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Research Article

Design, Synthesis and Antifungal Evaluation of N-Substituted Maleimide Derivatives with Imidazole and 2-Methyl Imidazole Moieties

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ABSTRACT

A series of *N*-substituted maleimide derivatives with an attached imidazole and 2-methyl imidazole heterocyclic rings were designed, synthesized and evaluated for their antifungal activity against four pathogenic fungi. ^{1}H-NMR, ^{13}C-NMR and mass spectra confirmed the chemical structures of all synthesized compounds. All compounds 4a-4g, 5b and 5f were initially screened for qualitative (zone of inhibition) antifungal activity against *C. albicans*, *A. fumigatus*, *A. niger*, and *A. oryzae*. The screening results indicate that most of the synthesized compounds showed significant antifungal activity against the tested fungi. Furthermore, the compounds that showed a significant zone of inhibition were selected and tested quantitatively (MIC_{50} and IC_{50}) against the same pathogenic fungi species. The MIC_{50} and IC_{50} results of selected compounds were analyzed. These results strongly suggest that compound 5f has shown promising antifungal activity. Furthermore, the structure–activity relationship of compound 5f revealed that the compound substituted with the –F group possess prominent antifungal activity.

INTRODUCTION

Fungi infect billions of people every year.[1] During the past two decades, the incidence and death rate due to deep fungal infection have drastically increased in life-threatening infections, especially in immune-compromised hosts suffering from tuberculosis, cancer, AIDS, bone marrow transplants and solid-organ transplants.[2-5] Despite the high morbidity and mortality associated with major diseases caused by fungal infections, treatment options are limited.

Now a days, the azole class of imidazole antifungal agents such as butoconazole, clotrimazole, econazole, ketoconazole, miconazole, oxiconazole, sulconazole, tioconazole,[6] bifonazole,[7] isoconazole,[8] lanoconazole, andluliconazole[9] are used clinically (Fig. 1). As a typical azole antifungal agent, it possesses a unique class of mechanism of action via blocking the conversion of lanosterol to ergosterol in fungal cell membranes by inhibiting cytochrome P_{450}. As a result, the lack of ergosterol in the fungal cell membrane makes it very unstable; it begins to break down and the fungal organism dies.[10-12] Furthermore, the structure-activity-relationship (SAR) studies of clinically available azole antifungal agents included the selection of some common pharmacophore features and their importance for antifungal activity. It includes: a) The heterocyclic region: At the molecular level, the electron pair of nitrogen atoms present at 3-position of imidazole and 4-position of 1,2,4-triazole is binding to the heme iron of enzyme-bound cytochrome P_{450} to inhibit

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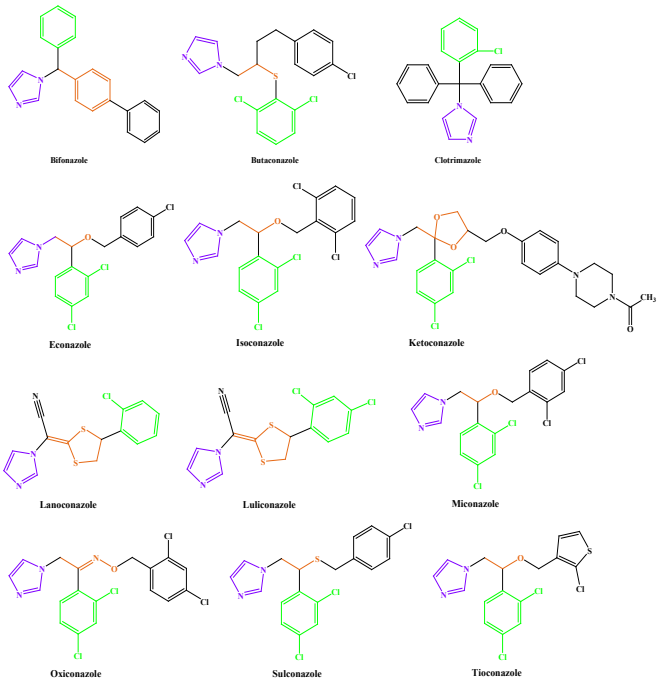


Fig. 1: Clinically available azole class of imidazole antifungal agents

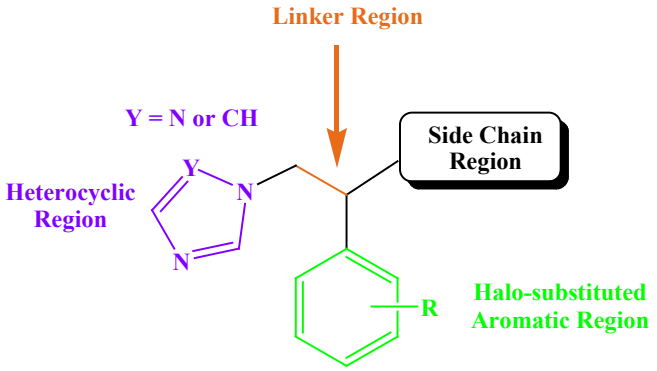


Fig. 2: Based on SAR studies, the selection of common pharmacophore.

