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Original scientific paper

Antimicrobial and anticancer evaluation of a novel synthetic tetracyclic system obtained by Dimroth rearrangement

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Abstract: A series of pyrido[3′,2′:4,5]thieno[2,3-*e*][1,2,4]triazolo[4,3-*c*]pyrimidines were prepared *via* oxidative cyclization of 4-(2-arylidenehydrazinyl)pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidines. Dimroth rearrangement of such a series yielded pyrido[3′,2′:4,5]thieno[2,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidines. A reaction mechanism was proposed and the products were screened for their antimicrobial and anticancer activities. From the antimicrobial screening results, it could be seen that compounds **8c, 9f** and **10c** showed excellent activity against Gram-positive bacteria while compounds **10d** and **8c** showed the highest activity against Gram-negative bacteria. The results of the anticancer activities showed that compound **9c** was the most active against HepG-2 and MCF-7 with IC_{50} values of 1.19 and 3.46 μg/mL, respectively.

Keywords: hydrazones; oxidative cyclizations; thienopyridines; reaction mechanism.

INTRODUCTION

The synthesis of fused triazolopyrimidine moieties has been described by many investigators and it was shown that these have pronounced biological activities.[1–6] Previous observations revealed that [1,2,4]triazolo[4,3-*c*]pyrimidine derivatives can isomerize under different suitable reaction conditions to the thermodynamically more stable [1,2,4]triazolo[1,5-*c*]pyrimidines.[7–9] This isomerization was first reported by Miller and Rose[10,11] when they treated [1,2,4]triazolo[4,3-*c*]pyrimidine derivatives with an acid, base, or thermally.

In continuation of an ongoing search for new bioactive heterocycles[12–20] based on the above-mentioned research results, the goal of this study was to synthesize some novel pyrido[3′,2′:4,5]thieno[2,3-*e*][1,2,4]triazolo[4,3-*c*]pyramidines not only to study their isomerization to pyrido[3′,2′:4,5]thieno[2,3-*e*][1,2,4]-

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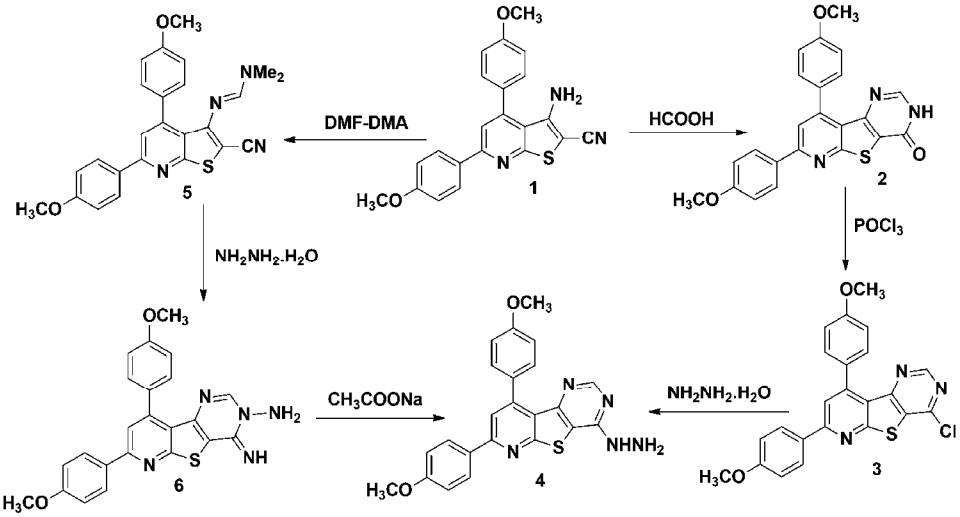
triazolo[1,5-*c*]pyrimidines, but also to obtain new compounds which were expected to find notable pharmacological applications.

RESULTS AND DISCUSSION

Chemistry

Detailed analytical and spectral data of the synthesized compounds are given in the Supplementary material to this paper.

Synthesis of compound **4** was achieved through two synthetic pathways. The first pathway involved the conversion of **1**[21] to 7,9-bis(4-methoxyphenyl)-pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**2**) through its cyclization with formic acid (Scheme 1). The IR spectra of **2** displayed no cyano group absorptions.



Scheme 1. Synthesis of the starting materials **4** and **6**.

