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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and antibacterial activity evaluation of 2,6-bis(6-substituted-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine derivatives

Haihua Xiao · Pingliang Li · Dongcai Guo · Jinhui Hu · Yuchao Chai · Wei He

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Abstract The reaction of 5,5′-(pyridine-2,6-diyl)bis(4-amino-3-mercapto-1,2,4-triazole) with various carboxylic acid in phosphorus oxychloride yielded the corresponding 2,6-bis(6-substituted-1,2,4-triazolo[3,4-*b*][1,3,4]thiadiazol-3-yl)pyridine derivatives **6a–l**. The structures of the newly synthesized compounds were confirmed by elemental analysis, ^{1}H NMR and ^{13}C NMR spectral, mass spectral, and Infrared spectral studies. All the synthesized target compounds have been investigated for their in vitro antibacterial activity, and the preliminary results revealed that the compounds **6b** and **6k** exhibited very promising activity against *Escherichia coli* and *Pseudomonas aeruginosa*.

Keywords Pyridine-2,6-dicarboxylic acid derivatives · 1,2,4-Triazole · Synthesis · Antibacterial activity

Introduction

1,2,4-Triazole derivatives, an important group of heterocyclic compounds, have been the subject of extensive study in the recent years (Eswaran *et al.*, 2009). It has been reported in literature that certain compounds bearing 1,2,4-triazole nucleus possess significant antimicrobial activities (Sahoo *et al.*, 2010; El-Ashry *et al.*, 2009; Feng *et al.*, 2012). Recently, triazole-fused thiadiazole compounds have been frequently found to display a broad spectrum of biological activities (Badr and Barwa, 2011; Mathew *et al.*, 2007). A triazolo-thiadiazole system may be viewed as a cyclic analog of two important components—thiosemicarbazide and biguanide (Fan *et al.*, 2010; Gilani *et al.*, 2010), which often display diverse biological activities including anti-inflammatory (Karegoudar *et al.*, 2008), antifungal (Sangshetti *et al.*, 2011), analgesic (Tozkoparan *et al.*, 2009), anticancer (Sunil *et al.*, 2010), antimicrobial (Almajan *et al.*, 2010a, b), and anti-tumor (Ibrahim, 2009) activities. In continuation to our efforts directed toward the synthesis of heterocyclic compounds containing nitrogen, sulfur, and bicyclic systems with anticipated antibacterial activities (Almajan *et al.*, 2010a, b; Dawood *et al.*, 2005), in this paper we report the synthesis and antimicrobial activities of 2,6-bis(6-substituted-1,2,4-triazolo[3,4-*b*][1,3,4]thiadiazol-3-yl)pyridine derivatives.

H. Xiao · P. Li · D. Guo (✉) · Y. Chai · W. He
College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, People's Republic of China
e-mail: dcguo2001@hnu.edu.cn

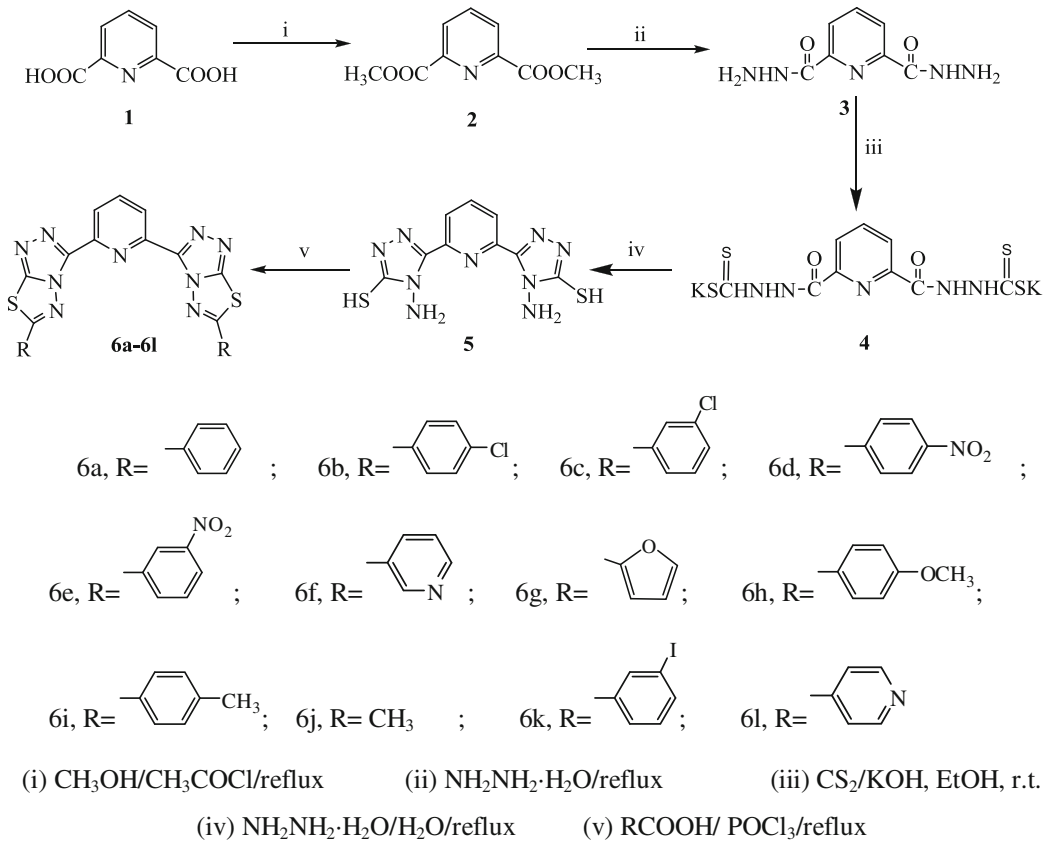
P. Li
Guangzhou Mechanical Engineering Research Institute Co., Ltd., Guangzhou 510700, People's Republic of China

