Medicinal Chemistry Research (2019) 28:1509–1528
https://doi.org/10.1007/s00044-019-02390-w

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design and synthesis of novel 4-hydrazone functionalized/1,2,4-triazole fused pyrido[2,3-*d*]pyrimidine derivatives, their evaluation for antifungal activity and docking studies

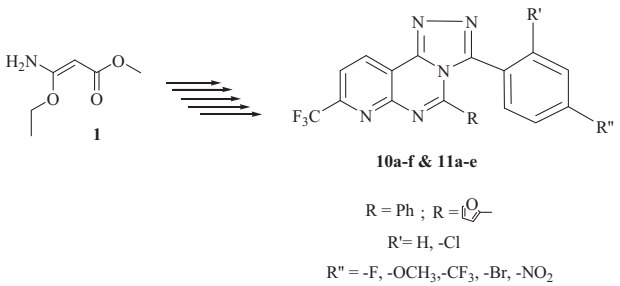
Nooka Ratnam Appna[1,2] · Ravi Kumar Nagiri[2] · Raghu Babu Korupolu[1] · Sirisha Kanugala[3] · Ganesh Kumar Chityal[3] · Ganapathi Thipparapu[4] · Narsaiah Banda[2]

Received: 28 March 2019 / Accepted: 25 June 2019 / Published online: 13 July 2019

Abstract

A series of novel 2-substituted 4-hydrazone functionalized pyrido[2,3-*d*]pyrimidine (**8a–f** and **9a–e**) and 1,2,4-triazole fused pyrido[2,3-*d*]pyrimidine derivatives (**10a–f** and **11a–e**) were prepared starting from ethyl 2-amino-6-(trifluoromethyl) nicotinate **3** via acylation, cyclization, chlorination, hydrazine reaction, hydrazone formation followed by intramolecular cyclization. All the final products were screened against various *Candida* strains for determining the antifungal activity, minimum fungicidal concentration and inhibition of ergosterol biosynthesis. Among the screened, compounds **8c**, **8f**, **9c**, **10f**, **11d** and **11e** were identified as promising antifungal agents. From a mechanistic perspective, the concomitant treatment of **10f, 11d** and **11e** on different *Candida* strains showed inhibition of ergosterol biosynthesis, which also revealed the possible antifungal action of these compounds on the ergosterol biosynthetic pathway. The binding mode of active compounds by docking studies showed that they fit well into the active site cavity of target protein. Further, the SAR and molecular docking studies data presumed that the presence of fluoro, trifluoromethyl, bromo and nitro groups on phenyl and furyl rings in pyrido[2,3-*d*]pyrimidine were found to be crucial to promote antifungal activity. All the strains for Miconazole a control drug showed MIC values equal to 3.9 µg/mL. Lipinski's parameters of all compounds are within the acceptable range defined for human use thereby indicating their potential as drug-like molecules.

Graphical Abstract



Keywords Pyrido[2,3-*d*]pyrimidine · 4-hydrazone · Triazole · Antifungal activity · Ergosterol · Molecular docking

✉ Narsaiah Banda
narsaiah@csiriict.in
narsaiahbanda84@gmail.com

[1] Andhra University College of Engineering, Visakhapatnam 530003 Andhra Pradesh, India

[2] Fluoroorganic division, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500007, India

[3] Medicinal Chemistry and Pharmacology Division, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad 500007, India

[4] Stem Cell Research Division, Department of Biochemistry, ICMR-National Institute of Nutrition (NIN), Hyderabad 500007 Telangana, India

Introduction

Fungal pathogens most often colonize the skin and cause dermal infections in human beings (Brand 2012). They directly affect internal organs like mucous membrane of the respiratory, gastrointestinal and urinary tract and cause infections. In general, the risk of infections caused by fungal pathogens has increased considerably over the past two decades (Jessup et al. 2000; Vandeputte et al. 2012), can be attributed to the increasingly growing number of individuals with weakened immune system. For example, those with cancer undergoing chemotherapy, leukemia, organ transplant, and patients with acquired immune deficiency syndrome (AIDS), diabetes or cystic fibrosis and patients who are receiving immunosuppressive drugs or antibiotics (Romani 2004; Neofytos et al. 2013; Al Mubarak et al. 2013; Tsai et al. 2013; Person et al. 2011; Tillotson and Tillotson 2015). The superficial mycosis which is caused by dermatophytes primarily the *Candida* species can be treated by administering anti-fungal drugs. Only a limited number of antifungal drugs approved by Food and Drug Administration (FDA) are available in the United States for the treatment of systemic fungal diseases (Ghannoum and Rice 1999), which include polyenes (e.g., Amphotericin-B), azoles (e.g., Fluconazole, Voriconazole, Itraconazole, etc.), pyrimidines (e.g., 5-Fluorocytosine), allylamines (ex: Terbinafine) and echinocandins (e.g., Caspofungin) (Fig. 1). Amphotericin-B is a most commonly used polyene class of antifungal drug which targets ergosterol, the principal sterol component of fungal membranes and used to treat systemic fungal infections due to its wide range of activity (Gibbs et al. 2005). The significant side effects associated with Amphotericin-B include acute reactions like fever, headache, vomiting, muscle pain, shaking chill, nausea, diarrhea and severe toxic side effects like renal, liver toxicities and neurotoxicity (Johnson and

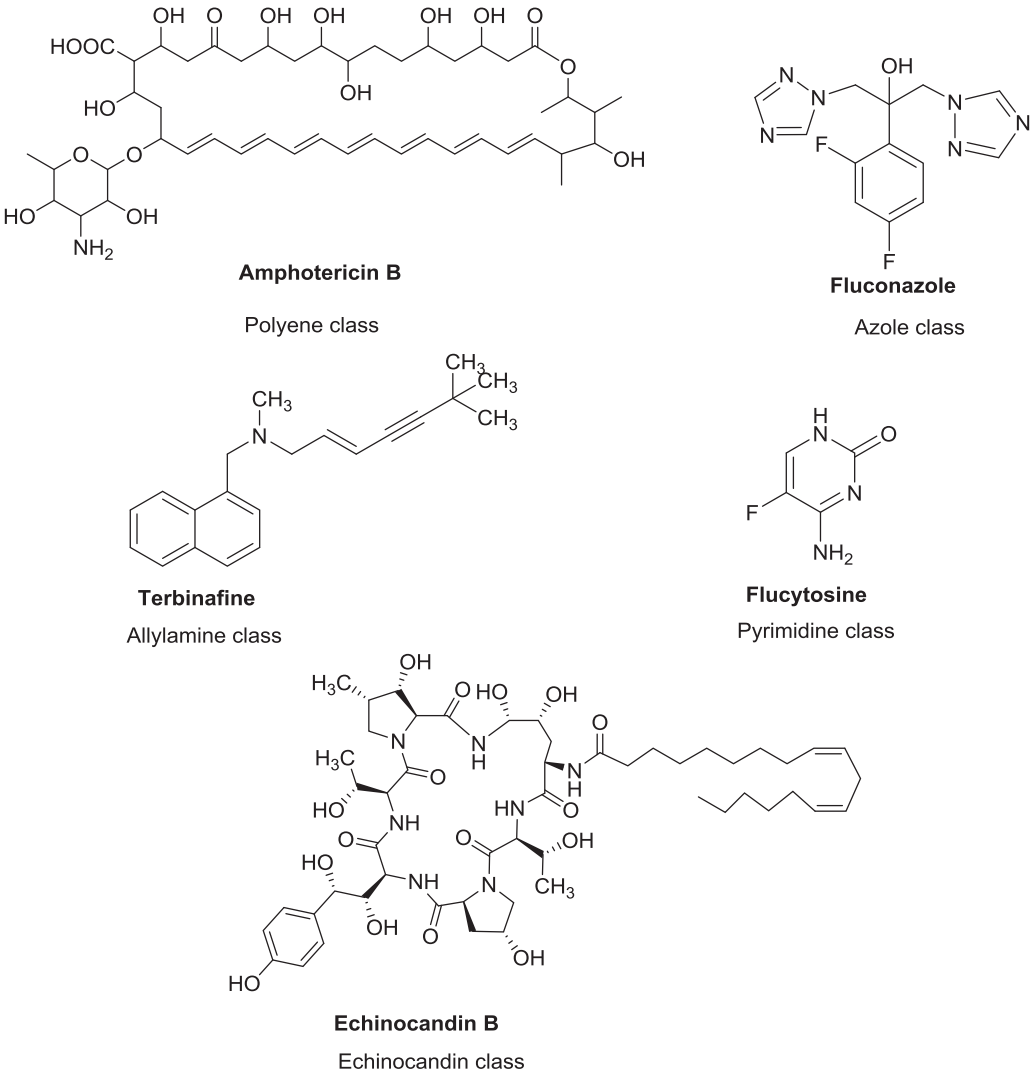


Fig. 1 Typical examples of pyrimidine, azole and polyene class of antifungal drugs

Einstein 2007). Fluconazole is an important azole class of antifungal drug used in managing systemic and superficial fungal infections due to its broad range of biological activity, high bioavailability and low protein binding nature (Grant and Clissold 1990). The common side effects associated with fluconazole include vomiting, dizziness, headache, nausea and long-term use will lead to severe liver toxicity. Major problem associated with azole drugs is their drug interaction with other classes of drugs like anticoagulants and some antihistamines (Hoesley and Dismukes 1997; Albengres et al. 1998). 5-Fluorocytosine is a fluorinated pyrimidine class of antifungal drug which has high fungicidal activity against various *Candida* strains and the major side effects include vomiting, headache, nausea, skin rash, and diarrhea and also causes severe side effects like renal dysfunction and bone marrow suppression (Bennett 1977). Terbinafine is an important synthetic antifungal drug of class allylamine that function as an ergosterol biosynthesis inhibitor. It showed promising inhibition against dermatophytes such as *C. albicans* and *Cryptococcus neoformans* under both in vitro and in vivo conditions (Ryder and Favre 1997). The common side effects of Terbinafine include headache, rashes, allergic reactions, dyspepsia, abdominal pain, flatulence, urticaria and constipation (Sinha et al. 2012). Echinocandins (e.g., Echinocandin B, Caspofungin, Micafungin and Anidulafungin) are synthetic derivatives of lipopeptides and a specific non-competitive inhibitor of fungal 3β-glucan synthase, an enzyme that catalyzes the polymerization of uridine diphosphate glucose into β(1–3)-glucan, the structural component is responsible for the maintenance of fungal cell wall integrity and rigidity. The inhibition of β (1–3)-glucan synthase leads to cell wall destabilization and to the leakage of intracellular components, resulting in fungal cell lysis (Denning 2002). It exhibits good fungicidal activity against *Candida* and *Aspergillus* species under both in vitro and in vivo conditions. However, they are fungicidal in *Candida* and fungistatic in *Aspergillus*, and the reasons for these differences are still unknown (Espinel-Ingroff 1998; Oakley et al. 1998). These drugs are poorly absorbed in the gastrointestinal tract due to their high molecular weights and are thus prescribed for intravenous use only. The major side effects of echinocandins include facial flushing, swelling, rash, pruritis, fever and derangement of hepatic transaminases (Keating and Figgitt 2003).

In this context, there is an imperative need for the discovery and development of new safer drugs with minimum side effects and promising activity against majority of the fungal pathogens. The rapid use of some triazoles as antifungal agents and development of resistance to existing antifungal drugs made us to focus our research on developing hybrid molecules with triazole including two or three scaffolds possessing antimicrobial and anti-fungal activities. Pyrido[2,3-*d*]pyrimidine derivatives are known to exhibit anti-tumor activity which may be attributed to inhibition of cyclin dependent kinase (VaanderWaal et al. 2005), mammalian target of rapamycin (mTOR) (Malagu et al. 2009). These are also known to possess wide spectrum of biological activities such as antibacterial, antifungal, antitubercular, anticancer, anticonvulsant, antidepressant, analgesic, anti-inflammatory, antioxidant, antiviral, anthelmintic and hypoglycemic activities (Cordeu et al. 2007; Bazgir et al. 2008; Nasr and Gineinah 2002; Wang et al. 2017). 1,2,4-Triazoles exhibit significant medicinal and pharmaceutical applications (Sahu et al. 2013; Pigaew et al. 2015; Kaur et al. 2016) due to their wide range of biological activities including antimicrobial (Bhat et al. 2009), anti-fungal (Holla et al. 2003), anticancer (Turan-Zitouni et al. 2005; Ezabadi et al. 2008; Nekkanti et al. 2017) and anti-viral, which established them as an important pharmacophores (Ozdemir et al. 2007; Roma et al. 2008). Based on these factors and following our continuous search for the synthesis of biologically active compounds (Adib et al. 2016; Jitender Dev et al. 2017), we synthesized a series of novel trifluoromethyl substituted triazole fused pyrido[2,3-*d*]pyrimidine derivatives. All the compounds were evaluated for antifungal activity and promising compounds were identified.

