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Research Article

Synthesis of Novel 6-Acylamino-Benzimidazole Derivatives Derived from Carbendazim

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ABSTRACT

Benzimidazole derivatives possess potential biological activity, and modern methods are required for the synthesis of new derivatives in high yields. The aim of this study was to obtain new benzimidazole derivatives through acylation of carbendazim (**1**) with aliphatic carboxylic acids, then carry out the reactions of nitration, reduction and acylation of acyl-derivatives, and studying chemical structure and cytotoxic activity of the obtained compounds. The resulting acyl compounds (**2** and **3**) were nitrated to synthesize 6-nitro derivatives (**4** and **5**) in high yields. Their reduction gave 6-aminobenzimidazole derivatives (**6** and **7**), respectively. The yield of the synthesized compound (**6**) was 72.1%, and that of compound (**7**) was 93%. Compounds (**6**) and (**7**) were acylated with aliphatic carboxylic acids to obtain bis-acyl products. It is worth noting that selective acylation of the amino group at position 6 of the benzimidazole molecule led to the formation of amides (**8**-**14**) in 79-92% yields. Only the reaction of compound (**6**) with glacial acetic acid led to the formation of triacetamide (**8**) in 92% yield. The synthesized all compounds were characterized by ^{1}H, ^{13}C NMR, IR spectral data. Additionally, synthesized benzimidazole derivatives were evaluated for their cytotoxic activity against triple-negative breast cancer cell lines (BT-20, MDA-MB-231, HCC1395) and the noncancerous HEK293 line using the MTT assay. Most compounds showed low cytotoxicity (IC_{50} > 50 μM), while derivatives (**9**) and (**10**) exhibited moderate activity suggesting that the presence of ethyl (**9**) or propyl (**10**) groups at position 2 of the imidazole ring influences the anticancer potential.

Keywords: benzimidazole derivatives; carbendazim; acylation; aliphatic carboxylic acids; nitration; reduction; acyl products

1. INTRODUCTION

Among heterocyclic compounds, benzimidazole and its derivatives are of particular importance in pharmaceutical practice due to their chemical structure and biological activity. Therefore, these compounds have attracted widespread attention from synthetic chemists and pharmaceutical researchers, as they serve as important structural frameworks for the design and development of new drugs (Lee et al., 2023). Benzimidazoles are of considerable interest due to the presence of reactive tautomeric forms in their molecules, which under certain conditions can transform into more labile tautomers. Benzimidazole is a bicyclic heteroaromatic organic compound, consisting of a benzene ring and an imidazole ring, which enable chemists to perform targeted electrophilic and nucleophilic substitution or addition reactions (Faheem et al., 2020). At the same time, 2,2′-bis-benzimidazoles and 2,5- or 2,6-disubstituted benzimidazoles are of particular interest, as these disubstituted derivatives have been studied using variable-temperature ^{1}H NMR spectroscopy. These studies indicate that reactions involving substituted benzimidazoles can lead to the formation of different tautomers, and the substituents on the aromatic or imidazole rings can influence the structural characteristics of the molecule (Dall'Oglio et al., 2002; Diaz et al., 2015). Carbendazim is a benzimidazole derivative with fungicidal activity (Chung et al., 2023; Aire, 2005). Therefore, carbendazim is the starting material for the synthesis of the compounds in this work. Figure 1 shows some drugs containing the benzimidazole moiety. In addition, an important feature of benzimidazoles is their wide spectrum of pharmacological activities.

