ABSTRACT

A series of bis heterocycles comprising of bis-1,2,4-triazoles and 1,3,4-thiadiazoles were synthesized from 3, 5-dimethyl-1H-pyrrole-2,4-dicarbohydrazide in two steps via respective thiosemicarbazide intermediates. The compounds 3a-g and 4a-g were evaluated for their anti fungal activity against a panel of four pathogenic fungal strains namely, Aspergillus niger, Aspergillus flavus, Aspergillus terrius and Candida albicans by two fold serial dilution method. The cytotoxic effect of these compounds was also studied against human lung carcinoma cells (A-549) using MTT assay method.

Keywords: Bis-triazole, Bis-thiadiazole, Antifungal activity, Cytotoxic activity, MTT assay.

INTRODUCTION

Bis-heterocyclic compounds are gaining increased interest in the recent past as the dimeric analogues have proven to have being better and potent biological activity than the corresponding monomer1-3. Many of the bis-1, 2, 4-triazoles 4-6 and bis-thiadiazoles7-9 have also been reported to possess wide spectrum of biological activity.

The triazoles such as fluconazole and terconazole are also being used clinically as potent antifungal agents. We have reported earlier the synthesis and cytotoxicity study of certain bis 1,2,4-triazole systems from our lab.10-12 It has been observed that the lipophilicity of the molecules seems to be important for the higher cytotoxic activity as evident from the lower IC50 values. Further the higher lipophilicity is also of importance to exert antifungal activity as high lipophilic compounds can easily penetrate through the fungal cell membrane. Keeping these observations in mind we report herein the synthesis and in vitro cytotoxic as well as antifungal activity of certain novel bis 1,2,4-triazoles and 1,3,4-thiadiazoles encompassing pyrrole.

MATERIALS AND METHODS

The melting points were determined in open glass capillaries and are uncorrected. IR spectra were recorded on Shimadzu FT-IR 8400-S spectrophotometer by KBr pellet technique. 1H-NMR and 13C-NMR spectra were recorded on AMX-400 NMR spectrophotometer at 400 MHz using DMSO-d6 as the solvent and tetra methyl silane (TMS) as internal standard. The chemical shifts are expressed in δ ppm. The splitting patterns were designated as follows; s: singlet; d: doublet; q: quartet; m: multiplet. LCMS were recorded by using Shimadzu LCMS-2010A instrument by ESI. Molecular ion (M+) value in m/z units is provided along with percent relative abundance in parenthesis.

Synthesis of the intermediate and target compounds was accomplished according to the steps depicted in Scheme 1.
Reagents and conditions: i) RNCS/EtOH and DMF reflux 4-6 h ii) 2N NaOH, heat for 4h neutralize with dil. HCl iii) dissolve in 5 ml Conc. H2SO4 stir below 5°C for 2h and neutralize with NaHCO3.

Preparation of 3, 5-dimethyl-1H-pyrrrole-2,4-dicarboxylic acid (2):

To the suspension of 0.005 mol of diethyl 3,5-dimethyl 1H pyrrole (3a) in 50 ml of absolute ethanol, 0.005 mol of appropriate thiosemicarbazide (2a-g): subtituted-1,2,4 triazol-3-yl) 3,5 dimethyl 1H pyrrole (3a-g):

A solution of 0.005 mol of appropriate thiosemicarbazide (2a-g) in 10 ml of 2N NaOH was refluxed for 4-6 h with constant stirring. The solid obtained after cooling was collected and crystallized with dichloromethane.

Yield- 85%; MP: 256°C; IR (ν cm⁻¹,KBr): 3210, 3072, 2980; 1H-NMR (DMSO-d6, δ ppm): 8.13 (s,1H, NH), 6.7-7.5 (m, 8H, Ar-H), 4.85 (s, 6H, OCH3), 2.48 (s, 3H, CH3), 2.19 (s, 3H, CH3), 2.10 (m, 8H, Cδ and Cβ protons of cyclohexyl), 1.70 (m, 8H Cδ and Cβ protons of cyclohexyl), 1.5 (m, 4H Cδ protons of cyclohexyl); LCMS m/z: 458 (M+45).