activation of molecular oxygen and prevent oxidation of steroidal substrates like lanosterol by the enzyme, b) The halo-substituted region: The most potent antifungal azoles possess two or three aromatic rings, at least one of which is halogen substituted and others are non-polar functional groups, c) Linker region: Clinically available maximum azoles have shown an ethane linker region present between the halo-substituted aromatic ring and heterocyclic rings such as imidazole or 1,2,4-triazole. This region showed the presence of various substituents at 2-position of the ethane linkage, such as 1,3-dioxolan-2-yl, tetrahydrofuran-2-yl, hydroxyl, oxy, thio, etc., and d) Side chain region: The side chain regions of clinically available antifungal agents have shown various substituents such as phenyl, 1,2,4-triazole, pyrimidine, thiazole, etc. These non-polar functionalities confer high lipophilicity of the antifungal agents (Fig. 2).[6,10,13]

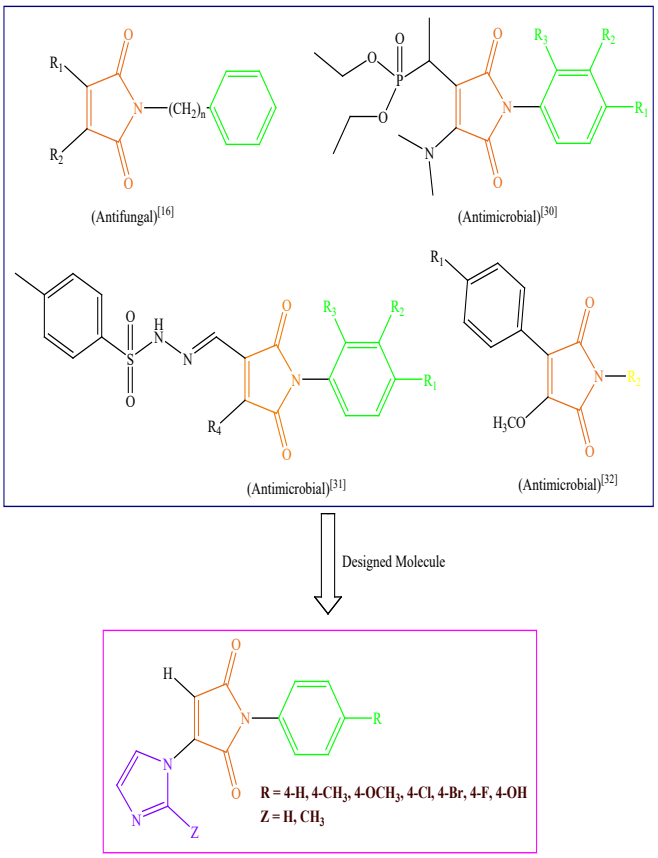


Fig. 3: Design strategy of *N*-substituted maleimide derivatives with an imidazole and 2-methyl imidazole moieties

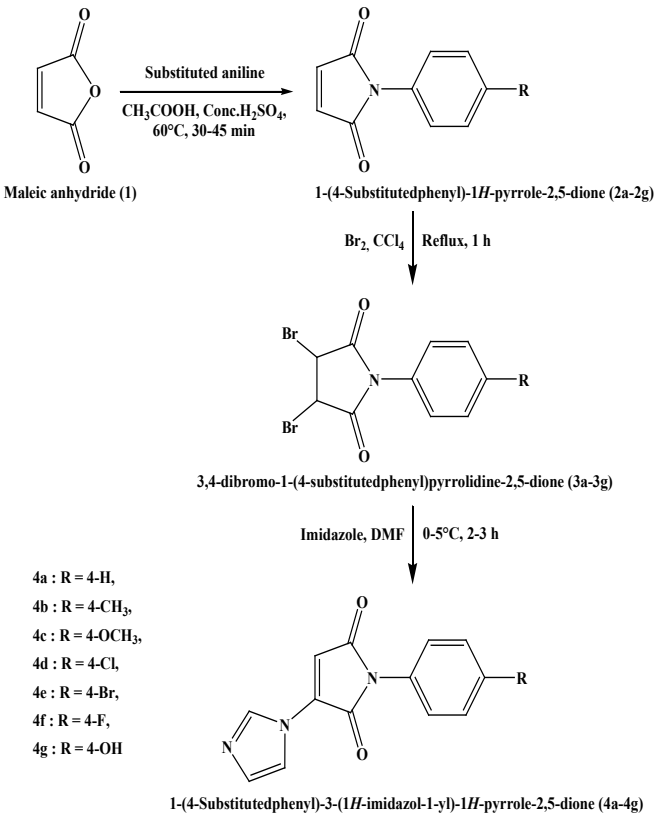
On the other hand, many researchers reported that *N*-substituted maleimide derivatives can be used as a medicinal agent with a range of biological activities such as antibacterial,[14,15] antifungal,[16] analgesic,[17] antistress,[18] antiprotozoal,[19] antiangiogenic, protein kinase inhibitor, antiproliferative,[20] antituberculosis,[21] anti-leishmanial, cytotoxic,[22] antiviral,[23] anticancer and antimicrobial.[24] Several derivatives of *N*-substituted maleimide are reported as selective inhibitors of monoglyceride lipase,[25] glycogen synthase kinase 3,[26] Cdc25B,[27] Bfl-1[28] and DNA methyltransferases[29] etc. Due to its wide biological applications, many researchers are motivated to synthesize maleimide derivatives.

