Organic & Biomolecular Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

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

Lingala Suresh[a], Y. Poornachandra[b], S. Kanakaraju[a], G. V. P. Chandramouli[a, *] and C. Ganesh Kumar[b]

[a] Department of Chemistry, National Institute of Technology, Warangal 506 004, Telangana, India.

[b] Medicinal Chemistry and Pharmacology Division, CSIR-Indian Institute of Chemical Technology, Uppal Road, Hyderabad 500 007, Telangana, India

[*]E-mail: gvpc2000@gmail.com

Abstract: A simple and facile synthesis of a series of novel pyrano[2,3-*d*]pyrimidines have been achieved successfully *via* 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-methylhydrogensulphate [Bmim]HSO_4 ionic liquid. This method has several advantages of producing 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 screened derivatives, the compounds **4c**, **4d**, **4h** and **4l** were found to be active against both bacterial and *Candida* strains with MIC values ranging between 3.9 to 31.2 µg mL^{-1}. In addition, the compound **4l** showed good minimum bactericidal concentration, minimum fungicidal concentration and anti-biofilm activities. Furthermore, the mode of antifungal action for the promising compound **4l** was evaluated in *C. albicans* MTCC 1637 through the ergosterol biosynthesis inhibition process.

Keywords: Pyrano[2,3-*d*]pyrimidines, DMF-DMA, [Bmim]HSO_4, Antimicrobial activity, Anti-biofilm activity.

1. Introduction

In the 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 antimicrobial resistance is the wide usage 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 and also *Candida* strains, there is a continuous demand and perusal 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, the pyranopyrimidine derivatives are known to be 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 were also identified as new HA14-1 analogues.[12] Some of the well-known pyranopyrimidine derivatives (**1**, **2**, **3** and **4**) were also reported as antimicrobial agents[13] are represented in **Fig. 1**.

Insert Figure 1

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, few researchers[14a, b] have developed some novel approaches to prepare 4-anilinoquinazoline derivatives. A good number of methods[14c-h] are also reported in the literature on the synthesis of pyranopyrimidines. 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 with an objective to achieve improved yields using 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 gained 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]

In continuation of our research work on the use of green reagents like ionic liquids, for the synthesis of biologically active heterocyclic compounds[16], herein we report the [Bmim]HSO_4 ionic liquid, which promoted 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 further screened for 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. In order 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 the ionic liquids under different temperatures. The results in this regard are depicted in (**Table 1, entry 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 acetontrile, ethanol, acetic acid (**Table 1, entry 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.

Insert Scheme 1 and Table 1

Subsequently, we examined the scope and efficiency of the reaction with respect to various amines under optimal conditions (**Table 2, entry 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 well in this reaction. We also investigated the reusability of the [Bmim]HSO_4 ionic liquid in the above model reaction. After completion of the reaction, the mixture was poured into water and stirred thoroughly. The solid product, thus obtained was isolated by filtration, and the filtrate containing ionic liquid was extracted with ethyl acetate (2 × 20 mL) to remove the non-ionic organic impurities. Then the water was evaporated under reduced pressure and the recovered ionic liquid was dried under vacuum and reused for four times in subsequent reactions without evident changes in the product yields (**Fig. 2**).

Insert Figure 2

All the synthesized compounds were confirmed by their spectral data (IR, Mass, ^{1}H NMR, and ^{13}C NMR) and elemental analysis. Spectral data for all the compounds were in full agreement

with the proposed structures. The structure of compound **4e** was further confirmed by single crystal X-ray diffraction analysis. The molecular structure of **4e** is shown in **Fig. 3**. A plausible mechanism for the formation of synthesized compounds, i.e pyrano[2,3-*d*]pyrimidin-6(5*H*)-one derivatives, **4a-l** is proposed in **Scheme 3**. 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**). Then 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 proton transformation from (**B**) to (**C**). The hydrolysis of the pyrimidine ring yielded the complex (**D**), which upon intramolecular cyclization and subsequent dehydration produced the corresponding pyrano[2,3-*d*] pyrimidin-6(5*H*)-one derivatives **4a-l**.

Insert Table 2, Figure 3 and Scheme 2

2.2. Biological evaluation

All the synthesized compounds were evaluated for various biological activity 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 all the derivatives screened, compound **4l** showed

promising activity (MIC values ranging between 3.9-15.6 µg/mL) against all the bacterial strains except *Pseudomonas aeruginosa* MTCC 2453; however, the compound **4h** and **4i** exhibited promising activity (MIC value 7.8 µg/mL) 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 which probably may be contributing to the antibacterial activity. In 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.