J. Hu
Hunan Province People's Hospital, Changsha 410005, People's Republic of China

Results and discussion

Chemistry

The target compounds were synthesized as outlined in Scheme 1. The key intermediate compound 5,5′-(pyridine-2,6-diyl)bis(4-amino-3-mercapto-1,2,4-triazole) (**5**) was prepared starting from pyridine-2,6-dicarboxylic acid (**1**) by esterification with absolute methanol to give pyridine-2,6-dicarboxylic acid dimethyl ester (**2**) (Yang *et al.*, 2012), then the compound **2** was treated with hydrazine hydrate in absolute ethanol to yield the pyridine-2,6-dimethylhydrazine



Scheme 1 Synthetic pathway of the target compounds

(**3**) (Duke *et al.*, 2012), which was allowed to react with carbon disulfide in the presence of potassium hydroxide to afford the corresponding potassium salt *N,N′*-(pyridine-2,6-dicarbonyl)dithiocarbazate (**4**) (Said *et al.*, 2011), this potassium salt underwent ring closure with hydrazine hydrate to yield the intermediate compound **5** (Tan *et al.*, 2007). The target compounds (**6a–l**) were prepared from the reaction of the compound **5** with various carboxylic acid under reflux in phosphorus oxychloride. All the synthesized compounds are novel except the compounds **6a, 6f, 6i**, and **6l** which have been reported (Tan *et al.*, 2007).

The structures of all the newly synthesized compounds were characterized by elemental analysis, ^{1}H NMR and ^{13}C NMR spectral, mass spectral, and Infrared spectral studies. The elemental analysis data along with other physical properties of these compounds are reported in Table 1. All the newly synthesized compounds were analyzed satisfactorily for their carbon, hydrogen, nitrogen, and sulfur content.

Since the structures of all the target compounds **6a–l** are very similar, only the compound **6b** has been selected for illustration. The ^{1}H NMR spectrum of compound **6b** showed that the peaks of aromatic protons appeared at 7.27–7.85 ppm and the peak of pyridine protons appeared at 8.27–8.41 ppm, the peaks of SH and NH_2 protons disappeared. This confirmed that the 5,5′-(pyridine-2,6-diyl)bis(4-amino-3-mercapto-1,2,4-triazole) **5** underwent ring closure to get the compound **6b**. The ^{13}C NMR spectral analyses of compound **6b** showed a number of signals that are consistent with the number of carbons in the molecule. The ^{13}C NMR signals of triazolo-thiadiazole-C-8,8′, triazolo-thiadiazole-C-3,3′, and triazolo-thiadiazole-C-6,6′ in compound **6b** were observed at 166.11, 156.13, and 145.84 ppm, respectively. The other signals present in ^{13}C NMR spectra of compound **6b** were recorded at the expected chemical shifts.

The mass spectra of the target compounds showed that all the fragmentation ions peaks were consistent with their structures and could be clearly assigned. For the compound **6b**, the molecular weight 547 was observed consistent with the molecular formula $C_{23}H_{11}Cl_2N_9S_2$. The values for the remaining compounds have been presented under the experimental part. The IR spectrum of the compound **6b** showed that the absorption band at 1,236 cm^{-1} was assigned to the N–N=C group and the absorption band at 1,461 cm^{-1} was attributed to the C=C group, and a very strong absorption band at 1,594 cm^{-1} was assigned to the C=N group. The new absorption band appeared at 677 cm^{-1} which was attributed to the stretching frequency of C–S–C group of the thiadiazole ring.

Table 1 Physico-chemical properties of the synthesized compounds

Compd.	R	Molecular formula	Molecular weight (g/mol)	M.p. (°C)	Yield (%)	Elemental analysis calc. (found)			
						C	H	N	S
5	–	$C_9H_9N_9S_2$	307.36	268–270	40	35.17 (35.08)	2.95 (2.96)	41.01 (41.06)	20.86 (20.80)
6a		$C_{23}H_{13}N_9S_2$	479	269–271	53	57.61 (57.36)	2.73 (2.75)	26.29 (26.24)	13.37 (13.56)
6b		$C_{23}H_{11}Cl_2N_9S_2$	547	>300	45	50.37 (50.45)	2.02 (2.27)	22.99 (22.76)	11.69 (11.58)
6c		$C_{23}H_{11}Cl_2N_9S_2$	547	>300	48	50.37 (50.64)	2.02 2.23)	22.99 (22.76)	11.69 (11.63)
6d		$C_{23}H_{11}N_{11}O_4S_2$	569	>300	55	48.50 (48.70)	1.95 (1.91)	27.05 (26.91)	11.26 (11.21)
6e		$C_{23}H_{11}N_{11}O_4S_2$	569	>300	56	48.50 (48.80)	1.95 (1.90)	27.05 (27.06)	11.26 (11.21)
6f		$C_{21}H_{11}N_{11}S_2$	481	>300	42	52.38 (52.14)	2.30 (2.35)	32.00 (32.05)	13.32 (13.93)
6g		$C_{19}H_9N_9O_2S_2$	459	>300	40	49.67 (49.44)	1.97 (2.00)	27.44 (27.40)	13.96 (13.23)
6h		$C_{25}H_{17}N_9O_2S_2$	539	249–251	42	55.65 (55.43)	3.18 (3.24)	23.36 (23.40)	11.88 (11.58)
6i		$C_{25}H_{17}N_9S_2$	507	>300	61	59.16 (59.42)	3.38 (3.44)	24.83 (24.75)	12.63 (12.49)
6j	CH_3	$C_{13}H_9N_9S_2$	355	279–281	40	43.93 (43.76)	2.55 (2.60)	35.47 (35.19)	18.04 (18.17)
6k		$C_{23}H_{11}I_2N_9S_2$	731	>300	50	37.77 (37.80)	1.52 (1.52)	17.24 (19.65)	8.77 (8.89)
6l		$C_{21}H_{11}N_{11}S_2$	481	>300	48	52.38 (52.49)	2.30 (2.36)	32.00 (32.25)	13.32 (12.72)

