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Synthesis, Structural Elucidation, and Evaluation of Antimicrobial Activity of 5-Ethoxy-2-Mercaptobenzimidazole Derivatives

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ABSTRACT

Objective: The aim of this study was to design and synthesize new amino acetylenic 5-ethoxy-2-mercaptobenzimidazole derivatives as potential antimicrobial agents. **Methods:** New series of 5-ethoxy-2-{[4-(t-amino-1-yl) but-2-yn-1-yl] sulfanyl}-1H-benzimidazole derivatives were synthesized by Mannich reaction, and investigated for their antimicrobial activity. Their structural confirmation was confirmed using the EuroEA elemental analyzer, and by Bruker FTIR, 1H-NMR, 13C-NMR. The antimicrobial activity was evaluated in-vitro by agar diffusion method and broth dilution test. The minimum inhibitory concentration and the minimum bactericidal/fungicidal concentration were determined. **Results:** The IR, 1H-NMR, 13C-NMR and elemental analysis were consistent with the assigned structures. All new amino acetylenic 5-Ethoxy-2-mercaptobenzimidazole derivatives showed good antibacterial activity against Bacillus subtilis with minimum inhibitory concentration value of 31.25 µg/ml except 5-ethoxy-2-{[4-(2.emethylpiperidin-1-yl] but-2-yn-1-yl]-sulfanyl}-1H-benzimidazole showed excellent antifungal activity against Candida albicans with the lowest minimum inhibitory concentration value of 31.25 µg/ml. **Conclusion:** The antimicrobial results promoted our interest to carry out further structural modifications, to enhance both antibacterial and antifungal activities, and their selectivity.

Keywords: 5-ethoxy-2-mercaptobenzimidazole, Aminoacetylenic, Mannich reaction, Alkylation

Abbreviations: MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; MFC: Minimum Fungicidal Concentration; SD: Standard Deviation; MHB: MUELLER Hinton Broth; MHA: Mueller Hinton Agar; SDB: Sabouraud Dextrose Broth; SDA: Sabouraud Dextrose Agar

INTRODUCTION

The progression in the bacterial resistance against antibiotic therapy and the emerging new pathogens is becoming a critical problem. Antimicrobial agents are the drugs, chemicals, or other substances that kill or inhibit the growth of the microbes, they include antibacterial drugs, antiviral agents, antifungal agents, and anti-parasitic drug [1,2]. Researchers use different structures to discover, synthesize and develop new antibacterial and antifungal agents [3]. Benzimidazole is considered a remarkable heterocyclic compound that has significance in medicinal chemistry and biological activity, 2-Mercaptobenzimidazole (2-MBI) consist of benzimidazole moiety which is a bicycle compound, includes a benzene ring fused with imidazole ring containing 2 nitrogen atoms at 1, 3 positions and the 2-substituted benzimidazole with thiol group SH (mercapto) gives the compound the antibacterial and other biological activities [4]. Shet, et al., designed and synthesized new 2-substituted alkyl thioarylbenzimidazole derivatives, these compounds were screened for *in-vitro* antibacterial activity against gram positive bacteria *Staphylococcus aureus* (*S. aureus*) and *Enterococcus fecalis* (*E. fecalis*), gram negative bacteria *Klebsiella* and *Escherichia coli* (*E. coli*), and the activity was compared to ciprofloxacin as a control, in addition, their antifungal activity was evaluated by using (*C. albicans* and

A. niger) and the activity were compared to fluconazole as a control. The results showed that 2-MBI derivatives with substituents -Cl, $-NO_2$, -H on thioaryl moiety have remarkable antimicrobial activity against gram positive bacteria and excellent antifungal activity against fungi [5] (Figure 1).



Figure 1 The structure of new 2-substituted alkyl thioarylbenzimidazole derivatives

In a recent study synthesis of novel benzimidazole derivatives (Figure 2) is involved. These novel compounds were assessed for their antibacterial and antifungal activities. Against gram positive (*S. aureus*) and gram negative (*E. coli*) bacteria, the researchers concluded the antimicrobial activity was low when there was no substitution, while when there is chlorine and dimethyl amine on the aromatic ring the antimicrobial activity was high, regarding the antifungal activity against (*C. albicans*) the researchers found that the derivative possessing p-dimethyl amine substituent showed a higher antifungal activity than the others [6].



In reviewing various structural features of different compounds under investigation as antimicrobials, the development of resistance, and selectivity, promoted our interest to synthesize amino acetylenic 5-ethoxy-2-mercaptobenzimidazole derivatives and investigate the role of the aminoacetylenic group on their antimicrobial activity. This will provide possibility to increase potency and decrease the resistance for the following reasons: the presence of sulfur group have a great contribution on the activity of the compounds due to hydrogen bonding or complexation, electrostatic interaction by the presence of 2-acetylenic group expected to enhance potency of the compounds against bacteria and fungi due to their overlap interaction with various sites in the microbes, in addition to their appropriate distance between 2-MBI and cyclic amine provide more binding sites with the microbes. The cyclic amine have several binding interaction such as, ionic interaction, improve lipophilicity, protonated nitrogen increases the compounds permeability through the outer membrane porins of gram negative bacteria and forms ionic bonding with their corresponding groups on receptors.

