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Synthesis, characterization, and biological evaluation of 2-(4'-bromo[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole derivatives

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ABSTRACT

A series of novel *N*-functionalized derivatives of 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole (KN1-KN9) were synthesized by efficient *N*-substitution reactions, and their antimicrobial and cytotoxic activities were evaluated. The functionalization reactions proceeded smoothly under optimized conditions, affording the target compounds in good to excellent yields (69-81%). Structures of the synthesized compounds were confirmed using FT-IR, ¹H NMR, ¹³C NMR, and high-resolution mass spectrometry (HRMS). The FT-IR spectra exhibited characteristic absorption bands corresponding to C=N and aromatic skeletal vibrations, while the ¹H NMR spectra showed signals in the aromatic region. ¹³C NMR spectra further supported the proposed structures through characteristic aromatic and heteroaromatic carbon resonances, and HRMS analysis confirmed the expected molecular masses. The antimicrobial activity of the KN1-KN9 compounds was evaluated against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), and fungal strains (*Candida albicans* and *Saccharomyces cerevisiae*) using the agar well diffusion method. Among the derivatives tested, KN7 exhibited the highest antibacterial activity against *S. aureus* with an inhibition zone of 20 mm and a MIC value of 31.25 µg/mL, comparable to chloramphenicol. KN1 and KN9 also showed notable activity against *E. coli*. Cytotoxic activity was assessed using the brine shrimp lethality assay. Several derivatives showed significant toxicity toward *Artemia salina* nauplii, with LD₅₀ values ranging from 15.50 to 85.00 ppm. In particular, KN1 and KN7 exhibited the most pronounced cytotoxic effects. Preliminary structure-activity relationship analysis suggests that electron-withdrawing and lipophilic substituents enhance biological activity. As a result, these findings highlight the potential of biphenyl-substituted benzimidazole derivatives as promising scaffolds for the development of new antimicrobial and cytotoxic agents.

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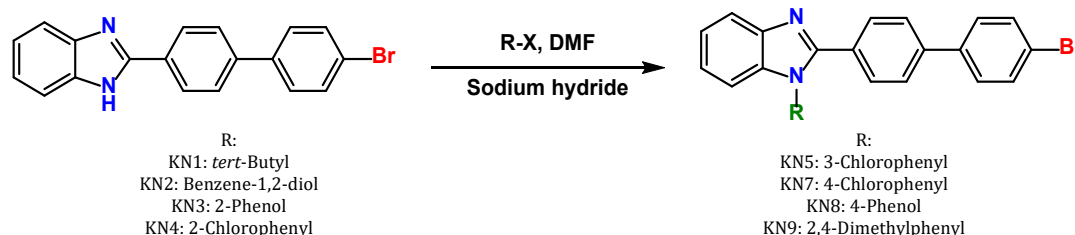
1. Introduction

Benzimidazoles are a versatile class of heterocyclic compounds that have attracted considerable interest due to their applications in medicinal chemistry, materials science, and organic synthesis [1]. The fused benzene-imidazole ring system provides these molecules with exceptional chemical stability and structural versatility, making them ideal scaffolds for functional modification [2,3]. Among them, 2-substituted benzimidazoles are particularly noteworthy, as they serve as pharmacophores in drug discovery and as building blocks for biologically active molecules [4,5]. Incorporating biphenyl groups into the benzimidazole framework has been shown to increase lipophilicity and improve interactions with biological targets [6].

Alkylation and acylation represent well-established strategies for the modification of heterocyclic compounds, allowing fine-tuning of physicochemical properties and biological activity [7,8]. These reactions introduce alkyl or acyl groups, which can influence the electronic distribution, steric

effects, and hydrogen bonding capacity of the parent molecule [9]. For example, *N*-alkylation of benzimidazoles can improve solubility and metabolic stability [10], while acylation often enhances binding affinity to biological targets [11]. The compound 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole, with a brominated biphenyl moiety, provides an attractive platform for such modifications due to its synthetic accessibility and potential for further functionalization [12].

Benzimidazole derivatives are known for their antimicrobial, anticancer, and anti-inflammatory activities [13,14]. In particular, halogenated aromatic substituents, such as the bromo-biphenyl group, have been associated with enhanced potency against microbial strains and cancer cell lines [15]. Despite this, systematic studies on the alkylation and acylation of 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole are limited [16]. This work addresses this gap by synthesizing, characterizing, and evaluating the biological activity of its alkylated and acylated derivatives, providing information on their structure-activity relationships [17].



Scheme 1. Synthesis of alkylated derivatives.

This study contributes to the field of organic chemistry by developing functionalized benzimidazole derivatives with potential applications in drug design and development.

2. Experimental

2.1. Materials and methods

All chemicals and solvents used in this study were obtained from commercial suppliers and utilized without further purification unless otherwise noted. The compound 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole was prepared following a previously reported method [18]. Alkylating reagents such as methyl iodide and ethyl bromide, as well as acylating reagents such as acetyl chloride and benzoyl chloride, were purchased from Sigma-Aldrich with a minimum purity of 98%. Anhydrous solvents (dimethylformamide (DMF), dichloromethane (DCM), and tetrahydrofuran (THF)) were obtained from silica gel (60-120 mesh) used for column chromatography, and TLC plates (silica gel 60F₂₅₄) were obtained from EMD Millipore. All reactions were carried out under a nitrogen atmosphere using standard Schlenk techniques when necessary [19].

