Organic & Biomolecular Chemistry

PAPER

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Cite this: *Org. Biomol. Chem.*, 2015, **13**, 7294

One-pot three-component domino protocol for the synthesis of novel pyrano[2,3-*d*]pyrimidines as antimicrobial and anti-biofilm agents†

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Received 7th April 2015,
Accepted 20th May 2015

DOI: 10.1039/c5ob00693g

www.rsc.org/obc

A simple and facile synthesis of a series of novel pyrano[2,3-*d*]pyrimidines has been achieved successfully *via* the one-pot three-component reaction of 2-amino-7-methyl-5-oxo-4-phenyl-4,5-dihydropyrano-[4,3-*b*]pyran-3-carbonitriles, DMF-DMA and arylamines in the presence of 1-butyl-3-methyl-hydrogensulphate [Bmim]HSO_4 ionic liquid. This method has several advantages such as high yields, clean reaction, simple methodology and short reaction times. The synthesized compounds were evaluated for their antimicrobial activity against Gram-positive, Gram-negative and different *Candida* strains. Among the derivatives screened, compounds **4c**, **4d**, **4h** and **4l** were found to be active against both bacterial and *Candida* strains with MIC values ranging from 3.9 to 31.2 μg mL^{-1}. In addition, compound **4l** showed a good minimum bactericidal concentration, minimum fungicidal concentration and anti-biofilm activities. Furthermore, the mode of the antifungal action for the promising compound **4l** was evaluated in *C. albicans* MTCC 1637 through an ergosterol biosynthesis inhibition process.

1. Introduction

In recent years, antimicrobial resistance has gained renewed interest globally and has been a serious public health concern, resulting in the incidence of various drugs-resistant microbial infections, such as community acquired infections like streptococcal infections, pneumonia, *etc.*, or hospital-acquired infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE) or extended spectrum beta-lactamase (BSLE) enzyme producing Gram-negative bacteria and azole-resistant *Candida* species. The primary reason for the antimicrobial resistance is the wide use or misuse of currently available antimicrobial agents, which is common in clinical practice.[1] In view of the increased threat from these drug-resistant Gram-positive and -negative bacterial strains as well as *Candida* strains, there is a continuous demand to identify new antimicrobial agents.

In this context, the pyrimidine entity is the essential ring found in the core structure of all essential nucleobases. In addition to pyrimidines, pyranopyrimidine derivatives are important synthetic bioactive compounds. In the recent years, the synthesis of novel pyrimidine derivatives has gained renewed interest in the area of medicinal chemistry for their diverse range of biological activities including antimicrobial,[2] antimalarial,[3] anti-inflammatory,[4] anti-viral,[5] anti-platelet,[6] anti-tumor,[7,8] anti-histamine,[9] anti-thrombotic[10] and antigenic properties.[11] Some of these compounds have also identified as new HA14-1 analogues.[12] Some of the well-known pyranopyrimidine derivatives (**1**, **2**, **3** and **4**) were also reported to be antimicrobial agents,[13] as represented in Fig. 1.

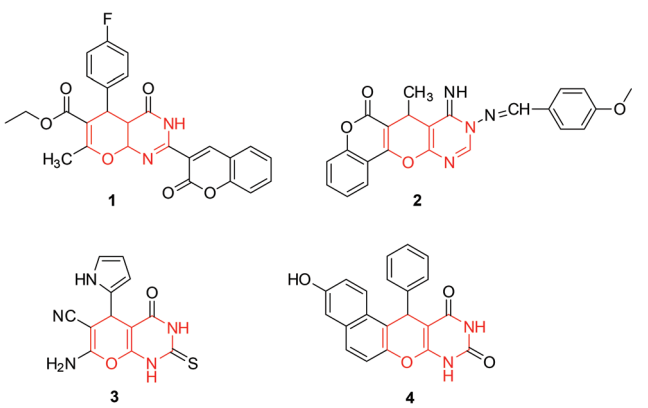

Fig. 1 Pyranopyrimidine-based antimicrobial agents.

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†Electronic supplementary information (ESI) available. CCDC 1401827. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c5ob00693g

One-pot multicomponent condensation represents a possible route for an ideal synthesis, enabling the synthesis of complex molecules with maximum simplicity. They offer significant advantages over conventional linear step synthesis by their convergence, productivity, facile execution and high yield. Recently, a few researchers[14a,b] developed some novel approaches to prepare 4-anilinoquinazoline derivatives. A large number of methods for the synthesis of pyranopyrimidines have also been reported.[14c–h] However, some of these methods suffer from drawbacks, such as harsh reaction conditions, unsatisfactory yields and prolonged reaction times. Hence, the development of a new methodology to achieve improved yields using a green chemistry approach for the synthesis of the title molecules is a welcome goal. Recently, ionic liquids, especially those based on 1,3-dialkyl imidazolium cations have attracted considerable interest as promising alternative green solvents and catalysts in organic synthesis. These ionic liquids have several interesting properties, such as non-volatility, high thermal stability, good solvating capability, wide liquid range and ease of recycling.[15]

As a continuation of our research work on the use of green reagents, such as ionic liquids, for the synthesis of biologically active heterocyclic compounds,[16] this paper reports the $[Bmim]HSO_4$ ionic liquid, which promotes the cyclization reaction of pyrano[2,3-*d*]pyrimidin-6(5*H*)-one derivatives. These pyrano[2,3-*d*]pyrimidin-6(5*H*)-one derivatives were screened further for their antibacterial, minimum bactericidal concentration, antifungal, minimum fungicidal concentration and anti-biofilm activities.

2. Results and discussion

2.1. Chemistry

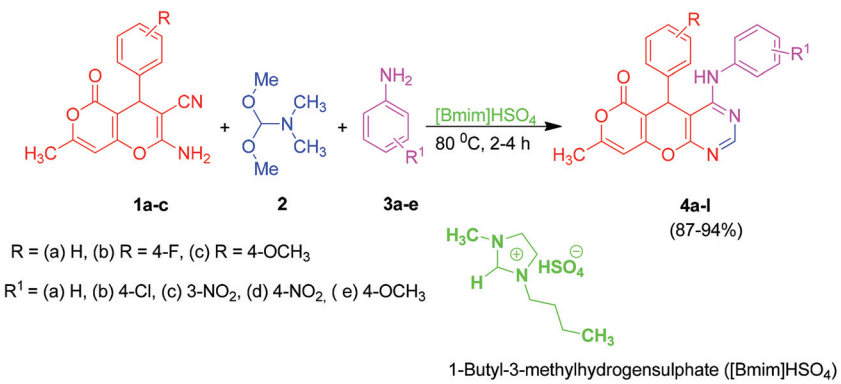
The target pyranopyrimidine derivatives **4a–l** were obtained in good yields (Scheme 1) by the reaction of 2-amino-7-methyl-5-oxo-4,5-dihydropyrano[4,3-*b*]pyran-3-carbonitriles **1a–c** (1.0 mmol) with *N*,*N*-dimethylacetaldehyde dimethyl acetal **2** (DMF-DMA) (1.2 mmol) and aromatic amines (1.0 mmol) **3a–e** in the presence of $[Bmim]HSO_4$ ionic liquid. To optimize the reaction conditions, a model reaction of 2-amino-7-methyl-5-oxo-4,5-dihydropyrano[4,3-*b*]pyran-3-carbonitriles **1a** (1 mmol), DMF-DMA **2** (1.2 mmol) and aniline **3a** (1 mmol) was performed in the presence of various solvents and ionic liquids under different temperatures. The results are depicted in (Table 1, entries 1–10). From these results, it was observed that the efficiency and the yield of the reaction using $[Bmim]HSO_4$ at 80 °C (Table 1, entry 6) was higher than those obtained in other solvents, such as acetonitrile, ethanol, acetic acid (Table 1, entries 1–3) and other ionic liquids like $[Bmim]BF_4$, [Bmim]Br and $[Bmim]PCl_5$ (Table 1, entries 8–10). It was inferred from the above results that the ionic liquid medium is an essential and crucial factor for promoting the reaction.