Insert Table 3

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 in comparison to ciprofloxacin as standard. Compound **4l** consistently showed promising minimum bactericidal concentration, activity against all the tested bacterial strains. The activity data in this regard are shown in **Table 4.**

Insert Table 4

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 since 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 adhesion mechanism and result 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 in comparison to the rational use of antibiotics and/or biocides. Considering these facts, a further step was undertaken to investigate whether these compounds exhibit a specific anti-biofilm activity or whether this observation was simply related to a general toxic effect on the Gram-positive bacterial strains. To this regard, the compounds **4a, 4b, 4e-k** and **4l** were screened for 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 common and important nosocomial pathogens having biofilm forming ability. The results summarized in **Table 5**, clearly reveal that not much information on the structure-activity relationship (SAR) can be highlighted at this stage; however, it was observed that compounds **4l** exhibited promising activity (IC_{50} values ranging between 2.5 – 11.5 μM) towards all the tested bacterial species, while 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 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 which antagonize the biofilm formation and probably may be contributing to the anti-biofilm activity. The activity data in this regard is shown in **Table 5.**

Insert Table 5

2.2. 3. Antifungal activity

Different *Candida* species are important opportunistic fungal pathogens and they 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. Due 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 get encased in this matrix to form biofilms, which are known to develop on the surfaces of prosthesis and medical devices. [23,24] Considering these above facts, we screened, selected compounds such as **4a, 4b, 4c, 4d, 4h** and **4l** against different *Candida* strains and among them, the compounds **4l** and **4h** showed promising anti-*Candida* activity against many *Candida* strains with a MIC value of 7.8 µg/meal comparable to the standard miconazole drug. While, the other compounds showed good to moderate activity (MIC values ranging between 7.8 – 62.5 µg/mL) against different *Candida* strains. The results of the antifungal activity are tabulated in **Table 6**.

Insert 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 further evaluated for 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. However, the standard miconazole drug exhibited MFC values ranging between 7.8 – 15.6 µg/mL. Among them, the compound **4l** proved promising against *Candida albicans* MTCC 1637 and *C. albicans* MTCC 4748 with a lower MFC value of 7.8 µg/mL. The MFC activity data in this regard is tabulated in **Table 7**.

Insert 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 was 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) of the test compound **4l** and the standard miconazole drug (see **Table 8**). Based on the results presented in Figure 4, it was observed that the ergosterol content decreased significantly with an increase in the concentration of test compound **4l**. Similarly, a dose-dependent decrease in ergosterol content was observed when the *C. albicans* MTCC 1637 strain was cultured in the presence of miconazole. Our findings suggest that the pyranopyrimidine derivative **4l** altered the sterol profile which probably may be contributing to its antifungal activity through 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 of the cell membrane. However, the exact mechanism of action of this compound needs to be further elucidated.

Insert Table 8

3. Conclusion

In summary, we have 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 of producing high yields, environmentally benign and milder reaction conditions. Further, among the screened pyranopyrimidine derivatives, the compound **4l** was quite promising and was identified as a lead compound exhibiting antibacterial, antifungal and anti-biofilm activities.

4. Experimental section

4.1 Chemistry

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 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). ^{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). Mass spectra (ESI) were carried out on a Jeo1 JMSD-300 spectrometer. CHN analysis was carried out using Carlo Erba EA 1108 automatic elemental analyzer. All the chemicals and solvents were of analytical or synthetic grade and were used devoid of further purification unless otherwise stated. The starting