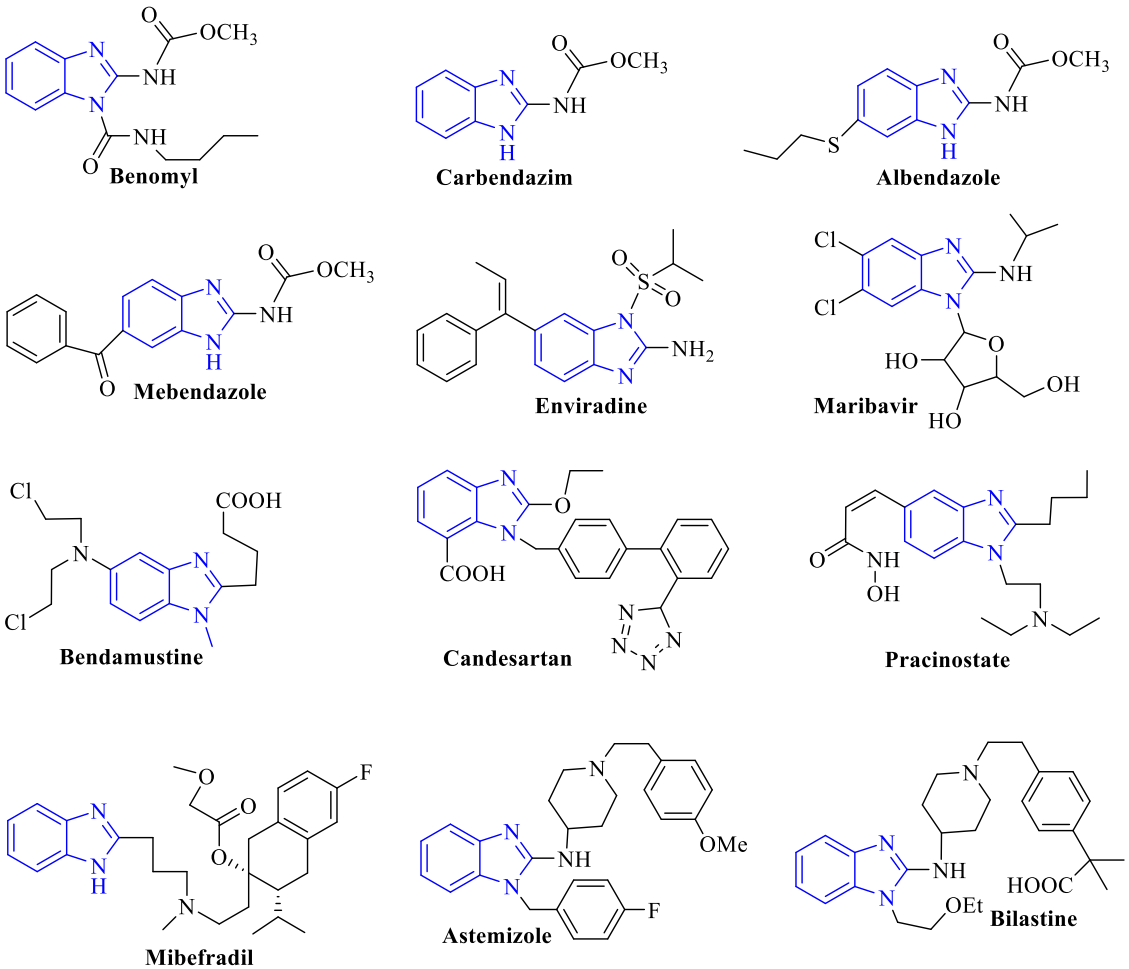


Figure 1. Drugs containing the benzimidazole moiety

Benzimidazole is a structurally diverse ligand which can bind to different receptor sites for the discovery of various immerging drugs. As shown in Figure 1, literature data indicate that benzimidazoles exhibit different biological activities depending on the type and location of the substituents in the molecule. In particular, the presence of different substituents at the N-1, C-2 and C-5 or C-6 positions in benzimidazole derivatives determines the biological activity of the molecule. For example, electron-withdrawing groups (F, Cl, Br, CN, NO_2, CO_2H or COR) and electron-donating groups (CH_3, NR_2 or OR) at the C-5(6) position have a significant impact on the structure-biological activity of the molecule (Hernández López et al., 2022). These drugs exhibit various pharmacological effects, including antimicrobial activity (benomyl) (Liu et al., 2019), however, compounds containing amines or alkylthio groups are used as antiparasitic agents, such us anthelmintic activity (albendazole, mebendazole) (Salahuddin et al., 2017), antiviral activity (enviradine, maribavir) (Vasantha et al., 2015; Faruk et al., 2014), act as anti-cancer and it has the ability to block nuclear division by binding with the spindle microtubules (bendamustine, pracinostat) (Pan et al., 2015; El-Gohary and Shaaban, 2017), antihypertensive activity (candesartan, mibefradil) (Abou Seri et al., 2011), and aliphatic substituents, sulfonyl or aromatic derivatives at the C-2 position of the molecule have antihistamine effects and are also used as anti-ulcer, antifungal and antibacterial agents, for example, antihistaminic activity (astemizole, bilastine) (Ziyadullaev et al., 2023; Bo et al., 2020). Cyclization products formed at the N-1 and C-2 positions of benzimidazole have biological activity against cancer cells, furthermore, the incorporation of different pharmacophore groups into benzimidazole derivatives allows the creation of new pharmaceutical compounds (Hernández López et al., 2022).

Based on these data, it is evident that expanding the synthetic potential of substituted benzimidazoles enables the preparation of molecules of greater chemical and biological interest. This study focuses on the synthesis of novel benzimidazole derivatives and the evaluation of their biological activities. Particular attention is paid to factors influencing the acylation reaction, including temperature, molar ratios of the reactants, and the selective formation of bis-acetylated products.

2. MATERIALS AND METHODS

Freshly distilled solvents: chloroform, hexane, benzene, ethanol, and methanol were used in this work. IR spectra were recorded on an FT-IR/NIR Spectrum 3 spectrometer (Perkin Elmer, Switzerland) using a frustrated total internal reflection (FTIR) system. NMR spectra were acquired on JNM-ECZ600R (600 MHz for ^{1}H, JEOL, Japan) and JNM-ECZ400R (400 MHz for ^{1}H, JEOL, Japan) spectrometers in DMSO-d_6, DMSO-d_6+CCl_4 and TFA-d. Tetramethylsilane (TMS, δ 0.00 ppm) was used as an internal standard for ^{1}H NMR shifts, while solvent signals (DMSO-d_6, 39.52 ppm; TFA-d, 116.60 ppm vs. TMS) served as references for ^{13}C NMR shifts. Thin layer chromatography (TLC) was performed on Silufol L/W 20 cm× 20 cm UV-254 plates (Sigma-Aldrich). The eluents used for TLC were as follows: chloroform: benzene: methanol = 2.5:1.5:0.5 (**A**); benzene: methanol=3:1 (**B**); acetone: benzene = 3:2 (**C**); acetone: benzene: methanol = 3:2:1 (**D**), and benzene: methanol = 1:3 (**E**). The melting points of the synthesized compounds were determined using BIOBASE BMP-M70 (China) and MEL-TEMP (USA) instruments.

2.1. Synthesis and Characterization

Synthesis of N-(1H-benzo[d]imidazol-2-yl) propionamide (3): 10 g (0.052 mol) of carbendazim (**1**) was added 35 mL of propionic acid (d = 0.99 g/mL) and the reaction mixture was refluxed for 8 h. Approximately 40% of the propionic acid was then removed, and the

mixture was allowed to stand overnight at room temperature. The resulting precipitate was filtered, washed with alcohol, and dried to yield 9.5 g (96%) of compound (**3**). m.p = 171-173ºC. R_f = 0.65 (B). IR spectrum (*v*, cm^{-1}): 3451 (NH_2), 1689 (C=O), 1631 (C=N), 1580 (C-N), 2851 va 2918 (CH_2, CH_3), 1516 (C=C). ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm, *J*/Hz): 0.95 (3H, t, *J* = 7.5, CH_3), 2.15 (2H, q, *J* = 7.5, CH_2), 6.86 (2H, m, H-5/H-7), 7.09 (2H, m, H-4/H-6), 9.14 (1H, br.s., NH). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 154.9 (C-2), 111.4 (C-4,7), 119.8 (C-5/C-6), 136.6 (C-8/C-9), 176.7 (C-11), 27.8 (C-12), 9.53 (C-13).