Subsequently, we examined the scope and efficiency of the reaction with respect to various amines under optimal conditions (Table 2, entries 1–12). It was observed that a variety of amines bearing either electron withdrawing or electron donating substitutions at the *ortho*-, *meta*- and *para*-positions participated in this reaction. The reusability of the $[Bmim]HSO_4$ ionic liquid in the above model reaction was also investigated. After completing the reaction, the mixture was poured into water and stirred thoroughly. The solid product obtained was

Table 1 Optimization of reaction parameters for the synthesis of **4a**

Entry[a]	Solvent	Temp (°C)	Time (h)	Yield[b] (%)
1	Ethanol	Reflux	10	28
2	Acetonitrile	Reflux	10	24
3	AcOH	Reflux	8	44
4	$[Bmim]HSO_4$	r.t.	2	48
5	$[Bmim]HSO_4$	60	2	76
6	**$[Bmim]HSO_4$**	**80**	**2**	**94**
7	$[Bmim]HSO_4$	100	2	92
8	$[Bmim]BF_4$	80	2	82
9	[Bmim]Br	80	2	74
10	$[Bmim]PCl_5$	80	2	62

[a] Reaction conditions: 2-Amino-7-methyl-5-oxo-4-phenyl-4,5-dihydropyrano[4,3-*b*]pyran-3-carbonitriles (1 mmol), DMF-DMA (1.2 mmol), aniline (1 mmol), $[Bmim]HSO_4$ (2 mL), 80 °C, 2 h. [b] Isolated yields after purification.



Scheme 1 Synthesis of pyrano[2,3-*d*] pyrimidines in a $[Bmim]HSO_4$ ionic liquid.

Table 2 Synthesis of pyrano[2,3-*d*]pyrimidine derivatives in [Bmim]HSO_4 ionic liquid

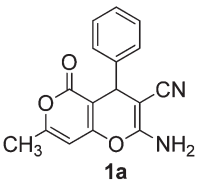
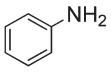
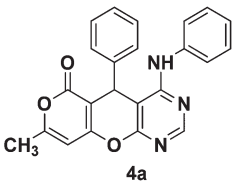
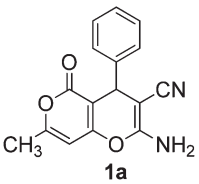
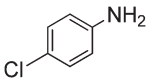
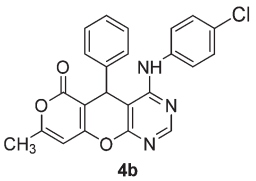
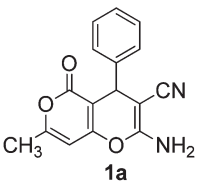
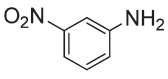
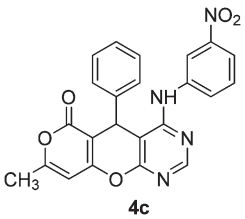
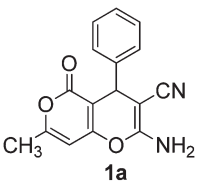
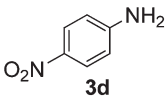
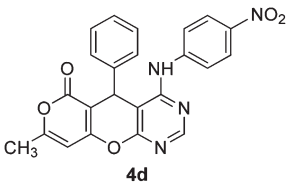
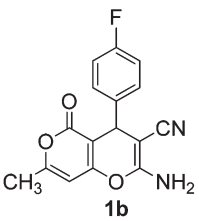
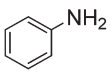
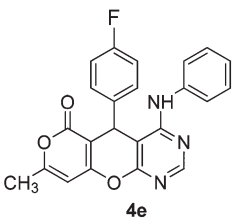
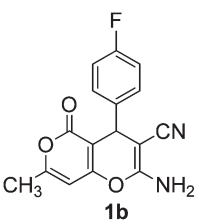
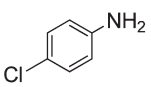
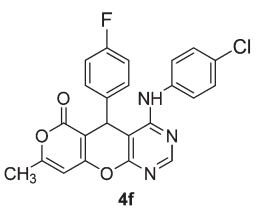
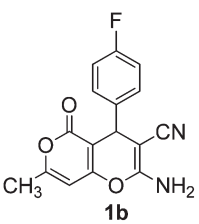
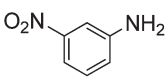
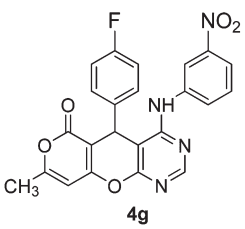
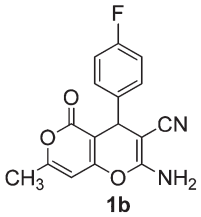
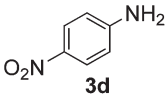
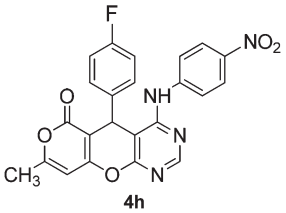
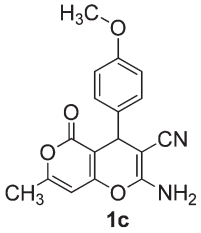
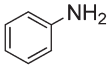
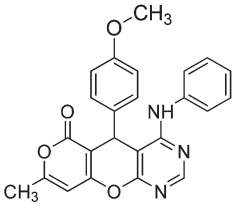
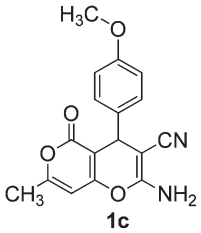
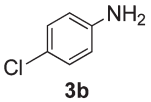
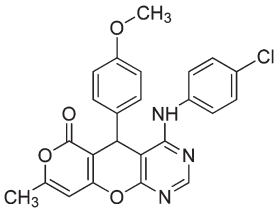
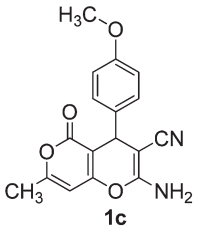
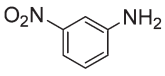
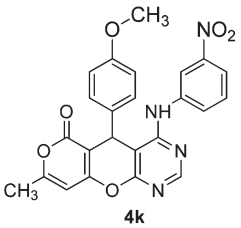
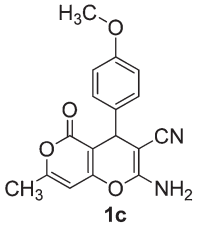
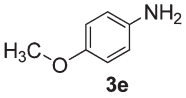
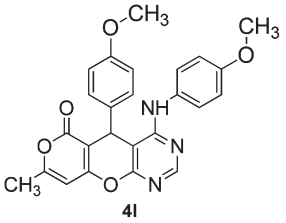
Entry	2-Amino dihydropyrano[4,3-*b*]pyran-3-carbonitriles (**1a–c**)	Aromatic amines (**3a–e**)	Products (**4a–l**)	Time (h)	Yield[a] (%)
1	 **1a**	 **3a**	 **4a**	**2**	**94**
2	 **1a**	 **3b**	 **4b**	3	90
3	 **1a**	 **3c**	 **4c**	2.5	88
4	 **1a**	 **3d**	 **4d**	2.5	90
5	 **1b**	 **3a**	 **4e**	4	92
6	 **1b**	 **3b**	 **4f**	3.5	89
7	 **1b**	 **3c**	 **4g**	2	90

Table 2 *(Contd.)*

Entry	2-Amino dihydropyrano[4,3-*b*]pyran-3-carbonitriles (**1a–c**)	Aromatic amines (**3a–e**)	Products (**4a–l**)	Time (h)	Yield[a] (%)
8	 **1b**	 **3d**	 **4h**	2	91
9	 **1c**	 **3a**	 **4i**	2.5	92
10	 **1c**	 **3b**	 **4j**	4	88
11	 **1c**	 **3c**	 **4k**	3.5	87
12	 **1c**	 **3e**	 **4l**	2	90

[a] Isolated yields after purification.

isolated by filtration, and the filtrate containing the ionic liquid was extracted with ethyl acetate (2 × 20 mL) to remove the non-ionic organic impurities. The water was then evaporated under reduced pressure and the recovered ionic liquid was dried under vacuum and reused four times in subsequent reactions without evident changes in the product yield (Fig. 2).

