Supporting Information

Discovery and Optimization of Potent, Selective, and in Vivo Efficacious 2-Aryl Benzimidazole BCATm Inhibitors

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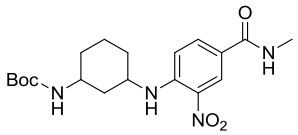
Contents:

Compound Synthesis

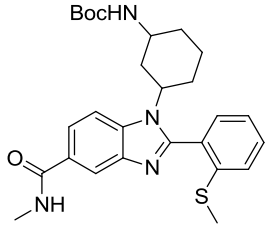
All solvents and reagents were used as obtained from commercial sources. ^{1}H NMR spectra were recorded on a Varian Mercury 400 Plus. Chemical shifts are expressed in parts per million (ppm, δ units). Coupling constants (*J*) are in units of hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), t (triplet), q (quartet), dd (double doublet), dt (double triplet), m (multiplet), br (broad). Analytical purity for a final compound was ≥ 95% unless stated otherwise. The purity of final compounds was checked using an Agilent 1100 HPLC system coupled with a Thermo Finnigan LCQ Mass Spectrometer. All mass spectra were performed by electrospray ionization (ESI). Two different HPLC conditions were used to analyze compound purity: LC-MS method A: 10-98% AcCN-H_2O (0.1%TFA) in 2.7 min, hold at 98% AcCN for 0.38 min, with the flow rate of 0.9 mL/min on a Phenomenex Luna 3μ C8(2) 100A 30 × 3.00 mm column. LC-MS method B: 10-95% AcCN-H_2O (0.1% formic acid) in 3.0 min, with the flow rate of 0.5 mL/min on a Kinetex 2.6 μ C18 100A 30 × 2.10 mm column. High resolution mass spectrometry (HRMS) was completed on a Waters qTOF Premiere mass spectrometer operating in W mode positive ionization with a resolving power of approximately 15000. Flow injection was completed using a Waters Nanoacquity LC. HRMS acceptable error is 3 mDa or 5 ppm, although most analyses are observed within 0.5 mDa with isotope fits in good agreement with the proposed structures. Purification of final compounds for biological testing was performed on a Gilson GX-281 system with a Phenomenex Luna 5μ C8(2) 100 × 21.20 mm 100A column running gradient of 5-95% MeCN/H_2O (+0.1% TFA or 0.1% formic acid) over 15-20 minutes with flow rate of 22 mL/min.

Tert-butyl (3-((4-(methylcarbamoyl)-2-nitrophenyl)amino)cyclohexyl)carbamate (3).



To a solution of 4-fluoro-3-nitrobenzoic acid (1.00 g, 5.40 mmol) in acetonitrile (40 mL) was added HATU (2.054 g, 5.40 mmol) and DIPEA (1.03 mL, 5.94 mmol). The reaction was stirred at room temperature for 15 min followed by addition of methanamine (HCl salt, 0.401 g, 5.94 mmol) and DIPEA (1.03 mL, 5.94 mmol). The reaction was continuously stirred for 3 h then was concentrated in vacuo. The residue was dissolved in water and extracted with ethyl acetate. The organic layer was washed with water, dried over Na_2SO_4, and condensed in vacuo to provide 4-fluoro-N-methyl-3-nitrobenzamide as a crude product. This crude product was dissolved in ethanol (40 mL) followed by addition of tert-butyl (3-aminocyclohexyl)carbamate (1.10 g, 5.13 mmol, racemic mixture of diastereomers) and DIPEA (1.03 mL, 5.94 mmol). The reaction was stirred at 85 °C for 6 h, then concentrated, and the residue was dissolved in ethyl acetate and washed with water. The organic layer was dried over Na_2SO_4 and condensed in vacuo to provide the title compound as a crude product with purity >95% (mixture of diatereomers). LC-MS (ESI) *m/z* $[M+1]^+$ = 392.7, Rt = 2.13 min (HPLC method A).