Sortino and co-workers have reported antifungal, cytotoxic andSAR studies of a series of N-aryl and N-alkylphenyl-maleimide compounds.[16] Patil and group synthesized α-hydroxy phosphonates clubbed derivative of N-aryl maleimides and investigated their antimicrobial activity towards *B. subtilis*, *E. coli* bacteria and *C. albicans*, *C. tropicalis*, *A. niger* and *A. clavatus* fungi.[30] Bhagare and co-workers reported a set of new schiff bases of N-aryl 3- and 4-substituted maleimides and tested for antimicrobial activity against *E. coli, S. aureus* bacteria and *A. niger, C. albicans* fungi.[31] Gosavi and group have reported the synthesis of 3-aryl-4-methoxy-N-alkyl maleimide and its

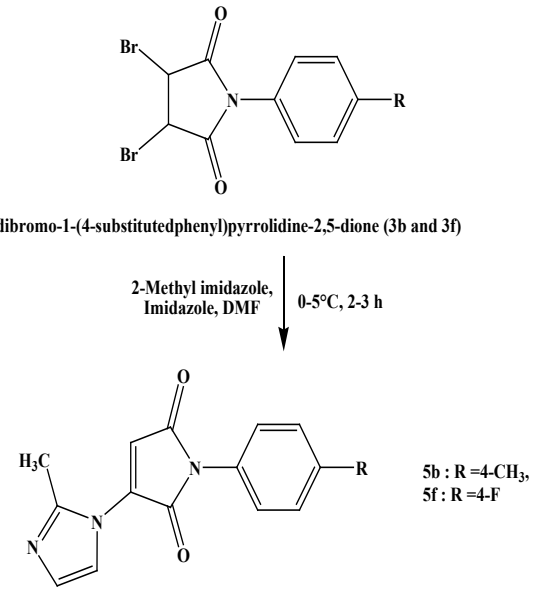
antimicrobial activity against gram positive bacteria S. aureus, gram negative bacteria *E. coli* and fungal strains *C. albicans, C. tropicalis, A. niger* and *A. clavatus* (Fig. 3).[32] In order to overcome the drawbacks of clinically available antifungal agents such as drug resistance, narrow spectrum activity and low bioavailability,[13,33-36] the design of new antifungal agents are highly desirable. In continuation of our work on *N*-substituted maleimide derivatives,[30] we have made an attempt to design and synthesize novel antifungal agents via incorporation of the *N*-substituted maleimide scaffold into the linker and side chain regions of the common pharmacophore (Fig.3).

Materials and Methods

All chemicals were purchased from Sigma-Aldrich and Alpha Chemika. Reactions were monitored and the purity of the products was checked by thin layer chromatography (TLC). TLC has been performed on Merck TLC Silica Gel 60 F254 plates (pre-coated aluminium sheets) with an appropriate solvent system used as the mobile phase and UV cabinet/iodine vapours used as the visualizing agent. Melting points were determined in capillary tubes using the DBK-Programmable melting point apparatus and were uncorrected. Infra-red (IR) spectrums of all synthesized compounds were recorded on Agilent Resolutions Pro and Shimadzu FTIR-84005. Nuclear magnetic resonance (NMR) spectra such as ^{1}H-NMR and ^{13}C NMR spectrum were recorded on a Bruker Avance Neo (500 MHz) NMR spectrometer with tetramethylsilane (TMS) used as an internal standard. The solvents used for recording NMR spectra were $CDCL_3$ and DMSO-d_6. The mass spectrum were recorded on Waters, Q-TOF Micromass with ESI-MS. Laboratory grade chemicals and solvents were used, purified as per literature methods.



Scheme 1: Scheme for synthesis of 1-(4-substitutedphenyl)-3-(1*H*-imidazol-1-yl)-1*H*-pyrrole-2,5-dione derivatives (4a-4g).



Scheme 2: Scheme for synthesis of 1-(4-substitutedphenyl)-3-(2-methyl-1*H*-imidazol-1-yl)-1*H*-pyrrole-2,5-dione derivatives (5b and 5f).

Synthetic Schemes

The synthetic work was done with the help of suitable synthetic routes, as mentioned in Schemes 1 and 2.

Procedures for the Synthesis of Targeted Analogs

General procedure for the synthesis of 1-(4-substitutedphenyl)-1H-pyrrole-2,5-dione (2a-2g)

To a stirred solution of the substituted anilines (0.01 mol) in acetic acid (10 mL), the maleic anhydride (0.01 mol) was added in one lot. To obtain a clear solution of the reaction mixture. The reaction mixture was stirred further at room temperature for 10 minutes. The obtained clear solution turned into a slurry due to the separation of the amic acid. In one lot, concentrated sulphuric acid (0.025 mol) was added to this stirred slurry. Due to the exothermic reaction, the reaction temperature was increased by 10 to 15°C, and the suspension turned into a clear solution. The temperature of the reaction mixture was maintained at 60°C for 30 to 45 minutes. After 30 to 45 minutes, the cooled reaction mixture was poured onto crushed ice to obtain a solid product. The solid was separated and washed with aqueous sodium bicarbonate and then with water. Finally, the obtained product was purified by the recrystallization method using aqueous ethanol.[30,37]

General procedure for synthesis of 3,4-dibromo-1-(4-substitutedphenyl)pyrrolidine-2,5-dione (3a-3g)