All synthesized compounds were confirmed by their spectral data (IR, Mass, ^{1}H NMR, and ^{13}C NMR) and elemental analysis. The spectral data for all the compounds were in full agreement with the proposed structures. The structure of compound **4e** was confirmed further by single crystal X-ray diffraction. The molecular structure of **4e** is shown in Fig. 3. A plausible mechanism for the formation of the synthesized compounds, *i.e.*, pyrano[2,3-*d*]pyrimidin-6(5*H*)-one derivatives, **4a–l** is proposed in Scheme 2. 2-Amino-7-methyl-5-oxo-4,5-dihydropyrano[4,3-*b*]pyran-3-carbonitrile upon condensation with DMF-DMA formed the key intermediate (**A**). The *in situ* formed activated imine attacked the aromatic amine to give intermediate (**B**) by the elimination of dimethylamine followed

by intermediate (**C**) formation. The proton of the imidazolium group (C2-H) formed a hydrogen bond with the nitrogen of the nitrile group, which increased its electrophilicity for the intramolecular nucleophilic attack, followed by a proton transformation from (**B**) to (**C**). The hydrolysis of the pyrimidine ring yielded complex (**D**), which upon intramolecular cyclization and subsequent dehydration, produced the corresponding pyrano[2,3-*d*] pyrimidin-6(5*H*)-one derivatives **4a–l**.

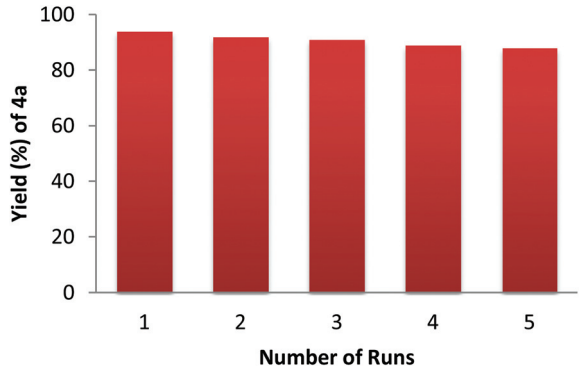


Fig. 2 Recyclability of the [Bmim]HSO_4 ionic liquid used for the synthesis of compound **4a**.

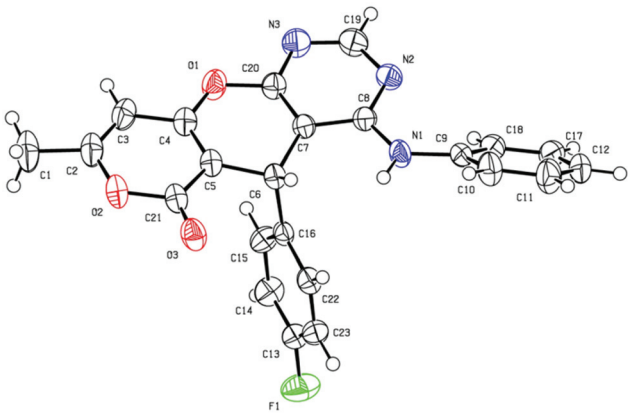
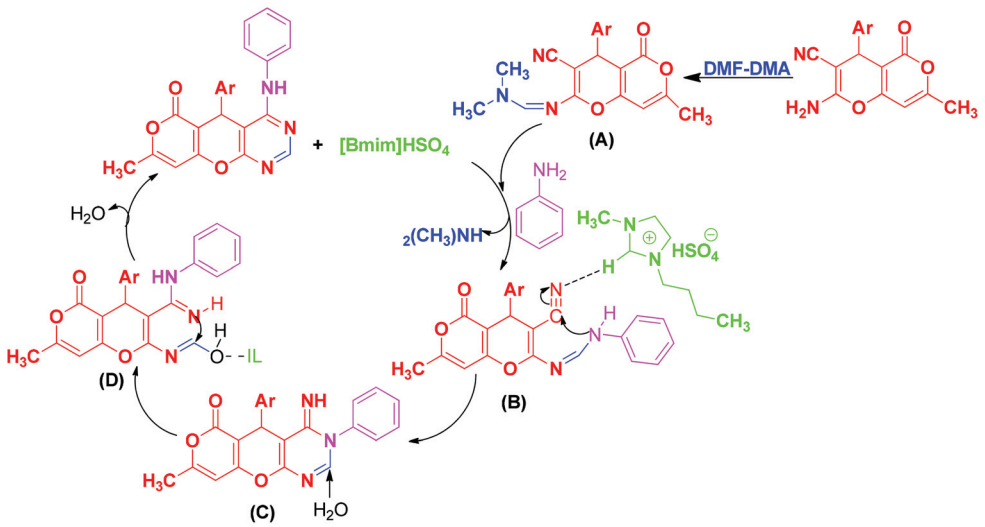


Fig. 3 ORTEP representation of compound **4e**.

2.2. Biological evaluation

All the synthesized compounds were evaluated for their various biological activities, such as antibacterial, minimum bactericidal concentration (MBC), anti-biofilm, antifungal, minimum fungicidal concentration (MFC), and inhibition of ergosterol biosynthesis.

2.2.1. Antibacterial activity. Compounds **4a–l** were screened for antibacterial activity[17] *in vitro* against different Gram-positive and Gram-negative bacterial strains, such as *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS-16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453, and *Klebsiella planticola* MTCC 530. Among the derivatives screened, compound **4l** showed promising activity (MIC values ranging between 3.9–15.6 μg mL^{-1}) against all the bacterial strains except *Pseudomonas aeruginosa* MTCC 2453; however, compound **4h** and **4i** exhibited promising activity (MIC value 7.8 μg mL^{-1}) specifically towards *Bacillus subtilis* MTCC 121 and *Staphylococcus aureus* MTCC 96, respectively. Based on the structure–activity relationship of the synthesized derivatives, it was observed that the compound **4l** has a methoxy substituent attached to the basic pyranopyrimidine scaffold, which has an electron donating property that might be contributing to the antibacterial activity. In the case of compound **4h**, a nitro substituent is attached to the basic pyranopyrimidine scaffold, while compound **4i** has a simple hydrogen atom attached to the basic pyranopyrimidine scaffold. The antibacterial activity results in this regard are tabulated in Table 3.



Scheme 2 Proposed mechanism for the formation of pyrano[2,3-*d*]pyrimidine derivatives **4a–l**.

Table 3 Antimicrobial activity of the synthesized pyrano[2,3-*d*]pyrimidine derivatives

	Minimum inhibitory concentration (μg mL^{-1})						
Test compound	*Micrococcus luteus* MTCC 2470	*Staphylococcus aureus* MTCC 96	*Staphylococcus aureus* MLS-16 MTCC 2940	*Bacillus subtilis* MTCC 121	*Escherichia coli* MTCC 739	*Pseudomonas aeruginosa* MTCC 2453	*Klebsiella planticola* MTCC 530
4a	—	—	31.2	—	—	—	—
4b	—	—	31.2	—	—	—	—
4c	—	31.2	31.2	—	—	—	—
4d	15.6	15.6	31.2	—	—	—	—
4e	—	15.6	—	—	—	—	—
4f	—	—	—	—	—	—	—
4g	—	15.6	—	—	—	—	—
4h	31.2	—	15.6	7.8	—	—	—
4i	—	7.8	—	—	—	—	—
4j	—	—	—	—	—	—	—
4k	—	—	—	—	—	—	—
4l	15.6	15.6	3.9	7.8	7.8	—	7.8
Ciprofloxacin (standard)	0.9	0.9	0.9	0.9	0.9	0.9	0.9

2.2.1.1. Minimum bactericidal concentration (MBC). Based on the antibacterial activity results, the compounds **4a**, **4b**, **4e–k**, and **4l** were screened for the minimum bactericidal concentration[18] against all the bacterial strains except *Pseudomonas aeruginosa* MTCC 2453 compared to ciprofloxacin as a standard. Compound **4l** consistently showed a promising minimum bactericidal concentration and activity against all the bacterial strains tested. Table 4 lists the activity data in this regard.

