

Efficient Synthesis and Biological Evaluation of a Novel Series of 1,5-Benzodiazepine Derivatives as Potential Antimicrobial Agents

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A series of novel 1,5-benzodiazepine derivatives were rationally designed and synthesized following the principle of the superposition of bioactive substructures by the combination of 1.5-benzodiazepine, pyridine (phenyl), and an ester group. The structures of the target compounds were determined by ¹H NMR, ¹³C NMR, MS, IR, and elemental analysis. All the synthesized compounds were evaluated for their antimicrobial activities in vitro against the fungi C. neoformans, C. neoformans clinical isolates (ATCC 32264), C. albicans (ATCC 10231), Gram-negative bacterium E. coli (ATCC 44752), and Gram-positive bacterium S. aureus (ATCC 25923). The results of the bioactive assay demonstrated that most of the tested compounds exhibited variable inhibitory effects on the growth of the tested microorganisms. All the active compounds showed better antifungal activity than antibacterial activity. Notably, compound 2b displayed the highest activity (MIC = 30 μ g/mL) against C. neoformans and (MIC = 31 μ g/mL) against C. neoformans clinical isolates. In addition, compound 2a also showed excellent activity against C. neoformans and C. neoformans clinical isolates with minimum inhibitory concentration of 35 and 36 µg/mL, respectively. Compounds 2a and 2b were further studied by evaluating their cytotoxicities, and the results showed that they have relatively low level cytotoxicity for BV2 and 293T cell. Preliminary structureactivity relationship study on three diverse sets (C-2, C-3, and C-8 positions) of 1,5-benzodiazepines was performed. The results revealed that the presence of a -CH₃ group at the C-8 position had a positive effect on the inhibitory activity of these compounds. Additionally, the 2-pyridyl group at the C-2 position may be a pharmacophore and $\text{-}\text{COOC}_2\text{H}_5$ at C-3 position is the best substituent for the maintenance of antimicrobial activities.

Key words: 1,5-benzodiazepine, antimicrobial activity, structure-activity relationship, synthesis Received 20 September 2015, revised 22 January 2016 and accepted for publication 26 January 2016

The effect of bacterial and fungal resistance to antimicrobial agents on human health is reaching alarming levels (1). Infectious disease caused by microorganisms is a primary cause of death worldwide. The search for new antibacterial and antifungal drugs is ongoing because of the increasing resistance of microbial pathogens. It is desirable to discover new drugs with superior potency and wide activity spectrum. Among various heterocyclic systems, 1,5-benzodiazepines have gained importance because of their pharmacological and biological activities. Over recent years, an increasing number of studies on the antimicrobial activity of 1,5-benzodiazepines have been reported. To date, a significant number of 1,5-benzodiazepines analogs with antimicrobial activity has been synthesized (2-5). For example, series of 1,5-benzodiazepines were described by Pandaram et al. as potent antimicrobial agents (6). Therefore, the research on the synthesis and antimicrobial activity of 1,5-benzodiazepines has become a popular topic.

On one hand, previous studies indicated that the free ester group at different positions of the nuclei of the molecules could enhance their pharmacological properties due to its high hydrophobicity and fat-solubility (7). On the other hand, the pyridine group is also known as an important scaffold in various biologically active molecules, possessing antimicrobial, antiviral, anti-HIV, anti fungal, and antimycobacterial activities (8–13).

Encouraged by these observations, we synthesized a series of novel 1,5-benzodiazepine derivatives based on the principle of superposition (14). The structures of these newly synthesized 1,5-benzodiazepine derivatives are shown in Figure 1. In our design, we emphasized the strategy of combining three molecule fragments, 1,5-benzodiazepine, pyridine and an ester group, into one frame. To the best of our knowledge, the synthesis and the antimicrobial activity of compounds **2–5** is reported first. This library compounds represent a new starting point in the synthesis of many other useful 1,5-benzodiazepine derivatives. The new compounds we synthesized were screened for their *in vitro* antimicrobial activity against *Cryptococcus neoformans*,



2: $R_2 = -CH_2CH_3$

2a: $R_1 = H = X = N$ **2b:** $R_1 = CH_3$ X = N **2c:** $R_1 = F$ $\mathbf{X} = \mathbf{N}$ **2e:** $R_1 = H$ X = C **2f:** $R_1 = CH_3$ X = C**2g:** $R_1 = Br$ X = C**2d:** $R_1 = Br X = N$ 3: $X = N R_2 = -CH_3$ **3a:** $R_1 = H$ **3b:** $R_1 = CH_3$ **3c:** $R_1 = F$ **3d:** $R_1 = Br$ 4: $X = N R_2 = -CH_2CH_2CH_3$ **4b:** $R_1 = CH_3$ **4c:** $R_1 = F$ **4d:** $R_1 = Br$ **4a:** $R_1 = H$ **5b:** $R_1 = CH_3$ **5c:** $R_1 = F$ **5d:** $R_1 = Br$ 5: $X = N R_2 = -CH(CH_3)_2$ **5a:** $R_1 = H$

Figure 1: Structures of compounds 2–5.

Cryptococcus neoformans clinical isolates, Candida albicans, Escherichia coli, and Staphylococcus aureus. The aim of the present research was to determine the exact moiety of the molecules that affects the activity and to determine whether the change of R₁, R₂, and X causes any significant changes in antimicrobial activity. The results of the bioactive assay showed that most of the synthesized compounds displayed variable inhibitory effects on the growth of all of the tested strains. Notably, compound **2b** showed the highest activity against *C. neoformans*, and *C. neoformans* clinical isolates.

Experimental Section

Starting materials were purchased from commercial sources and were used without further purification. Solvents were dried according to standard procedures. The reaction progress was monitored by thin layer chromatography. The melting of these new compounds was determined by an open capillary using a Veego Precision Digital Melting points apparatus (MP-D) and the results were uncorrected. The IR spectra (in KBr pellets) were recorded on a BIO-RAD PE-M-1730 IR spectrophotometer. ¹H NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer using Tetramethylsilane (TMS) as the internal standard with DMSO-d₆ as the solvent. Chemical shift values are expressed as parts per million downfield from TMS, and J values are in Hertz. Splitting patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet, and m, multiplet. Mass spectra were recorded on a Thermo DSQ Il mass spectrometer. The results of the elemental analysis were obtained on a Vario EL-III-CHN-0 elemental analyzer.

General method for the synthesis of 1,5benzodiazepines (2–5)

Synthesis of *N*-o-aryl amino- β -enamino esters 1

A mixture of ethyl acetoacetate (methyl acetoacetate, acetyl propyl acetate, or acetyl isopropyl acetate)

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(30 mmol) and various *o*-PDAs (25 mmol) was stirred for approximately 50 min at room temperature. The progress and situation of the reaction conditions were monitored by TLC. The solids were collected by filtration under reduced pressure to give the corresponding crude *N*-*o*-aryl amino- β -enamino esters **1** (~95%) after recrystallization with EtOH as a solvent for purification.

Synthesis of 1,5-benzodiazepines (2-5)

Ethyl acetoacetate and various o-PDAs were used to yield **1**. A mixture of compound **1** (20 mmol), a substituted pyridine aldehyde or benzonic aldehyde (20 mmol) and PMA (5 mol%) in dry ethanol (50 mL) was stirred at 0 °C for 15–60 min. The progress and situation of the reaction conditions were monitored by TLC. After the reaction was complete, the formed precipitate was filtered, washed with ethanol, and recrystallized from dry ethanol to yield compounds **2–5**.