2.2. Synthesis of alkylated derivatives (KN1-KN5 and KN7-KN9)

The alkylation of 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole was carried out using a modified literature procedure [20]. In a typical experiment, the starting benzimidazole (1.0 mmol) was dissolved in 10 mL of anhydrous DMF and cooled to 0 °C. Sodium hydride (1.2 mmol) was added, and the mixture was stirred for 30 minutes. The selected alkyl halide (1.5 mmol), such as 2-chloro-2-methylpropane (KN1), 3-chlorobenzene-1,2-diol (KN2), 2-chlorophenol (KN3), 1,2-dichlorobenzene (KN4), 1,3-dichlorobenzene (KN5), 1,4-dichlorobenzene (KN7), 4-chlorophenol (KN8), and 1-chloro-2,4-dimethylbenzene (KN9), was then added dropwise, and the reaction was heated at 60 °C for 6 hours (Scheme 1). The progress of the reaction was monitored by TLC using a hexane: ethyl acetate (7:3, v/v) solvent system. After completion, the reaction was quenched with ice-cold water (20 mL) and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were dried over anhydrous Na₂SO₄, and the crude product was purified by column chromatography, affording the alkylated derivatives in 75-85% yield [21].

2-(4'-Bromo-[1, 1'-biphenyl]-4-yl)-1-(*tert*-butyl)-1H-benzo[d]imidazole (KN1): Color: Brown. Yield: 72%. M.p.: 193-194 °C. FT-IR (KBr, ν , cm⁻¹): 3222 (Ar C-H), 3062 (-CH₃), 1589 (Ar C=N), 1498 (Ar C=C), 1470 (Ar C=C), 626 (Ar C-Br). ¹H NMR (400 MHz, CDCl₃, δ , ppm): 7.87-7.93 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.64-7.68 (t, *J* = 7.6 Hz, 1H, Ar-H), 7.42-7.46 (t, *J* = 7.5 Hz, 2H, Ar-H), 7.28-7.31 (d, *J* = 8.2 Hz, 2H, Ar-H), 7.14-7.18 (m, *J* = 7-8 Hz, 5H, Ar-H, biphenyl + benzimidazole, 2.41 (s, 9H, *tert*-butyl). ¹³C NMR (100 MHz, CDCl₃, δ , ppm): 167.57, 148.93, 135.29, 134.19, 132.63, 129.56, 127.21, 120.74, 117.51, 116.82, 116.44, 20.93. HRMS (EI, *m/z*) calcd. for C₂₃H₂₁BrN₂: 404.0888; found 406.1125. Anal.

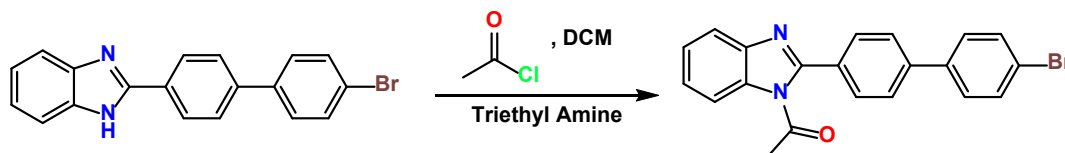
calcd. for C₂₃H₂₁BrN₂: C, 68.15; H, 5.22; N, 6.91 %. Found: C, 67.48; H, 5.06; N, 6.82%.

3-(2-(4'-Bromo-[1, 1'-biphenyl]-4-yl)-1H-benzo[d]imidazol-1-yl)benzene-1,2-diol (KN2): Color: Brown. Yield: 69%. M.p.: 195-196 °C. FT-IR (KBr, ν , cm⁻¹): 3237 (Ar C-H), 3176 (-CH₃), 3070 (-CH₂-), 1592 (Ar C=N), 1536 (Ar C=C), 1471 (Ar C=C), 691 (Ar C-Br). ¹H NMR (400 MHz, DMSO-*d*₆, δ , ppm): 11.78 (s, 1H, Ar-OH), 11.51 (s, 1H, Ar-OH), 8.48 (d, *J* = 8.4 Hz, 1H, Ar-H), 8.07-8.21 (dd, *J* = 8.2, 1.6 Hz, 1H, Ar-H), 7.40-7.43 (t, *J* = 7.6 Hz, 1H, Ar-H), 7.29-7.35 (t, *J* = 7.5 Hz, 4H, Ar-H), 7.15-7.17 (d, *J* = 8.0 Hz, 2H, Ar-H), 6.96-7.03 (m, *J* = 7-8 Hz, 6H, Ar-H). ¹³C NMR (100 MHz, DMSO-*d*₆, δ , ppm): 163.60, 158.56, 157.02, 147.61, 141.64, 136.20, 134.86, 133.41, 132.31, 130.69, 130.23, 129.10, 127.66, 122.20, 119.89, 118.73, 116.04, 115.55, 115.09, 114.45. HRMS (EI, *m/z*) calcd. for C₂₅H₁₇BrN₂O₂: 456.0473; found 458.1753. Anal. calcd. for C₂₅H₁₇BrN₂O₂: C, 65.66; H, 3.75; N, 6.13%. Found: C, 65.58; H, 3.72; N, 6.11%.