2.2.2. Biofilm inhibition assay. A biofilm is a structured consortium of bacteria embedded in a self-produced polymeric matrix consisting of polysaccharides, protein and DNA. Bacterial biofilms cause chronic infections in humans *via* hospital and community environments because they show increased tolerance to antibiotics and disinfectant chemicals as well as resisting phagocytosis and other components of the body's defence system.[19] In the medical sector, bacteria colonizes through an adhesion mechanism and results in biofilm formation on several biomedical implants, such as stents, heart valves, vascular grafts, and catheters.[20] In this context, the novel compounds that can specifically target and inhibit the biofilm formation would be of great interest compared to the rational use of antibiotics and/or biocides. Considering these facts, a further step was undertaken to determine if these compounds exhibit a specific anti-biofilm activity or whether this observation was related simply to a general toxic effect on the Gram-positive bacterial strains. To this regard, compounds **4a**, **4b**, **4e–k**, and **4l** were screened for their anti-biofilm activity[21] against *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* and *Klebsiella planticola* MTCC 530, which is a common and important nosocomial pathogens with biofilm forming ability. The results summarized in Table 5, clearly show that not much information on the structure–activity relationship (SAR) can be highlighted at this stage; however, it was observed that compound **4l** exhibited promising activity (IC_{50} values ranging between 2.5–11.5 μM) towards all the bacterial species tested, whereas compound **4i** showed specific activity towards *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MLS16 MTCC 2940, and *Bacillus subtilis* MTCC 121, and compound **4f** showed specific activity

Table 4 Minimum bactericidal concentration assay (MBC) of the synthesized pyrano[2,3-*d*]pyrimidine derivatives

	Minimum bactericidal concentration (μg mL^{-1})					
Test compound	*Micrococcus luteus* MTCC 2470	*Staphylococcus aureus* MTCC 96	*Staphylococcus aureus* MLS-16 MTCC 2940	*Bacillus subtilis* MTCC 121	*Escherichiacoli* MTCC 739	*Klebsiella planticola* MTCC 530
4a	>125	>125	62.5	>125	>125	>125
4b	>125	>125	62.5	>125	>125	>125
4c	>125	62.5	31.2	>125	>125	>125
4d	31.2	31.2	62.5	>125	>125	>125
4e	>125	31.2	>125	>125	>125	>125
4g	>125	31.2	>125	>125	>125	>125
4h	62.5	>125	31.2	15.6	>125	>125
4i	>125	15.6	>125	>125	>125	>125
4l	31.2	31.2	7.8	7.8	15.6	15.6
Ciprofloxacin (standard)	0.9	1.9	1.9	0.9	1.9	1.9

Table 5 Biofilm inhibition assay of the synthesized pyrano[2,3-*d*]pyrimidine derivatives

	IC_{50} values in (μM)					
Test compound	*Micrococcus luteus* MTCC 2470	*Staphylococcus aureus* MTCC 96	*Staphylococcus aureus* MLS-16 MTCC 2940	*Bacillus subtilis* MTCC 121	*Escherichia coli* MTCC 739	*Klebsiella planticola* MTCC 530
4a	—[a]	—	22.4 ± 0.52	—	—	—
4b	—	—	16.8 ± 0.44	—	—	—
4c	—	18.9 ± 0.38	15.6 ± 0.36	—	—	—
4d	10.2 ± 0.28	11.4 ± 0.26	22.4 ± 0.28	—	—	—
4e	—	9.8 ± 0.22	—	—	—	—
4g	—	8.6 ± 0.32	—	—	—	—
4h	17.4 ± 0.34	—	9.2 ± 0.24	4.5 ± 0.26	—	—
4i	—	4.9 ± 0.18	—	—	—	—
4l	11.5 ± 0.26	9.2 ± 0.24	2.5 ± 0.18	3.8 ± 0.26	4.2 ± 0.23	4.6 ± 0.18
Ciprofloxacin (standard)	0.5 ± 0.08	0.3 ± 0.11	0.4 ± 0.12	0.6 ± 0.08	0.4 ± 0.09	0.5 ± 0.10

[a] 125 μg mL^{-1} of IC_{50} value.

towards *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MTCC 96 and *Staphylococcus aureus* MLS16 MTCC 2940. Some of the compounds (**4g**, **4h** and **4j**) showed anti-biofilm activity specifically towards *Staphylococcus aureus* MLS16 MTCC 2940 with IC_{50} values of 9.8, 8.6 and 4.9 μM, respectively. The basic pyranopyrimidine scaffold of these compounds possesses different substituents, which exhibit electron donating or electron withdrawing properties that antagonize the biofilm formation and might contribute to the anti-biofilm activity. Table 5 lists the activity data in this regard.

2.2.3. Antifungal activity. Different *Candida* species are important opportunistic fungal pathogens that frequently cause infections within immunocompromised patients undergoing cancer chemotherapy, broad-spectrum antibiotics, and/or among HIV-infected individuals. Among the many pathogenic *Candida* species, *Candida albicans* is the major fungal pathogen of utmost importance to humans. Owing to its versatility, it can behave as a commensal organism posing a major problem from a clinical perspective, resulting in chronic infections.[22] Further, different *Candida* strains have the ability to produce extracellular polymeric substances (EPS) and become encased in this matrix to form biofilms, which develop on the surfaces of prosthesis and medical devices.[23,24] Considering these facts, we screened the selected compounds such as **4a**, **4b**, **4c**, **4d**, **4h** and **4l** against different *Candida* strains and among them, compounds **4l** and **4h** showed promising anti-*Candida* activity against many *Candida* strains with a MIC value of 7.8 μg per meal comparable to the standard miconazole drug. The other compounds showed good to moderate activity (MIC values ranging between 7.8–62.5 μg mL^{-1}) against different *Candida* strains. The results of the antifungal activity are tabulated in Table 6.

2.2.3.1. Minimum fungicidal concentration (MFC). Based on the antifungal activity results, the selected compounds **4a**, **4b**, **4c**, **4d**, **4h** and **4l** were evaluated further for their minimum fungicidal concentrations (MFC) against different *Candida* strains in comparison to the standard miconazole drug. All the compounds showed minimum fungicidal concentration (MFC) values ranging between 7.8–62.5 μg mL^{-1}. However, the standard miconazole drug exhibited MFC values ranging between 7.8–15.6 μg mL^{-1}. Among them, compound **4l** showed promising activity against *Candida albicans* MTCC 1637 and *C. albicans* MTCC 4748 with a lower MFC value of 7.8 μg mL^{-1}. The MFC activity data in this regard is tabulated in Table 7.

2.2.3.2. Inhibition of ergosterol biosynthesis in Candida albicans MTCC 1637. Candida albicans is now recognized as a major cause of hospital-acquired infections.[25] Most of the antifungal drugs currently available to treat *Candida* infections target the ergosterol biosynthetic pathway or its end product ergosterol. In view of this fact, we further investigated the promising test compound **4l** in comparison to the standard miconazole drug to delineate its mode of action in the ergosterol biosynthetic pathway for one of the susceptible strain of *C. albicans* MTCC 1637. In this regard, the UV spectral scans of the sterol profiles for the representative strain of *C. albicans* MTCC 1637 were determined and later the total ergosterol content was quantified from the data obtained on culturing the *C. albicans* MTCC 1637 strain with different concentrations (0, 2, 4, and 16 μg mL^{-1}) of the test compound **4l** and the standard miconazole drug (see Table 8). Based on the results presented in Fig. 4, it was observed that the ergosterol content decreased significantly with increasing concentration of test compound **4l**. Similarly, a dose-dependent decrease in the ergosterol content was observed when the *C. albicans* MTCC 1637 strain was cultured in the presence of miconazole. These findings suggest that the pyranopyrimidine derivative **4l** altered the sterol profile, which might contribute to its antifungal activity through the inhibition of ergosterol biosynthesis. The selective cytotoxic behavior of this compound hints at its affinity to the specific target site in the ergosterol biosynthetic pathway. The *Candida*-cidal activity of the compound **4l** might also be responsible for the direct damage to the cell membrane. However, the precise mechanism of action of this compound needs to be elucidated further.