Ethyl-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-benzodiazepine-3-carboxylate (2a, $C_{18}H_{19}N_3O_2$): White solid: yield (95%); mp: 129-131 °C; R_f = 0.16 (Petroleum ether: ethylacetate = 3:1); IR (KBr cm⁻¹): v = 3327 (N–H), 1672 (C=O), 1630 (C=C); ¹H NMR (DMSO-d₆, 500 MHz): $\delta = 1.07$ (t, J = 7.0 Hz, 3H, -CH₃), 2.48 (s, 3H, -CH₃), 3.93 (q, J = 7.0 Hz, 2H, -COOCH₂-), 5.65 (d, J = 6.0 Hz, 1H, -CH), 6.11 (d, J = 6.0 Hz, 1H, -NH), 6.47–8.36 (m, 8H, -C₆H₄, -C₅H₄N), 8.37 (s, 1H, -NH) p.p.m.; ¹³C NMR $(DMSO-d_6, 125 \text{ MHz}): \delta = 14.39, 23.51, 58.62, 61.64,$ 98.61, 119.55, 119.57, 120.21, 120.78, 121.17, 121.96, 131.90, 135.89, 138.00, 148.69, 152.10, 163.02, 167.68 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₈H₁₉N₃O₂ 310, found 310; Anal. Calcd for C₁₈H₁₉N₃O₂: C, 69.88; H, 6.19; N, 13.58; Found: C, 69.92; H, 6.14; N, 13.67.

Ethyl-4,8-dimethyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-benzo-diazepine-3-carboxylate (**2b**, $C_{19}H_{21}N_3O_2$): White solid: yield (95%); mp: 142–143 °C; $R_f = 0.09$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) v = 3317 (N–H), 1670

(C=O), 1618 (C=C). ¹H NMR (DMSO- d_6 , 500 MHz): $\delta = 1.07$ (t, J = 7.0 Hz, 3H, -CH₃), 1.98 (s, 3H, -CH₃), 2.46 (s, 3H, -CH₃), 3.96 (q, J = 7.0 Hz, 2H, -CH₂), 5.64 (d, J = 7.0 Hz, 1H, -CH), 6.03 (d, J = 7.0 Hz, 1H, -NH), 6.29–8.32 (m, 7H, -C₆H₃, -C₅H₄N), 8.37 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO- d_6 , 125 MHz): $\delta = 14.41$, 20.13, 23.56, 58.52, 61.40, 98.37, 119.56, 120.24, 120.35, 120.75, 121.16, 122.74, 130.71, 135.86, 137.78, 148.69, 152.17, 163.09, 167.68 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₉H₂₁N₃O₂ 324, found 324; Anal. Calcd for C₁₉H₂₁N₃O₂: C, 70.57; H, 6.55; N, 12.99; Found: C, 70.62; H, 6.59; N, 12.59.

Ethyl-8-fluoro-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-

benzodiazepine-3-carboxylate (**2c**, $C_{18}H_{18}FN_3O_2$): White solid: yield (93%); mp: 135–137 °C; $R_{\rm f}$ = 0.17 (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3359 (N–H), 1672 (C=O), 1623 (C=C). ¹H NMR (DMSO-d₆, 500 MHz): δ = 1.08 (t, J = 7.0 Hz, 3H, -CH₃), 2.46 (s, 3H, -CH₃), 3.94 (q, J = 6.5 Hz, 2H, -CH₂), 5.64 (d, J = 6.5 Hz, 1H, -CH), 6.31 (d, J = 6.5 Hz, 1H, -NH), 6.34–8.39 (m, 7H, -C₆H₃, -C₅H₄N), 8.45 (s, 1H, -NH), p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): δ = 14.86, 23.85, 59.09, 61.64, 99.10, 106.24, 120.86, 120.94, 121.27, 121.79, 128.85, 136.53, 140.20, 149.23, 152.48, 163.10, 168.02 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₈H₁₈FN₃O₂ 328, found 328.

Ethyl-8-bromo-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-

1,5-benzodiazepine-3-carboxylate (**2d**, $C_{18}H_{18}BrN_3O_2$): Brown solid: yield (93%); mp: 134–135 °C; $R_{\rm f}$ = 0.16 (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3341 (N–H), 1671 (C=O), 1618 (C=C). ¹H NMR (DMSO-d₆, 500 MHz): δ = 1.09 (t, J = 7.0 Hz, 3H, -CH₃), 2.46 (s, 3H, -CH₃), 3.93 (q, J = 7.0 Hz, 2H, -CH₂), 5.63 (d, J = 6.5 Hz, 1H, -CH), 6.44 (d, J = 6.5 Hz, 1H, -NH), 6.65–8.40 (m, 7H, -C₆H₃, -C₅H₄N), 8.45 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): δ = 14.87, 23.94, 59.05, 62.06, 99.35, 120.00, 120.64, 121.24, 121.63, 122.40, 132.34, 136.35, 138.45, 149.14, 152.52, 163.47, 168.12 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₈H₁₈BrN₃O₂ 390, found 390; Anal. Calcd for C₁₈H₁₈BrN₃O₂: C, 55.68; H, 4.67; N, 10.82; Found: C, 55.74; H, 4.63; N, 10.79.

Ethyl4-methyl-2-phenyl-2,5-dihydro-1,5-benzodiazepine-3carboxylate (**2e**, $C_{19}H_{20}N_2O_2$): White solid: yield (95%); mp: 130–132 °C; $R_f = 0.30$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3345 (N–H), 1667 (C=O), 1630 (C=C). ¹H NMR (DMSO-d₆, 500 MHz): δ = 1.12 (t, J = 7.0 Hz, 3H, -CH₃), 2.45 (s, 3H, -CH₃), 4.03 (q, J = 7.0 Hz, 2H, -CH₂), 5.62 (d, J = 6.5 Hz, 1H, -CH), 6.19 (d, J = 6.5 Hz, 1H, -NH), 6.47–7.12 (m, 8H, -C₆H₃, -C₅H₅), 8.28 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): δ = 14.50, 23.45, 58.96, 61.28, 100.38, 110.44, 121.10, 121.46, 121.51, 121.81, 124.18, 133.63, 136.27, 137.63, 148.96, 151.70, 162.69, 167.69 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₉H₂₀N₂O₂ 309,

found 309; Anal. Calcd for $C_{19}H_{20}N_2O_2{:}$ C, 74.00; H, 6.54; N, 9.08; Found: C, 74.02; H, 6.52; N, 9.11.

Ethyl4,8-dimethyl-2-phenyl-2,5-dihydro-1,5-benzodiaze-

pine-3-carboxylate (**2f**, $C_{20}H_{22}N_2O_2$): White solid: yield (95%); mp: 132–134 °C; $R_f = 0.30$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3306 (N–H), 1673 (C=O), 1630 (C=C). ¹H NMR (DMSO-d₆, 500 MHz): δ = 1.29 (t, J = 7.0 Hz, 3H, -CH₃), 2.46 (s, 3H, -CH₃), 4.23 (q, J = 7.0 Hz, 2H, -CH₂), 5.60 (d, J = 6.5 Hz, 1H, -CH), 6.13 (d, J = 6.5 Hz, 1H, -NH), 6.30–7.13 (m, 7H, -C₆H₃, -C₅H₄N), 8.23 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): δ = 14.92, 20.64, 23.89, 49.08, 59.02, 59.25, 100.46, 119.96, 120.44, 120.55, 126.24, 127.83, 128.21, 129.58, 131.13, 131.96, 138.45, 145.21, 152.49, 168.18 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₂₀H₂₂N₂O₂: C, 74.51; H, 6.88; N, 8.69; Found: C, 74.48; H, 6.87; N, 8.72.