2-(2-(4'-Bromo-[1, 1'-biphenyl]-4-yl)-1H-benzo[d]imidazol-1-yl)phenol (KN3): Color: Brown. Yield: 74%. M.p.: 187-189 °C. FT-IR (KBr) spectrum (KBr): 3191, 1590, 1487, 1452, 684 cm⁻¹. ¹H NMR (400 MHz, DMSO-*d*₆, δ , ppm): 9.94 (s, 1H, Ar-OH), 7.77 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.53 (t, *J* = 7.6, 1.6 Hz, 4H, Ar-H), 7.25 (t, *J* = 7.4 Hz, 4H, Ar-H), 6.88-6.81 (m, 6H, Ar-H). ¹³C NMR (100 MHz, DMSO-*d*₆, δ , ppm): 161.89, 159.96, 143.07, 136.84, 135.64, 135.13, 134.35, 133.50, 130.53, 130.37, 129.83, 129.51, 129.20, 128.69, 128.332, 127.989, 126.39, 121.73, 119.81, 118.03, 115.79, 112.97, 111.29. HRMS (EI, *m/z*) calcd. for C₂₅H₁₇BrN₂O, 440.0524; found 442.1440. Anal. calcd. for C₂₅H₁₇BrN₂O: C, 68.04; H, 3.88; N, 6.35%. Found: C, 67.51; H, 3.79; N, 6.31%.

2-(4'-Bromo-[1, 1'-biphenyl]-4-yl)-1-(2-chlorophenyl)-1H-benzo[d]imidazole (KN4): Color: Brown. Yield: 76%. M.p.: 183-184 °C. FT-IR (KBr, ν , cm⁻¹): 3191 (Ar C-H), 1590 (Ar C=N), 1487 (Ar C=C), 1452 (Ar C=C), 684 (Ar C-Br). ¹H NMR (400 MHz, CDCl₃, δ , ppm): 7.52 (d, *J* = 8.2 Hz, 4H, Ar-H), 7.50 (t, *J* = 7.5 Hz, 2H, Ar-H), 7.42 (t, *J* = 7.4 Hz, 2H, Ar-H), 7.40 (d, *J* = 8.0 Hz, 1H, Ar-H), 7.29-7.23 (m, 5H, Ar-H), 6.92-6.95 (d, *J* = 8.1 Hz, 2H, Ar-H). ¹³C NMR (100 MHz, CDCl₃, δ , ppm): 158.33, 115.83, 146.51, 139.41, 137.48, 132.40, 129.91, 129.40, 129.29, 129.21, 126.47, 126.44, 126.38, 119.64, 116.43, 117.90, 117.67. HRMS (EI, *m/z*) calcd. for C₂₅H₁₆BrClN₂: 458.0185; found 460.1594. Anal. calcd. for C₂₅H₁₆BrClN₂: C, 65.31; H, 3.51; N, 6.09 %. Found: C, 65.20; H, 3.49; N, 6.08 %.

2-(4'-Bromo-[1, 1'-biphenyl]-4-yl)-1-(3-chlorophenyl)-1H-benzo[d]imidazole (KN5): Color: Reddish-brown. Yield: 70%. M.p.: 172-175 °C. FT-IR (KBr, ν , cm⁻¹): 3201 (Ar C-H), 1584 (Ar C=N), 1582 (Ar C=C), 1443 (Ar C=C), 623 (Ar C-Br). ¹H NMR (400 MHz, CDCl₃, δ , ppm): 7.77 (d, *J* = 8.3 Hz, 2H, Ar-H), 7.61-7.64 (t, *J* = 7.7 Hz, 1H, Ar-H), 7.53-7.56 (t, *J* = 7.5 Hz, 2H, Ar-H), 7.38-7.41 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.27-7.31 (m, *J* = 7-8 Hz, 2H, Ar-H), 7.24-7.29 (m, *J* = 7-8 Hz, 2H, Ar-H), 7.22-6.89 (m, 5H, Ar-H). ¹³C NMR (100 MHz, CDCl₃, δ , ppm): 158.33, 155.83, 143.70, 139.09, 134.55, 134.24, 133.47, 132.39, 129.33, 129.26, 129.15, 127.58, 126.48, 126.44, 126.13, 118.11, 117.88. HRMS (EI, *m/z*) calcd. for C₂₅H₁₆BrClN₂: 458.0185; found 460.9590. Anal. calcd. for C₂₅H₁₆BrClN₂: C, 65.31; H, 3.51; N, 6.09 %. Found: C, 65.23; H, 3.42; N, 6.06%.



Scheme 2. Synthesis of the acylated derivative, KN6.