materials used in the present study **1a-c** were prepared based on literature methods and they were identified by comparison of 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 completion of the reaction, 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 silicagel (ethylacetate/n-hexane: 2/8) to afford 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) υ_{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) υ_{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) υ_{max} (cm^{-1}): 3394, 3005, 1702, 1650, 1565, 1384, 1248; ^{1}H 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) υ_{max} (cm^{-1}): 3374, 3081, 1696, 1608, 1570, 1438, 1247; ^{1}H 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) υ_{max} (cm^{-1}): 3370, 3065, 1691, 1599, 1498, 1397, 1260; ^{1}H 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) υ_{max} (cm^{-1}): 3352, 3083, 1694, 1667, 1505, 1490, 1229; ^{1}H 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) υ_{max} (cm^{-1}): 3377, 3081, 1696, 1609, 1570, 1439, 1248; ^{1}H 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) υ_{max} (cm^{-1}): 3334, 3092, 1691, 1601, 1509, 1438,1257; ^{1}H 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) υ_{max} (cm^{-1}): 3394, 3005, 1702, 1650, 1565, 1446, 1248; ^{1}H 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) υ_{max} (cm^{-1}): 3369, 2931, 1696, 1649, 1566, 1490, 1255; ^{1}H 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) υ_{max} (cm^{-1}): 3363, 3078, 1711, 1609, 1570, 1442, 125; ^{1}H 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) υ_{max} (cm^{-1}): 3381, 3058, 1695, 1645, 1571, 1509, 1244; ^{1}H 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, 32.0,8 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) alphaα = 90°
	b = 9.0798 (3) betaβ = 101.3230° (10)
	c = 16.4608 (5) gamma = 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, wR2 = 0.1191
R Indices (all data)	R = 0.0736, wR2 = 0.1442
Reflections collected	20635 / 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	110529f
Fine R indices [I>2sigma(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 were collected at room temperature using a Bruker Smart Apex CCD diffractometer with graphite monochromated MoKα radiation (λ = 0.71073 Å) by the ω-scan method.[27] Preliminary lattice parameters and orientation matrices were obtained from four sets of frames. Integration and scaling of 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 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 of 6.0 mm 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 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 $well^{-1}$, served as positive control, while the well containing DMSO served as negative control. The plates were incubated for 24 h at 37 °C for the different bacterial strains. The well containing the least concentration showing the inhibition zone is considered as the minimum inhibitory concentration. All the experiments were carried out in duplicates and mean values are represented.

4.3.2. Minimum bactericidal concentration (MBC) assay

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 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 ºC for 24 h. After 24 h of incubation, the minimum bactericidal concentration (MBC) was determined by sampling 10 µL of suspension from the tubes onto Mueller Hinton agar plates and were incubated for 24 h at 37 ºC to observe the growth of test organisms. MBC is the lowest concentration of test compound required to kill a particular bacterium strain. All the experiments were carried in duplicates and 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 of predetermined concentrations ranging from 0 to 250 µg mL^{-1} were mixed with the bacterial suspensions having 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 ºC 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 0.1% crystal violet solution followed by 30 min incubation at room temperature. Later, the crystal violet solution from the plates was discarded, thoroughly washed with distilled water for 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 TRIAD multimode reader (Dynex Technologies, Inc, Chantilly, VA, USA). Blank wells were employed as background check. The inhibition data were interpreted from the dose-response curves, where IC_{50} value is defined as the concentration of inhibitor required to inhibit 50% of biofilm formation under the above assay conditions. All the experiments were carried out in triplicates and the values are indicated as mean ± S.D.

4.3.4. Antifungal activity

The antifungal activity of the synthesized pyranopyrimidine derivatives was determined using 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 of 6.0 mm 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 were added in 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 well^{-1}, 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 the inhibition zone is considered as the minimum inhibitory concentration. All the experiments were carried out in duplicates 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 ºC for 24 h. After 24 h incubation, the MFC was determined by sampling 10 µL of 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 ºC. All the experiments were carried in the duplicates and mean values are represented, where MFC is the lowest concentration of 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 of 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 varying 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 8,000 rpm for 5 min and washed with sterile distilled water. The net wet weight of the cell pellet was determined. Three milliliters of 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 were incubated in a water bath at 85 ^{o}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. Ergosterol content was calculated as a percentage of the wet weight of the cell using the following equations:

% Ergosterol + % 24(28) DHE = [(A281.5/ 290) × F] /pellet weight

% 24(28) DHE = [(A230/518) × F] /pellet weight, and

% Ergosterol = [% Ergosterol + %24 (28) DHE] - %24 (28) 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.

References

1 (a) T. M. Barbosa, S. B. Levy, *Drug Resistance Updates.*, 2000, **3**, 303; (b) A. Kapil, *Indian Journal of Medical Research*., 2005, **121**, 83; (c) L. C. Balsalobre, M. Dropa, M. H. M. Brazilian, *Brazalian Journal of Microbiology.*, 2014, **45**, 1.