Material and methods

Melting points were recorded on Casia-Siamia (VMP-AM) melting point apparatus and are uncorrected. IR spectra were recorded on an Agilent Technologies Cary 630 FTIR spectrophotometer using KBr optics. ^{1}H NMR spectra were recorded on Bruker AV 300 MHz, AV 400 MHz and 500 MHz in $CDCl_3$ and DMSO-d_6 using TMS as internal standard. Electron impact (EI) and chemical ionization mass spectra were recorded on a VG 7070 H instrument at 70 eV. All the reactions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 F_{254} (mesh); spots were visualized with UV light. Merck silica gel (180-200 mesh) was used for column chromatography.

Preparation of ethyl 2-amino-6-(trifluoromethyl) nicotinate (3) (Onnis et al. 2009)

General procedure

1,1,1-trifluoro-4-butoxy-3-buten-2-one (1.96 g, 0.01 mol) was added to a solution of Ethyl 3-amino-3-ethoxypropenoate 2 (1.60 g, 0.01 mol) and ammonium acetate (0.77 g, 0.01 mol) in ethanol (20 mL). The reaction mixture was stirred at room temperature for 1 h and then

refluxed for 2 h. The solvent was removed and the resulting solid was dried and purified by column chromatography.

Preparation of ethyl 2-(aryl substituted amino)-6-(trifluoromethyl)nicotinate (4a, b) (Naresh Kumar et al. 2016)

General procedure

Ethyl 2-amino-6-(trifluoromethyl)nicotinate **3** (5.0 g, 0.02 mol) was taken in different aryl acid chlorides (phenyl or furyl, 30–40 ml) and was stirred at 60–120 °C for 6–8 h. The progress of the reaction was monitored by TLC. After completion of the reaction, reaction mixture was cooled and n-hexane was slowly added and the separated solid was filtered, washed with water. The resultant solid was dried and purified by column chromatography.

2-Substituted-7-(trifluoromethyl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one (5a–c) (Naresh Kumar et al. 2016)

General procedure

Ethyl 2-amino-6-(trifluoromethyl)nicotinate **3** (5.0 g, 0.02 mol) or Ethyl 2-substitutedamido-6-(trifluoromethyl) nicotinate **4a**, **b** (0.01 mol) was taken in formamide and heated upto 140–150 °C for 8–10 h. After completion of the reaction, the reaction mixture was cooled and then poured on to crushed ice. Filtered the solid, washed with water and dried under high vacuum to obtain respective products **5a**–**c**.

4-Chloro-2-substituted–7-(trifluoromethyl)-3,4-dihydropyrido[2,3-*d*]pyrimidine (6a–c)

2-substituted-7-(trifluoromethyl)pyrido[2,3-*d*]pyrimidin-4 (3*H*)-one **5a**–**c** (0.01 mol) and thionyl chloride were reacted at reflux for 3 h. After completion of reaction, mixture was cooled to room temperature and n-hexane was slowly added. The separated solid was filtered, washed with water and dried to obtain respective products **6a**–**c**.

4-Hydrazinyl-2-substituted-7-(trifluoromethyl)-3,4-dihydropyrido[2,3-*d*]pyrimidine (7b, c)

4-chloro-2-substituted-7-(trifluoromethyl)-3,4-dihydropyrido[2,3-*d*]pyrimidine **6a**–**c** (0.01 mol) was taken in ethanol (32 mL) and was added hydrazine hydrate (0.08 mol) and the reaction was allowed to reflux for 3–4 h. After completion of reaction, ethanol was removed under vacuum. The crude residue was treated with ice cold water, separated solid was collected by filtration, washed with water, dried and obtained products **7b**, **c**.

Preparation of 2-(4-substituted)-1-(7-(trifluoromethyl)-2-substitutedpyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (8a–f & 9a–e)

General procedure

Compound, 4-hydrazinyl-2-substituted-7-(trifluoromethyl)-3,4-dihydropyrido[2,3-*d*]pyrimidine **7b**, **c** (0.01 mol) and substituted aryl aldehyde (1.5 g, 0.01 mol) were taken in ethanol, stirred at room temperature for 10 min then catalytic amount of triethylamine (Et_3N) (5 mg, 0.007 mol) were added to the above mixture and refluxed for 2–4 h. The progress of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was allowed to cool and diluted with ice cold water and then extracted with ethyl acetate. The resultant solid was dried and purified by column chromatography to obtain respective products **8a**–**f** and **9a**–**e**.

2-(4-Fluorobenzylidene)-1-(7-(trifluoromethyl)-2-phenylpyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (8a)

Yield: 88% (brown solid); m.p:180–182 °C; IR (KBr) cm^{-1}: 3416 (NH), 3090 (aromatic-H), 1608 (C = N), 1563 (C = C); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 4.73 (br, s, 1 H, –NH), 7.24 (m, 1 H, Ar–H), 7.32 (m, 1 H, Ar–H), 7.67 (m, 3 H, Ar–H), 7.81 (s, 1 H, = C–H), 8.41 (t, 2 H, J = 9.781 Hz, Ar–H), 8.04 (d, 1 H, J = 8.192 Hz, Ar–H), 8.82 (t, 2 H, J = 9.781 Hz, Ar–H), 9.16 (d, 1 H, J = 8.192 Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 116.06, 116.28, 116.79, 120.42 (q, J = 275.098 Hz) ($\underline{C}F_3$), 124.99, 127.08, 129.21, 130.10, 130.18, 130.37, 131.87, 132.74, 135.00, 150.27 (q, J = 33.011 Hz) (C–CF_3), 154.56, 156.82; ESI-MS *m/z*: 412 (M + H), 434 (M + Na)

2-(4-Methoxybenzylidene)-1-(7-(trifluoromethyl)-2-phenylpyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (8b)

Yield: 86% (light yellow solid); m.p: 187–189 °C; IR (KBr) cm^{-1}: 3413 (NH); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.89 (s, 3 H, -OCH_3), 7.01 (t, 2 H, J = 8.545 Hz, Ar–H), 7.63 (m, 5 H, Ar–H), 7.80 (t, 2 H, J = 8.545 Hz, Ar–H), 8.19 (d, 1 H, J = 7.172 HZ, Ar–H), 8.56 (s, 1 H, = C–H), 8.74 (d, 1 H, J = 7.172 Hz, Ar–H), 10.62 (br, s, 1 H, -NH); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ p.p.m.: 55.13, 114.13, 119.20 (q, J = 275.090 Hz)($\underline{C}F_3$), 126.85, 127.08, 128.85, 129.62, 131.72, 132.32, 134.54, 144.10, 150.43 (q, J = 33.011 Hz, Ar–H) ($\underline{C}$–CF_3), 157.51, 161.83; ESI-MS *m/z*: 424 (M + H), 446 (M + Na)

2-(4-(Trifluoromethyl)benzylidene)-1-(7-(trifluoromethyl)-2-phenylpyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (8c)

Yield: 79% (off white solid); m.p: 164–166 °C; IR (KBr) cm^{-1}: 3428 (NH); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.01 (br, s, 1 H, -NH), 7.04 (t, 1 H, Ar–H), 7.05 (m, 1 H, Ar–H), 7.48 (d, 1 H, $J = 8.087$ Hz, Ar–H), 7.58 (t, 2 H, $J = 7.172$ Hz, Ar–H), 7.59 (m, 3 H, Ar–H), 8.04 (t, 2 H, $J = 7.172$ Hz, Ar–H), 8.48 (m, 1 H, Ar–H), 8.52 (d, 1 H, $J = 8.087$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 108.63, 122.92 (q, $J = 274.356$ Hz) ($\underline{C}F_3$), 127.25, 129.30, 132.19, 135.32, 141.13, 142.36, 143.25, 149.711, 150.01 (q, $J = 33.011$ Hz) ($\underline{C}$–CF_3), 157.14, 163.55, 168.71; ESI-MS *m/z*: 462 (M + H), 484 (M + Na)

2-(4-Nitrobenzylidene)-1-(7-(trifluoromethyl)-2-phenylpyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (8d)

Yield: 87% (yellow solid); m.p: 210–212 °C; IR(KBr) cm^{-1}: 3431 (NH), 1608 (C = N), 1563 (C = C); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 4.40 (br, s, 1 H, –NH), 7.73 (m, 3 H, Ar–H), 8.04 (d, 1 H, $J = 8.697$ Hz, Ar–H), 8.08 (t, 2 H, $J = 8.545$ Hz, Ar–H), 8.33 (d, 1 H, $J = 8.697$ Hz, Ar–H), 8.40 (t, 2 H, $J = 8.545$ Hz, Ar–H), 8.71 (s, 1 H, Ar–H), 8.79 (d, 1 H, Ar–H), 10.17 (s, 1 H, = C–CH); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ p.p.m.: 115.14, 122.87 (q, $J = 273.623$ Hz)($\underline{C}F_3$), 126.09, 127.64, 128.17, 128.86, 130.19, 139.57, 144.25, 151.31 (q, $J = 34.478$ Hz) (C–CF_3), 156.52, 162.81, 163.95, 169.11; ESI-MS *m/z*: 439 (M + H), 461 (M + Na)

2-(4-Bromobenzylidene)-1-(7-(trifluoromethyl)-2-phenylpyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (8e)

Yield: 76% (off white solid); m.p: 171–173 °C; IR (KBr) cm^{-1}: 3425 (NH); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.11 (br, s, 1 H, NH), 7.64 (m, 4 H, Ar–H), 7.75 (t, 2 H, $J = 8.087$ Hz, Ar–H), 7.80 (d, 1 H, $J = 8.240$ Hz, Ar–H), 8.18 (t, 2 H, $J = 8.087$ Hz, Ar–H), 8.57 (d, 1 H, Ar–H), 8.77 (d, 1 H, $J = 8.240$ Hz, Ar–H), 10.57 (s, 1 H, = C–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 120.21 (q, $J = 275.824$ Hz) ($\underline{C}F_3$), 125.17, 126.08, 127.75, 129.25, 130.58, 132.05, 133.09, 143.74, 150.17 (q, $J = 32.277$ Hz) (C–CF_3), 157.19, 160.84, 166.49, 169.85; ESI-MS *m/z*: 472 (M + H), 494 (M + Na)

2-(2-Chloro-4-fluorobenzylidene)-1-(7-(trifluoromethyl)-2-phenyl-pyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (8f)

Yield: 65% (dark yellow solid); m.p: 204–206 °C; IR (KBr) cm^{-1}: 3416 (NH); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.12 (br, s, 1 H, -NH), 7.12 (t, 2 H, Ar–H), 7.30 (m, 3 H, Ar–H), 7.35 (t, 2 H, $J = 7.934$ Hz, Ar–H), 7.48 (d, 1 H, $J = 8.240$ Hz, Ar–H), 7.71 (t, 2 H, $J = 7.934$ Hz, Ar–H), 8.78 (d, 1 H, $J = 8.240$ Hz, Ar–H), 8.95 (s, 1 H, = C–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 122.24 (q, $J = 277.291$ Hz)($\underline{C}F_3$), 126.56, 132.42, 134.68, 143.64, 144.36, 149.07, 151.14 (q, $J = 32.277$ Hz) (C–CF_3), 154.25, 156.51, 162.92, 165.42, 169.46; ESI-MS *m/z*: 446 (M + H), 468 (M + Na)

2-(4-Fluorobenzylidene)-1-(7-(trifluoromethyl)-2-(furan-2-yl)pyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (9a)

Yield: 89% (yellow brown solid); m.p: 224–226 °C; IR (KBr) cm^{-1}: 3427 (NH); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 4.74 (br, s, 1 H, -NH), 7.22 (d, 1 H, Ar–H), 7.67 (m, 2 H, Ar–H), 7.83 (s, 1 H, = C–H), 8.03 (d, 1 H, $J = 8.192$ Hz, Ar–H), 8.16 (d, 1 H, $J = 8.192$ Hz, Ar–H), 8.42 (t, 2 H, $J = 7.947$ Hz, Ar–H), 8.82 (t, 2 H, $J = 7.947$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 104.24, 106.74, 108.74, 121.91 (q, $J = 275.090$ Hz) ($\underline{C}F_3$), 129.86, 131.56, 132.42, 134.68, 149.07, 152.32 (q, $J = 33.744$ Hz, Ar–H) (C–CF_3), 154.25, 156.51, 162.92, 165.43, 168.08; ESI-MS *m/z*: 402 (M + H), 424 (M + Na)

2-(4-Methoxybenzylidene)-1-(7-(trifluoromethyl)-2-(furan-2-yl)pyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (9b)

Yield: 75% (dark brown solid); m.p: 179–181 °C; IR (KBr) cm^{-1}: 3415 (NH); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.89 (s, 3 H, -OCH_3), 7.00 (d, 1 H, $J = 7.934$ Hz, Ar–H), 7.62 (m, 2 H, Ar–H), 7.65 (t, 1 H, Ar–H), 7.79 (t, 2 H, $J = 8.392$ Hz, Ar–H), 8.18 (t, 2 H, $J = 8.392$ Hz, Ar–H), 8.57 (s, 1 H, = C–H), 8.74 (d, 1 H, $J = 7.934$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 55.76, 106.16, 108.04, 119.32 (q, $J = 274.356$ Hz)($\underline{C}F_3$), 127.65, 129.48, 130.25, 132.96, 135.33, 142.24, 144.44, 149.47 (q, $J = 33.743$ Hz) (C–CF_3), 155.83, 158.09, 162.46, 165.08, 167.91; ESI-MS *m/z*: 414(M + H), 436 (M + Na)