Benzimidazole is a heterocyclic group that varied from other heterocyclic such as oxazole or thiazole in affecting the antimicrobial activity. Benzimidazole showed their antibacterial activity by inhibiting the bacterial nucleic acid and protein synthesis, this ability of benzimidazole due to their structural similarities with the purine, the ethoxy group at 5-position expected to promote lipophilicity and activity [4,7]. The presence of these unique groups in our structure may provide a potent antimicrobial activity.

MATERIALS AND METHODS

Experimental

Chemistry: Chemicals

5-ethoxy-2-mercaptobenzimidazole 97% (2-MBI), propargyl bromide, pyrolidine 99%, 2-methylpiperidin 98%,

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1-methylpiperazine 99%, Cis-2, 6-dimethylpiperidine 98%, hexamethyleneimine 99%, morpholine reagent plus 99%, all of them were purchased from (Sigma Aldrich, USA), potassium carbonate anhydrous (Gainland chemical company (GCC), UK), cuprous chloride (East Anglia Chemicals Hadleigh lpswich), magnesium sulphate anhydrous (Lonover, UK), paraformaldehyde polymer (BDH chemicals Ltd Poole, England), acetonitrile 99.7% (PanReAc Quimca SA, EU), 1, 4-dioxane (FULL Time, China), acetone 99% (Scharlau, Spain), chloroform (TEDIA, USA), absolute ethanol 99.9% (Super Chem), distilled water (Ultra, Jordan), dimethyl sulfoxide (DMSO) (BBC Chemicals for lab, EU), diethyl ether (Lonover, England)/(RCL Labs can, Thailand).

Instruments

Analytical balance with a precision 0.01 mg (Phoenix instrument, USA), hot plate with magnetic stirrer (Dragon, China), rotary evaporator 0-100 Kpa/0-700 mmHg (Rocker 600, Germany), buchner funnel pump (Vacuubrand, Germany), gallenkamp melting point apparatus, bruker FT-IR spectrophotometer 7800 to 400 cm⁻¹ (evisa, Poland), NMR 300 MHz (Varian 300 MHz, USA), NMR 500 MHz (Varian 500 MHz, USA), elemental analyzer with variation range ($\pm 4\%$) (Euro Vector, Italy), balance (BoEco, Germany), autoclave machine (Rypa, Spain), incubator (EuroStar, EU), vortex mixer (Labinco, India), hot plate magnetic stirrer (Dragon, china), sterile tubes, sterile swabs (MWe, UK), micropipette (1 ml, 0.1 ml) (Oxford, USA).

Molecular Docking

The DHFR crystal structure was obtained from the protein data bank (PDB ID: IDFR) and was used in this docking study [8,9]. The DHFR structure was checked for any missing atoms or residues via the protein preparation module in the MOE software [10]. The checked protein was processed via the Maestro Protein Preparation Wizard in order to set up protonation states and partial charges on all protein atoms and also to remove any existing clashes [11,12]. The DHFR binding site was determined based on the coordinates of the co-crystallized inhibitor. A grid box was then generated via the glide receptor grid generation module [13].

Al ligands were prepared via the LigPrep module in order to assign partial charges and to generate a single low energy conformation [14]. Using glide all prepared ligands were docked into the previously prepared DHFR structure where the extra-precision (XP) algorithm was used for conformational sampling as well as scoring [13,15]. This extensive scoring function includes terms for Van der Waals, hydrogen bond, electrostatic interactions, desolvation penalty and penalty for intra-ligand contact. The best scoring pose of each ligand was selected for the final docking list. The binding mode picture of the best docked ligand was generated by MOE [16].

Synthesis

Synthesis of 5-ethoxy-2-(prop-2-yn-1-ylsulfanyl)-1H-benzimidazole (AZ-0): A mixture of 5-ethoxy-2-mercaptobenzimidazole (0.01 mole, 1.94 g), potassium carbonate anhydrous (>0.01 mole, 2 g) and acetonitrile (25 ml) has been heated and stirred under reflux for 30 min until the temperature reach 70°C, then propargyl bromide (>0.01 mole, 2 ml) was added drop wise, the reacted mixture was heated and stirred under reflux for 3 hours. After cooling, the mixture was filtered so the insoluble residues were removed by filtration, and then the solvent was removed under reduced pressure to give a dark brown liquid. After that, 25 ml chloroform and 25 ml distilled water was added 3 times and the filtrate were extracted by using separatory funnel, the chloroform layers were collected, dried by using magnesium sulphate and evaporated under reduced pressure. A brown viscous mixture were collected (AZ-0): C12H12N2OS, 1.5 g, 77% yield, Mp: 115°C -117°C, IR (KBr cm⁻¹), acetylenic C-H stretching (3280.32 cm⁻¹), C-H stretching Ar (3062.41 cm⁻¹), C=C stretching (2117.46 cm⁻¹), C=C stretching Ar. (1419.35 cm⁻¹), C=N stretching (1614 cm⁻¹), C-N stretching anomatic (1272.79), S-C stretching (651.82 cm⁻¹), N-H stretching (3382.59 cm⁻¹), C-O-C stretching (1029.80 cm⁻¹), CH₃ stretching aliphatic (2975.63 cm⁻¹), CH₂ stretching aliphatic (2919.70 cm⁻¹), C-H out of plane bending Ar (8157.74 cm⁻¹, 966.16 cm⁻¹), 1H-NMR (DMSO-d₆): δ , 6.75-7.48 (m, 3H, Aromatic protons), 5.05 (s, 1H, NH), 4-4.1 (m, 2H, O-CH₂ aliphatic), 1.38 (t, 3H, CH₃-CH₂ aliphatic), 3.19-3.21 (s, 2H, S-CH₂-C), 2.51 (s, 1H, C=CH).

