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Hybrids Of Fluconazole: Synthesis and Antimicrobial Activity

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Abstract

Based on the structure of the active site of cytochrome P450 14α-demethylase (CYP51) a series of 1-(1-(substituted phenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(substututed phenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine of fluconazole analogues were synthesized starting from phenacyl bromide (0.01 mol) and 1,2,4 triazole. All compounds were characterized by advanced tools like IR, ^{1}H-NMR, ^{13}C-NMR, mass spectroscopy and elemental analysis. All the synthesized compounds were tested qualitative (Zone of inhibition) and quantitative (MIC) antimicrobial activities against four pathogenic bacteria *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* and two pathogenic fungi *C. albicans* and *A. niger*. Most of the synthesized screened compounds showed potent antimicrobial activity against gram positive and gram negative bacteria as well as fungi species.

Keywords: Cytochrome P450, Fluconazole hybrids, Antimicrobial activity, Schiff bases, 14α-demethylation

Introduction

The chemistry of carbon–nitrogen double bond of schiff bases is becoming the backbone of condensation reaction in benzo-fused N-heterocycles.[1] Schiff bases (-N=C-) constitutes an important class of compounds for new drug development.[2] Many researchers have synthesized these compounds as target structures and evaluated their biological activities.[3-7] Development of a new chemotherapeutic Schiff bases is now attracting the attention of medicinal Chemist.[8] Many studies have reported regarding the biological activities of Schiff bases, including their anticancer,[9] antibacterial,[10] antifungal, and herbicidal activities. [11, 12] Schiff bases, derived from various heterocycles, were reported to possess cytotoxic,[13] anticonvulsant, [14] antiproliferative, [15] antianticancer and antifungal activities. [16]

For over a decade, azoles have been a mainstay of the antifungal therapy. Triazole derivatives (Figure 1) are known to exhibit various pharmacological properties such as antimicrobial,[17] antitubercular,[18] anticancer, [19] anticonvulsant,[20] anti-inflammatory, analgesic [21] and antiviral [22]. Triazoles have also been incorported in a wide variety of therapeutically interesting drugs including H_1/H_2 histamine receptor blockers, CNS stimulants, anti-anxiety agents and sedatives.[23] The most important use, however, is as antimycotics in drugs such as fluconazole, itraconazole and voriconazole. [24] The incidence of systemic fungal infections has been increasing dramatically due to the increasing number of immuno-compromised hosts, such as patients undergoing tuberculosis, cancer, AIDS and organ transplant case. [25, 26] Azole antifungal agents currently play a leading role in the treatment of invasive fungal infections.[27] These antifungal drugs act by competitive inhibition of cytochrome P450 14α-demethylase (CYP51), a necessary enzyme in the biosynthesis of ergosterol which is the primary membrane sterol in fungi. [28-30] However, their clinical application value has been limited by their relatively high risk of toxicity, the emergence of drug resistance, pharmacokinetic deficiencies and/or insufficiencies in their antifungal activities, what created an urgent need for the discovery of new antifungal compounds that have broader spectrum and lower toxicity.

CYP51 is a member of the cytochrome P450 super-family, which is widely distributed in different biological kingdoms, being found in animals, plants, fungi, yeast, lower eukaryotes and bacteria [31], and considered to be the most ancient member of the super-family. [32, 33] In all cases CYP51 catalyzes a three-step reaction of sterol 14α-demethylation. The 14α-methyl group is converted to an alcohol, then to an aldehyde, and is removed as formic acid in the final step [34] Ji et al.[35] built a homologous 3D model of CYP51 from *C. albicans* based on the crystal coordinates of all four known prokaryotic P450s. With this model they identified the structurally and functionally important residues such as the heme binding residues, the residue interacting with the redox partner protein and/or involved in electron transfer, the residues lining the substrate access

channel, and the substrate and inhibitor binding residues. Another 3D molecular model constructed by Lewis et al. [36] also showed that typical azole inhibitors were able to fit the putative active site of CYP51 by a combination of heme ligation, hydrogen bonding, π-π stacking and hydrophobic interactions within the heme environment of the enzymes. Ji's study indicated that the triazole ring in the scaffold of triazole antifungals was positioned perpendicularly to the porphyrin plane with a ring nitrogen atom coordinated to the heme iron of CYP51 and was of key importance for the antifungal activity. The halogenated phenyl group was deep in the same hydrophobic binding cleft in the active site of the enzyme CYP51 and long chains of some antifungals such as itraconazole and posaconazole surpassed the active site and interacted with residues in the substrate access channel.

Based on the structure of the active site of CYP51, we herein designed a series of 1-(1-(substituted phenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(substituted phenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine **(6-26)** containing a triazole ring, a halo substituted phenyl group and bis schiff base aiming to find alternative, displaying a broad antifungal spectrum and a lower potential of resistance development as in **Figure 1**. The nitrogen atom at 4-position of target triazole antifungal compounds was designed to be coordinated to iron atom of the heme, the difluorophenyl group could be located into the hydrophobic pocket and expected the bis schiff base interact with the residues of the narrow hydrophobic cleft.