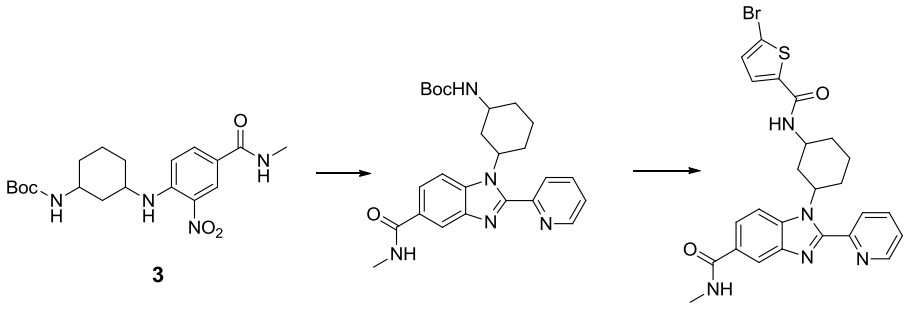
tert-butyl (3-(5-(methylcarbamoyl)-2-(2-(methylthio)phenyl)-1H-benzo[d]imidazol-1-yl)cyclohexyl)carbamate (4)



To a solution of 4-fluoro-N-methyl-3-nitrobenzamide (69 mg, 0.35 mmol) in 1,4-Dioxane (4 mL) was added 1,1-dimethylethyl (3-aminocyclohexyl)carbamate (54 mg, 0.25 mmol) and

DIPEA (87 μL, 0.5 mmol). The reaction was stirred at 80 °C for 24 h then was cooled to room temperature and added 2-(methylthio)benzaldehyde (38 mg, 0.25 mmol), sodium dithionite (131 mg, 0.75 mmol), and water (1.0 mL). The reaction was stirred at 80 °C for 24 h, then concentrated in vacuo. The residue was purified by preparative HPLC to afford the title compound **4** (48.9 mg, 39.5% yield). LC-MS (ESI) *m/z* $[M+1]^+$ = 495.2; Rt = 1.97 min (HPLC method A).

cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-methyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (8b).

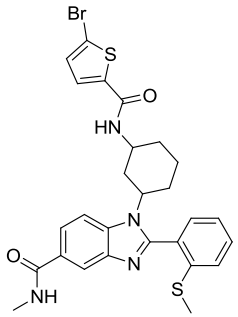


To a solution of the crude material **3** (5.4 mmol) in 1,4-dioxane (40 mL) was added picolinaldehyde (0.579 g, 5.40 mmol), sodium dithionite ($Na_2S_2O_4$) (5.64 g, 32.4 mmol) and water (10 mL). The reaction was stirred at 80 °C for 18 h, then concentrated in vacuo followed by extraction with ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and condensed *in vacuo*. The residue was purified by chromatography (silica gel column, UV detection at 298 nm, eluent system: 0-8% CH_3OH-CH_2Cl_2 over 40 min with flow rate of 60 mL/min) to yield intermediate tert-butyl (3-(5-(methylcarbamoyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazol-1-yl)cyclohexyl)carbamate as a mixture of diastereomers (1.5 g, 62% yield for 3 steps from 4-fluoro-3-nitrobenzoid acid). LC-MS (ESI) *m/z* $[M+1]^+$ = 450.0; Rt = 1.78 min (HPLC method A).

