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ORIGINAL ARTICLE

Sonochemical synthesis of pyrido[2,3-d:6,5-d′]-dipyrimidines catalyzed by $[HNMP]^+[HSO_4]^-$ and their antimicrobial activity studies

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In this study, the one-pot four-component reaction of aromatic aldehyde, 2-thiobarbituric acid, ammonium acetate in the presence of a catalytic amount of $[H\text{-}NMP]^+[HSO_4]^-$ under ultrasonic irradiation in water is reported. In the present procedure, the pyrido[2,3-d:6,5-d′]dipyrimidine derivatives were purely produced as valuable products. The process proved to be simple, environmentally friendly, efficient and high to excellent yielding. Moreover, some of the synthetic compounds were investigated and revealed important antimicrobial activity of prepared products.
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INTRODUCTION

The pyridopyrimidine scaffold is extensively described as heterocycles in many natural and synthetic biologically active compounds, as well as in different drug discovery programs. The properties of pyridopyrimidine depend on the position of the nitrogen atom in the fused ring scaffold.[1,2] Pyrido[2,3-d:6,5-d′]pyrimidines occupy a special place in four possible isomeric structures and their structure has been the subject of medical research, because this scaffold is associated with a wide range of pharmacological properties and biological activities, such as dihydrofolate reductase inhibitory activity,[3] antimicrobial activity,[4] antitumor activity,[5] anti-inflammatory,[6] tyrosine kinase inhibition,[7,8] calcium channel antagonists[9] and fibroblast growth factor receptor 3 inhibition.[10–12] Some of the pharmaceutically important compounds containing pyrido[2,3-d]pyrimidine nucleus such as adenosine kinase inhibitor (I) and cyclin-dependent kinase inhibitors (II)[13] are presented in Figure 1. Moreover, it is also reported that some heterocycles of this class have been found to possess activities of anti-HIV,[14] antifolate,[15] potassium sparing,[16] antibacterial,[17] analgesic,[6,18] antihypertensive,[19] antiallergic,[20] anticonvulsants[21] and tuberculostatic.[3]

Annulated pyrimidine derivatives have received great attention during the past years, because they exhibit useful biological activities. Uracil and its fused derivatives, such as pyrano[2,3-d]pyrimidines, pyrido[2,3-d]pyrimidines, pyrazo[3,4-d]pyrimidines or pyrimido[4,5-d] pyrimidines and annulated pyrido[2,3-d:6,5-d′]dipyrimidine derivatives based on diverse procedures such as Knoevenagel condensation, Michael addition followed by cyclodehydration strategy and finally heterocyclization.[22] These compounds have a wide range of biological activities such as antibacterial and antifungal activity, antiasthmatics, antiallergic, antihypertensive, cardiotonic, bronchodilator, antibronchitic or antitumor activity.[5,23–25] Therefore, great efforts have been directed toward the preparation of these fused molecules.

In the last few years, ultrasonic-assisted organic synthesis has been an important eco-friendly synthetic approach that is widely used in organic synthesis and has a profound impact on the chemical methods for the synthesis of complicated synthesis.[26] Ultra-sonication is based on generating the phenomenon of cavitation, nucleation, growth and implosive collapse that generates high pressures and temperatures in their surroundings. This performance leads to mass transfer improvement and performing chemical reactions.[27] Ultrasonic-assisted organic synthesis can be extremely applicable, to synthesize a wide range of practical synthesis. The notable features of the ultrasound approach are increase of reaction rate, facile manipulation and mild reaction conditions compared with traditional methods; this technique is more efficient and easily controlled, and is along with the goals of green chemistry.[28,29] However, the effect of using ultrasonic irradiation in the heterocyclic system is not fully explored.

Organic reactions in acidic ionic liquids media have received the considerable attention of synthetic organic chemists in recent years; ionic liquids is an environmentally friendly solvent with unique properties such as high ionic conductivity, non-volatility, high thermal stability, non-flammability and miscibility with organic compounds, especially with the heterocyclic compounds. Because of these useful properties, numerous works have been published in the last decades reporting the possibility to perform several organic reactions and catalyzed processes in ILs.[30,31]

As a part of our continuing efforts on the development of new simple and eco-compatible approach for the synthesis of biologically active heterocyclic compounds,[32,33] we wish to describe an efficient synthesis of novel pyrido[2,3-d]pyrimidines via a one-pot

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four-component reaction by using catalytic amount of $[H\text{-}NMP]^+[HSO_4]^-$ in water under ultrasonic irradiation at room temperature. Furthermore, the antibacterial activity of some of the prepared products was studied.