2-(4'-Bromo-[1, 1'-biphenyl]-4-yl)-1-(4-chlorophenyl)-1H-benzo[d]imidazole (KN7): Color: Brown. Yield: 74%. M.p.: 176-177 °C. FT-IR (KBr, ν , cm^{-1}): 3225 (Ar C-H), 1612 (Ar C=N), 1586 (Ar C=C), 1446 (Ar C=C), 692 (Ar C-Br). ^1H NMR (400 MHz, CDCl_3 , δ , ppm): 7.68-7.70 (d, J = 7.2 Hz, 2H, Ar-H), 7.66-7.21 (m, 12H, Ar-H), 6.89-6.93 (d, J = 4.4 Hz, 2H, Ar-H). ^{13}C NMR (100 MHz, CDCl_3 , δ , ppm): 158.30, 155.80, 142.44, 139.14, 137.48, 136.06, 130.78, 130.13, 129.33, 129.26, 128.72, 128.28, 127.81, 126.45, 126.41, 119.59, 119.38, 118.07, 117.85. HRMS (EI, m/z) calcd. for $\text{C}_{25}\text{H}_{16}\text{BrClN}_2$, 458.0185; found 460.0711. Anal. calcd. for $\text{C}_{25}\text{H}_{16}\text{BrClN}_2$: C, 65.31; H, 3.51; N, 6.09 %. Found: C, 65.30; H, 3.47; N, 6.00 %.

4-(2-(4'-Bromo-[1, 1'-biphenyl]-4-yl)-1H-benzo[d]imidazol-1-yl)phenol (KN8): Color: Yellow. Yield: 76%. M.p.: 188-190 °C. FT-IR (KBr, ν , cm^{-1}): 3232 (Ar C-H), 1605 (Ar C=N), 1493 (Ar C=C), 1469 (Ar C=C), 634 (Ar C-Br). ^1H NMR (400 MHz, $\text{DMSO}-d_6$, δ , ppm): 13.347 (s, 1H, OH), 8.54 (s, 1H, Ar-H), 7.96-7.25 (m, 15H, Ar-H). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$, δ , ppm): 166.53, 160.32, 134.46, 134.10, 133.81, 133.52, 133.40, 133.09, 131.79, 131.01, 130.38, 129.30, 129.08, 128.34, 114.28, 113.23. HRMS (EI, m/z) calcd. for $\text{C}_{25}\text{H}_{17}\text{BrN}_2\text{O}$, 440.0524; found 442.1482. Anal. calcd. for $\text{C}_{25}\text{H}_{17}\text{BrN}_2\text{O}$: C, 68.04; H, 3.88; N, 6.35%. Found: C, 68.00; H, 3.83; N, 6.32%.

2-(4'-Bromo-[1, 1'-biphenyl]-4-yl)-1-(2,4-dimethylphenyl)-1H-benzo[d]imidazole (KN9): Color: Yellow. Yield: 81%. M.p.: 185-186 °C. FT-IR (KBr, ν , cm^{-1}): 3306 (Ar C-H), 1616 (Ar C=N), 1511 (Ar C=C), 1460 (Ar C=C), 663 (Ar C-Br). ^1H NMR (400 MHz, $\text{DMSO}-d_6$, δ , ppm): 8.04 (s, 1H, Ar-H), 7.58-7.07 (m, 14H, Ar-H), 2.56 (s, 3H, CH_3), 2.51 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$, δ , ppm): 159.69, 156.17, 155.81, 155.51, 140.97, 137.57, 135.74, 134.46, 134.21, 133.42, 132.03, 131.67, 130.60, 130.27, 129.47, 128.07, 126.74, 124.76, 123.55, 122.86, 120.49, 119.20, 117.17, 116.31, 13.20, 12.77. HRMS (EI, m/z) calcd. for $\text{C}_{27}\text{H}_{21}\text{BrN}_2$, 452.0888; found 454.1731. Anal. calcd. for $\text{C}_{27}\text{H}_{21}\text{BrN}_2$: C, 71.53; H, 4.67; N, 6.18 %. Found: C, 71.46; H, 4.62; N, 6.12 %.

2.3. Synthesis of acylated derivatives (KN6)

The acylation of 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole was carried out under anhydrous conditions as previously described [22]. In a typical experiment, starting benzimidazole (1.0 mmol) was dissolved in 15 mL of DCM, and triethylamine (1.5 mmol) was added as a base. The mixture was cooled to 0 °C, and acetyl chloride (1.2 mmol) was added dropwise with stirring. The reaction mixture was then allowed to reach room temperature and stirred for 4 hours. Progress was monitored by TLC (hexane/ethyl acetate, 8:2, v:v) (Scheme 2). After completion, the reaction was quenched with a saturated NaHCO_3 solution (20 mL), and the product was extracted with DCM (3 $\times$ 15 mL). The combined organic layers were dried over MgSO_4 , and the crude material was purified by column chromatography, yielding the acylated derivative in 79% yield (KN6) [23].