Table 6 Antifungal activity of the synthesized pyrano[2,3-*d*]pyrimidine derivatives

	Minimum inhibitory concentration (μg mL^{-1})													
Test compound	*C. albicans* MTCC 183	*C. albicans* MTCC 227	*C. albicans* MTCC 854	*C. albicans* MTCC 1637	*C. albicans* MTCC 3017	*C. albicans* MTCC 3018	*C. albicans* MTCC 3958	*C. albicans* MTCC 4748	*C. albicans* MTCC 7315	*C. parapsilosis* MTCC 1744	*C. aaseri* MTCC 1962	*C. glabrata* MTCC 3019	*C. krusei* MTCC 3020	*Issatchenkia hanoiensis* MTCC 4755
4a	31.2	62.5	31.2	31.2	31.2	62.5	15.6	15.6	31.2	62.5	62.5	62.5	31.2	31.2
4b	62.5	62.5	31.2	31.2	31.2	62.5	31.2	15.6	31.2	31.2	62.5	31.2	62.5	62.5
4c	31.2	31.2	62.5	62.5	31.2	31.2	62.5	15.6	15.6	31.2	62.5	62.5	31.2	31.2
4d	31.2	31.2	15.6	15.6	31.2	31.2	15.6	62.5	31.2	15.6	7.8	15.6	15.6	7.8
4h	31.2	7.8	7.8	15.6	15.6	15.6	15.6	7.8	15.6	31.2	62.5	31.2	15.6	15.6
4l	7.8	15.6	7.8	7.8	7.8	15.6	7.8	7.8	15.6	31.2	15.6	7.8	15.6	7.8
Miconazole (standard)	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8

Table 7 Minimum fungicidal concentration (MFC) of the synthesized pyrano[2,3-*d*]pyrimidine derivatives

	Minimum inhibitory concentration (μg mL^{-1})												
Test compound	*C. albicans* MTCC 183	*C. albicans* MTCC 227	*C. albicans* MTCC 854	*C. albicans* MTCC 1637	*C. albicans* MTCC 3018	*C. albicans* MTCC 3958	*C. albicans* MTCC 4748	*C. albicans* MTCC 7315	*C. parapsilosis* MTCC 1744	*C. aaseri* MTCC 1962	*C. glabrata* MTCC 3019	*C. krusei* MTCC 3020	*Issatchenkia hanoiensis* MTCC 4755
4a	62.5	62.5	62.5	62.5	125	31.2	31.2	31.2	62.5	125	125	62.5	31.2
4b	62.5	125	62.5	62.5	125	62.5	15.6	62.5	62.5	62.5	62.5	125	125
4c	31.2	62.5	125	125	62.5	125	31.2	15.6	62.5	125	125	62.5	31.2
4d	62.5	62.5	31.2	15.6	62.5	31.2	125	62.5	15.6	15.6	31.2	31.2	15.6
4h	31.2	15.6	15.6	31.2	15.6	31.2	15.6	15.6	62.5	62.5	62.5	31.2	31.2
4l	15.6	15.6	15.6	7.8	31.2	15.6	7.8	15.6	62.5	31.2	15.6	15.6	15.6
Miconazole (standard)	15.6	7.8	7.8	15.6	15.6	7.8	15.6	7.8	7.8	7.8	15.6	7.8	7.8

Table 8 Ergosterol biosynthesis inhibition (*C. albicans* MTCC 1637)

	Mean ergosterol content of cells grown with compounds at a concentration (μg ml^{-1})				
Compound	0	2	4	8	16
4l	1.62 ± 0.12	1.18 ± 0.09	0.78 ± 0.09	0.39 ± 0.07	0.19 ± 0.01
Miconazole (standard)	1.62 ± 0.12	0.98 ± 0.11	0.54 ± 0.06	0.22 ± 0.03	0.11 ± 0.009

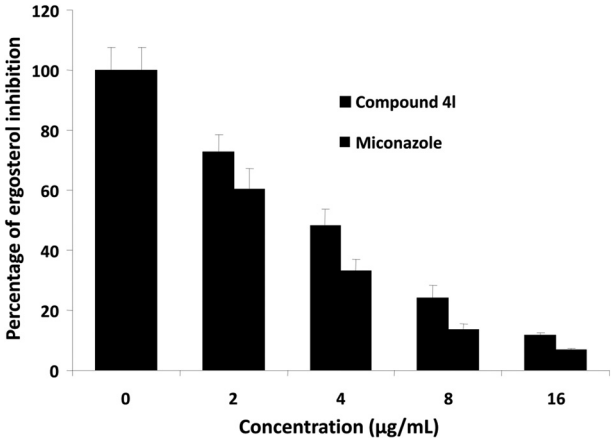


Fig. 4 Effect of compound **4l** on the inhibition of ergosterol biosynthesis in *Candida albicans* MTCC 1637.

3. Conclusion

In summary, we achieved an efficient protocol for a one-pot three-component reaction of 2-amino-7-methyl-5-oxo-4-phenyl-4,5-dihydropyrano[4,3-*b*]pyran-3-carbonitriles, DMF-DMA and arylamines using [Bmim]HSO_4 ionic liquid. This method has several advantages such as high yields, environmentally benign and milder reaction conditions. Furthermore, among the screened pyranopyrimidine derivatives, compound **4l** was considerably promising and was identified as a lead compound exhibiting antibacterial, antifungal and anti-biofilm activities.

4. Experimental section

4.1. Chemistry

The melting points were recorded on a Stuart SMP30 melting point apparatus and were uncorrected. Column chromatography was performed using silica gel (60–120 mesh size) purchased from Thomas Baker and Thin layer chromatography (TLC) was carried out using the aluminium sheets pre-coated with silica gel $60F_{254}$ purchased from Merck. IR spectra (KBr) were taken on Bruker WM-4 (X) spectrometer (577 model). The ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker WM-400 spectrometer at 400 MHz and 100 MHz, respectively, in DMSO-d_6 with TMS as an internal standard. The chemical shifts were reported in ppm (δ). The mass spectra (ESI) were carried out on a Jeol JMSD-300 spectrometer. CHN analysis was carried out using a Carlo Erba EA 1108 automatic elemental analyzer. All the chemicals and solvents were of analytical or synthetic grade and used as received unless stated otherwise. The starting materials used in the present study **1a–c** were prepared based on the literature methods and were identified by a comparison of the physical data (mp) with the literature.[26]

4.1.1. General procedure for the synthesis of pyrano[2,3-*d*]-pyrimidine derivatives (4a–l). A dry 50 mL flask was charged with 2-amino-7-methyl-5-oxo-4-phenyl-4,5-dihydropyrano[4,3-*b*]-pyran-3-carbonitriles **1** (1 mmol), DMF-DMA **2** (1.2 mmol) and ionic liquid [Bmim]HSO_4 (2 mL). The reaction mixture was stirred at 80 °C for 60–90 min. The progress of the reaction was monitored by TLC and after completion of the reaction (single spot on TLC), aromatic amine **3** (1 mmol) was added and the reaction was continued for an additional 60–150 min. The progress of the reaction was monitored by TLC (eluent = *n*-hexane/ethyl acetate: 8/2). After the reaction was complete, the reaction mixture was cooled to RT and poured into ice cold water, the solid separated was filtered, washed with water, dried, and purified by column chromatography using silica gel (ethyl acetate/*n*-hexane: 2/8) to afford the title compounds **4a–l** in good yields.