RESULTS AND DISCUSSION

Development of new highly effective synthetic methods and procedures with environmental friendly tools is one of the priority goals of chemical research. In the present study, to help achieve this goal, it should be considered the highly significant pharmacological activities of pyrido[2,3-d]pyrimidine. In continuation of our research on the efficient preparation of different heterocycles via a simple and environmentally benign synthetic method, a four-component synthesis was planned based on foreseeing a one-pot reaction among benzaldehyde, 2-thiobarbituric acid and ammonium acetate with 1:2:1 molar ratio for the synthesis of pyrido[2,3-d]dipyrimidine under ultrasonic irradiation. Some limited previously reported works were found in the literature related to preparation of hexahydropyrido [2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione.[34–36]

At the beginning, choosing a convenient and green reaction medium is extremely important for successful synthesis. To this aim, due to valuable properties of ionic liquids in recent times[37,38], and our experience in the efficacy of the catalyst $[HNMP]^+[HSO_4]^-$ as an acidic green catalyst, we examined its catalytic activity in a model reaction between 4-chlorobenzaldehyde, 2-thiobarbituric acid and ammonium acetate with 1:2:1 mole in water under ultrasonic irradiation at power of 26.5 W cm^{-2} (Scheme 1). When the model reaction was carried out in the presence of 12 mol% of catalyst, the product is obtained in low yield. However, it was found that increase the catalyst loadings to 15 mol%, improved the product yield over the 98%. Thus, 15 mol% of catalyst amount was chosen as the maximum quantity of the catalyst for this reaction (Table 1, entries 2 and 3).

To achieve suitable conditions, a model reaction performed in various solvents in the presence of a catalytic amount of $[HNMP]^+[HSO_4]^-$ ionic liquid at room temperature. Among the different solvents screened, using water gave the products in high to excellent yields in a shorter reaction time and mixture of EtOH and water was also equally effective, but in longer time. The reaction in the other solvents gave the desired products in lower yields (Table 2, entries 1–3).

In continuation of this research, the effect of various powers of ultrasonic irradiation has been surveyed. Product **4f** was synthesized as a model reaction in order to optimize the best-suited reaction conditions. It was observed that the reaction in the presence of $[H\text{-}NMP]^+[HSO_4]^-$ as catalyst and ultrasonic irradiation with power 26.5 W cm^{-2} provides the best result as obtained product with 98% isolated yield during 10 min (Table 3, entry 3). In general, increase of ultrasonic power means that higher intensity of ultrasound was introduced into the reaction vessel, which would accelerate the reactions. As it can be seen in this table, the increase of ultrasonic

Table 1 Different amounts of the $[H\text{-}NMP]^+[HSO_4]^-$ as catalyst in model reaction[a]

Entry	*Catalyst (mol%)*	*Time (min)*	*Yield (%)[b]*
1	0	20	30
2	12	12	80
3	15	5	98

[a]Reaction conditions: 4-cholorobenzaldehyde (0.25 mmol), 2-thiobarbituric acid (0.5 mmol), ammonium acetate (0.3 mmol), H_2O (10 ml), 26.5 W cm^{-2}.
[b]Isolated yields.

Table 2 Screening of solvents for the synthesis of 4f[a]

Entry	*Solvent*	*Time (min)*	*Yield[b] (%)*
1	EtOH	10	70
2	Acetone	15	40
3	Acetonitrile	15	40
4	H_2O/EtOH (4:1)	10	99
5	H_2O	5	98

[a]Reaction conditions: 4-cholorobenzaldehyde (0.25 mmol), 2-thiobarbituric acid (0.5 mmol), ammonium acetate (0.3 mmol), Solvent (5 ml), catalyst (15 mol%), 26.5 W W cm^{-2}.
[b]Isolated yields.

Table 3 Power optimization for the synthesis of 4f[a]

Entry	*Power (W cm^{-2})*	*Time (min)*	*Yield (%)[b]*
1	20	10	85
2	26.5	5	98
3	33	10	98
4	39.5	15	80

[a]Reaction conditions: 4-cholorobenzaldehyde (0.25 mmol), 2-thiobarbituric acid (0.5 mmol), ammonium acetate (0.3 mmol), H_2O (10 ml), catalyst(15 mol%).
[b]Isolated yields

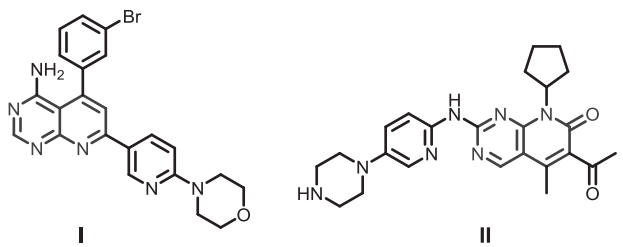
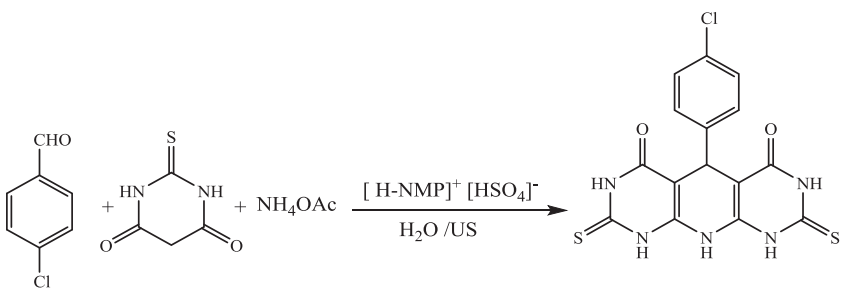


Figure 1 Representative compounds containing pyrido[2,3-d]pyrimidine. A full color version of this figure is available at *The Journal of Antibiotics* journal online.



