxorubicin	5.5	3.73

3. Conclusion

A new series of triazoles **3**–**18**, have been synthesized and evaluated for their antibacterial activity. Compared with the reference drug, Ampicillin, our compounds are more sensitive to Gram-positive bacteria than Gram-negative bacteria. Compounds, **11–13** found to be as effective as reference drug against *S. aureus*. Furthermore, compounds, **11–13** were evaluated for their in vitro cytotoxic activity against Hep-G2 and HCT-116 cell lines. Compounds **11** and **13** displayed stronger cytotoxicity against Hep-G2 cell line compared to standard.

4. Experimental

Unless otherwise noted, all materials were obtained from commercial suppliers (Aldrich and Merck companies) and used without further purification. Melting points were determined on Fisher–Johns melting point apparatus and are uncorrected. Elemental analytical data (in accordance with the calculated values) were performed at the microanalytical unit of Cairo University and were within ± 0.4% of theoretical values. The IR spectra were recorded on Mattson 5000 FT-IR spectrometer (ν in cm^{-1}) using a KBr disk at the Faculty of Science, Mansoura University. ^1H NMR spectra were recorded on FT-NMR JNM-LA spectrometer (300 MHZ) in $CDCl_3$ or DMSO-d_6 as solvent, using TMS as an internal standard (chemical shifts in ppm, δ units). The mass spectra were performed using JEOL JMS-600H spectrometer at Cairo University, Cairo, Egypt. All reactions were followed by *TLC* on silica gel-protected aluminum sheets (type 60 F_{254}, Merk).

4.1. Synthesis of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol 3

A mixture of 5-nitro-2-furoic acid (0.01 mol) and thiocarbohydrazide (0.015 mol) was heated in an oil bath at 170–180 °C for 30 min. The product obtained on cooling was triturated with hot water, filtered, dried and recrystallized from chloroform/methanol to give a yellow compound. Yield 78%, mp 220–222 °C. IR (KBr, ν, cm^{-1}): 3292, 3275 (NH_2), 1640 (C=N), 2600 (SH); ^1H NMR ($CDCl_3$): δ 5.46 (s, 2H, NH_2 D_2O exchangeable), 7.48 (d, J = 3.7 Hz, 1H, furan), 7.90 (d, J = 3.7 Hz, 1H, furan), 13.19 (s, 1H, SH); ^{13}C NMR ($CDCl_3$): δ 115.6, 130.2, 154.4 and 163.1 (C-furan), 149.8 and 152.6 (C-triazole); Mass m/z: 227 [M^+]. Anal. Calcd (%) for $C_6H_5N_5O_3S$: C, 31.72; H, 2.22; N, 30.82. Found: C, 31.51; H, 2.38; N, 30.62.

4.2. General procedure for the synthesis of 6-(aryl)-3-(5-nitrofuran-2-yl)-5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives 4–8

A mixture of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** (0.005 mol), appropriate hetero aromatic aldehyde (0.005 mol), anhydrous dimethylformamide (20 ml) and a catalytic amount of *p*-toluene sulfonic acid (0.2 g) was refluxed for 12–14 h, then cooled to room temperature. The cooled mixture was poured onto crushed ice with stirring. The mixture was allowed to stand overnight. The separated solid was filtered, washed thoroughly with water, dried and recrystallized from ethanol/dioxan to yield the title compounds **4**–**8**.

4.2.1. 3-(5-Nitrofuran-2-yl)-6-(1*H*-pyrrol-2-yl)-5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole 4

Brown compound, yield 70%, mp 192–194 °C. IR (KBr, ν, cm^{-1}): 3352 (NH), 2950 (CH), 1632 (C=N); ^1H NMR (DMSO-d_6): δ 5.70 (s, 1H, CH), 6.40 (s, 1H, NH, D_2O exchangeable), 6.76 (d, J = 2.92 Hz, 1H, pyrole), 6.91 (dd, J = 2.92 Hz, 1.69 Hz, 1H, pyrole), 7.34 (d, J = 1.69 Hz, 1H, pyrole), 7.51 (d, J = 3.7 Hz, 1H, furan), 7.86 (d, J = 3.7 Hz, 1H, furan), 9.82 (s, 1H, NH pyrole); ^{13}C NMR (DMSO-d_6): δ 117.8, 131.1, 154.3 and 162.7 (C-furan), 90.8, 156.3 and 160.8 (C-dihydrotriazolothiadiazole), 119.1, 120.8, 129.8 and 142.8 (C-pyrrol); Mass m/z: 304 [M^+]. Anal. Calcd (%) for $C_{11}H_8N_6O_3S$: C, 43.42; H, 2.65; N, 27.62. Found: C, 43.63; H, 2.34; N, 27.45.