2 (a) M. M. Khafagy, A. H. Abd El-Wahab, F. A. Eid and A. M. El-Agrody, *Il Farmaco.*, 2002, **57**, 715; (b) A. H. Bedair, H. A. Emam, N. A. El-Hady, K. A. Ahmed and A. M. El-Agrody, *Il Farmaco.*, 2001, **56**, 965; (c) U. Sankappa Rai, A. M. Isloor, P. Shetty, A. M. Vijesh, N. Prabhu, S. Isloor, M. Thiageeswaran and H. K. Fun, *European Journal of Medicinal Chemistry.*, 2010, **45**, 2695.

3 A. Agarwal, K. Srivastava, S. K. Puri and P. M. S. Chauhan, *Bioorganic and Medicinal Chemistry.*, 2005, **13**, 4645.

4 B. Tozkoparan, M. Ertan, P. Kelicen and R. Demirdamar, *Farmaco.*, 1999, **54**, 588.

5 X. Fan, D. Feng, Y. Qu, X. Zhang, J. Wang, P. M. Loiseau, G. Andrei, R. Snoeck and E. De Clercq, *Bioorganic and Medicinal Chemistry Letters.*, 2010, **20**, 809.

6 O. Bruno, C. Brullo, S. Schenone, F. Bondavalli, A. Ranise, M. Tognolini, M. Impicciatore, V. Ballabeni and E. Barocelli, *Bioorganic and Medicinal Chemistry.*, 2006, **14**, 121.

7 G. R. Roehrig, J. H. Billman, J. Marcec, P. Fritz and F. Shea, *Journal of Pharmaceutical Sciences.*, 1980, **69**, 1232.

8. M. M. Ghorab, F. a. Ragab, H. I. Heiba, R. K. Arafa and E. M. El-Hossary, *European Journal of Medicinal Chemistry.*, 2011, **20**, 388.

9 H. Cerecetto, *European Journal of Medicinal Chemistry.*, 2000, **35**, 343.

10 O. Bruno, C. Brullo, S. Schenone, F. Bondavalli, A. Ranise, M. Tognolini, V. Ballabeni and E. Barocelli, *Bioorganic and Medicinal Chemistry.*, 2004, **12**, 553.

11 F. Chabchoub, M. Messaâd, H. Ben Mansour, L. Chekir-Ghedira and M. Salem, *European Journal of Medicinal Chemistry.*, 2007, **42**, 715.

12 L. Moafi, S. Ahadi and A. Bazgir, *Tetrahedron Letters.*, 2010, **51**, 6270.

13 (a) M. Ghashang and S. S. Mansoor, *Journal of Advanced Research.*, 2014, **5**, 209; (b) A. Bedair, N. A. El-Hady, M. S. A. El-Latif, A. Fakery and A. El-Agrody, *Il Farmaco.*, 2000, **55**, 708; (c) H. M. Aly and M. M. Kamal, *European Journal of Medicinal Chemistry.*, 2012, **47**, 18. (d) J. M. Khurana, A. Lumb, A. Chaudhary and B. Nand, *RSC Advances.*, 2013, **3**, 1844.

14 (a) Z. Wang, C. Wang, Y. Sun, N. Zhang, Z. Liu and J. Liu, *Tetrahedron*, 2014, **70**, 906; (b) V. Chandregowda, G. V. Rao and G. C. Reddy, *Org. Process Res. Dev.*, 2007, **11**, 813; (c) H. -Y. Guo, X.-J. Li and Y. Yu, *Synthetic Communications.*, 2012, **42**, 3011; (d) M. Ghashang and S. S. Mansoor, *Journal of Advanced Research.*, 2014, **5**, 209; (e) I. R. Siddiqui, A. Srivastava, S. Shamim, A. Srivastava, M. a. Waseem, Shireen, Rahila, A. a H. Abumhdi, A. Srivastava and P. Rai, *Journal of Molecular Catalysis A: Chemical.*, 2014, **382**, 126; (f) J. M. Khurana, A. Lumb, A. Chaudhary and B. Nand, *RSC Advances.*, 2013, **3**, 1844; (g) M. M. Heravi, A. Ghods, K. Bakhtiari and F. Derikvand, *Synthetic*

Communications., 2010, **40**, 1927; (h) M. Kidwai, R. Venkataramanan and B. Dave, *Synthetic Communications.*, 2002, **32**, 2161.