Scheme 1 Model reaction for the synthesis of 5-(4-chlorophenyl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (**4f**).

power led to relatively higher yield until the ultrasound power intensity to reach 39.5 W and then the yield slightly decreased with increasing ultrasound power intensity (Table 3, entry 4).

In order to survey the recyclability and reusability of the catalyst, the reaction mixture filtered to separate the solid product. Then, diethyl ether was added to the filtrate for removing the remained organic compounds. The remained $[H\text{-}NMP]^+[HSO_4]^-$ catalyst in water easily extracted from mentioned mixture. After that, the water was evaporated under vacuum, to separate the $[H\text{-}NMP]^+[HSO_4]^-$. The recovered catalyst was reused for subsequent reactions. As indicated in Figure 2, it was shown no major loss of efficiency with regard to the reaction yield after four successive runs.

To show the merit of this method, a comparison of the present work with very few previously reported methods was provided for the formation of pyrido[2,3-d:6,5-d′]pyrimidine is presented in Table 4. These methods suffer from one or more disadvantages such as; long reaction times, low yields and harsh and unclean reaction conditions.

To explore the scope and limitation of this reaction, we have extended the reaction with a range of aromatic aldehydes (Scheme 2). The results are indicated in Table 5.

As shown in Scheme 3, hexahydropyrido[2,3-d:6,5-d′]dipyrimidine could be synthesized via sequential condensation, addition, hydrolysis, cyclization and tautomerization. The reaction may proceed in a stepwise manner, in which the intermediate **3** was formed by fast Knoevenagel condensation reaction of the activated aldehyde and 2-thiobarbituric acid. Subsequently, Michael-type addition of compound **2** to the intermediate **3** and tautomerization produce the intermediate **3**. Then, the amine group in intermediate **3** attacked to activated carbonyl group and the corresponding products were obtained by intramolecular cyclization and dehydration. Brønsted acidic ionic liquid ($[H\text{-}NMP]^+[HSO_4]^-$) can activate carbonyl groups via hydrogen bonding to decrease the energy of transition state.

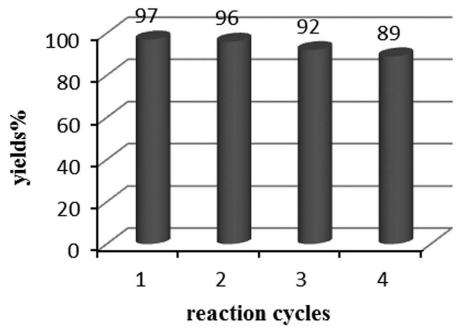


Figure 2 Catalyst recyclability on the synthesis of pyridopyrimidine.

The results of antimicrobial activity of compounds are summarized in Tables 6 and 7. Our results indicated that none of synthesized compounds had antimicrobial activity against gram negative bacteria (Table 6). Compounds **4i** and **4m** were active against gram positive bacteria. Although compound **4i** had the highest inhibition zone against *S. epidermidis* in comparison with compound **4m**, it did not have strongest MIC value.

As displayed in Tables 6 and 7, compounds **4c**, **4f** and **4h** had activity against Gram-positive bacteria and fungi. Compound **4h** revealed better effects against Gram-positive bacteria and fungi in comparison with compounds **4c** and **4f**. In addition, as indicated in Table 7, MIC values of the compounds **4c**, **4f**, **4m** and **4h** ($<31.2\ \mu g\ ml^{-1}$) were evaluated.

CONCLUSION

In this study, we have described a successful strategy for the efficient and convenient synthesis of pyrido[2,3-d:6,5-d′]dipyrimidine derivatives in the one-pot, four-component condensation reaction of 2-thiobarbituric acid, ammonium acetate and aromatic aldehydes using the inexpensive, nontoxic and easily available $[H\text{-}NMP]^+[HSO_4]^-$ as a catalyst under ultrasonic irradiation. The advantages of this protocol are such as the economically reaction procedure, easy workup, high product yields and short reaction times. In addition, antimicrobial activities of different compounds were investigated.

EXPERIMENTAL PROCEDURE

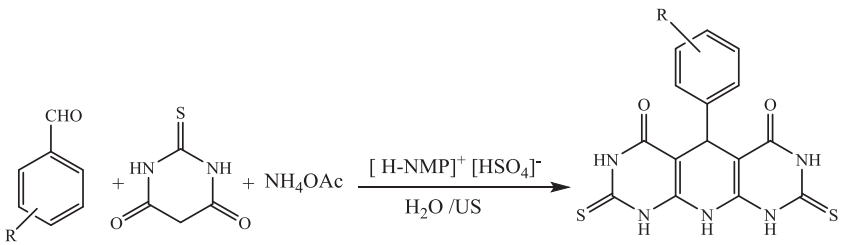
Chemistry

Materials. All commercially available reagents were used without further purification and purchased from the Merck Chemical Company (Darmstadt, Germany) in high purity.