*4.1.1.1. 8-Methyl-5-phenyl-4-(phenylamino)pyrano[3,4,5,6]-pyrano[2,3-d]pyrimidin-6(5H)-one (**4a**).* White powder; mp: 262–264 °C; IR (KBr) ν_{max} (cm^{-1}): 3352, 3086, 1694, 1597, 1571, 1398, 1260; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H), 5.55 (s, 1H), 6.52 (s, 1H), 7.03 (t, 1H), 7.17 (t, 1H), 7.25–7.29 (m, 4H), 7.41 (d, 2H), 7.54 (d, 2H), 8.35 (s, 1H), 8.74 (s, 1H)); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.36, 32.27, 98.63, 102.23, 121.65, 123.48, 127.22, 128.21, 138.98, 142.08, 156.32, 159.29, 160.92, 161.70, 162.87; MS-ESIMS: *m*/*z* 384 (M + 1)$^+$; Anal. Calcd for $C_{23}H_{17}N_3O_3$: C, 72.05; H, 4.47; N, 10.96; found: C, 72.11; H, 4.42; N, 10.91.

*4.1.1.2. 4-((4-Chlorophenyl)amino-8-methyl-5-phenylpyrano-[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4b**).* Yellow powder; mp: 272–275 °C; IR (KBr) ν_{max} (cm^{-1}): 3372, 3086, 1699, 1606, 1567, 1439, 1262; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H), 5.54 (s, 1H), 6.53 (s, 1H), 7.17 (t, 1H), 7.26 (t, 2H), 7.33 (d, 2H), 7.40 (t, 2H), 7.61 (d, 2H), 8.38 (s, 1H), 8.86 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.38, 21.05, 32.23, 98.38, 98.61, 102.10, 122.97, 125.59, 127.07, 128.11, 128.07, 128.14, 128.32, 128.45, 128.75, 138.02, 142.01, 156.31, 159.25, 161.32, 162.95; MS-ESIMS: *m*/*z* 418 (M + 1)$^+$; Anal. Calcd for $C_{23}H_{16}ClN_3O_3$: C, 66.11; H, 3.86; N, 10.06; found: C, 66.06; H, 3.89; N, 10.13.

*4.1.1.3. 8-Methyl-4-((3-nitrophenyl)amino)-5-phenylpyrano-[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4c**).* White powder; mp: 268–270 °C; IR (KBr) ν_{max} (cm^{-1}): 3394, 3005, 1702, 1650, 1565, 1384, 1248; ^1H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 5.62 (s, 1H), 6.55 (s, 1H), 7.16 (t, 1H), 7.26 (t, 2H), 7.41 (d, 2H), 7.57 (t, 1H), 7.88 (t, 1H), 8.06 (d, 1H), 8.48 (s, 1H), 8.60 (s, 1H), 9.24 (s, 1H)); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.39, 32.22, 98.61, 100.09, 102.24, 115.00, 117.58, 127.12, 127.31, 128.26, 129.80, 140.38, 141.39, 147.84, 156.33, 158.51, 159.36, 161.29, 161.73, 163.02; MS-ESIMS: *m*/*z* 430 $(M + 1)^+$; Anal. Calcd for $C_{23}H_{16}N_4O_5$: C, 64.48; H, 3.76; N, 13.08; found: C, 64.40; H, 3.71; N, 13.02.

*4.1.1.4. 8-Methyl-4-((4-nitrophenyl)amino)-5-phenylpyrano-[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4d**).* White powder; mp: 273–275 °C; IR (KBr) ν_{max} (cm^{-1}): 3374, 3081, 1696, 1608, 1570, 1438, 1247; ^1H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 5.68 (s, 1H), 6.55 (s, 1H), 7.13–7.18 (m, 1H), 7.25 (t, 2H), 7.39 (d, 2H), 7.91 (d, 2H), 8.17 (d, 2H), 8.53 (s, 1H), 9.39 (s, 1H)); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.92, 36.46, 100.91, 101.94, 112.24, 119.24, 124.73, 125.67, 129.80, 137.90, 142.34, 147.02, 153.26, 156.45, 159.68, 162.62, 164.27, 165.34, 174.64; MS-ESIMS: *m*/*z* 429 $(M + 1)^+$; Anal. Calcd for $C_{23}H_{16}N_4O_5$: C, 64.48; H, 3.76; N, 13.08; found: C, 64.39; H, 3.71; N, 13.01.

*4.1.1.5. 5-(4-Fluorophenyl)-8-methyl-4-(phenylamino)pyrano-[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4e**).* White powder; mp: 285–287 °C; IR (KBr) ν_{max} (cm^{-1}): 3370, 3065, 1691, 1599, 1498, 1397, 1260; ^1H NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H), 5.55 (s, 1H), 6.52 (s, 1H), 7.04–7.12 (m, 3H), 7.28 (t, 2H), 7.43–7.47 (m, 2H), 7.52 (d, 2H), 8.36 (s, 1H), 8.74 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.41, 31.66, 98.69, 98.81, 102.09, 115.09, 115.30, 121.83, 123.65, 128.48, 130.13, 130.21, 138.21, 138.96, 156.47, 158.90, 159.19, 159.99, 160.94, 161.76, 162.41, 163.03; MS-ESIMS: *m*/*z* 402 $(M + 1)^+$; Anal. Calcd for $C_{23}H_{16}FN_3O_3$: C, 68.82; H, 4.02; N, 10.47; found: C, 68.75; H, 4.09; N, 10.42.

*4.1.1.6. 4-((4-Chlorophenyl)amino)-5-(4-fluorophenyl)-8-methylpyrano[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4f**).* Light yellow powder; mp: 280–282 °C; IR (KBr) ν_{max} (cm^{-1}): 3352, 3083, 1694, 1667, 1505, 1490, 1229; ^1H NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H), 5.55 (s, 1H), 6.52 (s, 1H), 7.09 (t, 2H), 7.34 (d, 2H), 7.42–7.46 (m, 2H), 7.60 (d, 2H), 8.39 (s, 1H), 8.86 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.38, 31.57, 98.62, 99.17, 102.04, 115.18, 123.09, 123.65, 125.59, 127.18, 128.15, 128.33, 130.05, 130.13, 138.05, 156.41, 159.34, 161.00, 162.37, 163.03; MS-ESIMS: *m*/*z* 436 $(M + 1)^+$; Anal. Calcd for $C_{23}H_{15}ClFN_3O_3$: C, 63.38; H, 3.47; N, 9.64; found: C, 63.29; H, 3.41; N, 9.69.

*4.1.1.8. 5-(4-Fluorophenyl)-8-methyl-4-((3-nitrorophenyl)-amine)pyrano[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4g**).* Yellow powder; mp: 281–283 °C; IR (KBr) ν_{max} (cm^{-1}): 3377, 3081, 1696, 1609, 1570, 1439, 1248; ^1H NMR (400 MHz, DMSO-d_6): δ 2.05 (s, 3H), 5.66 (s, 1H), 6.82 (d, 1H), 7.05 (t, 1H), 7.22 (t, 1H), 7.30 (t, 1H), 7.41 (d, 1H), 7.53 (d, 1H), 7.58 (d, 1H), 7.73 (t, 1H) 8.02 (d, 1H), 8.41 (s, 1H), 8.80 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.52, 33.42, 98.53, 98.77, 102.42, 116.41, 122.07, 129.13, 130.85, 131.06, 138.72, 139.61, 147.81, 157.17, 158.88, 159.42, 159.91, 160.92, 161.92, 162.51, 163.12; MS-ESIMS: *m*/*z* 447 $(M + 1)^+$; Anal. Calcd for $C_{23}H_{15}FN_4O_5$; C, 61.88; H, 3.39; N, 12.55; found: C, 61.77; H, 3.31; N, 12.46.

*4.1.1.7. 5-(4-Fluorophenyl)-8-methyl-4-((4-nitrorophenyl)-amine)pyrano[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4h**).* Yellow powder; mp: 289–291 °C; IR (KBr) ν_{max} (cm^{-1}): 3334, 3092, 1691, 1601, 1509, 1438, 1257; ^1H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 5.63 (s, 1H), 6.55 (s, 1H), 7.09 (t, 2H), 7.47–7.43 (m, 2H), 7.57 (t, 1H), 7.88 (d, 1H), 8.05 (d, 1H), 8.49 (s, 1H), 8.59 (s, 1H), 9.23 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 19.49, 32.36, 98.44, 98.91, 102.19, 115.13, 121.37, 128.83, 130.15, 130.26, 138.91, 147.91, 156.57, 158.93, 159.22, 159.99, 160.97, 161.86, 162.48, 163.02; MS-ESIMS: *m*/*z* 447 $(M + 1)^+$; Anal. Calcd for $C_{23}H_{15}FN_4O_5$; C, 61.88; H, 3.39; N, 12.55; found: C, 61.79; H, 3.34; N, 12.48.