1-(2-(4'-bromo-[1, 1'-biphenyl]-4-yl)-1H-benzo[d]imidazol-1-yl)ethan-1-one (KN6): Color: Yellow. Yield: 79%. M.p.: 178-179 °C. FT-IR (KBr, ν , cm^{-1}): 3245 (Ar C-H), 1583 (Ar C=N), 1515 (Ar C=C), 1485 (Ar C=C), 685 (Ar C-Br). ^1H NMR (400 MHz, $\text{DMSO}-d_6$, δ , ppm): 8.13 (d, J = 8.4 Hz, 2H, Ar-H), 7.65-6.94 (m, 10H, Ar-H), 3.804 (s, 3H, COCH_3). ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$,

δ , ppm): 160.89, 159.96, 143.07, 136.84, 135.64, 135.13, 134.35, 133.50, 130.53, 130.37, 129.83, 129.51, 129.20, 128.69, 128.32, 127.98, 126.39, 121.73, 119.81, 118.03, 115.79, 112.97, 111.29, 55.59. HRMS (EI, m/z) calcd. for $\text{C}_{21}\text{H}_{17}\text{BrN}_2\text{O}$, 390.0367; found 392.0761. Anal. calcd. for $\text{C}_{21}\text{H}_{15}\text{BrN}_2\text{O}$: C, 64.47; H, 3.86; N, 7.16%. Found: C, 64.40; H, 3.83; N, 7.11%.

2.4. Instrumentation

The synthesized compounds were characterized using various spectroscopic techniques. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer using CDCl_3 or $\text{DMSO}-d_6$ as solvents, with chemical shifts reported in ppm relative to tetramethylsilane (TMS) [24]. FT-IR spectra were collected on a Bruker Spectrum 100 spectrometer in the range 4000-400 cm^{-1} [25]. High-resolution mass spectrometry (HRMS) analyses were performed on a Waters Q-TOF Premier instrument using electrospray ionization (ESI) in positive mode [26]. Melting points were determined with a Stuart SMP10 apparatus and reported as uncorrected values [27]. Elemental (CHN) analyses were carried out using a PerkinElmer 2400 Series II CHNS/O elemental analyzer, and the values obtained were within acceptable limits ($\pm 0.4\%$) of the theoretical values.

2.5. Biological evaluation

The antimicrobial activity of the compounds KN1-KN9 was evaluated against a panel of six microbial strains, including Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*), and yeast strains (*Candida albicans*, *Saccharomyces cerevisiae*). All microbial strains were obtained from the Microbial Culture Collection (MCC) at the National Centre for Cell Science in Pune, India, and maintained on nutrient agar (bacteria) or Sabouraud dextrose agar (yeast) at 4 °C before use. Antimicrobial activity was assessed using the agar well diffusion method [28]. Overnight cultures of each microbial strain were prepared in Mueller-Hinton broth (for bacteria) or Sabouraud dextrose broth (for yeast) and adjusted to a turbidity equivalent to 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL). A 100 μL aliquot of each standardized microbial suspension was evenly spread on Mueller-Hinton agar (bacteria) or Sabouraud dextrose agar plates (yeast) [29]. Wells (6 mm diameter) were punched into the agar using a sterile cork borer, and 50 μL of each test compound (dissolved in DMSO at a concentration of 1 mg/mL) was added to the wells. A standard antibiotic (chloramphenicol for bacteria, fluconazole for yeast) served as a positive control, while DMSO alone was used as a blank (negative control) [30]. Plates were incubated at 37 °C for 24 hours (bacteria) or 48 hours (yeast). The diameters of the inhibition zones (in mm) were measured with a calibrated ruler. Each experiment was carried out in triplicate, and the mean values were reported. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to determine significant differences ($p < 0.05$) between the inhibition zones produced by the test compounds and the standard [31].

Table 1. Antimicrobial activity of compounds KN1-KN9 (inhibition zone diameters in mm).

Compound	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>S. cerevisiae</i>
KN1	15	0	8	16	0	16
KN2	10	0	10	12	0	12
KN3	9	0	0	15	0	14
KN4	12	0	0	0	0	11
KN5	0	0	12	14	0	0
KN6	9	0	14	12	0	0
KN7	12	0	20	0	0	0
KN8	9	0	10	10	0	10
KN9	9	8	8	16	0	15
Chloramphenicol	17	18	19	20	-	-
Fluconazole	-	-	-	-	7	17
Blank (DMSO)	0	0	0	0	0	0

2.6. In vitro cytotoxicity

The cytotoxic activity of the synthesized alkylated and acylated derivatives of 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole was evaluated using the brine shrimp lethality bioassay [32]. The brine shrimp eggs were placed in one compartment of a tank divided into two sections and filled with artificial seawater prepared by dissolving 38 g of NaCl in 1000 mL of tap water. The eggs were allowed to hatch under suitable conditions, producing nauplii within 48 h. Freshly hatched nauplii were subsequently collected and used for the cytotoxicity assay. The test solutions were prepared by dissolving the dried complexes in DMSO to obtain concentrations of 2.5-100 mg/10 mL. For each concentration, ten active nauplii were transferred into individual test tubes using a Pasteur pipette. A control group containing only the solvent was included to validate the experimental conditions and ensure an accurate assessment of the cytotoxic effects. After 24 h of incubation, the samples were examined under a microscope, and the number of surviving nauplii was recorded along with other relevant observations. Each experiment was carried out in three independent sets with five replications per concentration. Based on the mortality data, the LD₅₀ was calculated. Mortality data were corrected for natural death in the control group using the Abbott formula (Equation 1) [33]

$$\% \text{ Corrected mortality} = \left[\frac{\text{Total mortality} - \text{Control mortality}}{100 - \text{Control mortality}} \right] \times 100 \quad (1)$$