2-(4-Bromobenzylidene)-1-(7-(trifluoromethyl)-2-(furan-2-yl)pyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (9c)

Yield: 88% (white solid); m.p: 204–206 °C; IR (KBr) cm^{-1}: 3428 (NH); ^1H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.11 (br, s, 1 H, -NH), 6.72 (s, 1 H, Ar–H), 7.12 (t, 1 H, Ar–H), 7.28 (m, 2 H, Ar–H), 7.35 (d, 1 H, $J = 7.934$ Hz, Ar–H), 7.48 (d, 1 H, $J = 8.240$ Hz, Ar–H), 7.72 (d, 1 H, $J = 7.934$ Hz, Ar–H), 8.78 (d, 1 H, $J = 8.240$ Hz, Ar–H), 8.94 (s, 1 H, = C–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm:

106.47, 107.859, 108.85, 114.16, 119.02 (q, J = 275.090 Hz)($\underline{C}F_3$), 124.18, 127.06, 132.20, 135.37, 136.52, 143.60, 144.56, 151.91 (q, J = 35.945 Hz) (C–CF_3), 153.92, 156.68, 160.56, 164.27, 168.36; ESI-MS *m/z*: 463 (M + H), 485 (M + Na)

2-(4-Nitrobenzylidene)-1-(7-(trifluoromethyl)-2-(furan-2-yl)pyrido[2,3-*d*]pyrimidin-4-yl)hydrazine (9d)

Yield: 68% (brown yellow solid); m.p: 151–153 °C; IR (KBr) cm^{-1}: 3416 (NH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.16 (br, s, 1 H, -NH), 7.73 (m, 2 H, Ar–H), 8.04 (d, 1 H, J = 8.697 Hz, Ar–H), 8.08 (t, 2 H, J = 8.545 Hz, Ar–H), 8.33 (d, 1 H, J = 8.697 Hz, Ar–H), 8.40 (t, 2 H, J = 8.545 Hz, Ar–H), 8.71 (s, 1 H, = C–H), 8.79 (t, 1 H, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 104.25, 104.98, 107.76, 120.56 (q, J = 276.557 Hz)(CF_3), 127.75, 129.32, 130.45, 132.05, 133.09, 149.09, 150.82 (q, J = 35.935 Hz) (C–CF_3), 151.02; ESI-MS m/z: 429(M + H), 451 (M + Na)

2-(2-Chloro-4-fluorobenzylidene)-1-(7-(trifluoromethyl)-2-(furan-2-yl)pyrido[2,3-*d*] pyrimidin-4-yl)hydrazine (9e)

Yield: 89% (yellow solid); m.p: 183–185 °C; IR (KBr) cm^{-1}: 3418 (NH); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.11 (br, s, 1 H, -NH), 6.72 (s, 1 H, Ar–H), 7.12 (t, 1 H, Ar–H), 7.28 (m, 2 H, Ar–H), 7.35 (d, 1 H, J = 7.934 Hz, Ar–H), 7.48 (d, 1 H, J = 8.240 Hz, Ar–H), 7.72 (d, 1 H, J = 7.934 Hz, Ar–H), 8.78 (d, 1 H, J = 8.240 Hz, Ar–H), 8.94 (s, 1 H, Ar–H), 8.94 (s, 1 H, = C–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 103.10, 106.74, 108.30, 118.91 (q, J = 275.090 Hz)($\underline{C}F_3$), 126.76, 129.78, 131.68, 134.68, 142.26, 143.83, 148.90 (q, J = 35.945 Hz) (C–CF_3) 149.07, 154.25, 154.44, 156.50, 162.43, 168.52; ESI-MS *m/z*: 436 (M + H), 458 (M + Na)

Preparation of 3-(substituted)-5-substituted-8-(trifluoromethyl pyrido[3,2-*e*][1,2,4]triazolo [4,3-*c*] pyrimidine derivatives (10a–f & 11a–e)

General procedure

Compound, 2-(4-substituted)-1-(7-(trifluoromethyl)-2-substituted pyrido[2,3-*d*]pyrimidin-4-yl)hydrazine **8a–f** and **9a–e** (54.5 mmol) were dissolved in dry THF (150 mL), and Chloramine-T (18.5 g, 65.6 mmol) was added at room temperature. The reaction mixture was heated to 60 °C for 1.5 h. The reaction mixture was cooled to room temperature and the organic phase was washed with 10 wt% aq. sodium sulfite solution and then evaporated to dryness. The resultant solid was purified by column chromatography to obtain respective products **10a–f** and **11a–e**.

3-(Fluorophenyl)-5-phenyl-8-(trifluoromethyl)pyrido[3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives (10a)

Yield: 83% (yellow solid); m.p: 183–185 °C; IR (KBr) cm^{-1}: 1608 (C = N), 1563 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.49 (m, 2 H, Ar–H), 7.66 (m, 3 H, A-H), 8.04 (d, 1 H, J = 8.19``2 Hz, Ar–H), 8.41 (t, 2 H, J = 9.781 Hz, Ar–H), 8.82 (t, 2 H, J = 9.781 Hz, Ar–H), 9.17 (d, 1 H, J = 8.192 Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 116.08, 119.93 (q, J = 272.156 Hz)($\underline{C}F_3$), 124.61, 126.69, 129.950, 130.77, 139.07, 145.12, 149.89 (q, J = 35.945 Hz) (C–CF_3), 151.42, 155.10, 155.69, 165.61, 167.96, 171.02; ESI-MS *m/z*: 410 (M + H), 432 (M + Na)

2-(4-Methoxybenzylidene)-1-(7-(trifluoromethyl)-2-(furan-2-yl)pyrido[2,3-*d*]pyrimidin-4-yl)hydrazine derivatives (10b)

Yield: 75% (dark brown solid); m.p: 179–181 °C; IR(KBr) cm^{-1}: 1601 (C = N), 1578 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.89 (s, 3 H, -OCH_3), 7.00 (d, 1 H, J = 7.934 Hz, Ar–H), 7.62 (m, 2 H, Ar–H), 7.65 (t, 1 H, Ar–H), 7.79 (t, 2 H, J = 8.392 Hz, Ar–H), 8.18 (t, 2 H, J = 8.392 Hz, Ar–H), 8.57 (s, 1 H, = C–H), 8.74 (d, 1 H, J = 7.934 Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 55.769, 106.160, 108.047, 119.327 (q, J = 274.356 Hz) ($\underline{C}F_3$), 127.653, 129.489, 130.256, 132.960, 135.330, 142.247, 144.443, 149.473 (q, J = 33.743 Hz) (CF_3), 155.830, 158.095, 162.464, 165.089, 167.914; ESI-MS *m/z*: 414 (M + H), 436 (M + Na)

3-(4-(Trifluoromethyl)phenyl)-5-phenyl-8-(trifluoromethyl)pyrido[3,2-*e*][1,2,4]triazolo[4,3-*c*] pyrimidine derivatives (10c)

Yield: 85% (light yellow solid); m.p: 173–175 °C; IR(KBr) cm^{-1}: 1612 (C = N), 1568 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.08 (t, 1 H, Ar–H), 7.42 (d, 1 H, J = 8.087 Hz, Ar–H), 7.56 (m, 3 H, Ar–H), 7.62 (t, 2 H, J = 7.172 Hz, Ar–H), 8.06 (t, 2 H, J = 7.172 Hz, Ar–H), 8.48 (m, 1 H, Ar–H), 8.52 (d, 1 H, J = 8.087 Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 114.09, 119.27 (q, J = 275.824 Hz)($\underline{C}F_3$), 124.56, 125.81, 126.08, 127.61, 128.05, 129.34, 130.19, 134.93, 135.10, 137.85, 138.72, 143.24, 149.08 (q, J = 38.146 Hz) (C–CF_3), 150.26, 151.04, 151.66, 154.59, 155.69, 165.65; ESI-MS *m/z*: 460 (M + H), 482 (M + Na)

3-(Nitrophenyl)-5-phenyl-8-(trifluoromethyl)pyrido [3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives (10d)

Yield: 89% (yellow solid); m.p: 232–234 °C; IR(KBr) cm^{-1}: 1614 (C = N), 1558 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.72 (m, 3 H, Ar–H), 8.04 (d, 1 H, $J = 8.697$ Hz, Ar–H), 8.08 (t, 2 H, $J = 8.545$ Hz, Ar–H), 8.33 (d, 1 H, $J = 8.697$ Hz, Ar–H), 8.40 (t, 2 H, $J = 8.545$ Hz, Ar–H), 8.74 (m, 2 H, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 123.17 (q, $J = 275.82$ Hz)($\underline{C}F_3$), 124.85, 126.01, 127.025, 128.93, 129.86, 130.26, 132.77, 134.25, 149.75 (q, $J = 38.879$ Hz) (C–CF_3), 150.92, 152.60, 153.34, 155.88, 156.87, 160.52, 163.92, 166.17; ESI-MS *m/z*: 437 (M + H), 459 (M + Na).

3-(Bromophenyl)-5-phenyl-8-(trifluoromethyl)pyrido [3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives (10e)

Yield: 69% (yellow solid); m.p: 196–198 °C; IR(KBr) cm^{-1}: 1602 (C = N), 1566 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.62 (m, 3 H, Ar–H), 7.71 (m, 2 H, Ar–H), 7.75 (d, 1 H, $J = 8.240$ Hz, Ar–H), 7.80 (d, 1 H, $J = 8.240$ Hz, Ar–H), 8.17 (t, 2 H, $J = 7.324$ Hz, Ar–H), 8.77 (t, 2 H, $J = 7.324$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 114.82, 117.61 (q, $J = 277.291$ Hz)($\underline{C}F_3$), 125.70, 126.43, 127.85, 129.875, 130.84, 132.91, 135.92, 143.93, 150.93 (q, $J = 32.277$ Hz) (C–CF_3), 155.24, 156.20, 159.89, 165.75, 166.51; ESI-MS *m/z*: 470 (M + H), 492 (M + Na)

3-(2-Chloro-4-fluorophenyl)-5-phenyl-8-(trifluoromethyl)pyrido[3,2-*e*][1,2,4]triazolo[4,3-*c*] pyrimidine derivatives (10f)

Yield: 81% (brown solid); m.p: 247–249 °C; IR(KBr) cm^{-1}: 1611 (C = N), 1559 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.12 (m, 2 H, Ar–H), 7.32 (m, 5 H, Ar–H), 7.49 (d, 1 H, $J = 8.087$ Hz, Ar–H), 7.72 (s, 1 H, Ar–H), 8.78 (d, 1 H, $J = 8.087$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 114.07, 118.71 (q, $J = 275.824$ Hz)($\underline{C}F_3$), 124.29, 125.27, 127.54, 128.44, 129.92, 130.45, 132.52, 135.86, 138.98, 144.04, 149.94 (q, $J = 36.679$ Hz) (C–CF_3), 152.60, 155.37, 165.12, 167.64; ESI-MS *m/z*: 444 (M + H), 466 (M + Na)

3-(4-Fluorophenyl)-5-furyl-8-(trifluoromethyl)pyrido [3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives (11a)

Yield: 90% (yellow solid); m.p: 236–238 °C; IR(KBr) cm^{-1}: 1603 (C = N), 1560 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.67 (m, 3 H, Ar–H), 8.03 (d, 1 H, $J = 8.192$ Hz, Ar–H), 8.16 (d, 1 H, $J = 8.192$ Hz, Ar–H), 8.41 (t, 2 H, $J = 14.305$ Hz, Ar–H), 8.82 (t, 2 H, $J = 14.305$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 104.25, 107.76, 116.89 (q, $J = 275.824$ Hz)($\underline{C}F_3$), 124.15, 125.17, 127.75, 129.25, 133.09, 142.40, 143.52, 150.12 (q, $J = 33.011$ Hz) (C–CF_3), 152.57, 153.69, 155.32, 163.19, 166.49; ESI-MS *m/z*: 400 (M + H), 422 (M + Na)

3-(4-Methoxyphenyl)-5-furyl-8-(trifluoromethyl) pyrido[3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives (11b)

Yield: 83% (brown yellow solid); m.p: 189–191 °C; IR (KBr) cm^{-1}: 1612 (C = N), 1561 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 3.89 (s, 3 H, -OCH_3), 8.73 (d, 1 H, $J = 7.934$ Hz, Ar–H), 8.17 (t, 2 H, $J = 15.432$ Hz), 7.79 (t, 2 H, $J = 15.432$ Hz, Ar–H), 7.62 (m, 3 H, Ar–H), 7.01 (d, 1 H, $J = 7.934$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ p.p.m.: 55.76, 106.16, 108.60, 114.76, 121.53 (q, $J = 274.356$ Hz)($\underline{C}F_3$), 127.49, 129.48, 130.25, 132.96, 135.22, 141.38, 143.51, 149.04 (q, $J = 37.412$ Hz) (C–CF_3), 152.03, 156.29, 158.14, 162.52, 165.79; ESI-MS *m/z*: 412 (M + H), 434 (M + Na)

3-(4-Bromophenyl)-5-furyl-8-(trifluoromethyl)pyrido [3,2-*e*][1,2,4]triazolo[4,3-*c*]pyrimidine derivatives (11c)