Synthesis of 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6): A mixture of 5-ethoxy-2-(prop-2-yn-1-ylsulfanyl)-1H-benzimidazole (AZ-0), paraformaldehyde (0.46 g, 0.01 mole in excess), the cyclic amine 0.01 mole (pyrolidine, 2-methylpiperidine, 1-methylpiperazine, 2,6-dimethylpiperidine, azepane, morpholine), and a catalytic amount of cuprous chloride in 1, 4-dioxane (25 ml) was stirred at a room temperature for about 10 min then heated and stirred under reflux in a range between 70-75°C for about three hours. The resulted brown mixture was cooled, and then filtered to remove the insoluble residue and concentrated under a

reduced pressure to give a brown, semisolid product, which was dissolved in a least amount of diethyl ether, then filtered and concentrated under a reduced pressure. The final products were (AZ-1, AZ-2, AZ-3, AZ-4, AZ-5, and AZ-6).

5-ethoxy-2-{[4-(pyrrolidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-1): The titled compound was prepared following the similar procedure for the synthesis of 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6) by Mannich reaction in 1.5 g yield 47%, mp: (89°C -91°C), FT-IR (KBr cm⁻¹): C-H stretching aromatic (3066.26 cm⁻¹), C-H stretching with tertiary amine (2339.23 cm⁻¹), C=C stretching aromatic (1442.50 cm⁻¹), C=N stretching (1617.38 cm⁻¹), C-N tertiary cyclic amine (1145.51 cm⁻¹), C-N stretching aromatic (1324.86 cm⁻¹), C=O stretching (1037.52 cm⁻¹), N-H stretching (3424.96 cm⁻¹), CH₃ stretching aliphatic (2938.98 cm⁻¹), CH₂ stretching aliphatic (2798.21 cm⁻¹), C-H out of plane bending aromatic (962.31 cm⁻¹, 827.31 cm⁻¹, 711.60 cm⁻¹), 1H-NMR (DMSO-d₆): δ , 1.33-1.37 (t, 3H, CH₃-CH₂ aliphatic), 1.64-2.72 (m, 8H), 3.31-3.34 (s, 2H, C-CH₂-N), 3.57 (s, 2H, S-CH₂-C), 4-4.17 (m, 2H, O-CH₂ aliphatic), 5.07 (s, 1H, NH), 6.78-7.47 (m, 3H, aromatic proton), 13C-NMR (DMSO d₆): δ , , 15.16 ppm (5-Ethoxy-2-MBI), 23.54 ppm (SCH₂), 23.68 ppm (cyclic amine carbons), 42.72 ppm (CH₂N), 52.14, 64.09 ppm, 78.36 ppm (C≡C), 79.9 ppm, 102.05 ppm, 110.7 ppm,112.23 ppm, 130.52 ppm, 137.81 ppm, 147.8 ppm, 155.07 ppm. Elemental analysis: C₁₇H₂₁N₃OS, Calcd: C, 64.67%; H, 6.65%; N, 13.31%. Found: C, 64.41%; H, 6.44%; N, 13.04%.

5-ethoxy-2-{[4-(2-methylpiperidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole(AZ-2): The titled compound was prepared following the similar procedure for the synthesis of 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl] sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6) by Mannich reaction in 2.3 g yield 67%, mp: (105°C - 108°C), FT-IR (KBr cm⁻¹): C-H stretching aromatic (3046.98 cm⁻¹), C-H stretching with tertiary amine (2329.59 cm⁻¹), C=C stretching aromatic (1452.14 cm⁻¹), C=N stretching (1617.38 cm⁻¹), C-N stretching tertiary cyclic amine (1145.51 cm⁻¹), C-N stretching aromatic (1346.07 cm⁻¹), C-O-C stretching (1039.44 cm⁻¹), N-H stretching (3393.96 cm⁻¹), CH₃ stretching aliphatic (2944.77 cm⁻¹), CH₂ stretching aliphatic (2809.78 cm⁻¹), C-H out of plane bending aromatic (958.45 cm⁻¹, 815.74 cm⁻¹). 1H-NMR (DMSO-d₆): δ , 1.33-1.36 (t, 3H, CH3-CH₂ aliphatic), 1.1-2.5 (m, 12H), 3.14-3.3 (s, 2H, C-CH₂-N), 3.4-3.57 (m, 2H, S-CH₂-C), 4-4.16 (m, 2H, O-CH₂ aliphatic), 5.08 (s, 1H, NH), 6.78-7.47 (m, 3H, aromatic proton), 13C-NMR (DMSO d₆): δ , 15.15 ppm, 17.41 ppm (cyclic amine CH3 carbons), 22.23 ppm (SCH2), 22.42 (cyclic amine carbons), 25.48 ppm, 33.53 ppm, 43.81 ppm (CH₂N), 50.92 ppm, 54.41 ppm, 64.1 ppm, 79.19 ppm, 79.35 ppm, 102.17 ppm, 110.8 ppm, 112.27 ppm, 136.5 ppm, 137.84 ppm, 149.43 ppm (5-Ethoxy-2-MBI), 154.96 ppm. Elemental analysis: C₁₉H₂₅N₃OS, Calcd: C, 66.37%; H, 7.27%; N, 12.22%; Found: C, 66.54%; H, 7.64%; N, 11.98%.