To the above intermediate (1.5 g, 3.34 mmol) in DCM (30 mL) was added dropwise TFA (2.57 mL, 33.4 mmol). The reaction was stirred at room temperature for 18 h. The reaction was condensed in vacuo and the resulting residue was dissolved in acetonitrile (30 mL) to provide solution A. In a separate vial, a reaction of 5-bromothiophene-2-carboxylic acid (0.691 g, 3.34 mmol), HATU (1.269 g, 3.34 mmol), and DIPEA (1.276 mL, 7.34 mmol) in acetonitrile (30 mL) was stirred at room temperature for 30 min. To this reaction was added the solution A. The resultant was stirred at room temperature for 4 h then was condensed in vacuo. The residue was participated between ethyl acetate and water. The aqueous layer was extracted twice by ethyl acetate and the combined ethyl acetate solution was washed with saturated $NaHCO_3$ solution followed by brine, then dried over Na_2SO_4 and concentrated to provide a crude product. The crude product was first purified by silica gel chromatography (ISCO system, detection at 298 nm, eluting with 0-8% CH_3OH-CH_2Cl_2 over 50 min at flow rate of 85 mL/min) to yield *trans*-isomer (800 mg) and a mixture of *cis*- and *trans*- isomers (857 mg). The combined yield for *cis*- and *trans*- isomers is 1.65 g (92% yield over 2 steps). The 857 mg of *cis*- and *trans*- mixture was further purified by preparative HPLC system (Column: Phenomenex Gemini C18 110A, Ax1A, 100 x 30.00 mm, 5 µ column; flow rate: 40 mL/min; UV detection: 254 nm; gradient: 20-55% AcCN-H_2O (0.2% formic acid as modifier) in 20 min) to yield pure *cis*-isomer product (**8b**, 305 mg). MS (ESI) *m/z* $[M+1]^+$ = 538.1 and 540.1; Rt = 2.24 min (HPLC method B); ^{1}H NMR NMR (400 MHz, DMSO-d_6) δ 8.79-8.75 (m, 1H), 8.51-8.44 (m, 2H), 8.23 (s, 1H), 8.16 (dd, 1H, *J* = 7.7, 1.4 Hz), 8.06-8.00 (m, 1H), 7.91-7.81 (m, 2H), 7.59-7.54 (m, 2H), 7.24 (dd, 1H, *J* = 3.9, 0.8 Hz), 5.57-5.47 (m, 1H), 3.92-3.80 (m, 1H), 2.81 (d, 3H, *J* = 4.3 Hz), 2.49-2.34 (m, 1H), 2.32-2.19 (m, 1H), 2.17-2.09 (m, 1H), 2.01-1.83 (m, 3H), 1.54-1.38 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{25}H_{24}BrN_5O_2S + H]$ 538.0812; found 538.0815. For the *trans*-isomer, MS (ESI) *m/z*

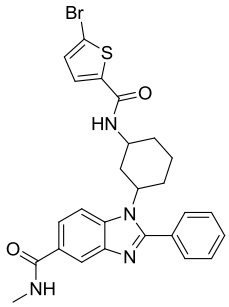
$[M+1]^+$ = 538.0 and 540.1; Rt = 2.10 min (HPLC method B); ^{1}H NMR NMR (400 MHz, DMSO-d_6) δ 8.43-8.42 (m, 1H), 8.19 (d, 1H, *J* = 4.0 Hz), 8.15 (s, 1H), 8.05-8.01 (m, 2H), 7.95 (d, 1H, *J* = 9.2 Hz), 7.89 (dd, 1H, *J* = 8.0, 1.2 Hz), 7.75 (dd, 1H, *J* = 8.8, 1.2 Hz), 7.67 (d, 1H, *J* = 8.0 Hz), 7.38-7.35 (m, 1H), 7.28 (d, 1H, *J* = 4.0 Hz), 5.55-5.49 (m, 1H), 4.26 (s, 1H), 2.76 (d, 3H, *J* = 4.4), 2.44-2.42 (m, 1H), 2.38-2.28 (m, 2H), 1.93-1.92 (m, 2H), 1.90-1.83 (m, 1H), 1.77-1.74 (m, 1H), 1.67-1.63 (m. 1H). HRMS $(M+H)^+$ calcd for $[C_{25}H_{24}BrN_5O_2S + H]$ 538.0812; found 538.0814.

cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-methyl-2-(2-(methylthio)phenyl)-1H-benzo[d]imidazole-5-carboxamide (1).



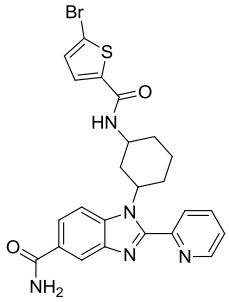
Using 4 as a starting material, the title compound was prepared analogously to the synthesis of **8b** as a white solid in 14% overall yield. LC-MS (ESI) *m/z* $[M+1]^+$ = 583.1; ^{1}H NMR NMR (400 MHz, DMSO-d_6) δ 8.50-8.38 (m, 2H), 8.19 (s, 1H), 7.86-7.78 (m, 2H), 7.62-7.48 (m, 3H), 7.40 (d, 1H, *J* = 7.1 Hz), 7.36-7.29 (m, 1H), 7.24 (dd, 1H, *J* = 3.9, 0.7 Hz), 3.96-3.86 (m, 1H), 3.72-3.60 (m, 1H), 2.81 (d, 3H, *J* = 4.3 Hz), 2.42 (s, 3H), 2.36-2,00 (m, 3H), 1.99-1.74 (m, 3H), 1.48-1.17 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{27}H_{27}BrN_4O_2S_2 + H]$ 583.0837; found 583.0842.

cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-methyl-2-phenyl-1H-benzo[d]imidazole-5-carboxamide (8a).



The title compound was prepared analogously to the synthesis of **8b** as a white solid in 12.5% overall yield. LC-MS (ESI) *m/z* $[M+1]^+$ = 537.3; ^{1}H NMR NMR (400 MHz, CD_3OD) δ 8.27-8.25 (m, 1H), 8.14 (d, 1H, *J* = 8.8 Hz), 8.03 (dd, 1H, *J* = 8.8, 1.2 Hz), 7.83-7.71 (m, 5H), 7.48 (dd, 1H, *J* = 3.9, 0.8 Hz), 7.13 (d, 1H, *J* = 3.9 Hz), 4.70-4.60 (m, 1H), 3.97-3.88 (m, 1H), 2.98 (s, 3H), 2.52-2.33 (m, 3H), 2.17-1.99 (m, 3H), 1.64-1.45 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{26}H_{25}BrN_4O_2S + H]$ 537.0960; found 537.0961.

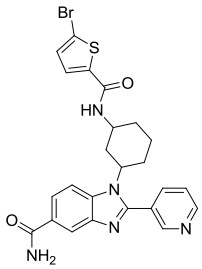
cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (8c).



The title compound was prepared analogously to the synthesis of **8b** as a white solid in 4.0% overall yield. MS (ESI) *m/z* $[M+1]^+$ = 524.4 and 526.5; ^{1}H NMR NMR (400 MHz, DMSO-d_6) δ 8.78 (dd, 1H, *J* = 5.9, 0.8 Hz), 8.47 (d, 1H, *J* = 7.8 Hz), 8.29 (s, 1H), 8.16 (d, 1H, *J* = 7.8 Hz), 8.05-8.01 (m, 2H), 7.88 (s, 2H), 7.58-7.55 (m, 2H), 7.31 (s, 1H), 7.23 (d, 1H, *J* = 4.3 Hz), 5.55-5.49 (m, 1H), 3.85-3.69 (m, 1H), 2.47-2.39 (m, 1H), 2.36-2.25 (m, 1H), 2.13-2.10 (m, 1H), 1.96

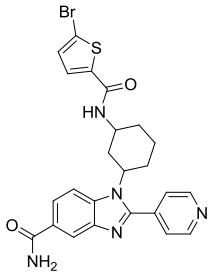
– 1.88 (m, 3H), 1.48-1.44 (m, 2H). HRMS $(M+H)^+$ calcd for [$C_{24}H_{22}BrN_5O_2S$ + H] 524.0756; found 524.0757.

cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-2-(pyridin-3-yl)-1H-benzo[d]imidazole-5-carboxamide (8d).



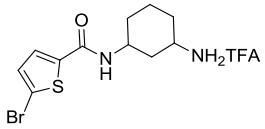
The title compound was prepared analogously to the synthesis of **8b** as a white solid in 4.6% overall yield for 5 steps (from 4-fluoro-3-nitro-benzoid acid). MS (ESI) *m/z* $[M+1]^+$ = 524.6 and 526.1; ^{1}H NMR NMR (400 MHz, DMSO-d_6) δ 8.88 (d, 1H, *J* = 2.4Hz), 8.79 (d, 1H, *J* = 4.2 Hz), 8.45 (d, 1H, *J* = 7.8Hz), 8.29 (s, 1H), 8.12 (m, 1H), 8.03 (s, 1H), 7.88 (s, 2H), 7.66 (d, 1H, *J* = 5.1 Hz), 7.64 (d, 1H, *J* = 5.8 Hz), 7.55 (d, 1H, *J* = 3.9 Hz), 7.32 (m, 1H), 7.24 (d, 1H, *J* = 3.9 Hz), 4.38 (m, 1H), 3.82 (m, 1H), 1.98-1.95 (m, 1H), 2.38-2.35 (m, 1H), 2.48-2.42 (m, 1H), 1.90-1.83 (m, 2H), 1.42-1.44 (m, 2H). HRMS $(M+H)^+$ calcd for [$C_{24}H_{22}BrN_5O_2S$ + H] 524.0756; found 524.0753.

cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-2-(pyridin-4-yl)-1H-benzo[d]imidazole-5-carboxamide (8e).



The title compound was prepared analogously to the synthesis of **8b** as a white solid in 4.0% overall yield for 5 steps (from 4-fluoro-3-nitro-benzoid acid). MS (ESI) *m/z* $[M+1]^+$ = 524.6 and 526.1; ^{1}H NMR NMR (400 MHz, DMSO-d_6) δ 8.82 (d, 2H, *J* = 5.5 Hz), 8.45 (d, 1H, *J* = 8.4 Hz), 8.30 (s, 1H), 8.03 (m, 1H), 7.88 (s, 2H), 7.74 (d, 2H, *J* = 5.5 Hz), 7.55 (d, 1H, *J* = 3.5 Hz), 7.33 (m, 1H), 7.24 (d, 1H, *J* = 3.2 Hz), 4.44 (m, 1H), 3.88 (m, 1H), 2.42-2.39 (m, 1H), 2.36-2.33 (m, 1H), 2.22-2.12 (m, 1H), 1.99-1.96 (m, 1H), 1.99-1.96 (m, 2H), 1.43 (m, 2H). HRMS $(M+H)^+$ calcd for [$C_{24}H_{22}BrN_5O_2S$ + H] 524.0756; found 524.0757.

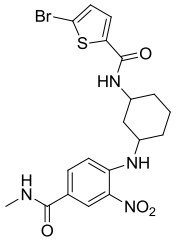
cis N-(3-aminocyclohexyl)-5-bromothiophene-2-carboxamide (6, TFA salt).



To a solution of 5-bromothiophene-2-carboxylic acid (19.27 g, 93 mmol) in DCM (200 mL) was added HOBt (16.29 g, 106 mmol), EDCI (20.40 g, 106 mmol) and triethylamine (29.7 mL, 213 mmol). The mixture was stirred at room temperature for 30 minutes. Then tert-butyl (3-aminocyclohexyl)carbamate (19.0 g, 89 mmol) was added . The reaction was stirred at room temperature overnight. The precipitate was filtered, washed with DCM to give the *cis*-compound *cis*-tert-butyl-3-(5-bromothiophene-2-carboxamido)cyclohexyl)carbamate (mixture of two cis-isomers, 12.0 g, 29.8 mmol, 33.6 % yield) as a white solid (the filtrate contains mainly the trans isomers). MS (ESI) *m/z* = 405.0 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.37 (d, *J*

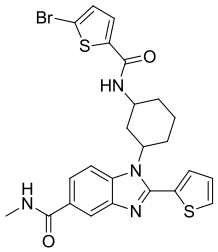
= 7.78 Hz, 1H), 7.62 (d, *J* = 3.99 Hz, 1H), 7.26 (d, *J* = 3.99 Hz, 1H), 6.85 (d, *J* = 7.78 Hz, 1H), 3.7 (m, 1H), 3.26 (m, 1H), 1.93 (d, *J* = 11.58 Hz, 1H), 1.73 (m, 3H), 1.38 (br. s., 9H), 1.31 – 1.14 (m, 4H). To the above Boc protected intermediate *cis*-tert-butyl-3-(5-bromothiophene-2-carboxamido)cyclohexyl)carbamate (12.0 g, 29.8 mmol) in DCM (100 mL) was added TFA (22.10 mL, 298 mmol). The mixture was stirred at room temperature for 4 h then concentrated *in vacuo* to give a brown viscous oil. This oil was triturated with diisopropyl ether to give the title compound **6** (12.0 g, 97 % yield) as a beige powder. MS (ESI) *m/z* = 304.9 $(M+H)^+$.

cis 5-bromo-N-3-((4-(methylcarbamoyl)-2-nitrophenyl)amino)cyclohexyl)thiophene-2-carboxamide (7).