Yield: 78% (white solid); m.p: 183–185 °C; IR(KBr) cm^{-1}: 1614 (C = N), 1559 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.50 (d, 1 H, Ar–H), 7.64 (m, 2 H, Ar–H), 7.80 (d, 1 H, $J = 8.240$ Hz, Ar–H), 8.18 (t, 2 H, $J = 137.324$ Hz, Ar–H), 8.77 (d, 1 H, J = 8.240 Hz, Ar–H), 7.70 (t, 2 H, $J = 17.324$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ p.p.m.: 105.97, 107.37, 120.30 (q, $J = 275.090$ Hz)($\underline{C}F_3$), 127.40, 129.53, 132.19, 135.19, 142.89, 143.71, 149.90 (q, $J = 35.212$ Hz) (C–CF_3),152.22, 154.88, 157.14, 166.06; ESI-MS *m/z*: 459 (M + H), 481 (M + Na)

3-(4-Nitrophenyl)-5-furyl-8-(trifluoromethyl)pyrido [3,2-*e*][1,2,4]triazolo[4,3-*c*] pyrimidine derivatives (11d)

Yield: 67% (yellow solid); m.p: 204–206 °C; IR(KBr) cm^{-1}: 1606 (C = N), 1564 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 6.73 (d, 1 H, Ar–H), 7.73 (m, 2 H, Ar–H), 8.04 (d, 1 H, $J = 8.697$ Hz, Ar–H), 8.08 (t, 2 H, $J = 14.325$ Hz, Ar–H), 8.33 (d, 1 H, $J = 8.697$ Hz, Ar–H), 8.40 (t, 2 H, $J = 14.325$ Hz, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 106.57, 108.05, 118.52 (q, $J = 275.090$ Hz)(CF_3), 124.37, 126.72, 128.69, 135.74, 138.50, 143.88, 145.67, 149.81 (q, $J = 38.146$ Hz) (C–CF_3),

152.30, 154.18, 155.23, 166.29; ESI-MS *m/z*: 427 (M + H), 449 (M + Na)

3-(2-Chloro-4-fluorophenyl)-5-furyl-8-(trifluoromethyl)pyrido[3,2-*e*][1,2,4] triazolo[4,3-*c*] pyrimidine derivatives (11e)

Yield: 82% (yellow solid); m.p: 232–234 °C; IR(KBr) cm^{-1}: 1609 (C = N), 1565 (C = C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 7.12 (m, 2 H, Ar–H), 7.32 (m, 3 H, Ar–H), 7.49 (d, 1 H, $J = 8.392$ Hz, Ar–H), 7.72 (d, 1 H, $J = 8.392$ Hz, Ar–H), 8.78 (s, 1 H, Ar–H); ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100 MHz) δ ppm: 105.24, 107.44, 115.97 (q, $J = 276.557$ Hz)(CF_3), 124.40, 130.22, 131.73, 132.77, 134.48, 143.67, 144.611, 150.09 (q, $J = 37.412$ Hz) (C–CF_3), 152.60, 153.34, 155.04, 165.87, 166.17; ESI-MS *m/z*: 434 (M + H), 456 (M + Na)

Biological assay

Antifungal activity assay

Both the synthesized novel 4-hydrazone functionalized pyrido[2,3-*d*]pyrimidine derivatives (**8a–f** and **9a–e**) and 1,2,4-triazole fused pyrido[2,3-*d*]pyrimidine derivatives (**10a–f** and **11a–e**) were evaluated for their anti-*Candida* activity (Kamal et al. 2015; Document M27, 4th Ed. CLSI 2006, 2017) against various *Candida* strains including *Candida albicans* MTCC 227, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C. albicans* MTCC 3018, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020 and *Issatchenkia hanoiensis* MTCC 4755. All the yeast strains were cultured in Muller Hinton broth for 24 h at 37 °C. The cells of the grown *Candida* strains (equivalent to 0.5 McFarland standard) of 1×10^6 cfu/ml were seeded onto the Muller Hinton agar plates. Wells of 6 mm were made with a sterile borer and the derivatives (**8a–f** and **9a–e**) and (**10a–f** and **11a–e**) were added in a concentration range of 250–0.9 µg/ml. The derivative loaded plates were incubated at 37 °C for 24 h in an incubator. Later the wells with lowest concentration of the derivatives showing zone of inhibition are considered to represent the minimum inhibitory concentration (MIC). All the experiments were performed in triplicates and the average values are represented in Tables 3–5.

Minimum Fungicidal Concentration assay

Micro centrifuge tubes of 2 mL were used for determining the minimum fungicidal concentration (Kamal et al. 2015) for novel 4-hydrazone functionalized pyrido[2,3-*d*]pyrimidine derivatives and 1,2,4-triazole fused pyrido[2,3-*d*] pyrimidine derivatives. The dose ranges were prepared in Muller Hinton broth to which the overnight grown cells of the above listed *Candida* strains were added to attain a density of 1×10^6 cfu mL^{-1}. Later the compound treated suspension was seeded onto the Muller Hinton agar plates to incubate at 37 °C for 24 h. The least concentration of the test derivative that killed the *Candida* strains was considered as MFC. All test experiments were carried in triplicates and the mean values were represented as MFC in the Tables 6–8.

Ergosterol quantification assay

Procedure

The derivatives **10f**, **11d** and **11e** were analyzed for quantification of the ergosterol content in *Candida parapsilosis* MTCC 1744 and *C. krusei* MTCC 3020. The total sterol content in the *Candida* strains treated with the derivatives namely, **10f**, **11d** and **11e** was quantified by extraction method of Breivik and Owades (1957) with slight modifications. The tested *Candida* strains were inoculated into conical flasks with 50 mL of Sabouraud dextrose broth containing 0, 5, 10, and 20 µg/mL of each of the derivatives, **10f**, **11d** and **11e**. Further, these cultures were incubated overnight at 37 °C and agitation at 150 rpm. After incubation, the cell pellet of each *Candida* strain was collected into separate glass tubes and the net weight was measured. Alcoholic potassium hydroxide (25%) was added to the pellets, vortexed well and heated at 85 °C for 1 h in water bath. The resulting suspensions of each *Candida* strain was homogenized in distilled water: n-heptane (1:3) by vortexing. This enables the separation of the sterols into the heptane layer. These heptane fractions containing sterols were collected and stored at −20 °C for 24 h. These heptane fractions were subsequently diluted with ethanol and subjected to absorbance measurements. Absorbance of the extracted sterols was recorded between 240 and 300 nm employing UV-Visible spectrophotometer (Lambda 25, PerkinElmer, Shelton, CT, USA). These spectra are characteristic patterns of the ergosterol and a late sterol pathway intermediate [24(28) dehydroergosterol] and their quantities. At 281.5 nm, ergosterol and 24(28)dehydroergosterol (DHE) showed absorbance. While 24(28) DHE showed higher absorbance at 230 nm. Thus, the percentage of total ergosterol present in the control and untreated *Candida* strains can be quantified by calculating the total ergosterol + 24(28) DHE content, and later eliminating this by subtraction from the total characteristic absorption of 24(28)DHE. The ergosterol content in the test *Candida* strains treated with **10f**, **11d** and **11e** derivatives was calculated using the following equations:

Percentage of ergosterol + Percentage of 24(28) DHE = [(Absorbance at 281.5/ Absorbance at 290) × F] / Cell pellet weight of *Candida* strains

Percentage of 24(28) DHE = [(Absorbance at 230/ 518) × F] / Cell pellet weight of *Candida* strains

Thus, Percentage of ergosterol = [Percentage of ergosterol + Percentage of 24(28) DHE] - Percentage of 24(28) DHE

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

Computational studies

Docking studies were performed to identify the interactions of the most active compounds viz., **10f**, **11d** and **11e** with the crystal structure of sterol 14-alpha demethylase (CYP51) from a pathogenic yeast *Candida albicans* strain SC5314 in complex with the antifungal drug posaconazole (PDB ID: 5FSA) using Molegro Virtual Docker (Thomsen and Christensen 2006) (installed on an Intel Centrino Machine, Intel Corporation, Santa Clara, CA, USA). All the ligand structures were constructed using Chem3D ultra8.0 software, and then these structures were energetically minimized by using MOPAC (semi-empirical quantum mechanics), Jop Type with 100 iterations and minimum RMS gradient of 0.01, and saved as protein data bank (.pdb) format. The pre-downloaded PDB structure of protein (5FSA) was imported to the workspace. To obtain better potential binding sites in the protein, a maximum of five cavities were detected using parameters such as molecular surface (expanded Vander Waals), maximum number of cavities ($n = 5$), minimum cavity volume (10), maximum cavity volume (10000), probe size (1.20), maximum number of ray checks ($n = 16$), minimum number of ray hits ($n = 12$), and grid resolution (0.30). The chosen cavity was further refined using side-chain minimization by selecting the add-visible option at a maximum of steps per residue (10000) and a maximum of global steps (10000). The setup for side-chain flexibility by selection of the add-visible option, the setting for the selected flexible side chain residues of maximum 15 during the docking option, and other parameters, all were kept in default. The binding site on the receptor was defined as extending in $X = 15.90$, $Y = 11.73$, and $Z = 18.01$ directions around the Dock molecule with a radius of approximately 15.00 A°. The Mol Dock optimization search algorithm with a maximum of ten runs was used through the calculations, with all other parameters kept as defaults. One pose per run was retained based on root mean square division clustering using a heavy atom threshold set at 1.0 A° and an energy penalty of 100. Docking results showed that compounds and having highest MolDock scores, Rerank scores, hydrogen bond energies and protein-ligand interactions are shown in the Table 9. Validation of docking studies was done using active co-crystal ligand as shown in Fig. 2.

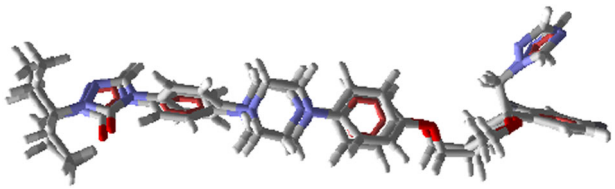


Fig. 2 Validation of docking with active co-crystal ligand

Results and discussion

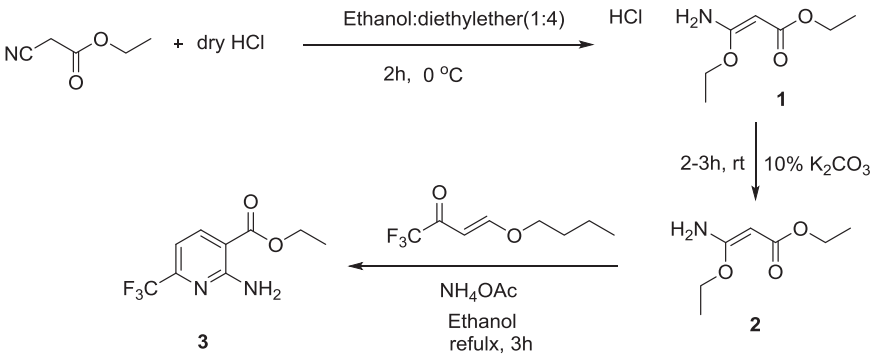
Chemistry

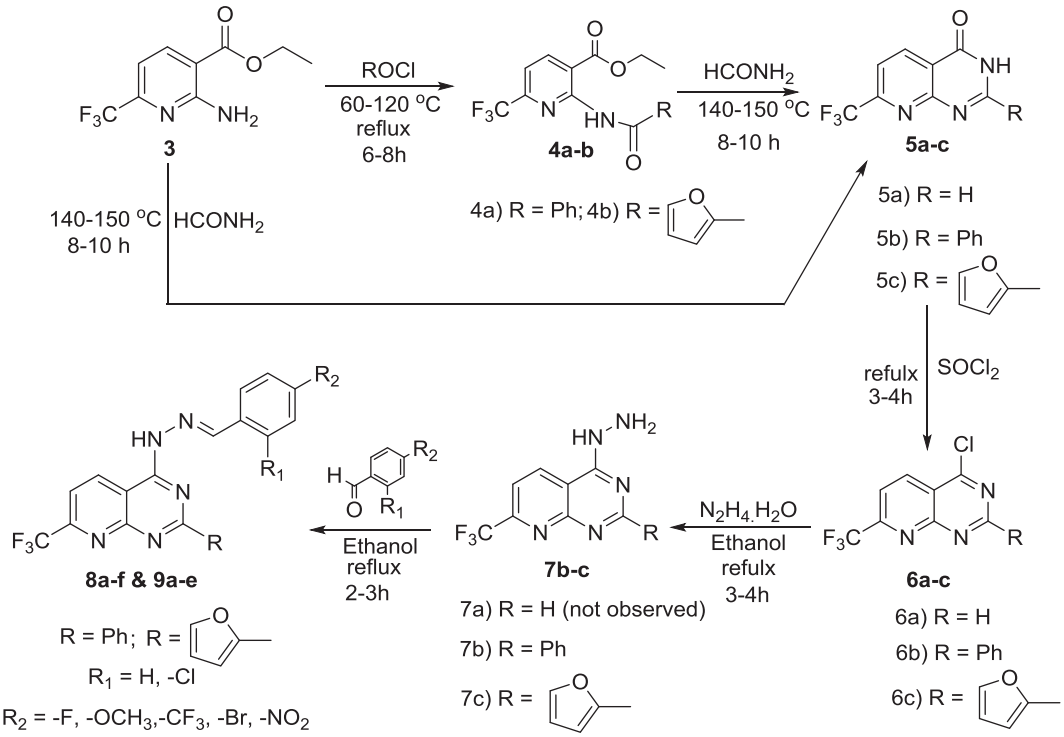
Synthesis of novel pyrido[2,3-*d*]pyrimidine 4-hydrazone derivatives (**8a–f** and **9a–e**) involve a series of steps. Ethyl 2-cyanoacetate on reaction with HCl gas in ethanol and diethyl ether (1:4) resulted hydrochloride salt of ethyl 3-amino-3-ethoxyacrylate **1** on neutralization obtained ethyl 3-amino-3-ethoxyacrylate **2**. Compound **2** on reaction with 4-butoxy-1,1,1-trifluorobut-3-en-2-one in ethanol in the presence of ammonium acetate resulted ethyl 2-amino-6-(trifluoromethyl) nicotinate **3**. The sequence of reaction mainly involves the attack of ammonia on *β*-carbon of compound **2** from ammonium acetate followed by elimination of ethanol to form 3,3-diamino ethyl acrylate in situ. This was followed by reaction with *n*-butyl vinyl acetyl ether to form product **3**. The 3,3-diamino ethyl acrylate amine nucleophile selectively attacks on vinyl carbon of *n*-butyl vinyl trifluoro acetyl ether followed by elimination of n-butanol and cyclization to form product **3**. (Scheme 1)

