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Synthesis and biological evaluation of 4-(2',4'-difluorobiphenyl-4-yl)-6-arylpyrimidin-2-amine derivatives

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4-(2',4'-difluorobiphenyl-4-yl)-6-arylpyrimidin-2-amines **5a–g**.

Abstract: 4-(2',4'-Difluorobiphenyl-4-yl)-6-arylpyrimidin-2-amines **5a–g** were synthesized and evaluated for their ability to inhibit various bacterial and fungal strains. The cytotoxicity of the compounds was investigated by a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against the Hep-2 cell line. Molecular docking studies were also conducted to locate the binding interaction with the target protein 4LRH.

Keywords: antimicrobial activity; cytotoxic activity; molecular docking; MTT assay.

Introduction

Pyrimidine is a key structural moiety of natural products, such as vitamins, coenzymes, and uric acid, as well as of many synthetic drugs including veronal, sulfadiazine, fluorouracil, glivec, and rosuvastatin [1, 2]. DNA and RNA contain pyrimidine fragments [3]. A wide range of biological activities have been noted for pyrimidine-containing natural products and synthetic molecules [4–8]. In particular, 2-aminopyrimidine derivatives have found important applications in pharmacology and as synthetic precursors in medicinal chemistry [9, 10]. Many fluorine-containing organic compounds including fluorinated pyrimidines are known to exhibit a broad range of biological activities [11–14]. Herein we report the synthesis and biological evaluation of new

Results and discussion

Chemistry

The desired compounds **5a–g** were synthesized as shown in Scheme 1 using the synthetic methodology described previously for similar derivatives [15, 16]. The key intermediates **3a–g** were prepared by the Claisen-Schmidt condensation method as reported previously [17]. The structures of the synthesized compounds were characterized by elemental analysis, IR, ^1H NMR, ^{13}C NMR spectroscopy, and mass spectrometry. The spectral analysis is fully consistent with the given structures. In several cases, the tentative assignments made by analysis of ^1H NMR and ^{13}C NMR spectra were verified by analysis of the ^1H - ^{13}C COSY spectra. The electrospray ionization mass spectra of all products show the expected peak at $[\text{M}+1]^+$.

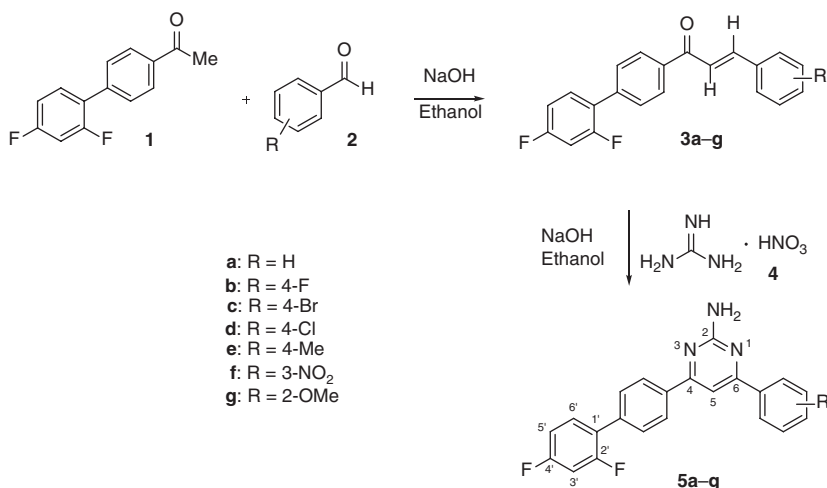
Antimicrobial evaluation

Compounds **5a–g** were tested against Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*), and fungi (*Candida albicans*) by the disc diffusion method. Additional evaluation was carried out on compounds **5a–g** to determine their minimum inhibitory concentration (MIC) values using the two-fold serial dilution method. The results are illustrated in Figures 1 and 2 (Tables S1 and S2 in the Supplementary Material). The mean zone of inhibition produced by these compounds against tested bacterial and fungal strains ranged from 7 mm to 16 mm. Under similar conditions, gentamycin, used as a positive control, produced a mean zone of inhibition ranging between 17 mm and 20 mm. It can be seen that the fluorine-substituted

