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Using 2-Aminobenzimidazole Derivatives to Inhibit *Mycobacterium smegmatis* Biofilm Formation

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General Experimental: All reagents used for chemical synthesis were purchased from commercially available sources and used without further purification. Chromatography was performed using 60 Å mesh standard grade silica gel from Sorbtech. NMR solvents were obtained from Cambridge Isotope Labs and used as is. ^{1}H NMR (300 MHz or 400 MHz) and ^{13}C NMR (75 MHz or 100 MHz) spectra were recorded at 25 °C on Varian Mercury spectrometers. Chemical Shifts (δ) are given in ppm relative to tetramethylsilane or respective NMR solvent; coupling constants (*J*) are in hertz (Hz). Abbreviations used are s = singlet, bs = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, bt = broad triplet, qt = quartet, m = multiplet, bm = broad multiplet and br = broad. High and low resolution mass spectra were obtained at the NCSU Department of Chemistry Mass Spectrometry Facility. Infrared Spectra were obtained on an FT/IR-4100 spectrophotometer (νmax in cm^{-1}). UV absorbance was recorded on a Genesys 10 scanning UV/visible spectrophotometer (λmax in nm). The purities of the tested compounds were all verified to be >95% by LC-MS analysis on a Shimadzu LC-MS 2020 with Kinetex, 2.6 mm, C18 50 x 2.10 mm.

Synthetic procedures

Compounds were synthesized following previously reported procedures[1, 2] described below.

N-(4-fluoro-3-nitrophenyl)-4-pentylbenzamide was synthesized as previously reported.[1] To a solution of 4-fluoro-3-nitroaniline (10.0 g, 64.1 mmol) in 100 mL of dry dichloromethane was added 4-pentylbenzoyl chloride (16.9 mL, 83.3 mmol) and 4-dimethylaminopydridine (7.82 g, 10.0 mmol) under an inert atmosphere and the reaction was allowed to stir overnight. The mixture was then washed with water (2 x 100 mL), saturated sodium bicarbonate (2 x 100mL),

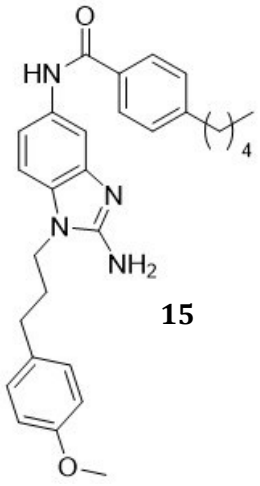
and brine (2 x 100mL). The organic layer was dried over sodium sulfate and purified via silica gel column chromatography (0 – 25% ethyl acetate in hexanes). The product was concentrated in vacuo to a white solid (81% yield). ^{1}H NMR, ^{13}C NMR, and LRMS aligned with previously reported spectral and MS data.

General procedure for nucleophilic aromatic substitution reaction: *N*-(4-fluoro-3-nitrophenyl)-4-pentylbenzamide (**3.7**) (1 mmol) was dissolved in 10 mL of ethanol. To this mixture was added the corresponding amine (5 mmol) dropwise and the reaction mixture was heated to 97 °C and allowed to stir until completion via TLC analysis. The mixture was cooled in an ice bath at 0 °C and water was added to precipitate the product. The product was filtered and washed with cold water and dried overnight on a high vacuum pump.

General procedure for the formation of *N*-1 substituted 2-aminobenzimidazoles:

tert-butyl (4-(benzylamino)-3-nitrophenyl)carbamate (15.0 g, 31.81 mmol) was dissolved in ethanol (0.5 M). To the solution was added 0.1 equivalents of Pd/C, and the reaction mixture was heated to reflux. Ammonium formate (20.58 g, 318.1 mmol) was then added to the refluxing solution slowly over a 1 h period. The reaction was allowed to stir until completion, via TLC analysis. The reaction mixture was cooled to room temperature, and filtered through a pad of celite, which was washed with DCM. The crude product was placed under an inert atmosphere. Solid cyanogen bromide (42.78 g, 318.1 mmol) was then added to the crude product and allowed to stir overnight. The reaction mixture was then concentrated and purified via flash chromatography (1-5% MeOH Sat. NH_3/DCM). The product was dried under vacuum overnight.

