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# Synthesis and antimicrobial activity of *N*-alkyl and *N*-aryl piperazine derivatives

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**Abstract**—A series of substituted piperazine derivatives have been synthesized and tested for antimicrobial activity. The antibacterial activity was tested against *Staphylococcus aureus* (MTCCB 737), *Pseudomonas aeruginosa* (MTCCB 741), *Streptomyces epidermidis* (MTCCB 1824) and *Escherichia coli* (MTCCB 1652), and antifungal activity against *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*. All synthesized compounds showed significant activity against bacterial strains but were found to be less active against tested fungi. In vitro toxicity tests demonstrated that compounds **4d** and **6a** showed very less toxicity against human erythrocytes.


## 1. Introduction

Antimicrobial diseases are now more frequent than during the first half of the century, being still difficult to diagnose clinically. During the later half of the century, particularly during the past two decades, a number of different classes of antibacterial[1–7] and antifungal agents[8–14] have been discovered. Although, since the discovery of several synthetic and semi-synthetic antibacterial sulfa drugs, nitrofuranes, penicillins, cephalosporins, tetracyclines, macrolides, and oxazolidinones, and antifungal agents such as fluconazole, ketoconazole and miconazole, including amphotericin B, there has been much progress in this field.[1–4,11–14] Despite advances in antibacterial and antifungal therapies, many problems remain to be solved for most antimicrobial drugs available. For example, appearance of multidrug resistant Gram-positive bacteria, in particular, methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* is causing a serious menace. The use of amphotericin B, known as the 'gold standard,' is limited because of its infusion-related reactions and nephrotoxicity.[15,16] Also the use of azoles, such as fluconazole, ketoconazole and miconazole, has resulted in clinically resistant strains of *Candida* spp.[17,18] A 3.6–7.2% of vaginal isolates of *Candida albicans* from women with candidal vaginitis is resistant to fluconazole.[19] This situation highlights the need for advent of safe, novel and effective antibacterial and antifungal compounds.

Piperazines and substituted piperazines are important pharmacophores that can be found in many marketed drugs, such as the Merck HIV protease inhibitor Crixivan,[20] and drugs under development.[21] Piperazinyl-linked ciprofloxacin dimers reported as potent antibacterial agents against resistant strains,[22] a novel class of mixed $D_2/D_4$ receptor antagonists,[23] dual calcium antagonist,[24] antimalarial agents[25] and potential antipsychotic agents.[26] Recently, piperazine derivatives containing tetrazole nucleus have been reported as an antifungal agent.[27]Herein, we have described the synthesis of a series of *N*-alkyl and *N*-aryl piperazine derivatives as a new class of synthetic antimicrobial agents along with their in vitro antimicrobial activity [zone of inhibition for antibacterial and minimum inhibitory concentration (MIC) for antifungal activity].

## 2. Results and discussion

### 2.1. Chemistry

*N*-Alkyl and *N*-aryl substituted piperazine derivatives have been prepared by a well-established three compo-

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nent condensation reaction of *N*-alkyl and *N*-aryl substituted piperazines **1a-d** with benzotriazole and formaldehyde to construct the key benzotriazolyl intermediate **2a–d**, followed by nucleophilic substitutions of the benzotriazolyl group to perform N-functionalization. The reactive C-*N* bond of the key benzotriazolyl intermediate **2a–d** allows easy replacement the of benzotriazolyl group with other functionalities via nucleophilic substitutions, elimination and reduction.[28]