To a solution of 1-(4-substitutedphenyl)-1*H*-pyrrole-2,5-diones (0.01 mol) in CCl_4 (8 mL) was added dropwise a solution of bromine (0.011 mol) in CCl_4 (4 mL) at 25°C and refluxed for 1 to 1.5 hours. The completion of the reaction was checked with the help of TLC by using the mobile phase. The obtained white solid product was separated, filtered, and washed with cold CCl_4 (4–8 mL). Then, the product was dried and recrystallized using ethanol.[30,37]

General procedure for synthesis of 1-(4-substitutedphenyl)-3-(1H-imidazol-1-yl)-1H-pyrrole-2,5-dione (4a-4g)

A solution of compounds 3a–3g (0.01 mol) was prepared in purified DMF (10 mL) at 0 to 5°C. After 10 minutes, imidazole (0.03 mol) was added with constant stirring at 0 to 5°C for 2 to 3 hours. TLC monitored the reaction's progress. After completion of the reaction, the reaction mixture was poured into crushed ice. The solid product was separated and collected by filtration. The crude product was recrystallized by using an appropriate solvent.

General procedure for synthesis of 1-(4-substitutedphenyl)-3-(2-methyl-1H-imidazol-1-yl)-1H-pyrrole-2,5-dione (5b and 5f)

The compounds 5b and 5f (0.01 mol) were prepared by using the same procedure as 4a-4g. But instead of imidazole, use 2-methyl imidazole in purified DMF. The obtained crude product was recrystallized by using an appropriate solvent.

Spectral Data of Synthesized Analogues (4a-4g, 5b and 5f)

3-(1H-imidazol-1-yl)-1-phenyl-1H-pyrrole-2,5-dione (4a)

Yield: 70%; Yellow solid; M.P.: 186–188°C; IR (ν cm^{-1}): 3105, 3061 (C-H Str., Ar), 1669, 1622 (C=O Str., pyrrole-2,5-dione), 1583, 1490 (C=C Str., Ar), 1332, 1259, 1205, 1132, 1060, 1006 (C-N Str.); ^{1}H-NMR (DMSO, 500 MHz, δppm): 8.55 (s, 1H, 2-CH of imidazole), 7.95 (s, 1H, ,5-CH ofimidazole), 7.75–7.72 (m, 2H, 2- and 6–CH of Ar), 7.54–7.51 (m, 1H, 4 –CH of Ar), 7.41–7.36 (m, 2H, 3- and 5 –CH of Ar), 7.24 (s, 1H, 4-CH of imidazole), 7.17-7.16 (d,*J* = 6.9 Hz, 1H, 4-CH of pyrrole-2,5-dione); ^{13}C-NMR (DMSO, 500 MHz, δppm): 167.97, 164.99, 138.69, 136.80, 131.84, 131.10, 130.73, 130.44, 128.82, 128.75, 127.93, 126.87, 120.77, 118.28, 109.97, 109.91; ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{13}H_9N_3O_2$ calcd. 239.2285, found 240.0759 [M + 1].

3-(1H-imidazol-1-yl)-1-(4-methylphenyl)-1H-pyrrole-2,5-dione (4b)

Yield: 84%; Yellow solid; M.P.: 168–170°C; IR (ν cm^{-1}): 3132, 3064 (C-H Str., Ar), 1710, 1639 (C=O Str., pyrrole-2,5-dione), 1479 (C=C Str., Ar), 1296, 1234, 1153, 1041 (C-N Str.); ^{1}H NMR (DMSO, 500 MHz, δppm): ^{1}H-NMR (DMSO, 500 MHz, δppm): 8.54 (s, 1H, 2-CH of imidazole), 7.53 (s, 1H, 5-CH of imidazole), 7.30-7.28 (d,*J* = 8.3 Hz, 2H, 2- and 6 –CH of Ar), 7.26 (s, 1H, 4-CH of imidazole), 7.24–7.22 (d, *J* = 8.4 Hz, 2H, 3- and 5 –CH of Ar), 6.39 (s, 1H, 4-CH of pyrrole-2,5-dione), 2.40 (s, 3H, 4-CH_3 of Ar); ^{13}C NMR (DMSO, 500 MHz, δppm):167.69, 164.74, 138.65, 138.33, 137.01, 132.04, 129.95, 127.91, 126.13, 117.37, 108.69; ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{14}H_{11}N_3O_2$ calcd. 253.2549, found 254.1763 [M+1], 256.2478 [M+3].

3-(1H-imidazol-1-yl)-1-(4-methoxyphenyl)-1H-pyrrole-2,5-dione (4c)

Yield: 73%; Yellow solid; M.P.: 164–166°C; IR (ν cm^{-1}): 3136, 3100, 3060 (C-H Str., Ar), 3000, 2960, 2833 (C-H Str., CH_3), 1691, 1634 (C=O Str., pyrrole-2,5-dione), 1600. 1475 (C=C Str., Ar), 1320, 1285, 1249, 1200, 1125, 1100, 1060 (C-N and C-O Str.); ^{1}H-NMR (DMSO, 500 MHz, δppm): 8.54 (s, 1H, 2-CH of imidazole), 7.53 (t,*J* = 1.3 Hz, 1H, 5-CH of imidazole), 7.26 (m, 2H, 2- and 6 –CH of Ar), 7.26 – 7.25 (m, 1H, 4-CH of imidazole), 7.01–6.99 (m, 2H, 3- and 5–CH of Ar), 6.39 (s, 1H, 4-CH of pyrrole-2,5-dione), 3.84 (s, 3H, 4-OCH_3 of Ar); ^{13}C-NMR (DMSO, 500 MHz, δppm): 167.85, 164.88, 159.53, 138.31, 136.99, 132.03, 127.73, 123.11, 117.35, 114.64, 108.62, 55.55. ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{14}H_{11}N_3O_3$ calcd. 269.2543, found 270.1900 [M+1], 271.2327 [M+2], 272.2533 [M+3].