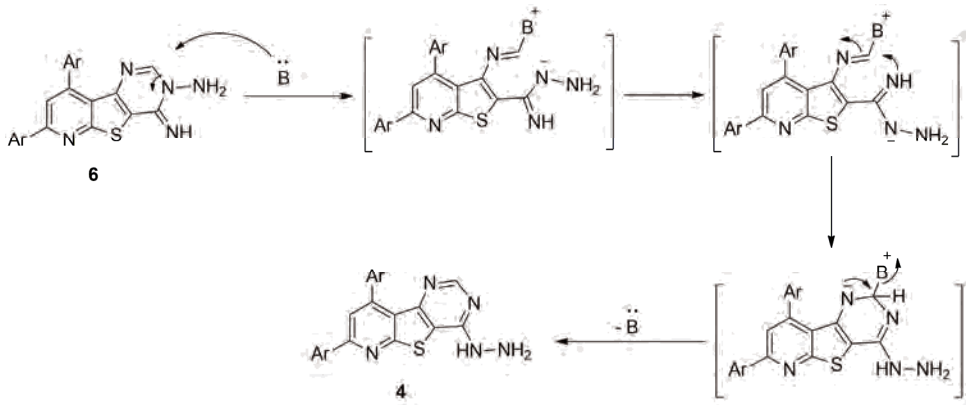
Chlorination of **2** with phosphorus oxychloride under reflux afforded **3**. Nucleophilic displacement of the 4-chloro group in **3** was achieved by heating under reflux with hydrazine hydrate to give compound **4**.[22] The IR spectrum of the titled compound **4** showed the appearance of absorption bands at 3424–3291 cm^{-1} for NH and NH_2), its ^{1}H-NMR spectrum showed the presence of characteristic peaks at δ 5.01 and 9.02 ppm of NH_2 and NH groups, respectively, while the mass spectrum showed a molecular ion peak at *m*/*z* 429 corresponding to its molecular formula $C_{23}H_{19}N_5O_2S$. Detailed analytical and spectral data are given in the Supplementary material to this paper. These results confirmed the chemical structure of compound **4** (Scheme 1).

The second pathway was the reaction of **1** with DMF–DMA under reflux for 4 h to give *N′*-(2-cyano-4,6-bis(4-methoxyphenyl)thieno[2,3-*b*]pyridin-3-yl)--*N*,*N*-dimethylformimidamide (**5**).

IR spectrum of **5** showed an absorption band at 2191 cm^{-1} assigned to the cyano group (CN). Furthermore, its ^{1}H-NMR spectrum showed two singlet signals at 2.64 and 2.87 ppm, which were indicative of the $N(CH_3)_2$ group in this structure. The mass spectrum of **5** showed the molecular ion peak at *m*/*z* 442 corresponding to its molecular formula $C_{25}H_{22}N_4O_2S$.

Reaction of the latter compound with hydrazine hydrate (99 %) in ethanol afforded **6**. The formation of compound **6** was assumed to proceed *via* the loss of a dimethylamine from **5** followed by intramolecular cyclization into **6.** The IR spectrum of **6** revealed the absence of the cyano group and the appearance of absorption bands at 3472, 3341 and 3220 cm^{-1} for NH and NH_2. Its ^{1}H-NMR spectrum showed three singlets at δ 4.92, 9.02 and 8.44 ppm which indicated the presence of NH_2, NH and the pyrimidine proton, respectively.

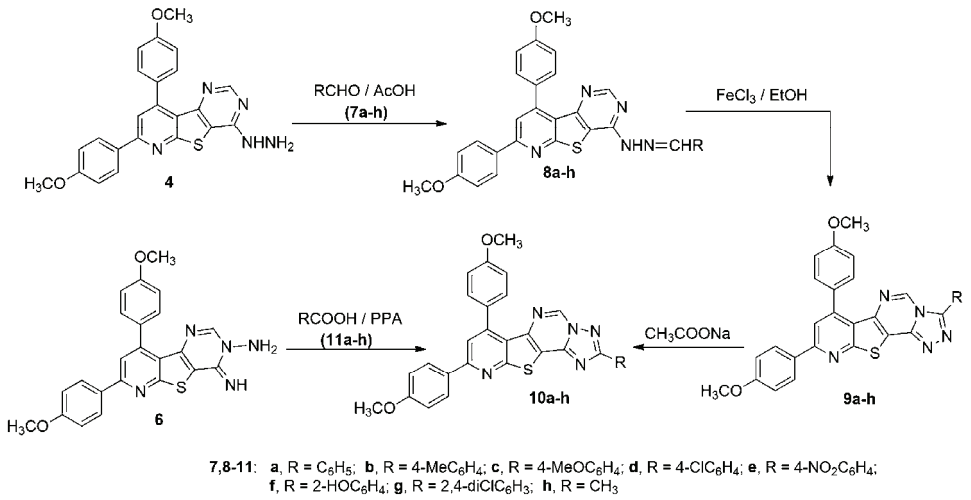
Isomerization of **6** to its corresponding more thermodynamically stable hydrazine compound **4** was realized by refluxing **6** in dioxane in the presence of sodium acetate through a Dimroth-type rearrangement, which involves a sequence of ring opening and ring closure reactions under basic conditions, as shown in Scheme 2.[23,24]



Scheme 2. Mechanism of the rearrangement of compound **6** to **4**.