*4.1.1.9. 5-(4-Methoxyphenyl)-8-methyl-4-(phenylamino)-pyrano[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4i**).* White powder; mp: 263–265 °C; IR (KBr) ν_{max} (cm^{-1}): 3394, 3005, 1702, 1650, 1565, 1446, 1248; ^1H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 3.65 (s, 3H), 5.66 (s, 1H), 6.82 (d, 2H), 7.05 (t, 1H), 7.30 (t, 2H), 7.41 (d, 1H), 7.54–7.47 (d, 1H), 7.59 (d, 1H), 7.76–7.71 (m, 1H), 8.04 (m, 1H), 8.41 (s, 1H), 8.80 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.98, 36.87, 55.8, 100.92, 101.24, 112.26, 114.21, 117.86, 122.40, 129.52, 130.02, 134.67, 140.43, 153.29, 157.64, 158.32, 162.48, 164.69, 167.06, 173.38; MS-ESIMS: *m*/*z* 414 $(M + 1)^+$; Anal. Calcd for $C_{24}H_{19}N_3O_4$; C, 69.72; H, 4.63; N, 10.16; found: C, 69.64; H, 4.67; N, 10.09.

*4.1.1.10. 4-((4-Chlorophenyl)amino)-5-(4-methoxyphenyl)-8-methylpyrano[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4j**).* Yellow powder; mp: 267–269 °C; IR (KBr) ν_{max} (cm^{-1}): 3369, 2931, 1696, 1649, 1566, 1490, 1255; ^1H NMR (400 MHz, DMSO-d_6): δ 1.91 (s, 3H), 3.65 (s, 3H), 5.66 (s, 1H), 6.82 (d, 2H), 7.41–7.39 (m, 2H), 7.47–7.53 (m, 1H), 7.67 (d, 2H), 7.73 (t, 1H), 8.02 (d, 1H), 8.44 (s, 1H), 8.92 (s, 1H);); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.14, 36.50, 55.89, 100.92, 101.90, 114.26, 118.45, 119.49, 122.17, 129.60, 130.03, 134.69, 139.06, 148.05, 157.65, 158.33, 159.87, 162.33, 167.09, 175.56; MS-ESIMS: *m*/*z* 448 $(M + 1)^+$; Anal. Calcd for $C_{24}H_{18}ClN_3O_4$; C, 64.36; H, 4.05; N, 9.38; found: C, 64.29; H, 4.11; N, 9.28.

*4.1.1.11. 5-(4-Methoxyphenyl)-8-methyl-4-((3-nitrophenyl)-amino)pyrano[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4k**).* Yellow powder; mp: 272–274 °C; IR (KBr) ν_{max} (cm^{-1}): 3363, 3078, 1711, 1609, 1570, 1442, 125; ^1H NMR (400 MHz, DMSO-d_6): δ 1.91 (s, 3H), 3.65 (s, 3H), 5.73 (s, 1H), 7.41 (d, 2H), 7.53–7.40 (m, 1H), 7.59 (t, 1H), 7.73 (t, 1H), 7.90 (t, 1H), 8.02 (d, 1H), 8.12 (d, 1H), 8.54 (s, 1H), 8.65 (s, 1H), 9.29 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.01, 32.09, 54.99, 100.19, 105.79, 113.32, 113.96, 115.07, 116.61, 117.63, 122.61, 124.97, 127.16, 129.40, 129.82, 132.97, 133.63, 140.40, 147.86, 152.09, 154.21, 156.28, 158.44, 158.53, 159.90, 161.04, 172.12; MS-ESIMS: *m*/*z* 459 $(M + 1)^+$; Anal. Calcd for $C_{24}H_{18}N_4O_6$; C, 62.88; H, 3.96; N, 12.22; found: C, 62.95; H, 3.90; N, 12.12.

*4.1.1.12. 5-(4-Methoxyphenyl)-4-(4-methoxyphenyl)amino)-8-methylpyrano[3,4,5,6]pyrano[2,3-d]pyrimidin-6(5H)-one (**4l**).* White powder; mp: 256–259 °C; IR (KBr) ν_{max} (cm^{-1}): 3381, 3058, 1695, 1645, 1571, 1509, 1244; ^1H NMR (400 MHz,

DMSO-d_6): δ 2.22 (s, 3H), 3.66 (s, 3H), 3.72 (s, 3H), 5.57 (s, 1H), 6.83 (d, 2H), 6.88 (d, 2H), 7.40–7.43 (m, 1H), 7.47–7.53 (m, 2H), 7.70–7.75 (m, 1H), 8.00–8.02 (m, 1H), 8.34 (s, 1H), 8.70 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.10, 32.08 54.94, 55.17, 98.33, 105.77, 113.36, 113.60, 113.78, 116.52, 122.54, 123.78, 124.83, 129.39, 131.81, 132.80, 133.73, 152.03, 154.19, 155.73, 156.25, 158.33, 158.93, 159.81, 160.48; MS-ESIMS: m/z 444 $(M + 1)^+$; Anal. Calcd for $C_{25}H_{21}N_3O_5$; C, 67.71; H, 4.77; N, 9.48; found: C, 67.63; H, 4.72; N, 9.39.

4.2. X-ray crystallographic study

	4e
Formula weight	401.39
Temperature	298 (2) K
Wavelength	0.71073 Å
Crystal system, space group	Monoclinic, $P21/n$
Unit cell dimension	a = 12.6990 (4) α = 90°
	b = 9.0798 (3) β = 101.3230° (10)
	c = 16.4608 (5) λ = 90°
Volume	1861.06 (10) A^3
Z, Density (calculated)	4, 1.282 Mg m^{-3}
Adsorption coefficient	0.084 mm^{-1}
$F_{(000)}$	584
Crystal size	0.40 mm
Theta range for data collection	1.86–28.3°
R Indices [$I > 2\sigma(I)$]	R = 0.0443, wR_2 = 0.1191
R Indices (all data)	R = 0.0736, wR_2 = 0.1442
Reflections collected	20 635/4583 [R(int) = 0.0323]
Independent reflections	856
Completeness to theta max	0.996
Absorption correction	0.9900
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	4583/0/272
Max. and min. transmission	0.9796 and 0.9646
Goodness of fit	1.060
Data/restraints/parameters	110 529f
Fine R indices [$I > 2\sigma(I)$]	0.0738
R indices (all data)	0.1259
Largest diff. peak and hole	0.242 and −0.266 A^{-3}

X-ray data for the compounds was collected at room temperature using a Bruker Smart Apex CCD diffractometer with graphite monochromated MoKα radiation (λ = 0.71073 Å) using the ω-scan method.[27] Preliminary lattice parameters and orientation matrices were obtained from four sets of frames. Integration and scaling of the intensity data were accomplished using the SAINT program.[27] The structure was solved by direct methods using SHELXS9735, and refinement was carried out by the full-matrix least-squares technique using SHELXL97.

4.3. Biological evaluation

4.3.1. Antibacterial activity. The antibacterial activity of the synthesized pyranopyrimidine derivatives was determined using the well diffusion method[17] against different pathogenic bacterial strains procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. The pathogenic reference strains were seeded on the surface of the Muller-Hinton agar Petri plates with 0.1 ml of previously prepared microbial suspensions individually containing 1.5 × 10^8 cfu ml^{-1} (equal to 0.5 McFarland standard). Wells, 6.0 mm in diameter, were prepared in the media plates using a cork borer and the synthesized compounds dissolved in 10% DMSO at a dose range of 125–0.97 μg mL^{-1} were added in each well under sterile conditions in a laminar air flow chamber. Standard antibiotic solutions of ciprofloxacin at a dose range of 125–0.97 μg per well served as a positive control, while the well containing DMSO served as the negative control. The plates were incubated for 24 h at 37 °C for the different bacterial strains. The well containing the lowest concentration showing the inhibition zone is considered the minimum inhibitory concentration. All the experiments were carried out in duplicates and the mean values are represented.