4.2.2. 6-(Furan-2-yl)-3-(5-nitrofuran-2-yl)-5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole 5

Red compound, yield 68%, mp 241–243 °C. IR (KBr, ν, cm^{-1}): 3345 (NH), 2958 (CH), 1625 (C=N); ^1H NMR (DMSO-d_6): δ 5.61 (s, 1H, CH), 6.64 (s, 1H, NH, D_2O exchangeable), 7.10 (d, J = 3.6 Hz, 1H, furan), 7.30 (dd, J = 3.6 Hz, 1.7 Hz, 1H, furan), 7.48 (d, J = 3.3 Hz, 1H, furan), 7.67 (d, J = 1.7 Hz, 1H, furan), 7.92 (d, J = 3.3 Hz, 1H, furan); Mass m/z: 305 [M^+]. Anal. Calcd (%) for $C_{11}H_7N_5O_4S$: C, 43.28; H, 2.31; N, 22.94. Found: C, 43.12; H, 2.14; N, 23.15.

4.2.3. 3,6-Bis(5-nitrofuran-2-yl)- 5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole 6

Brown compound, yield 60%, mp 268–270 °C. IR (KBr, ν, cm^{-1}): 3365 (NH), 2915 (CH), 1630 (C=N); ^1H NMR (DMSO-d_6): δ 5.70 (s, 1H, CH), 6.40 (s, 1H, NH, D_2O exchangeable), 7.51 (d, J = 3.3 Hz, 2H, furan), 7.88 (d, J = 3.3 Hz, 2H, furan); Mass m/z: 350 [M^+]. Anal. Calcd (%) for $C_{11}H_6N_6O_6S$: C, 37.72; H, 1.73; N, 23.99. Found: C, 37.42; H, 1.93; N, 23.67.

4.2.4. 3-(5-Nitrofuran-2-yl)-6-(thiophen-2-yl)-5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole 7

Brown compound, yield 63%, mp 256–258 °C. IR (KBr, ν, cm^{-1}): 3350 (NH), 2963 (CH), 1620 (C=N); ^1H NMR (DMSO-d_6): δ 5.74 (s, 1H, CH), 6.42 (s, 1H, NH, D_2O exchangeable), 6.98 (d, J = 5.1 Hz, 1H, thiophene), 7.22 (dd, J = 5.1 Hz, 3.2 Hz, 1H, thiophene), 7.51 (d, J = 3.7 Hz, 1H, furan), 7.77 (d, J = 3.2 Hz, 1H, thiophene), 7.96 (d, J = 3.7 Hz, 1H, furan); ^{13}C NMR (DMSO-d_6): δ 117.2, 130.6, 157.8 and 165.3 (C-furan), 92.4, 159.1 and 162.2 (C-dihydrotriazolothiadiazole), 124.2, 126.2, 127.3 and 138.4 (C-thiophene); Mass m/z: 321 [M^+]. Anal. Calcd (%) for $C_{11}H_7N_5O_3S_2$: C, 41.12; H, 2.20; N, 21.79. Found: C, 41.45; H, 2.45; N, 21.56.

4.2.5. 3-(5-Nitrofuran-2-yl)-6-(5-nitrothiophen-2-yl)-5,6-dihydro[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole 8

Brown compound, yield 60%, mp 285–287 °C. IR (KBr, ν, cm^{-1}): 3370 (NH), 2958 (CH), 1625 (C=N); ^1H NMR (DMSO-d_6): δ 5.65 (s, 1H, CH), 6.33 (s, 1H, NH, D_2O exchangeable), 7.36 (d, J = 4.8 Hz, 1H, thiophene), 7.53 (d, J = 3.4 Hz, 1H, furan), 7.83 (d, J = 4.8 Hz, 1H, thiophene), 7.98 (d, J = 3.4 Hz, 1H, furan); Mass m/z: 366 [M^+]. Anal. Calcd (%) for $C_{11}H_6N_6O_5S_2$: C, 36.06; H, 1.65; N, 22.94. Found: C, 36.27; H, 1.48; N, 22.63.