Compound **3** on reaction with different aryl acid chlorides in the range of 60–120 °C resulted ethyl 2-(substituted)-6-(trifluoromethyl) pyridine-3-carboxylate **4a–b**. Further, compounds **3** or **4a–b** in formamide at 140–150 °C obtained 2-substituted-7-(trifluoromethyl) pyrido[2,3-*d*] pyrimidin-4(3 *H*)-one derivatives **5a–c**. Compounds **5a–c** were treated with thionyl chloride under reflux for 3 h, resulted 4-chloro-2-substituted–7-(trifluoromethyl)-3,4-dihydro pyrido[2,3-*d*]pyrimidine derivatives **6a–c** and was further reacted with hydrazine hydrate in ethanol under reflux condition obtained 4-hydrazinyl-2-substituted-7-(trifluoromethyl)-3,4-dihydropyrido[2,3-*d*]pyrimidine derivatives **7b–c**. Compounds **7b** and **7c** were independently reacted with different substituted aryl aldehydes in ethanol in presence of triethyl amine at reflux and obtained 2-substituted pyrido[2,3-*d*]pyrimidine 4-hydrazone derivatives **8a–f** and **9a–e**, respectively. Synthetic pathways for the formation of all the derivatives represented in Scheme 2 and the physical properties of products were tabulated in Table 1.

Chloramine-T was prepared in situ by treating *p*–toluene sulfonamide and aqueous sodium hypochlorite at 0 °C for 1–2 h, followed by addition of compounds **8a–f** & **9a–e** in

Scheme 1 Synthesis of ethyl 2-amino-6-(trifluoromethyl) nicotinate





Scheme 2 Synthetic pathways for 2-phenyl-7-(trifluoromethyl)pyrido[2,3-*d*]pyrimidine derivatives (**8a**–**f**) and 2-furyl-7-(trifluoromethyl)pyrido [2,3-*d*]pyrimidine derivatives (**9a**–**e**)

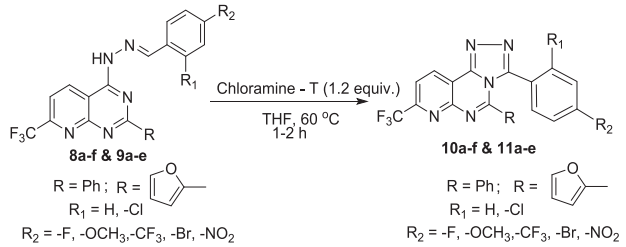
dry THF yielded 3-substituted 1,2,4-triazole fused pyrido [2,3-*d*]pyrimidine derivatives **10a**–**f** and **11a**–**e**. Chloramine-T afforded rapid conversions and high yields in most solvents. Synthetic pathways of all the derivatives were represented in Scheme 3 and the physical properties of products were tabulated in Table 2.

Antifungal activity

Two sets of final compounds such as 4-hydrazone functionalized pyrido[2,3-*d*]pyrimidine derivatives (**8a**–**f, 9a**–**e**) and 1,2,4-triazole fused pyrido[2,3-*d*]pyrimidine derivatives (**10a**–**f**, **11a**–**e**) were screened in vitro against ten fungal pathogenic strains such as *C. albicans* MTCC 227, *C. albicans* MTCC 1637, *C. albicans* MTCC 3017, *C.*

Table 1 Physical properties of 2-substituted 4-hydrazone functionalized pyrido[2,3-*d*]pyrimidine (**8a**–**f** and **9a**–**e**)

S.No.	Compd No.	R	R^1	R^2	Yield (%)
1	**8a**	C_6H_5	H	F	88
2	**8b**	C_6H_5	H	OCH_3	86
3	**8c**	C_6H_5	H	CF_3	79
4	**8d**	C_6H_5	H	NO_2	87
5	**8e**	C_6H_5	H	Br	76
6	**8 f**	C_6H_5	Cl	F	65
7	**9a**	C_4H_3O	H	F	89
8	**9b**	C_4H_3O	H	OCH_3	75
9	**9c**	C_4H_3O	H	Br	88
10	**9d**	C_4H_3O	H	NO_2	68
11	**9e**	C_4H_3O	Cl	F	89



Scheme 3 Synthetic pathways for triazole fused 7-(trifluoromethyl) pyrido[2,3-*d*]pyrimidine derivatives (**10a–f** and **11a–e**)

Table 2 Physical properties of triazole functionalized pyrido[2,3-*d*] pyrimidine derivatives (**10a–f** and **11a–e**)

S.No.	Compd No.	R	R^1	R^2	Yield (%)
1	**10a**	C_6H_5	H	F	83
2	**10b**	C_6H_5	H	OCH_3	79
3	**10c**	C_6H_5	H	CF_3	85
4	**10d**	C_6H_5	H	NO_2	89
5	**10e**	C_6H_5	H	Br	69
6	**10 f**	C_6H_5	Cl	F	81
7	**11a**	C_4H_3O	H	F	90
8	**11b**	C_4H_3O	H	OCH_3	83
9	**11c**	C_4H_3O	H	Br	67
10	**11d**	C_4H_3O	H	NO_2	78
11	**11e**	C_4H_3O	Cl	F	82

albicans MTCC 3018, *C. albicans* MTCC 4748, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, *C. glabrata* MTCC 3019, *C. krusei* MTCC 3020 and *Issatchenkiahanoiensis* MTCC 4755 using Miconazole as standard. Among all the 4-hydrazone functionalized pyrido [2,3-*d*]pyrimidine derivatives (**8a–f**, **9a–e**), compounds **8c**, **8f** and **9c** showed promising activity and more specifically, compound **8f** showed excellent antifungal activity against all the fungal strains at MIC value of 3.9 µg/mL, except for *C. albicans* MTCC 227, *C. albicans* MTCC 7315 and *C. parapsilosis* MTCC 1744 and is equal to standard Miconazole. Similarly, compound **9c** showed high activity against *C. albicans* MTCC 3018, *C. albicans* MTCC 4748 and *C. glabrata* MTCC 3019 with MIC value of 3.9 µg/mL. Compound **8c** also showed high activity against *C. glabrata* MTCC 3019 and *Issatchenkiahanoiensis* MTCC 4755 with MIC value of 3.9 µg/mL. The order of activity of compounds is found to be **8c < 9c < 8f**. Further, among all the 1,2,4-triazole fused pyrido[2,3-*d*]pyrimidine derivatives (**10a–f**, **11a–e**), compounds **10f**, **11d** and **11e** showed excellent activity against all the fungal strains at the MIC value of 3.9 µg/mL except for *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744, which is equal to the standard Miconazole. The order of activity is found to be **10f < 11d = 11e**. The antifungal activity data of compounds **8a–f** and **9a–e** were tabulated in Table 3 and the antifungal activity data of compounds **10a–f** and **11a–e** were tabulated in Table 4 and the antifungal activity data of promising compounds (**8c**, **8f**, **9c**, **10f**, **11d** and **11e**) were tabulated in Table 5.

Minimum fungicidal Concentration (MFC)

Compounds in each series were further screened for minimum fungicidal concentration against all the ten *Candida* strains. In 4-hydrazone functionalized pyrido[2,3-*d*]pyrimidine series, compound **8f** showed excellent fungicidal activity against all the organisms except *C. albicans* MTCC 227, *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744 at MFC value of 7.8 µg/mL and is equal to the standard Miconazole. Compound **9c** showed high activity against *C. albicans* MTCC 3018, *C. albicans* MTCC 4748 and *C. glabarata* MTCC 3019 at MFC value of 7.8 µg/mL. Similarly, compound **8c** showed activity against *C. glabrata* MTCC 3019 and *Issatchenkia hanoiensis* MTCC 4755 at MFC value of 7.8 µg/mL. The order of activity is confirmed as **8f > 9c > 8c**. Screening of 1,2,4-triazole functionalized pyrido[2,3-*d*]pyrimidine derivatives against these various *Candida* strains showed that, compound **11d** showed excellent activity against all the tested strains at MFC value of 3.9 µg/mL, except for *C. albicans* MTCC 7315, *C. parapsilosis* MTCC 1744 which is more than the standard Miconazole. Compounds **11e** and **10f** also showed good activity against all the *Candida* strains at MFC value of 7.8 µg/mL. The order of activity is confirmed as **11d > 11e = 10 f**. The minimum fungicidal concentration (MFC) data of compounds **8a–f** and **9a–e** were tabulated in Table 6 and the minimum fungicidal concentration (MFC) data of compounds **10a–f** and **11a–e** were tabulated in Table 7 and the minimum fungicidal concentration (MFC) data of promising compounds (**8c**, **8f**, **9c**, **10f**, **11d** and **11e**) were tabulated in Table 8.

Structure-Activity Relationship (SAR)

The structure verses activity of promising 4-hydrazone functionalized pyrido[2,3-*d*]pyrimidine derivatives **8f**, **9c** and **8c** revealed that, the presence of 2-chloro and 4-fluoro combination (compound **8f**) promoted promising antifungal activity. In the absence of chlorine and fluorine, a furyl substituent in 2nd position of pyrido[2,3-*d*]pyrimidine and 4-bromo in 4-hydrazone phenyl (compound **9c**) also favoured high antifungal activity. Alternatively, the presence of 4-CF_3 in 4-hydrazone phenyl (compound **8c**) also showed high activity. Similarly, the structure verses activity of promising 1,2,4-triazole fused pyrido[2,3-*d*]pyrimidine derivatives (**10f**, **11d** and **11e**) revealed that, the presence of 4-fluoro-2-chlorophenyl triazole and 2-furyl substituent in

Table 3 Antifungal activity of the synthesized derivatives (**8a–f** and **9a–e**)

Test Compounds	Minimum inhibitory concentration (MIC, µg/ml)									
	C.a[a]	*C.a*[b]	*C.a*[c]	*C.a*[d]	*C.a*[e]	*C.a*[f]	*C.p*[g]	*C.g*[h]	*C.k*[i]	*I.h*[j]
8a	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
8b	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
8c	>125	7.8	>125	>125	>125	>125	>125	3.9	7.8	3.9
8d	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
8e	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
8f	>125	3.9	3.9	3.9	3.9	>125	>125	3.9	3.9	3.9
9a	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
9b	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
9c	>125	7.8	>125	3.9	3.9	>125	>125	3.9	>125	>125
9d	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
9e	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
Miconazole (Standard)	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9

[a]*C. albicans* MTCC 227
[b]*C. albicans* MTCC 1637
[c]*C. albicans* MTCC 3017
[d]*C. albicans* MTCC 3018
[e]*C. albicans* MTCC 4748
[f]*C. albicans* MTCC 7315
[g]*C. parapsilosis* MTCC 1744
[h]*C. glabrata* MTCC 3019
[i]*C. krusei* MTCC 3020
[j]*Issatchenkia hanoiensis* MTCC 4755

Table 4 Antifungal activity of the synthesized derivatives (**10a–f** and **11a–e**)

Test Compounds	Minimum inhibitory concentration (MIC, µg/ml)									
	C.a[a]	*C.a*[b]	*C.a*[c]	*C.a*[d]	*C.a*[e]	*C.a*[f]	*C.p*[g]	*C.g*[h]	*C.k*[i]	*I.h*[j]
10a	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10b	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10c	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10d	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10e	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10f	3.9	7.8	3.9	3.9	3.9	7.8	15.6	3.9	3.9	3.9
11a	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
11b	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
11c	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
11d	3.9	3.9	3.9	3.9	3.9	7.8	15.6	3.9	3.9	3.9
11e	3.9	3.9	3.9	3.9	3.9	7.8	15.6	3.9	3.9	3.9
Miconazole (Standard)	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9