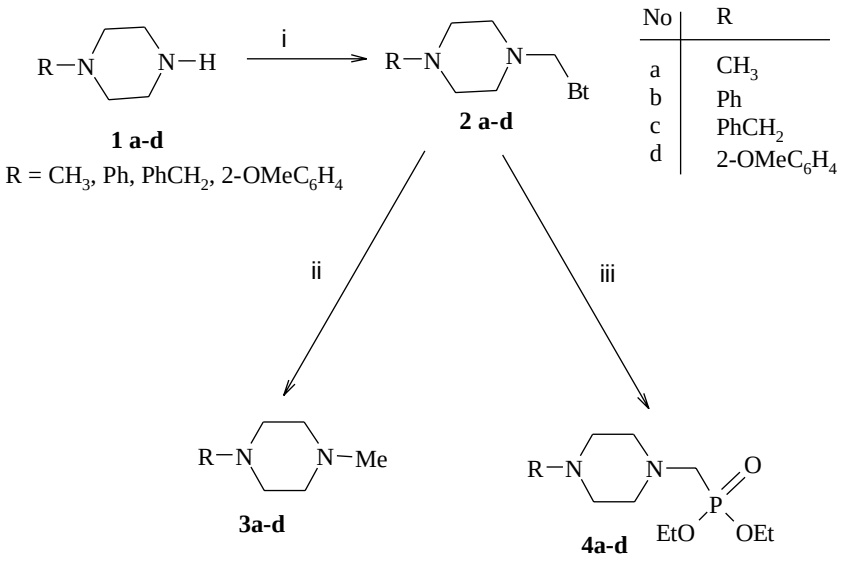
The condensation of 1-methylpiperazine **1a**, 1-phenyll-piperazine **1b**, 1-benzylpiperazine **1c** and 1-(2-methoxyphenyl)piperazine **1d** with 1 equiv of benzotriazole and 1 equiv of formaldehyde (37% aqueous solution) in MeOH/$H_2O$ (9:1) at 25 °C gave 1-[(4-methylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2a**, 1-[(4-phenylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2b**, 1-[(4-benzylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2c** and 1-{[4-(2-methoxyphenyl)piperazin-1-yl]methyl}-1*H*-1,2,3-benzotriazole **2d** in 87-94% yields as a sole benztoriazol-1-yl ($bt^1$) isomer. Treatment of **2a–d** with 2 equiv of sodium borohydride in refluxing THF replaced the benzotriazole group with hydrogen to give 1,4-dimethylpiperazine **3a**, 1-phenyl-4- methylpiperazine **3b**, 1-benzyl-4- methylpiperazine **3c** and 1-(2-methoxyphenyl)-4-methylpiperazine **3d** in 76–88% yields (Scheme 1). The benzotriazolyl group of **2a–d** was also replaced by triethylphosphite in the presence of $ZnBr_2$ to afford diethyl(4-methylpiperazin-1-yl)methyl phosphonate **4a**, diethyl(4-phenylpiperazin-1-yl)methyl phosphonate **4b**, diethyl(4-benzylpiperazin-1-yl)methyl phosphonate **4c** and diethyl [4-(2-methoxyphenyl)piperazin-1-yl]methylphosphonate **4d** in 74–91% yields (Scheme 1).

Nucleophilic substitution of **2a–d** with various Grignard reagents 1-propynyl-, phenylethynyl-, *p*-tolyl- and isopropyl magnesium bromides in dry THF furnished 1-but-2-ynyl-4-methylpiperazine **5a**, 1-phenyl-4-but-2-ynylpiperazine **5b**, 1-benzyl-4-but-2-ynylpiperazine **5c**, 1-but-2-ynyl-4-(2-methoxyphenyl)-piperazine **5d**, 1-methyl-4-(3-phenylprop-2-ynyl)piperazine **6a**, 1-phenyl-4-(3-phenylprop-2-ynyl)piperazine **6b**, 1-benzyl-4-(3-phenylprop-2-ynyl)piperazine **6c,** 1-(2-methoxyphenyl)-4-(3-phenylprop-2-ynyl)piperazine **6d**, 1-isobutyl-4-methylpiperazine **7a**, 1-phenyl-4-isobutylpiperazine **7b**, 1-benzyl-4-isobutylpiperazine **7c**, 1-isobutyl-4-(2-methoxyphenyl)piperazine **7d,** 1-methyl-4-benzyl-piperazine **8a,** 1-phenyl-4-benzyl-piperazine **8b**, 1,4-dibenzyl-piperazine **8c** and 1-(2-methoxyphenyl)-4-(4-methylbenzyl)piperazine **8d**, respectively, in 68–87% yields (Scheme 2).

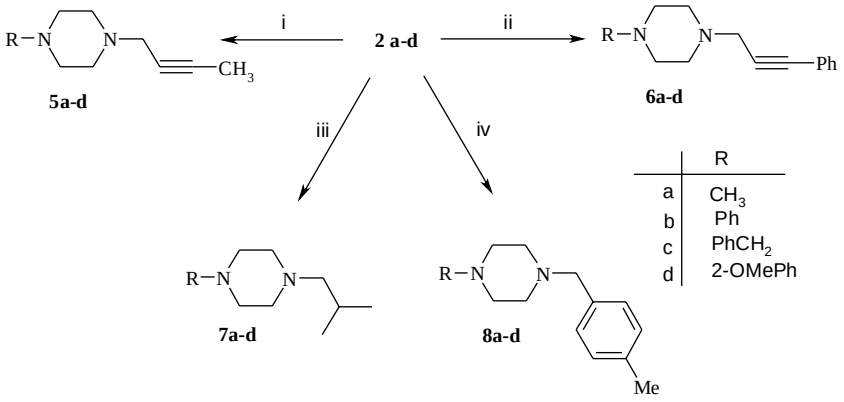
The condensation of piperazine **9** with 2 equiv of benzotriazole and 2 equiv of formaldehyde (37% aqueous solution) in MeOH/$H_2O$ (9:1) at 25 °C gave 1,4-bis-[1,2,3]benzotriazol-1-ylmethylpiperazine **10** in 92% yields as a sole $bt^1$ isomer. Varied nucleophiles and Grignard reagents in varied solvents were tried to substitute the benzotriazolyl group with nucleophiles, but one failed to obtain substituted compounds due to insolubility of compound **10** in various solvents (Scheme 3).