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Scheme 1

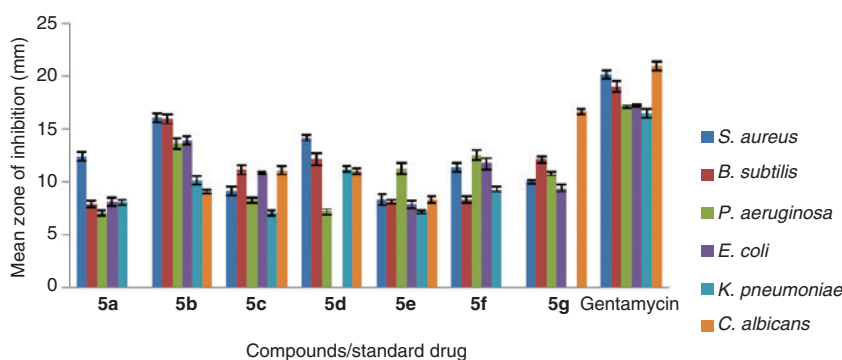


Figure 1 Antimicrobial activity of compounds 5a–g obtained by disc diffusion method.

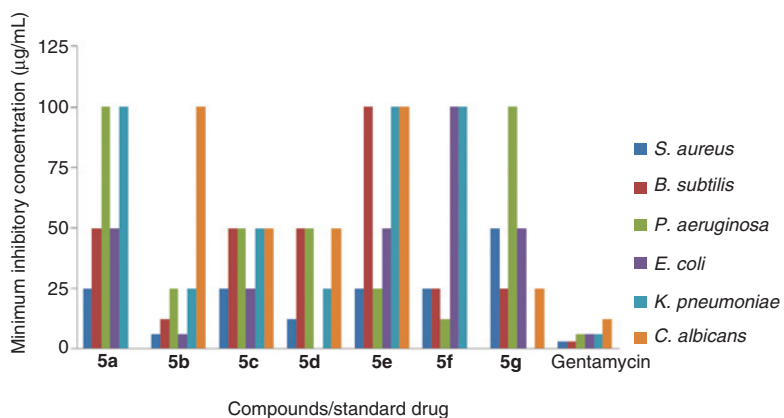


Figure 2 Antimicrobial activity of compounds 5a–g obtained by two-fold serial dilution method.

derivative **5b** possesses 75%–80% of the activity of gentamycin against all bacterial strains. Compound **5g** with methoxy substitution at the *ortho* position exhibits superior activity towards the fungal strain *C. albicans*. The MIC

results support the conclusion above that compound **5b** has a pronounced antibacterial activity. All other derivatives, **5a** and **5c–g**, exert only moderate activities against the tested organisms.

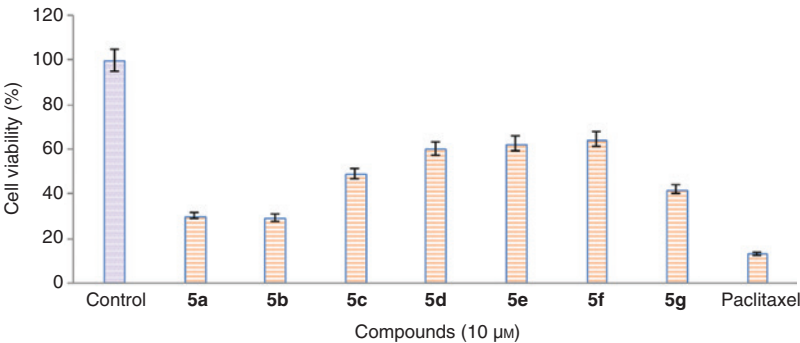


Figure 3 Cell viability of compounds 5a–g.

Table 1 Docking scores of compounds 5a–g.

Compound	5a	5b	5c	5d	5e	5f	5g
G score	−10.0	−9.9	−9.9	−9.9	−10.0	−9.9	−10.9
Interacting residues	TRP 171, TRP 140, HIE 135, ARG 103	TRP 171, TYR 60, TYR 85, THR 82	TRP 171, TRP 140, HIE 135, ARG 103	TRP 171, TRP 140, HIE 135, ARG 103	TRP 171, TRP 140, HIE 135, ARG 103	TRP 171, TRP 140, HIE 135, ARG 103	TRP 171, TRP 140, HIE 135, H ₂ O