5-ethoxy-2-{[4-(4-methylpiperazin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-3): The titled compound was prepared following the similar procedure for the synthesis of 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl] sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6) by Mannich reaction in 2.2 g, yield 63%, FT-IR (KBr cm⁻¹): C-H stretching aromatic (3075.91 cm⁻¹), C-H stretching with tertiary amine (2339.14 cm⁻¹), C=C stretching aromatic (1448.28 cm⁻¹), C=N stretching (1699.41 cm⁻¹), C-N tertiary cyclic amine (1280.50, 1145.51 cm⁻¹), C-N stretching aromatic (1346.07 cm⁻¹), C-O-C stretching (1006.66 cm⁻¹), N-H stretching (3399.89 cm⁻¹), CH₃ stretching aliphatic (2944.77 cm⁻¹), CH₂ stretching aliphatic (2798.21 cm⁻¹), C-H out of plane bending aromatic (941.09 cm⁻¹, 815.74 cm⁻¹). 1H-NMR (DMSO-d₆): δ , 1.33-1.38 (t, 3H, CH₃-CH₂ aliphatic), 2.108-2.5 (m, 11H), 3.05 (s, 2H, C-CH₂-N), 3.62 (s, 2H, S-CH2-C), 4-4.18 (m, 2H, O-CH₂ aliphatic), 5.09 (s, 1H, NH), 6.78-7.48 (m, 3H, aromatic proton),. 13C-NMR (DMSO d₆): δ , 15.17 ppm, 22.18 ppm (SCH₂), 45.11 ppm (cyclic amine CH₃ carbon), 46.53 ppm (CH₂N), 51.37 ppm, 54.37 ppm (cyclic amine carbons), 64.06 ppm, 79.15 ppm, 80.03 ppm (C≡C), 102.08 ppm, 110.72 ppm, 112.34 ppm, 131.71 ppm, 137.79 ppm, 149.38 ppm, 155.11 ppm (5-Ethoxy-2-MBI). Elemental analysis: C₁₈H₂₄N₄OS, Calcd: C, 62.76%; H, 6.96%; N, 16.25%. Found: C, 62.56%; H, 7.29%; N, 15.97%.

5-ethoxy-2-{[4-(2,6-dimethylpiperidin-1-yl)but-2-yn-1-yl]-sulfanyl}-1H-benzimidazole (AZ-4): The titled compound was prepared following the similar procedure for the synthesis of 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6) by Mannich reaction in 1.9 g, yield 53%, FT-IR (KBr cm-1): C-H stretching Aromatic (3089.58 cm⁻¹), C-H stretching with tertiary amine (2264.02 cm⁻¹), C=C stretching Aromatic (1430.92 cm⁻¹), C=N stretching (1627.63 cm⁻¹), C-N tertiary cyclic amine (1180.22 cm⁻¹, 1110.80 cm⁻¹), C-N stretching Aromatic (1328.71 cm⁻¹), C-O-C stretching (1099.44 cm⁻¹), N-H stretching (3201.26 cm⁻¹), CH₃ stretching aliphatic (2925.48 cm⁻¹), CH₂ stretching aliphatic (2856.06 cm⁻¹), C-H out of plane bending aromatic (952.66 cm⁻¹, 809.96 cm⁻¹, 728.96 cm⁻¹). 1H-NMR (DMSO-d₆): δ , 1.12-2.5 (m, 14H), 1.32-1.35 (t, 3H, CH₃-CH₂ aliphatic), 3.32-3.42 (s, 2H, C-CH₂-N), 3.5-3.57 (s, 2H, S-CH₂-C), 4-4.15 (m, 2H, O-CH, aliphatic), 5.08 (s, 1H, NH), 6.8-7.45 (m,

3H, aromatic proton). Elemental analysis: C₂₀H₂₇N₃OS, Calcd: C, 67.13%; H, 7.55%; N, 11.74%. Found: C, 67.53%; H, 7.15%; N, 11.44%.

5-ethoxy-2-{[4-(azepan-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-5): The titled compound was prepared following the similar procedure for the synthesis of 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6) by Mannich reaction in 2 g, yield 58%, mp (118°C -119°C), FT-IR (KBr cm⁻¹): C-H stretching aromatic (3075.905 cm⁻¹), C-H stretching with tertiary amine (2350.80 cm⁻¹), C=C stretching aromatic (1432.85 cm⁻¹), C=N stretching (1604.48 cm⁻¹), C-N tertiary cyclic amine (1184.08 cm⁻¹), C=N stretching aromatic (1355.71 cm⁻¹), C=O-C stretching (1099.23 cm⁻¹), N-H stretching (3377.99 cm⁻¹), CH₃ stretching aliphatic (2910.06 cm⁻¹), CH₂ stretching aliphatic (2842.56 cm⁻¹), C-H out of plane bending aromatic (977.80 cm⁻¹, 802.24 cm⁻¹, 711.60 cm⁻¹). 1H-NMR (DMSO-d₆): δ , 1.34-1.37 (t, 3H, CH₃-CH₂ aliphatic), 1.41-2.6 (m, 12H), 3.02 (s, 2H, C-CH₂-N), 3.26-3.36 (s, 2H, S-CH₂-C), 4-4.16 (m, 2H, O-CH₂ aliphatic), 5.062 (s, 1H, NH), 6.8-7.47 (m, 3H, aromatic proton), Elemental analysis: C₁₉H₂₅N₃OS, Calcd: C, 66.37%; H, 7.27%; N, 12.22%. Found: C, 66.40%; H, 6.97%; N, 12.30%.