Ethyl-8-bromo-4-methyl-2-phenyl-2,5-dihydro-1,5-benzo-

diazepine-3-carboxylate (**2g**, $C_{19}H_{19}BrN_2O_2$): Brown solid: yield (93%) mp: 134–135 °C; $R_{\rm f}$ = 0.36 (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3303 (N–H), 1682 (C=O), 1627 (C=C). ¹H NMR (DMSO-d₆, 500 MHz): δ = 1.11 (t, J = 7.0 Hz, 3H, -CH₃), 2.45 (s, 3H, -CH₃), 3.96 (q, J = 7.0 Hz, 2H, -CH₂), 5.59 (d, J = 6.5 Hz, 1H, -CH), 6.48 (d, J = 6.5 Hz, 1H, -NH), 6.64–7.17 (m, 7H, -C₆H₃, -C₅H₄N), 8.39 (s, 1H, -NH), 6.64–7.17 (m, 7H, -C₆H₃, -C₅H₄N), 8.39 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): δ = 14.68, 23.65, 56.54, 59.28, 99.88, 113.87, 121.59, 122.24, 122.96, 123.99, 128.45, 132.23, 139.87, 152.52, 159.76, 161.71, 167.58 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₉H₁₉BrN₂O₂ 388, found 388; Anal. Calcd for C₁₉H₁₉BrN₂O₂: C, 58.93; H, 4.95; N, 7.23; Found: C, 58.89; H, 4.90; N, 7.28.

Methyl-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-benzodiazepine-3-carboxylate (**3a**, $C_{17}H_{17}N_3O_2$): White solid: yield (95%); mp:135–137 °C; $R_f = 0.08$ (Petroleum ether: ethylacetate = 3:1); IR(KBr cm⁻¹): v = 3327 (N–H), 1674 (C=O), 1637 (C=C); ¹H NMR (DMSO-d₆, 500 MHz): $\delta = 2.49$ (s, 3H, -CH₃), 3.48 (s, 3H, -COOCH₃), 5.65 (d, J = 6.5 Hz, 1H, -CH), 6.13 (d, J = 6.5 Hz, 1H, -NH), 6.48–8.38 (m, 8H, -C₆H₄, -C₅H₄N), 8.41 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSOd₆, 125 MHz): $\delta = 23.48$, 50.50, 61.47, 98.61, 119.55, 119.59, 120.20, 120.80, 121.26, 122.01, 131.77, 135.97, 138.00 148.74, 152.74, 162.84, 168.05 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₇H₁₇N₃O₂ 296, found 296; Anal. Calcd for C₁₇H₁₇N₃O₂: C, 69.14; H, 5.80; N, 14.23; Found: C, 69.03; H, 5.92; N, 14.11.

Methyl-4,8-dimethyl-2-(pyridin-2-yl)-2,5-dihydro-1,5diaze-

pine-3-carboxylate (**3b**, $C_{18}H_{19}N_3O_2$): White solid: yield (95%); mp:129–130 °C; $R_f = 0.10$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3318 (N–H), 1672 (C=O), 1617 (C=C). ¹H NMR (DMSO-*d*₆, 500 MHz): δ = 1.98 (s, 3H, -CH₃), 2.47 (s, 3H, -CH₃), 3.47 (s, 3H, -CH₃), 5.63 (d, *J* = 6.5 Hz, 1H, -CH), 6.03 (d, *J* = 6.5 Hz,

1H, -NH), 6.30–8.35 (m, 7H, $-C_6H_3$, $-C_5H_4N$), 8.38 (s, 1H, -NH) p.p.m.; ^{13}C NMR (DMSO- d_6 , 125 MHz): δ = 20.56, 23.98, 49.09, 50.86, 61.75, 98.57, 120.02, 120.82, 121.22, 121.70, 129.68, 131.25, 136.42, 138.24, 149.19, 153.01, 163.38, 168.53 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for $C_{18}H_{19}N_3O_2$ 310, found 310; Anal. Calcd for $C_{18}H_{19}N_3O_2$: C, 69.88; H, 6.19; N, 13.58; Found: C, 69.80; H, 6.27; N, 13.67.

Methyl-8-fluoro-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-

benzodiazepine-3-carboxylate (**3c**, $C_{17}H_{16}FN_3O_2$): White solid: yield (93%); mp:155–156 °C; $R_f = 0.09$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3326 (N–H), 1670 (C=O), 1637 (C=C). ¹H NMR (DMSO-d₆, 500 MHz): δ = 2.47 (s, 3H, -CH₃), 3.49 (s, 3H, -CH₃), 5.63 (d, J = 6.5 Hz, 1H, -CH), 6.32 (d, J = 6.5 Hz, 1H, -NH), 6.34–8.40 (m, 7H, -C₆H₃, -C₅H₄N), 8.43 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): δ = 23.86, 50.97, 61.58, 98.83, 106.30, 120.90, 120.98, 121.28, 121.85, 128.77, 136.57, 140.24, 149.29, 152.87, 162.96, 168.41 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₇H₁₆FN₃O₂ 314, found 314.

Methyl-8-bromo-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5benzodiazepine-3-carboxylate (**3d**, $C_{17}H_{16}BrN_3O_2$): Brown solid: yield (93%); mp:154–155 °C; $R_f = 0.12$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3336 (N–H), 1673 (C=O), 1638 (C=C). ¹H NMR (DMSO- d_6 , 500 MHz): δ = 2.46 (s, 3H, -CH₃), 3.50 (s, 3H, -CH₃), 5.62 (d, J = 6.0 Hz, 1H, -CH), 6.45 (d, J = 6.5 Hz, 1H, -NH), 6.65– 8.41 (m, 7H, -C₆H₃, -C₅H₄N), 8.48 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO- d_6 , 125 MHz): δ = 23.77, 51.08, 61.45, 99.92, 113.67, 121.36, 121.55, 121.95, 122.19, 131.64, 136.68, 140.29, 149.37, 152.67, 162.75, 168.40 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₇H₁₆BrN₃O₂ 374, found 374; Anal. Calcd for C₁₇H₁₆BrN₃O₂: C, 54.56; H, 4.31; N, 11.23; Found: C, 54.47; H, 4.38; N, 11.29.