The identity of compounds **4** and **6** was proven based on their melting points (m.p.), thin layer chromatography (TLC) and spectral data.

Condensation of equimolar quantities of 4-hydrazinylpyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidine (**4**) with the aldehydes **7a**–**h** gave the corresponding 4-(2--benzylidenehydrazinyl)-pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidines **8a**–**h** (Scheme 3). The structures of the products **8a**–**h** were confirmed based on their elemental analysis and spectral data (see the Supplementary material to this paper).



Scheme 3. Synthesis of fused [1,2,4]triazolo[1,5-*c*]pyrimidine derivatives **10a**–**h**.

The IR spectrum of **8a**, taken as a typical example of the prepared series, revealed an absorption band at 3440 cm^{-1} due to the NH group. Their ^{1}H-NMR spectra showed the presence of hydrazone (–CH=N–NH–)protons as two singlets at δ 8.82 and 12.16 ppm, respectively (see Supplementary material).

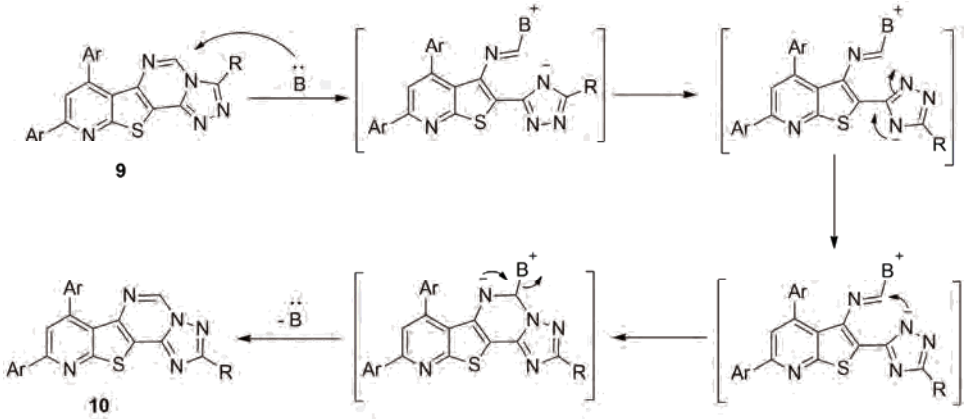
Next, the oxidative cyclization of the hydrazone derivatives **8a**–**h** with iron (III) chloride in ethanol yielded the respective triazolo[4,3-*c*]pyrimidine derivatives **9a**–**h** (Scheme 3).

TLC analysis of the crude products isolated from the foregoing dehydrogenative cyclization method indicated that only one product was formed in each ease. The mass spectra of the isolated products showed high intensity molecular ion peaks at the expected *m*/*z* values that were less by two than those of the corresponding hydrazone **8**. Their IR spectra showed the disappearance of the NH group. Their ^{1}H-NMR spectra lacked the characteristic signals for the azomethine (–CH=N–) and the hydrazone (–C=N–NH–) protons present in the spectra of **8**.

The conversion of **8** into **9** is reminiscent of other related oxidative cyclization of aldehyde *N*-heteroarylhydrazones with bromine in acetic acid in the presence of sodium acetate or iron(III) chloride, which have been reported to proceed *via* the generation of the respective nitrilimines, which undergo *in situ* 1,5-electrocyclization to give the respective fused heterocycles.[25–27]

When each of the triazolo[4,3-*c*]pyrimidine derivatives **9a**–**h** was heated in ethanol in the presence of sodium acetate, they isomerized to the thermodynamically more stable triazolo[1,5-*c*]pyrimidine derivatives **10a**–**h** through tandem ring opening and ring closure reactions, as shown in Scheme 4. This rearrangement is consistent with those reported in some earlier reports.[8,28] The structure

elucidation of all compounds **10a**–**h**, which have not been reported, hitherto, was based on their spectral and analytical data (see the Supplementary material). In the mass spectra, all compounds gave the molecular ions at the expected *m*/*z* values, which in most cases are not the base peaks. The ^{1}H-NMR spectra were also consistent with their proposed structures.



Scheme 4. Mechanism of the rearrangement of compounds **9a**–**h** to **10a**–**h**.