Synthesis of N-(6-nitro-1H-benzo[d]imidazol-2-yl) propionamide (5): 10 g (0.057 mol) of (**3**) was dissolved in 20 mL of concentrated sulfuric acid at 0+5ºC with stirring. Then, the nitrating mixture (15 mL of HNO_3 and 15 mL of concentrated H_2SO_4) was added dropwise to the reaction mixture with stirring. The reaction mixture was stirred for another 3 h at a temperature of (0+5ºC). The reaction mixture was left at room temperature for 12 h. Then, the reaction mixture was poured onto ice, the precipitate was filtered, washed with water until neutral, dried, and recrystallized from ethanol. As a result, 9.3 g (74%) of (**5**) was obtained. m.p = 244-246ºC. R_f = 0.58 (B). IR spectrum (*v*, cm^{-1}): 3451 (NH_2), 1935, 1945 (CH_2, CH_3), 1689 (C=O), 1632 (C=N), 1580 (C-N), 1516 (C=C). ^{1}H NMR (600 MHz, DMSO-d_6+CCl_4, δ, ppm, *J*/Hz): 1.16 (3H, t, *J* = 7.5, CH_3), 2.51 (2H, q, *J* = 7.5, CH_2), 6.05 (2H, br.s., 2-NH), 7.56 (1H, d, *J* = 8.8, H-4), 8.01 (1H, dd, *J* = 8.8 and 2.3, H-5), 8.30 (1H, d, *J* = 2.3, H-7). ^{13}C NMR (150 MHz, DMSO-d_6+CCl_4, δ, ppm): 148.49 (C-2), 113.69 (C-4), 117.85 (C-5), 142.77 (C-6), 109.9 (C-7), 138.8 (C-8), 131.4 (C-9), 173.3 (C-11), 29.0 (C-12), 8.6 (C-13).

Synthesis of N-(6-amino-1H-benzo[d]imidazol-2-yl) acetamide (6): 10 g (0.045 mol) of 5-nitro-2-acetylaminobenzimidazole (**4**) was dissolved in 84.0 mL of ethyl alcohol and 25 g (0.11 mol) of tin II chloride dihydrate were added and heated at 78º C for 2 h under an inert (nitrogen) atmosphere. The reaction mixture was left at room temperature for 12 h and neutralized with 10% NaOH solution. The resulting suspension solution was stirred for 1 h using a mechanical stirrer. The reaction mixture was then filtered and the filtrate was extracted with ethyl acetate and the solvent was evaporated. The residue was recrystallized from ethyl alcohol and dried. As a result, 6.21 g (72.1 %) (**6**) was obtained. m.p = 344-346ºC. R_f = 0.29 (B). ^{1}H NMR (600 MHz, DMSO-d_6+CCl_4, δ, ppm, *J*/Hz): 2.12 (3H, s, CH_3), 4.33 (2H, br.s, NH_2), 6.39 (1H, dd, *J* = 8.3 and 1.9, H-5), 6.64 (1H, br.s, H-7), 7.06 (1H, d, *J* = 8.3, H-4), 11.31 (2H, br.s., 2-NH).

Synthesis of N-(6-amino-1H-benzo[d]imidazol-2-yl) propionamide (7): 5 g (0.021 mol) of (**5**) was dissolved in 84 mL of ethyl alcohol and 25 g (0.11 mol) of tin II chloride dihydrate was added and heated at 78ºC for 2 h under an inert (nitrogen) atmosphere. The reaction mixture was left at room temperature for 12 h and neutralized with 10% NaOH solution. The resulting suspension was stirred for 1 h with a mechanical stirrer, then the reaction mixture was filtered and the solvent was extracted with ethyl acetate and the solvent was evaporated. The residue was recrystallized from ethanol and dried. As a result 4.05 g (93.0%) (**7**) was obtained. m.p = 268-270ºC. R_f = 0.27 (B). IR spectrum (KBr, *v*, cm^{-1}): 3376 (NH), 1965 (CH_3), 1598 (C=O), 1522 (C=N), 1489 (C-N). ^{1}H NMR (600 MHz, DMSO-d_6+CCl_4, δ, ppm, *J*/Hz): 1.12 (3H, t, *J* = 7.6, CH_3), 2.39 (2H, q, *J* = 7.6, CH_2), 6.41 (1H, dd, *J* = 8.4 and 2.4, H-5), 6.66 (1H, d, *J* = 2.1, H-7), 7.06 (1H, d, *J* = 8.4, H-4). ^{13}C NMR (150 MHz, DMSO-d_6+CCl_4, δ, ppm): 145.4 (C-2), 114.3 (C-4), 110.5 (C-5), 141.7 (C-6), 99.0 (C-7), 136.2 (C-8), 129.4 (C-9), 172.7 (C-11), 28.7 (C-12), 9.2 (C-13).

Synthesis of N-(6-acetamido-1H-benzo[d]imidazol-2-yl)-N-acetylacetamide (8): 0.1 g (0.52 mmol) of (**6**) was dissolved in 2 mL of glacial acetic acid (d = 1.0498 g/mL), the reaction mixture was refluxed for 5 h. The solution was then neutralized with 17% NH_3 solution and the formed precipitates were filtered off. The residue was recrystallized from ethanol, and 0.12 g (95.0%) of amide (**8**) was obtained. m.p = 344-346ºC. R_f = 0.58 (E). IR spectrum (KBr, *v*, cm^{-1}): 3305 (NH), 1980 (CH_3), 1652 (C=O), 1533 (C=N), 1370 (C-N). ^{1}H

NMR (600 MHz, DMSO-d_6+CCl_4, δ, ppm, *J*/Hz): 2.14 (3H, s, $COCH_3$), 2.16 (6H, s, 2-$N(COCH_3)_2$), 6.81 (1H, dd, *J* = 8.4 and 1.8, H-5), 7.19 (1H, d, *J* = 1.8, H-7), 7.43 (1H, d, *J* = 8.4, H-4), 11.55 (1H, br.s, NH), 12.04 (1H, br.s, NH). ^{13}C NMR (150 MHz, DMSO-d_6+CCl_4, *δ*, ppm): 147.6 (C-2), 116.6 (C-4), 121.2 (C-5), 140.3 (C-8/C-9), 112.7 (C-7), 132.6 (C-6), 172.1 (C-11/C-13), 26.4 (C-12/C-14), 169.1 (C-16), 23.0 (C-17).