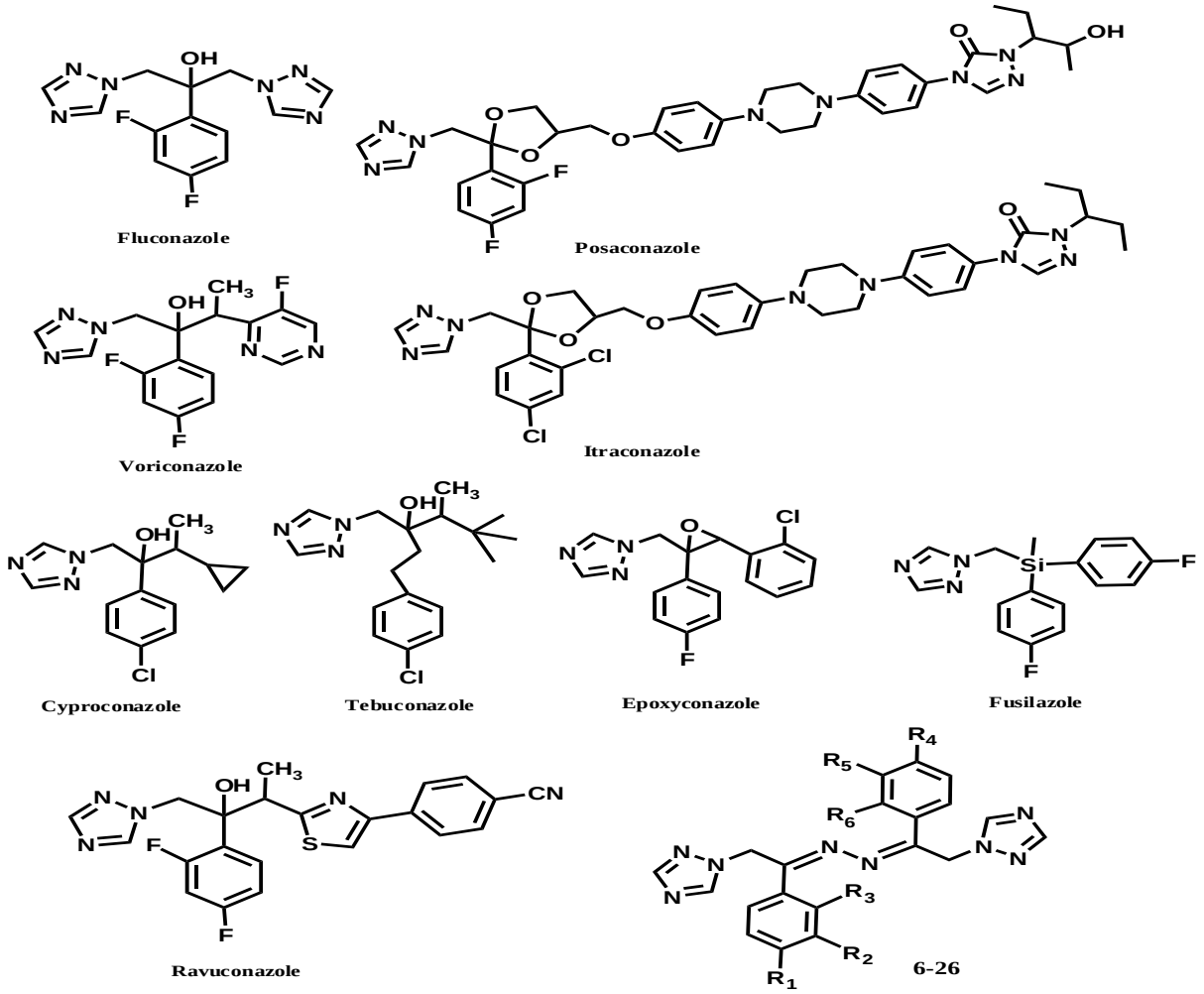


Figure 1 Reported analogues structures containing a triazole ring, a halo substituted phenyl group and bis schiff base.

Results and Discussion

Considering the importance of schiff bases and triazoles it was thought worthwhile to synthesize bis schiff bases containing triazole derivatives. In the present work we report the synthesis 1-(1-(substituted

phenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(substututed phenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine **(6-26).**

Compound **6-26**, were synthesized (Scheme I) from the substituted phenacyl bromide **1a-g** and **4 a-g** which were converted into their corresponding 2-(1H- [1,2,4] triazol-1-yl) -1-(substituted phenyl) ethanone **2a-g** and **5a-g** respectively. **2a-g** was converted into their hydrazine derivatives **3a-g.** which upon reaction with **5a-g** gives 1-(1-(substituted phenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(substututedphenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine **(6-26).**

The structures of products **6-26** were deduced by ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry, and Fourier transform–infrared (FT-IR). In MS spectra, molecular ion peaks of all title compounds were obtained from EI-MS, the presence of M+2 peaks are characteristics for the compound having chlorine and bromine atoms. The ^{1}H NMR spectrum of **6-26** exhibited one singlet, arising from the methylene group (δ 5.53-5.66 ppm), also 2,4 difluoro substituted phenyl protons show signal at δ 6.90-7.00 (ddd, 2H, *J* = 11.0 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz), 7.00-7.10 (ddd, 2H, *J* = 8.6 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz), 7.90-8.04 (ddd, 2H, *J* = 5.5 Hz, *J* = 8.6 Hz) and 4-fluoro substituted phenyl protons show signal at δ 7.1 (t, 2H, *J* = 11.0 Hz, *J* = 8.6 Hz, Ar-H), 7.7 (t, 2H, *J* = 8.6 Hz and 5.5 Hz, Ar-H). With regard to the ^{13}C NMR spectra, the presence of the methylene carbon at δ 51-53 ppm and imine's carbon was observed at δ 164 ppm, which confirms the proposed structure. In 4-fluoro phenyl ($\underline{C}_4$-F) carbon showed doublet at 163.5, 161.7 (*J* = 244 Hz), while a doublet due to ortho ($\underline{C}_3$-F) carbon appeared at 114.5, 116.4 (*J* = 21 Hz) and doublet due to meta $\underline{C}_2$-F appeared at 129.6, 130.8 (*J* = 7.7 Hz) and 2,4 difluoro phenyl carbon ($\underline{C}_2$ and $\underline{C}_4$-F) showed doublet at 166.5, 164.4 (*J* = 244 Hz), while doublet of doublet due to carbon ($\underline{C}_3$-F) in between two fluorine appeared at 109.3, 110.2 , 111.5, 112.7 (*J* = 21 Hz) and doublet due to $\underline{C}_1$-F $\underline{C}_5$-F carbon appeared at 114.2, 115.5 (*J* = 21 Hz), and carbon ($\underline{C}_6$-F) showed doublet at 129.4, 130.6 (*J* = 7.7 Hz). In the IR spectra, the presence of signal around 1600 cm^{-1} is due to C=N related to the imine group.

EXPERIMENTAL

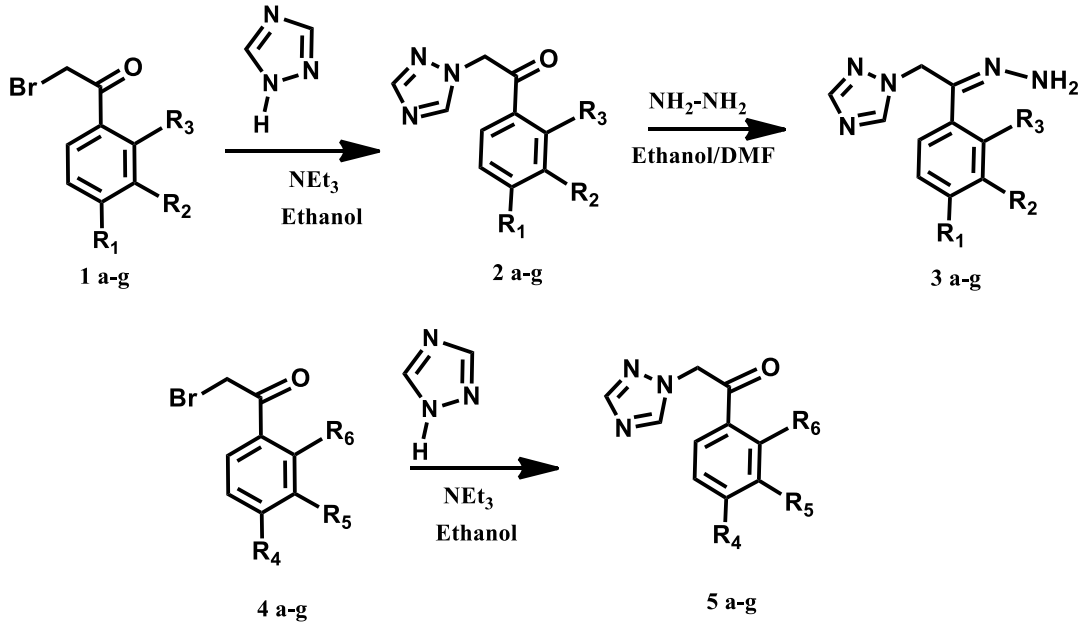
Chemicals were procured from Aldrich Chemical Co. Reactions were monitored and purity of the products was checked by thin layer chromatography (TLC). TLC was performed on Merck 60 F-254 silica gel plates with visualization by UV-light. Melting points were determined in capillary tubes in silicon oil bath using a veego melting point apparatus and are uncorrected. ^{1}H (300 MHz) NMR and ^{13}C (75 MHz) NMR spectra were recorded on Varian mercury XL-300. Chemical shifts are reported from internal tetramethyl silane standard and are given in δ units. The solvent for NMR spectra was $CDCl_3$ and DMSO-d_6. Infra red spectra were taken on schimadzu FTIR – 408 in KBr. The mass spectra were recorded on Shimadzu GC-MS QP 2010A mass spectrometer with an ionization potential of 70 eV. Elemental analysis was performed on a Hosli CH-analyzer and was within 0.4 of the theoretical percentage. Column chromatography was performed on silica gel (230–400 mesh) supplied by Acme Chemical Co. The chemicals and solvents used were laboratory grade and were purified as per literature methods.