Table 4 Comparison condition used for the synthesis of pyrido[2,3-d:6,5-d']dipyrimidine

Entry	*Catalyst*	*Condition*	*Time*	*Yield (%)*[a]
1	$[H\text{-}NMP]^+[HSO_4]^-$[b]	us	5 min	89
2	Al_2O_3(solid phase)[c]	MW	6 h	86 (Kidwai *et al.*[40])
3	Al_2O_3(liquid phase)[c]	Δ	48 h	98 (Kidwai *et al.*[40] and Vafaeezadeh *et al.*[43])

[a]Isolated yields.
[b]Reaction conditions: 4-cholorobenzaldehyde (0.25 mmol), 2-thiobarbituric acid (0.5 mmol), ammonium acetate (0.3 mmol), H_2O (10 ml), catalyst (15 mol%), 26.5 W cm^{-2}.
[c]Reaction conditions: benzaldehyde, 2-thiobarbituric acid, ammonium acetate 1:2:1, Al_2O_3 (acid).



Scheme 2 The reaction leading to the synthesis of novel pyrido[2,3-d]dipyrimidine derivatives.

Table 5 Synthesis of pyrido[2,3-d:6,5-d']dipyrimidine derivatives under ultrasonic irradiation[a]

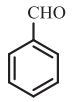
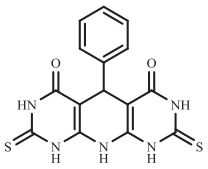
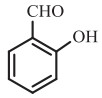
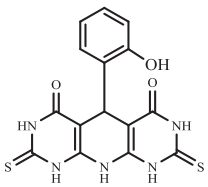
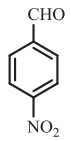
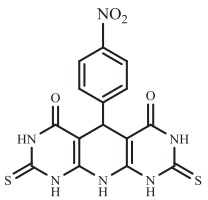
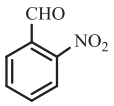
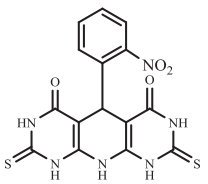
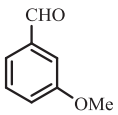
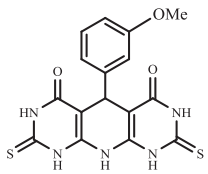
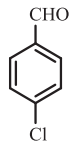
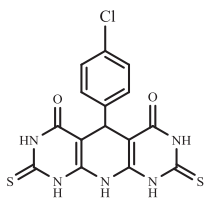
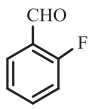
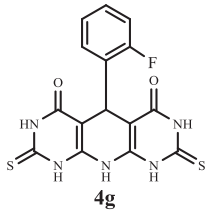
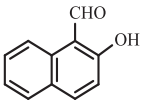
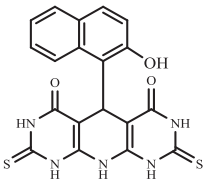
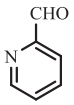
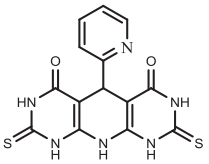
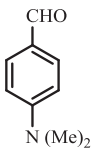
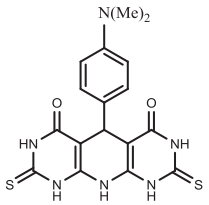
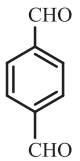
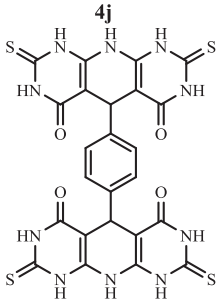
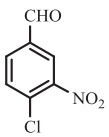
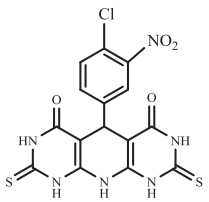
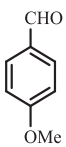
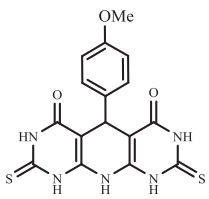
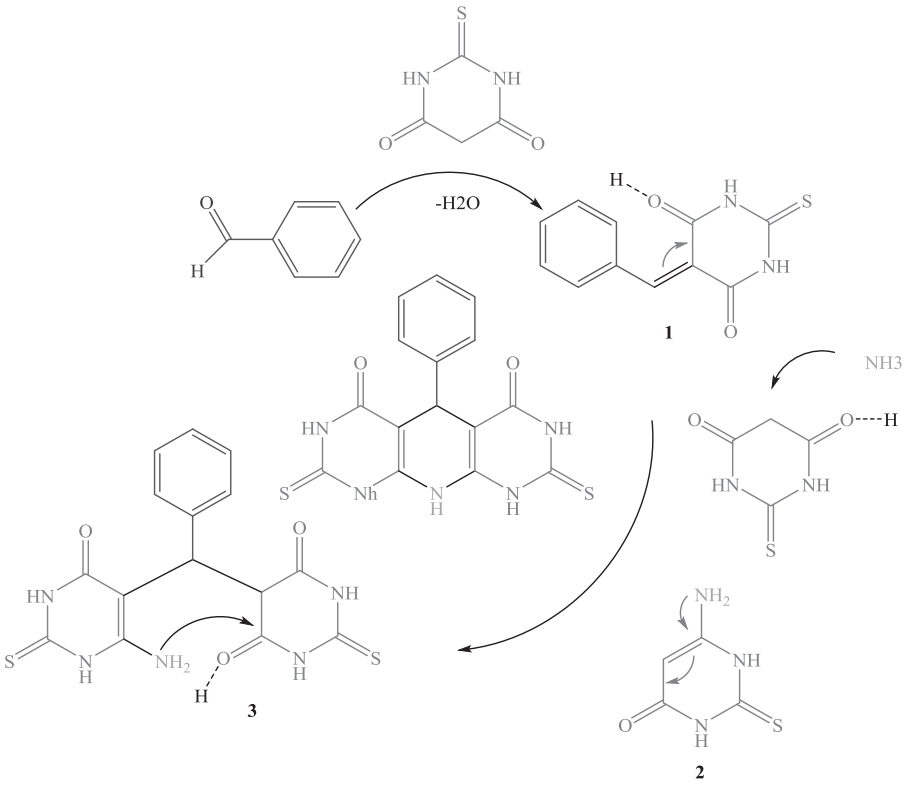
Entry	*Aldehyde*	*Product*	*Time (min)*	*Yield (%)[b]*
1		 **4a**	8	95
2		 **4b**	30	< 5
3		 **4c**	8	98
4		 **4d**	10	95
5		 **4e**	8	98
6		 **4f**	5	98
7		 **4g**	8	90