Synthesis of N-(2-acetamido-1H-benzo[d]imidazol-6-yl)propionamide (9): Similar to the above method, 0.1 g (0.52 mmol) of (**6**) was dissolved in 2 mL of propionic acid (d = 0.99 g/mL), and the reaction mixture was refluxed for 5 h. The reaction mixture was left overnight at room temperature. Then the solution was neutralized with 17% NH_3 solution and the precipitated crystals were filtered off. The mixture was recrystallized from ethyl alcohol and dried to give 0.11 g (78.5%) of (**9**). m.p = 302-304ºC. R_f = 0.67 (E). IR spectrum (KBr, *v*, cm^{-1}): 3344 (NH), 1969 (CH_3), 1643 (C=O), 1592 (C=N), 1518 (C-N). ^{1}H NMR (600 MHz, DMSO-d_6+CCl_4, δ, ppm, *J*/Hz): 1.14 (3H, t, *J* = 7.6, CH_2-CH_3), 2.16 (3H, s, $COCH_3$), 2.29 (2H, q, *J* = 7.6, CH_2-CH_3), 7.13 (1H, dd, *J* = 8.4 and 1.9, H-5), 7.26 (1H, d, *J* = 8.4, H-4), 7.82 (1H, d, *J* = 1.9, H-7), 9.5 (1H, br.s, NH), 11.5 (1H, br.s, NH). ^{13}C NMR (150 MHz, DMSO-d_6+CCl_4, δ, ppm): 147.1 (C-2), 114.3 (C-4), 114.3 (C-5), 133.9 (C-6,9), 105.9 (C-7), 135.4 (C-8), 169.4 (C-11), 23.5 (C-12), 171.4 (C-14), 30.0 (C-15), 10.3 (C-16).

Synthesis of N-(2-acetamido-1H-benzo[d]imidazol-6-yl)butyramide (10): The reaction was carried out similarly to the above method, 0.1 g (0.52 mmol) of (**6**) was dissolved in 2 mL of butyric acid (d = 0.9563 g/mL), the reaction mixture was refluxed for 5 h. The reaction mixture was left overnight at room temperature. Then the solution was neutralized with 17% NH_3 solution and the precipitated crystals were filtered off. The mixture was recrystallized from ethanol and dried to give 0.11 g (62.7%) of (**10**). m.p = 278-280ºC. R_f = 0.73 (E). IR spectrum (KBr, *v*, cm^{-1}): 3317 (NH), 1968 (CH_3), 1686 (C=O), 1593 (C=N), 1528 (C-N). ^{1}H NMR (600 MHz, TFA-d, ppm, δ, *J*/Hz): 1.15 (3H, t, *J* = 7.4, H-17), 1.93 (2H, m, H-16), 2.60 (3H, s, H-12), 2.69 (2H, t, *J* = 7.7, H-15), 7.61 (1H, dd, *J* = 8.8 and 1.9, H-5), 7.80 (1H, d, *J* = 8.8, H-4), 8.20 (1H, d, *J* = 1.9, H-7). ^{13}C NMR (150 MHz, TFA-d, ppm, δ): 145.1 (C-2), 116.2 (C-4), 123.7 (C-5), 130.0 (C-6), 110.0 (C-7), 136.6 (C-8), 127.8 (C-9), 176.4 (C-11), 21.4 (C-12), 180.5 (C-14), 40.4 (C-15), 24.3 (C-16), 13.9 (C-17).

Synthesis of N-(2-acetamido-1H-benzo[d]imidazol-6-yl)-3-methylbutanamide (11): Similar to the above method, 0.1 g (0.52 mmol) of (**6**) was dissolved in 2 mL of isovaleric acid (d = 0.9286 g/mL), and the reaction mixture was refluxed for 5 h. The reaction mixture was left overnight at room temperature. The solution was then neutralized with 17% NH_3 solution and the precipitated crystals were filtered off. The mixture was recrystallized from ethyl alcohol and dried to yield 0.10 g (57.6%) of (**11**). m.p = 325-327ºC. R_f = 0.77 (E). IR spectrum (KBr, *v*, cm^{-1}): 3353 (NH), 1970 (CH_3), 1650 (C=O), 1591 (C=N), 1531 (C-N). ^{1}H NMR (600 MHz, TFA-d, ppm, δ, *J*/Hz): 1.15 (6H, d, *J* = 6.6, H-17/H-18), 2.30 (1H, m, H-16), 2.58 (2H, d, *J* = 7.6, H-15), 2.60 (3H, s, H-12), 7.60 (1H, dd, *J* = 8.8 and 2.0, H-5), 7.79 (1H, d, *J* = 8.8, H-4), 8.20 (1H, d, *J* = 2.0, H-7). ^{13}C NMR (150 MHz, TFA-d, ppm, δ): 145.2 (C-2), 116.3 (C-4), 123.8 (C-5), 130.0 (C-6), 110.1 (C-7), 136.5 (C-8), 127.9 (C-9), 176.4 (C-11), 24.2 (C-12), 180.1 (C-14), 47.5 (C-15), 29.3 (C-16), 22.8 (C-17/C-18).

Synthesis of N-(6-acetamido-1H-benzo[d]imidazol-2-yl)propionamide (12): 0.1 g (0.49 mmol) of (**7**) was dissolved in 5 mL of glacial acetic acid (d = 1.0498 g/mL), the reaction mixture was refluxed for 5 h. Then the precipitated crystals were filtered off and recrystallized from ethanol and dried to give 0.101 g (84.2%) of (**12**). m.p = 308-310ºC. R_f = 0.35 (E). IR spectrum (KBr, *v*, cm^{-1}): 3286 (NH), 1966 (CH_3), 1675 (C=O), 1588 (C=N), 1483 (C-N). ^{1}H NMR spectrum (600 MHz, DMSO-d_6+CCl_4, δ, ppm, *J*/Hz): 1.19 (3H, t, *J* = 7.5, CH_2CH_3), 2.03 (3H, s, $COCH_3$), 2.46 (2H, q, *J* = 7.5, CH_2CH_3), 7.11 (1H, dd, *J* = 8.5 and 1.9, H-5), 7.26 (1H, d, *J* = 8.5, H-4), 7.82 (1H, d, *J* = 1.9, H-7), 9.59 (1H, br.s, 14-NH), 11.54 (1H, br.s, NH). ^{13}C NMR (150 MHz, DMSO-d_6+CCl_4, δ, ppm): 146.7 (C-2), 113.6 (C-4),

113.6 (C-5), 133.2 (C-6,9), 105.1 (C-7), 135.7 (C-8), 172.6 (C-11), 28.7 (C-12), 9.0 (C-13), 167.0 (C-15), 23.6 (C-16).