### 2.2. In vitro antibacterial activity

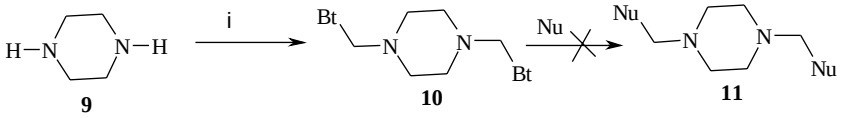
Antibacterial activity of the synthesized compounds was tested against pathogenic bacterial strains such as *Staphylococcus aureus* (MTCCB 737), *Pseudomonas aeruginosa* (MTCCB 741), *Staphylococcus epidermidis* (MTCCB 1824) and *Escherichia coli* (MTCCB 1652) using the disc diffusion method.[29] Gentamicin was used as reference drug for bacteria. The zone of inhibition (mm) of tested compounds against pathogenic bacterial strains is shown in Table 1. In general, the compounds showed significant to moderate antibacterial activity, whereas the compounds **5c** and **7b** were inactive at the highest tested concentration against Gram-negative and Gram-positive bacteria. Diethyl(4-benzylpiperazin-1-yl)methylphosphonate **4c,** diethyl [4-(2-methoxyphenyl)piperazin-1-yl]methylphosphonate **4d** and 1-methyl-4-(3-phenylprop-2-ynyl)piperazine **6a** had highest antibacterial activities against pathogenic bacterial strains with the zone of inhibition in the range of 9–18 mm. The reason for this higher antibacterial activity might be related to the presence of phosphate group in **4c**, **4d** and triple bond in **6a** (Table 1). Compound **4d** exhibited significant activity against *S. aureus* (with the zone of inhibition, 18 mm), *S. epidermidis* (16 mm) and *P. aeruginosa* (15 mm), but moderate activity against *E. Coli* (13 mm). Compound **6a** showed significant activity against *S. aureus* (with the zone of inhibition, 18 mm), *P. aeruginosa* (16 mm) and *E. coli* (15 mm), but poor activity against *S. epidermidis* (9 mm). Diethyl(4-benzylpiperazin-1-yl)methylphosphonate **4c** showed significant activity against *S. aureus* and *S. epidermidis* with 16 and 17 mm of the zone of inhibition, respectively. By considering the considerable activity of substituted 1,2,4-triazoles heterocyles against Gram-negative and Gram-positive bacteria as well as fungi,[30–33] a new series of benzotriazole derivatives of *N*-alkyl and *N*-aryl piperazine were also synthesized and their antimicrobial properties evaluated. 1-[(4-Methylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2a** showed moderate activity against Gram-negative and Gram-positive bacteria with the zone of inhibition in the range of 11-14 mm. 1-[(4-Benzylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2c** and 1-{[4-(2-methoxyphenyl) piperazin-1-yl] methyl}-1*H*-1,2,3-benzotriazole **2d** showed moderate activity against bacterial strains. However, 1-[(4-phenylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2b** shows mild antibacterial activity. This might be due to the presence of benzotriazolyl group (triazole fused with benzene nucleus). 1-But-2-ynyl-4-methylpiperazine **5a** and 1-isobutyl-4-(2-methoxyphenyl)piperazine **7d** showed significant activity against antibacterial strains with an inhibition zone diameter of 11–17 mm. These might be due to the presence of the triple group in **5a** and the isopropyl group in **7d**. The other related compounds in this study demonstrated weak antibacterial activity. The preliminary results of antimicrobial activities indicated that some of the compounds exhibited a moderate




**Scheme 1.** Reagents and conditions: (i) BtH/$CH_2O$, MeOH/$H_2O$ (9:1), 25 °C, 6 h; (ii) $NaBH_4$, THF, reflux, 8–12 h; (iii) triethylphosphite/$ZnBr_2$, 25 °C, 12–16 h.