2, 4 bis (5-mercapto-1,2,4 triazol-3-yl) 3,5 dimethyl 1H pyrrole (3e)

The above method was followed using 0.005 mol of 2e.

Yield- 60%; MP: 185°C; IR (ν cm⁻¹,KBr): 3233, 2905; 1H-NMR (DMSO-d6, δ ppm): 12.15 (s,2H, SH), 8.18 (s,1H, NH), 3.87 (s, 2H, protons of butyl), 2.40 (s, 3H, CH3), 2.27 (s,3H, CH3), 2.18 (m, 4H Cδ protons of butyl), 1.90 (m, 4H Cδ protons of butyl), 1.2 (m, 6H, Cδ protons of butyl); LCMS m/z: 406 (M+40).

2, 4 bis (5-mercapto-1,2,4 triazol-3-yl) 3,5 dimethyl 1H pyrrole (3f)

The above method was followed using 0.005 mol of 2f.

Yield- 66%; MP: 212°C; IR (ν cm⁻¹,KBr): 3225, 3065, 2978; 1H-NMR (DMSO-d6, δ ppm): 12.32 (s,2H, SH), 8.14 (s,1H, NH), 6.6-7.4 (m, 8H, Ar-H), 3.7 (s, 6H, OCH3), 2.4 (s, 3H, CH3), 2.20 (s, 3H, CH3); LCMS m/z: 482 (M+45).

General method for the preparation of bis-thiosemicarbazides (2a-g):

A mixture of 0.005 mol of 2 and 0.01 mol of appropriate isothiocyanates in 20 ml of absolute ethanol was refluxed for 8 h on boiling water bath. The resulting solution was concentrated and cooled to get the bis-thiosemicarbazides in quantitative yield. The colourless solid thus obtained were washed with a mixture of DMF and ethanol and were used without further purification.

General method for the preparation of 2, 4 bis (5-mercapto-1,2,4 triazol-3-yl) 3,5 dimethyl 1H pyrrole (3a-g):

A solution of 0.005 mol of appropriate thiosemicarbazide (2a-g) in 10 ml of 2N NaOH was refluxed for 4-6 h with constant stirring. The reaction mixture was cooled, filtered and the filtrate was acidified with cold dilute HCl to get the solid triazole. Recrystallization was done by ethanol.

2, 4 bis (5-mercapto-1,2,4 triazol-3-yl) 3,5 dimethyl 1H pyrrole (3a)

The above method was followed using 0.005 mol 2a.

Yield- 80%; MP: 225°C; IR (ν cm⁻¹,KBr): 3215, 3050; 1H-NMR (DMSO-d6, δ ppm): 12.3 (s,2H, SH), 8.1 (s,1H, NH), 6.8-7.3 (m, 10H, Ar-H), 2.4 (s, 3H, CH3), 2.25 (s, 3H, CH3); 13C-NMR (DMSO-d6, δ ppm): 169.7, 153.9, 149.7, 147.5, 138.0, 129.4, 128.2, 125.6, 124.9, 122.2, 120.5, 14.2, 13.1; LCMS m/z: 446 (M–55).

2, 4 bis (5-mercapto-1,2,4 triazol-3-yl) 3,5 dimethyl 1H pyrrole (3b)

The above method was followed using 2b (0.005 mol).

Yield- 85%; MP: 256°C; IR (ν cm⁻¹,KBr): 3210, 3045; 1H-NMR (DMSO-d6, δ ppm): 12.2 (s,2H, SH), 8.12 (s,1H, NH), 6.6-7.5 (m, 8H, Ar-H), 2.35 (s, 3H, CH3), 2.23 (s, 3H, CH3), 2.12 (s, 6H, tolyl CH3); LCMS m/z: 474 (M+75).