Propyl-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-benzodiazepine-3-carboxylate (4a, $C_{19}H_{21}N_3O_2$): White solid: yield (90%); mp:132-134 °C; R_f = 0.31 (Petroleum ether: ethylacetate = 3:1); IR(KBr cm⁻¹): v = 3325 (N–H), 1671 (C=O), 1622 (C=C); ¹H NMR (DMSO-d₆, 500 MHz): $\delta = 0.73$ (t, J = 7.0 Hz, 3H, -CH₃), 1.44–1.46 (m, 2H, $-CH_2$), 2.49 (s, 3H, $-CH_3$), 3.84 (t, J = 6.0 Hz, 2H, -COOCH₂), 5.66 (d, J = 6.5 Hz, 1H, -CH), 6.10 (d, J = 6.5 Hz, 1H, -NH), 6.45–8.36 (m, 8H, -C₆H₄, -C₅H₄N), 8.38 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): $\delta = 10.43, 21.80, 23.44, 61.74, 64.27, 98.64, 119.53,$ 119.60, 120.20, 120.67, 121.17, 121.97, 131.99, 135.86, 137.95, 148.64, 152.06, 163.09, 167.62 p.p.m.; MS (ESI-TOF) m/z: $[M + H^+]$ Calcd for $C_{19}H_{21}N_3O_2$ 324, found 324; Anal. Calcd for C19H21N3O2: C, 70.57; H, 6.55; N, 12.99; Found: C, 70.65; H, 6.48; N, 12.89.

Propyl-4,8-dimethyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-benzodiazepine-3-carboxylate (**4b**, $C_{20}H_{23}N_3O_2$): White solid: yield (90%); mp:130–131 °C; $R_f = 0.31$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3319 (N–H), 1671 (C=O), 1617 (C=C). ¹H NMR (DMSO-*d*₆, 500 MHz): δ = 0.73 (t, *J* = 7.0 Hz, 3H, -CH₃), 1.42–1.47 (m, 2H, -CH₂), 1.98 (s, 3H, -CH₃), 2.47 (s, 3H, -CH₃), 3.84 (t, *J* = 6.5 Hz, 2H, -COOCH₂), 5.65 (d, *J* = 6.0 Hz, 1H, -CH), 6,02 (d, *J* = 6.0 Hz, 1H, -NH), 6.28–8.32 (m, 7H, -C₆H₃, -C₅H₄N), 8.37 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-*d*₆, 125 MHz): δ = 10.90, 20.60, 22.28, 23.96, 62.04, 64.67, 98.63, 120.82, 121.11, 121.61, 123.22, 129.89, 131.19, 136.32, 138.20, 149.10, 152.57, 163.66, 168.11 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₂₀H₂₃N₃O₂ 338, found 338; Anal. Calcd for C₂₀H₂₃N₃O₂: C, 71.19; H, 6.87; N, 12.45; Found: C, 71.24; H, 6.94; N, 12.36.

Propyl-8-fluoro-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5benzodiazepine-3-carboxylate (**4c**, $C_{19}H_{20}FN_3O_2$): White solid: yield (90%); mp: 139–141 °C; $R_f = 0.18$ (Petroleum ether: ethylacetate = 3:1); IR (KBr cm⁻¹): v = 3358 (N–H), 1675 (C=O), 1635 (C=C); ¹H NMR (DMSO- d_6 , 500 MHz): $\delta = 0.74$ (t, J = 7.0 Hz, 3H, -CH₃), 1.42–1.48 (m, 2H, -CH₂), 2.47 (s, 3H, -CH₃), 3.86 (t, J = 6.0 Hz, 2H, -COOCH₂), 5.65 (d, J = 6.5 Hz, 1H, -CH), 6.11 (d, J = 6.5 Hz, 1H, -NH), 6.30–8.42 (m, 7H, -C₆H₃, -C₅H₄N), 8.47 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO- d_6 , 125 MHz): $\delta = 10.42$, 21.79, 23.35, 61.38, 64.30, 98.38, 105.84, 120.52, 120.71, 120.83, 121.31, 128.51, 136.02, 139.79, 148.73, 152.06, 156.60, 162.74, 167.55 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₉H₂₀FN₃O₂ 342, found 342.

Propyl-8-bromo-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5benzodiazepine-3-carboxylate (**4d**, $C_{19}H_{20}BrN_3O_2$): Brown solid: yield (90%); mp:132-133 °C; R_f = 0.22 (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm^{-1}) v = 3338 (N–H), 1670 (C=O), 1618 (C=C). ¹H NMR (DMSO-*d*₆, 500 MHz): $\delta = 0.74$ (t, J = 7.5 Hz, 3H, -CH₃), 1.43–1.50 (m, 2H, -CH₂), 2.47 (s, 3H, -CH₃), 3.87 (t, J = 7.5 Hz, 2H, - $COOCH_2$), 5.64 (d, J = 6.5 Hz, 1H, -CH), 6.45 (d, J = 6.5 Hz, 1H, -NH), 6.66–8.40 (m, 7H, -C₆H₃, -C₅H₄N), 8.47 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-*d*₆, 125 MHz): $\delta = 10.90, 22.25, 23.75, 61.74, 64.90, 99.98, 113.67,$ 121.26, 121.55, 121.86, 122.23, 131.85, 136.58, 140.23, 149.28, 152.27, 163.00, 168.00 p.p.m.; MS (ESI-TOF) m/ z: [M + H⁺] Calcd for C₁₉H₂₀BrN₃O₂ 404, found 404; Anal. Calcd for C₁₉H₂₀BrN₃O₂: C, 56.73; H, 5.01; N, 10.45; Found: C, 56.79; H, 5.08; N, 10.50.

Isopropyl-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-benzodiazepine-3-carboxylate (**5a**, $C_{19}H_{21}N_3O_2$): White solid: yield (88%); mp:133–134 °C; $R_f = 0.16$ (Petroleum ether: ethylacetate = 3:1); IR (KBr cm⁻¹): v = 3330 (N–H), 1672 (C=O), 1621 (C=C); ¹H NMR (DMSO-d₆, 500 MHz): $\delta = 0.96$ (d, J = 6.0 Hz, 3H, -CH₃), 1.14 (d, J = 6.0 Hz, 3H, -CH₃), 2.47 (s, 3H, -CH₃), 4.77–4.82 (m, 1H, -CH), 5.64 (d, J = 6.5 Hz, 1H, -CH), 6.07 (d, J = 6.5 Hz, 1H, -NH), 6.45–8.33 (m, 8H, -C₆H₄, -C₅H₄N,) 8.37 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): $\delta = 21.78$, 22.02, 23.46, 61.82, 65.39, 99.11, 119.52, 119.56, 120.20, 120.70, 121.09, 121.89, 132.03, 135.80, 137.95, 148.61, 151.63, 163.17, 167.25 p.p.m.; MS (ESI-TOF) m/ z: $[M + H^+]$ Calcd for $C_{19}H_{21}N_3O_2$ 324, found 324; Anal. Calcd for $C_{19}H_{21}N_3O_2$: C, 70.57; H, 6.55; N, 12.99; Found: C, 70.65; H, 6.48; N, 12.90.

Isopropyl-4,8dimethyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-benzodiazepine-3-carboxylate (**5b**, $C_{20}H_{23}N_3O_2$): White solid: yield (88%); mp:135–136 °C; $R_{\rm f}$ = 0.20 (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm^{-1}) v = 3337 (N–H), 1671 (C=O), 1620 (C=C). ¹H NMR (DMSO-*d*₆, 500 MHz): $\delta = 0.97$ (d, J = 6.0 Hz, 3H, -CH₃), 1.13 (d, J = 6.5 Hz, 3H, -CH₃), 1.98 (s, 3H, -CH₃), 2.45 (s, 3H, -CH₃), 4.76-4.81 (m, 1H, -CH), 5.62 (d, J = 6.0 Hz, 1H, -CH), 5.99 (d, J = 6.5 Hz, 1H, -NH), 6.28–8.27 (m, 7H, -C₆H₃, -C₅H₄N), 8.37 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): $\delta = 20.61, 22.28, 22.51, 23.99, 62.05, 65.74, 99.10,$ 120.80, 121.16, 121.56, 129.91, 131.09, 136.29, 138.20, 149.08, 152.16, 163.73, 167.72 p.p.m.; MS (ESI-TOF) m/ z: $[M + H^+]$ Calcd for $C_{20}H_{23}N_3O_2$ 338, found 338; Anal. Calcd for C₂₀H₂₃N₃O₂: C, 71.19; H, 6.87; N, 12.45; Found: C, 71.25; H, 6.95; N, 12.49.