Synthesis of N,N'-(1H-benzo[d]imidazole-2,6-diyl)dipropionamide (13): In a similar manner to the method described above, 0.1 g (0.49 mmol) of (**7**) was dissolved in 5 mL of propionic acid (d = 0.99 g/mL), and the reaction mixture was refluxed for 5 h. The reaction mixture was left overnight at room temperature, and the precipitated crystals were filtered off. Recrystallization from ethanol yielded 0.098 g (77.2%) of compound (**13**). m.p = 327-329ºC. R_f = 0.36 (E). IR spectrum (KBr, *v*, cm^{-1}): 3275 (NH), 1973 (CH_3), 1642 (C=O), 1510 (C=N), 1484 (C-N). ^{1}H NMR spectrum (600 MHz, DMSO-d_6+CCl_4, δ, ppm, *J*/Hz): 1.11 (3H, t, *J* = 7.8, CH_2CH_3), 1.15 (3H, t, *J* = 7.8, CH_2CH_3), 2.25 (2H, q, *J* = 7.8, CH_2CH_3), 2.42 (2H, q, *J* = 7.8, CH_2CH_3), 7.08 (1H, br.d, *J* = 8.4, H-5), 7.22 (1H, d, *J* = 8.4, H-4), 7.78 (1H, d, *J* = 1.8, H-7), 9.45 (1H, br.s, NH), 11.41 (1H, br.s, NH), 11.69 (1H, br.s, NH). ^{13}C NMR (150 MHz, DMSO-d_6+CCl_4, δ, ppm): 146.6 (C-2), 113.6 (C-4), 113.6 (C-5), 133.1 (C-6/C-8/C-9), 102.9 (C-7), 172.5 (C-11), 28.6 (C-12), 9.0 (C-13), 170.7 (C-15), 29.4 (C-16), 9.6 (C-17).

Synthesis of N-(2-propionamido-1H-benzo[d]imidazol-6-yl)butyramide (14): The reaction was carried out similarly to the above method, 0.1 g (0.49 mmol) of (**7**) was dissolved in 5 mL of butyric acid (d = 0.9563 g/mL), the reaction mixture was refluxed for 5 h. The reaction mixture was left overnight at room temperature. Then the precipitated crystals were filtered off and recrystallized from ethanol. 0.097 g (72.4%) of (**14**) was obtained. m.p = 305-307ºC. R_f = 0.38 (E). IR spectrum (KBr, *v*, cm^{-1}): 3277 (NH), 1981 (CH_3), 1640 (C=O), 1485 (C=N), 1370 (C-N). ^{1}H NMR spectrum (600 MHz, DMSO-d_6+CCl_4, δ, ppm, *J*/Hz): 0.98 (3H, t, *J* = 7.5, $CH_2CH_2CH_3$), 1.18 (3H, t, *J* = 7.5, CH_2CH_3), 1.67 (2H, sext, *J* = 7.5, $CH_2CH_2CH_3$), 2.25 (2H, t, *J* = 7.5, $CH_2CH_2CH_3$), 2.45 (2H, q, *J* = 7.5, CH_2CH_3), 7.12 (1H, d, *J* = 8.5, H-5), 7.26 (1H, d, *J* = 8.5, H-4), 7.82 (1H, br.s, H-7), 9.48 (1H, br.s, NH), 11.44 (1H, br.s, NH), 11.71 (1H, br.s, NH). ^{13}C NMR (150 MHz, DMSO-d_6+CCl_4, δ, ppm): 146.6 (C-2), 113.6 (C-4), 113.63 (C-5), 133.1 (C-6/C-8/C-9), 102.5 (C-7), 172.5 (C-11), 28.6 (C-12), 9.0 (C-13), 169.8 (C-15), 38.2 (C-16), 18.5 (C-17), 13.5 (C-18).

2.2. Cell Cultures

The cytotoxic activity of the samples was screened against the human triple-negative breast cancer BT-20 (HTB-19), HCC1395 (ATCC®CRL-2324), MDA-MB-231 (CRM-HTB-26), and HEK293 (CRL-1573) embryonic kidney cell lines (Mamadalieva et al., 2024a). The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 4.5 g/L glutamine and D-glucose, 10% fetal bovine serum, and a 1% penicillin/streptomycin mixture and incubated at 37°C under a 5% CO_2 humidified atmosphere. Cell passage and seeding performed after washing the adherent cells with PBS and detaching them using a Trypsin solution. The cells were detached and resuspended at 160,000 cells/mL and added into each well of a transparent 384-well plate in the final volume of 25 µL/well (4000 cells per well). The cells were maintained in DMEM. The next day, the 384-well plate was examined under an inverted microscope to identify cell seeding errors, growth characteristics, morphology and equal distribution. Then, the medium in each well was removed using a washer–dispenser (Biotek FX, Belgium) and replaced by 40 µL of the fresh medium containing the indicated concentrations of compounds and incubated for 72 h.

2.3. Sample Preparation for Bioassays and Treatment of Cells

Stock solutions (50 mM) of the individual compounds were prepared in DMSO (Sigma-Aldrich, Germany) and subsequently diluted with complete medium to the required working concentrations prior to use. A complete medium was used to prepare this solution. The

samples were further serially diluted in DMSO into eleven different concentrations and then added to the complete cell culture medium so as to attain the final concentrations ranging from 50 to 1.45 μM for the individual compounds in 384-well plates, which were added in four replicates. A 40 μL sample containing medium from each concentration was dispensed into each well. DMSO concentration was kept constant at 0.05% in all wells. As a negative control, cells were treated with DMEM containing DMSO. Docetaxel (0.02 to 6.9 μM) was used as a positive control (Mamadalieva et al., 2024a).