To provide decisive evidence for this rearrangement, the products **10a**–**h** were compared with authentic samples prepared by an alternative synthesis. Thus, treatment of **6** with the respective aromatic carboxylic acids **11a**–**h** in presence of polyphosphoric acid gave products **10a**–**h**, which proved identical in all respects (m.p., mixed m.p., IR and ^{1}H-NMR spectra) with those obtained above from base-catalyzed rearrangement of **9a**–**h** (Scheme 3). This finding confirmed the base-catalyzed rearrangement of **9** into **10** (Scheme 3). The driving force for the observed rearrangement is the fact that the [1,2,4]triazolo[1,5-*c*]pyrimidine ring system is thermodynamically more stable than its isomer, namely [1,2,4]-triazolo[4,3-*c*]pyrimidine.[29]

Antimicrobial evaluation

The synthesized compounds were evaluated for their *in vitro* antimicrobial activity at 5 mg mL^{-1} using the agar well diffusion method against a representative panel of pathogenic strains, *i.e.*, *Staphylococcus aureus* and *Bacillis subtilis* as examples of Gram-positive bacteria as well as *Pseudomonas aeruginosa* and *Escherichia coli* as examples of Gram-negative bacteria while *Aspergillus fumigatus* and *Candida albicans* were used as the fungal strains. DMSO was used for dissolving the tested compounds and showed no inhibition zones, confirming that it has no influence on growth of the tested microorganisms. The results of the testing for antibacterial and antifungal effects summarized in Table I showed that

the new derivatives tested displayed variable *in vitro* antibacterial and antifungal actions. In general, the chemical structure of the whole molecule, comprising the nature of the heterocyclic system as well as the type of the substituted function present in the heterocyclic ring structure, has a pronounced effect on the antimicrobial activity. Most of the substituted analogues produced high inhibitory effects against bacteria, which were comparable to the effects of reference drugs.

TABLE I. *In vitro* antibacterial and antifungal activity of the compounds tested by the well-diffusion agar assay expressed as the diameter (mm) of the inhibition zone in the form of mean ± *SD*

Tested compound	Gram-positive bacteria		Gram-negative bacteria		Fungi	
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. fumigatus*	*C. albicans*
8a	6.7±0.4	7.8±0.5	11.8±0.9	9.4±0.7	0	0
8b	6.1±0.3	6.4±0.4	9.2±0.3	7.3±0.6	0	0
8c	22.4±0.7	20.1±0.9	21.9±0.8	18.2±0.7	0	6.9±0.3
8f	6.5±0.4	6.8±0.6	7.1±0.4	6.9±0.5	22.1±0.8	13.3±0.7
9e	6.3±0.3	6.6±0.4	6.2±0.4	6.7±0.6	0	0
9f	20.9±0.6	21.4±0.8	16.9±0.9	12.1±0.7	19.3±0.4	7.4±0.3
9h	17.3±0.7	15.9±0.5	13.7±0.6	8.4±0.5	16.8±0.6	6.7±0.4
10c	19.8±0.7	13.8±0.8	7.2±0.5	6.5±0.4	19.7±0.8	10.6±0.6
10d	14.3±0.6	17.0±0.6	24.8±0.7	19.5±0.8	11.2±0.6	0
10f	18.9±0.5	14.1±0.6	6.7±0.4	6.6±0.5	10.4±0.7	0
10g	0	0	15.4±0.7	13.6±0.7	0	0
Penicillin G	26.4±0.7	24.6±0.6	–	–	–	–
Streptomycin	–	–	26.7±0.9	20.4±0.5	–	–
Amphotericin B	–	–	–	–	25.9±0.8	20.1±0.7

From the screening results, it could be seen that compounds **8c**, **9f** and **10c** showed excellent activity against the Gram positive bacteria and compounds **10f**, **9h** and **10d** showed moderate effects. The highest activity against Gram negative bacteria were exerted by **10d** and **8c**. Selective antimicrobial activity was observed under these screening conditions for compound **10g** against the tested Gram-negative bacteria. Compounds **4**, **6**, **8d**, **8e**, **8g**, **8h**, **9a**–**d**, **9g**, **10a**, **10b**, **10e** and **10h** were completely inactive under these screening conditions. The rest of the compounds showed non-significant activity against the tested bacteria compared with the standard drugs.

Interestingly, compounds **8f, 10c** and **9h** showed promising inhibitory activity against *A. fumigatus* and *C. albicans* compared with amphotericin B as a reference drug.