Isopropyl-8-fluoro-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-

1,5-benzodiazepine-3-carboxylate (**5c**, $C_{19}H_{20}FN_3O_2$): White solid: yield (85%); mp:159–160 °C; $R_f = 0.19$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3329 (N–H), 1667 (C=O), 1615 (C=C). ¹H NMR (DMSO-d₆, 500 MHz): δ = 0.98 (d, J = 6.0 Hz, 3H, -CH₃), 1.14 (d, J = 6.0 Hz, 3H, -CH₃), 2.45 (s, 3H, -CH₃), 4.79–4.82 (m, 1H, -CH), 5.62 (d, J = 6.5 Hz, 1H, -CH), 6.30 (d, J = 6.5 Hz, 1H, -CH), 6.30 (d, J = 6.5 Hz, 1H, -NH), 6.32–8.35 (m, 7H, -C₆H₃, -C₅H₄N), 8.39 (s, 1H, -NH) p.p.m.; ¹³C NMR (DMSO-d₆, 125 MHz): δ = 22.27, 22.48, 23.84, 61.82, 65.92, 99.38, 106.25, 106.45, 120.85, 120.92, 121.24, 121.73, 128.98, 136.47, 140.17, 149.17, 152.07, 163.26, 167.63 p.p.m.; MS (ESI-TOF) m/z: [M + H⁺] Calcd for C₁₉H₂₀FN₃O₂ 342, found 342.

Isopropyl-8-bromo-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-

1,5-benzodiazepine-3-carboxylate (**5d**, $C_{19}H_{20}BrN_3O_2$): Brown solid: yield (85%); mp:141–142 °C; $R_f = 0.25$ (Petroleum ether: ethylacetate = 3:1); IR (KBr, cm⁻¹) ν = 3329 (N–H), 1671 (C=O), 1617 (C=C). ¹H NMR (DMSO- d_6 , 500 MHz): δ = 0.99 (d, J = 6.0 Hz, 3H, -CH₃), 1.15 (d, J = 6.0 Hz, 3H, -CH₃), 2.45 (s, 3H, -CH₃), 4.79–4.84 (m, 1H, -CH), 5.62 (d, J = 6.0 Hz, 1H, -CH), 6.42 (d, J = 6.0 Hz, 1H, -NH), 6.66–8.40 (m, 7H, -C₆H₃, -C₅H₄N), 8.42 (s, 1H, -NH) p.p.m. ¹³C NMR (DMSO- d_6 , 125 MHz): δ = 21.69, 21.88, 23.19, 61.11, 65.52, 99.88, 112.97, 120.76, 120.94, 121.24, 121.58, 121.64, 131.27, 135.99, 139.65, 148.68, 151.27, 162.49, 167.02 p.p.m.; MS (ESITOF) m/z: [M + H⁺] Calcd for C₁₉H₂₀BrN₃O₂ 404, found 404; Anal. Calcd for C₁₉H₂₀BrN₃O₂: C, 56.73; H, 5.01; N, 10.45; Found: C, 56.65; H, 5.09; N, 10.50.

Biological evaluation

Assay of antimicrobial activity *in vitro*. The standardized disc diffusion methods were used to test the antimicrobial



(antibacterial and antifungal) activities of 19 1,5-benzodiazepine derivatives we synthesized. Five microorganisms have been used including *C. neoformans* (ATCC 32264), *C. neoformans* clinical isolates, *C. albicans* (ATCC 10231), *E. coli* (ATCC 44752), and *S. aureus* (ATCC 25923).

In the disc diffusion method, sterile paper discs (Φ 6 mm) impregnated with compounds dissolved in DMSO at concentrations of 200 mg/disc were used. Discs containing DMSO were used as controls. Paper discs impregnated with a solution of the compound tested were placed on the surface of the media inoculated with the microorganisms. The plates were incubated at 37 °C for 24 h for culture of microorganisms. After incubation, the growth inhibition zones around the discs were observed, which indicated that the examined compound inhibited the growth of microorganisms. Each assay in this experiment was repeated three times.

MIC assays were performed in sterile 96-well plates using the method described by Sarmiento (15). The MIC is defined as the minimum concentration of a compound required to exhibit complete inhibition of bacterial and fungal growth. MIC₈₀ was recorded as the concentration that produced 80% growth reduction compared with wells with no compound present. The MFC is the concentration at which fungi failed to grow in the liquid nutrient medium. It was determined that the solvent had no antifungal activities against any of the tested microorganisms. MFC assays were also conducted in sterile 96-well plates with different compound concentrations inoculated with 200 μ L of fungi suspension (2 \times 10³ CFU/mL). The plates were incubated for 36 h at 30 °C, and growth was visually observed. Approximately 200 μ L of fungi suspension from the wells that did not show growth was plated on nutrient agar. The MFC is the concentration at which fungi failed to grow in the liquid nutrient medium and nutrient agar inoculated with 200 μ L of suspension. The MIC and MFC assays were repeated three times.

The BV2 cells were cultured at 37 °C in 96-well plates at a density of 4 \times 10⁴ cells per well with 5% CO₂ in Dulbecco's Modified Eagle Medium (Invitrogen, GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen) for 72 h. The 293T cells were incubated in the same conditions with BV2 cells in RPMI 1640 supplemented with 10% fetal bovine. Compounds 2a, 2b, and fluconazole were added to each well at final concentrations of 10.0, 20.0, 35.0, 50.0, 100.0, and 200.0 µg/mL. An equivalent volume of DMSO without the compound was added as a control. The cells were incubated for 48 h. Subsequently, 10 μ L of aqueous MTT solution (5.0 mg/mL) and the mixture were incubated at 37 °C for 4 h. The MTT solution was carefully decanted off, and 100 µL of DMSO was added to each well. The color was measured with an Epoch microplate spectrophotometer at 490 nm with the reference filter set to 620 nm. All MTT assays were repeated three times. Each measurement contained six parallel treatments (wells).



Scheme 1: Synthesis of this novel series of 1,5-benzodiazepines 2–5.

a:Solvent free, Catalyst free, Room tempeature $R_1 = H, CH_3, F, Br$ X = N, Cb:Dry ethanol, Ice bath (0 °C), PMA $R_2 = CH_3, CH_2CH_3, CH_2CH_3, CH(CH_3)_2$

Relative survival rate

= (treatment A490/negative control A620) \times 100%

Results and Discussion

Chemistry

The novel 1,5-benzodiazepine derivatives **2–5** were synthesized in two steps as shown in Scheme 1. First, the condensation of substituted 1,2-phenylenediamines and ethyl acetoacetate was performed under solvent-free, catalyst-free, and room temperature conditions to yield approximately 95% *N-o*-aminoaryl- β -enamino esters **1**. The addition of the solvents leads to a decrease of the yield of compound **1**. It is worth mentioning that adding catalyst or increasing the temperature promotes the formation of the by-product benzimidazole (Figure 2). Therefore, the reaction was conducted under solvent-free, catalyst-free, and room-temperature conditions.