Table 5 (Continued)

Entry	*Aldehyde*	*Product*	*Time (min)*	*Yield (%)[b]*
8		 **4h**	12	95
9		 **4i**	8	96
10		 **4j**	30	< 5
11		 **4k**	5	98
12		 **4l**	10	95
13		 **4m**	14	93

[a]Reaction conditions: aldehyde (0.25 mmol), 2-thiobarbituric acid (0.5 mmol), ammonium acetate (0.3 mmol), H_2O (10 ml), catalyst (15 mol%), 26.5 W cm^{-2}.
[b]Isolated yields.



Scheme 3 Proposed reaction mechanism for the formation of **4a.**

Table 6 ***In vitro*** **antimicrobial activity of the prepared compounds 4c, 4f, 4i, 4m and 4h by agar diffusion assayAbbreviation: NT, not tested.**

	Diameter of zone of inhibition in mm						
Test microorganisms	*4c*	*4f*	*4i*	*4m*	*4h*	*Tetracycline*	*Nystatin*
P. aeruginosa	–	–	–	–	–[a]	8±0.0	NT
E. coli	–	–	–	–	–	19.5±0.5	NT
P. vulgaris	–	–	–	–	–	19±1	NT
B. subtilis	–	–	10±0.0	–	13.5±0.5	18±0.0	NT
S. aureus	17±1	11±1	18.5±0.5	11±0.0	19.5±1.5	24.5±0.5	NT
S. epidermidis	14±2	15.5±0.5	22.5±0.5	13.5±0.5	26.5±0.5	38±1	NT
C. albicans	–	14±0.0	–	–	23.5±0.5	NT	26±1
A. niger	10±0.0	12.5±1.5	–	–	17.5±0.5	NT	31.5±0.5
A. brasiliensis	–	14±1	–	–	18.5±0.5	NT	31.5±1.5

[a]A dash (–) indicates no antimicrobial activity.

Table 7 Minimum inhibitory concentration (MIC in μg ml^{-1}) values of the effective synthesized compounds 4c, 4f, 4i, 4m and 4h.

	Bacteria			*Fungus*		
Compound	*S. epidermidis*	*S. aureus*	*B. subtilis*	*C. albicans*	*A. niger*	*A. brasiliensis*
4c	3.9	250	–[a]	–	500	–
4f	1.95	500	–	>2000	>2000	>2000
4i	62.5	1000	>2000	–	–	–
4m	3.9	2000	–	–	–	–
4h	1.95	62.5	1000	>2000	1000	1000
Tetracycline	250	250	7.8	NT	NT	NT
Nystatin	NT[b]	NT	NT	100	12.5	25

Abbreviation: NT, not tested.
[a]A dash (–) indicates no antimicrobial activity.