General Procedure for the synthesis of 2-(1H-benzo[d][1,2,3]triazol-1-yl)-1-(substituted phenyl) ethanone 2a-g and 5a-g

A mixture of phenacyl bromide (0.01 mol) **1a-g** or **4a-g** and 1,2,4 triazole (0.01 mol) was stirred at room temperature in presence of ethyl alcohol (15 ml) and triethyl amine for 60 min, the solid product **2a-g** or **5a-g** appeared during stirring was collected in good yield according to the known procedure as represented in **Scheme 1**. Products are recrystalized using ethanol to get pure crystals.

General Procedure for the synthesis of 1,2-bis(2-(1H-[1,2,4]triazol-1-yl)-1-(substituted phenyl) ethylidene)hydrazine (3a-g)

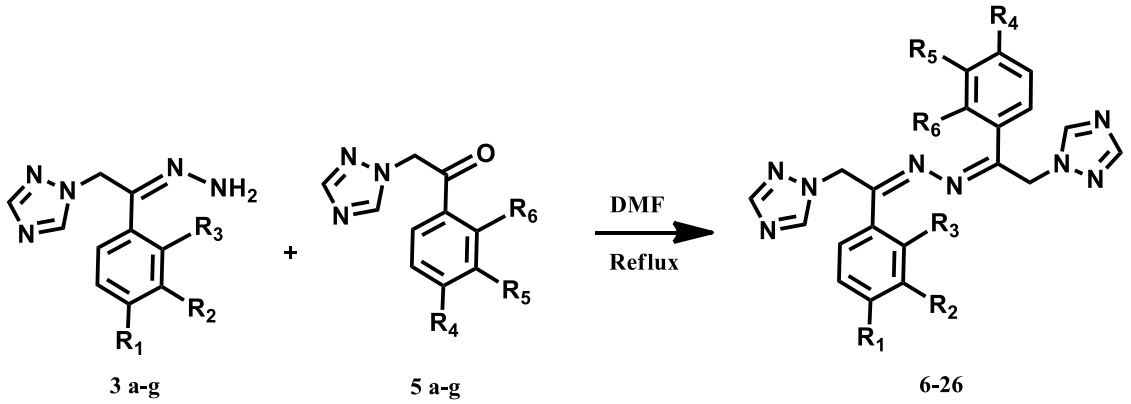
A mixture of hydrazine (10 ml) and **2a-g** (1 gm) in dry etahnol (20 ml) was refluxed for 30 min, and then this reaction mixture was poured over crushed ice as reactions represented in **Scheme 1**. The solid product **3a-g** thus obtained was filtered, washed with water and dried. The product was purified by recrystallization from ethanol.



Scheme I: Synthesis of 1,2-bis(2-(1H-[1,2,4]triazol-1-yl)-1-(substituted phenyl) ethylidene)hydrazine (3a-g) from 1a-g or 4a-g.

General Procedure for the synthesis of 1,2-bis(2-(1H-[1,2,4]triazol-1-yl)-1-(substituted phenyl) ethylidene)hydrazine (6-26)

A mixture of hydrazone **3a-g** (0.001 mol) and **5a-g** (0.001 mol) in dry methanol (20 ml) was refluxed for 3 hrs, the progress of reaction was monitored by TLC as represented in **Scheme II**. After cooling and filtration, the crystalline powder of bis-Schiff bases was collected, then washed with methanol and dried to afford compounds **6-26** in high yields. The product was purified on silica gel column chromatography using n-hexane and ethyl acetate (8:2 v/v) as a eluent.



Scheme II: Synthesis of 1,2-bis(2-(1H-[1,2,4]triazol-1-yl)-1-(substituted phenyl) ethylidene)hydrazine (6-26) from 3 a-g and 5 a-g.

All synthesised 2-bis(2-(1H-[1,2,4]triazol-1-yl)-1-(substituted phenyl) ethylidene)hydrazine (6-26) moieties are represented in **Table 1.**

Table 1 2-bis(2-(1H-[1,2,4]triazol-1-yl)-1-(substituted phenyl) ethylidene)hydrazine derivatives.

Sr. No.	R_1	R_2	R_3	R_4	R_5	R_6
6	F	H	F	F	H	F
7	F	H	F	Cl	H	Cl
8	F	H	F	F	H	H
9	F	H	F	Cl	H	H
10	F	H	F	Br	H	H
11	F	H	F	NO_2	H	H
12	Cl	H	Cl	Cl	H	Cl
13	Cl	H	Cl	F	H	H
14	Cl	H	Cl	Cl	H	H
15	Cl	H	Cl	Br	H	H
16	Cl	H	Cl	NO_2	H	H
17	F	H	H	F	H	H
18	F	H	H	Cl	H	H
19	F	H	H	Br	H	H
20	F	H	H	NO_2	H	H
21	Cl	H	H	Cl	H	H
22	Cl	H	H	Br	H	H
23	Cl	H	H	NO_2	H	H
24	Br	H	H	Br	H	H
25	Br	H	H	NO_2	H	H
26	NO_2	H	H	NO_2	H	H

Characterisation of 1,2-bis(2-(1H-[1,2,4]triazol-1-yl)-1-(substituted phenyl) ethylidene)hydrazine (6-26) derivatives

3.4.1 1,2-bis(1-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (6)

Yield: 71 % mp: 266 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.60 (s, 4H, CH_2), 7.00 (ddd, 2H, *J* = 11.0 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 7.07 (ddd, 2H, *J* = 8.6 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 8.04 (ddd, 2H, *J* = 5.5 Hz, *J* = 5.5 Hz, *J* = 8.6 Hz, Ar-H), 8.06 (s, 2H), 8.53 (s, 2H) ^{13}C NMR(75 MHz, $CDCl_3$): δ 48.7, 165.6, 166.8 (*J* = 245 Hz), 109.3, 110.2, 111.5, 112.7 (*J* = 21 Hz), 114.2, 115.5 (*J* = 21 Hz), 129.4, 130.6 (*J* = 7.7 Hz), 151.3 (2C), 143.6 (2C), 164.7 (2C); Anal. Calcd for: $C_{20}H_{14}F_4N_8$: C, 54.30; H, 3.19; N, 25.33 Found: C, 54.30; H, 3.19; N, 25.33; m/z (70 eV): 443.1(M+1), 444.1 (M+2), 445.1 (M+3).