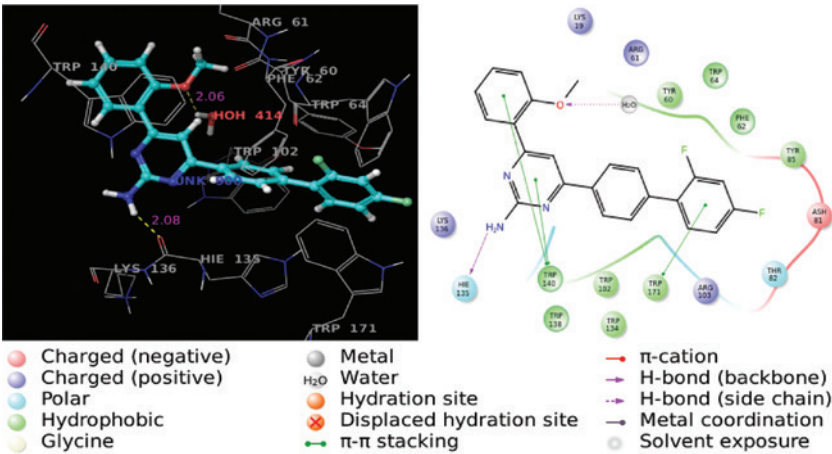


Figure 4 3D and 2D images of the complex of compound 5g with 4LRH protein residues.

Cytotoxicity

Compounds **5a–g** were assayed for their *in vitro* cytotoxic activity against the Hep-2 laryngeal carcinoma cell line by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay with maintaining a control (with solvent only). The results are shown in Figure 3. In general, the pyrimidine derivatives exhibit good cytotoxic activity against various cell lines like melanoma, colon cancer, ovarian cancer, and breast cancer

[18]. The results also indicate that the compounds are strongly inhibitory against the Hep-2 cell line. Based on the substitution on the phenyl ring, the cytotoxic activity decreases in the order of 4-F > H > 2-OCH₃ > 4-Br > 4-Cl > 4-CH₃ > 3-NO₂. It is evident that compound **5b** with lipophilic fluorine substitution exhibits the most potent inhibitory activity with a cytotoxicity of 70.5%. The results are in agreement with previous findings that incorporation of fluorine enhances the pharmacological activity of the modified molecule [19].

Molecular docking studies

Molecular docking is a promising tool to gain insight into the binding of the ligand and protein. Docking studies were made with protein carotenoid dehydrosqualene synthase from *S. aureus* complexed with BPH-673 (3ACX) and human folate receptor alpha in complex with folic acid (4LRH). Docking results of the ligands were expressed as a G score (Table 1), which is an empirical function that includes many factors like hydrophobicity, hydrogen bonding, and rotation penalty, among others. The docked ligands were ranked based on various types of interactions between the ligand and protein. The results reveal that all ligands can accommodate the active pocket of the receptor and show an excellent G score ranging from -10.9 to -9.9. The 3D- and 2D-binding modes of the best ligand **5g** are shown in Figure 4.

Conclusions

Compounds **5a–g** were synthesized and assessed for *in vitro* antimicrobial and cytotoxic activities, with the fluorine-substituted compound **5b** having the highest activity. The *in silico* docking studies revealed that compounds **5a–g** can inhibit the protein 4LRH and thus have great potential for development as anticancer agents.

Experimental

All reagents and solvents were of analytical grade and used without further purification. Melting points were measured in open glass capillaries and were uncorrected. The FT-IR spectra were recorded on an AVATAR-330 FT-IR spectrophotometer using KBr pellets. The NMR spectra were recorded in CDCl₃ on a BRUKER Avance III 400 spectrometer operating at 400 MHz for ¹H and 100 MHz for ¹³C. Mass spectra were recorded on a SCIEX-API 2000 spectrometer. Elemental analysis was carried out using a VARIO-MICRO V2.2.0 CNH analyzer.

Antimicrobial assays

The clinical isolates of bacterial strains *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, and a fungal strain *Candida albicans* were obtained from the Department of Microbiology, Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalai Nagar, Tamilnadu, India. The strains were inoculated on a sterile medium and sub-cultured on Mueller Hinton agar plates and maintained on agar at 4°C. Gentamycin was used as a positive control for bacteria and fungi.

Disc diffusion assay

Antimicrobial activity was assayed using the standard disc diffusion method. Inhibition zones were measured and compared with the standard positive controls. All the tests were carried out in triplicate.

Minimum inhibitory concentration (MIC)

The dilution susceptibility testing method was used for MIC determination with reference to the literature [20]. The test compounds were dissolved in 1 mL of chloroform. The growth was indicated by the measure of a white pellet on a well bottom.