Then. the target compounds 1,5-benzodiazepine derivatives 2 were obtained via the reaction between 2pyridinecarboxaldehyde or benzaldehyde and compound 1 (N-o-aminoaryl- β -enamino esters). This step occurred under various reaction conditions (e.g., catalyst, solvent, and reaction temperature), which were investigated in preliminary experiments. At the beginning of this study, the reaction of 2-pyridinecarboxaldehyde and N-o-aminoaryl- β -enamino esters was selected as a model to establish optimum reaction conditions. The results are displayed in Table 1. In the absence of the catalyst, only a trace amount of the desired product was observed even after 1 h (Table 1, entry 1), which demonstrated that catalyst plays an important role in this reaction. Then, we tried to optimize the reaction conditions with different catalysts and found that the reaction gave satisfying results in the presence of phosphomolybdic acid (PMA). Other catalysts, such as p-TsOH, acetic acid (HOAc), silicotungstic acid $(STA, H_4SiW_{12}O_{40})$, CeCl₃·7H₂O, NiCl₂·6H₂O and l₂ afforded the target compounds in low yields. Several solvents including acetonitrile, dichloromethane, chloroform, methanol, ethanol, benzene, and toluene were tested for the reaction. Compared with other solvents, EtOH was found to be superior in terms of reaction time, environmental protection, and significant yields. Although the rate of the transformation could be enhanced and the reaction time could be shortened by refluxing or increasing the dosage of the catalyst, the application of milder conditions (0 °C, PMA as a catalyst) proved to be more favorable which can avoid the formation of the by-product benzimidazole (Figure 2). In conclusion, higher temperature, high acidity, and excess catalyst would reduce the yields of 1,5-benzodiazepines. Therefore, the reaction was conducted using PMA as a catalyst in the appropriate amount under optimal conditions (0 °C and EtOH as a solvent). The products 2 were obtained after stirring of the mixtures for 15-60 min at 0 °C in yields of 93-95%. Compounds 3, 4 and 5 were obtained via a similar synthetic route in which ethyl acetoacetate was replaced by the corresponding ester (methyl acetoacetate, acetyl propyl acetate, and acetyl isopropyl acetate) in yields of 85-95%. Simple operation, short reaction time, high yields, and environment friendliness are the advantages of this method. These features make this method an attractive choice for the synthesis of 1,5-benzodiazepines. The structures of the synthesized analogs 2-5 were confirmed by ¹H NMR, ¹³C NMR, IR, MS, and elemental analysis.

Overall, we have proposed an efficient, clean, simple, and economical procedure for the synthesis of novel 1,5-benzodiazepine. Mild reaction conditions, short reaction time, simple work-up, high selectivity, and excellent yields of the products make this methodology highly significant. This procedure represents a powerful green technology using an environmentally solvent and avoids unwanted waste production for the synthesis of new 1,5-benzodiazepine derivatives. Surprisingly, the products were isolated without column chromatography, which is beneficial for industrial production.

Based on the above results and previous studies, a plausible mechanism for this reaction is presented in Scheme 2. The first step involves the nucleophilic attack of the *para*-amino group in substituted 1,2-phenylenediamines on the carbonyl carbon of the β -keto ester to generate compounds **1** by the loss of a water molecule. Compounds **1**



Figure 2: Structure of by-product benzimidazole.

Table 1: Optimization of the reaction conditions for the synthesis of 2aª





Entry	Catalyst	Amount of catalyst (mol%)	Solvent	Temperature (°C)	Time (min)	Yield ^b (%)	
1	None	_	EtOH	0 °C	60	_	
2	HOAc	5	EtOH	0 °C	25	72	
3	STA	5	EtOH	0 °C	30	88	
4	CeCl ₃ ·7H ₂ O	5	EtOH	0 °C	32	76	
5	p-TsOH	5	EtOH	0 °C	30	85	
6	NiCl ₂	5	EtOH	0 °C	38	70	
7	I ₂	5	EtOH	0 °C	50	75	
8	PMA	5	EtOH	0 °C	15	95	
9	PMA	5	CH3CN	0 °C	40	73	
10	PMA	5	CH ₂ Cl ₂	0 °C	35	75	
11	PMA	5	MeOH	0 °C	20	90	
12	PMA	5	Toluene	0 °C	50	55	
13	PMA	5	Benzene	0 °C	45	50	
14	PMA	5	EtOH	r. t	10	75	
15	PMA	5	EtOH	Reflux	10	70	

PMA, phosphomolybdic acid.

^aReaction conditions: *N*-o-aminoaryl-β-enamino esters (1 mmol), 2-pyridinecarboxaldehyde (1 mmol), Solvent (5 mL).

^blsolated yield.

were confirmed by IR, ¹H NMR, MS, and elemental analysis. Then, the other amino group of substituted 1,2-phenylenediamines attacks the carbonyl carbon of 2-pyridinecarboxaldehyde or benzaldehyde to give the intermediate I. The latter may undergo a cyclization reaction by hydrogen transfer to yield the target product **2–5** (16,17).

Antimicrobial activity

In this study, all the synthesized 1,5-benzodiazepine derivatives were tested against five microbial strains using disc diffusion methods, as follows: *C. neoformans* (ATCC 32264), *C. neoformans* clinical isolates, *C. albicans* (ATCC 10231), *E. coli* (ATCC 44752), and *S. aureus* (ATCC 25923). To facilitate visualization, the zone scope from these assays indicates the average diameter (from three

trails) of the growth inhibition. The margin of error of these measurements is ± 1 mm. The antimicrobial activity was classified as highly active (>14 mm), moderately active (10–14 mm), slightly active (6–10 mm), and inactive (≤6 mm). The zone data of the 19 1,5-benzodiazepines are reported in Table 2.

From Table 2, it could be observed that most of the synthesized 1,5-benzodiazepines showed different inhibitory effects on the growth of all tested microbial strains. The synthesized compounds with antimicrobial activity exhibited better antifungal activities than antibacterial activities. The results suggest that these new series of 1,5-benzodiazepines are biologically active and have obvious specificity to the tested fungi, especially *C. neoformans* and *C. neoformans* clinical isolates.



Scheme 2: Plausible mechanism for the synthesis of products 1–5.