1-(4-chlorophenyl)-3-(1H-imidazol-1-yl)-1H-pyrrole-2,5-dione (4d)

Yield: 93%; Yellow solid; M.P.: 230-232°C; IR (ν cm^{-1}): 3121, 3059 (C-H Str., Ar), 1716, 1637 (C=O Str., pyrrole-2,5-dione), 1485 (C=C Str., Ar), 1324, 1248, 1226, 1194, 1145, 1047 (C-N Str.), 769,730 (C-Cl Str.); ^{1}H-NMR (DMSO, 500 MHz, δppm): 8.55 (s, 1H, 2-CH of imidazole), 7.94 (t,*J*= 1.4 Hz, 1H, 5-CH of imidazole), 7.60–7.57 (m, 2H, 2- and 6 –CH of Ar), 7.45 – 7.43 (m, 2H, 3- and 5 –CH of Ar), 7.23 (d,*J*= 0.6 Hz, 1H, 4-CH of imidazole), 7.17 (s, 1H, 4-CH of pyrrole-2,5-dione); ^{13}C NMR (DMSO, 500 MHz, δppm): 168.02, 164.65, 138.68, 136.79, 132.34, 130.73, 130.00, 128.90, 128.48, 118.27, 109.96. ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{13}H_8ClN_3O_2$ calcd. 273.6737, found 274.0452 [M + 1], 276.0383 [M + 3], 277.0376 [M + 4].

1-(4-bromophenyl)-3-(1H-imidazol-1-yl)-1H-pyrrole-2,5-dione (4e)

Yield: 84%; Yellow solid; M.P.: 196–198°C; IR (ν cm^{-1}): 3118, 3051 (C-H Str., Ar), 1710, 1627 (C=O Str., pyrrole-2,5-dione), 1475 (C=C Str., Ar), 1301, 1163, 1139, 1056 (C-N Str.), 626(C-Br Str.); ^{1}H-NMR (DMSO, 500 MHz) δ:8.55 (s, 1H, 2-CH of imidazole), 7.95 (t, *J* = 1.4 Hz, 1H, 5-CH of imidazole), 7.75–7.72 (m, 2H, 2- and 6 –CH of Ar), 7.39–7.36 (m, 2H, 3- and 5–CH of Ar), 7.24 (s, 1H, 4-CH of imidazole), 7.17 (s, 1H, 4-CH of pyrrole-2,5-dione); ^{13}C NMR (DMSO, 500 MHz, δppm): 167.97, 164.39, 138.39, 136.79, 131.84, 130.73, 130.44, 128.74, 120.78, 118.27, 109.96. ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{13}H_8BrN_3O_2$ calcd. 318.1247, found 319.6484 [M + 1], 320.7175 [M + 2],

1-(4-fluorophenyl)-3-(1H-imidazol-1-yl)-1H-pyrrole-2,5-dione (4f)

Yield: 80%; Brown solid; M.P.: 200–202°C. IR (ν cm^{-1}): 3124, 3088 (C-H Str., Ar), 1693, 1625 (C=O Str., pyrrole-2,5-dione), 1475 (C=C Str., Ar), 1404 (C-F Str.), 1234, 1149, 1091 (C-N Str.); ^{1}H-NMR (DMSO, 500 MHz, δppm): 8.56 (s, 1H, 2-CH of imidazole), 7.95 (s, 1H, 5-CH of imidazole), 7.74–7.73 (m, 2H, 2- and 6 –CH of Ar), 7.39 – 7.37 (m, 2H, 3- and 5–CH of Ar), 7.24 (s, 1H, 4-CH of imidazole), 7.17 (s, 1H, 4-CH of pyrrole-2,5-dione). ^{13}C-NMR (DMSO, 500 MHz, δppm): 167.98, 164.60, 138.70, 136.79, 131.85, 130.73, 130.44, 128.82, 128.76, 126.88, 120.78, 118.27, 109.97. ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{13}H_8FN_3O_2$calcd. 257.2191, found 257.4069 [M^+], 258.1320[M + 1], 259.4985 [M + 2].