**Scheme 2.** Reagents and conditions: (i) 1-propynyl magnesium bromides, THF, reflux, 8 h; (ii) phenylethynyl magnesium bromides, THF, reflux, 6–8 h; (iii) isopropyl magnesium bromides, THF, reflux, 8–10 h; (iv) *p*-tolyl-magnesium bromides, THF, reflux, 6–8 h.




**Scheme 3.** Reagents and conditions: (i) BtH/$CH_2O$, MeOH/$H_2O$ (9:1), 25 °C, 6 h.

to significant activity against Gram-positive and Gram-negative strains.

### 2.3. In vitro antifungal activity

Antifungal activity of the synthesized compounds was tested against pathogenic fungal strains such as *Aspergillus fumigatus* (ITCC 4517), *Aspergillus flavus* (ITCC 5192) and *Aspergillus niger* (ITCC 5405). The anti-*Aspergillus* activity of all the synthesized compounds was evaluated by the disc diffusion (DDA)[34] microbroth dilution (MDA),[34] and percentage spore germination inhibition (PSGI) assays.[35] Amphotericin B was used as a standard drug. The minimum inhibitory concentrations (MICs, $mgml^{-1}$, $mgdisc^{-1}$) of tested compounds against pathogenic fungi are shown in Table 2. Results of in vitro anti-*Aspergillus* activity demonstrate that **2c**, **2d**, **4c**, **6c** and **11** show mild to moderate anti-*Aspergillus* activities against pathogenic strains used in the experiment. 1-[(4-Phenylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2b** was found to be a potential inhibitor of the growth of *A. fumigatus*, *A. flavus* and *A. niger* result of the anti-*Aspergillus* activity. 1-[(4-Phenylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2b** has significant MIC values in the range of 23.43 μg/disc in DDA, 62.50–125.0 μg/ml in MDA and

**Table 1.** Antibacterial activity of substituted piperazine derivatives

| Compound | Zone of inhibition (mm) | | | |
|---|---|---|---|---|
| | *S. aureus* | *S. epidermidis* | *P. aeruginosa* | *E. coli* |
| **2a** | 13 | 11 | 12 | 14 |
| **2b** | 9 | — | 9 | 9 |
| **2c** | 9 | 14 | 9 | 8 |
| **2d** | 8 | 10 | 10 | 11 |
| **3c** | 12 | 10 | 8 | 7 |
| **4c** | 16 | 17 | 14 | 10 |
| **4d** | 18 | 16 | 15 | 13 |
| **5a** | 16 | 13 | 11 | 14 |
| **5b** | 9 | 6 | 7 | 6 |
| **5c** | — | — | — | — |
| **6a** | 18 | 9 | 16 | 15 |
| **6c** | 8 | 13 | 7 | 8 |
| **7b** | — | — | 10 | — |
| **7d** | 17 | 14 | 13 | 17 |
| **8d** | 8 | 7 | 8 | 8 |
| **10** | 10 | 7 | 8 | — |
| Gentamicin | 16 | 17 | 16 | 10 |

62.50–125.0 μg/ml in the PSGI assay against *A. flavus*, *A. niger* and *A. fumigatus* (Table 2). Compound 1-[(4-methylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2a** has MIC values in the range of 46.75 μg/disc in DDA, 250–500 μg/ml in MDA and 250 μg/ml in the PSGI assay against *A. flavus*, *A. niger* and *A. fumigatus*. Enhanced activity of compounds **2a**, **2b** might be due to the presence of the benzotriazolyl group. Compounds **2c** and **2d** showed moderate anti-*Aspergillus* activity with MIC values in the range of 187.5–187.5 μg/disc in DDA, 500–1000 μg/ml in MDA and 500–500 μg/ml in PSGI assay against *A.flavus*, *A. niger* and *A. fumigatus* (Table 2). Although it was not possible to established the structure–activity relationship with respect to fungi.

### 2.4. Cytotoxicity study on diethyl [4-(2-methoxyphenyl) piperazin-1-yl] methylphosphonate (4d) and 1-methyl-4-(3-phenylprop-2-ynyl)piperazine (6a)

The in vitro cell cytotoxicity of diethyl [4-(2-methoxyphenyl) piperazin-1-yl] methylphosphonate **4d** and 1-but-2-ynyl-4-methylpiperazine **6a** was investigated using the haemolytic assay.[34,36] Compounds **4d** and **6a** were found to be less toxic upto the tested concentration, that is, 1000 μg/ml and lysed only 10% **4d** and 17.4% **6a** of human erythrocytes, respectively, whereas standard drug Gentamicin lysed 31% of erythrocytes at a concentration of 1000 μg/ml (Figs. 1 and 2).