4.3. General procedure for the synthesis of 6-alkyl/arylamino-3-(5-nitrofuran-2-yl)[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazole derivatives 9–13

A mixture of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** (0.005 mol) and alkyl/aryl isothiocyanate (0.005 mol) in anhydrous dimethylformamide (20 ml) was refluxed with stirring for 18–20 h. The reaction mixture was cooled to room temperature and then poured onto crushed ice with stirring. The mixture was allowed to stand overnight. The separated solid was filtered, washed thoroughly with water, dried and recrystallized from ethanol/pet. ether to afford the title compounds **9**–**13**.

4.3.1. *N*-ethyl-3-(5-nitrofuran-2-yl) [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-amine 9

Red compound, yield 70%, mp 160–162 °C. IR (KBr, ν, cm^{-1}): 3410 (NH), 2981, 2962, 2879 (CH_3 and CH_2), 1625 (C=N); ^1H NMR ($CDCl_3$): δ 1.54 (t, J = 7.0 Hz, 3H, CH_3), 3.42 (q, J = 7.0 Hz, 2H, N-CH_2), 7.51 (d, J = 3.7 Hz, 1H, furan), 7.96 (d, J = 3.7 Hz, 1H, furan), 9.14 (br s, 1H, NH, D_2O exchangeable); ^{13}C NMR ($CDCl_3$): δ 14.8 (CH_3), 38.6 (CH_2), 117.4, 130.0, 154.6 and 164.6 (C-furan), 150.0, 152.2 and 161.2 (C-triazolothiadiazole); Mass m/z: 280 [M^+]. Anal. Calcd (%) for $C_9H_8N_6O_3S$: C, 38.57; H, 2.88; N, 29.99. Found: C, 38.31; H, 2.74; N, 29.76.

4.3.2. *N*-butyl-3-(5-nitrofuran-2-yl) [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-amine 10

Red compound, yield 74%, mp 181–183 °C. IR (KBr, ν, cm^{-1}): 3428 (NH), 2981, 2958, 2870 (CH_3 and CH_2), 1630 (C=N); ^1H NMR ($CDCl_3$): δ 0.96 (t, J = 6.7 Hz, 3H, CH_3), 1.55–1.71 (m, 4H, CH_2), 3.01 (t, J = 6.7 Hz, 2H, N-CH_2), 7.58 (d, J = 3.3 Hz, 1H, furan), 7.92 (d, J = 3.3 Hz, 1H, furan), 9.34 (br s, 1H, NH, D_2O exchangeable); Mass m/z: 308 [M^+]. Anal. Calcd (%) for $C_{11}H_{12}N_6O_3S$: C, 42.85; H, 3.92; N, 27.26. Found: C, 42.61; H, 3.74; N, 27.52.

4.3.3. 3-(5-Nitrofuran-2-yl)-*N*-phenyl [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-amine 11

Brown compound, yield 65%, mp 237–239 °C. IR (KBr, ν, cm^{-1}): 3347 (NH), 3070 (C=C aryl), 1620 (C=N); ^1H NMR (DMSO-d_6): δ 7.01 (t, J = 8.5 Hz, 1H, Ar-H), 7.23 (t, J = 8.5 Hz, 2H, Ar-H), 7.40 (d, J = 8.5 Hz, 2H, Ar-H), 7.62 (d, J = 3.4 Hz, 1H, furan), 7.94 (d, J = 3.4 Hz, 1H, furan), 9.45 (br s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): δ 116.5, 130.7, 156.0 and 163.1 (C-furan), 151.2, 154.0 and 161.4 (C-triazolothiadiazole), 127.3 (C), 129.0 (2C), 129.8 (2C) and 132.8 (C) (C-aryl); Mass: m/z 328 [M^+]. Anal. Calcd (%) for $C_{13}H_8N_6O_3S$: C, 47.56; H, 2.46; N, 25.60. Found: C, 47.69; H, 2.23; N, 25.34.

4.3.4. *N*-(4-fluorophenyl)-3-(5-nitrofuran-2-yl) [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-amine 12

Red compound, yield 60%, mp 280–282 °C. IR (KBr, ν, cm^{-1}): 3338 (NH), 3079 (C=C aryl), 1627 (C=N); ^1H NMR (DMSO-d_6): δ 7.32 (d, J = 7.5 Hz, 2H, Ar-H), 7.50 (d, J = 3.6 Hz, 1H, furan), 7.71 (d, J = 7.5 Hz, 2H, Ar-H), 7.92 (d, J = 3.6 Hz, 1H, furan), 9.96 (br s, 1H, NH, D_2O exchangeable); Mass m/z: 346 [M^+]. Anal. Calcd (%) for $C_{13}H_7FN_6O_3S$: C, 45.09; H, 2.04; N, 24.27. Found: C, 45.38; H, 2.31; N, 24.46.