1-(4-hydroxyphenyl)-3-(1H-imidazol-1-yl)-1H-pyrrole-2,5-dione (4g)

Yield 65%; Greyish white solid; M.P.: 120–122°C. IR (ν cm^{-1}): 3124 (C-H Str., Ar), 1712, 1637 (C=O Str., pyrrole-2,5-dione), 1497 (C=C Str., Ar), 1290, 1223, 1147, 1110, 1048, 1004 (C-N Str.); ^{1}H-NMR (DMSO, 500 MHz, δppm): 8.54 (s, 1H, 2-CH of imidazole), 7.94 (t, *J* = 1.4 Hz, 1H, 5-CH of imidazole), 7.54–7.53 (m, , 2H, 2- and 6 –CH of Ar), 7.23 (s, 1H, 4-CH of imidazole), 7.22–7.20 (m, 2H, 3- and 5–CH of Ar), 7.12 (s, 1H, 4-CH of pyrrole-2,5-dione). 7.05 (d, 1H, 4-OH of Ar); ^{1}H-NMR (DMSO, 500 MHz, δppm): 168.38, 165.00, 153.99, 138.50, 136.76, 131.51, 130.69, 127.76, 122.90, 118.24, 115.92, 108.87, 108.52; ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{13}H_9N_3O_3$ calcd. 256.2166, found 256.2166 [M^+].

1-(4-methylphenyl)-3-(2-methyl-1H-imidazol-1-yl)-1H-pyrrole-2,5-dione (5b)

Yield: 98%; Golden orange solid; M.P.: 204–206°C; IR (ν cm^{-1}): 3105 (C-H Str., Ar), 1707, 1634 (C=O Str., pyrrole-2,5-dione), 1289, 1138 (C-N Str.);^{1}H-NMR (DMSO, 500 MHz, δppm): 7.91 (d, *J* = 1.0 Hz, 1H, 4-CH of imidazole), 7.30-7.28 (d,*J* = 8.2 Hz, 2H, 2H, 2- and 6 –CH of Ar), 7.24 – 7.22 (m, 2H, 2H, 3- and 5–CH of Ar), 7.05 (s, 1H, 5-CH of imidazole), 6.46 (s, 1H, 4-CH of pyrrole-2,5-dione), 2.62 (s, 3H, 2-CH_3 of imidazole), 2.40 (s, 3H, 4-CH_3 of Ar); ^{13}C NMR (DMSO, 500 MHz, δppm): 167.84, 165.41, 146.61, 138.60, 138.42, 129.93, 129.14, 128.00, 126.10, 119.33, 111.97, 21.19, 16.19. ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{15}H_{13}N_3O_2$calcd. 267.2813, found 268.1303 [M+1], 269.1325 [M + 2], 270.1308 [M + 3].

1-(4-fluorophenyl)-3-(2-methyl-1H-imidazol-1-yl)-1H-pyrrole-2,5-dione (5f)

Yield: 92 %; Golden orange solid; M.P.: 184–186°C; IR (ν cm^{-1}): 3032 (C-H Str., Ar), 1711, 1632 (C=O Str., pyrrole-2,5-dione), 1384 (C-F Str.), 1287, 1220, 1134, 1090(C-N Str.);^{1}H NMR (DMSO, 500 MHz, δppm): 7.90 (d, *J* = 1.7 Hz, 1H, 4-CH of imidazole), 7.37 – 7.34 (m, 2H, 2- and 6–CH of Ar), 7.20–7.17 (m, 2H, 3- and 5 –CH of Ar), 7.06 (d, *J* = 1.7 Hz, 1H, 5-CH of imidazole), 6.47 (s, 1H, 3-CH of pyrrole-2,5-dione), 2.63 (s, 3H, 4-CH_3 of Ar). ^{13}C-NMR (DMSO, 500 MHz, δppm): 167.55, 165.22, 146.62, 138.48, 129.25, 128.10, 128.03, 126.60, 126.57, 119.26, 116.45, 116.25, 111.85, 16.22. ESI-MS (TOF MS ES$^+$, *m/z*): (M^+) for $C_{14}H_{10}FN_3O_2$ calcd. 271.2455; found 272.1039 [M+1], 273.1077 [M + 2], 274.1948 [M + 3].

Evaluation of Antifungal Activity

Fungal strains

The fungal strains used were *C. albicans* (MTCC 183), *A. fumigatus* (MTCC 5453), *A. niger* (NCIM 616) and *A. oryzae* (NCIM 634); the respective strains were procured from MTCC, Chandigarh and NICM, Pune, India. The direct contact method studied the antifungal effect of various synthesized compounds.

Preparation of the fungal suspension

The spores from new culture (5 day culture) of the above mentioned strains were recovered after scraping surface of petri plates in sterile physiological water (3 mL). The spores were counted using Malassez cell and a microscope from this suspension. Then, the optical density of fungal suspension was measured by a microplate reader (EPOCH, BioTek-Agilent, US) at wavelength 630 nm in order to determine the concentration of spore suspension at 10^7 spores/mL. An optical density of 0.04 was estimated, corresponding to a 107 spores/mL concentration.

Preparation of culture medium containing test compounds with varying concentrations

Various test compounds were dissolved in DMSO. Vehicles containing different concentrations of test compounds were incorporated into potato dextrose agar (v/v) after sterilizing media in the autoclave at 121°C at 15 psi for 15 minutes. Then, test culture (10 µL) was spot inoculated on potato dextrose agar plate, except for the negative control, which contains potato dextrose agar media with DMSO. The petri plates were then incubated at 28°C for 72 hours. Meanwhile, fluconazole and clotrimazole were utilized as positive controls at different concentrations.[38]

Parameters studied for the evaluation of fungal growth

In this study, the effects of test compounds on fungal growth were compared to estimate the inhibition diameter by using a colony counter (Equitec, India). This reading always made in comparison with those of the controls, which are conducted under the same test conditions. Growth inhibition rates in relation to the control are calculated according to the following formula:

Inhibition of fungal growth (%) = 5[(A-B)/A] ×100

In the formula, *A* represents the diameter of the colony in petri dishes - initial disc diameter (mm) and *B* represents the diameter of the colony in the petri dishes in the medium containing samples - initial diameter of the disc (mm).
MIC is the lowest concentration of test compound that

inhibits the visible growth of fungi after overnight incubation. Petri dishes containing various test compound concentrations and showing the absence of fungal growth have been selected to determine the minimum inhibitory concentration. The inhibitory concentrations of selected, tested compounds were calculated using GraphPad Prism software version 7.0.