The mean values of the inhibition zone diameter obtained for these compounds suggested that some of the synthesized compounds possess significant antimicrobial activity against the tested organisms used in these assays, and therefore, the minimum inhibitory concentration (*MIC*) of the active compounds were evaluated *in vitro* using the microdilution technique, while the lowest con-

centration showing no growth was taken as the *MIC*. The fungicides amphotericin B and griseofulvin as well as the bactericides penicillin G and streptomycin were used as reference drugs to evaluate the potency of the tested compounds under the same conditions. The results of the *MIC* determinations reported in Table II showed that compounds **8f**, **9f**, **9h**, **10c**, **10d** and **10f** exhibited broad spectrum action against both Gram-positive and Gram-negative bacteria as well as fungi. Compound **8c** had broad-spectrum antibacterial activity. Compound **9f** reached the highest potency with *MIC* values of 15.6 and 31.25 μg mL^{-1} against the Gram-positive bacteria *B. subtilis* and *S. aureus*, respectively. The highest activity (31.25 μg mL^{-1}) against *E. coli* was detected for compound **10d**. Moreover, significant *MIC* values were determined for compounds **8f** and **9f** against *A. fumigatus* compared with the reference drugs.

TABLE II. Antimicrobial activity expressed as minimum inhibitory concentration (*MIC* / μg mL^{-1}) of the synthesized bioactive compounds compared with standard drugs

Tested compound	Microorganism					
	Gram-positive bacteria		Gram-negative bacteria		Fungi	
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. fumigatus*	*C. albicans*
8c	62.5	62.5	62.5	125	–	–
8f	500	500	500	500	31.25	250
9f	15.63	31.25	250	125	31.25	500
9h	125	125	125	250	125	500
10c	62.5	250	250	250	62.5	500
10d	125	125	31.25	125	250	–
10f	125	250	500	500	250	–
Penicillin G	0.03	0.06	–	–	–	–
Streptomycin	–	–	0.03	3.9	–	–
Amphotericin B	–	–	–	–	0.3	2.9
Griseofulvin	–	–	–	–	100	250

Cytotoxic activity

The *in vitro* growth inhibitory activity of the synthesized compounds was investigated in comparison with the well-known standard anticancer drug doxorubicin using the crystal violet colorimetric viability assay. For comparison purposes, the cytotoxicity of imatinib (2-substituted aminopyrimidine derivative; Gleevec®), a standard antitumor drug used for the treatment of gastrointestinal tract tumors, was also evaluated under the same conditions. Data generated were used to plot a dose response curve of which the concentration of test compounds required to kill 50 % of the cell population (IC_{50}) was determined. The cytotoxic activity was expressed as the mean IC_{50} of three independent experiments (Table III) and the results revealed that all the tested compounds showed inhibitory activity to the tumor cell lines in a concentration dependent manner.

The results presented in Table III and Figs. S-1–S-3 of the Supplementary material showed that compounds **9c**, **8h, 9f**, **8g** and **10h** had significant anti-cancer activity against the two tumor cell lines MCF-7 and HepG-2, compared with reference drug imatinib. Interestingly, compound **9c** was the most active against HepG-2 and MCF-7 with IC_{50} values of 1.19 and 3.46 μg mL^{-1}, respectively, comparable to doxorubicin. However, compounds **8a**–**f**, **6**, **9a**, **9e**, **9g**, **9h** and **10b**–**g** were less active than imatinib. Moreover, compound **10a** was almost inactive under the employed screening conditions.

TABLE III. The *in vitro* inhibitory activity of tested compounds against tumor cell lines expressed as IC_{50} values (μg mL^{-1}) ± standard deviation from six replicates

Tested compound	Tumor cell line	
	MCF-7	HepG2
4	15	20
6	> 50	35.5
8a	32.6	40.5
8b	37.7	27.9
8c	34.3	31.1
8d	44.9	49.7
8e	22.9	21.7
8f	38.2	43.4
8g	19.7	10.7
8h	8.67±0.31	2.94±0.12
9a	39.7	40.6
9b	20.1	29.4
9c	3.46±0.24	1.19±0.07
9d	39.9	43.6
9e	24.6	35.1
9f	7.7	17.6
9g	22.5	27.9
9h	48.6	> 50
10a	> 50	> 50
10b	45.6	36.9
10c	29.1	25.4
10d	30.4	35.2
10e	44.3	47.4
10f	36.1	39.3
10g	33.9	41.1
10h	22.4	16.1
Doxorubicin	0.46	0.42
Imatinib	24.6	18.9

EXPERIMENTAL

Chemistry

The melting points were recorded on a Gallenkamp electrothermal apparatus. The structures of the synthesized compounds were confirmed by their spectral (MS, IR and ^{1}H-NMR)

data and elemental analyses. The infrared spectra (KBr) were determined on a Pye Unicam SP-3000 infrared spectrophotometer. The ^{1}H-NMR spectra were obtained on a Varian Gemini 300 spectrometer (300 MHz) in DMSO-d_6 with TMS as the internal standard. The mass spectra were recorded on a GCMS-QP 1000 EX Shimadzu spectrometer. Elemental analyses were realized at the Microanalytical Center, University of Cairo, Giza, Egypt. The biological evaluation of the products was performed at the Regional Center for Mycology and Biotechnology at Al-Azhar University, Cairo, Egypt.