4.3.5. *N*-(4-methylbenzyl)-3-(5-nitrofuran-2-yl) [1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazol-6-amine 13

Brown compound, yield 67%, mp 200–202 °C. IR (KBr, ν, cm^{-1}): 3375 (NH), 3060 (C=C aryl), 2980, 2956, 2860 (CH_3 and CH_2), 1630 (C=N); ^1H NMR (DMSO-d_6): δ 1.21 (t, J = 6 Hz, 3H, CH_3), 1.93 (q,

J = 6 Hz, 2H, CH_2), 7.02 (d, J = 7.8 Hz, 2H, Ar-H) 7.32 (d, J = 7.8 Hz, 2H, Ar-H), 7.52 (d, J = 3.6 Hz, 1H, furan), 7.90 (d, J = 3.6 Hz, 1H, furan), 9.73 (br s, 1H, NH, D_2O exchangeable); Mass m/z: 356 [M^+]. Anal. Calcd (%) for $C_{15}H_{12}N_6O_3S$: C, 50.56; H, 3.39; N, 23.58. Found: C, 50.22; H, 3.62; N, 23.83.

4.4. General procedure for the synthesis of 6-(4-substituted phenyl)-3-(5-nitrofuran-2-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine derivatives 14–18

A mixture of 4-amino-5-(5-nitrofuran-2-yl)-4*H*-1,2,4-triazole-3-thiol **3** (0.01 mol) and 4-substituted phenacyl bromides (0.01 mol) in absolute ethanol (40 ml) was refluxed with stirring for 6–8 h. The reaction mixture was cooled; the precipitated solid was filtered, washed thoroughly with water, dried and recrystallized from absolute ethanol to afford the title compounds **14**–**18**.

4.4.1. 3-(5-Nitrofuran-2-yl)-6-phenyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 14

Yellow compound, yield 73%, mp 195–197 °C. IR (KBr, ν, cm^{-1}): 2981, 2962 (CH_2), 1620 (C=N); ^{1}H NMR (DMSO-d_6): δ 4.16 (s, 2H, CH_2), 7.10 (t, J = 8 Hz, 1H, Ar-H), 7.32 (t, J = 8 Hz, 2H, Ar-H), 7.50 (d, J = 3.7 Hz, 1H, furan), 7.62 (d, J = 8 Hz, 2H, Ar-H), 7.90 (d, J = 3.7 Hz, 1H, furan); ^{13}C NMR (DMSO-d_6): δ 115.6, 131.4, 158.3 and 164.2 (C-furan), 41.2, 160.2, 162.6 and 167.1 (C-triazolothiadiazine), 128.2 (C), 129.9 (2C), 133.1 (2C) and 138.2 (C) (C-aryl); Mass m/z: 327 [M^+]. Anal. Calcd (%) for $C_{14}H_9N_5O_3S$: C, 51.37; H, 2.77; N, 21.40. Found: C, 51.11; H, 2.41; N, 21.63.

4.4.2. 6-(4-Chlorophenyl)-3-(5-nitrofuran-2-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 15

Yellow compound, yield 65%, mp 245–247 °C. IR (KBr, ν, cm^{-1}): 2930, 2876 (CH_2), 1630 (C=N); ^{1}H NMR (DMSO-d_6): δ 4.20 (s, 2H, CH_2), 7.42 (d, J = 3.3 Hz, 1H, furan), 7.60 (d, J = 7.5 Hz, 2H, Ar-H), 7.75 (d, J = 7.5 Hz, 2H, Ar-H), 7.93 (d, J = 3.3 Hz, 1H, furan); Mass m/z: 361 [M^+], 363 [M^+ +2]. Anal. Calcd (%) for $C_{14}H_8ClN_5O_3S$: C, 46.48; H, 2.23; N, 19.36. Found: C, 46.72; H, 2.01; N, 19.16.

4.4.3. 6-(4-Bromophenyl)-3-(5-nitrofuran-2-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 16

Yellow compound, yield 68%, mp 260–262 °C. IR (KBr, ν, cm^{-1}): 2963, 2895 (CH_2), 1610 (C=N); ^{1}H NMR (DMSO-d_6): δ 4.18 (s, 2H, CH_2), 7.34 (d, J = 8 Hz, 2H, Ar-H), 7.50 (d, J = 3.7 Hz, 1H, furan), 7.63 (d, J = 8 Hz, 2H, Ar-H), 7.90 (d, J = 3.7 Hz, 1H, furan); ^{13}C NMR (DMSO-d_6): δ 116.4, 130.2, 157.5 and 165.1 (C-furan), 42.0, 160.4, 162.1 and 167.8 (C-triazolothiadiazine), 124.8 (C), 127.6 (2C), 132.3 (2C) and 136.5 (C) (C-aryl); Mass m/z: 406 [M^+], 408 [M^+ +2]. Anal. Calcd (%) for $C_{14}H_8BrN_5O_3S$: C, 41.39; H, 1.99; N, 17.24. Found: C, 41.68; H, 2.17; N, 17.51.