Compound characterization



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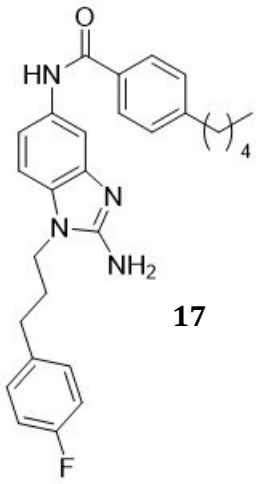
***N*-(2-Amino-1-(3-(4-methoxyphenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide**: *N*-(2-Amino-1-(3-(4-methoxyphenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide was synthesized following the general procedure for the formation of *N*-1 substituted 2-aminobenzimidazoles.[1] Following purification via silica gel column chromatography, the product was obtained as a brown solid (36%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.99 (d, *J* = 1.9 Hz, 1H), 7.91 – 7.82 (m, 2H), 7.50 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.30 (dd, *J* = 15.9, 8.5 Hz, 3H), 7.08 (dd, *J* = 9.2, 2.7 Hz, 2H), 6.79 (td, *J* = 6.1, 2.4 Hz, 2H), 4.15 (t, *J* = 7.4 Hz, 2H), 3.73 (s, 3H), 2.66 (d, *J* = 7.4 Hz, 4H), 2.10 (p, *J* = 7.7 Hz, 2H), 1.64 (p, *J* = 7.6 Hz, 2H), 1.45 – 1.25 (m, 4H), 0.98 – 0.82 (m, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 168.8, 159.8, 159.6, 148.7, 136.9, 133.7, 133.3, 130.3, 130.1, 129.7, 128.7, 128.3, 118.1, 114.9, 111.3, 105.8, 55.7, 43.6, 36.7, 32.7, 32.6, 32.1, 30.4, 23.5, 14.3. IR (KBr) *ν*(cm^{-1}) 3104, 2822, 11708, 1193, 1038; λ_{max} = 230 nm; HRMS (ESI): *m/z*: Calcd for $C_{29}H_{34}N_4O_2$: 471.2755 $[M+H]^+$, found 471.2755



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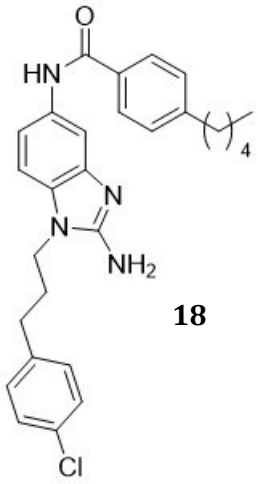
***N*-(2-Amino-1-(3-(4-fluorophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide**:

N-(2-Amino-1-(3-(4-fluorophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide was synthesized following the general procedure for the formation of *N*-1 substituted 2-aminobenzimidazoles. Following purification via silica gel column chromatography, the product was obtained as a dark red solid (32%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.99 (d, *J* = 1.9 Hz, 1H), 7.86 (d, *J* = 8.3 Hz, 2H), 7.52 (dd, *J* = 8.7, 2.0 Hz, 1H), 7.31 (t, *J* = 8.4 Hz, 3H), 7.21 – 7.14 (m, 2H), 6.94 (t, *J* = 8.8 Hz, 2H), 4.16 (t, *J* = 7.3 Hz, 2H), 2.72 (t, *J* = 7.8 Hz, 1H), 2.66 (t, *J* = 7.7 Hz, 1H), 2.10 (m, 2H), 1.70 – 1.56 (m, 2H), 1.32 (m, 4H), 0.89 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 168.8, 164.0, 162.7 (d, *J* = 222.4 Hz), 151.5, 148.7, 137.7, 136.9, 133.3, 130.9 (d, *J* = 7.8 Hz),130.3, 129.7, 128.7, 128.2, 118.1, 116.0 (d, *J* = 21.4 Hz),, 111.2, 105.9, 43.6, 36.7, 32.7, 32.5, 32.1, 30.5, 23.5, 14.3. IR (KBr) *ν*(cm^{-1}) 3096, 1780, 1242, 1174; λ_{max} = 248 nm; HRMS (ESI): *m/z*: Calcd for $C_{28}H_{31}FN_4O$: 459.2555 $[M+H]^+$, found 459.2555