## 3. Conclusion

In conclusion, in an effort to discover new piperazine analogues with antibacterial activity we found that compounds **4c**, **4d**, **5a**, **6a** and **7d** show significant antibacterial activity. Compounds **2a** and **2b** were found show significant anti-*Aspergillus* activity. Compounds **4d** and **6a** were found to be less toxic to human erythrocytes when compared with Gentamicin. The results of the present study indicate that compounds **2a**, **2b**, **4c**, **5a**, **6a** and **7d** might be of interest for the identification of new antimicrobial molecules.

## 4. Experimental

All reagents used were of AR grade. THF was distilled from sodium/benzophenone prior to use. Melting points were determined with a Thomas Hoover melting point apparatus and are uncorrected. $^{1}H$ (300 MHz) and $^{13}C$ NMR (75 MHz) spectra were recorded on a Bruker 300 NMR spectrometer in $CDCl_3$ with TMS for $^{1}H$ and chloroform-*d* for $^{13}C$ as internal references) unless otherwise stated. Mass Spectrum was recorded on a Hybrid Quadrupole-TOF LC\MS\MS mass spectrometer (Q. Star XL). Column chromatography was performed on silica gel (230–400 mesh). Microanalyses were obtained with an Elemental Analysensysteme GmbH VarioEL V3.00 element analyser. The reactions were monitored by thin layer chromatography (TLC) using aluminium sheets with silica gel 60 $F_{254}$ (Merck). All of the reactions were carried out under nitrogen atmosphere.

**Table 2.** Antifungal activity of substituted piperazine derivatives

| Compound | MIC | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DDA (μg/disc) | | | MDA (μg/ml) | | | PSG1 (μg/ml) | | |
| | *A. flavus* | *A. niger* | *A. fumigatus* | *A. flavus* | *A. niger* | *A. fumigatus* | *A. flavus* | *A. niger* | *A. fumigatus* |
| **2a** | 46.75 | 46.75 | 46.75 | 500 | 500 | 250 | 250 | 250 | 250 |
| **2b** | 23.43 | 23.43 | 23.43 | 62.50 | 125.0 | 125.0 | 62.50 | 125.0 | 62.50 |
| **2c** | 187.5 | 375.5 | 187.5 | 500 | 1000 | 500 | 500 | 500 | 500 |
| **2d** | 187.5 | 187.5 | 187.5 | 500 | 500 | 500 | 500 | 500 | 500 |
| **4b** | — | — | — | — | — | — | — | — | — |
| **4c** | — | — | — | — | — | 1000 | — | — | 1000 |
| **4d** | — | — | — | — | — | — | — | — | — |
| **5c** | — | — | — | — | — | — | — | — | — |
| **6c** | 500 | 500 | 250 | 250 | 500 | 500 | 500 | 500 | 500 |
| **7d** | — | — | — | — | — | — | — | — | — |
| **8a** | — | — | — | — | — | — | — | — | — |
| **11** | — | — | 187.5 | — | — | 1000 | — | — | 500 |
| Amphotericin B | 2.5 | 2.5 | 2.5 | 5 | 5 | 5 | 5 | 5 | 5 |

(—) means no activity.