2, 4 bis (5-mercapto-1,2,4 triazol-3-yl) 3,5 dimethyl 1H pyrrole (3c)

The above method was followed using 0.005 mol 2c.

Yield- 70%; MP: 234°C; IR (ν cm⁻¹,KBr): 3212, 3070, 2980; 1H-NMR (DMSO-d6, δ ppm): 12.17 (s,2H, SH), 8.0 (s,1H, NH), 6.5-7.4 (m, 8H, Ar-H), 4.1 (q, 4H, OCH2), 2.31 (s, 3H, CH3), 2.23 (s, 3H, CH3), 1.1(t, 6H, CH3), 13C-NMR (DMSO-d6, δ ppm): 168.3, 153.7, 148.9, 147.35, 139.20, 130.4, 128.12, 125.6, 124.9, 122.2, 120.5, 59.2, 14.5, 13.11, 10.34; LCMS m/z: 534 (M40).

2, 4 bis (5-mercapto-1,2,4 triazol-3-yl) 3,5 dimethyl 1H pyrrole (3d)

The above method was followed using 0.005 mol 2d.

Yield- 65%; MP: 205°C; IR (ν cm⁻¹,KBr): 3230, 2975; 1H-NMR (DMSO-d6, δ ppm): 12.05 (s,2H, SH), 8.15 (s,1H, NH), 3.65 (s, 2H, Cδ protons of cyclohexyl), 2.32 (s, 3H, CH3), 2.19 (s, 3H, CH3), 2.10 (m, 8H, Cδ and Cβ protons of cyclohexyl), 1.70 (m, 8H Cδ and Cβ protons of cyclohexyl), 1.5 (m, 4H Cδ protons of cyclohexyl); LCMS m/z: 458 (M+45).
2, 4 bis (5-(n-butylamino)-1,3,4-thiadiazol-2-yl) 3,5 dimethyl 1H pyrrole (4d)

Yield- 60%; MP: 170°C; IR (v cm⁻¹,KBr): 3330, 3214, 2980; ¹H-NMR (DMSO-d₆, δ ppm): 8.15 (s,1H, NH), 4.66 (s, 2H, NH), 3.87 (s, 2H, protons of butyl), 2.43 (s, 3H, CH₃), 2.21 (s, 3H, CH₃), 2.10 (m, 4H, C₂ protons of butyl), 1.82 (m, 4H, C₃ protons of butyl), 1.15 (m, 6H, C₄ protons of butyl); LCMS m/z: 406 (M⁺, 35).

2, 4 bis (5-(4-methoxyphenyl amino)-1,3,4-thiadiazol-2-yl) 3,5 dimethyl 1H pyrrole (4f)

Yield- 70%; MP: 230°C; IR (v cm⁻¹,KBr): 3342, 3230, 3068, 2986; ¹H-NMR (DMSO-d₆, δ ppm): 8.21 (s,1H, NH), 6.4 -7.3 (m, 8H, Ar-H), 4.85 (s, 6H, OCH₃), 2.38 (s, 3H, CH₃), 2.23 (s, 3H, CH₃); LCMS m/z: 506 (M⁺, 65).

In vitro antifungal activity

In vitro antifungal activity of the synthesized compounds was evaluated by two fold serial dilution method. Media used was Potato Dextrose Broth (PDB). Initially, the stock culture of Aspergillus niger, Aspergillus flavus, Aspergillus terreus and Candida albicans were revived by inoculating in broth media and grown at 37°C for 48 hrs. The tubes of the above media PDB (5 ml) were prepared and each tube was added with compounds (10-500 µg) and inoculated with 100 µl of 48 hr old cultures. The control tubes with fluconazole and DMSO were also prepared. All the tubes were incubated at 37°C for 48 h with constant shaking and the absorbance of biomass were measured 660 nm against autoclaved, uninoculated media as blank. The result of in vitro anti fungal activity is expressed as Minimum Inhibitory concentration (MIC) and is given in Table 2.