Antibacterial activity

With gentamicin and ampicillin sodium as the reference drug molecules, the newly synthesized compounds **6a–l** were tested for in vitro antibacterial activity against two Gram-negative and three Gram-positive bacterial strains.

The diameter of growth inhibition zone of the preliminary antimicrobial testing of both the compounds **6a–l** (250 μg/tube) and the antibacterial antibiotic gentamicin and ampicillin sodium (100 μg/tube) are shown in Table 2. The minimum inhibitory concentration (MIC) of the compounds **6a–l**, the antibacterial antibiotic gentamicin and ampicillin sodium (Table 3) were in accordance with the results obtained in the primary screening. Moderate antibacterial acticity was observed for the tested compounds **6a–l** against *Staphylococcus aureus* in comparison to reference gentamicin and ampicillin, the diameter of the inhibition zone of the most active compound **6k** (MIC = 32 μg/mL) is 16 mm. The results indicated that the compounds **6b, 6h**, and **6k** have a certain antibacterial activity against *Bacillus*

Table 2 Antibacterial activity of the compounds **6a–l** as diameter of growth inhibition zone

Compd.	Diameter of growth inhibition zone (mm)				
	Gram-positive bacteria[a]			Gram-negative bacteria[b]	
	SA	BS	SP	EC	PA
6a	12	10	12	12	10
6b	15	14	16	17	15
6c	14	13	16	15	14
6d	13	11	15	15	12
6e	13	11	14	14	12
6f	12	11	13	14	13
6g	14	12	12	15	15
6h	14	14	14	16	12
6i	13	10	13	14	10
6j	12	10	12	14	12
6k	16	14	14	18	15
6l	12	10	13	14	13
Gentamicin	22	22	21	25	21
Ampicillin sodium	23	21	18	17	17

[a] *SA* (*S. aureus*); *BS* (*B. subtilis)*; *SP* (*S. pneumoniae*)

[b] *EC* (*E. coli*); PA (*P. aeruginosa*)

Table 3 Antibacterial activities of the compounds **6a–l** as MIC values (μg/mL)

Compd.	Minimum inhibitory concentrations (MIC, μg/mL)				
	Gram-positive bacteria			Gram-negative bacteria	
	SA	BS	SP	EC	PA
6a	128	–	128	128	–
6b	32	64	32	32	32
6c	64	64	32	64	64
6d	128	128	64	64	128
6e	128	128	64	64	128
6f	128	128	128	64	128
6g	64	128	128	64	32
6h	64	64	64	32	128
6i	128	–	128	64	–
6j	128	–	128	64	128
6k	32	64	64	32	64
6l	128	–	128	64	128
Gentamicin	2	2	2	0.5	1
Ampicillin sodium	1	0.5	2	2	2

(–) MIC values >128 μg/mL

subtilis and the rest of the compounds have low antibacterial activity. Most of the tested compounds have a good antibacterial activity against *Streptococcus pneumoniae,* the compounds **6b** (MIC = 32 μg/mL) and **6c** (MIC = 32 μg/mL) were found to be as effective as reference drug ampicillin. In addition, all the target compounds **6a–l** displayed potential antibacterial activity against *Escherichia coli,* the most active compounds are **6b** (MIC = 32 μg/mL) and **6k** (MIC = 32 μg/mL) with 17 and 18 mm diameters of the inhibition zone, respectively. It can be observed that the compounds **6b, 6g**, and **6k** exhibited a good antibacterial activity against *Pseudomonas aeruginosa.*

The relationship between the structures and biological activity was discussed. The antimicrobial activity results of the compounds **6a–l** revealed that the antimicrobial activity of aryl substituted derivatives was better than that of the aliphatic substituted derivatives, and the phenyl substituted derivatives were better than that of the heterocyclic substituted derivatives. The results indicated that the antibacterial activity of the derivatives with substituent in phenyl was higher than that of the derivatives without substituent in phenyl for the target compounds with the same sketch structure. For example, the antibacterial activity of the derivatives **6b–e, 6h, 6i**, and **6k** were better than that of the compound **6a**. The antibacterial activity of the para-substituent derivatives were better than that of the ortho- and meta-substituent derivatives. For example, the antibacterial activity of the compound **6b** was better than that of the compound **6c**. Otherwise, the antibacterial activity of the derivatives substituted by the donative electron group was better than that of the derivatives substituted by the electrophilic group, the antibacterial activity of the compound **6h** was better than that of the compound **6e**. The preliminary results revealed that the compounds **6b** and **6k** exhibited very promising activity against *E. coli* and *P. aeruginosa.*