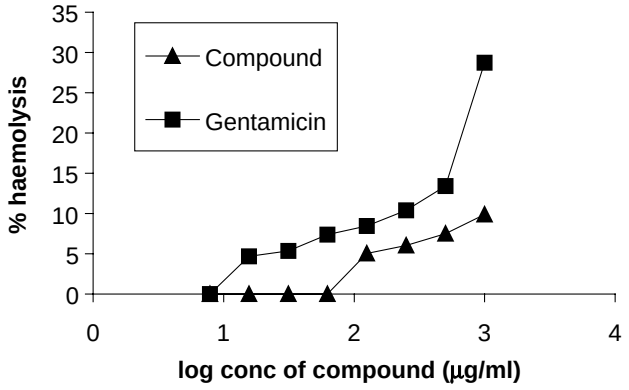



**Figure 1.** Haemolytic activity of diethyl [4-(2-methoxyphenyl) piperazin-1-yl] methylphosphonate **4d**.

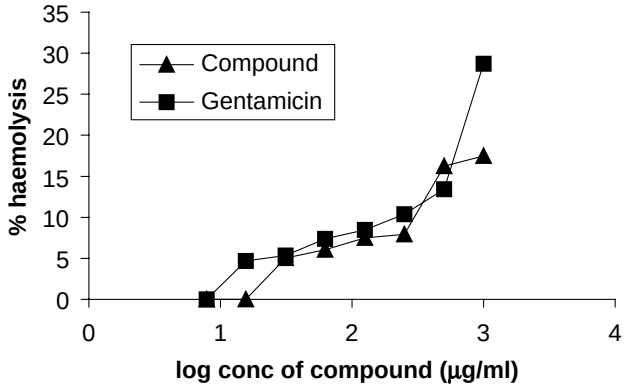



**Figure 2.** Haemolytic activity of 1-methyl-4-(3-phenylprop-2-ynyl) piperazine **6a**.

### 4.1. General procedure for the synthesis of benzotriazolyl derivatives of *N*-alkyl and *N*-aryl piperazines (2a–d)

To a solution of *N*-alkyl and *N*-aryl piperazines **1a**–**d**, [0.35 g, 2 mmol] and benzotriazole (0.2148 g, 2 mmol) in $CH_3OH/H_2O$ (9:1, 10 ml) was added formaldehyde (37% aqueous solution, 4 mmol). The mixture was stirred at room temperature for 6–8 h. The precipitate formed was filtered and washed with cold $Et_2O$ to give pure product 1-[(4-methylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2a**, 1-[(4-phenylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2b**, 1-[(4-benzylpiperazin-1-yl)methyl]-1*H*-1,2,3-benzotriazole **2c** and 1-{[4-(2-methoxyphenyl) piperazin-1-yl] methyl}-1*H*-1 and 2,3-benzotriazole **2d** in 87–94% yields as a sole bt[1] isomer, which was directly used for subsequent reactions. For microanalysis purposes, the precipitate was recrystallised from $CHCl_3$/hexanes (1:1).

Spectral data of the synthesized compounds **2a**–**d** were consistent with the reported values.

### 4.2. General procedure for the reduction of 2a–d with sodium borohydride

To a solution of benzotriazolyl derivative of *N*-alkyl and *N*-aryl piperazines **2a**–**d** (0.31 g, 1.0 mmol) and $NaBH_4$ 0.076 g, 2.0 mmol) refluxed in dry THF (10 ml) 8–12h, after removal of the solvent in vacuo, the residue was diluted with EtOAc. The mixture was washed with 1 N NaOH, and brine and dried over $Na_2SO_4$ and evopration of the solvent in vacuo, the residue was purified by column chromatography with hexanes/EtOAc (6:1 to 3:1) as an eluent to give **3a**–**d** in 76–88% yields.

Spectral data of the synthesized compounds **3a**–**d** were consistent with the reported values.

### 4.3. General procedure for the nucleophilic substitutions of 2a–d with triethyl phosphite

To a solution of benzotriazolyl derivative of *N*-alkyl and *N*-aryl piperazines **2a**–**d** (0.31 g, 0.69 mmol) in dry $CH_2Cl_2$ 20 ml) at 0 °C were successively added $ZnBr_2$ 0.186 g, (0.827 mmol) and triethyl phosphite (0.137 ml, 0.827 mmol). The reaction mixture was stirred at 0 °C for 2 h and then at 25 °C for 12–16 h (completion of the reaction was monitored by TLC), and the reaction was quenched with $H_2O$. After extraction with $CH_2Cl_2$, the combined organic layers were washed with 1 N NaOH, brine and dried over anhydrous $Na_2SO_4$. After removal of the solvent in vacuo, the residue was purified by column chromatography with hexanes/EtOAc (4:1) as an eluent to afford **4a**–**d** in 74–91% yields.

### 4.4. Diethyl(4-phenylpiperazin-1-yl)methyl phosphonate (4b)

The general synthetic method described above affords **4b**, as a colourless oil. Yield 98%; $^1$H NMR ($CDCl_3$, 300 MHz,): 7.27 (t, $J = 9$ Hz, 2H), 6.91 (d, $J = 9$ Hz, 1H), 6.85 (d, $J = 6$ Hz, 2H), 4.22 (s, 2H), 4.17 (m, 4H), 3.20 (m, 4H), 2.82 (m, 4H), 1.34 (t, $J = 6$ Hz, 6H); $^{13}$C NMR (75 MHz, $CDCl_3$): 151.15, 129.05, 119.7, 116.02, 62.17, 54.89, 54.77, 49.14, 16.48; LCMS *m*/*z* found 312.8 (M+), 160, 175.

### 4.5. Diethyl[4-(2-methoxyphenyl)piperazin-1-yl]methylphosphonate (4d)

The general synthetic method described above affords **4d** as a colourless oil. Yield 98%; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.07 (d, $J = 8.1$ Hz, 1H), 7.89 (d, $J = 9$ Hz, 1H), 7.61–7.69 (m, 1H), 7.36–7.51 (m, 1H), 5.48 (s, 2H), 3.18 (s, 3H), 2.81–2.90 (m, 8H), 1.43–1.46 (m, 4H), 1.24–1.26 (m, 6H); $^{13}$C NMR (75 MHz, $CDCl_3$): 141.2, 127.9, 119.2, 118.1, 116.2, 115.2, 77.0, 60.9, 56.0, 54.9, 48.08, 15.098; LCMS: *m*/*z* found 341.2 ($M^+$), 342.2 (M+1) peak.