3.4.2 2-(1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-1-(1-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (7)

Yield: 68 % mp: 279 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.58 (s, 4H, CH_2), 7.00 (ddd, 1H, *J* = 11.0 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 7.07 (ddd, 1H, *J* = 8.6 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 8.04 (ddd, 1H, *J* = 5.5 Hz, *J* = 5.5 Hz, *J* = 8.6 Hz Ar-H), 7.67 (dd, 1H, *J* = 8.6 Hz, *J* = 2.4 Hz, Ar-H), 7.85 (d, 1H, *J* = 2.4 Hz, Ar-H), 8.00 (d, 1H, *J* = 8.6 Hz, Ar-H), 8.06 (s, 2H), 8.53 (s, 2H); ^{13}C NMR: δ 48.7, 104.8, 165.6, 166.8 (*J* = 245 Hz), 109.3, 110.2, 111.5, 112.7 (*J* = 21 Hz), 114.2, 115.5 (*J* = 21 Hz), 129.4, 130.6 (*J* = 7.7 Hz), 135.3, 135.8, 130.5, 138.0, 127.1, 132.0, 151.3, (2C) 143.6, (2C) 164.7, (2C); Anal. Calcd for: $C_{20}H_{14}Cl_2F_2N_8$: C, 50.54; H, 2.97; N, 23.58 Found: C, 50.87; H, 3.08; N, 23.33; m/z (70 eV): 474.07 (M+1), 475.10 (M+2).

3.4.3 1-(1-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (8)

Yield: 72 % mp: 255 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.56 (s, 4H, CH_2), 7.00 (ddd, 1H, *J* = 11.0 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 7.07 (ddd, 1H, *J* = 8.6 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 8.04 (ddd, 1H, *J* = 5.5 Hz, *J* = 5.5 Hz, *J* = 8.6 Hz, Ar-H), 7.2 (dd, 2H, *J* = 11.0 Hz, *J* = 8.6 Hz, Ar-H), 7.8 (dd, 2H, *J* = 5.5 Hz and 8.6 Hz, Ar-H), 8.06 (s, 2H), 8.18 (dd, 2H, *J* = 8.6 Hz, *J* = 5.5 Hz, Ar-H), 8.53 (s, 2H)); ^{13}C NMR: δ 48.7, 104.8, 165.6, 166.8 (J = 245 Hz), 109.3, 110.2, 111.5, 112.7 (*J* = 21 Hz), 114.2, 115.5 (*J* = 21 Hz), 129.4, 130.6 (*J* = 7.7 Hz), 114.5, 116.4 (*J* = 21 Hz) 129.6,

130.8 (*J* = 7.7 Hz) 163.5, 161.7 (*J* = 244 Hz), 151.3, (2C) 143.6, (2C) 164.7, (2C); Anal. Calcd for: $C_{20}H_{15}F_3N_8$: C, 56.60; H, 3.56; N, 26.40 Found: C, 56.28; H, 3.64; N, 26.57; m/z (70 eV): 424.31(M+1), 425.41 (M+2).

3.4.4 2-(1-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-1-(1-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (9)

Yield: 67 % mp: 264 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.58 (s, 4H, CH_2), 6.94 (ddd, 1H, *J* = 11.0 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 7.07 (ddd, 1H, *J* = 8.6 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 7.77 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.04 (d, 1H, *J* = 8.6 Hz, Ar-H), 8.06 (s, 2H), 8.00 (ddd, 2H, *J* = 5.5 Hz, *J* = 5.5 Hz, *J* = 8.6 Hz, Ar-H), 8.53 (s, 2H), ^{13}C NMR: δ 48.7, 104.8, 165.6, 166.8 (J = 245 Hz), 109.3, 110.2, 111.5, 112.7 (*J* = 21 Hz), 114.2, 115.5 (*J* = 21 Hz), 129.4, 130.6 (*J* = 7.7 Hz), 151.3, (2C) 143.6, (2C) 164.7, (2C), 132.1, 130.6, 129.0, 136.6; Anal. Calcd for: $C_{20}H_{15}ClF_2N_8$: C, 54.49; H, 3.43; N, 25.42 Found: C, 54.82; H, 3.65; N, 25.55; m/z (70 eV): 441.84 (M+1), 442.81 (M+2).

3.4.5 2-(1-(4-bromophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-1-(1-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (10)

Yield: 69 % mp: 278 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.53 (s, 2H, CH_2), 5.62 (s, 2H, CH_2), 6.91 (ddd, 1H, *J* = 11.0 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 6.97 (ddd, 1H, *J* = 8.6 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 7.87 (d, 2H, J = 8.6 Hz, Ar-H), 8.02 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.90 (ddd, 1H, *J* = 5.5 Hz, *J* = 5.5 Hz, *J* = 8.6 Hz, Ar-H), 8.06 (s, 2H), 8.53 (s, 2H), ^{13}C NMR: δ 48.7, 104.8, 165.6, 166.8 (J = 245 Hz), 109.3, 110.2, 111.5, 112.7 (*J* = 21 Hz), 114.2, 115.5 (*J* = 21 Hz), 129.4, 130.6 (*J* = 7.7 Hz), 151.3, (2C) 143.6, (2C) 164.7, (2C), 133.0, 131.4, 132.0, 125.4; Anal. Calcd for: $C_{20}H_{15}BrF_2N_8$: C, 49.50; H, 3.12; N, 23.09 Found: C, 49.28; H, 3.00; N, 23.18; m/z (70 eV): 485.18(M+1), 486.20 (M+2), 487.20 (M+3).