Table 1: List of compounds prepared

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Compound</th>
<th>R</th>
<th>Mol Wt</th>
<th>Mol formula</th>
<th>C log P</th>
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<tr>
<td>1</td>
<td>3a</td>
<td>Phenyl</td>
<td>446</td>
<td>C₂₂H₁₁N₃S₂</td>
<td>4.60</td>
</tr>
<tr>
<td>2</td>
<td>3b</td>
<td>p-tolyl</td>
<td>474</td>
<td>C₂₂H₁₁N₃S₂</td>
<td>5.59</td>
</tr>
<tr>
<td>3</td>
<td>3c</td>
<td>n-propoxyphenyl</td>
<td>534</td>
<td>C₂₂H₁₁N₃O₂S₂</td>
<td>5.61</td>
</tr>
<tr>
<td>4</td>
<td>3d</td>
<td>Cyclohexyl</td>
<td>458</td>
<td>C₂₂H₁₁N₃S₂</td>
<td>4.49</td>
</tr>
<tr>
<td>5</td>
<td>3e</td>
<td>n-butyl</td>
<td>406</td>
<td>C₂₂H₁₁N₃S₂</td>
<td>3.61</td>
</tr>
<tr>
<td>6</td>
<td>3f</td>
<td>p-methoxyphenyl</td>
<td>506</td>
<td>C₂₂H₁₁N₃O₂S₂</td>
<td>5.23</td>
</tr>
<tr>
<td>7</td>
<td>3g</td>
<td>2-fluoro phenyl</td>
<td>482</td>
<td>C₂₂H₁₁F₂N₃S₂</td>
<td>4.89</td>
</tr>
<tr>
<td>8</td>
<td>4a</td>
<td>Phenyl</td>
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<td>C₂₂H₁₁N₃S₂</td>
<td>5.66</td>
</tr>
<tr>
<td>9</td>
<td>4b</td>
<td>p-toluyl</td>
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<tr>
<td>10</td>
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<td>11</td>
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<tr>
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<td>13</td>
<td>4f</td>
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</tr>
<tr>
<td>14</td>
<td>4g</td>
<td>2-fluoro phenyl</td>
<td>482</td>
<td>C₂₂H₁₁F₂N₃S₂</td>
<td>5.95</td>
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</table>

Table 2: In vitro antifungal and cytotoxic activity of test compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC in µg/ml</th>
<th>Antifungal activity</th>
<th>Cytotoxic Activity IC₅₀ in µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. niger</td>
<td>A. flavus,</td>
<td>A. terreus,</td>
</tr>
<tr>
<td>3a</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3b</td>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>3c</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>3d</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3e</td>
<td>25</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>3f</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>3g</td>
<td>12.5</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>4a</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4b</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>4c</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4d</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4e</td>
<td>100</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>4f</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>4g</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>6.25</td>
<td>6.25</td>
<td>6.25</td>
</tr>
</tbody>
</table>

The cytotoxicity of the compounds was evaluated in vitro against the human lung carcinoma cancer cell line (A-549). The cells were procured from National Centre for Cell Sciences, Pune, India, and were cultured in DMEM medium supplemented with 10%FBS, 1%glutamine and 50µg/ml gentamycin sulphate in a CO₂ incubator in a humidified atmosphere of 5%CO₂ and 95% air. Effect of test compounds on cell proliferation of cancer cells was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were cultured in duplicates at a density of 1 x 10⁵ cells/well. After 24 h, the test compounds were added at a concentration of 10, 20 and 50 µM and incubated for 48 h. Cells were harvested and incubated with MTT reagent (5 mg/ml, Sigma-Aldrich, USA). The resulting insoluble MTT formazan product was then dissolved in detergent containing 50%
N,N-dimethylformamide (Sigma-Aldrich, USA) and 10% of sodium dodecyl sulphate (Amresco, USA). Absorbance of the resulting colored solution was measured at 570 nm on a multiwell ELISA plate reader. Cells treated with DMSO grown in culture media were used as a vehicle control. The experiment was repeated three independent times and the percentage cytotoxicity was calculated using the following formula.