Experimental

Materials and methods

The five bacteria used were provided by the Hunan University of Chinese Medicine (HNUCM). Melting points were determined with TECH XT-4 melting point apparatus and were uncorrected. Infrared spectra were obtained in solid state as KBr pallet on a PERKIN-ELMER Spectrum One. ^{1}H NMR spectra were registered on a Varian-400 NMR instrument (400 MHz) and ^{13}C NMR spectra were registered on a Bruker spectrometer at 400 MHz using DMSO-d_6 as solvent and the tetramethylsilane (TMS) as an internal standard, chemical shifts are reported as δ values in units of ppm. The mass spectra were recorded on LC–MS-Agilent 1,100 series. Elemental analyses were performed on VARIO EL (III) elemental analyzer.

Chemistry

*Synthesis of pyridine-2,6-dicarboxylic acid dimethylester (**2**)*

A mixture of pyridine-2,6-dicarboxylicacid (10.1 g, 60 mmol) and absolute methanol (80 mL) was heated to 30 °C with stirring in a 150-mL three-neck flask, then acetyl chloride (3.5 mL, 90 mmol) was added portionwise. The reaction mixture was refluxed for 20 h at 90 °C, cooled to the room temperature (RT), and then filtered. The filter cake was washed with absolute ethanol, and dried under vacuum to get the pyridine-2,6-dicarboxylicaciddimethylester. Yield 86 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 2.10 (s, 6H, $2CH_3$), 8.18 (t, 1H, $J = 7.8$ Hz, pyridine proton); 8.41 (d, 2H, $J = 7.8$ Hz, pyridine protons).

*Synthesis of pyridine-2,6-dimethylhydrazine (**3**)*

A mixture of pyridine-2,6-dicarboxylicaciddimethylester (9.75 g, 50 mmol) and 80 % hydrazine hydrate (20 mL) in 100 mL of ethanol was refluxed for 10 h at 80 °C with stirring in a 150-mL three-neck flask. After cooled to RT, the crude product was obtained. Which was filtered and washed thoroughly with 10 mL ethanol and 10 mL diethyl ether, then dried under vacuum to get a white needle crystal. Yield 90 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 2.12 (s, 4H, NH_2), 7.84 (s, 2H, NH), 8.23 (t, 1H, $J = 8.0$ Hz, pyridine proton); 8.31 (d, 2H, $J = 8.0$ Hz, pyridine protons); MS (ESI) *m/z* (%): 194 (M−1, 100), 195 (M, 5).

*Synthesis of potassium pyridine-2,6-dithiocarbazates (**4**)*

Pyridine-2,6-dimethylhydrazine (0.975 g, 5 mmol), potassium hydroxide (1.12 g, 20 mmol), and absolute ethanol (30 mL) were added to a 150-mL single-neck flask and then carbon disulfide (1.52 g, 20 mmol) was added dropwise. The reaction mixture was stirred for 14 h at RT, and then diluted with 30 mL of dry ether. The solid product was collected by filtration and washed with 2 × 10 mL ether, then dried at 65 °C under vacuum. The potassium salt was used for the next reaction directly without purification.

*Synthesis of 5,5′-(pyridine-2,6-diyl)bis(4-amino-3-mercapto-1,2,4-triazole) (**5**)*

A mixture of potassium salt (**4**) (1.30 g, 5 mmol) and 85 % hydrazine hydrate (2 mL, 35 mmol) was refluxed for 6 h at 110 °C, then diluted with 30 mL of cold water and acidified with hydrochloric acid (6 mol/L) to pH 6. The white precipitate was filtered and washed with 2 × 15 mL portions of cold water, then recrystallized from ethanol–water, and dried under vacuum. Yield 64 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 6.382 (s, 4H, NH_2); 8.124 (d, 2H, $J = 6.8$ Hz, pyridine protons); 8.169 (dd, 1H, $J_1 = 9.6$ Hz, $J_2 = 6.0$ Hz, pyridine proton); 10.950 (s, 2H, SH); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 165.65 ($C_{3,3'}$-triazole ring); 146.55 ($C_{2,6}$-pyridine); 145.14 ($C_{5,5'}$-triazole ring); 139.67 (C_4-pyridine); 124.50 ($C_{3,5}$-pyridine).

*General synthesis procedure of 2,6-bis(6-substituted-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine (**6a–l**)*

A mixture of 5,5′-(pyridine-2,6-diyl)bis(4-amino-3-mercapto-1,2,4-triazole) **5** (0.307 g, 1 mmol) and the substituted carboxylic acid (4 mmol) in 10 mL $POCl_3$ was refluxed for 6 h at 110 °C in a 50-mL single-neck flask. The reaction mixture was poured into 50 mL cold water with vigorous stirring, alkalized with sodium hydroxide (2 mol/L) to pH 10, and then filtered. The filter cake was washed thoroughly with cold water, and then recrystallized from dimethylformamide (DMF) to yield the target compounds **6a–l**.