3.4.6 1-(1-(2,4-difluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(4-nitrophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (11)

Yield: 78 % mp: 280 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.57 (s, 4H, CH_2), 6.92 (ddd, 1H, *J* = 11.0 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 6.99 (ddd, 1H, *J* = 8.6 Hz, *J* = 11.0 Hz, *J* = 2.4 Hz, Ar-H), 8.00 (ddd, 1H, *J* = 5.5 Hz, *J* = 5.5 Hz, *J* = 8.6 Hz, Ar-H), 8.06 (s, 2H), 8.53 (s, 2H), 7.92 (2H, d, *J* = 8.6 Hz, Ar-H), 8.43 (2H, d, *J* = 8.6 Hz, Ar-H); ^{13}C NMR: δ 48.7, 104.8, 165.6, 166.8 (J = 245 Hz), 109.3, 110.2, 111.5, 112.7 (*J* = 21 Hz), 114.2, 115.5 (*J* = 21 Hz), 129.4, 130.6 (*J* = 7.7 Hz), 151.3, (2C) 143.6, (2C) 164.7, (2C), 140.1, 130.1, 121.2, 150.7; Anal. Calcd for: $C_{20}H_{15}F_2N_9O_2$: C, 53.22; H, 3.35; N, 27.93; Found: C, 53.43; H, 3.58; N, 28.07; m/z (70 eV): 452.39(M+1), 450.40 (M-3).

3.4.7 1,2-bis(1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (12)

Yield: 75 % mp: 282 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.58 (s, 4H, CH_2), 7.67 (dd, 2H, *J* = 8.6 Hz, *J* = 2.4 Hz, Ar-H), 7.85 (d, 2H, *J* = 2.4 Hz), 8.00 (d, 2H, *J* = 8.6 Hz), 8.01 (s, 2H), 8.58 (s, 2H); ^{13}C NMR: 48.7, 135.3, 135.8, 130.5, 138.0, 127.1, 132.0, 151.3, (2C) 143.6, (2C) 164.7, (2C); Anal. Calcd for: $C_{20}H_{14}Cl_4N_8$: C, 47.27; H, 2.78; N, 22.05Found: C, 47.44; H, 2.97; N, 22.22; m/z (70 eV): 508.19 (M+1), 509.19 (M+2).

3.4.8 1-(1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (13)

Yield: 65 % mp: 261 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.60 (s, 4H, CH_2), 7.1 (dd, 2H, *J* = 9.2 Hz, *J* = 8.6 Hz, Ar-H),7.67 (dd, 1H, *J* = 8.6 Hz, *J* = 2.4 Hz, Ar-H),7.85 (d, 1H, *J* = 2.4 Hz, Ar-H), 8.00 (d, 1H, *J* = 8.6 Hz, Ar-H), 8.01 (s, 2H), 7.7 (dd, 2H, *J* = 8.6 Hz and 5.5 Hz, Ar-H), 8.58 (s, 2H); ^{13}C NMR: 114.5, 116.4 (*J* = 21 Hz) 129.6, 130.8 (*J* = 7.7 Hz) 163.5, 161.7 (*J* = 244 Hz), 48.7, 135.3, 135.8, 130.5, 138.0, 127.1, 132.0, 151.3, (2C) 143.6, (2C) 164.7, (2C); Anal. Calcd for: $C_{20}H_{15}Cl_2FN_8$: C, 52.53; H, 3.31; N, 24.50 Found: C, 52.82; H, 3.47; N, 24.70; m/z (70 eV): 457.29 (M+1), 458.30 (M+2), 459.30 (M+3).

3.4.9 1-(1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (14)

Yield: 68 % mp: 266ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.56 (s, 4H, CH_2), 7.67 (dd, 1H, *J* = 8.6 Hz, *J* = 2.4 Hz, Ar-H), 7.77 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.85 (d, 1H, *J* = 2.4 Hz, Ar-H), 8.00 (d, 1H, *J* = 8.6 Hz, Ar-H), 8.01 (s, 2H), 8.10 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.58 (s, 2H) ^{13}C NMR: 48.7, 135.3, 135.8, 130.5, 138.0, 127.1, 132.0, 151.3, (2C) 143.6, (2C) 164.7, (2C), 132.1, 130.6, 129.0, 136.6; Anal. Calcd for: $C_{20}H_{15}Cl_3N_8$: C, 50.71; H, 3.19; N, 23.65 Found: C, 50.98; H, 3.41; N, 23.80; m/z (70 eV): 473.75(M+1), 474.76 (M+2).

3.4.10 2-(1-(4-bromophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-1-(1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (15)

Yield: 71 % mp: 286 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.65 (s, 4H, CH_2), 7.67 (dd, 1H, *J* = 8.6 Hz, *J* = 2.4 Hz, Ar-H), 7.85 (d, 1H, *J* = 2.4 Hz, Ar-H), 7.87 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.00 (d, 1H, *J* = 8.6 Hz, Ar-H), 8.01 (s, 2H), 8.02 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.58 (s, 2H); ^{13}C NMR: 48.7, 135.3, 135.8, 130.5, 138.0, 127.1, 132.0, 151.3, (2C) 143.6,

(2C) 164.7, (2C), 133.0, 131.4, 132.0, 125.4; Anal. Calcd for: $C_{20}H_{15}BrCl_2N_8$: C, 46.36; H, 2.92; N, 21.62 Found: C, 46.131.4, 66; H, 3.10; N, 21.93; m/z (70 eV): 518.21(M+1), 519.20 (M+2).

3.4.11 1-(1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(4-nitrophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (16)

Yield: 78 % mp: >300 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.61 (s, 4H, CH_2), 7.67 (dd, 1H, *J* = 8.6 Hz, *J* = 2.4 Hz, Ar-H), 7.85 (d, 1H, *J* = 2.4 Hz, Ar-H), 8.00 (d, 1H, *J* = 8.6 Hz, Ar-H), 8.01 (s, 2H), , 8.58 (s, 2H), 7.92 (2H, d, *J* = 8.6 Hz, Ar-H), 8.43 (2H, d, *J* = 8.6 Hz, Ar-H), ^{13}C NMR: 48.7, 135.3, 135.8, 130.5, 138.0, 127.1, 132.0, 151.3, (2C) 143.6, (2C) 164.7, (2C), 140.1, 130.1, 121.2, 150.7; Anal. Calcd for: $C_{20}H_{15}Cl_2N_9O_2$: C, 49.60; H, 3.12; N, 26.03; Found: C, 49.71; H, 3.28; N, 27.83; m/z (70 eV): 484.31(M+1), 485.30 (M+2).

3.4.12 1,2-bis(1-(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (17)

Yield: 72 % mp: 264 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): δ 5.64 (s, 4H, CH_2), 7.1 (dd, 4H, *J* = 11.0 Hz, *J* = 8.6 Hz, Ar-H), 7.7 (dd, 4H, *J* = 8.6 Hz and 5.5 Hz, Ar-H), 7.95 (s, 2H), 8.42 (s, 2H);^{13}C NMR: 48.7, 114.5, 116.4 (*J* = 21 Hz) 129.6, 130.8 (*J* = 7.7 Hz) 163.5, 161.7 (*J* = 244 Hz), 151.3, (2C) 143.6, (2C) 164.7, (2C), Anal. Calcd for: $C_{20}H_{16}F_2N_8$: C, 59.11; H, 3.97; N, 27.57 Found: C, 59.38; H, 4.07; N, 27.66; m/z (70 eV): 407.39(M+1), 408.40 (M+2), 409.39 (M+3).