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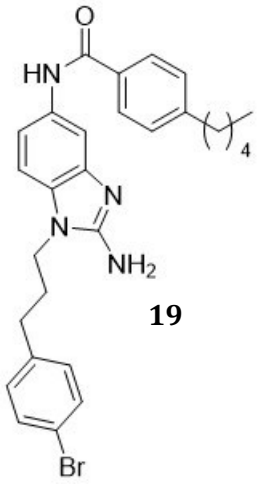
***N*-(2-Amino-1-(3-(4-chlorophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide**: *N*-(2-Amino-1-(3-(4-chlorophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide was synthesized following the general procedure for the formation of *N*-1 substituted 2-aminobenzimidazoles. Following purification via silica gel column chromatography, the product was obtained as a pale yellow solid (55%). ^{1}H NMR (400 MHz, DMSO-d6) δ 10.45 (s, 1H), 8.94 (s, 2H), 8.10 – 7.89 (m, 2H), 7.64 (d, *J* = 8.8 Hz, 2H), 7.45 (d, *J* = 8.7 Hz, 1H), 7.41 – 7.19 (m, 3H), 4.25 (m, 2H), 2.78 – 2.57 (m, 4H), 1.97 (s, 2H), 1.59 (t, *J* = 7.4 Hz, 2H), 1.25 (m, 4H), 0.85 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 167.2, 153.8, 149.1, 143.0, 142.7, 134.6, 134.2, 132.0, 129.9, 129.3, 128.5, 128.4, 128.3, 126.7, 115.8, 109.8, 107.9, 41.3, 37.8, 34.7, 334.2, 33.5, 30.1, 22.3, 13.8. IR (KBr) *v*(cm^{-1}) 3374, 2833, 1823; λ_{max} = 284, 296 nm; HRMS (ESI): *m/z*: Calcd for $C_{28}H_{31}ClN_4O$: 475.2259 $[M+H]^+$, found 475.2259



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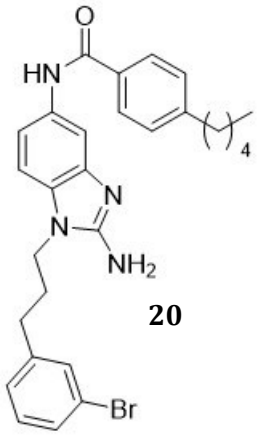
***N*-(2-Amino-1-(3-(4-bromophenyl)propyl)-1*H*-benzoimidazole-5-yl)-4-pentylbenzamide**: *N*-(2-amino-1-(3-(4-bromophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide was synthesized following the general procedure for the formation of N-1 substituted 2-aminobenzimidazoles. Following purification via silica gel column chromatography, the product was obtained as a brown solid (47%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.93 (s, 1H), 10.44 (d, J = 2.8 Hz, 1H), 8.95 (s, 3H), 8.07 (d, J = 2.6 Hz, 1H), 7.93 (dd, J = 8.2, 2.9 Hz, 2H), 7.65 (dt, J = 8.7, 2.7 Hz, 1H), 7.42 (ddd, J = 22.6, 8.6, 2.9 Hz, 3H), 7.28 (dd, J = 8.3, 2.8 Hz, 2H), 7.15 (dd, J = 8.5, 2.9 Hz, 2H), 4.24 (t, J = 7.2 Hz, 2H), 2.83 – 2.51 (m, 4H), 2.07 – 1.88 (m, 2H), 1.59 – 1.49 (m, 2H), 1.24 (m, 4H), 0.81 (t, J = 6.9Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.36, 161.16, 149.89, 146.31, 140.48, 135.56, 132.14, 131.10, 130.41, 128.85, 128.17, 127.81, 126.30, 118.90, 115.70, 109.88, 103.89, 42.10, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89, 34.93, 31.21, 30.81, 30.36, 29.23, 21.92, 13.88. IR (KBr) ν(cm^{-1}) 3324, 2983, 1673, 1128; λ_{max} = 254 nm; HRMS (ESI): *m/z*: Calcd for $C_{28}H_{31}BrN_4O$: 519.1754 $[M+H]^+$, found 519.1754