*Synthesis of 7,9-bis(4-methoxyphenyl)pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**2**)*. A mixture of 3-amino-4,6-bis(4-methoxyphenyl)thieno[2,3-*b*]pyridine-2-carbonitrile (**1**) (3.87 g, 10 mmol) and formic acid (20 mL) was refluxed for 2 h. The solution was cooled and then poured onto water. The resulting solid was collected and recrystallized from ethanol to give **2** as yellow crystals.

*Synthesis of 4-chloro-7,9-bis(4-methoxyphenyl)pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidine (**3**)*. A mixture of compound **2** (4.15 g, 10 mmol) and phosphorus oxychloride (20 mL) was heated at reflux for 12 h and the excess of phosphorus oxychloride was removed by distillation under reduced pressure. The residue was treated with dry benzene (10 mL), the solvent was distilled off under vacuum to remove the last traces of phosphorus oxychloride and the resultant gummy residue was triturated with ice and sodium bicarbonate solution. The thus obtained solid was collected, dried and taken for the next step without any purification.

*Synthesis of 4-hydrazinyl-7,9-bis(4-methoxyphenyl)pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidine (**4**)*. A mixture of compound **3** (4.33 g, 10 mmol) and hydrazine hydrate (30 mL) was refluxed for 4 h. The reaction mixture was poured onto crushed ice. The thus obtained white solid was filtered, dried and recrystallized from dioxane as white crystals.

Synthesis of N′*-(2-cyano-4,6-bis(4-methoxyphenyl)thieno[2,3-*b*]pyridin-3-yl)-*N,N*-dimethylformimidamide (**5**)*. A mixture of compound **1** (3.87 g, 10 mmol) and dimethylformamide dimethylacetal (DMF-DMA) (1.33 mL,10 mmol) in dry dioxane (30 mL) was heated under reflux for 6 h, then allowed to cool and poured into cold water (40 mL). The solid product was collected and recrystallized from ethanol to give **5** as brown crystals.

*Synthesis of 4-imino-7,9-bis(4-methoxyphenyl)pyrido[3′,2′:4,5]thieno[3,2-*d*]pyrimidin-3(4*H*)-amine (**6**)*. A mixture of compound **5** (4.42 g, 10 mmol) and hydrazine hydrate (10 mL) in ethanol (30 mL) was refluxed for 3 h, and then allowed to cool. The solid product was collected and recrystallized to give **6** as white crystals.

*Preparation of hydrazones **8a**–**h***

General procedure. A mixture of hydrazine **4** (0.858 g, 2 mmol) and the appropriate aldehyde (2 mmol) in acetic acid (30 mL) was heated under reflux for 4 h and then cooled. The mixture was diluted with water and the solid that precipitated was filtered off, washed with water, dried and crystallized from dioxane to give the respective hydrazones **8a**–**h**. The physical constants of the hydrazones **8a**–**h** are listed in the Supplementary material.

*Synthesis of pyrido[3′,2′:4,5]thieno[2,3-*e*]-1,2,4-triazolo[4,3-*c*]pyrimidines **9a**–**h***

General procedure. To the appropriate hydrazone **8** (14 mmol) in ethanol (40 mL), a solution of iron(III) chloride (2M, 5 mL) was added. The mixture was refluxed for 20 min, and then left overnight at room temperature. The excess solvent was distilled off under reduced pressure, and solid residue remaining was washed with water several times, dried and finally crystallized from the appropriate solvent to give the respective products **9**. The physical constants of the products **9a**–**h** are given in the Supplementary material.

*Rearrangement of **9a–h** to **10a–h***

General procedure. To a solution of the appropriate **9a**–**h** (1 mmol) in ethanol (50 mL) was added sodium acetate (0.164 g, 2 mmol) and the mixture was refluxed for 6 h and then cooled. The precipitated solid was filtered off, washed with water and then with ethanol and finally crystallized from dimethylformamide to give pyrido[3′,2′:4,5]thieno[2,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines **10a**–**h**.