Apparatus

IR spectra were obtained as KBr pellets on a Perkin–Elmer 781 spectrophotometer (Waltham, MA, USA) and on an impact 400 Nicolet FT–IR spectrophotometer (Thermofisher, Waltham, MA, USA). ^{1}H NMR and ^{13}C NMR were recorded in dimethul sulfoxide (DMSO)-d_6 solvents on a Bruker DRX-400 spectrometer (Karlsruhe, Germany) with tetramethylsilane as internal reference. UV–Vis spectra were obtained with a Perkin–Elmer 550 spectrophotometer recorded in EtOH solvent. Melting points were obtained with a Yanagimoto micro melting point apparatus and are uncorrected. Mass spectra were recorded on an Agilent Technology HP 5973 Network Mass Selective Detector Mass spectrometer (Agilent Technology, Santa Clara, CA, USA) operating at an ionization potential of 70 eV (EI). The BANDELIN ultrasonic HD 3200 (BANDELIN electronic GmbH & Co. KG, Berlin, Germany) with probe model KE 76, 6 mm diameter was used to produce ultrasonic irradiation. The purity determination of the substrates and reaction monitoring were accomplished by TLC on silica-gel poly gram SILG/UV 254 plates (from Merck Company). All the newly synthesized compounds were screened for antimicrobial activity. The microorganisms used in this research were: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 10536) and *Proteus vulgaris* (PTCC 1182) as examples of Gram-negative bacteria, *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29737) and *Staphylococcus epidermidis* (ATCC 12228) as examples of Gram-positive bacteria, *Candida albicans* (ATCC 10231), *Aspergilluasniger* (ATCC 16404) and *Aspergilluasbrasiliensis* (ATCC 16404) as examples of fungal strains.

Typical procedure for the synthesis of $[H\text{-}NMP]^+[HSO_4]^-$

$[H\text{-}NMP]^+[HSO_4]^-$ was synthesized by following procedure. The *N*-methyl-2-pyrrolidone (9.9 g, 0.1 mol, 9.71 ml) was charged into a 250 ml three necked flask with magnetic stirrer, then sulfuric acid (9.6 g, 0.1 mol, 5.5 ml) was slowly added dropwise into the flask at 0–5 °C. After stirring for 4 h at room temperature, the reaction mixture was washed with ethyl acetate (3 × 10 ml) and dried at 80 °C in vacuum.[39] The ionic liquid was prepared in quantitative yield and characterized by ^{1}H NMR data before using in the reaction as followed: ^{1}H NMR (DMSO-d_6, 400 MHz): δ (p.p.m.) 3.28–3.31 (t, 2H, J = 7.2 Hz), 2.75 (s, 3H), 2.15–2.19 (t, 2H, J = 8.1 Hz), 1.88–1.96 (q, 2H, J = 7.6 Hz).

General procedure for synthesis of pyrido[2,3-d:6,5-d′]dipyrimidine derivatives

To a mixture of an aromatic aldehyde (0.25 mmol), 2-thiobarbituric acid (0.5 mmol), ammonium acetate (0.3 mmol), $[H\text{-}NMP]^+[HSO_4]^-$ (12 mol%) and water (2 ml) was added. Then, ultrasonic probe was directly immersed in the resulting mixture. The progress of the reactions was monitored by TLC until conversion of the starting materials was satisfactory. After completion of the reaction, the solvent was evaporated and the precipitate was washed with EtOH and hot water to afford the pure product. All products were identified by physical and spectroscopic data. The synthesis of the compounds **4b** and **4j** was investigated but unfortunately even after 24 h, only a trace amount of product was observed by TLC, that is why we could not reported the spectroscopic data of them.

5-Phenyl-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4a). Cream powder; m.p.: 211 °C Lit[40] (m.p.rep: 218 °C). IR (KBr) (ν_{max}/cm^{-1}): 3452 (NH), 3054 (C–H, sp^2 stretch), 2898 (C–H, sp^3), 1637 (C = O), 1440, 1559 (C = C, Ar). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 11.33–12.00 (3H, s, NH), 7.91 (1H, s, NH), 7.60 (1H, s, NH), 7.15 (2H, s, H-Ar), 7.05 (1H, s, H-Ar), 6.98-6.99 (2H, d, J = 6.0, H-Ar), 5.93 (1H, s, CH).

5-(4-Nitrophenyl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine 4,6(1H,5H)-dione (4c). Light brown powder. m.p.: 330 °C Lit[41] (m.p.rep: > 300 °C decompose). IR (KBr) (ν_{max}/cm^{-1}): 3447 (NH), 3175 (C–H, .sp^2 stretch), 1602 (C = O), 1432, 1509 (C = C, Ar). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 17.10 (1H, s, NH), 11.75–11.97 (2H, s, NH), 11.62 (2H, s, NH), 8.04–8.28 (2H, d, J = 8.8 Hz, H-Ar), 7.23–7.25 (2H, d, J = 8.4 Hz, H-Ar), 6.04 (1H, s, CH), ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 173.5, 164.0, 163.1, 152.5, 145.7, 128.2, 123.5, 95.6, 31.6.