### 4.6. General procedure for the nucleophilic substitutions of 2a–d with Grignard reagents (5a–d, 6a–d, 7a–d and 8a–d)

To a solution of benzotriazolyl derivative of *N*-alkyl and *N*-aryl piperazines **2a**–**d** (1.0 mmol) in dry THF

(10 ml) at 0 °C was added dropwise a solution of an appropriate Grignard reagent (1.2 mmol). The reaction mixture was allowed to warm to 25 °C and stirred for 0.5 h. Then, the mixture was refluxed for 6–8 h. After the mixture was cooled, the reaction was quenched with water and the mixture was extracted with ether. The combined extracts were washed with 1 N NaOH, brine and dried over $Na_2SO_4$. After removal of the solvent in vacuo, the residue was purified by column chromatography with hexanes/EtOAc (6:1 to 3:1) as an eluent gave **5a–d**, **6a–d**, **7a–d** and **8a–d** in 68–87% yields.

### 4.7. 1-But-2-ynyl-4-methylpiperazine (5a)

The general synthetic method described above affords **5a** as a colourless oil. Yield 91%; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 2.73–2.69 (m, 6H), 2.54–2.40 (m, 6H), 2.24 (s, 3H); $^{13}$C NMR (75 MHz, $CDCl_3$) 77.5, 77.1, 77.0, 52.0, 48.4, 44.2, 1.5; LCMS: *m*/*z* found 153.3 (M+1) as peak.

### 4.8. 1-But-2-ynyl-4-phenylpiperazine (5b)

The general synthetic method described above affords **5b** as a colourless oil. Yield 92%; $^1$H NMR (300 MHz, $CDCl_3$,): 7.23–7.33 (m, 5H), 1.89 (s, 3H), 1.45 (s, 2H), 1.43 (m, 4H), 1.25 (m, 4H); $^{13}$C NMR (75 MHz, $CDCl_3$): 129.1, 118.4, 113.2, 80.5, 57.2, 50.0, 42.6, 1.5; LCMS *m*/*z* found 215.29 (M+1), 175.29, 162.

### 4.9. 1-Benzyl-4-but-2-ynylpiperazine (5c)

The general synthetic method described above affords **5c** as a colourless oil. Yield 93%; $^1$H NMR (300 MHz, $CDCl_3$): 7.38–7.24 (m, 5H), 3.57–3.42 (m, 6H), 2.59–2.61 (m, 5H), 2.53–2.48 (m, 4H); $^{13}$C NMR (75 MHz, $CDCl_3$): 137.9, 132.9, 129.2, 128.1, 125.2, 77.0, 52.8, 52.0, 41.4, 29.7, 3.5; LCMS *m*/*z* found: 229.3 (M+1) peak.

### 4.10. 1-(2-Methoxyphenyl)-4-(3-phenylprop-2-ynyl)piperazine (6d)

The general synthetic method described above affords **6d** as a colourless oil. Yield 93%; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 7.50–7.89 (m, 5H), 6.29–6.95 (m, 4H), 3.75 (s, 3H), 3.22–3.29 (m, 4H), 2.80 (s, 2H), 2.73–2.79 (m, 4H); $^{13}$C NMR (75 MHz, $CDCl_3$) 142.7, 132.3, 129.2, 128.5, 128.3, 127.1, 122.1, 118.9, 116.7, 112.2, 82.5, 77.2, 76.9, 54.4, 50.0, 32.3; LCMS: *m*/*z* found 307.4 (M+1) as peak.

### 4.11. 1-Benzyl-4-isobutylpiperazine (7c)

The general synthetic method described above affords **7c** as a colourless oil. Yield 91%; $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 6.84–7.01 (m, 5H), 3.86 (s, 2H), 3.12 (m, 4H), 2.7 (m, 4H), 2.26 (s, 2H), 1.82 (m, 1H), 0.9003 (d, $J$ = 7.6, 6H); $^{13}$C NMR (75 MHz, $CDCl_3$): 138.8, 129.6, 128.4, 127.5, 77.4, 76.5, 62.6, 52.8, 29.6, 20.9; LCMS *m*/*z* found: 233.23 (M+1), 115.4.