4.4.4. 3-(5-Nitrofuran-2-yl)-6-(4-nitrophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 17

Yellow compound, yield 62%, mp 275–277 °C. IR (KBr, ν, cm^{-1}): 2961, 2889 (CH_2), 1627 (C=N); ^{1}H NMR (DMSO-d_6): δ 4.32 (s, 2H, CH_2), 7.43 (d, J = 3.7 Hz, 1H, furan), 7.62 (d, J = 7.5 Hz, 2H, Ar-H), 7.81 (d, J = 7.5 Hz, 2H, Ar-H), 7.98 (d, J = 3.7 Hz, 1H, furan); Mass m/z: 372 [M^+]. Anal. Calcd (%) for $C_{14}H_8N_6O_5S$: C, 45.16; H, 2.17; N, 22.57. Found: C, 45.37; H, 2.00; N, 22.33.

4.4.5. 6-(4-Methoxyphenyl)-3-(5-nitrofuran-2-yl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine 18

Orange compound, yield 84%, mp 175–177 °C. IR (KBr, ν, cm^{-1}): 2976, 2866 (CH_2), 1605 (C=N); ^{1}H NMR ($CDCl_3$): δ 3.57 (s, 3H, CH_3), 4.29 (s, 2H, CH_2), 7.03 (d, J = 7.8 Hz, 2H, Ar-H), 7.44 (d, J = 3.4 Hz, 1H, furan), 7.60 (d, J = 7.8 Hz, 2H, Ar-H), 7.89 (d, J = 3.4 Hz, 1H, furan); Mass m/z: 357 [M^+]. Anal. Calcd (%) for $C_{15}H_{11}N_5O_4S$: C, 50.42; H, 3.10; N, 19.60. Found: C, 50.20; H, 3.31; N, 19.47.

4.5. Antibacterial screening

The newly synthesized compounds were assayed in vitro for antibacterial activity against *S. aureus* (representative for Gram-positive bacteria) and *E. coli* (representative for Gram-negative bacteria). The organisms were obtained from the Microbiology Laboratory, Department of Microbiology, Faculty of Pharmacy, Mansoura University. The antibacterial assay was carried out using NCCLS broth dilution method.[35–38] The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^5 c.f.u./ml. Compounds to be investigated were dissolved in DMSO. The microplates were incubated for 24 h at 37 °C. In order to ensure that the solvent had no effect on bacterial growth, a control test was performed containing broth supplemented with only DMSO. The lowest concentrations without visible growth were defined as concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by plating out the contents of the wells showing no visible growth of bacteria onto Mueller Hinton agar (MHA) plates and incubating for 24 h at 37 °C. The lowest concentrations that prevent any residual colony formation were defined as the MBCs.

Ampicillin was used as a reference standard to compare the antibacterial activity. All experiments were performed in duplicate and repeated three times. The MIC and MBC values were expressed in μg/ml as shown in Table 1.

4.6. Cytotoxicity screening

In vitro cytotoxic activity against Hep-G2 and HCT-116 cell lines was determined using Sulforhodamine B assay.[39,40] Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the compounds to allow attachment of cell to the wall of the plate. Different concentrations of the compound under test (0, 1, 2.5, 5, and 10 μg/ml) were added to the cell monolayer wells which prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37 °C in a humidified incubator with 5% CO_2. Cells were fixed with trichloroacetic acid and stained for 30 min with 0.4% (wt/vol) Sulforhodamine B (SRB) stain dissolved in 1% acetic acid. Unbound dye was washed with 1% acetic acid and protein bound dye was extracted with Tris EDTA. The absorbance of each well was determined by an ELISA reader. For IC_{50} determination, the relation between surviving fraction and drug (compound) concentration was plotted to get the survival curve of each tumor cell line after the specified compound (Table 2).

Acknowledgments

The authors would like to express their gratitude and thanks to the National Cancer Institute (NCI), Cancer Biology Department, Pharmacology Unit, Cairo University, Egypt, for doing the cytotoxic activity.

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