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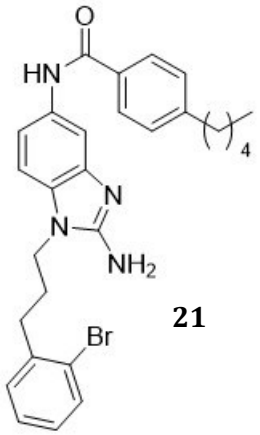
***N*-(2-Amino-1-(3-(3-bromophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide**: *N*-(2-Amino-1-(3-(3-bromophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide was synthesized following the general procedure for the formation of *N*-1 substituted 2-aminobenzimidazoles. Following purification via silica gel column chromatography, the product was obtained as a dark brown solid (46%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H), 7.81 (d, *J* = 8.0 Hz, 2H), 7.50 (s, 1H), 7.32 – 7.23 (m, 2H), 7.20 (d, *J* = 7.7 Hz, 2H), 7.06 (t, *J* = 7.8 Hz, 1H), 6.95 (d, *J* = 7.7 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 3.76 (m, 2H), 2.61 (t, *J* = 7.7 Hz, 2H), 2.50 (t, *J* = 7.9 Hz, 2H), 1.97 – 1.85 (m, 2H), 1.60 (p, *J* = 7.6 Hz, 2H), 1.30 (m, 4H), 0.94 – 0.83 (t, *J* = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.9, 155.1, 147.2, 143.0, 135.9, 132.3, 131.4, 130.2, 129.4, 128.8, 127.4, 127.1, 122.6, 116.8, 114.8, 107.6, 42.0, 35.9, 32.4, 31.6, 31.0, 29.8, 22.7, 14.2. IR (KBr) *v*(cm^{-1}) 3228, 2931, 1534, 1279, 1135; λ_{max} = 272 nm; HRMS (ESI): *m/z*: Calcd for $C_{28}H_{31}BrN_4O$: 519.1754 $[M+H]^+$, found 519.1754



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***N*-(2-Amino-1-(3-(2-bromophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide**:

N-(2-Amino-1-(3-(2-bromophenyl)propyl)-1*H*-benzo[d]imidazole-5-yl)-4-pentylbenzamide was synthesized following the general procedure for the formation of *N*-1 substituted 2-aminobenzimidazoles. Following purification via silica gel column chromatography, the product was obtained as a dark red solid (41%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.93 (s, 1H), 10.44 (d, J = 2.8 Hz, 1H), 8.95 (s, 1H), 8.15 – 7.84 (m, 2H), 7.65 (m, 1H), 7.42 (m, 3H), 7.22 (m, 3H), 4.24 (d, J = 7.5 Hz, 2H), 3.51 (m, 2H), 2.75 – 2.53 (m, 4H), 1.95 (t, J = 8.0 Hz, 2H), 1.55 (m, 2H), 1.33 – 1.16 (m, 4H), 0.81 (t, J = 6.9, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.8, 170.6, 159.3, 155.8, 149.9, 145.0, 141.6, 140.5, 139.9, 138.3, 137.6, 137.3, 135.7, 128.3, 125.1, 119.3, 113.3, 51.5, 44.4, 40.7, 40.3, 39.8, 38.7, 31.4, 23.3. IR (KBr) v(cm^{-1}) 3181, 3045, 1723, 1366, 1243; λ_{max} = 268 nm; HRMS (ESI): *m/z*: Calcd for $C_{28}H_{31}BrN_4O$: 519.1754 $[M+H]^+$, found 519.1747