Cell viability assay

The cytotoxicity of the synthesized compounds was tested against Hep-2 cell lines using the MTT assay [21]. The MTT assays were performed four times independently, and each independent experiment was done in triplicate. The percentage of survival was calculated using the formula:

$$\% \text{ Survival} = \frac{\text{live cell number (test)}}{\text{live cell number (control)}} \times 100.$$

$$\text{Cytotoxicity (\%)} = 100 - \% \text{ survival}$$

Molecular docking

Molecular docking was performed with the aid of Maestro v. 9.3.5 of the Schrödinger software suite, 2011. The 3D crystallographic structures of proteins (PDB ID: 4LRH, 3ACX) were retrieved from Protein Data Bank (www.rcsb.org/pdb). The protein structures were pre-processed and refined by Protein Preparation Wizard. Further, they were minimized by Optimized Potential for Liquid Simulations (OPLS)-2005 force field until the root mean square deviation (RMSD) reached the value of 0.3 Å. The ligands were optimized by a LigPrep program using the OPLS-2005 force field to generate the lowest energy state of ligands. The molecular docking studies of the ligand and protein were carried out by GLIDE. The best fit ligands with the target protein were ranked based on G score.

Synthesis of 4-(2',4'-difluorobiphenyl-4-yl)-6-arylpyrimidin-2-amines **5a–g**

A solution of (2*E*)-1-(2',4'-difluorobiphenyl-4-yl)-3-arylprop-2-en-1-one (**3a–g**, 0.01 mol) and guanidine nitrate (**4**, 0.01 mol) in ethanol (30 mL) was treated with aqueous sodium hydroxide (10%, 2 mL) and the mixture was heated under reflux for 2 h. An additional amount of the sodium hydroxide solution was added and the mixture was heated for an additional 6 h. Progress of the reaction was monitored by thin layer chromatography (TLC). After cooling, the mixture was poured into crushed ice and the resultant solid was filtered out, washed with water, dried, and crystallized from ethanol.

4-(2',4'-Difluorobiphenyl-4-yl)-6-phenylpyrimidin-2-amine (5a) Yellow solid; yield 85%; mp 104–106°C; IR: 3495 (N-H), 1631

(C=N), 2845–3047 (C-H), 1103, 1141 cm^{-1} (C-F); ^1H NMR: δ 6.78 (s, 2H, NH_2), 7.77 (s, 1H, H5), 7.20–8.34 (m, 12H, Ar-H); ^{13}C NMR: δ 101.9 (C5), 104.3–137.3 (Ar-C), 159.2 (C4'), 162.1 (C2'), 163.9 (C2), 164.2 (C4), 164.9 (C6); ESI-MS: m/z 360.5 $[\text{M}+1]^+$. Anal. Calcd for $\text{C}_{22}\text{H}_{15}\text{F}_2\text{N}_3$: C, 73.53; H, 4.21; N, 11.69. Found: C, 73.34; H, 4.45; N, 11.38.

4-(2',4'-Difluorobiphenyl-4-yl)-6-(4-fluorophenyl)pyrimidin-2-amine (5b) Pale yellow solid; yield 87%; mp 144–146°C; IR: 3428 (N-H), 1639 (C=N), 2849–2958 (C-H), 1098, 1137 (C-F); ^1H NMR: δ 6.80 (s, 2H, NH_2), 7.78 (s, 1H, H5), 7.04–8.34 (m, 11H, Ar-H); ^{13}C NMR: δ 101.6 (C5), 104.4–136.7 (Ar-C), 159.1 (C4'), 161.5 (C2'), 163.8 (C2), 163.9 (C4), 164.3 (C6); ESI-MS: m/z 378.2 $[\text{M}+1]^+$. Anal. Calcd for $\text{C}_{22}\text{H}_{14}\text{F}_3\text{N}_3$: C, 70.02; H, 3.74; N, 11.14. Found: C, 70.35; H, 3.98; N, 11.07.

4-(2',4'-Difluorobiphenyl-4-yl)-6-(4-bromophenyl)pyrimidin-2-amine (5c) Yellow solid; yield 79%; mp 96–98°C; IR: 3423 (N-H), 1637 (C=N), 2849–2958 (C-H), 1101, 1140 cm^{-1} (C-F); ^1H NMR: δ 6.84 (s, 2H, NH_2), 7.80 (s, 1H, H5), 7.22–8.34 (m, 11H, Ar-H); ^{13}C NMR: δ 101.8 (C5), 104.3–140.6 (Ar-C), 159.1 (C4'), 161.9 (C2'), 163.8 (C2), 163.9 (C4), 164.5 (C6); ESI-MS: m/z 438.0 $[\text{M}+1]^+$. Anal. Calcd for $\text{C}_{22}\text{H}_{14}\text{F}_2\text{N}_3\text{Br}$: C, 60.29; H, 3.22; N, 9.59. Found: C, 60.26; H, 3.59; N, 9.31.