4.3.2. Minimum bactericidal concentration (MBC) assay. A minimum bactericidal concentration assay[18] was performed in sterile 2.0 mL microfuge tubes against a panel of pathogenic bacterial strains, including *Micrococcus luteus* MTCC 2470, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS-16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739, *Pseudomonas aeruginosa* MTCC 2453, and *Klebsiella planticola* MTCC 530, cultured overnight in Mueller Hinton broth. Serial dilutions of the test compounds at different concentrations, ranging from 0 to 125 μg mL^{-1}, were prepared in Mueller Hinton broth. To the test compounds, 100 μL of overnight cultured bacterial suspensions were added to reach a final concentration of 1.5 × 10^8 cfu mL^{-1} (equal to 0.5 McFarland standard) and incubated at 37 °C for 24 h. After 24 h incubation, the minimum bactericidal concentration (MBC) was determined by sampling 10 μL of the suspension from the tubes onto Mueller Hinton agar plates and incubated for 24 h at 37 °C to observe the growth of the test organisms. MBC is the lowest concentration of the test compound required to kill a particular bacterium strain. All the experiments were carried in duplicate and the mean values are represented.

4.3.3. Biofilm inhibition assay. The test compounds were screened in sterile 96 well polystyrene microtiter plates using the modified biofilm inhibition assay[21] against a panel of pathogenic bacterial strains including *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS16 MTCC 2940, *Bacillus subtilis* MTCC121, *Pseudomonas aeruginosa* MTCC 2453, and *Klebsiella planticola* MTCC 530, which were cultured overnight in tryptone soy broth (supplemented with 0.5% glucose). The test compounds at predetermined concentrations ranging from 0 to 250 μg mL^{-1} were mixed with the bacterial suspensions with an initial inoculum concentration of 5 × 10^5 cfu mL^{-1}. Aliquots of 100 μL were distributed in each well and then incubated at 37 °C for 24 h under static conditions. The medium was then discarded and washed with phosphate buffered saline to remove the non-adherent bacteria. Each well of the microtiter plate was stained with 100 μL of a 0.1% crystal violet solution followed by 30 min incubation at room temperature. Later, the crystal violet solution from the plates was discarded, washed thoroughly with distilled water 3 to 4

times and air dried at room temperature. The crystal violet stained biofilm was solubilized in 95% ethanol (100 μL) and the absorbance was recorded at 540 nm using a TRIAD multimode reader (Dynex Technologies, Inc., Chantilly, VA, USA). Blank wells were employed as a background check. The inhibition data was interpreted from the dose–response curves, where the IC_{50} value is defined as the concentration of the inhibitor required to inhibit 50% of biofilm formation under the above assay conditions. All the experiments were carried out in triplicate and the values are indicated as the mean ± S.D.

4.3.4. Antifungal activity. The antifungal activity of the synthesized pyranopyrimidine derivatives was determined using a well diffusion method[17] against different *Candida* strains such as *Candida albicans* MTCC 183, *C. albicans* MTCC 227, *C. albicans* MTCC 854, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, *C. albicans* MTCC 3958, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. aaseri* MTCC 1962, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020 and *Issatchenika hanoiensis* MTCC 4755 procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. The pathogenic reference strains were seeded on the surface of the Muller-Hinton agar Petri plates with 0.1 ml of previously prepared microbial suspensions individually containing 1.5×10^8 cfu ml^{-1} (equal to 0.5 McFarland standard). Wells, 6.0 mm in diameter, were prepared in the media plates using a cork borer and the synthesized compounds dissolved in 10% DMSO at a dose range of 125–0.97 μg mL^{-1} were added to each well under sterile conditions in a laminar air flow chamber. Standard antibiotic solutions of Miconazole at a dose range of 125–0.97 μg per well, served as positive control, while the well containing DMSO served as negative control. The plates were incubated for 24 h at 30 °C for different *Candida* strains. The well containing the least concentration showing that the inhibition zone is considered as the minimum inhibitory concentration. All the experiments were carried out in duplicate and mean values are represented.

4.3.5. Minimum fungicidal concentration (MFC) assay. Fungicidal assays were performed in sterile 2.0 ml microfuge tubes. Different *Candida* strains, such as *Candida albicans* MTCC 183, *C. albicans* MTCC 227, *C. albicans* MTCC 854, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, *C. albicans* MTCC 3958, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. aaseri* MTCC 1962, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020 and *Issatchenika hanoiensis* MTCC 4755, were cultured overnight in Sabouraud dextrose broth. Serial dilutions of test compounds in different concentrations ranging from 0 to 150 μg mL^{-1} were prepared in Sabouraud dextrose broth. To the test compounds, 100 μL of overnight cultured bacterial suspensions were added to reach a final concentration of 1.5×10^8 cfu mL^{-1} (equal to 0.5 McFarland standard) and incubated at 30 °C for 24 h. After 24 h incubation, the MFC was determined by sampling 10 μL of the suspension from the tubes onto fresh plates of Sabouraud dextrose agar to observe the growth of the fungi. The plates were incubated for 24 h at 30 °C. All the experiments were carried in duplicate and the mean values are represented, where MFC is the lowest concentration of the compound required to kill a particular *Candida* strain.

4.3.6. Quantification of ergosterol content in Candida albicans MTCC 1637. The total intracellular sterols from *Candida albicans* MTCC 1637 were extracted using the method reported by Breivik and Owades[28] with slight modifications. A single *C. albicans* colony cultured overnight in Sabouraud dextrose agar was inoculated with 50 ml of Sabouraud dextrose broth containing various concentrations of the test compounds, including 0, 2, 4, and 16 μg mL^{-1}. The culture was incubated at 30 °C for 20 h with continuous shaking. The stationary phase cells were harvested by centrifugation at 8000 rpm for 5 min and washed with sterile distilled water. The net wet weight of the cell pellet was determined. Three milliliters of a 25% alcoholic potassium hydroxide solution were added to each pellet and vortexed for 1 min. The cell suspensions were transferred to sterile glass screw-cap tubes and incubated in a water bath at 85 °C for 1 h and then allowed to cool to room temperature. Sterols were then extracted by the addition of a mixture of sterile distilled water and *n*-heptane (1 : 3) followed by vigorous vortexing for 3 to 4 min. The heptane layer was transferred to a clean glass tube and stored at −20 °C for 24 h duration. An aliquot (20 μL) of the sterol extract diluted five-fold in 100% ethanol was scanned spectrophotometrically from 240 to 300 nm. The presence of ergosterol and 24(28) dehydroergosterol [24(28) DHE, a late sterol pathway intermediate] in the extracted sample exhibited a characteristic four-peaked curve. The absence of detectable ergosterol content in the extracts was indicated by a flat line. A dose-dependent decrease in the height of the absorbance peaks was evident, which corresponded to the decreased ergosterol concentration. The ergosterol content was calculated as a percentage of the wet weight of the cell using the following equations:

$$\%\ \text{Ergosterol} + \%\ 24(28)\ \text{DHE} = [(A281.5/290)F]/\text{pellet weight}$$

$$\%\ 24(28)\ \text{DHE} = [(A230/518)F]/\text{pellet weight, and}$$

$$\%\ \text{Ergosterol} = [\%\ \text{Ergosterol} + \%24(28)\ \text{DHE}] - \%24(28)\ \text{DHE},$$

where F is the factor for dilution in ethanol and 290 and 518 are the E values (in percentages per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively.

Acknowledgements

The authors L. S. and S. K. are thankful to the Director, National Institute of Technology, Warangal for providing facilities and financial support. The authors are also thankful to the STIC, Cochin for performing the single crystal X-ray analysis.

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