5-ethoxy-2-{[4-(morpholin-4-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-6): The titled compound was prepared following the similar procedure for the synthesis of 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6) by Mannich reaction in 1.7 g, yield 51%, mp (129°C-131°C), FT-IR (KBr cm⁻¹): C-H stretching aromatic (3075.91 cm⁻¹), C-H stretching with tertiary amine (2335.37 cm⁻¹), C=C stretching aromatic (1452.14 cm⁻¹), C=N stretching (1612.20 cm⁻¹), C-N tertiary cyclic amine (1268.99 cm⁻¹, 1128.16 cm⁻¹), C-N stretching aromatic (1336.43 cm⁻¹), C-O-C stretching (1006.66 cm⁻¹), N-H stretching (3409.59 cm⁻¹), CH₃ stretching aliphatic (2923.56 cm⁻¹), CH₂ stretching aliphatic (2825.20 cm⁻¹), C-H out of plane bending aromatic (850.45 cm⁻¹, 792.6 cm⁻¹). 1H-NMR (DMSO-d6): δ , 1.33-1.37 (t, 3H, CH₃-CH₂ aliphatic), 2.2, 2.4, 2.5 (m, 8H), 3.22-3.31 (s, 2H, C-CH₂-N), 3.46-3.57 (s, 2H, S-CH₂-C), 4-4.19 (m, 2H, O-CH₂ aliphatic), 6.8-7.48 (m, 3H, aromatic proton), 5.097 (s, 1H, NH), . Elemental analysis: C₁₇H₂₁N₃O₂S, Calcd: C, 61.55%; H, 6.33%; N, 12.67%. Found: C, 61.25%; H, 6.68%; N 12.95%.

Docking Results

Docking results were shown in Table 1 and Figure 3.

Molecule	Gride XP Score (kcal/mol)	Number of heavy atoms	Ligand efficiency (kcal/mol)
AZ-1	-7.04	22	-0.32
AZ-2	-5.18	24	-0.216
AZ-3	-5.6	24	-0.233
AZ-4	-5.54	24	-0.231
AZ-5	-5.28	23	-0.23
AZ-6	-6.68	25	-0.23
Co-crystallized ligand	-10.24	32	-0.32

Table 1 The Glide XP scores of docked compounds

AZ-1: 5-ethoxy-2-{[4-(pyrrolidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-2: 5-ethoxy-2-{[4-(2-methylpiperidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-3: 5-ethoxy-2-{[4-(4-methylpiperazin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-4: 5-ethoxy-2-{[4-(2,6-dimethylpiperidin-1-yl)but-2-yn-1-yl]-sulfanyl}-1H-benzimidazol;

AZ-5: 5-ethoxy-2-{[4-(azepan-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole; AZ-6: 5-ethoxy-2-{[4-(morpholin-4-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole



Figure 3 The binding mode of 5-ethoxy-2-{[4-(pyrrolidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-1) with the DHFR active site. Hydrogen bonding is shown as orange dotted lines whereas intramolecular cation-π interactions are shown as blue dotted lines

Culture Media

Mueller Hinton agar (MHA) (Mastgrp Ltd, UK)/(HiMedia, India), Mueller Hinton broth (MHB) (Mastgrp Ltd, UK), Sabouraud dextrose agar (SDA) (Mastgrp Ltd, UK), Sabouraud dextrose broth (SDB) (HiMedia, India).

Test Microorganisms

Staphylococcus aureus (S. aureus ATCC[®] 6538TM), Bacillus subtilis (B. Subtilis ATCC[®] 6633 TM), Pseudomonas aeruginosa (P. aeruginosa ATCC[®] 9027 TM), Escherichia coli (E. coli ATCC[®] 8739 TM), and Candida albicans (C. albicans ATCC[®] 10231 TM), all these pure cultures of bacterial strains were obtained from Dar Al Dawa (Na'ur, Jordan).

Antimicrobial Activity Evaluation

The antimicrobial activity of the newly synthesized compounds 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6) were tested against gram positive bacteria, gram negative bacteria and fungi (*S. aureus* ATCC[®] 6538TM, *B. subtilis* ATCC[®] 6633TM, *P. aeruginosa* ATCC[®] 9027TM, *E. coli* ATCC[®] 8739TM, *C. albicans* ATCC[®] 10231TM) using agar diffusion and broth dilution methods. Starting agar diffusion method by 0.1 ml (1×106 CFU/ml) from overnight cultures of bacterial or fungal broth suspension were spread uniformly over the surface of MHA plates for bacteria and SDA for fungi, then wells were cut into the media filled with 0.2 ml of serial compounds concentrations (AZ1-AZ6) (1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml). The plates were incubated for 24 hours at 37°C for bacteria and for 48 hours at 25°C for fungi. Then, by measuring the diameter formed around each well, the zone of inhibition were measured and compared with the positive control (5 µg of ciprofloxacin disk, fluconazole 150 µg/ml dissolved in DMSO), in addition they were compared with negative control (DMSO).