Results

The key intermediates 3a-3g were used for the synthesis of 4a-4g, 5b and 5f. The substituted *N*-arylmaleimide derivatives 2a-2g were synthesized using anhydrous maleic anhydride 1, reacting with corresponding substituted anilines in glacial acetic and concentrated sulphuric acid. The dibromo compounds 3a-3g were obtained by refluxing 2a-2g with bromine in CCl_4.[30,37] The key intermediates 3a-3g were reacted further with imidazole in DMF at 0 to 5°C to afford the desired compounds 4a-4g (Scheme 1). The protocol was extended for the reaction of 3b and 3f intermediates with 2-methylimidazole in DMF at 0 to 5°C to afford 5b and 5f products, respectively (Scheme 2).

^{1}H-NMR, ^{13}C-NMR and ESI-MS spectra characterized all synthesized compounds 4a-4g, 5b and 5f. The *N*-substituted maleimide series of 4a-4g, representative compound 4a showed an IR stretching frequency of 2- and 5-positions of carbonyl functional groups of the maleimide ring at 1669 and 1622 cm^{-1}, respectively. The ^{1}H-NMR spectra of 4a, containing a 4-positon proton of maleimide, exhibited a singlet at 7.24 ppm. The ^{13}C-NMR spectra of 4a containing two carbonyl carbons showed 167 and 164 ppm values, respectively. In contrast, 2-methyl imidazole is attached to *N*-substituted maleimide, containing compounds 5b and 5f. The representative compound 5b showed an IR stretching frequency of 2- and 5-positions, where the carbonyl functional groups of the maleimide ring appeared at 1707 and 1634 cm^{-1}, respectively. The ^{1}H-NMR spectra of 5b, containing a 4-positon proton of maleimide, exhibited a singlet at 6.46 ppm. The CH_3 groups of 5b present at 2- and 4-positions of imidazole and phenyl rings containing protons ppm values appeared at 2.62 and 2.40, respectively. The ^{13}C-NMR spectra of 5b containing carbons present at 4-position of the phenyl ring and at 2-position of the imidazole ring were characterized by 21 and 16 ppm values, respectively. Additionally, the exact molecular weight of all synthesized compounds 4a-4g, 5b and 5f was confirmed by the ESI–MS spectra.

The antifungal activity of synthesized compounds 4a-4g, 5b and 5f was evaluated by the direct contact method *in-vitro* against *C. albicans*, *A. fumigatus*, *A. niger* and *A. oryzae*. To evaluate the activity of synthesized compounds, the percentage zone of inhibition at 200 µg/mL in dimethyl sulphoxide (DMSO) and minimum inhibitory concentrations (MICs) at 1, 10, 50, 100 and 200 µg/mL in DMSO are used. The tested compounds' inhibitory concentrations (ICs) were calculated using GraphPad Prism software version 7.0. Finally, the results of antifungal activity were compared with the standard antifungal drugs fluconazole and clotrimazole (Tables 1 and 2).

Table 1: Antifungal activity of synthesized compounds in percentage zone of inhibition.

Compounds	*Percentage zone of inhibition at 200 µg/mL concentration*			
	Candida albicans	*Aspergillus fumigatus*	*Aspergillus niger*	*Aspergillus oryzae*
4d	61	-	44	-
4e	65	81	81	-
4f	35	77	82	-
4g	66	88	67	
5b	-	79.9	-	-
5f	85.9	75	82	54
Fluconazole	94	90	88	87
Clotrimazole	99	96	95	88

(-) – Inactive

Discussion

Nine analogues 4a-4g, 5b and 5f of *N*-substituted maleimide-bearing imidazole and 2-methyl imidazole moieties were

Table 2: Antifungal activity of selected compounds in terms of MIC_{50} and IC_{50}

Compound	*Candida albicans*		*Aspergillus fumigatus*		*Aspergillus niger*		*Aspergillus oryzae*	
	MIC_{50}	IC_{50}	MIC_{50}	IC_{50}	MIC_{50}	IC_{50}	MIC_{50}	IC_{50}
4d	50	71.93	-	-	50	50.67	-	-
4e	50	67.37	100	102.5	25	30.2	-	-
4f	100	176.47	50	91.15	25	69.20	-	-
4g	25	54.30	25	35.75	50	72.82	-	-
5b	-	-	50	53.12	-	-	-	-
5f	12.9	16.71	10	17.15	10	12.94	25	43.2
Fluconazole	1	1.91	5	7.51	5	14.77	5	17.89
Clotrimazole	1	20.41	5	16.41	1	2.31	5	7

(-) – Inactive

designed, synthesized and evaluated for their antifungal activity. In view of the high degree of bioactivity shown by both imidazole or 2-methyl imidazole and N-substituted maleimides, we have focused on the design of new structural entities that incorporate both of these structural and functional moieties into a single molecule scaffold (4a-4g, 5b and 5f) to evaluate the potential additive effects of these two systems on biological activity, especially with regard to antifungal activity. Figs 1 and 2 describe the clinically available azole class of imidazole antifungal agents and a selection of their significant common pharmacophore features. Fig. 3 also describes the biological activities of various maleimide derivatives containing *N*-substituted maleimide scaffolds and their incorporation into the azole agents containing a selected common pharmacophore. To date, adequate efforts have not been made to combine *N*-substituted maleimide and a selected common pharmacophore as a single molecular scaffold.