3.4.13 2-(1-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-1-(1-(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (18)

Yield: 77 % mp: 275ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): 5.60 (s, 4H, CH_2), 7.1 (dd, 2H, *J* = 11.0 Hz, *J* = 8.6 Hz, Ar-H), 7.3 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.6 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.7 (dd, 2H, *J* = 8.6 Hz and 5.5 Hz, Ar-H), 7.95 (s, 2H), 8.42 (s, 2H); ^{13}C NMR: 48.7, 114.5, 116.4 (*J* = 21 Hz) 129.6, 130.8 (*J* = 7.7 Hz) 163.5, 161.7 (*J* = 244 Hz), 151.3, (2C) 143.6, (2C) 164.7, (2C), 132.1, 130.6, 129.0, 136.6; Anal. Calcd for: $C_{20}H_{16}ClFN_8$: C, 56.81; H, 3.81; N, 26.50 Found: C, 56.63; H, 4.00; N, 26.77; m/z (70 eV): 423.85(M+1), 424.84 (M+2).

3.4.14 2-(1-(4-bromophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-1-(1-(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (19)

Yield: 67 % mp: 287 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): 5.66 (s, 4H, CH_2), 7.2 (dd, 2H, *J* = 11.0 Hz, *J* = 8.6 Hz, Ar-H), 7.6 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.7 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.8 (dd, 2H, *J* = 5.5 Hz and 8.6 Hz, Ar-H), 8.01 (s, 2H), 8.44 (s, 2H); ^{13}C NMR: 48.7, 114.5, 116.4 (*J* = 21 Hz) 129.6, 130.8 (*J* = 7.7 Hz) 163.5, 161.7 (*J* = 244 Hz), 151.3, (2C) 143.6, (2C) 164.7, (2C), 133.0, 131.4, 132.0, 125.4; Anal. Calcd for: $C_{20}H_{16}BrFN_8$: C, 51.40; H, 3.45; N, 23.98 Found: C, 51.55; H, 3.67; N, 23.93; m/z (70 eV): 468.30 (M+1), 469.29 (M+2).

3.4.15 1-(1-(4-fluorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(4-nitrophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (20)

Yield: 70 % mp: 276 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): 5.62 (4H, s, CH_2), 7.2 (dd, 2H, *J* = 11.0 Hz, *J* = 8.6 Hz, Ar-H), 7.7 (d, 2H, *J* =8.6 Hz, Ar-H), 7.8 (dd, 2H, *J* = 5.5 Hz and 8.6 Hz, Ar-H), 8.2 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.0 (s, 2H), 8.46 (s, 2H); ^{13}C NMR: 48.7, 114.5, 116.4 (*J* = 21 Hz) 129.6, 130.8 (*J* = 7.7 Hz) 163.5, 161.7 (*J* = 244 Hz), 151.3, (2C) 143.6, (2C) 164.7, (2C), 140.1, 130.1, 121.2, 150.7; Anal. Calcd for: $C_{20}H_{16}FN_9O_2$: C, 55.43; H, 3.72; N, 29.09; Found: C, 55.73; H, 3.66; N, 29.10; m/z (70 eV): 433.14(M+1), 434.18 (M+2).

3.4.16 1,2-bis(1-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (21)

Yield: 67 % mp: 270º C IR: 1600 cm^{-1} ^{1}H NMR: (300 MHz, $CDCl_3$): 5.66 (s, 4H, CH_2), 7.3 (d, 4H, *J* = 8.6 Hz, Ar-H), 7.6 (d, 4H, *J* = 8.6 Hz, Ar-H), 8.0 (s, 2H), 8.5 (s, 2H); ^{13}C NMR: 48.7, 132.1, 130.6, 129.0, 136.6, 151.3, (2C) 143.6, (2C) 164.7, (2C), Anal. Calcd for: $C_{20}H_{16}Cl_2N_8$: C, 54.68; H, 3.67; N, 25.51 Found: C, 54.77; H, 3.51; N, 25.68; m/z (70 eV): 439.09 (M+1), 440.11 (M+2).

3.4.17 2-(1-(4-bromophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-1-(1-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (22)

Yield: 70 % mp: 268 ºC IR: 1600 cm^{-1} ^{1}H NMR: (300 MHz, $CDCl_3$): 5.65 (s, 4H, CH_2), 7.3 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.6 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.7 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.8 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.0 (s, 2H), 8.4 (s, 2H); ^{13}C NMR: 48.7, 132.1, 130.6, 129.0, 136.6, 151.3, (2C) 143.6, (2C) 164.7, (2C), 133.0, 131.4, 132.0, 125.4; Anal. Calcd for: $C_{20}H_{16}BrClN_8$: C, 49.66; H, 3.33; N, 23.16 Found: C, 49.81; H, 3.43; N, 23.38; m/z (70 eV): 484.75(M+1), 485.80 (M+2).

3.4.18 1-(1-(4-chlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(4-nitrophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (23)

Yield: 77 % mp: 270 ºC; ^{1}H NMR: (300 MHz, $CDCl_3$): 5.65 (4H, s, CH_2), 7.3 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.6 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.8 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.2 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.1 (s, 2H), 8.4 (s, 2H); ^{13}C NMR: 48.7,

132.1, 130.6, 129.0, 136.6, 151.3, (2C) 143.6, (2C) 164.7, (2C), 140.1, 130.1, 121.2, 150.7; Anal. Calcd for: $C_{20}H_{16}ClN_9O_2$: C, 53.40; H, 3.58; N, 28.02; Found: C, 53.54; H, 3.72; N, 28.11; m/z (70 eV): 450.85(M+1), 451.85 (M+2).

3.4.19 1,2-bis(1-(4-bromophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (24)

Yield: 79 % mp: >300ºC IR: 1600 cm^{-1} ^1H NMR: (300 MHz, $CDCl_3$): 5.65 (s, 4H, CH_2), 7.6 (d, 4H, *J* = 8.6 Hz, Ar-H), 7.8 (d, 4H, *J* = 8.6 Hz, Ar-H), 8.1 (s, 2H), 8.5 (s, 2H),^{13}C NMR: 48.5, 133.0, 131.4, 132.2, 125.4, 151.3, (2C) 143.6, (2C) 164.7, (2C); Anal. Calcd for: $C_{20}H_{16}Br_2N_8$: C, 48.60; H, 3.26; N, 25.50; Found: C, 48.78; H, 3.43; N, 25.62; m/z (70 eV): 528.20 (M+1), 529.21 (M+2).

3.4.20 1-(1-(4-bromophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)-2-(1-(4-nitrophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (25)

Yield: 71 % mp: >300 ºC IR: 1600 cm^{-1} ^1H NMR: (300 MHz, $CDCl_3$): 5.64 (4H, s, CH_2), 7.6 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.8 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.0 (d, 2H, *J* = 8.6 Hz, Ar-H), 8.3 (d, 2H, *J* =8.6 Hz, Ar-H), 8.0 (s, 2H), 8.5 (s, 2H); ^{13}C NMR: 48.5, 133.0, 131.4, 132.2, 125.4, 151.3, (2C) 143.6, (2C) 164.7, (2C), 140.1, 130.1, 121.2, 150.7; Anal. Calcd for: $C_{20}H_{16}BrN_9O_2$: C, 48.60; H, 3.26; N, 25.50; Found: C, 48.78; H, 3.41; N, 25.30; m/z (70 eV): 494.30 (M+1), 495.31 (M+2).