\[
\% \text{ Cytotoxicity} = \frac{(\text{Control abs} - \text{Test abs}) \times 100}{\text{Control abs}}.
\]

RESULTS AND DISCUSSION

Chemistry

The synthesis of bis-triazoles and thiazololes encompassing pyrrole moiety was carried out in three steps in good yield as depicted in the scheme of the synthesis. The di-carboxyhydrazone (1) was prepared by refluxing respective diester in presence of excess of hydrxazine hydrate under inert atmosphere. The absence of characteristic ethoxy signals and appearance of CONH and NH3 signals at 10.5 and 4.3 ppm in the PMR, indicate the conversion of ester to hydrazide.

The above dicarboxyhydrazone was further treated with different aryl, cycloalkyl and alky1 isothiocyanates to obtain respective thiosemicarbazides (2a-g) in almost quantitative yield. The compounds when subjected to cyclization under different reaction conditions yielded bis 1,2,4-triazoles (3a-g) and 1,3,4-thiadiazoles (4a-g).

In PMR spectrum of the bis-1,2,4-triazole derivative 3a, absence of the group signals corresponding to the CONH, CNH and NH-NH group of the respective thiosemicarbazide and appearance of a signal due SH at 12.5 ppm confirms the cyclization into mercapto triazoles. The high down field signal for the SH proton is due to the thiophene thiol tautomerism of the 5-mercapto 1,2,4-triazoles system. The LCMS data showed M+ peak at 446 m/z.

The thiazole derivative 4a, which was prepared by reacting 2a with cold concentrated sulphuric acid exhibited a broad peak at 4.94 ppm due to aryl amino proton while the group signals of thiosemicarbazide were absent. The MS data showed a stable base peak at 446 m/z units representing the molecular mass of the compound. The fragmentation was very minimum as the spectra were recorded in ESI mode. Similarly other compounds were characterized and found to be in agreement with the proposed structure.

Lipophilicity

The efficiency of an anti infective drug will depend in part on its ability to accumulate in microorganisms. Further, certain resistant strains of microorganism develop resistance by decreasing the accumulation of the drug substance in the cytoplasm of the microorganism. Lipophilicity (Log P) of the drugs hence, plays a vital role in the antimicrobial effect of the compounds. ClogP of the compounds was determined by the fragment based prediction software and found to be in the range of 3.61 to 5.61 for bis-triazoles; while that of bis-thiazole were found to be between 4.84 to 6.62.

The in vitro anti fungal activity was carried out against different fungal species by two fold serial dilution method. From the MIC data presented in Table 2, it is clear that the triazole derivatives exhibited better antifungal activity than the thiazole derivatives. The lower MIC values suggested that the compounds 3c and 3g showed significant activity as compared to the standard drug.

The compounds were screened for their cytotoxicity against human lung carcinoma cells (A-549) by using standard MTT assay protocol14-17. The inhibition concentration (IC50) defined as the concentration of the drug that causes 50% cell growth inhibition after 48 h of continuing exposure to the test compounds and the mean of the results obtained from triplicate assays are shown in Table 2. The IC50 values were compared with that of anticancer antibiotic doxorubicin. From the evaluation of the data reported in Table 2, the following observations can be made. All the synthesized compounds were far less cytotoxic than the standard drug doxorubicin (IC50 value 0.09 ± 0.01μM) as evident from higher IC50 values. However, among the synthesized compounds 3c & 4c and 3f & 4f which contain electron releasing functional groups such as ethoxy and methoxy respectively exhibited moderate activity; however the fluoro substituted compounds 3g and 4g were found to be more potent cytotoxic compound. The compounds with cyclohexyl and n-butyl substitution exhibited poor cytotoxicity.

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Authors declare no conflict of interest.

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