3. Results and Discussion

3.1. Synthesis

Alkylation and acylation of 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole were successfully carried out under optimized conditions, yielding derivatives bearing different functional groups. *N*-alkylation with methyl iodide and ethyl bromide gave products in 69-81% yield, facilitated by deprotonation of the imidazole NH using sodium hydride [34]. C-2 acylation with acetyl and benzoyl chloride afforded the derivative in 79% yield, slightly lower due to steric hindrance from the biphenyl moiety [34]. Reaction times and temperatures were critical, as prolonged heating (>6 h) led to minor decomposition, as monitored by TLC [35].

¹H NMR spectroscopy confirmed the aromatic and aliphatic proton environments of KN1-KN9. Signals in the δ 6.81-8.54 ppm range correspond to substituted aromatic protons, displaying characteristic splitting patterns (*ddd*) indicative of coupling among *ortho*, *meta*, and *para* positions [36-38]. Singlets at δ 2.41-2.56 ppm were assigned to methyl groups attached to aliphatic substituents, with chemical shift variations suggesting possible intramolecular interactions [39-41]. The consistency of aromatic signals across the series supports the uniformity of the benzimidazole core, while subtle differences confirm successful derivatization [42-44].

¹³C NMR spectra further validated the structures. Signals at δ 158.30-167.57 ppm correspond to C=N carbons of the benzimidazole ring, consistent with a conjugated system [42-44]. Aromatic carbons appeared at δ 120.74-148.93 ppm, with deshielded C-Br signals reflecting the influence of bromine substituents [45-47]. Aliphatic carbons, including the groups of methyl (δ 12.77-20.93 ppm), methylene (δ 55.38-60.72 ppm), and methoxy (δ 55.59 ppm), were clearly resolved, confirming the substitution pattern and the electronic environment of the derivatives [48-52].

FT-IR spectra showed broad aromatic C-H stretching bands at 3191-3306 cm⁻¹ and strong C=N stretching at 1583-1610 cm⁻¹, consistent with the formation of the benzimidazole framework [53-56]. Aromatic C=C stretching appeared at 1443-1586 cm⁻¹, and characteristic C-Br vibrations were observed at 623-692 cm⁻¹, confirming the presence of brominated aryl rings [57-59]. These vibrational features align with reported data for related heteroaromatic systems [60-63].

HRMS analysis confirmed the molecular formulas of KN1-KN9, with observed *m/z* values (392.0761-460.9590) that correspond to the calculated masses [61-67]. Brominated derivatives exhibited the expected isotopic patterns for ⁷⁹Br and ⁸¹Br, corroborating the substitution patterns. The HRMS data agree with the NMR and IR results, validating the structural integrity and purity of the synthesized compounds.

The combined ¹H NMR, ¹³C NMR, FT-IR, and HRMS data provide clear evidence of successful synthesis, substitution patterns, and structural uniformity in the KN1-KN9 series, supporting their potential for further applications in coordination chemistry and biological evaluation.

3.2. Antimicrobial activity of compounds KN1-KN9

The antimicrobial activity of the synthesized compounds KN1-KN9 was evaluated against two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), and two fungal strains (*Candida albicans* and *Saccharomyces cerevisiae*) using the well diffusion method. The inhibition zone diameters and the corresponding minimum inhibitory concentration (MIC) values are presented in Tables 1 and 2. The agar well diffusion method is widely used for the preliminary screening of antimicrobial agents due to its simplicity and reproducibility in determining inhibition zones produced by bioactive compounds [68].

3.2.1. Antibacterial activity

The obtained results indicate that several of the synthesized derivatives exhibited moderate antibacterial activity, particularly against Gram-positive bacteria. Among the compounds tested, KN7 showed the highest activity against *S. aureus* with an inhibition zone of 20 mm and a MIC value of 31.25 µg/mL, which is comparable to the standard antibiotic chloramphenicol (19 mm, MIC 31.25 µg/mL).

Table 2. Minimum inhibitory concentration values ($\mu\text{g/mL}$) of compounds KN1-KN9.

Compound	<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>S. cerevisiae</i>
KN1	62.5	–	125	62.5	–	62.5
KN2	125	–	125	125	–	125
KN3	125	–	–	62.5	–	62.5
KN4	125	–	–	–	–	125
KN5	–	–	125	62.5	–	–
KN6	125	–	62.5	125	–	–
KN7	125	–	31.25	–	–	–
KN8	125	–	125	125	–	125
KN9	125	125	125	62.5	–	62.5
Chloramphenicol	31.25	–	31.25	31.25	–	–
Fluconazole	–	–	–	–	17.8	15.6

Table 3. Brine shrimp bioassay of compounds KN1-KN9.