*2,6-Bis(6-phenyl-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl) pyridine (**6a**)* A light brown solid, Yield 53 %, m.p. 269–271 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.361 (t, 4H, $J = 7.8$ Hz, aromatic protons); 7.506 (t, 2H, $J = 7.4$ Hz, aromatic protons); 7.938 (d, 4H, $J = 7.2$ Hz, aromatic protons); 8.306 (dd, 1H, $J_1 = 8.8$ Hz, $J_2 = 7.2$ Hz, pyridine proton); 8.436 (d, 2H, $J = 7.6$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 167.36 ($C_{8,8'}$-triazolo-thiadiazole ring); 155.85 ($C_{3,3'}$-triazolo-thiadiazole ring); 150.15 ($C_{2,6}$-pyridine); 145.91 ($C_{6,6'}$-triazolo-thiadiazole ring); 139.58 (C_4-pyridine); 133.23 ($C_{1,1'}$-aromatic ring); 129.82 ($C_{2,2',6,6'}$-aromatic ring); 129.34 ($C_{4,4'}$-aromatic ring); 127.71 ($C_{3,3',5,5'}$-aromatic ring); 123.62 ($C_{3,5}$-pyridine); IR (KBr) ν/cm^{-1}: 3081 (Aromatic C–H), 1591 (C=N), 1572 (Aromatic C–H), 1519 (Aromatic C–H), 1457 (C=C), 1365 (Aromatic C–H), 1237 (N–N=C), 686 (C–S–C); MS (EI) *m/z* (%): 481 (M+2, 16), 480 (M+1, 31), 479 (M, 100), 478 (M−1, 15), 406 (5), 304 (10), 187 (4), 173 (5), 161 (18), 129 (9), 121 (46), 103 (85), 77 (16), 76 (26), 51 (7).

*2,6-Bis(6-(4-chlorophenyl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine (**6b**)* A light brown solid, Yield 45 %, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.279 (d, 4H, $J = 8.0$ Hz, aromatic protons); 7.842 (d, 4H, $J = 8.4$ Hz, aromatic protons); 8.279 (t, 1H, $J = 8.0$ Hz, pyridine proton); 8.406 (d, 2H, $J = 8.0$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 166.11 ($C_{8,8'}$-triazolo-thiadiazole ring); 156.13 ($C_{3,3'}$-triazolo-thiadiazole ring); 145.96 ($C_{2,6}$-pyridine); 145.84

($C_{6,6'}$-triazolo-thiadiazole ring); 139.52 (C_4-pyridine); 137.84 ($C_{4,4'}$-aromatic ring); 129.56 ($C_{3,3',5,5'}$-aromatic ring); 129.29 ($C_{2,2',6,6'}$-aromatic ring); 128.10 ($C_{1,1'}$-aromatic ring); 123.27 ($C_{3,5}$-pyridine); IR (KBr) ν/cm^{-1}: 3087 (Aromatic C–H), 1594 (C=N), 1572 (Aromatic C–H), 1523 (Aromatic C–H), 1461 (C=C), 1364 (Aromatic C–H), 1236 (N–N=C), 677 (C–S–C); MS (EI) *m/z* (%): 551 (M+4, 9), 550 (M+3, 11), 549 (M+2, 35), 548 (M+1, 17), 547 (M, 46), 512 (11), 338 (4), 195 (11), 155 (38), 139 (39), 137 (100), 102 (39), 77 (6), 76 (12), 75 (16), 51 (7).

*2,6-Bis(6-(3-chlorophenyl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine (**6c**)* A gray white solid, Yield 48 %, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.328 (t, 2H, $J = 7.8$ Hz, aromatic protons); 7.461 (d, 2H, $J = 8.0$ Hz, aromatic protons); 7.830 (d, 2H, $J = 7.6$ Hz, aromatic protons); 7.872 (s, 2H, aromatic protons); 8.280 (q, 1H, $J = 5.2$ Hz, pyridine proton); 8.397 (d, 2H, $J = 8.0$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 165.74 ($C_{8,8'}$-triazolo-thiadiazole ring); 156.04 ($C_{3,3'}$-triazolo-thiadiazole ring); 146.04 ($C_{2,6}$-pyridine); 145.75 ($C_{6,6'}$-triazolo-thiadiazole ring); 139.67 (C_4-pyridine); 134.40 ($C_{3,3'}$-aromatic ring); 132.75 ($C_{1,1'}$-aromatic ring); 131.50 ($C_{5,5'}$-aromatic ring); 131.14 ($C_{6,6'}$-aromatic ring); 126.77 ($C_{4,4'}$-aromatic ring); 126.59 ($C_{2,2'}$-aromatic ring); 123.29 ($C_{3,5}$-pyridine); IR (KBr) ν/cm^{-1}: 3063 (Aromatic C–H), 1592 (C=N), 1572 (Aromatic C–H), 1517 (Aromatic C–H), 1454 (C=C), 1366 (Aromatic C–H), 1234 (N–N=C), 683 (C–S–C); MS (EI) *m/z* (%): 551 (M+4, 14), 550 (M+3, 16), 549 (M+2, 59), 548 (M+1, 26), 547 (M, 75), 512 (11), 338 (13), 195 (21), 155 (70), 139 (34), 137 (100), 102 (40), 77 (15), 76 (16), 75 (22), 51 (11).