Agar diffusion method was designed to allow statistical analysis and was performed in triplicates. Experimental data presented in this study represent the mean \pm SD of those triplicate data sets. The second method was broth dilution method which is one of the most basic methods for determination of minimum inhibitory concentration (MIC) of the synthesized compounds [17]. Stock solutions of the 5-ethoxy-2-{[4-(t-amino-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6) were diluted in MHB for each bacteria and in SDB for fungi to give concentrations ranging from 1000 µg/ml to 31.25 µg/ml in test tubes, the final volume in each tube (compound and medium) was 5 ml, 0.1 ml of overnight bacterial culture or 0.2 of 48 hours fungal culture were added to each specified tubes. The positive control test tube contain 5 ml of sterile MHB/SDB and 0.1 ml of bacterial or 0.2 ml fungal culture, while the negative control contain 5 ml of the tested compound dilution in sterile MHB/SDB. All test tubes were

incubated for 24 hours at 37°C for bacteria and for 48 hours at 25°C for fungi. The determination of MIC was done by comparing the turbidity of the positive control with the turbidity of each tested tube. MIC tube was having the lowest concentration of the compound in which no turbidity was observed.

The minimum bactericidal/fungicidal concentration (MBC/MFC) is the lowest concentration of antimicrobial agent required to kill the microorganism [18]. The determination of minimum bactericidal/fungicidal concentration (MBC/MFC) was done by sub-culturing the MIC tube and the tubes with concentration more than MIC on MHA/SDA using sterile swabs, the plates were incubated for 24 hours at 37°C for bacteria and for 48 hours at 25°C for fungi. The test tube having the lowest concentration which gave no growth was the MBC/MFC, the test were performed in triplicates.

Statistical Analysis

Statistical analysis was carried by using statistical packages for social science software (SPSS version 21) for student's t-test. Values are expressed as mean \pm SD.

RESULTS

Antimicrobial Activity

The newly synthesized compounds (AZ1-AZ6) showed antimicrobial activity against all types of microorganisms being tested (Tables 2 and 3). After 24 hours incubation at 37°C for bacteria and after 48 hours incubation at 25°C for fungi, the zone of inhibition diameter and the minimum inhibitory concentration (MIC) were determined. Compounds 5-ethoxy-2-{[4-(pyrrolidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-1), 5-ethoxy-2-{[4-(4methylpiperazin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-3), 5-ethoxy-2-{[4-(2,6-dimethylpiperidin-1-yl)but-2-yn-1-yl]-sulfanyl}-1H-benzimidazole 5-ethoxy-2-{[4-(azepan-1-yl)but-2-yn-1-yl]sulfanyl}-(AZ-4), 1H-benzimidazole (AZ-5), and 5-ethoxy-2-{[4-(morpholin-4-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-6) exhibited the highest antibacterial activity against B. subtilis with lowest MIC value 31.25 μ g/ml, all the synthesized compounds did not exhibited zone of inhibition at a concentration of 31.25 µg/ml against B. subtilis. Compounds 5-ethoxy-2-{[4-(pyrrolidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-1), 5-ethoxy-2-{[4-(4methylpiperazin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-3), and 5-ethoxy-2-{[4-(morpholin-4-yl)but-2yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-6) revealed the highest antibacterial activity against S. aureus with lowest MIC value of 125 μ g/ml and zone of inhibition diameter of 10.5 \pm 2.1 mm at concentration 125 μ g/ml for (AZ-1) and 11 ± 2.8 mm at concentration of 125 µg/ml for (AZ-3), while compound (AZ-6) did not showed zone of inhibition. All the synthesized compounds showed the same antibacterial activity against E. coli and P. aeruginosa with MIC values of (500 µg/ml, 250 µg/ml) respectively. Compound 5-ethoxy-2-{[4-(2,6-dimethylpiperidin-1-yl)but-2-yn-1yl]-sulfanyl}-1H-benzimidazole (AZ-4) showed the best antifungal activity against C. albicans with the lowest MIC value of 31.25 μ g/ml and zone of inhibition diameter of 6 ± 1.4 mm.

For MBC values, all the synthesized derivatives showed the same MBC value ($62.5 \ \mu g/ml$) against *B. subtilis* except compound 5-ethoxy-2-{[4-(2-methylpiperidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole (AZ-2) which exhibited the highest MBC value of 500 $\mu g/ml$. All the synthesized compounds had the same values against *E. coli* and *P. aeruginosa*, the MBC value against *E. coli* was (1000 $\mu g/ml$) compared to the standard (ciprofloxacin) which showed MBC value of (125 $\mu g/ml$), while against *P. aeruginosa* the value was 500 $\mu g/ml$, while the positive control showed MBC value of 250 $\mu g/ml$. All the MBC values against bacteria were higher than the standard ciprofloxacin. For *C. albicans*, compound (AZ-4) exhibited MFC value of 62.5 $\mu g/ml$, which was the same as the MFC value for the positive control (fluconazole). All synthesized compounds (AZ1-6) exhibited good antibacterial and antifungal activity.