2.4. MTT Assay

The cytotoxicity of the samples was determined in triplicate using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Mamadalieva et al., 2024b; Eshboev et al., 2023). The next day, the medium of the 384-well plates treated with samples was removed. Then, 25 μL of MTT solution (0.5 mg MTT in 1 mL PBS) was added to each well and incubated at 37°C for 2 h. After incubation with MTT for 2 h, the solution was removed from the wells by the washer–dispenser, and the formed formazan crystals were then dissolved in 25 μL of DMSO. After a further 10 min of incubation at room temperature, the samples were mixed briefly, and the absorbance was detected at 510 nm with a Tecan Infinite 200 Pro Reader (Tecan Group Ltd., Switzerland). The cell viability rate (%) was calculated by the following formula:

$$\text{Cell viability rate (\%)} = [\ (OD_{\text{treated cells}} - OD_{\text{media blank}} / (OD_{\text{control cells}} - OD_{\text{media blank}}\ ] \times 100\%$$

2.5. TOPFlash Assay

The TOPFlash assay was performed as described (Boudou et al., 2023; Koval et al., 2021). TNBC BT-20 cell line stably transfected with TopFlash reporter plasmid was seeded at 450,000 cells/mL in a white opaque 384-well plate in the final volume of 25 μL. The cells were maintained and incubated at 37°C with 5% CO_2 overnight for attachment. Subsequently, they were transfected by a plasmid encoding Renilla luciferase under the CMV promoter using 12 μg/mL of DNA and 40 μL/mL XtremeGENE 9 reagent as described in the manufacturer's protocol. The next day, the medium in each well was replaced with a 20 μL fresh medium containing Wnt3a (2.5 μg/mL) and compound dilution. Compound dilutions were prepared by serial dilution in DMSO and diluted with the amount of medium necessary to obtain their final concentrations indicated on the figures and tables and maintain a concentration of DMSO of 0.05% in all assay points. Wnt3a was added after 1 h of preincubation with compound dilution. After overnight incubation, the supernatant in each well was removed by the washer–dispenser, and the luciferase activity was measured as described (Boudou et al., 2023; Koval et al., 2021). Briefly, the culture medium was completely removed from all wells of the plate. Next, the luciferase activity of the firefly and Renilla luciferases was detected sequentially in individual wells of a 384-well plate through the injection of corresponding measurement solutions in Tecan Infinite 200 Pro multifunctional plate reader with an injection module.

2.6. Statistical Analysis

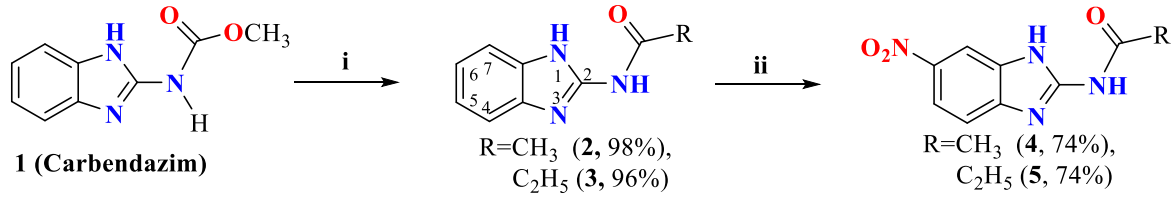
The experiments were carried out in four replicates. Continuous variables were presented as the mean ±SD. The IC_{50} was determined as the drug concentration which resulted in a 50% reduction in cell viability or the inhibition of biological activity. The level of significance was set at $p < 0.05$. The IC_{50} was estimated using the linear regression method

of plots of the percent of cell viability against the concentration of the tested compounds using GraphPad Prism 8.0.1 software (San Diego, USA).

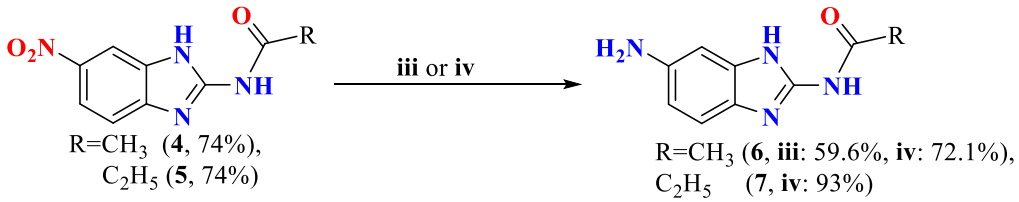
3. RESULTS AND DISCUSSION

3.1. Synthesis of Novel 2,6-Disubstituted Benzimidazoles

In our recent studies, the interaction of carbendazim (**1**) with aliphatic carboxylic acids was investigated, leading to the formation of the N-acetyl derivative (**2**) (Abdurazakov et al., 2021). Continuing our research, nitration of N-acetyl derivative (**2**) using a nitrating mixture afforded the corresponding 6-nitro derivative (**4**) (Saidov, 2020). Subsequent reduction of this compound with $SnCl_2 \cdot 2H_2O$ in EtOH/HCl produced the 6-amino derivative (**6**) in 84.3% yield, demonstrating high synthetic potential (Kubayev et al., 2022; Bellamy and Ou, 1984). To further expand these studies, the corresponding 3,5-disubstituted derivatives were synthesized following the procedures reported in the literature (Kubayev et al., 2022; Abdurazakov et al., 2021; Saidov, 2020).



i) 1: AcOH (or $C_2H_5CO_2H$) in excess), reflux, 5 h.
ii) 2/3:HNO_3+H_2SO_4; 0+5^{0}C, 3 h; then (20-25^{0}C).



iii) 4:$SnCl_2$*$2H_2O$ (EtOH, HCl); 78^{0}C, 3 h; 20-25^{0}C, overnight.
iv) 4/5:$SnCl_2$*$2H_2O$ (EtOH); 78^{0}C, 2 h; 20-25^{0}C, overnight.