*2,6-Bis(6-(4-nitrophenyl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine (**6d**)* A aurantiaceous solid, Yield 55 %, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.910 (d, 4H, $J = 8.0$ Hz, aromatic protons); 8.085 (d, 4H, $J = 8.4$ Hz, aromatic protons); 8.346 (t, 1H, $J = 8.8$ Hz, pyridine proton); 8.463 (d, 2H, $J = 7.2$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 166.66 ($C_{8,8'}$-triazolo-thiadiazole ring); 154.68 ($C_{3,3'}$-triazolo-thiadiazole ring); 149.42 ($C_{6,6'}$-triazolo-thiadiazole ring); 146.38 ($C_{2,6}$-pyridine); 141.17 ($C_{4,4'}$-aromatic ring); 139.76 (C_4-pyridine); 138.27 ($C_{1,1'}$-aromatic ring); 128.93 ($C_{2,2',6,6'}$-aromatic ring); 124.27 ($C_{3,3',5,5'}$-aromatic ring); 119.95 ($C_{3,5}$-pyridine); IR (KBr) ν/cm^{-1}: 3060 (Aromatic C–H), 1605 (C=N), 1576 (Aromatic C–H), 1528 (Aromatic C–H), 1459 (C=C), 1348 (Aromatic C–H), 1263 (N–N=C), 688 (C–S–C); MS (EI) *m/z* (%): 571 (M+2, 8), 570 (M+1, 14), 569 (M, 43), 207 (14), 206 (8), 166 (15), 148 (50), 137 (19), 129 (16), 118 (36), 102 (100), 90 (28), 77 (17), 76 (31), 75 (46), 64 (33), 51 (28).

*2,6-Bis(6-(3-nitrophenyl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine (**6e**)* A light yellow solid, Yield 56 %, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.550 (t, 2H, $J = 8.0$ Hz, aromatic protons); 8.074 (d, 2H, $J = 8.0$ Hz, aromatic protons); 8.224 (d, 2H, $J = 8.0$ Hz, aromatic protons); 8.293 (dd, 1H, $J_1 = 8.4$ Hz, $J_2 = 7.2$ Hz, pyridine proton); 8.417 (d, 2H, $J = 8.4$ Hz, pyridine protons); 8.443 (s, 2H, aromatic protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 165.15 ($C_{8,8'}$-triazolo-thiadiazole ring); 156.16 ($C_{3,3'}$-triazolo-thiadiazole ring); 147.46 ($C_{3,3'}$-aromatic ring); 146.08 ($C_{2,6}$-pyridine); 145.67 ($C_{6,6'}$-triazolo-thiadiazole ring); 139.62 (C_4-pyridine); 133.90 ($C_{6,6'}$-aromatic ring); 131.52 ($C_{1,1'}$-aromatic ring); 130.68 ($C_{5,5'}$-aromatic ring); 126.91 ($C_{4,4'}$-aromatic ring); 123.16 ($C_{2,2'}$-aromatic ring); 121.16 ($C_{3,5}$-pyridine); IR (KBr) ν/cm^{-1}: 3083 (Aromatic C–H), 1592 (C=N), 1574 (Aromatic C–H), 1533 (Aromatic C–H), 1457 (C=C), 1345 (Aromatic C–H), 1242 (N–N=C), 674 (C–S–C); MS (EI) *m/z* (%): 571 (M+2, 15), 570 (M+1, 34), 569 (M, 100), 207 (6), 206 (15), 166 (34), 148 (20), 137 (11), 129 (12), 120 (30), 102 (45), 90 (8), 77 (7), 76 (16), 75 (18), 64 (9), 51 (10).

*2,6-Bis(6-(pyridin-3-yl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine (**6f**)* A dark brown solid, Yield 42 %, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.336 (dd, 2H, $J_1 = 8.0$ Hz, $J_2 = 5.2$ Hz, pyridine protons), 7.943 (s, 2H, pyridine protons), 8.281 (d, 2H, $J = 7.6$ Hz, pyridine protons), 8.323 (t, 1H, $J = 8.0$ Hz, pyridine proton), 8.472 (d, 2H, $J = 7.6$ Hz, pyridine protons), 8.613 (d, 2H, $J = 4.4$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 164.88 ($C_{8,8'}$-triazolo-thiadiazole ring); 156.07 ($C_{3,3'}$-triazolo-thiadiazole ring); 152.72 ($C_{6,6'}$-triazolo-thiadiazole ring); 149.10 ($C_{2,2'}$ of 6-substituted pyridine); 148.09 ($C_{6,6'}$ of 6-substituted pyridine); 146.00 ($C_{2,6}$-pyridine); 145.83 ($C_{4,4'}$ of 6-substituted pyridine); 139.69 (C_4-pyridine); 135.23 ($C_{3,3'}$ of 6-substituted pyridine); 125.87 ($C_{5,5'}$ of 6-substituted pyridine); 124.51 ($C_{3,5}$-pyridine); IR (KBr) ν/cm^{-1}: 3083 (Aromatic C–H), 1590 (C=N), 1573 (Aromatic C–H), 1519 (Aromatic C–H), 1457 (C=C), 1360 (Aromatic C–H), 1260 (N–N=C), 670 (C–S–C); MS (EI) *m/z* (%): 483 (M+2, 6), 482 (M+1, 13), 481 (M, 56), 305 (15), 207 (26), 173 (7), 162 (4), 129 (14), 122 (41), 104 (100), 103 (22), 77 (60), 64 (6), 51 (17).