15 (a) AaaN. Isambert, M. del M. Sanchez Duque, J. Plaquevent, Y. Génisson, J. Rodriguez and T. Constantieux, *Chemical Society Reviews.*, 2011, **40**, 1347; (b) H. Olivier-Bourbigou, L. Magna and D. Morvan, *Applied Catalysis A: General.*, 2010, **373**, 1; (c) M. A. P. Martins, C. P. Frizzo, D. N. Moreira, N. Zanatta and H. G. Bonacorso, *Chemical Society Reviews.*, 2008, **108** , 2015.

16 (a) S. Kanakaraju, B. Prasanna, S. Basavoju and G. V. P. Chandramouli, *Journal of Molecular Structure.*, 2012, **1017**, 60; (b) S. Kanakaraju, B. Prasanna, P. Venkatanarayana and G. V. P. Chandramouli, *Journal of the Iranian Chemical Society.*, 2012, **9**, 933; (c) M. Maradolla and G. V. P. Chandramouli, *Phosphorus, Sulfur, and Silicon and the Related Elements.*, 2011, **186**, 1650.

17 D. Amsterdam, Susceptibility testing of antimicrobials in liquid media. In: V. Loman, (Ed.) Antibiotics in Laboratory Medicine, 4th Edition, Williams and Wilkins, Baltimore, MD, 1996, 52.

18. National Committee for Clinical Laboratory Standards, NCCLS. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard fifth edition, NCCLS, Wayne, PA, 2000.

19 L. Hall-Stoodley, J. W. Costerton, P. Stoodley, *Nature Reviews in Microbiology., 2004,* **2**, 95.

20 J. W. Costerton, L. Montanaro, C. R. Arciola, *International Journal of Artificial Organs,*. 2005, **28**, 1062.

21 A. Kamal, R. Abdul, S. Riyaz, Y. Poornachandra, B. Moku, C. G. Kumar, S. M. Hussaini, B. Sridhar, P. K. Machiraju, *Organic and Biomolecular Chemistry.,* 2015**,** ***13,*** 1347.

22 G. Ramage, S. P. Saville, D. P. Thomas, J. L. Lopez-Ribot, *Eukaryotic Cell*., 2005, **4**, 633.

23 L. J. Douglas, *Trends in Microbiology.,* 2003, **11**, 30.

24 E. M. Kojic, R. O. Darouiche, *Clinical Microbiology Reviews.,* 2004, **17**, 255.

25 J. Berman, P. E. Sudbery, *Nature Reviews in Genetics.,* 2002, **3**, 918.

26 X. Fan, D. Feng, Y. Qu, X. Zhang, J. Wang, P. M. Loiseau, G. Andrei, R. Snoeck and E. De Clercq, *Bioorganic and Medicinal Chemistry Letters*, 2010, **20**, 809.

27 SMART & SAINT, Software reference manuals. Versions 6.28a & 5.625, Bruker Analytical X-ray Systems Inc., Madison, Wisconsin, USA, 2001.

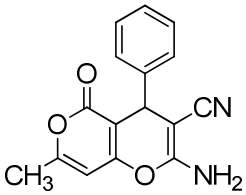
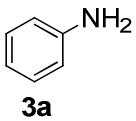
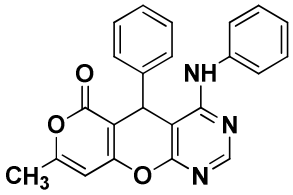
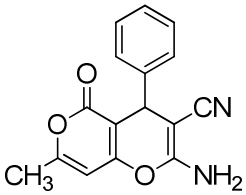
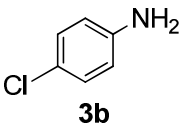
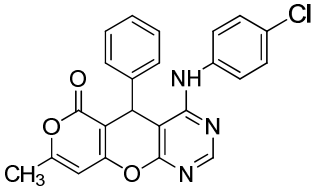
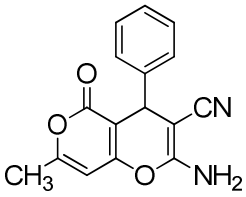
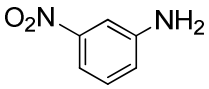
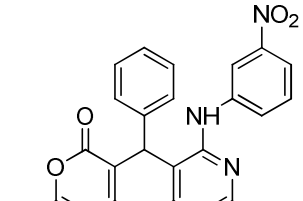
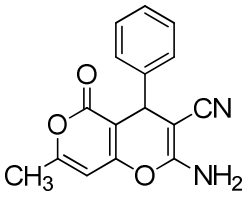
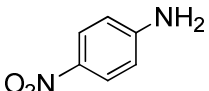
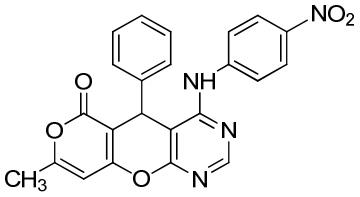
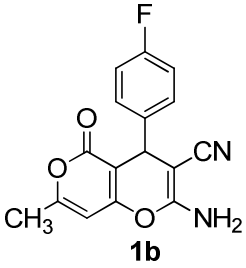
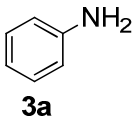
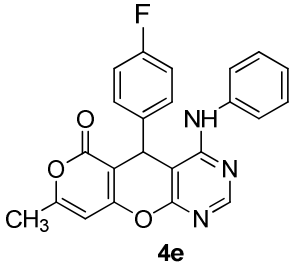
28 O. N. Breivik, J. L. Owades, *Journal of Agriculture and Food Chemistry*., 1957, **3**, 360.