3.4.21 1,2-bis(1-(4-nitrophenyl)-2-(1H-1,2,4-triazol-1-yl)ethylidene)hydrazine (26)

Yield: 78 % mp: 212ºC; ^1H NMR: (300 MHz, $CDCl_3$): 5.60 (4H, s, CH_2), 7.6 (d, 4H, *J* = 8.6 Hz, Ar-H), 8.2 (d, 4H, *J* = 8.6 Hz Ar-H), 8.0 (s, 2H), 8.4 (s, 2H), ^{13}C NMR: 48.5, 140.1, 130.1, 121.2, 150.7, 151.3, (2C) 143.6, (2C) 164.7, (2C); Anal. Calcd for: $C_{20}H_{16}N_{10}O_4$: C, 52.17; H, 3.50; N, 30.42; Found: C, 52.27; H, 3.66; N, 30.48; m/z (70 eV): 461.41(M+1), 459.40 (M-2).

Biological Results and Discussion

All the synthesized compounds were tested for antibacterial activity against bacteria *B. subtilis* (2250), *S. aureus* (2079), *E. coli* (2109) and *P. aeruginosa* (2036) and antifungal activity against two fungi *C. albicans* (3471) and *A. niger* (545) To evaluate the activity of the synthesized compounds the zone of inhibition (128 µg/mL in DMSO) and minimum inhibitory concentrations (MICs) (at 128, 64, 32, 16, 8, 4, 2 and 1 ug/ml in DMSO) were determined using agar diffusion method [37-39]. Known antibiotic Chloromphenicol (the reference for antibacterial drugs) and Nystatin (the reference for antifungal drug) were used for comparison. The zone of inhibition and MIC against micro organisms tested is reported in Tables 2 and 3, respectively. As shown in Table 2 most of the compounds were active against gram positive and gram negative bacteria as well as both the fungi species**.**

Table 2 Antimicrobial screening of synthesized compounds **6-26** (zone diameter of growth inhibition in mm).

Compounds[a]	Microorganisms					
	S. aureus	*E. coli*	*B. subtilis*	*P. aeruginosa*	*A. niger*	*C. albicans*
6	21.22	22.00	24.17	11.13	19.01	22.25
7	24.18	26.52	27.21	21.75	20.84	20.18
8	22.15	22.33	20.55	22.52	18.72	19.74
9	18.11	20.63	25.71	20.35	19.25	21.54
10	17.76	11.01	19.97	18.44	15.62	17.48
11	16.58	16.63		14.12	11.72	18.75
12	27.78	22.44	28.32		21.50	20.85
13	23.04	17.08	25.47		23.88	18.92
14	22.80	14.76	26.82	19.24	18.44	22.31
15	11.74	09.25	13.24	16.54	11.65	17.66
16	-	-	15.12	12.33	12.74	15.41
17	26.98	21.84	21.58	17.43	18.25	19.74
18	22.44	23.18	24.77	21.92	17.66	20.15
19	12.11	-		11.48	16.44	16.32
20	-	14.52	-	-	14.11	13.62
21	23.48					

	24.17	26.30		18.72	18.76	19.02		22	18.23	-		
	11.92		-	13.53	15.80		23	-	-			
	15.22		-	11.78	15.25		24	14.25	17. 76	-		
	12.27	16.96	17.34		25	-		-	13.65			
-	-	-		26	-	-		-	-	-		
-												
Nystatin	NA	NA	NA	NA		20.32	22.03					
Chloramphenicol	31.4	27.55	29.24	23.87		NA	NA					

[a]Chloromphenicol (128 µg/disc), [a]Nystatin (128 µg/disc) were used as reference; synthesized compounds (128 µg/disc); NA = Not Applicable; (-) = Inactive.

Table 3 Antimicrobial screening of synthesized compounds **6-26** minimum inhibitory concentration (MIC) in µg/mL.

Compounds[a]	Microorganisms					
	S. aureus	*E. coli*	*B. subtilis*	*P. aeruginosa*	*A. niger*	*C. albicans*
6	32	32	32	32	16	16
7	32	32	32	16	16	32
8	32	32	64	16	16	32
9	64	32	32	32	16	16
10	64	128	64	32	32	32
11	-	128	64	128	64	32
12	16	32	32	16	16	16
13	32	64	32	16	16	32
14	32	128	32	32	16	16
15	128	128	128	64	64	32
16	-	-	128	128	64	64
17	32	64	64	64	16	32
18	32	32	32	16	32	32
19	-	128	-	64	32	64
20	-	128	-	-	128	128
21	32	32	32	32	32	32
22	64	-	128	-	64	64
23	-	-	128	-	128	128
24	128	64	-	128	64	32
25	-	-	128	-	-	-
26	-	-	-	-	-	-
Nystatin	NA	NA	NA	NA	16	16
Chloramphenicol	16	16	16	16	NA	NA

[a]Chloromphenicol (µg/mL), [a]Nystatin (µg /mL) were used as reference;
NA = Not Applicable; (-) = Inactive.

Careful analysis of the MICs in Table 2 provides some lead molecules with good antibacterial and antifungal activity. Of the compounds **6-26** tested, compounds with electron-withdrawing F and Cl at the phenyl ring expressed a moderate to good activity against most of the tested pathogens, they inhibited the Gram-negative and Gram-positive pathogens equally. Compounds **6-26** required about 16-128 µg/mL against Gram positive and Gram negative bacteria as well as both the fungi species, whereas **6, 7, 8, 13, 14, 18, 21** required 32 µg/mL and **9, 10, 22** required 64 µg/mL against *S. aureus*. Also compounds **6, 7, 8, 9, 12, 18, 21** required 32 µg/mL, **13, 17, 24** required 64 µg/mL and **10, 11, 14, 15, 19, 20** registered their MIC at 128 µg/mL against *E.coli*. However, the NO_2 substituent did not enhance the activity. Introduction of the F and Cl substituent on the phenyl ring showed an improvement in its activity. Compounds **6, 7, 9, 12, 13, 14, 18, 21** required 32 µg/mL, **8, 10, 11, 17, r**equired 64 µg/mL and **15, 16, 22, 23, 25** showed MIC at 128 µg/mL against

B. *subtilis*. Compounds **7, 8, 9, 12, 13** required 16 µg/mL, **6, 9, 13, 14, 18, 21 r**equired 32 µg/mL also Compounds **15, 17, 19** and **11, 16, 24** registered MIC at 64 and 128 µg/mL against *P. aeruginusa* (they are four and eightfold less potent than chloramphenicol).