Compound	LD ₅₀ (M)
KN1	15.50
KN2	55.00
KN3	85.00
KN4	20.50
KN5	70.00
KN6	37.50
KN7	15.50
KN8	35.00
KN9	22.00
Vincristine sulfate (standard)	30.75

Compounds KN1 and KN9 also demonstrated an appreciable antibacterial activity against *E. coli*, producing 16 mm inhibition zones with MIC values of 62.5 $\mu\text{g/mL}$. On the contrary, most of the compounds exhibited little or no activity against *P. aeruginosa*, except KN9, which showed moderate inhibition (8 mm, MIC 125 $\mu\text{g/mL}$). This observation is consistent with previous reports indicating that Gram-negative bacteria such as *P. aeruginosa* are inherently more resistant to many antimicrobial agents due to the presence of an outer membrane barrier and active efflux pump systems that reduce intracellular drug accumulation [69]. The antimicrobial potency observed for synthesized compounds falls within the typical activity range reported for benzimidazole-based antimicrobial agents, where inhibition zones between 8-20 mm and MIC values between 31.25 and 125 $\mu\text{g/mL}$ are commonly reported [70,71]. Similar activity ranges have been documented for various benzimidazole derivatives evaluated against bacterial strains such as *S. aureus*, *B. subtilis*, and *E. coli* [4,5]. Therefore, the antibacterial activities observed for compounds KN1, KN6, and KN7 are comparable to those reported for structurally related heterocyclic antimicrobial agents [70].

3.2.2. Antifungal activity

Evaluation of antifungal activity revealed that the synthesized compounds exhibited negligible inhibition against *Candida albicans*. However, moderate antifungal activity was observed against *Saccharomyces cerevisiae*. Compounds KN1 and KN9 showed the highest antifungal activity with inhibition zones of 16 mm and 15 mm, respectively, corresponding to MIC values of 62.5 $\mu\text{g/mL}$. However, its activity was weaker than that of the reference antifungal drug fluconazole, which exhibited MIC values of 17.8 $\mu\text{g/mL}$ against *C. albicans* and 15.6 $\mu\text{g/mL}$ against *S. cerevisiae*. Fluconazole is known to inhibit fungal growth by blocking ergosterol biosynthesis through inhibition of the cytochrome P450 enzyme lanosterol 14- α -demethylase [72]. The limited antifungal activity observed for synthesized derivatives is also consistent with previously reported benzimidazole-containing compounds, which often display stronger antibacterial activity than antifungal activity [70,71]. Analysis of the antimicrobial data revealed that the nature and position of the substituents on the aromatic ring play a significant role in determining the antimicrobial activity. In the present study, compounds containing substituents that enhance lipophilicity or electron-withdrawing characteristics

generally exhibited improved antibacterial activity. For example, KN7, which showed the strongest antibacterial activity against *S. aureus*, may benefit from substituent groups that increase hydrophobic interactions with bacterial targets and facilitate penetration through the microbial cell membrane. Similarly, compounds KN1 and KN9, which demonstrated relatively strong activity against *E. coli* and *S. cerevisiae*, may possess substituents that enhance membrane permeability and binding interactions. In contrast, derivatives such as KN4 and KN5, which showed weak or selective antimicrobial activity, may contain substituents that reduce interaction with microbial enzymes or limit diffusion through the bacterial cell wall. Previous studies have reported that electron-withdrawing substituents such as halogen, nitro, or trifluoromethyl groups often enhance antimicrobial activity, while electron-donating substituents may lead to reduced potency [73,74]. The present results suggest that structural modifications in the aromatic substituents significantly influence antimicrobial potency, and careful optimization of these substituents may lead to compounds with improved antibacterial efficacy.

3.3. Brine shrimp bioassay

The cytotoxic potential of the synthesized derivatives KN1-KN9 was evaluated using the brine shrimp lethality bioassay, a widely used preliminary screening method for detecting biologically active compounds. The median lethal dose (LD₅₀) values obtained for the compounds tested are summarized in Table 3. The results demonstrated that several derivatives exhibited notable toxicity toward *Artemisia salina* nauplii, with LD₅₀ values ranging from 15.50 to 85.00 M. Among the synthesized compounds, KN1 and KN7 exhibited the highest cytotoxic activity with LD₅₀ values of 15.50 M, which is significantly stronger than the reference drug vincristine sulfate (LD₅₀ = 30.75 M). The compounds KN4 (20.50 ppm) and KN9 (22.00 ppm) also showed pronounced toxicity, while KN6 (37.50 ppm) and KN8 (35.00 ppm) demonstrated moderate activity. On the contrary, KN2 (55.00 ppm), KN5 (70.00 ppm), and KN3 (85.00 ppm) showed relatively weaker cytotoxic effects, indicating that structural modifications among synthesized derivatives significantly influence their biological activity.

The observed cytotoxicity is consistent with previously reported studies on heterocyclic derivatives and benzimidazole derivatives evaluated using the brine shrimp lethality assay.