### 4.12. 1,4-Bis-[1,2,3]benzotriazol-1-ylmethyl-piperazine (10)

Compound **10** as a white solid. Yield 92%; $^1$H NMR (300 MHz, $CDCl_3$): $\delta$ 8.04 (d, $J$ = 9 Hz, 2× 1H), 7.64 (d, $J$ = 9 Hz, 2× 1H), 7.48 (t, $J$ = 6 Hz, 2× 1H), 7.35 (t, $J$ = 6 Hz, 2× 1H), 4.84 (s, 4H), 2.75–2.68 (m, 4H), 2.42–2.23 (m, 4H); $^{13}$C NMR (75 MHz, $CDCl_3$): 133.6, 129.6, 127.2, 126.2, 123.6, 118.04, 80.56, 54.52, 49.96; LCMS: *m*/*z* found 348.4 (M+1) as peak.

### 4.13. Disc diffusion method

The in vitro antibacterial activity was tested by the disc diffusion method[29] using pathogenic strains of *S. aureus* (MTCCB 737), *P. aeruginosa* (MTCCB 741), *S. epidermidis* (MTCCB 1824) and *E. coli* (MTCCB 1652). The concentration 30 μg/disc of compounds was impregnated on the discs. These discs were placed on the surface of the agar plates already inoculated with pathogenic bacteria. The plates were incubated at 37 °C and examined at 48 h for zone of inhibition, if any, around the discs. Gentamicin was used in the assay as a standard control drug. An additional control disc without any sample but impregnated with an equivalent amount of solvent (DMSO) was also used in the assay. The result of antibacterial activity indicated that some of the compounds exhibited mild to moderate activity.

### 4.14. Antifungal activity assay

The anti-*Aspergillus* activity of all the compounds was studied by disc diffusion, microbroth dilution and percentage spore germination inhibition assays.[34] The pathogenic strains of *Aspergillus fumigatus* (ITCC 4517), *A. flavus* (ITCC 5192) and *A. niger* (ITCC 5405) were used in the study for performing various experiments.

### 4.15. Microbroth dilution

The test was performed in 96-well culture plates (Nunc, Nunclon). Various concentrations of synthetic compounds in the range of 1000–7.81 μg/ml were prepared in the wells by a 2-fold dilution method. Assay was performed as per the standard method described earlier.[34]

### 4.16. Disc diffusion

The disc diffusion assay was performed in radiation sterilized petri plates of 10.0 cm diameter (Tarsons) as described.[34] Different concentrations in the range of 750–1.46 μg of the test compounds were impregnated on the sterilized discs (5.0 mm in diameter) of Whatman filter paper No. 1. The discs were placed on the surface of the agar plates already inoculated with *Aspergillus* spores. The plates were incubated at 37 °C and examined at 24, 48 and 96 h for zone of inhibition, if any, around the discs. The concentration, which developed the zone of inhibition of at least 6.0 mm diameter, was considered as minimum inhibitory concentration (MIC).

### 4.17. Percentage spore germination inhibition

Various concentrations of the test compounds in 90.0 μl culture medium were prepared in 96-well culture plates (Nunc, Nunclon) by the double dilution method.[35] The wells were prepared in triplicates for each concentration. Each well was then inoculated with 10.0 μl of spore suspension containing 100 ± 5 spores. The plates were incubated at 37 °C for 16 h and then examined for spore germination with an inverted microscope (Nikon, Diphot). The number of germinated and non-germinated spores was counted. The lowest concentration of the compound, which resulted in >90% inhibition of germination of spores in the wells, was considered as $MIC_{90}$.

### 4.18. Haemolytic assay

The toxicity of piperazine derivatives having antibacterial and antifungal potential was investigated by using the haemolytic assay.[34,36] A slight modification was employed to determine the haemolytic effect of antibacterial diethyl [4-(2-methoxyphenyl) piperazin-1-yl] methylphosphonate **4d** and 1-methyl -4-(3-phenyl-prop-2-ynyl) piperazine **6a**. Human erythrocytes collected from apparently healthy volunteers were washed thrice with PBS and 2.0% (v/v) suspension of erythrocytes was prepared in phosphate-buffered saline at pH 7.2. Half millilitres of (2.0%) human erythrocyte suspension in 16 duplicate sets of tubes was treated with compound **4c** at a concentration of 1.22 μg/ml for 1 h at 37 °C. After incubation, tubes were centrifuged at 5000 rpm for 10 min. The supernatant was collected and the OD was measured at $A_{415}$ nm using a spectrophotometer (UV–vis Spect Lambda Bio 20 Perkin Elmer). Results were expressed as % haemolysis by the compound. Only a buffer of pH 7.2 was used for background lysis in negative control sets, whereas in positive controls, lysis buffer was used for completely lysing the erythrocytes.

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