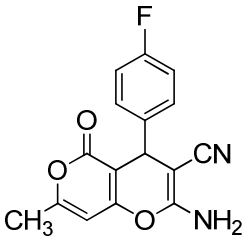
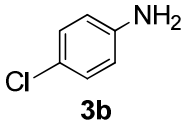
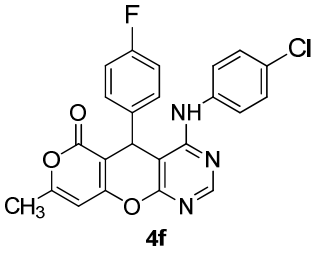
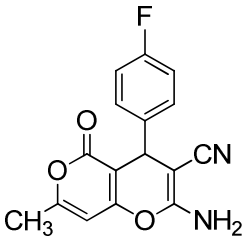
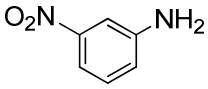
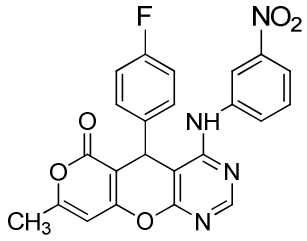
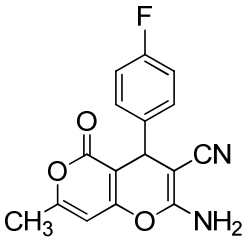
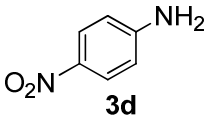
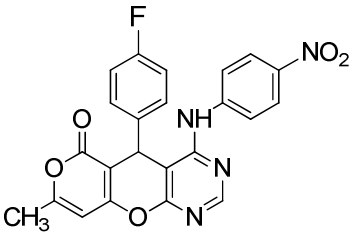
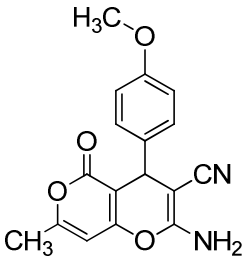
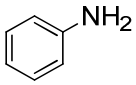
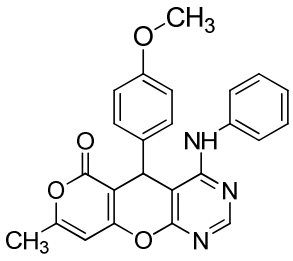
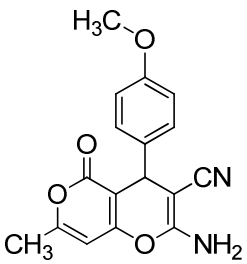
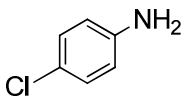
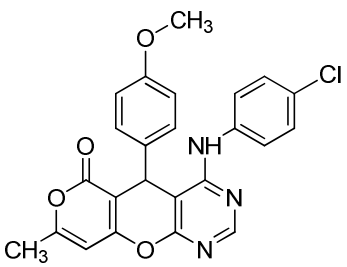
Table 1 Optimization of reaction parameters for the synthesis of **4a**