Figure 2. Synthesis of 6-acylamino-benzimidazole derivatives based on carbendazim

It should be emphasized that the reduction of compound (**4**) by the method reported in the literature afforded the product in a relatively low yield (59.6%), which is likely due to partial hydrolysis of the acetyl group in the starting material (Kubayev et al., 2022). Therefore, alternative methods for reducing the nitro group were explored. By following the procedure described by (Bellamy and Ou, 1984), the amino product (**6**) was obtained in a significantly improved yield of 72.1%. Therefore, we used this same method of reducing the nitro product (**5**) and obtained a high yield (93.0%) of the corresponding amino derivative (**7**) (Dreikorn et al., 2000). However, it should be emphasized that in this work only 2,5-disubstituted nitro (**4**, **5**) and amino derivatives (**6**, **7**) were synthesized, and the formation of 2,6-disubstituted tautomers (**B**) was not observed. The present work is a continuation of the previous studies (Kubayev et al., 2022; Abdurazakov et al., 2021; Saidov, 2020). In particular, it was interesting to carry out further acylation of compounds (**6**, **7**) containing *endo*- and *exocyclic* amino groups. From a theoretical perspective, this reaction could lead to the

formation of di- and tri-acetamides. The reaction of N-(6-amino-1H-benzo[d]imidazol-2-yl) acetamide (**6**) with aliphatic carboxylic acids, such as acetic, propionic, butyric and isovaleric acids, proceeds along different pathways. The reaction with glacial acetic acid (118°C, reflux, 5 h) was found to produce the triacetamide N-(6-acetamido-1H-benzo[d]imidazol-2-yl)-N-acetylacetamide (**8**) in high yield (95.0%) without the formation of the diacetamide.

The substance (**6**) can initially form N,N'-(1-acetyl-1H-benzo[d]imidazole-2,6-diyl)diacetamide of type (**a**) in the presence of glacial acetic acid. Structurally similar substances to this compound have been reported in the literature to undergo migration of the N1-acetyl group under the influence of high temperature, which can be stabilized by attaching to the exocyclic NH-group in position 2, forming the corresponding triacetamide (**8**) (Kadyrov et al., 1980). However, we expected the formation of 2,6-diacetamide. Contrary to expectations, it was isolated triacetamide in high yield. Reactions with other aliphatic carboxylic acids (propionic, butanoic, and *iso*-butanoic) may proceed at very high temperatures and not form the corresponding triacetamides due to the strong influence of steric effects in the molecules. However, reactions with other carboxylic acids (propionic, butyric and isovaleric acids) occur at the free aromatic amino group at position 6 of the benzene ring. As a result, 6-(N-acetyl) derivatives (**9-11**) were obtained in 78.5-57.6% yields.

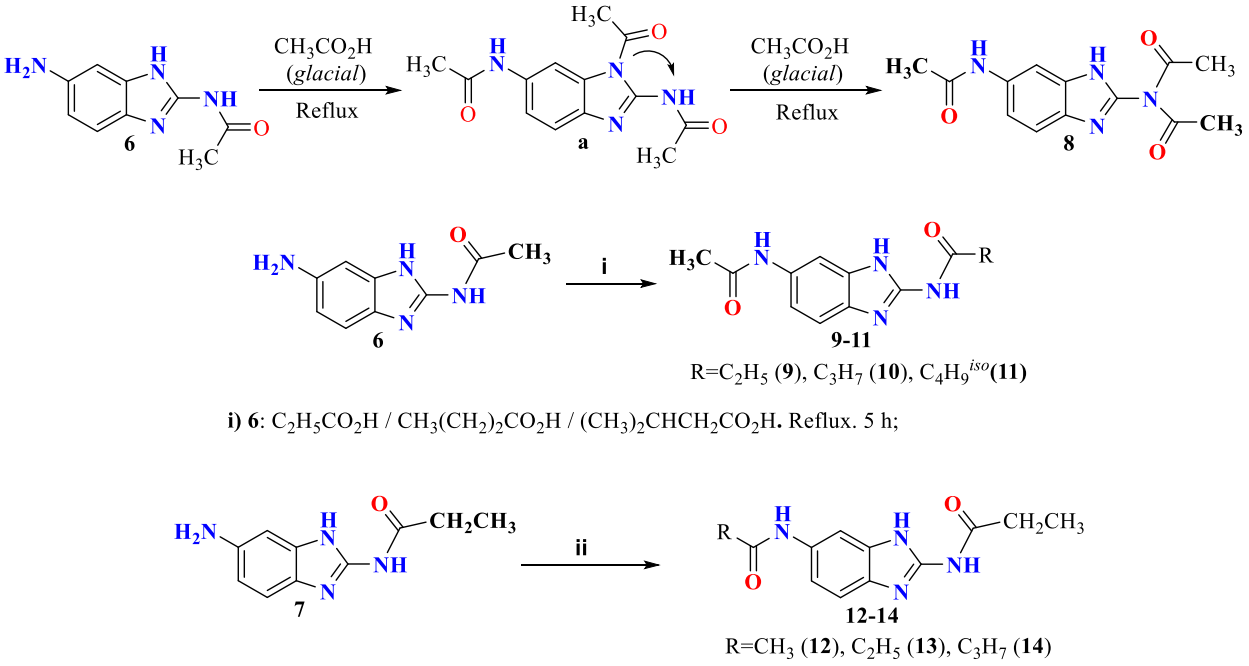


Figure 3. Synthesis of 2,6-disubstituted-benzimidazole derivatives

It should be noted that the yields of the target products (**8–11**) synthesized in the presence of carboxylic acids were found to be as follows, respectively: (**8**) (95.0%) →(**9**) (78.5%) →(**10**) (62.7%) →**11** (57.6%). This effect is apparently due to the decreasing acidity of the carboxylic acids in this series and the increased rate of decomposition of the 6-(N-acetyl) derivatives (**8-11**) at relatively high temperatures. The reaction of N-(6-amino-1H-benzo[d]imidazol-2-yl)propionamide (**7**) with acetic, propionic, butyric acids occurs mainly via the aromatic exocyclic amino group with the formation of the corresponding amides (**12-14**) in 84.2-72.4% yields. It is also noteworthy that, as the reaction temperature increases, the yields of diamides decrease in series **12** (84.2%) →**13** (77.2%) →**14** (72.4%). From a theoretical point, acylation reactions usually proceed smoothly at not very high temperatures, and the decrease in hydrophilicity of carboxylic acids in the C_1-C_5 (for example C_1-C_4-

miscible with water, C_5- 5 g/100 g of H_2O) series makes it difficult to isolate products from the reaction medium, which leads to a decrease in the yields of 6-(N-acetyl) derivatives (**8-14**).