5-(2-Nitrophenyl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4d). Yellow powder; m.p.: 230 °C decompose. IR (KBr) (ν_{max}/cm^{-1}): 3437 (NH), 3137 (C–H, sp^2 stretch), 1610 (C = O), 1535, 1445, (C = C, Ar). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 11.57–1.99 (5H, s, NH), 7.45 (1H, m, H-Ar), 7.30–7.32 (1H, m, H-Ar), 7.19 (1H, s, H-Ar), 7.06 (1H, s, H-Ar), 6.09–6.21 (1H, s, CH). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 173.4, 163.1, 150.1, 136.0, 131.5, 129.7, 127.1, 124.0, 95.2, 29.1; Anal. Calcd for $C_{15}H_{10}N_6O_4S_2$: C, 44.77; H, 2.50; N, 20.88,%; Found C, 44.81; H, 2.53; N, 20.91%.

5-(3-Methoxyphenyl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4e). Yellow powder; m.p.: 242 °C decompose. IR (KBr) (ν_{max}/cm^{-1}): 3591, 3447 (NH), 3166 (C–H, sp^2 stretch), 2922 (C–H, sp^3), 1632 (C = O), 1437, 1536 (C = C, Ar). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 11.56–11.73 (4H, m, NH), 7.19–7.23 (1H, s, NH), 6.93 (1H, s, H-Ar), 6.62–6.64 (1H, m, H-Ar), 6.56–6.57 (m, 1H), 6.49–6.52 (1H, s, H-Ar), 5.91–5.97 (1H, s, CH), 3.63 (3H, s, CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 173.2, 159.4, 145.2, 129.0, 119.6, 113.6, 109.8, 96.2, 55.2, 30.8; Anal. Calcd for $C_{16}H_{15}N_5O_3S_2$: C, 49.34; H, 3.88; N, 17.98,%; Found C, 49.38; H, 3.90; N, 18.02%.

5-(4-Chlorophenyl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4f). Yellow powder; m.p.: 257 °C decompose. IR (KBr) (ν_{max}/cm^{-1}): 3433 (NH), 3130 (C–H, sp^2 stretch), 1626 (C = O), 1434, 1534 (C = C, Ar). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 11.67 (5H, s, NH), 7.19 (1H, s, H-Ar), 7.06 (1H, s, H-Ar), 6.94 (2H, d, H-Ar), 5.91 (1H, s, CH). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 175.5, 165.2, 162.9, 144.0, 136.5, 133.8, 130.1, 122.0, 119.4, 93.1,31.2.

5-(2-Fluorophenyl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4g). White powder; m.p.: 240 °C decompose. IR (KBr) (ν_{max}/cm^{-1}): 3432 (NH), 3108 (C–H, sp^2 stretch), 1623, 1687 (C = O), 1433, 1544 (C = C, Ar); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 17.00 (1H, s, NH), 11.49–11.75 (4H, m, NH), 7.08–7.12 (2H, m, H-Ar), 6.91–7.00 (2H, m, H-Ar), 6.01 (1H, s, CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 173.2, 163.3 (m, 1C), 162.0, 159.6, 130.4–130.5, 130.0 (d, 1C), 127.5, 115.2, 95.4, 26.8, 23.6; Anal. Calcd for $C_{15}H_{12}FN_5O_2S_2$: C, 47.74; H, 3.20; N, 18.56,%; Found C, 47.76; H, 3.24; N, 18.60%.

5-(2-Hydroxynaphthalen-1-yl)2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4h). Light brown powder; m.p.: 308 °C decompose. IR (KBr) (ν_{max}/cm^{-1}): 3530 (NH), 3069 (C–H, sp^2 stretch), 2892 (C–H, sp^3), 1680 (C = O), 1450, 1562 (C = C, Ar); ^{1}H NMR(DMSO-d_6, 400 MHz) δ (p.p.m.): 17.1 (1H, s, NH), 11.56 (2H, s, NH), 11.40 (2H, s, NH), 7.96 (1H, d, H-Ar), 7.77 (2H, m, H-Ar), 7.39-7.41 (2H, m, H-Ar), 7.19–7.22 (1H, m, H-Ar), 5.39–5.41 (1H, m, CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 173.7, 161.6, 154.5, 147.8, 131.2, 129.2, 127.6, 125.4, 123.4, 116.8, 115.4, 97.5, 91.6, 24.8. Anal. Calcd for $C_{19}H_{13}FN_5O_3S_2$: C, 53.89; H, 3.09; N, 16.54,%; Found C, 53.92; H, 3.12; N, 16.56%.

5-(Pyridin-2-yl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4i). Red powder; m.p.: 280 °C decompose. IR (KBr) (ν_{max}/cm^{-1}): 3398 (NH), 3210 (C–H, sp^2 stretch), 2881 (C–H, sp^3), 1605 (C = O), 1455, 1533 (C = C, Ar). ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 14.00–16.00 (1H, s, NH), 11.84 (4H, s, NH), 8.60 (1H, m, H-Ar), 8.42 (1H, m, H-Ar), 7.83 (2H, s, H-Ar), 6.20 (1H, s, CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 174.2, 163.4, 158.9, 146.8, 141.9, 126.2, 124.8, 92.9, 32.0. EI–MS (70 eV) *m/z*: 358 (0.06), 352 (3.91), 228 (100), 126 (4.96), 124 (37.53), 77 (23.27), 51 (8.31).