[a]*C. albicans* MTCC 227
[b]*C. albicans* MTCC 1637
[c]*C. albicans* MTCC 3017
[d]*C. albicans* MTCC 3018
[e]*C. albicans* MTCC 4748
[f]*C. albicans* MTCC 7315
[g]*C. parapsilosis* MTCC 1744
[h]*C. glabrata* MTCC 3019
[i]*C. krusei* MTCC 3020
[j]*Issatchenkia hanoiensis* MTCC 4755

Table 5 Antifungal activity of compounds **8c**, **8f**, **9c**, **10f**, **11d** and **11e**

Test compounds	Minimum inhibitory concentration (μg/mL)									
	C.a[a]	*C.a*[b]	*C.a*[c]	*C.a*[d]	*C.a*[e]	*C.a*[f]	*C.p*[g]	*C.g*[h]	*C.k*[i]	*I.h*[j]
8c	—	7.8	-	—	—	—	—	3.9	7.8	3.9
8 f	—	3.9	3.9	3.9	3.9	—	—	3.9	3.9	3.9
9c	—	7.8	—	3.9	3.9	—	—	3.9	—	—
10 f	3.9	7.8	3.9	3.9	3.9	7.8	15.6	3.9	3.9	3.9
11d	3.9	3.9	3.9	3.9	3.9	7.8	15.6	3.9	3.9	3.9
11e	3.9	3.9	3.9	3.9	3.9	7.8	15.6	3.9	3.9	3.9
Miconazole (control)	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9	3.9

[a]*C. albicans* MTCC 227
[b]*C. albicans* MTCC 1637
[c]*C. albicans* MTCC 3017
[d]*C. albicans* MTCC 3018
[e]*C. albicans* MTCC 4748
[f]*C. albicans* MTCC 7315
[g]*C. parapsilosis* MTCC 1744
[h]*C. glabrata* MTCC 3019
[i]*C. krusei* MTCC 3020
[j]*Issatchenkia hanoiensis* MTCC 4755

Table 6 Minimum fungicidal concentration (MFC) of the synthesized derivatives (**8a–f** and **9a–e**)

Test Compounds	Minimum fungicidal concentration (MFC, μg/ml)									
	C.a[a]	*C.a*[b]	*C.a*[c]	*C.a*[d]	*C.a*[e]	*C.a*[f]	*C.p*[g]	*C.g*[h]	*C.k*[i]	*I.h*[j]
8a	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
8b	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
8c	>125	15.6	>125	>125	>125	>125	>125	7.8	15.6	7.8
8d	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
8e	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
8f	>125	7.8	7.8	7.8	7.8	>125	>125	7.8	7.8	7.8
9a	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
9b	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
9c	>125	15.6	>125	7.8	7.8	>125	>125	7.8	>125	>125
9d	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
9e	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
Miconazole (Standard)	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8

[a]*C. albicans* MTCC 227
[b]*C. albicans* MTCC 1637
[c]*C. albicans* MTCC 3017
[d]*C. albicans* MTCC 3018
[e]*C. albicans* MTCC 4748
[f]*C. albicans* MTCC 7315
[g]*C. parapsilosis* MTCC 1744
[h]*C. glabrata* MTCC 3019
[i]*C. krusei* MTCC 3020
[j]*Issatchenkia hanoiensis* MTCC 4755

Table 7 Minimum fungicidal concentration (MFC) of the synthesized derivatives (**10a–f** and **11a–e**)

Test Compounds	Minimum fungicidal concentration (MFC, μg/ml)									
	C.a[a]	*C.a*[b]	*C.a*[c]	*C.a*[d]	*C.a*[e]	*C.a*[f]	*C.p*[g]	*C.g*[h]	*C.k*[i]	*I.h*[j]
10a	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10b	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10c	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10d	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10e	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
10f	7.8	15.6	7.8	7.8	7.8	15.6	31.2	7.8	7.8	7.8
11a	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
11b	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
11c	>125	>125	>125	>125	>125	>125	>125	>125	>125	>125
11d	7.8	7.8	7.8	7.8	7.8	15.6	15.6	7.8	7.8	7.8
11e	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8
Mic	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8

Mic Miconazole (standard)

[a]*C. albicans* MTCC 227

[b]*C. albicans* MTCC 1637

[c]*C. albicans* MTCC 3017

[d]*C. albicans* MTCC 3018

[e]*C. albicans* MTCC 4748

[f]*C. albicans* MTCC 7315

[g]*C. parapsilosis* MTCC 1744

[h]*C. glabrata* MTCC 3019

[i]*C. krusei* MTCC 3020

[j]*Issatchenkia hanoiensis* MTCC 4755

Table 8 Minimum fungicidal concentration (MFC) of compounds **8c**, **8f**, **9c**, **10f**, **11d** and **11e**

Test compounds	Minimum fungicidal concentration (μg/mL)									
	C.a[a]	*C.a*[b]	*C.a*[c]	*C.a*[d]	*C.a*[e]	*C.a*[f]	*C.p*[g]	*C.g*[h]	*C.k*[i]	*I.h*[j]
8c	—	15.6	—	—	—	—	—	7.8	15.6	7.8
8 f	—	7.8	7.8	7.8	7.8	—	—	7.8	7.8	7.8
9c	—	15.6	—	7.8	7.8	—	—	7.8	—	—
10 f	7.8	15.6	7.8	7.8	7.8	15.6	31.2	7.8	7.8	7.8
11d	3.9	3.9	3.9	3.9	3.9	7.8	15.6	3.9	3.9	3.9
11e	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8
Miconazole (control)	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8	7.8

[a]*C. albicans* MTCC 227

[b]*C. albicans* MTCC 1637

[c]*C. albicans* MTCC 3017

[d]*C. albicans* MTCC 3018

[e]*C. albicans* MTCC 4748

[f]*C. albicans* MTCC 7315

[g]*C. parapsilosis* MTCC 1744

[h]*C. glabrata* MTCC 3019

[i]*C. krusei* MTCC 3020

[j]*Issatchenkia hanoiensis* MTCC 4755

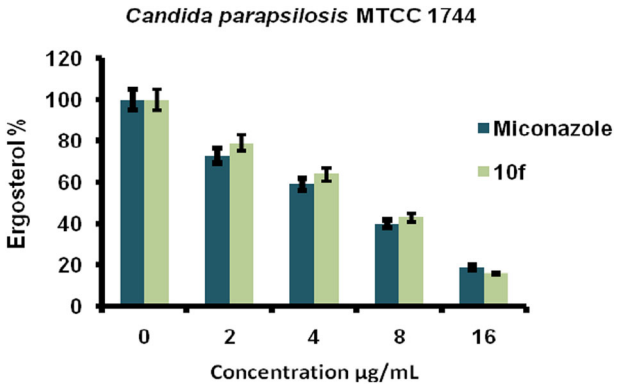


Fig. 3 Inhibition of ergosterol biosynthesis of derivative **10f**

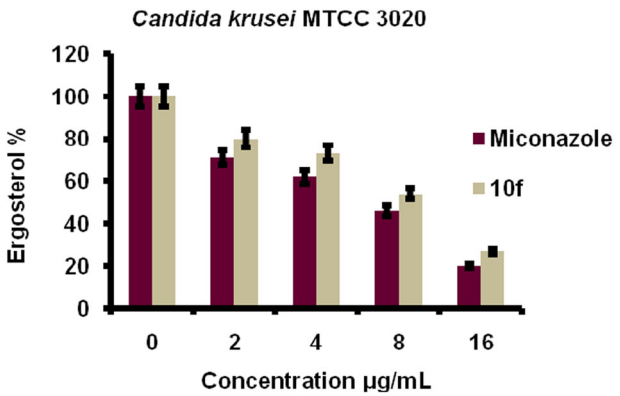


Fig. 4 Inhibition of ergosterol biosynthesis of derivative **10f**

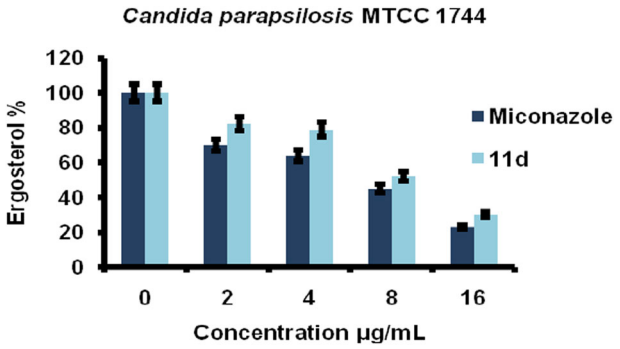


Fig. 5 Inhibition of ergosterol biosynthesis of derivative **11d**

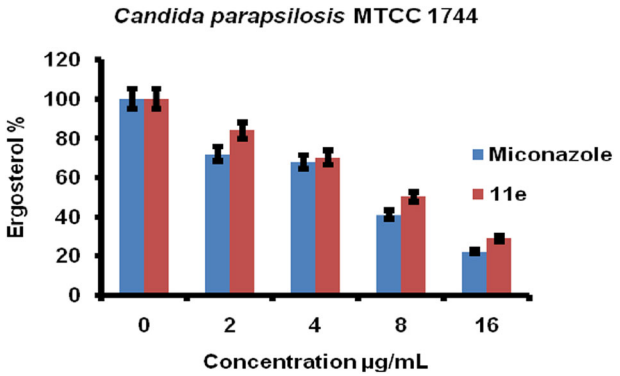


Fig. 6 Inhibition of ergosterol biosynthesis of derivative **11e**

pyrido[2,3-*d*]pyrimidine (compound **11e**) showed excellent antifungal activity. The 4-nitrophenyl triazole in combination with 2-furyl pyrido[2,3-*d*]pyrimidine (compound **11d**) also showed equal antifungal activity. The 2-chloro-4-fluoro phenyl triazole with 2-phenyl pyrido[2,3-*d*]pyrimidine (compound **10f**) showed promising antifungal activity. Based on the activity verses structure, it was felt that the presence of fluoro, trifluoromethyl, bromo and nitro groups on phenyl and furyl in pyrido[2,3-*d*]pyrimidine was crucial to promote antifungal activity. Further optimization is underway in order to find a lead molecule. All the strains showed MIC values equal to 3.9 µg/mL for Miconazole and therefore all the strains were considered as Miconazole susceptible isolates.

Ergosterol quantification assay

As ergosterol plays vital role in the cell wall of yeast, it becomes an important target of many antifungal drugs. Thus, the derivatives **10f**, **11d** and **11e** were assessed for their potential ergosterol inhibition. **10f**, **11d** and **11e** exhibited a dose-dependent decrease in ergosterol content as comparable to the Miconazole. Where, **10f** was effective in reducing the ergosterol content of *Candida parapsilosis* MTCC 1744 (Fig. 3) and *Candida krusei* MTCC 3020 (Fig. 4) in a dose-dependent manner. While, **11d** and **11e** reduced the ergosterol content of *Candida parapsilosis* MTCC 1744 as shown the figures (Figs. 5 and 6).

Computational studies

The ergosterol quantification assay confirmed that derivatives **10f**, **11d** and **11e** inhibited the ergosterol biosynthesis pathway which has prompted us to study the binding modes of these compounds with the crystal structure of lanosterol 14-alpha demethylase (CYP51) from a pathogenic yeast *Candida albicans* in complex with the antifungal drug posaconazole (PDB ID: 5FSA) using computational techniques. The results indicated that the amino acid residues present in the active binding site of the protein (PDB code: 5FSA) can frequently interact with compounds as demonstrated earlier (Thomsen and Christensen 2006). The docked poses of the compounds with the protein 5FSA are shown in Fig. 7, which clearly demonstrates the binding positions of ligands with the protein. The docked ligands and the active co-crystal ligand within the cavity are shown in Fig. 8. Analysis of the receptor ligand complex models generated after successful molecular docking of the synthesized compounds with the protein (5FSA) was done

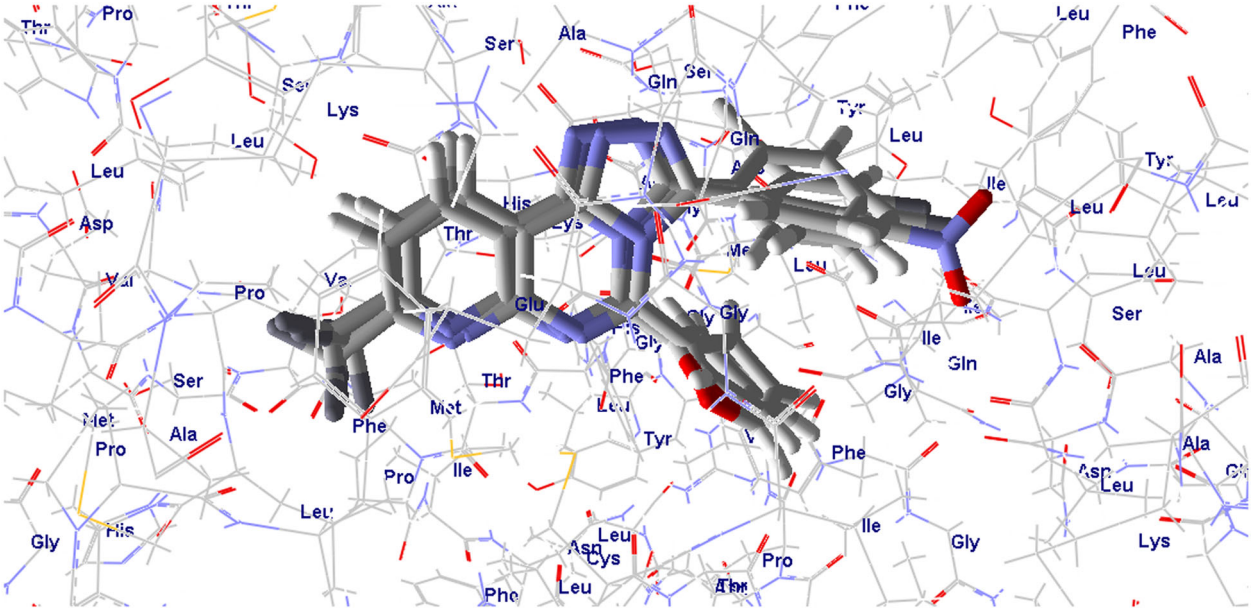


Fig. 7 Docked ligands with protein (PDB ID: 5FSA)