*Alternate synthesis of **10a–g***

A mixture of **6** (0.429 g, 1 mmol) and polyphosphoric acid (10 mL) was heated to 50–60 °C under stirring. A substituted benzoic acid (1 mmol) was added portionwise. The mixture was then heated at 180–200 °C for 3 h under stirring. After completion of the reaction, the reaction mixture was poured into ice and neutralized with concentrated aqueous ammonia solution. The crude product was filtered, washed with water and recrystallized from DMF to afford a product that was found to be identical in all respects (m.p., mixed m.p. and IR) with product **10a**–**g** but in higher yields.

*Alternate synthesis of **10h***

To a solution of **6** (0.429 g, 1 mmol) in glacial acetic acid (10 mL), acetic anhydride (0.204 g, 2 mmol) was added and the mixture was refluxed for 2 h. After refluxing, the reaction mixture was cooled and poured into ice-cold water. The solid product that was formed was collected by filtration and crystallized from DMF to give a product that was identical in all respects with **10h** prepared from the rearrangement of **9h**.

Antimicrobial activity assay

The preliminary antimicrobial activity was investigated on a dozen of the newly synthesized compounds in order to increase the selectivity of these derivatives towards the test microorganisms. All microbial strains were provided from culture collection of the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

The antimicrobial profile was tested against two Gram-positive bacterial species (*Bacillus subtilis* and *Staphylococcus aureus*), two Gram-negative bacterial species (*Escherichia coli* and *Pseudomonas aeruginosa*) and two fungi (*Aspergillus fumigatus* and *Candida albicans*) using a modified well diffusion method.[30,31] Briefly, 100 μL of the test bacteria/fungi were grown in 10 mL of fresh media Mueller–Hinton and Sabouraud agar (Oxoid, UK), respectively, until they reached a count of approximately 10^8 cells mL^{-1} for the bacteria or 10^5 cells mL^{-1} for the fungi. One hundred μL of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained and tested for susceptibility by the well diffusion method. One hundred μL of each sample (at 5 mg ml^{-1}) was added to each well (10 mm diameter holes cut in the agar gel). The plates were incubated for 24–48 h at 37 °C (for the bacteria and yeast) and for 48 h at 28 °C (for the filamentous fungi). After incubation, the growth of the microorganism was observed. The plates were performed in triplicate and the resulting inhibition zone diameters were measured in mm and used as criterion for the antimicrobial activity. The size of the clear zone is proportional to the inhibitory action of the compound under investigation. A solvent control (DMSO) was included in every experiment as a negative control. Penicillin G and streptomycin (Sigma–Aldrich, USA) were used as a positive control against the Gram-positive and Gram-negative bacteria, respectively. Amphotericin B (Sigma–Aldrich, USA) was used as a positive control for the fungi.

MIC *determination using the broth microdilution method*. All the newly synthesized compounds were screened *in vitro* for their antibacterial and antifungal activities by the broth dilution method as described by CLSI[32] to determine the lowest concentration inhibiting the

growth of an organism, which was recorded as the *MIC* value. DMSO was used as the diluent. Stock solutions at 1000 μg mL^{-1} were prepared, from which serial dilutions were prepared for screening the tested compounds. Mueller–Hinton broth was used as the nutrient medium to grow and dilute the drug suspensions for the tested bacteria, and Sabouraud dextrose broth was used for fungal nutrition. The inoculum size for test strain was adjusted to 10^8 CFU (colony forming units) per mL by comparing the turbidity. For the broth microdilution test, 50 μL of each microbial suspension in suitable growth medium was added to the wells of a sterile 96-well microtiter plate already containing 50 μL of two-fold serially diluted tested compound. Control wells were prepared with culture medium, microbial suspension only, tested compound only and DMSO in amounts corresponding to the highest quantity present. The contents of each well were mixed on a microplate shaker (Eppendorf, Hamburg, Germany) at 900 rpm for 1 min prior to incubation for 24–48 h under the above-described cultivation conditions. The *MIC* was the lowest concentration where no viability was observed after 24––48 h based on the metabolic activity. To indicate respiratory activity, the presence of color was determined after the addition of 10 μL $well^{-1}$ of TTC (2,3,5-triphenyltetrazolium chloride, Sigma) dissolved in water (20 mg mL^{-1}) and incubation under appropriate cultivation conditions for 30 min in the dark.[33,34] After incubation, the optical density was measured using a Microplate Reader (Sunrise, Tecan, Inc., USA). Positive controls were wells with a microbial suspension in an appropriate growth medium in amounts corresponding to the highest quantity present in the broth microdilution assay. Negative controls were wells with growth medium and a tested compound. All measurements of the *MIC* values were performed in triplicate. Penicillin G and streptomycin (Sigma Aldrich, USA) were used as standard antibacterial while griseofulvin and amphotericin B (Sigma Aldrich, USA) were used as standard antifungal drugs.