Compound	P. aeruginosa mean ± SD	<i>E. coli</i> mean ± SD	<i>S. aureus</i> mean ± SD	<i>B. subtilis</i> mean ± SD	<i>C. albicans</i> mean ± SD
Concentration	500 μg/ml	500 μg/ml	500 μg/ml	500 μg/ml	500 μg/ml
AZ-1	7.5 ± 2.1	6.5 ± 2.1	12.5 ± 2.1	15.5 ± 0.7	13 ± 0
AZ-2	8 ± 2.8	9 ± 1.4	17 ± 0.7	12.5 ± 2.1	17.5 ± 2.1
AZ-3	-	-	17.5 ± 2.1	12 ± 1.4	17 ± 0
AZ-4	-	-	8.5 ± 2.1	14.5 ± 0.7	21 ± 2.8
AZ-5	-	-	10 ± 1.4	14.5 ± 0.7	14.5 ± 0.5

Table 2 The zone of inhibition diameter (in mm) of the synthesized compounds (AZ1-AZ6) at 500 µg/ml concentration

AZ-6	-	-	10 ± 1.4	12 ± 0	15.5 ± 0.7
Ciprofloxacin (5 µg/ml)	28 ± 1.4	26.5 ± 2.1	22.5 ± 2.1	24 ± 2.8	-
Fluconazole (500 μg/ml)	-	-	-	-	25 ± 1.4
Negative control	-	-	-	-	-

Values are mean \pm SD (n=3), (-): no growth

AZ-1: 5-ethoxy-2-{[4-(pyrrolidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-2: 5-ethoxy-2-{[4-(2-methylpiperidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-3: 5-ethoxy-2-{[4-(4-methylpiperazin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-4: 5-ethoxy-2-{[4-(2,6-dimethylpiperidin-1-yl)but-2-yn-1-yl]-sulfanyl}-1H-benzimidazole;

AZ-5: 5-ethoxy-2-{[4-(azepan-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole; AZ-6:5-ethoxy-2-{[4-(morpholin-4-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole

Table 3 The minimum inhibitory concentration (MIC) of the synthesized compounds (AZ1-AZ6) in µg/ml against *S. aureus, B. subtilis, E. coli, P. aeruginosa* and *C. albicans*

Compound	P. aeruginosa	E. coli	S. aureus	B. subtilis	C. albicans
Concentration	MIC/MBC (µg/ml)				
AZ-1	250/500	500/1000	125/250	31.25/62.5	250/500
AZ-2	250/500	500/1000	500/1000	250/500	125/500
AZ-3	250/500	500/1000	125/250	31.25/62.5	62.5/125
AZ-4	250/500	500/1000	500/1000	31.25/62.5	31.25/62.5
AZ-5	250/500	500/1000	500/1000	31.25/62.5	125/250
AZ-6	250/500	500/1000	125/250	31.25/62.5	125/250
Ciprofloxacin	125/250	62.5/125	62.5/125	15.6/31.25	-
Fluconazole	-	-	-	-	31.25/62.5
Negative control	-	-	-	-	-

AZ-1: 5-ethoxy-2-{[4-(pyrrolidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole,

AZ-2: 5-ethoxy-2-{[4-(2-methylpiperidin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-3: 5-ethoxy-2-{[4-(4-methylpiperazin-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-4: 5-ethoxy-2-{[4-(2,6-dimethylpiperidin-1-yl)but-2-yn-1-yl]-sulfanyl}-1H-benzimidazole;

AZ-5: 5-ethoxy-2-{[4-(azepan-1-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole;

AZ-6: 5-ethoxy-2-{[4-(morpholin-4-yl)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole. MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericida Concentration, MFC: Minimum Fungicidal Concentration

DISCUSSION

Chemistry

The designed compounds were prepared as shown in Figures 4 and 5. Figure 4 involved direct neuclophilic displacement by the anionic sulfur in the 2-mercaptobenzimidazole to bromide on the β carbon of the propargyl bromide to generate 5-ethoxy-2-(prop-2-yn-1-ylsulfanyl)-1H-benzimidazole (AZ-0). The Mannich reaction of 2-(prop-2-yn-1-ylsulfanyl)-1,3-benzothiazole AZ-0, paraformaldehyde, appropriate cyclic amines, and a catalytic amount of cuprous chloride in peroxide-free 1,4-dioxane was heated at 70-75°C to yield the designed compounds (AZ1-AZ6).



Figure 4 Alkylation reaction of 5-ethoxy-2-MBI

The yield obtained ranged from 47% to 67% as semi solid products. The proposed mechanism for Mannich reaction is shown in Figure 5, the mechanism first showed the formation of the Schiff base from condensation of formaldehyde and the appropriate cyclic amine. The attack of the acetylenic carbanion on the Schiff base and the migration of the double bond to the nitrogen resulted in the final products (AZ1-AZ6). These structures were verified through FT-IR, 1H-NMR, 13C-NMR, and elemental analysis.