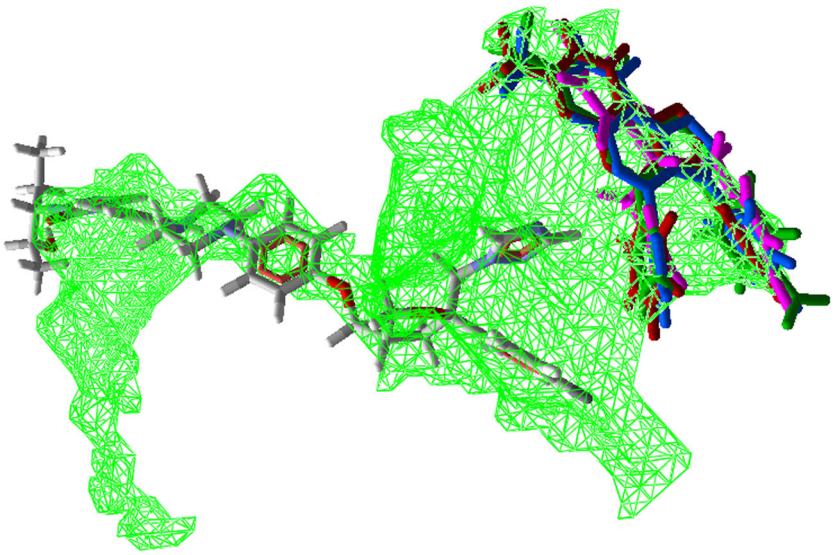


Fig. 8 Docked ligands and active co-crystal ligand within the cavity

based on the parameters such as H-Bond Energy, Protein-Ligand Interactions, Mol Dock Score of the docked compound within the active site. Protein-ligand interactions like hydrogen bonds, steric and electrostatic interactions between the ligand and the active site of the receptor were found to be responsible for mediating the biological activity. The results on H-Bond Energy, MolDock Score and Protein-Ligand Interactions are shown in Table 9 and the ligand interactions with the specific amino acid residues were represented in Figs. 9–11. All the potent compounds were docked in the active site of the target protein. Interestingly, all these docked poses showed interaction with the target protein in a similar manner. The superimposition of all these poses illustrates the binding mode of the compounds which is depicted in Fig. 7. It was observed that compound **10f** interacted with various amino acid residues such as Gly308, Gly472, Leu204, Cys470, Ala476, Ser312, Pro375 residues, while compound **11d** interacted with amino acid residues such as Leu276, Leu150, Gly472, Cys470, Ser312, Pro375, Pro462 and compound **11e** interacted with the amino acid residues such as Leu204, Gly308, Gly472, Cys470, Ala476, Ser312, Pro375, Thr311 of the target protein. While the standard Miconazole drug (control) interacted with amino acid residues such as Leu370, Gly308, Gly472, Cys470, Ser312, Ile304 of the target protein (Fig. 12).

Table 9 Protein-ligand interactions of the compounds **10f**, **11d** and **11e** with the target protein (PDB ID: 5FSA)

Ligand	MolDock score	Rerank score	H-bond	Protein-ligand interactions
10 f	−161.541	−131.865	−3.172	Gly 308, Gly 472, Leu 204, Cys 470, Ala 476, Ser 312, Pro 375
11d	−158.089	−134.206	−1.529	Leu 276, Leu 150, Gly 472, Cys 470, Ser 312, Pro 375, Pro 462
11e	−146.700	−117.478	−1.300	Leu 204, Gly 308, Gly 472, Cys 470, Ala 476, Ser 312, Pro 375, Thr 311
Miconazole	−116.067	−32.638	0.000	Leu 370, Gly 308, Gly 472, Cys 470, Ser 312, Ile 304

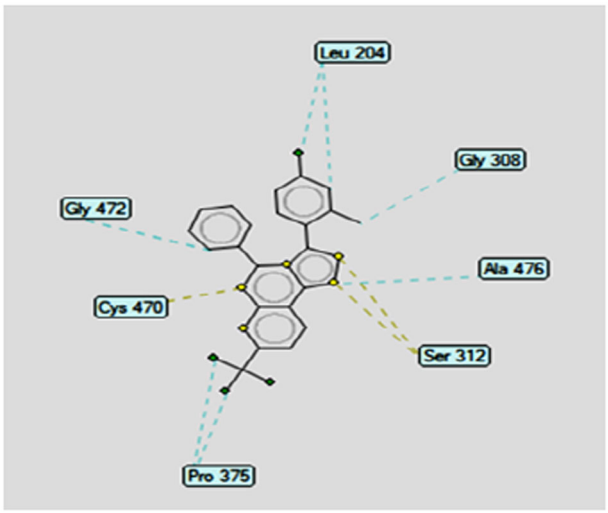


Fig. 9 Protein-ligand interactions of **10f** with protein (PDB ID: 5FSA)

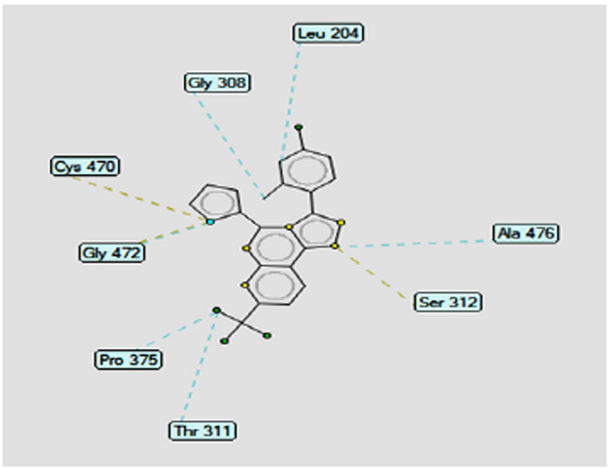


Fig. 11 Protein-ligand interactions of **11e** with protein (PDB ID: 5FSA)

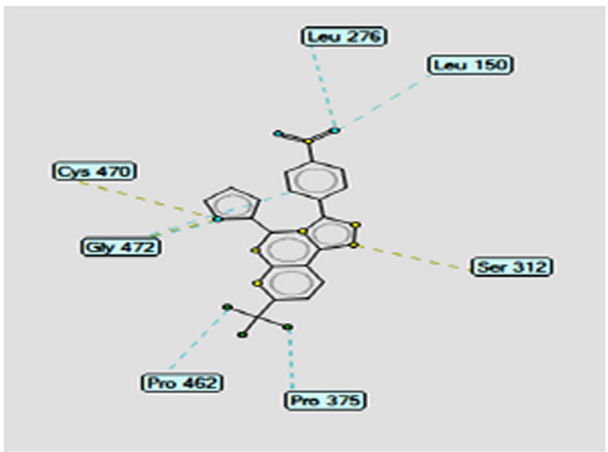


Fig. 10 Protein-ligand interactions of **11d** with protein (PDB ID: 5FSA)

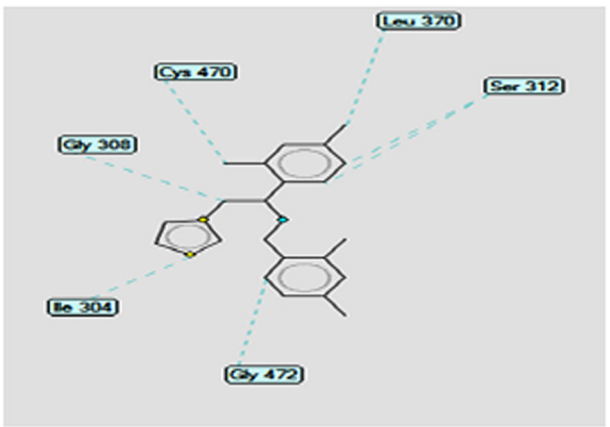


Fig. 12 Protein-ligand interactions of Miconazole (Standard control) with protein (PDB ID: 5FSA)

The docking study also revealed that the nitrogen of triazole and pyrimidine ring formed hydrogen bonding interactions with enzyme in compound **10f** suggesting that triazole and pyrimidine ring were important for inhibiting the 14-alpha-demethylase of *C. albicans* when in combination with pyridine ring (Fig. 9). In compounds **11d** and **11e**, the nitrogen of triazole and oxygen of furyl ring were responsible for hydrogen bonding interactions with enzyme suggesting that triazole and furyl ring is important for inhibiting the 14-alpha-demethylase of *C. albicans* when in combination with pyrido[2,3-*d*]pyrimidine ring (Figs. 10 and 11). Compound **10f** showed the best hydrogen bond interaction (-3.17) with SER312 and CYS470 amino acid residues. In case of compound **11d** and **11e**, the nitrogen of 1,2,4-triazole ring and oxygen of furyl ring formed hydrogen bond with SER312 and CYS470 amino acid residues, respectively. On the basis of activity data and docking results, it was observed that compounds **10f**, **11d** and **11e**

Table 10 Lipinski's parameter data for the synthesized compounds (**8a–f**, **9a–e**, **10a–f** and **11a–e**) and Miconazole (Control)

Code	Molecular weight	LogP	Hydrogen bond donor	Hydrogen bond acceptor	Molar refractivity
8a	411.000	5.10	1	5	104.72
8b	423.000	4.97	1	6	111.32
8c	461.000	5.98	1	5	109.77
8d	438.000	4.87	1	7	111.42
8e	471.000	5.72	1	5	112.46
8 f	445.500	4.98	1	5	106.80
9a	401.000	4.50	1	6	96.33
9b	413.000	4.37	1	7	102.92
9c	461.000	5.13	1	6	104.07
9d	428.000	4.27	1	8	103.02
9e	435.500	4.39	1	6	98.41
10a	409.000	4.91	0	4	101.02
10b	421.000	4.78	0	5	107.61
10c	459.000	5.79	0	4	106.06
10d	436.000	4.55	0	6	107.28
10e	469.000	5.53	0	4	108.76
10 f	443.500	4.79	0	4	103.10
11a	399.000	4.18	0	5	92.19
11b	411.000	4.05	0	6	98.79
11c	459.000	4.81	0	5	99.94
11d	426.000	4.27	0	7	99.98
11e	433.500	4.38	0	5	95.37
Mic	416.127	3.37	0	2	90.95

Mic Miconazole

showed interaction with the target in a similar manner and had the potential to inhibit CYP51 of *C. albicans* and therefore can be considered as lead moieties for further optimization.

Compounds bearing a phenyl ring at 2nd position of pyrido[2,3-*d*]pyrimidine moiety, and bearing 4-methoxy phenyl, 4-nitrophenyl ring at position-3 of 1,2,4-triazole moiety (compounds **10b** and **10d**), did not show any appreciable antifungal activity. Presence of 4-trifluoromethyl phenyl or 4-bromo phenyl at the same position (compounds **10c** and **10e**) also resulted in same. Presence of 2-chloro-4-fluoro substituted phenyl ring at this position (compound **10f**) brought an appreciable change in the antifungal activity of the compounds. Substitution of 2-chloro-4-fluoro phenyl with a 4-fluoro phenyl ring resulted in the loss of the activity (compound **10a**) which revealed the presence of chloro group had an appreciable role in biopotency of molecules. Substitution of a furyl ring with phenyl ring at 2nd position of pyrido[2,3-*d*]pyrimidine moiety also increases the antifungal activity of compounds having 4-nitro phenyl, 2-chloro-4-fluoro phenyl ring at position-3 of 1,2,4-triazole moiety (compounds **11d** and **11e**). The structure of the designed compounds needs to be further optimized by placing different substituents at position-3 of the 1,2,4-triazole moiety or position-2 of the pyrido[2,3-*d*]pyrimidine moiety. Another strategy could be the replacement of the 1,2,4-triazole ring with a different potent pharmacophore to achieve the best molecular scaffold, which can find use as a future drug.

Lipinski's rule of five

The Lipinski's rule of five (Lipinski 2004; Jayaram et al. 2012) was used to analyze drug likeness of compounds. It was found that all the synthesized compounds complied with these rules with the exception of **8a**, **8c**, **8e**, **9c**, **10c** and **10e**. All Lipinski's parameters are within the acceptable range, and hence they are considered as drug-like molecules and the results are depicted in Table 10.