5,5′-(1,4-Phenylene)bis(2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione) (4k). Red powder; m.p.: 300 °C decompose. IR (KBr) (ν_{max}/cm^{-1}): 3428 (NH), 3200 (C–H, sp^2 stretch), 2923 (C–H, sp^3), 1623 (C = O), 1434, 1535 (C = C, Ar); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 13.41 (2H, s, NH), 12.40 (2H, s, NH), 12.09 (1H, s, NH), 11.61 (1H, s, NH), 7.98 (2H, m, NH), 7.86-7.91 (4H, m, H-Ar), 7.51 (2H, m, NH), 5.59 (2H, s, CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 173.4, 164.0,

163.0, 126.2, 115.3, 96.4, 30.8. EI-MS (70 eV) *m*/*z*: 636 (0.55), 637 (1.06), 289 (27.58), 264 (35.19), 135 (55.28).

5-(4-Chloro-3-nitrophenyl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4l). Yellow powder; m.p.: 249 °C decompose. IR (KBr) (ν_{max}/cm^{-1}): 3450 (NH), 3090 (C–H, sp^2 stretch), 1615 (C = O), 1542, 1434 (C = C, Ar); ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 11.63–11.70 (4H, m, NH), 7.53–7.56 (1H, m, NH), 7.28–7.30 (1H, m, H-Ar), 7.14–7.18 (1H, m, H-Ar), 7.05 (1H, s, H-Ar), 5.98 (1H, s, CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 173.5, 163.9, 163.1, 147.7, 145.2, 132.7, 131.4, 123.7, 121.9, 95.3, 30.8. EI–MS (70 eV) *m*/*z*: 436 (0.30), 431 (3.07), 311 (85.72), 144 (96.27), 116 (42.61), 69 (100), 43 (85.88); Anal. Calcd for $C_{15}H_{10}ClN_5O_2S_2$: C, 45.98; H, 2.57; N, 17.87,%; Found C, 46.01; H, 2.60; N, 17.92%.

5-(4-Methoxyphenyl)-2,8-dithioxo-2,3,7,8,9,10-hexahydropyrido[2,3-d:6,5-d′]dipyrimidine-4,6(1H,5H)-dione (4m). Orange powder; m.p.: 280 °C decompose; IR (KBr) (ν_{max}/cm^{-1}): 3432, 3090, 1629, 1542, 1442; ^{1}H NMR (DMSO-d_6, 400 MHz) δ (p.p.m.): 11.62 (m, 2H, NH), 11.49 (m, 2H, NH), 7.11–7.09 (s, 1H, NH), 7.86–6.88 (m, 2H, H-Ar), 6.70–6.72 (m, 2H, H-Ar), 5.88 (s, 1H, CH), 3.66 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ (p.p.m.): 191.7, 164.6, 157.3, 132.2, 130.0, 121.2, 114.9, 107.8, 56.1, 16.5.

In vitro antimicrobial activity

Agar diffusion assay. The preliminary antimicrobial activity of compounds was determined by agar diffusion method.[42] Stock solution of 30 mg ml^{-1} of each compound was prepared in DMSO in separate tubes. One hundred microliters of suspension containing 10^8 CFU per ml of bacteria, 10^6 CFU per ml of yeast and fungi spread (10^4 spore per ml) on the nutrient agar, sabouraud dextrose agar and potato dextrose agar medium, respectively. Uniformly sized wells (6 mm diameters) were punched on the media plates and filled with 10 µl of the test compounds. Tetracycline (10 µg per well) was used as positive control for bacteria and Nystatine (100 IU per well) for fungi. DMSO was used as a negative control. The inoculated plates were incubated for 24 h at 37 °C for bacterial strains and 48 h and 72 h at 30 °C for yeast and mold isolated, respectively. The diameter of inhibition zone for each compound on the surface of plates was recorded in mm and the results reported as mean ± s.d. of a triplicate experiment.

Micro-well dilution assay

Bacterial strains sensitive to the compounds in agar diffusion assay were studied for their MIC values using micro-well dilution assay method. The compounds dissolved in 10% DMSO were first diluted to the highest concentration (2000 µg ml^{-1}) to be tested and then serial two fold dilutions were made in a concentration range from 31.3 to 2000 µg ml^{-1} in 10 ml sterile test tubes containing brain heart infusion broth. Tetracycline was used as standard drug for positive control in conditions identical to tests materials. Turbidity indicated growth of microorganism and the MIC were defined as the lowest concentrations of the compounds that prevented visible growth.

MIC agar dilution assay

MIC values of the compounds for the fungus isolate sensitive to them were evaluated based on the agar dilution method. The serial concentration of these compounds were added to sterile molted sabouraud dextrose agar medium and poured into petri plates. The plates were spot inoculated with 5 µl (10^4 spore per ml) of fungus isolate. The inoculated plates were incubated at 30 °C for 72 h. Nystatin was used as reference antifungal drug. The MIC was defined as the lowest concentration of the compounds needed to inhibit the growth of microorganisms.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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