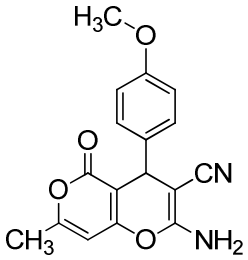
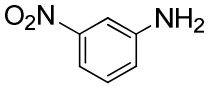
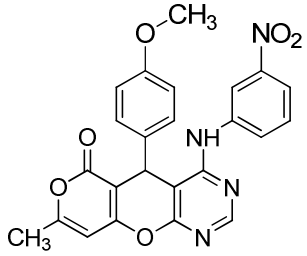
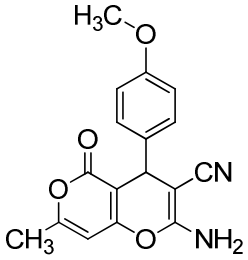
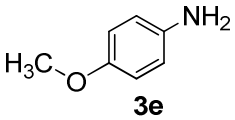
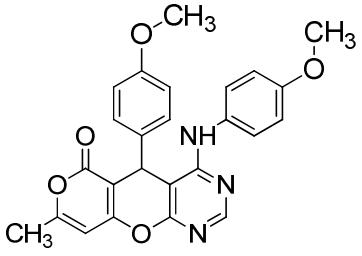
Entry[a]	Solvent	Temp (°C)	Time (h)	Yield[b] (%)
1	Ethanol	Reflux	10	28
2	Acetonitrile	Reflux	10	24
3	AcOH	Reflux	8	44
4	[Bmim]HSO_4	r.t.	2	48
5	[Bmim]HSO_4	60	2	76
6	**[Bmim]HSO_4**	**80**	**2**	**94**
7	[Bmim]HSO_4	100	2	92
8	[Bmim]BF_4	80	2	82
9	[Bmim]Br	80	2	74
10	[Bmim]PCl_5	80	2	62

[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.

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)
1	 **1a**	 **3a**	 **4a**	**2**
2	 **1a**	 **3b**	 **4b**	3
3	 **1a**	 **3c**	 **4c**	2.5
4	 **1a**	 **3d**	 **4d**	2.5
5	 **1b**	 **3a**	 **4e**	4

6	 **1b**	 **3b**	 **4f**	3.5
7	 **1b**	 **3c**	 **4g**	2
8	 **1b**	 **3d**	 **4h**	2
9	 **1c**	 **3a**	 **4i**	2.5
10	 **1c**	 **3b**	 **4j**	4

11	 **1c**	 **3c**	 **4k**	3.5
12	 **1c**	 **3e**	 **4l**	2

[a]Isolated yields after purification.

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

Test Compound	Minimum inhibitory concentration (µg/mL)						
	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

Table 4. Minimum Bactericidal Concentration Assay (MBC) of the synthesized pyrano[2,3-*d*]pyrimidine derivatives

Test Compound	Minimum bactericidal concentration (µg/mL)					
	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	> 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

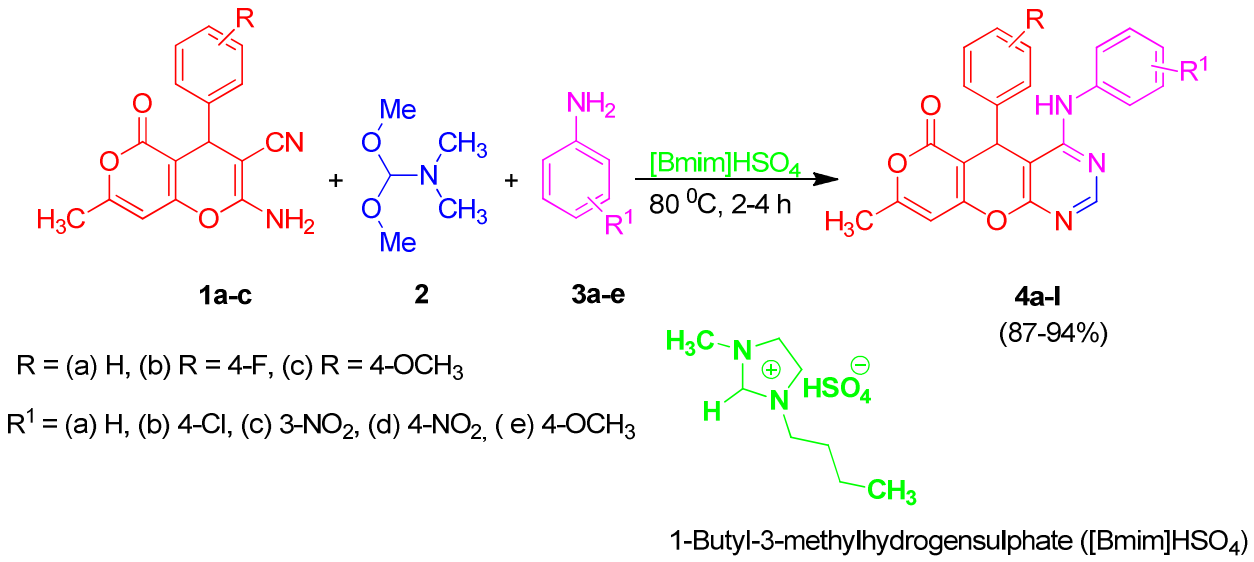
Test Compound	IC_{50} values in (μM)					
	Micrococcus luteus MTCC 2470	*Staphylococcus aureus* MTCC 96	*Staphylococcus aureus* MLS-16 MTCC 2940	*Bacillus subtilis* MTCC 121	*Escherichia coli* MTCC 739	*Klebsiella planticola* MTCC 530
4a	-[a]	-	22.4 ± 0.52	-	-	-
4b	-	-	16.8 ± 0.44	-	-	-
4c	-	18.9 ± 0.38	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