Thus, it was found that the yield of products decreases with an increase in the amount of methylene group in the homologous series of acids. The structures of all compounds (**3**, **5**, **6**, **7**, **8-14**) were characterized by IR, ^{1}H, ^{13}C NMR spectroscopy. The details are provided in the experimental section and supporting information. For example, in the ^{1}H NMR spectrum (in DMSO-d_6+CCl_4) of N-(6-acetamido-1H-benzo[d]imidazol-2-yl)-N-acetylacetamide (**8**), at δ 2.14 a three-protonic singlet was detected and δ 2.16, one six-protonic singlets of two $COCH_3$ groups in position C-2. In the aromatic part of the spectrum, three signals were detected in the form of the ABX spin system at δ 6.81 (dd, *J* = 8.4, 1.8, H-5), 7.19 (d, *J* = 1.8, H-7), 7.43 (d, *J* = 8.4, H-4), which were assigned to the protons of the benzene ring. Further, in the low-field of the spectrum, signals of two NH groups were detected in the form of single-proton broadened singlets at δ 11.55 and 12.04. At the same time, signals of 13 carbon atoms were detected in the ^{13}C NMR spectrum. These data confirm the proposed the chemical structure of compound (**8**).

3.2. Cytotoxicity and Wnt Signaling Assay

The synthesized benzimidazole derivatives were evaluated for their cytotoxic activity against three triple-negative breast cancer (TNBC) cell lines - BT-20, MDA-MB-231, and HCC1395 -as well as the noncancerous human embryonic kidney cell line HEK293 (Table 1). The MTT assay was employed after 72 h exposure at varying concentrations, using docetaxel as the positive control. Most of the synthesized compounds displayed IC_{50} values greater than 50 μM against all tested cell lines, indicating low cytotoxicity under the tested conditions. However, compounds (**9**) and (**10**) exhibited moderate cytotoxic effects, particularly against BT-20 and HCC1395 cells, with IC_{50} values of 40.7 and 28.6 μM, respectively. These results suggest that substitution at the 5-position of the benzimidazole ring influences the biological activity, likely due to electronic or steric factors affecting interaction with cellular targets.

Table 1. *In vitro* cytotoxic activity of benzimidazole derivatives against BT-20, HCC1395, MDA-MB-231 and HEK293 cell lines for exposure of 72 h (MTT test)

Compound	BT-20	MDA-MB-231	HCC1395	HEK293
			IC_{50}, μM	
3	>50	>50	>50	>50
6	>50	>50	>50	>50
7	>50	>50	>50	>50
8	>50	>50	>50	>50
9	40.7±1.93	>50	28.6±0.93	37.8±2.86
10	39.1±2.71	>50	35.5±3.01	34.2±1.65
11	>50	>50	>50	>50
Docetaxel	4.4 ± 0.0008	17.7 ± 0.0029	6.5 ± 0.0007	4.47 ± 0.0013

The majority of the derivatives exhibited weak or no cytotoxic activity (IC_{50} >50 μM), which limits the extent to which meaningful structure-activity relationships can be established. Their low activity may stem from poor cell permeability or reduced electrophilicity of the benzimidazole core, factors that can diminish interactions with intracellular targets (Lee et al., 2022). The consistently low toxicity toward the noncancerous HEK293 cell line (IC_{50} >34 μM) further supports the conclusion that these compounds are largely inactive under the tested conditions. To further assess possible mechanistic effects, selected derivatives were tested in a TOPFlash luciferase assay using the BT-20 Wnt3a-

responsive cell line. None of the tested compounds significantly inhibited Wnt3a-induced β-catenin signaling at concentrations up to 20 μM, suggesting that their cytotoxicity is not related to modulation of the Wnt/β-catenin pathway. This finding implies that other signaling mechanisms may be involved or that the tested structural framework does not favor Wnt pathway interference. Overall, these results highlight that the introduction of suitable substituents at the benzimidazole nucleus can modulate cytotoxic activity and compounds (**9**) and (**10**) represent promising scaffolds for future structural refinement aimed at enhancing anticancer potency and selectivity (Badawy et al., 2025).

4. CONCLUSION

A convenient and efficient method for the synthesis of di- and triacetamides in a series of potentially bioactive benzimidazole derivatives has been developed. The approach involves the nitration of amides (**2**, **3**) to yield 6-nitro derivatives (**4**, **5**), followed by reduction with tin (II) chloride in an acidic medium to produce the corresponding 6-amino derivatives (**6**, **7**) in high yields. Subsequent acylation of the aromatic amino group in position 6 with various aliphatic carboxylic acids afforded amides (**8-11**) in good to excellent yields. The reaction of compound (**6**) with glacial acetic acid, unexpectedly, led to the formation of triacetamide (**8**) in excellent yield. It was observed that increasing the boiling point or chain length of the carboxylic acids reduced product yields in the homologous series. The synthesized compounds were characterized by 1H, ^{13}C NMR, and IR spectroscopy. Evaluation of their cytotoxic activity against triple-negative breast cancer cell lines (BT-20, MDA-MB-231, HCC1395) and the noncancerous HEK293 line revealed generally low cytotoxicity, with compounds (**9**) and (**10**) showing moderate effects. Substitutions at the 5-position of the benzimidazole ring appeared to influence biological activity, likely through electronic or steric factors. The observed selectivity toward noncancerous cells suggest low inherent toxicity. Overall, this study presents a simple, cost-effective synthetic route to benzimidazole-based amides with promising biological potential. These findings support further structural optimization to enhance anticancer activity while maintaining selectivity and safety.

Conflict of Interest
The authors declare no conflicts of interest.

Author Contribution Statement
Sh.Kubayev, S.Saidov, E.Rakhmatov: Data curation, methodology, and formal analysis. A.Abdurazakov: Conceptualization, methodology, writing original draft, review, and editing. A.Koval, V.Katanaev: Visualization, investigation, writing, review, and editing. K.Bobakulov: Software and data curation. N.Mamadalieva: Conceptualization, investigation, data curation, writing original draft, review, and editing, B.Elmuradov: Project administration and resources.

Data Availability Statement
All supporting data are available in the supplementary materials and can be provided upon request.

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