Conclusions

In conclusion, a series of novel 4-hydrazone functionalized pyrido[2,3-*d*]pyrimidine and 1,2,4-triazole fused pyrido [2,3-*d*]pyrimidine derivatives were prepared under mild conditions, screened for antifungal activity and promising compounds in each case have been identified. Among the compounds tested, **8c**, **8f**, **9c**, **10f**, **11d** and **11e** exhibited significant antifungal activities, while all other 4-hydrazone

functionalized pyrido[2,3-*d*]pyrimidine and 1,2,4-triazole fused pyrido[2,3-*d*]pyrimidine derivatives showed moderate antifungal activity as compared with the standard. In addition, the binding mode of the tested compounds in the active site of target protein was predicted using a docking technique. SAR and molecular docking studies provided a clear understanding that the presence of fluoro, trifluoromethyl, bromo and nitro groups on phenyl and furyl ring in pyrido [2,3-*d*]pyrimidine may result in the development of novel triazole antifungal agents. Further optimization is underway in order to find a lead molecule. Lipinski's parameters of all compounds are within the acceptable range defined for human use thereby indicating their potential as drug-like molecules.

Acknowledgements N.R.A. is thankful to the Council of Scientific and Industrial Research (CSIR), New Delhi, India for the award of Senior Research Fellowship. N.R.A., N.R.K., K.S. and T.G. are also thankful to Council of Scientific and Industrial Research (CSIR), New Delhi, India for providing financial assistance in the form of Research Fellowship and contingency grant.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Adib M, Ayashi N, Heidari F, Mirzaei P (2016) Reaction between 4-nitro-1,3-diarylbutan-1-ones and ammonium acetate in the presence of morpholine and sulfur: an efficient synthesis of 2,4-Diarylpyrroles. Synlett 27(11):1738–1742

Adib M, Sheikhi E, Yazzaf R, Bijanzadeh HR, Mirzaei P (2016) An efficient, three-component synthesis of isoindolin-1-one-3-phosphonates under mild and solvent-free conditions. Tetrahedron Lett 57(8):841–844

Adib M, Yasaei Z, Mirzaei P (2016) A one-pot, multicomponent synthesis of 5′-amino-2,2′-dioxospiro[indoline-3,3′-pyrrole]-4′-carbonitriles. Synlett 27(03):383–386

Adib M, Zainali M, Kim I (2016) An efficient three-component synthesis of benzimidazo[1,2-a]-quinoline-6-carbonitriles. Synlett 27:1844–1847

Albengres E, Louet H, Tillement JP (1998) Drug interactions of systemic antifungal agents. Drug Safety 18(2):83–97

Al Mubarak S, Robert AA, Baskaradoss JK, Al-Joman K, Al Sohail A, Alsuwyed A, Ciancio S (2013) The prevalence of oral Candida infections in periodontitis patients with type 2 diabetes mellitus. J Infect Public Health 6(4):296–301

Bazgir A, Khanaposhtani MM, Soorki AA (2008) One-pot synthesis and antibacterial activities of pyrazolo[4',3':5,6]pyrido[2,3-*d*] pyrimidine-dione derivatives. Bioorg Med Chem Lett 18 (21):5800–5803

Bennett JE (1977) Flucytosine. Ann Intern Med 86(3):319–322

Bhat KS, Poojari B, Prasad DJ, Naik P, Holla BS (2009) Synthesis and antitumor activity studies of some new fused 1,2,4-triazole derivatives carrying 2,4-dichloro-5-fluorophenyl moiety. Eur J Med Chem 44:5066–5070

Brand A (2012) Hyphal growth in human fungal pathogens and its role in virulence. Int J Microbiol 2012:517529. https://doi.org/10.1155/2012/517529

Breivik ON, Owades JL (1957) Yeast analysis, spectrophotometric semimicro determination of ergosterol in yeast. J Agric Food Chem 5(5):360–363

CLSI Clinical and Laboratory Standards Institute (2006) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. CLSI documents M27-S3. Wayne, PA, USA.

CLSI (2017) Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. Document M27, 4th Ed., Clinical and Laboratory Standards Institute, Wayne, PA.

Cordeu L, Cubedo E, Bandres E, Rebollo A, Saenz X, Chozas H, Victoria Dominguez M, Echeverria M, Mendivil B, Sanmartin C, Palop JA, Font M, Garcia-Foncillas J (2007) Biological profile of new apoptotic agents based on 2,4-pyrido[2,3-*d*]pyrimidine derivatives. Bioorg Med Chem 15(4):1659–1669

Denning DW (2002) Echinocandins: a new class of antifungal. J Antimicrob Chemother 49(6):889–891

Espinel-Ingroff A (1998) Comparison of in vitro activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. J Clin Microbiol 36(10):2950–2956

Ezabadi IR, Camoutsis C, Zoumpoulakis P, Geronikaki A, Sokovic M, Glamocilija J, Ciric A (2008) Sulfonamide-1,2,4-triazole derivatives as antifungal and antibacterial agents: synthesis, biological evaluation, lipophilicity, and conformational studies. Bioorg Med Chem 16:1150–1161

Ghannoum MA, Rice LB (1999) Antifungal agents: Mode of action, mechanisms of resistance, correlation of these mechanisms and bacterial resistance. Clin Microbiol Rev 12:501–517

Gibbs WJ, Drew RH, Perfect JR (2005) Liposomal amphotericin B: clinical experience and perspectives. Expert Rev Anti Infect Ther 3(2):167–181

Grant SM, Clissold SP (1990) Fluconazole: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in superficial and systemic mycoses. Drugs 39(6):877–916

Hoesley C, Dismukes WE (1997) Overview of oral azole drugs as systemic antifungal therapy. Semin Resp. Crit Care Med 18 (03):301–309

Holla BS, Veerendra B, Shivananda MK, Poojary B (2003) Synthesis characterization and anticancer activity studies on some Mannich bases derived from 1,2,4-triazoles. Eur J Med Chem 38:759–767

Jayaram B, Singh T, Mukherjee G, Mathur A, Shekhar S, Shekhar V (2012) Sanjeevini: a freely accessible web-server for target directed lead molecule discovery. BMC Bioinformatics 13(Suppl 17):S7

Jessup CJ, Warner J, Isham N, Hasan I, Ghannoum MA (2000) Antifungal susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. J Clin Microbiol 38(1):341–344

Jitender Dev G, Poornachandra Y, Ratnakar Reddy K, Naresh Kumar R, Ravikumar N, Krishna Swaroop D, Ranjithreddy P, Shravan Kumar G, Nanubolu JB, Ganesh Kumar C, Narsaiah B (2017) Synthesis of novel pyrazolo[3,4-b]quinolinyl acetamide analogs, their evaluation for antimicrobial and anticancer activities, validation by molecular modeling and COMFA analysis. Eur J Med Chem 130:223–239

Johnson RH, Einstein HE (2007) Amphotericin B and coccidioidomycosis. Ann N Y Acad Sci 1111:434–441

Kamal A, Rahim A, Riyaz S, Poornachandra Y, Moku B, Kumar CG, Hussaini SM, Sridha B, Machiraju PK (2015) Regioselective synthesis, antimicrobial evaluation and theoretical studies of 2-styryl quinolines. Org Biomol Chem 13:1347–1357

Kaur R, Dwivedi AR, Kaur B, Kumar V (2016) Recent developments on 1,2,4-triazole nucleus in anticancer compounds: a review. Anticancer Agents Med Chem 16:465–489

Keating G, Figgitt D (2003) Caspofungin: a review of its use in oesophageal candidiasis, invasive candidiasis and invasive aspergillosis. Drugs 63:2235–2263

Lipinski CA (2004) Lead- and drug-like compounds: the rule-of-five revolution. Drug Discov Today Technol 1(4):337–341

Malagu K, Duggan H, Menear K, Hummersone M, Gomez S, Bailey C, Edwards P, Drzewiecki J, Leroux F, Quesada MJ, Hermann G, Maine S, Martin N, Smith G, Pass M (2009) The discovery and optimisation of pyrido[2,3-*d*]pyrimidine-2,4-diamines as potent and selective inhibitors of mTOR kinase. Bioorg Med Chem Lett. 19(20):5950–5953

Naresh Kumar R, Jitender Dev G, Ravi Kumar N, Krishna Swaroop D, Debanjan B, Bharat G, Narsaiah B, Nishanth Jain S, Gangagni Rao A (2016) Synthesis of novel triazole/isoxazole functionalized 7-(trifluoromethyl)pyrido[2,3-*d*]pyrimidine derivatives as promising anticancer and antibacterial agents. Bioorg Med Chem Lett 26(12):2927–2930

Nasr MN, Gineinah MM (2002) Pyrido[2,3-*d*]pyrimidines and pyrimido[5',4':5, 6]pyrido[2,3-*d*]pyrimidines as new antiviral agents: synthesis and biological activity. Arch Pharm 335(6):289–295

Nekkanti S, Tokala R, Shankaraiah N (2017) Targeting DNA minor groove by hybrid molecules as anticancer agents. Curr Med Chem 24(26):2887–2907

Neofytos D, Lu K, Hatfield-Seung A, Blackford A, Marr KA, Treadway S, Ostrander D, Nussenblatt V, Karp J (2013) Epidemiology, outcomes, and risk factors of invasive fungal infections in adult patients with acute myelogenous leukemia after induction chemotherapy. Diagn Microbiol Infect Dis 75(2):144–149

Oakley KL, Moore CB, Denning DW (1998) In vitro activity of the echinocandin antifungal agent LY303,366 in comparison with itraconazole and amphotericin B against *Aspergillus* spp. Antimicrob Agents Chemother 42(10):2726–2730

Onnis V, Cocco TMC, Fadda R, Congiu C (2009) Synthesis and evaluation of anticancer activity of 2-arylamino-6-trifluoromethyl-3-(hydrazonocarbonyl)pyridines. Bioorg Med Chem 17(17):6158–6165

Ozdemir A, Turan-Zitouni G, Kaplancikli ZA, Chevallet P (2007) Synthesis of some 4-arylidenamino-4H-1,2,4-triazole-3-thiols and their antituberculosis activity. J Enzyme Inhib Med Chem 22:511–516

Person K, Kontoyiannis DP, Alexander BD (2011) Fungal infections in transplant and oncology patients. Hematol Oncol Clin N Am 25:193–213

Pigaew R, Prachayasittikul V, Mandi P, Nantasenamat C, Prachayasittikul S, Ruchirawat S, Prachayasittikul V (2015) synthesis and molecular docking of 1,2,3-triazole-based sulfonamides as aromatase inhibitors. Bioorg Med Chem 23:3472–3480

Roma G, Grossi G, Braccio MD, Piras D, Ballabeni V, Tognolini M, Bertoni S, Barocelli E (2008) 1,8-Naphthyridines VII. New substituted 5-amino[1,2,4]triazolo[4,3-*a*][1,8] naphthyridine-6-carboxamides and their isosteric analogues, exhibiting notable anti-inflammatory and/or analgesic activities, but no acute gastrolesivity. Eur J Med Chem 43(8):1665–1680

Romani L (2004) Immunity to fungal Infections. Nat Rev Immunol 4(1):1–23

Ryder N, Favre B (1997) Antifungal activity and mechanism of action of terbinafine. Rev. Contemp. Pharmacother. 8:275–287

Sahu JK, Ganguly S, Kaushik A (2013) Triazoles: a valuable insight into recent developments and biological activities. Chin J Nat Med 11(5):456–465

Sinha R, Sharma P, Kumar P, Kuchhal V (2012) Terbinafine-induced taste impairment - report of two cases. J. Pak Assoc Dermatol 22(4):363–365

Thomsen R, Christensen MH (2006) MolDock: a new technique for high-accuracy molecular docking. J Med Chem 11:3315–3321

Tillotson J, Tillotson GS (2015) the regulatory pathway for antifungal drugs. Clin Infect Dis 61:678–683

Tsai PW, Chen YT, Hsu PC, Lan CY (2013) Study of *Candida albicans* and its interactions with the host. BioMedicine 3:51–64

Turan-Zitouni G, Kaplancikli ZA, Yildiz MT, Chevallet P, Kaya D (2005) synthesis and antimicrobial activity of 4-phenyl/cyclohexyl-5-(1-phenoxyethyl)-3-[N-(2-thiazolyl) acetamide]-thio-4*H*-1,2,4-triazole derivatives. Eur J Med Chem 40:607–613

VaanderWaal SN, Harvey PJ, McNamara DJ, Repine JT, Keller PR, Quin III J, Booth RJ, Elliott WL, Dobrusin EM, Fry DW, Toogood PL (2005) Pyrido[2,3-*d*]pyrimidin-7-ones as specific inhibitors of cyclin-dependent kinase 4. J Med Chem 48(7): 2371–2387

Vandeputte P, Ferrari S, Coste AT (2012) Antifungal resistance and new strategies to control fungal infections. Int J Microbiol. 2012:713687. https://doi.org/10.1155/2012/713687

Wang M, Yang J, Yuan M, Xue L, Tian Hli C, Wang X, Liu J, Zhang Z (2017) Synthesis and antiproliferative activity of a series of novel 6-substituted pyrido[3,2-*d*]pyrimidines as potential non-classical lipophilic antifolates targeting dihydrofolate reductase. Eur J Med Chem 128:88–97