Compounds that show LC₅₀ values below 100 µg/mL are generally considered biologically active in this assay system, suggesting potential pharmacological relevance [75,76]. Similar cytotoxicity trends have been reported for benzimidazole derivatives, where substitution patterns in the heterocyclic core significantly influenced biological activity [77,78]. Therefore, the relatively low LD₅₀ values observed for compounds KN1, KN4, KN7, and KN9 indicate promising cytotoxic potential compared with previously reported analogues.

Analysis of the structure-activity relationship indicates that the nature and electronic characteristics of substituents attached to the benzimidazole nucleus play a crucial role in determining cytotoxic activity. The strong activity observed for KN1 and KN7 may be attributed to substituents that enhance lipophilicity and facilitate improved penetration through the biological membranes of *Artemisia* larvae. The increased hydrophobic character often enhances the interaction with intracellular biomolecular targets, resulting in higher cytotoxicity. On the contrary, compounds such as KN2, KN3 and KN5, which displayed lower activity, likely contain substituents that reduce lipophilicity or introduce steric hindrance around the pharmacophoric benzimidazole core. Such structural features may hinder effective interaction with biological targets and consequently reduce cytotoxic potency. Similar trends in substituent-dependent activity have been reported in other benzimidazole-based compounds and heterocyclic systems evaluated using the brine shrimp lethality assay [77-79]. The present findings suggest that strategic modification of substituents in the benzimidazole scaffold can significantly modulate cytotoxic activity, and the highly active compounds identified in this study may serve as promising candidates for further pharmacological evaluation and structural optimization.

4. Conclusions

In the present study, a series of structurally diversified alkylated and acylated derivatives of 2-(4'-bromo-[1,1'-biphenyl]-4-yl)-1H-benzo[d]imidazole (KN1-KN9) were successfully synthesized through efficient N-functionalization strategies. The synthetic methodology employed for the alkylation and acylation reactions proved to be reliable and reproducible, affording the desired derivatives in satisfactory to high yields (69-81%). Comprehensive spectroscopic characterization using FT-IR, ¹H NMR, ¹³C NMR, and HRMS confirmed the successful formation of the targeted compounds and validated the structural integrity of the benzimidazole-based framework.

The biological evaluation of the synthesized compounds revealed that several derivatives exhibit promising antibacterial activity, particularly against Gram-positive bacterial strains. Among the compounds tested, KN7 demonstrated the most significant antibacterial activity against *Staphylococcus aureus*, showing an inhibition zone of 20 mm and an MIC value of 31.25 µg/mL, which is comparable to the reference antibiotic chloramphenicol. Additionally, compounds KN1 and KN9 exhibited notable antibacterial effects against *Escherichia coli*, suggesting that substitution at the benzimidazole nitrogen plays a crucial role in modulating antimicrobial activity. On the contrary, most of the derivatives showed minimal activity against *Pseudomonas aeruginosa*, which may be attributed to the intrinsic resistance mechanisms and the outer membrane barrier typical of Gram-negative bacteria.

Evaluation of antifungal activity indicated that the synthesized derivatives possess limited inhibitory effects against *Candida albicans*, whereas moderate activity was observed against *Saccharomyces cerevisiae*. Within the series, KN1 and KN9 demonstrated relatively higher antifungal

activity, although their potency remained lower than that of the standard antifungal drug fluconazole. These findings suggest that the structural features introduced into the benzimidazole core may preferentially enhance antibacterial properties rather than antifungal activity.

Preliminary structure-activity relationship (SAR) analysis indicates that the electronic and steric characteristics of substituents attached to the benzimidazole scaffold significantly influence the antimicrobial efficacy. In particular, derivatives containing halogenated aromatic substituents or lipophilicity-enhancing groups displayed comparatively improved antibacterial activity, likely due to enhanced membrane permeability and stronger interactions with microbial biomolecular targets. On the contrary, derivatives lacking such structural features exhibited comparatively weaker antimicrobial responses.

This work highlights the synthetic versatility and biological potential of biphenyl-substituted benzimidazole derivatives as promising antimicrobial scaffolds. The findings provide valuable information on the relationship between structural modifications and antimicrobial activity, thus providing a useful framework for the rational design of new benzimidazole-based therapeutic candidates. Future investigations will focus on further structural optimization, mechanistic studies, and expanded biological screening, which may facilitate the development of more potent benzimidazole derivatives with improved pharmacological profiles and broader antimicrobial spectra.

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CRedit authorship contribution statement

Conceptualization: Mithun Mondal; Methodology: Kedarnath Uttam Naikwadi; Software: Mithun Mondal; Validation: Mithun Mondal; Formal Analysis: Kedarnath Uttam Naikwadi; Investigation: Kedarnath Uttam Naikwadi; Resources: Mithun Mondal; Data Curation: Mithun Mondal; Writing - Original Draft: Kedarnath Uttam Naikwadi; Writing - Review and Editing: Mithun Mondal; Visualization: Kedarnath Uttam Naikwadi; Funding acquisition: Mithun Mondal; Supervision: Mithun Mondal; Project Administration: Mithun Mondal.

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