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

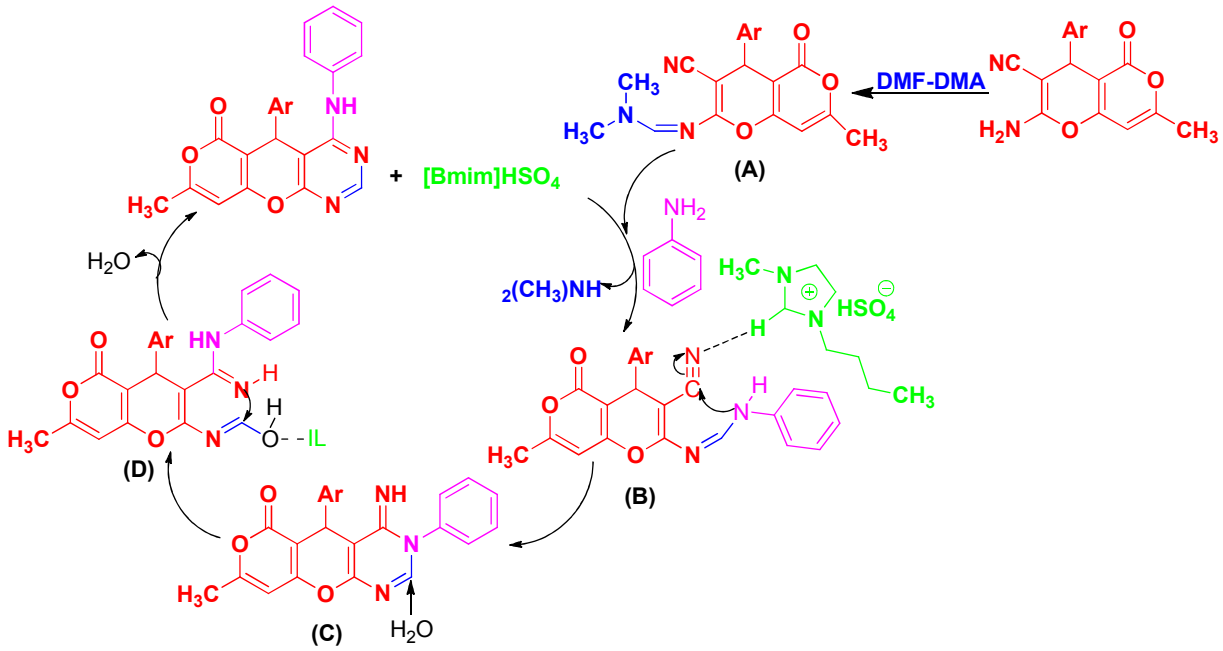
Test Compound	Minimum inhibitory concentration (µg/mL)													
	C. albicans MTCC 183	*C. albicans* MTCC 227	*C. albicans* MTCC 854	*C. albicans* MTCC 1637	*C. albicans* MTCC 3017	*C. albicans* MTCC 3018	*C. albicans* MTCC 3958	*C. albicans* MTCC 4748	*C. albicans* MTCC 7315	*C. parapsilosis* MTCC 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

Test Compound	Minimum inhibitory concentration (µg/mL)												
	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. albi cans* 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



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



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