4-(2',4'-Difluorobiphenyl-4-yl)-6-(4-chlorophenyl)pyrimidin-2-amine (5d) Yellow solid; yield 82%; mp 128–130 °C; IR: 3493 (N-H), 1636 (C=N), 2855–3080 (C-H), 1101, 1141 (C-F) cm^{-1} ; ^1H NMR: δ 6.75 (s, 2H, NH_2), 7.78 (s, 1H, H5), 7.22–8.33 (m, 11H, Ar-H); ^{13}C NMR: δ 101.8 (C5), 104.4–140.2 (Ar-C), 159.1 (C4'), 161.9 (C2'), 163.7 (C2), 163.9 (C4), 164.5 (C6); MS: m/z 394.2 $[\text{M}+1]^+$. Anal. Calcd for $\text{C}_{22}\text{H}_{14}\text{F}_2\text{N}_3\text{Cl}$: C, 67.10; H, 3.58; N, 10.67. Found: C, 67.03; H, 3.83; N, 10.42.

4-(2',4'-Difluorobiphenyl-4-yl)-6-(4-methylphenyl)pyrimidin-2-amine (5e) Pale brown solid; yield 76%; mp 136–138°C; IR: 3482 (N-H), 1628 (C=N), 2918–3041 (C-H), 1104, 1142 cm^{-1} (C-F); ^1H NMR: δ 2.38 (s, 3H, CH_3), 6.74 (s, 2H, NH_2), 7.73 (s, 1H, H5), 7.0–8.32 (m, 11H, Ar-H); ^{13}C NMR: δ 20.9 (CH_3), 101.5 (C5), 104.4–140.3 (Ar-C), 159.1 (C4'), 162.1 (C2'), 163.9 (C2), 164.1 (C4), 164.9 (C6); ESI-MS: m/z 374.1 $[\text{M}+1]^+$. Anal. Calcd for $\text{C}_{23}\text{H}_{17}\text{F}_2\text{N}_3$: C, 73.98; H, 4.59; N, 11.25. Found: C, 73.87; H, 4.77; N, 10.93.

4-(2',4'-Difluorobiphenyl-4-yl)-6-(3-nitrophenyl)pyrimidin-2-amine (5f) Brown solid; yield 89%; mp 162–164°C; IR: 3491 (N-H), 1637 (C=N), 2851–3082 (C-H), 1101, 1141 cm^{-1} (C-F); ^1H NMR: δ 6.97 (s, 2H, NH_2), 7.96 (s, 1H, H5), 7.22–9.07 (m, 11H, Ar-H); ^{13}C NMR: δ 102.2 (C5), 104.3–148.3 (Ar-C), 159.1 (C4'), 161.7 (C2'), 163.1 (C2), 163.9 (C4), 164.9 (C6); ESI-MS: m/z : 405.4 $[\text{M}+1]^+$. Anal. Calcd for $\text{C}_{22}\text{H}_{14}\text{F}_2\text{N}_5\text{O}_2$: C, 65.35; H, 3.49; N, 13.86. Found: C, 64.97; H, 3.71; N, 13.98.

4-(2',4'-Difluorobiphenyl-4-yl)-6-(2-methoxyphenyl)pyrimidin-2-amine (5g) Yellow solid; yield 78%; mp 130–132°C; IR: 3450 (N-H), 1597 (C=N), 2837–3072 (C-H), 1100, 1138 cm^{-1} (C-F); ^1H NMR: δ 3.85 (s, 3H, OCH_3), 6.69 (s, 2H, NH_2), 7.79 (s, 1H, H5), 7.01–8.06 (m, 11H, Ar-H); ^{13}C NMR: δ 55.4 (OCH_3), 96.9 (C5), 104.1–138.8 (Ar-C), 159.1 (C4'), 161.6 (C2'), 163.0 (C2), 163.9 (C4), 164.5 (C6); ESI-MS: m/z 392.3 $[\text{M}+3]^+$. Anal. Calcd for $\text{C}_{23}\text{H}_{17}\text{F}_2\text{N}_3\text{O}$: C, 70.94; H, 4.40; N, 10.79. Found: C, 70.58; H, 4.69; N, 10.50.

Supplementary information

Tabulated biological activities of all compounds, 2-D NMR of compound **5a**, and docking poses of all compounds are given in the online Supplement.

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