After being designed, the synthesis of target molecules was done by conventional routes of synthesis, as depicted in Schemes 1 and 2. In both schemes, the key intermediates of 3,4-dibromo-1-(4-substitutedphenyl)pyrrolidine-2,5-diones were used for the synthesis of 4a-4g, 5b and 5f. The key intermediate obtained by the bromination reaction takes place at the 3- and 4-positions of *N*-substituted maleimides. Finally, the targeted molecules were achieved by a simple dehalogenation reaction that takes place in the presence of imidazole or 2-methyl imidazole using DMF as solvent. Further, all synthesized compounds 4a-4g, 5b and 5f were structurally confirmed by using spectroscopic techniques such as IR, NMR and mass.

The antifungal activity of all compounds 4a-4g, 5b and 5f was evaluated against *C. albicans*, *A. fumigatus*, *A. niger* and *A. oryzae*. The activity results indicate that, most of the compounds 4d, 4e, 4f, 4g, 5b and 5f have significant antifungal activity. While compounds 4a, 4b, and 4c did not show any zones of inhibition against the fungi species C. *albicans*, *A. fumigatus*, *A. niger* and *A. oryzae* at a 200 µg/mL concentration, so the antifungal activity of these compounds was not reported in Table 1. Based on the zone of inhibition results, compounds with moderate to good activity were selected further to find out their MIC_{50} and IC_{50} values against C. *albicans*, *A. fumigatus*, *A. niger* and *A. oryzae.*

According to the results in Table 2, the compounds 4d, 4e and 4f showed less potent activity against *C. albicans* than The compound 4g (MIC_{50} = 25 µg/mL and IC_{50} = 54.30 µM) has moderate activity against *C. albicans*. Whereas, with the -F atom substituent present at 4-position of phenyl ring, compound 5f showed appreciable activity against *C. albicans* as compared to fluconazole and clotrimazole, *with MICs of 12.9* µg/mL and IC_{50} of 16.71 µM. The compounds 4e, 4f and 5b show less potent activity against *A. fumigatus*. The compound 4g (MIC_{50} = 25 µg/mL and IC_{50} = 35.75 µM) has moderate activity against *A. fumigatus*. Compound 5f (MIC50 = 10 µg/mL and IC_{50} = 17.15 µM) showed more significant activity against *A. fumigatus* than fluconazole and clotrimazole.

According to the results illustrated in Table 2, the compounds 4e and 4f (MIC_{50} = 25 µg/mL and IC_{50} in the range of 30–70 µM) have moderate activity against *A. niger*. The compounds 4d and 4g (MIC_{50} = 50 µg/mL) were shown to have less potent activity against *A. niger. While* compound 5f (MIC_{50} = 10 µg/mL) showed more appreciable activity against *A. niger* as compared with fluconazole. Furthermore, compound 5f (IC_{50} = 12.94 µM) also showed good activity against *A. niger* as compared with fluconazole. As compared to fluconazole, compound 5f (MIC_{50} = 10 µg/mL) was shown to have ten times less activity against *A. niger*. Compound 5f, with MIC_{50} = 25 µg/mL and IC_{50} = 43.2 µM was shown to have moderate activity against *A. oryzae*.

Further, the structure-activity relationship (SAR) studies of *N*-substituted maleimide derivatives and their significant antifungal activity against four pathogenic fungi, whose MIC_{50} values were less than 25 µg/mL indicates that, the electronic effect (electron donating or electron withdrawing) of 4-position substituted phenyl ring, 2-position substituted imidazole ring and incorporation of maleimide heterocyclic ring at linker and side chain regions played a key role in the antifungal activity.

The outcome of biological evaluation revealed that compounds 4d, 4e, 4f and 4g were substituted with electronegative or electropositive groups at 4-position of phenyl ring attached to 1-position of maleimide and imidazole ring attached to 3-position of maleimide didn't show antifungal activity against *A. oryzae* as compared to fluconazole and clotrimazole. Compound 5b was substituted with -CH_3 electron donating group present at 4-position of phenyl ring attached to 1-position of maleimide and 2-position of imidazole ring attached to 3-position of maleimide didn't show antifungal activity against *C. albicans*, *A. niger* and *A. oryzae*. Compound 5f was synthesized by replacement of CH_3 group present in 5b with F, showing significant antifungal activity concerning fluconazole and clotrimazole against *C. albicans*, *A. fumigatus and A. niger. Finally,* based on antifungal activity and SAR studies of synthesized compounds, we confirmed the importance of *N*-substituted maleimide scaffolds incorporation at linker and side chain regions of designed common pharmacophore for antifungal activity.

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