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 $R_1 \xrightarrow{9} H$ $R_1 \xrightarrow{1} 2$ R_1

Compd.	R ₁		R_2	Zone of inhibition ^a (mm) Dose (200 μ g/disc)							
		Х		<i>C. n.</i> ^b	<i>C. n.C.</i> ^b	<i>C. a.</i> ^b	<i>E. c.</i> ^b	<i>S. a.</i> ^b			
2a	Н	Ν	CH ₂ CH ₃	23.53 ± 0.05	24.80 ± 0.08	14.33 ± 0.12	12.20 ± 0.15	13.73 ± 0.06			
2b	CH ₃	Ν	CH ₂ CH ₃	25.13 ± 0.03	26.97 ± 0.05	17.77 ± 0.11	13.10 ± 0.09	13.30 ± 0.10			
2c	F	Ν	CH ₂ CH ₃	21.63 ± 0.12	21.49 ± 0.09	13.90 ± 0.10	11.67 ± 0.15	13.40 ± 0.10			
2d	Br	Ν	CH ₂ CH ₃	20.67 ± 0.07	20.13 ± 0.05	12.78 ± 0.10	10.00 ± 0.13	10.67 ± 0.15			
2e	Н	С	CH ₂ CH ₃	6.00 ± 0.00	14.30 ± 0.05	7.20 ± 0.10	6.00 ± 0.00	10.02 ± 0.11			
2f	CH₃	С	CH ₂ CH ₃	6.00 ± 0.00	15.60 ± 0.10	16.60 ± 0.05	6.00 ± 0.00	11.17 ± 0.12			
2g	Br	С	CH ₂ CH ₃	6.00 ± 0.00	15.60 ± 0.12	11.33 ± 0.10	10.10 ± 0.11	10.30 ± 0.12			
3a	Н	Ν	CH3	21.50 ± 0.09	21.09 ± 0.05	12.93 ± 0.12	9.81 ± 0.12	12.00 ± 0.05			
3b	CH₃	Ν	CH ₃	23.50 ± 0.05	23.23 ± 0.12	13.50 ± 0.10	11.71 ± 0.12	12.80 ± 0.11			
3c	F	Ν	CH ₃	19.60 ± 0.09	19.23 ± 0.12	12.47 ± 0.05	6.00 ± 0.00	6.00 ± 0.00			
3d	Br	Ν	CH ₃	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00			
4a	Н	Ν	CH ₂ CH ₂ CH ₃	15.57 ± 0.11	15.43 ± 0.05	11.13 ± 0.09	9.63 ± 0.10	6.00 ± 0.00			
4b	CH₃	Ν	CH ₂ CH ₂ CH ₃	21.67 ± 0.09	22.13 ± 0.05	12.33 ± 0.10	11.57 ± 0.09	11.03 ± 0.12			
4c	F	Ν	CH ₂ CH ₂ CH ₃	11.07 ± 0.12	11.90 ± 0.11	11.10 ± 0.05	6.00 ± 0.00	6.00 ± 0.00			
4d	Br	Ν	CH ₂ CH ₂ CH ₃	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00			
5a	Н	Ν	CH(CH ₃) ₂	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00			
5b	CH ₃	Ν	CH(CH ₃) ₂	13.03 ± 0.09	12.93 ± 0.05	8.47 ± 0.12	8.10 ± 0.10	9.54 ± 0.11			
5c	F	Ν	CH(CH ₃) ₂	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00			
5d	Br	Ν	CH(CH ₃) ₂	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00			

^aThe values indicate the average diameters in mm (of three trials) for the zone of growth inhibition observed after 24 h of incubation at 37 °C.

^bC. n.: C. neoformans ATCC 32264; C. n. C.: C. neoformans clinical strain; C. a: C. albicans ATCC 10231; E. c.: E. coli ATCC 44752; S. a.: S. aureus ATCC 25923.

Among compounds 2a-2d containing a 2-pyridyl group at the C-2 position. 2b was the most active derivative with inhibition zone data of 25.13 and 26.97 mm against C. neoformans and C. neoformans clinical isolates, respectively. The derivatives 2a, 2c, 2d also exhibited high activities against C. neoformans and C. neoformans clinical isolates. Compounds 2e-2g containing a phenyl group at the C-2 position, displayed only weak or moderate activity against C. neoformans and C. neoformans clinical isolates. The results demonstrated that the pyridine ring was essential for the antifungal (C. neoformans and C. neoformans clinical isolates) activity of this family of compounds. All the compounds 2 showed moderate to low antimicrobial activity against C. albicans, E. coli and S. aureus. Particularly, compounds 2a-2b inhibited all the tested microorganisms and showed a broad-spectrum inhibitory effect.

Compounds **3–5** with a 2-pyridyl unit were synthesized by modifying the length and size of R_2 to further explore its influence on the antimicrobial activity of this series of 1,5-

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benzothiazepines. Compounds 3, 4, and 5 contain R_2 groups of -CH₃, -CH₂CH₂CH₃, and -CH(CH₃)₂, respectively. Compounds 3a-3c also showed good activity against C. neoformans and C. neoformans clinical isolates and moderate activity against C. albicans, E. coli, and S. aureus, but compound **3d** ($R_1 = -Br$) did not exhibit any activity against any of the tested microorganism. Compound **4b** ($R_1 = -CH_3$) is also an efficient broad-spectrum antibiotic and exhibits excellent inhibition activities against the C. neoformans and C. neoformans clinical isolates. In contrast, compounds 4a, 4c, and 4d showed weak or moderate activity against the tested microorganisms. Compounds 5 failed to show any significant inhibition with the exception of compound **5b** ($R_1 = -CH_3$), which showed low to moderate antimicrobial activity against all the tested microorganisms. In conclusion, compounds 2a-2d with an ethyl ester group at the C-3 position of the seven-membered ring display better antimicrobial activity than compounds 3-5. This indicated that the ethyl ester group in the region is favorable.

Table 3: Inhibition zones at different dose levels for compounds 2a, 2b, 2c, 2d, 3a, and 3b



	(Zone of inhibition/mm)											
	2a		2b		2c		2d		3a		3b	
Dose (µg/disc)	С. п.	C. n. C.	С. п.	C. n. C.	С. п.	C. n. C.	С. п.	C. n. C.	С. п.	C. n. C.	С. п.	C. n. C.
200	23.53	24.80	25.13	26.97	21.63	21.49	20.67	20.13	21.50	21.09	23.50	23.23
100	18.73	18.50	19.33	19.47	17.00	17.97	16.63	16.23	16.27	16.20	18.40	18.27
50	12.27	12.30	13.23	13.90	11.67	11.23	11.93	11.13	11.60	11.03	12.97	12.47
35	7.63	7.83	8.90	8.70	6.90	6.93	6.50	6.00	6.00	6.23	6.97	6.37
30	6.90	6.10	7.00	6.86	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
25	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00

C. n.: C. neoformans ATCC 32264; C. n. C.: C. neoformans clinical strain.

To explore the antimicrobial activity of 1,5-benzodiazepine and to identify promising lead compounds. Compounds **2a**, **2b**, **2c**, **2d**, **3a**, and **3b**, which exhibited remarkable antifungal activity in this study, were selected to investigate the effect of dose levels on antifungal activity against *C*. *neoformans*, *C*. *neoformans* clinical isolates. The inhibition zones (Table 3) showed that the concentrations of the compounds were ranged from 25 μ g to 200 μ g. It was also found that the antimicrobial activity of the compounds was reduced with decreasing in concentration. Compounds **2a** and **2b** exhibited slight activity at 30 μ g/disc, but **2c**, **2d**, **3a**, and **3b** did not exhibit any activity at 30 μ g/disc. Compounds **2a** and **2b** exhibited inactivity at 25 μ g/disc.