*2,6-Bis(6-(furan-2-yl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine (**6g**)* A brown solid, Yield 40 %, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 6.674 (dd, 2H, $J_1 = 3.6$ Hz, $J_2 = 2$ Hz, furan protons); 7.428 (d, 2H, $J = 2$ Hz, furan protons), 8.068 (d, 2H, $J_2 = 2$ Hz, furan protons); 8.338 (q, 1H, $J = 7.4$ Hz, pyridine

proton); 8.397 (d, 2H, $J = 7.2$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 166.44 ($C_{8,8'}$-triazolo-thiadiazole ring); 161.64 ($C_{6,6'}$-triazolo-thiadiazole ring); 153.22 ($C_{3,3'}$-triazolo-thiadiazole ring); 148.14 ($C_{2,6}$-pyridine); 145.91 ($C_{5,5'}$-furan); 145.84 ($C_{2,2'}$-furan); 143.41 (C_4-pyridine); 123.70 ($C_{3,5}$-pyridine); 115.56 ($C_{3,3'}$-furan); 113.77 ($C_{4,4'}$-furan); IR (KBr) ν/cm^{-1}: 3114 (Aromatic C–H), 1592 (C=N), 1572 (Aromatic C–H), 1505 (Aromatic C–H),1453 (C=C), 1360 (Aromatic C–H), 1269 (N–N=C), 682 (C–S–C); MS (EI) *m/z* (%): 461 (M+2, 14), 460 (M+1, 28), 459 (M, 100), 405 (6), 294 (15), 173 (4), 151 (17), 129 (12), 111 (37), 93 (36), 76 (4), 64 (15).

2,6-Bis(6-(4-methoxyphenyl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine ***(6h)*** A dark brown solid, Yield 42 %, m.p. 249–251 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 3.773 (s, 6H, OCH_3); 6.839 (d, 4H, $J = 8.4$ Hz, aromatic protons); 7.836 (d, 4H, $J = 8.4$ Hz, aromatic protons); 8.282 (t, 1H, $J = 8.0$ Hz, pyridine proton), 8.406 (d, 2H, $J =$ 7.2 Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 166.92 ($C_{8,8'}$-triazolo-thiadiazole ring); 163.14 ($C_{4,4'}$-aromatic ring); 155.77 ($C_{3,3'}$-triazolo-thiadiazole ring); 145.95 ($C_{2,6}$-pyridine); 139.44 (C_4-pyridine); 129.52 ($C_{6,6'}$-triazolo-thiadiazole ring); 128.89 ($C_{2,2',6,6'}$-aromatic ring); 123.39 ($C_{1,1'}$-aromatic ring); 121.66 ($C_{3,5}$-pyridine); 115.08 ($C_{3,3',5,5'}$-aromatic ring); 56.00 (–OCH_3); IR (KBr) ν/cm^{-1}: 3083 (Aromatic C–H), 1606 (C=N), 1574 (Aromatic C–H), 1527 (Aromatic C–H), 1457 (C=C), 1364 (Aromatic C–H), 1263 (N–N=C), 683 (C–S–C); MS (EI) *m/z* (%): 541 (M+2, 15), 540 (M+1, 38), 539 (M, 100), 538 (M−1, 34), 436 (5), 334 (12), 191 (11), 165 (7), 151 (56), 133 (49), 103 (24), 90 (18), 76 (7).

2,6-Bis(6-(4-methylphenyl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine ***(6i)*** A yellow solid, Yield 61 %, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 2.256 (s, 6H, CH_3); 7.052 (d, 4H, $J = 8.0$ Hz, aromatic protons); 7.714 (d, 4H, $J = 8.0$ Hz, aromatic protons); 8.220 (t, 1H, $J = 7.6$ Hz, pyridine proton); 8.344 (d, 2H, $J = 8.0$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 167.22 ($C_{8,8'}$-triazolo-thiadiazole ring); 155.72 ($C_{3,3'}$-triazolo-thiadiazole ring); 149.43 ($C_{2,6}$-pyridine); 145.84 ($C_{6,6'}$-triazolo-thiadiazole ring); 143.44 ($C_{4,4'}$-aromatic ring); 139.39 (C_4-pyridine); 130.11 ($C_{3,3',5,5'}$-aromatic ring); 127.54 ($C_{2,2',6,6'}$-aromatic ring); 126.53 ($C_{1,1'}$-aromatic ring); 123.37 ($C_{3,5}$-pyridine); 21.46 (–CH_3); IR (KBr) ν/cm^{-1}: 3050 (Aromatic C–H), 1592 (C=N), 1575 (Aromatic C–H), 1523 (Aromatic C–H), 1456 (C=C), 1383 (Aromatic C–H), 1237 (N–N=C), 685 (C–S–C); MS (EI) *m/z* (%): 509 (M+2, 17), 508 (M+1, 33), 507 (M, 100), 434 (4), 318 (10), 175 (11), 151 (6), 135 (47), 117 (34), 116 (25), 103 (18), 90 (16), 77 (6).

2,6-Bis(6-methyl-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl) pyridine ***(6j)*** A light brown solid, Yield 40 %, m.p. 279–281 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 2.819 (s, 6H, CH_3); 8.261 (q, 1H, $J = 7.4$ Hz, pyridine proton); 8.331 (d, 2H, $J = 6.8$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 167.24 ($C_{8,8'}$-triazolo-thiadiazole ring); 156.76 ($C_{3,3'}$-triazolo-thiadiazole ring); 145.95 ($C_{2,6}$-pyridine); 145.37 ($C_{6,6'}$-triazolo-thiadiazole ring); 139.35 (C_4-pyridine); 123.48 ($C_{3,5}$-pyridine); 18.54 (-CH_3); IR (KBr) ν/cm^{-1}: 3040 (Aromatic C–H), 1596 (C=N), 1575 (Aromatic C–H), 1537 (Aromatic C–H), 1460 (C=C), 1389 (Aromatic C–H), 1242 (N–N=C), 682 (C–S–C); MS (EI) *m/z* (%): 357 (M+2, 11), 356 (M+1, 20), 355 (M, 98), 244 (14), 243 (100), 242 (18), 187 (9), 173 (18), 147 (4), 130 (12), 129 (25), 103 (32), 99 (2), 76 (11), 59 (32).