Bacterial strains, media, and antibiotics

The *Mycobacterium smegmatis* strain (ATCC 700084, mc^2155) was obtained from ATCC (Manassas, VA). Stock cultures were stored in glycerol stock media (50% v/v glycerol and 7H9, ADC, Tween 80) and maintained at -80 °C. Prior to use, colonies were grown on 7H10 agar (OADC, glyercol) for 2 days, and single colonies were subcultured in 7H9 (ADC, Tween 80) for 2 days. 7H10, 7H9, OADC, and ADC were purchased from BD Diagnostics. Glycerol and Tween 80 were purchased from Sigma Aldrich. Ampicillin, carbenicillin, cefaclor, cefadroxil, cefmetazole, cefotaxime, cefoxitin, ceftazidime, cephalothin, methicillin, naficillin, and penicillin were purchased from Sigma Aldrich. Oxacillin was purchased from TCI America. All assays were run in duplicate and repeated at least two separate times. All compounds were dissolved as their HCl salts in molecular biology grade DMSO-D6 as 100 mM stock solutions and stored at -20 °C.

Broth microdilution method for determination of minimum inhibitory concentrations

Mycobacterium smegmatis was grown in 7H9 (ADC, 0.5% Tween 80) for 48 h, and this culture was used to inoculate fresh 7H9 (5×10^5 CFU mL^{-1}, OD600 = 0.006). Aliquots (1 mL) were placed in culture tubes, and compound was added from 100 mM stock solutions in DMSO-D6, such that the compound concentration equaled the highest concentration tested. Antibiotics, from a water stock, were added at the highest concentration tested to 1 mL aliquots of cultures. Inoculated media not treated with compound served as the control. Samples were then aliquoted (200 μL) into the first wells of a 96-well plate, with all remaining wells being filled with 100 μL of initial bacterial subculture. Row 1 wells were mixed 8 times, before 100 μL was transferred to

row 2. Row 2 was then mixed 8 times, and 100 μL was transferred to row 3. This process was repeated to serially dilute the rest of the rows. One row with bacteria subculture served as the control. Plates were then covered with GLAD Press n' Seal and incubated under stationary conditions at 37 °C. After 48 h, 10 μL of alamarBlue was added to each well, and the plate was resealed and incubated under stationary conditions at 37 °C. After 6 h, the minimum inhibitory concentration (MIC) values were recorded as the lowest concentration of compound or antibiotic at which no visible growth of bacteria was observed.

General inhibition assay protocol for *M. smegmatis*

Cultures were incubated for 48 hr and then subcultured to 0.01 in Difco M9 minimal salts media. 100 μL of subculture containing the appropriate concentration of compound was added to all of the wells in columns 2–4 and 9–11 of 96- well PVC microtiter plate. Columns 1 and 12 were filled with 100 μL of sterile M9 minimal salts media to serve as controls. Plates were covered with Glad Press n' Seal and were incubated under stationary conditions at 37°C for 48 hr. After 48 hr, the media were discarded, and the plates were washed thoroughly with water. 110 μL of a 0.1% aqueous solution of crystal violet was added to every well, and the plates were left at ambient temperature for 30 min. After 30 min, the crystal violet was disposed, and the plates were washed thoroughly with water. 200 μL of 95% ethanol was added to each well, and the plates were left at ambient temperature for 10 min. 125 μL of the ethanol solution was transferred to a fresh polystyrene microtiter plate, and the plate was quantified by measuring the OD_{540}. The percent inhibition was calculated by comparing the OD540 of the treated wells with the OD_{540} of the untreated wells. The first and last column, which had only sterile media, were used as blanks and those values were subtracted from the OD_{540} obtained in the other columns.

References

1. Lindsey, E.A., et al., *The discovery of N-1 substituted 2-aminobenzimidazoles as zinc-dependent S. aureus biofilm inhibitors.* Medchemcomm, 2012. **3**(11): p. 1462-1465.
2. Nguyen, T.V., et al., *The Discovery of 2-Aminobenzimidazoles That Sensitize Mycobacterium smegmatis and M. tuberculosis to beta-Lactam Antibiotics in a Pattern Distinct from beta-Lactamase Inhibitors.* Angew Chem Int Ed Engl, 2017. **56**(14): p. 3940-3944.