Table 3 also describes the MIC of synthesized compounds for their antifungal activity. The introduction of F, Cl, Br, NO_2 substituents on phenyl ring exhibited moderate to good activity against *C. albicans* and *A. niger*, whereas, except the **25, 26** all other compounds showed moderate to good activity against *A. niger* and *C. albican.* Of the fluoro and chloro substituted compounds **6, 7, 8, 9, 12, 13, 14, 17** registered a excellent activity against *A. niger* at 16 µg/mL, also compounds **10, 18, 19, 21** recorded good activity at 32 µg/mL and **11, 15, 16, 20, 22, 24** and **23** recorded moderate activity at 64 µg/mL and 128 µg/mL respectively which is fourfold and eight fold lower than standard Nystatin. In addition compounds **6, 9, 14** registered the MIC at 16 µg/mL, **7, 8, 10, 11, 13, 15, 17, 18, 21, 24** recorded at 32 µg/mL and **16, 19, 20, 23** registered the MIC at 64 µg/mL against *C. albicans*. Introduction of the halo substituent on the phenyl ring inhibited the growth of *A. niger* and *C. albican*.

Antibacterial Activity

4.2.1 Medium

Solid medium used for the study were Muller-Hinton agar (Hi media) MHA of the following composition beef infusion 300 gL^{-1}, casein acid hydrolysate 17.5 gL^{-1}, starch 1.5 g/mL, agar-agar 17 g/L and sterillized distilled water 1000 mL adjusted to pH 7.4. and soyabean casein digest agar (SCDA; casein enzymatic hydrolysate 17.0 g/L, papain digest of soyabean 3.0 g/L, NaCL 5.0 g/L, dipotassium phosphate 2.5 g/L and distilled water 1000 mL, adjusted to pH 7.3), were used for biological assays.

4.2.2 Test microorganisms

Microorganisms used were *B. subtilis* (2250), *S. aureus* (2079), *E. coli* (2109) and *P. aeruginosa* (2036). All of them were obtained from microbial type culture collection (MTCC) at the NCIM, Pune, India.

4.2.3 Primary screening

The antibacterial activity of all the newly synthesized compounds was done by the agar-well diffusion assay technique by following the method of Kumr et al.[37, 38] 24-h-old bacterial cultures of all test microorganisms were used as inoculums, which was adjusted to 0.5 McFarland standard, that is, 1.5 X 10^8 CFU/mL. ref The stock solution of all test compounds (100 µg/mL) were prepared by dissolving 100 µg of the test compound in DMSO (1 mL). Chlorampenicol and DMSO were used as positive and negative controls, respectively.

20 mL of molten and cooled MHA and 320 µL of each test bacterial culture were mixed (separate flasks were used for each bacterial culture) and poured in sterilized and labeled petri plates. The wells of 6 mm were punched in the solidified petri plates, aseptically 50 µL from stock solutions of all compounds as well as controls was added to each well of labeled petri plates and incubated at 35°C for 24 h. The diameter of the zone of growth inhibition around each well was measured after incubation using Vernier Caliper.

4.2.4 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of the antimicrobial agent that prevents the development of visible growth after overnight incubation. MIC of compounds against Gram-positive and Gram-negative test bacteria was determined by the method of NCCLS.[39] All the test cultures were streaked on SCDA and incubated overnight at 37°C. Turbidity of all the bacterial cultures was adjusted to 0.5 McFarland standard by preparing bacterial suspension of 3-5 well isolated colonies of the same morphological type selected from an agar plate culture. The cultures were further diluted 10-fold to get an inoculums size of 1.2 x 10^7 CFU/mL. Stock solutions of 4 mg/mL of each compound was prepared in DMSO and was appropriately diluted to get a final concentration of 128, 64, 32, 16, 8, 4, 2, 1 mg/mL.

Standard antibiotic Chlorampenicol were also diluted to get a final concentration in the same manner. Three hundred and twenty microliters of each dilution was added to 20mL molten and cooled MHA (separate flasks was taken for each dilution). After thorough mixing, the medium was poured in sterilized petri plates. The test bacterial cultures were spotted in a predefined pattern by ascetically transferring 5 ml of each bacterial culture on the surface of solidified agar plates and incubated at 35°C for 24 h.

4.3 Antifungal activity:

4.3.1 Biological assay

Solid medium used for the study were Potato dextrose agar (Hi media) of the following composition potato 250g, Dextrose 10g, agar-agar 20 g, sterilled distilled water 100 mL adjusted to pH 7.3.

4.3.2 Test microorganisms

Microorganisms used were C*. albicans* (3471) and *A. niger* (545). All of them were obtained from microbial type culture collection (MTCC) at the NCIM, Pune, India.

4.3.3 Primary screening

Sliced potatoes were taken with 500 mL of distilled water in a pan and boiled for half an h till a spoon when placed on a slice can pierce into it. Filtered it while hot and broth was again taken in a pan with rest of the distilled water. Dextrose dissolved in distilled water and weighed agar was added to the broth and heated it to boil. The medium thus obtained was sterilled in pressure cooker for 30 min. Sterillized medium (15 mL) each was pipette out into flat petridishes. When it solidified 15 mL of warm seeded agar was applied over it. The seeded agar was made by cooling the medium to 40°C and then adding spore suspension to seeded medium. The spores were obtained from ten days culture of *C. albicans* and *A. niger* species. The final inoculums size was adjusted to $1x10^6$ spore mL^{-1}. Nystatin and DMSO were used as positive and negative controls, respectively.

Before the solidification of agar, the plate was tilted to ensure that coverage should be even. These petridishes were then put into the refrigerator upside down to prevent condensation of moisture. Concentration 100 µg mL^{-1} of the synthesized compounds were prepared by dissolving the required quantity of compounds in DMSO, Sterilized whatmann filter paper number 541 discs were prepared by cutting 6 mm diameter with cork borer and were spread individually with needle and planted upon the chilled seeded medium. The culture plates were then incubated for 24 to 72 h at 37ºC and inhibition zone around each disc was measured from the centre of the discs. The diameter of growth inhibition zone was calculated by vernier caliper.

4.3.4 Minimum inhibitory concentration

The minimum inhibitory concentrations (MIC) of synthesized compounds 5–37 were determined in the range of concentrations from 128 to 1 µg/mL. The standardized micro broth dilution methods, were used according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical and LaboratoryStandards NCCLS).[39] Table 2 summarizes the minimum concentration of each derivative necessary to completely inhibit (MIC 90) the growth of two standardized opportunistic pathogenic fungi including *C. albicans* and *A. Niger*

Conclusion

In conclusion, a series of new **6-26** compounds were synthesized. The pharmacological studies were undertaken to evaluate the effect of substituents for their antimicrobial activities. Most of the synthesized compounds exhibited moderate to good activity towards gram-positive and gram-negative bacteria as well as both the fungi species. The enhancement in antibacterial and antifungal activity can be attributed to the presence of pharmacologically active F and Cl groups irrespective of their position in the molecule.

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