Figure 5 Mannich reaction proposed

Docking our compounds along with the co-crystallized ligand into the DHFR enzyme active pocket has resulted in energetically favorable binding energies (Table 1). Compound 1 (Glide-XP score=-7.04 kcal/mol) had the lowest docking score, although it was higher than the co-crystallized inhibitor (Glide-XP score=-10.24 kcal/mol). However, analyzing compounds docking scores in relationship with their size, compound AZ-1 was able to obtain as good ligand efficiency score (0.32 kcal/mol) as the co-crystallized ligand. As shown in Figure 3, compound AZ-1 was able to adopt a U-shape conformation that fits nicely on the DHFR active site. Such a conformation was assisted by the intra-molecular interactions formed between the pyrrolidine protonated amine and the bicyclic aromatic system. Compound AZ-1 was able to make hydrogen bonding with the backbone amide of Leu22 and multiple vdW contacts with surrounding hydrophobic residues (e.g. Ile07, Leu22, Phe34, Ile60 and Val115). These compounds, particularly compound 1, are predicted to be good binders to the DHFR enzyme and could potentially act as a good starting point for future antibacterial agents.

Antimicrobial Activity

The evaluation of the antimicrobial activity of the newly synthesized compounds was through agar diffusion test and broth dilution test for MIC determination as they are the most common method used in evaluating the antimicrobial activity [18], the results of the agar diffusion method and the broth dilution method were variable [19]. The variability of the results using the agar diffusion method and the MIC method may be due to the selectivity of the 5-ethoxy-2{[4- (t- cyclic amine)but-2-yn-1-yl]sulfanyl}-1H-benzimidazole derivatives (AZ1-AZ6), and their general reactivity, resulted in reporting a low antimicrobial activity against some microorganisms especially *E. coli* and *P. aeruginosa* (gram negative bacteria), because of their low solubility in water that made their diffusion in agar medium weak, so the accuracy of agar diffusion is unsatisfactory. The results are frequently inadequate in comparison with the results of the broth dilution test that lack these difficulties [17].

The best antifungal activity against *C. albicans* was (AZ-4), which gave antifungal activity similar to the positive control antifungal activity, this activity may attributed to the flexibility enlargement of the cyclic amine (AZ-4), electronegativity, lipophilicity, ionic nature, and the appropriate distance between ionic head aryl imidazole and cyclic amine in these compounds. The proposed mechanism for the antifungal selectivity of these compounds was through inhibition of CA-CYP51 resulted in blocking of the ergosterol formation due to the inhibition of lanosterol 14- α -demethylase that facilitate permeability through the cell membrane, which was the same mechanism of action of the positive control (flucanozole) [20].

All synthesized compounds showed better antibacterial activity against gram positive than gram negative bacteria, which may be due to the differences in the cell wall structures, causing greater inhibition of cell growth in gram positive bacteria [17]. The difference between them in the nature of peptidoglycan layer, in which the cell wall of gram positive bacteria were more receptive to the synthesized compounds, due to the absence of outer membrane, while gram-negative bacteria showed greater resistance, associated with the presence of the outer membrane that contain phospholipids bilayer, pore-forming proteins and lipopolysaccharides (LPS), which hinders access to the bacterial inner membrane, which is the probable cellular target of the compound.

These synthesized compounds showed antibacterial activity against *B. subtilis* better than the antibacterial activity against *S. aureus*, which may be due to the virulence nature and high resistance of *S. aureu*. All the synthesized compounds gave good antimicrobial activity against *B. subtilis* with MIC value 31.25 μ g/ml, except (AZ-2) which showed MIC value of 250 μ g/ml as showed in Table 2, this may be due to the steric factor generated by the methyl

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group neighboring basic cyclic amine of 2-methylpiperidine. The diffusion of these compounds through the cell wall is facilitated due to their good lipophilicity, which play important role in facilitating the passage through the cell wall of gram positive bacteria. Surprisingly, the antimicrobial activity of the synthesized compound against *P. aeruginosa* was better than the antimicrobial activity against *E. coli*, which may be attributed to the good permeability of the synthesized compounds through *P. aeruginosa* outer membrane by dual pathway (porins and phospholipids bilayer), in which *E. coli* lacks porins in the outer membrane, for that with *E. coli* the synthesized compounds used only one pathway for entering into *E. coli* bacterial cell, resulted in decreasing the accumulation of the compounds inside bacteria thus decreasing their antimicrobial activity [21].

CONCLUSION

We have reported the synthesis of novel series of amino acetylenic 5-ethoxy-2-mercaptobenzimidazole derivatives. A unique amino acetylenic side chain provides additional forces of interaction with various microorganisms. These amino acetylenic 5-ethoxy-2-mercaptobenzimidazole derivatives showed effective binding with DHFR as seen from the docking results and showed promising antimicrobial activity against fungi and against gram positive bacteria better than the antimicrobial activity against gram negative bacteria. Compound AZ-4 showed the highest antifungal activity against *C. albicans* with MIC value of $31.25 \mu g/ml$ which is similar to the antifungal activity of fluconazol. Future research, structural modifications are required for enhancing both antibacterial and antifungal activity, and selectivity to either pure antifungal agent.

DECLARATIONS

Conflict of Interest

The authors have disclosed no conflict of interest, financial or otherwise.

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