2,6-Bis(6-(3-iodophenyl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine ***(6k)*** A yellow solid, Yield 50 %, m. p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.108 (t, 2H, $J = 7.8$ Hz, aromatic protons); 7.751 (d, 2H, $J =$ 8.4 Hz, aromatic protons); 7.888 (d, 2H, $J = 7.6$ Hz, aromatic protons); 8.153 (s, 2H, aromatic protons); 8.302 (q, 1H, $J = 5.2$ Hz, pyridine proton); 8.408 (d, 2H, $J = 8.0$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 165.63 ($C_{8,8'}$-triazolo-thiadiazole ring); 156.00 ($C_{3,3'}$-triazolo-thiadiazole ring); 146.05 ($C_{2,6}$-pyridine); 145.75 ($C_{6,6'}$-triazolo-thiadiazole ring); 141.47 ($C_{2,2'}$-aromatic ring); 139.64 (C_4-pyridine); 135.16 ($C_{1,1'}$-aromatic ring); 131.36 ($C_{4,4'}$-aromatic ring); 131.12 ($C_{5,5'}$-aromatic ring); 127.43 ($C_{6,6'}$-aromatic ring); 123.21 ($C_{3,5}$-pyridine); 95.87 ($C_{3,3'}$-aromatic ring); IR (KBr) ν/cm^{-1}: 3040 (Aromatic C–H), 1596 (C=N), 1573 (Aromatic C–H), 1513 (Aromatic C–H), 1452 (C=C), 1364 (Aromatic C–H), 1239 (N–N=C), 681 (C–S–C); MS (EI) *m/z* (%): 754 (M + 23, 42), 755 (10), 1484 (2M+23, 100), 1485 (59), 1486 (41), 1487 (15).

2,6-Bis(6-(pyridin-4-yl)-1,2,4-triazolo[3,4-b][1,3,4]thiadiazol-3-yl)pyridine ***(6l)*** A dark brown solid, Yield 48 %, m.p. >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm: 7.841 (d, 4H, $J = 6.0$ Hz, pyridine protons), 8.327 (t, 1H, $J = 8.0$ Hz, pyridine protons), 8.462 (d, 2H, $J =$ 8.0 Hz, pyridine protons), 8.518 (d, 4H, $J = 5.6$ Hz, pyridine protons); ^{13}C NMR (400 MHz, DMSO-d_6) δ/ppm: 165.46 ($C_{8,8'}$-triazolo-thiadiazole ring); 157.13 ($C_{3,3'}$-triazolo-thiadiazole ring); 151.10 ($C_{2,2',6,6'}$ of 6-substituted pyridine); 148.24 ($C_{2,6}$-pyridine); 145.80 ($C_{6,6'}$-triazolo-thiadiazole ring); 140.65 (C_4-pyridine); 136.41 ($C_{4,4'}$ of 6-substituted pyridine); 123.52 ($C_{3,5}$-pyridine); 121.18 ($C_{3,3',5,5'}$ of 6-substituted pyridine); IR (KBr) ν/cm^{-1}: 3030 (Aromatic C–H), 1596 (C=N), 1573 (Aromatic C–H), 1512 (Aromatic C–H), 1457 (C=C), 1369 (Aromatic C–H), 1243 (N–N=C), 685 (C–S–C); MS (EI) *m/z* (%): 483 (M+2, 10),

482 (M+1, 23), 481 (M, 78), 305 (6), 207 (23), 173 (9), 162 (8), 129 (18), 122 (47), 104 (100), 103 (29), 77 (64), 64 (26), 51 (20).

Antibacterial activity

The newly synthesized compounds were assayed in vitro for antibacterial activity against the Gram-positive bacteria (*S. aureus; B. subtilis; S. pneumoniae*) and Gram-negative bacteria (*E. coli; P. aeruginosa*) by using the Oxford cup method (cylinder-plate method) (Plein and Plein, 1957; Mohammadzadeh *et al.*, 2012). Gentamicin and ampicillin sodium were used as the control drugs to compare the antibacterial activity. The target compounds were dissolved in dimethylsulfoxide (DMSO) of specific concentration (250 μg/tube), the antibacterial antibiotics Gentamicin and Ampicillin Sodium (100 μg/tube) were carefully placed on the agarose gel culture plates that have been previously inoculated separately with the microorganisms, and incubated at 36 °C for 24 h. The diameter of the growth inhibition zone was measured. In order to ensure that the solvent had no effect on bacterial growth, a control test was carried out with only DMSO on the agarose gel culture plates, the test results showed that the solvent had no antibacterial activity against any of the test microorganisms.

The MIC was determined by using the serial dilutions method. The synthesized compounds **6a–l**, gentamicin, and ampicillin sodium were dissolved in DMSO at concentration of 128 μg/mL. Nine different dilutions of the test compounds between 128 and 0.5 μg/mL were prepared in plates by serial dilutions from top to bottom. Then the microorganisms suspensions at 10^6 CFU/mL (colony forming unit/mL) concentrations were inoculated to the corresponding wells. The MIC was defined as the lowest concentration of the test compounds that inhibited visible growth of microorganisms on the plate.

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