In addition, the MIC, MIC₈₀, and MFC values were determined to explore the antifungal activity of compounds 2a and 2b, which show the highest activity. Flucanazole was used as the standard drug against fungi. The results are summarized in Table 4. Surprisingly, the MIC values of compound **2b** were 30 µg/disc and 31 µg/disc against C. neoformans and C. neoformans clinical isolates, respectively. Compound 2a displayed similar fungicidal effect with MIC values of 35 μ g/disc and 36 μ g/disc against C. neoformans and C. neoformans clinical isolates, respectively. By comparison, the MIC values of compounds 2a and 2b were 3-4 fold more potent than the reference drug fluconazole (MIC >128.0 μ g/mL). The MFC involves an additional set of steps performed once the MIC is determined. The antimicrobials are typically regarded as bactericidal/fungicidal if the MFC is not >4 times the MIC (18). The MFC values of compounds 2a and 2b were approximately 2× MIC over the range of 5264 μ g/disc in the case of *C. neoformans* and *C. neoformans* clinical isolates. These results indicated that compounds **2a** and **2b** may become lead molecules of antifungal drug against *C. neoformans*.

Cytotoxicities of compounds 2a and 2b

The synthesized compounds 2a and 2b were screened for their cytotoxicity against two cell lines (BV2 and 293T) using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cytotoxicity of DMSO was used as a control. The results are given in Figures 3 and 4. A similar tendency was observed for the cytotoxic activity of compounds 2a and 2b against the two cell lines. It was found that compounds 2a and 2b showed no cytotoxic activity at concentrations \leq 35 μ g/mL, which was not significantly different from fluconazole. This result indicated that compounds 2a and 2b did not affect the cell viability of the tested cell lines (BV2 and 293T) at their MIC values (35 and 30 µg/mL) against C. neoformans and MIC values (36 and 31 µg/mL) against the C. neoformans clinical strain, respectively. The relative survival rate of BV2 significantly decreased with compounds 2a and 2b at concentrations \geq 50.0 μ g/mL (p < 0.01). Compounds **2a** and **2b** showed significant cytotoxic effects against 293T at concentrations \geq 100.0 μ g/mL (p < 0.01). The results indicated that compounds 2a and 2b showed slightly lower cytotoxic activity against 293T than BV2. The disparity between the cytotoxicities and antimicrobial activities of compounds 2a and 2b suggested that these compounds exhibited their high in vitro antibacterial activities at noncytotoxic concentrations.

Table 4: MIC, MIC₈₀ and MFC values for compounds **2a**, **2b**, and fluconazole (µg/mL)

Fungal strain ^a	2a			2b			Fluconazole		
	MIC	MIC ₈₀	MFC	MIC	MIC ₈₀	MFC	MIC	MIC ₈₀	MFC
C. n	35.0	33.0	60.0	30.0	25.5	52.0	>128.0	2.0	>128.0
C. n. C.	36.0	35.0	64.0	31.0	28.0	54.0	>128.0	2.0	>128.0

^aC. n.: C. neoformans ATCC 32264; C. n. C.: C. neoformans clinical strain.



Figure 3: Effects of compounds 2a, 2b, and fluconazole on the survival of BV2. *Significantly different between compounds 2a, 2b and standard drug fluconazole.



Figure 4: Effects of compounds 2a, 2b, and fluconazole on the survival of 293T. *Significantly different between compounds 2a, 2b and standard drug fluconazole.

Structure-activity relationship study

The structure-activity relationship (SAR) was studied, considering the following: the nature of the substitution at the C-2 position, the length and size of the side chain at the C-3 position, and the electronic properties of the substitution of R_1 at the C-8 position.

First, the influences of the substituent at the C-2 position were explored. Generally, the compounds **2a–2d** with a 2-pyridyl group at the C-2 position exhibit higher antimicrobial activity than the compounds **2e–2g** with a phenyl group at the C-2 position. In this study, the presence of a phenyl moiety at the C-2 position induces only a slightly improvement of antimicrobial activity. However, the 2-pyridyl group at the C-2 position significantly increased the antimicrobial effectiveness, especially against *C. neoformans* and *C. neoformans* clinical isolates. The results suggest that the 2-

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pyridyl unit plays an important role in the antifungal activity against *C. neoformans* and *C. neoformans* clinical isolates.

The subsequent SAR analysis focused on the ester group at the C-3 positions of the seven-membered ring of 1,5benzodiazepine. Compounds 3, 4, and 5 have R₂ groups of -CH₃, -CH₂CH₂CH₃ and -CH(CH₃)₂ at the C-3 positions, respectively. Overall, compounds 3-5 displayed lower antimicrobial activity than compounds 2a-2d containing the -CH₂CH₃ group. A decrease or increase in the length of R₂ can result in low or no activity. Both in the $-CH_2CH_2CH_3$ series (compounds 4) and in the $-CH(CH_3)_2$ series (compounds 5), the activity generally decreases as the size of the side carbon chain increases. The results indicate that the substituent size appears to be an important factor for biological activity. This is presumably because the bulky groups at these positions hindered the crossing of the molecular over the bacterial cell wall or hindered its interaction with intracellular bacterial molecular (19).

In another variation, an SAR study was conducted to explore R₁ at the C-8 position of the 1.5-benzodiazepine. Hence, we planned to introduce electron withdrawing and donating groups at the C-8 position to study their influence on activity. As shown in Table 2, compounds **2–5** carrying R_1 with different electronic properties (-CH₃, -H, -F and -Br) exhibited different inhibitory activities. This indicated that the electronic properties of R1 exerted important influence on their antimicrobial activity. The antibacterial results lead to the assumption that the activity of the compounds was in the order of $R_1 = CH_3 > R_1 = -H > R_1 = -F > R_1 = -Br$. It should point out that the compounds (2b, 3b, 4b, and 5b) with a -CH₃ group at the R₁ position also display a broad-spectrum inhibitory effect against all the tested microorganisms. The electron-donating substituent $(R_1 = -CH_3)$ exhibited high inhibitory activity, indicating that -CH₃ group exert an enhanced positive influence on the antimicrobial activity. However, compounds with electron-withdrawing groups, such as fluorine and bromine (3d, 4c, 4d, 5c, and 5d) exhibited low to no activity. The results revealed that the electron withdrawing groups attached at the C-8 position reduce the activity. This loss in activity may be because the high electronegativity impedes the intracellular transport (20).

Thus, the various substituent groups exhibited diverse effects on the biological activity of the synthesized compounds. However, the effects on biological activity were caused by the joint action of these substituent groups.

Conclusion

In this study, 19 novel 1,5-benzodiazepine derivatives were synthesized and their biological activities were preliminarily evaluated *in vitro*. The synthesis scheme is sim-

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ple and has only a limited number of steps, making this economical process. The results of the primary *in vitro* antimicrobial bioactivity assay and the preliminary SAR analysis revealed that the 1,5-benzodiazepine derivatives containing pyridine ring had good antimicrobial activity. The results also showed that the $-COOC_2H_5$ group at the C-3 position is the best substituent for the maintenance of biological activities. In addition, the electronic properties of the R₁ at the C-8 position exert an important influence on the activity of this series of 1,5-benzodiazepines. Among all the synthesized compounds, **2b** showed the highest antimicrobial activity, and compounds **2a**, **2c**, **2d**, **3a**, **3b**, **3c**, and **4b** showed excellent antimicrobial activity.

Because the ester-4-methyl-2-(pyridin-2-yl)-2,5-dihydro-1,5-benzodiazepine -3-carboxylate represents a new type of 1,5-benzodiazepine with antimicrobial activity, further structural modifications and optimizations are required to obtain more information about the SARs. The results of this study will aid the design and development of new antimicrobial agents. Further evaluation of the *in vitro* antimicrobial activity properties of the compounds, particularly the mechanisms underlying their enhanced antimicrobial activity, should be performed in future investigations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Spectrum data of target compounds.