Evaluation of the antitumor activity using a viability assay. Human breast carcinoma (MCF-7) and human hepatocellular carcinoma (HepG2) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown in RPMI-1640 medium supplemented with 10 % inactivated fetal calf serum and 50 μg ml^{-1} gentamicin. The cells were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 and were subcultured two to three times a week.

The potential cytotoxicity of the compounds was evaluated on tumor cells using the method of Gangadevi and Muthumary.[35] The cells were grown as monolayers in growth RPMI-1640. The monolayers of 10^4 cells adhered at the bottom of the wells in a 96-well microtiter plate incubated for 24 h at 37 °C in a humidified incubator with 5 % CO_2. The monolayers were then washed with sterile phosphate-buffered saline (0.01 M, pH 7.2) and simultaneously the cells were treated with 100 μL from different dilutions of the tested sample in fresh maintenance medium and incubated at 37 °C. A control of untreated cells was made in the absence of the tested sample. Positive controls containing the drugs imatinib or doxorubicin were also tested as references for comparison. Six wells were used for each concentration of the test sample. Every 24 h, the cells were observed under an inverted microscope. The number of surviving cells was determined by staining the cells with crystal violet[35,36] followed by cell lysing using 33 % glacial acetic acid and the absorbance at 590 nm was read using a microplate reader (Sunrise, Tecan, Inc, USA) after well mixing. The absorbance values from untreated cells were considered as corresponding to 100 % proliferation.

The number of viable cells was determined using microplate reader as previously mentioned before and the percentage viability was calculated as:

$$\text{Cell viability, \%} = 100\frac{OD_t}{OD_c}$$

where OD_t is the mean optical density of the wells treated with the tested sample and OD_c is the mean optical density of untreated cells. The relation between surviving cells and drug concentration was plotted to obtain the survival curve of each tumor cell line after treatment with a specified compound. The 50 % inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50 % of intact cells, was estimated from graphical plots.

CONCLUSIONS

In conclusion, a general and convenient method for the synthesis of novel pyrido[3′,2′:4,5]thieno[2,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine derivatives in moderate to excellent yields was reported herein. The protocol features initial oxidative cyclization of the respective pyrimidinylhydrazones followed by Dimroth rearrangement under basic conditions. The synthesized compounds were evaluated for their *in vitro* antimicrobial activity at 5 mg mL^{-1} against a representative panel of pathogenic strains using the agar well diffusion method and the results indicated that compounds **8c**, **9f**, **10c** and **10d** showed excellent activity against bacteria. In addition, the *in vitro* growth inhibitory activity of the synthesized compounds was investigated in comparison with the well-known anticancer standard drug doxorubicin using the crystal violet colorimetric viability assay and the results indicated that compounds **9c**, **8h**, **9f**, **8g** and **10h** had significant anticancer activity against the two tumor cell lines MCF-7 and HepG-2. Interestingly, compound **9c** was the most active against HepG-2 and MCF-7 with IC_{50} values of 1.19 and 3.46 μg mL^{-1}, respectively.

SUPPLEMENTARY MATERIAL

The analytical and spectral data of the synthesized compounds and *in vitro* inhibitory activities, Figs. S-1–S-3, are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

ИЗВОД

ИСПИТИВАЊЕ АНТИМИКРОБНЕ И АНТИКАНЦЕРСКЕ АКТИВНОСТИ НОВИХ ТЕТРАЦИКЛИЧНИХ СИСТЕМА ДОБИЈЕНИХ ДИМРОТОВИМ ПРЕМЕШТАЊЕМ

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Синтетисана је серија деривата пиридо[3',2':4,5]тиено[2,3-*e*]-1,2,4-триазоло[4,3-*c*]-пиримидина оксидативном циклизацијом 4-(2-арилиденхидразинил)пиридо [3',2':4,5]-тиен[3,2-*d*]пиримидина. Добијени интермедијери Димротовим премештањем дају пиридо[3',2':4,5]тиено[2,3-*e*]-1,2,4-триазоло[1,5-*c*]пиримидине. Испитана је антимикробна и антиканцерска активност добијених деривата. На основу добијених резултата једињења **8c**, **9f** и **10c** показују изузетну активност према Грам-позитивним бактеријама, а деривати **10d** и **8c** показују најбољу активност према Грам-негативним бактеријама. Испи-

тивања антиканцерске активности су показала да дериват **9c** показује најбољу активност према HepG-2 и MCF-7, са IC_{50} вредностима 1,19 и 3,46 μg mL^{-1}, редом.

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