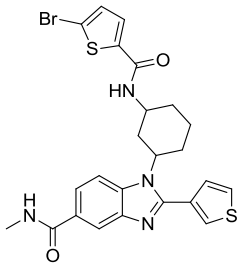
To a solution of 4-fluoro-N-methyl-3-nitrobenzamide (4.99 g, 25.2 mmol) in ethanol (100 mL), was added compound **6** (TFA salt, 10.0 g, 23.97 mmol) followed by DIEA (8.71 mL, 52.7 mmol). The resultant was heated at 85 °C for 6 h, then at room temperature overnight . The reaction was concentrated in vacuo, and the residue was treated with water and ethyl acetate (200 mL). The yellow solid formed was filtered and washed with diisopropyl ether to give, after drying, the title compound **7** (8.8 g, 76 % yield) as a yellow powder. LC-MS: Rt = 3.07 min, MS (ESI) *m/z* = 482.8 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.63 (s, 5H), 8.45 (m, 2H), 8.10 (d, *J* = 7.78 Hz, 1H), 7.98 (d, *J* = 8.73 Hz, 1H), 7.62 (d, *J* = 3.80 Hz, 1H), 7.25 (m, 2H), 3.91 (m, 1H), 3.87 (m, 1), 2.77 (d, *J* = 3.99 Hz, 3H), 2.22 (m, 1H), 2.02 (m, 1H), 1.9 – 1.79 (m, 2H), 1.47 (m, 2H), 1.31 (m, 2H).

cis 1-3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-methyl-2-(thiophen-2-yl)-1H-benzo[d]imidazole-5-carboxamide (8f).



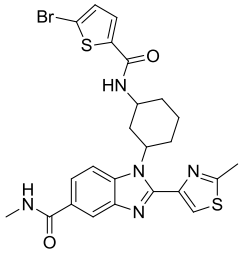
Compound **7** (241 mg, 0.5 mmol), thiophene-2-carbaldehyde (72.9 mg, 0.650 mmol), and sodium dithionite (0.261 g, 1.500 mmol) were mixed in 1,4-dioxane (4 mL) and H_2O (2 mL). The reaction mixture was heated at 130 °C for 1h under microwave irradiation. The reaction mixture was then concentrated in vacuo. Water was added to the residue and the aqueous phase was extracted with ethyl acetate (2 × 50 mL). The organic phase was washed with water, then dried over Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on a silic gel column eluted with CH_2Cl_2/MeOH = 94/6 (v/v) to afford the title compound **8f** in 29.4% yield (80 mg) as a yellow solid. MS (ESI) *m/z* = 544.8 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.49 (m, 2H), 8.19 (br s, 1H), 7.96 – 7.75 (m, 3H), 7.60 (br s, 2H), 7.3 (m, 2H), 4.79 (br s, 1H), 3.94 (br s, 1H), 2.83 (br s, 3H), 2.46 – 2.22 (m, 2H), 2.16 (m, 1H), 1.94 (m, 3H), 1.54 (m, 2H); HRMS $(M+H)^+$ calcd for $[C_{24}H_{23}BrN_4O_2S_2+H]$ 543.0524; found 543.0547.

cis 1-3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-methyl-2-(thiophen-3-yl)-1H-benzo[d]imidazole-5-carboxamide (8g).



The title compound was prepared analogously to the synthesis of **8f** as a yellow solid in 33.1% yield. MS (ESI) *m/z* = 544.8 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.49 (m, 2H), 8.20 (s, 1H), 8.05 (br s, 1H), 7.83 (br s, 3H), 7.59 (m, 1H), 7.50 (d, *J* = 4.18 Hz, 1H), 7.26 (m, 1H), 4.59 (br s, 1H), 3.88 (br s, 1H) 2.83 (m, 3H), 2.43-2.26 (m, 2H), 2.12 (m, 1H), 2.05-1.80 (m, 3H), 1.48 (m, 2H); HRMS $(M + H)^+$ calcd for $[C_{24}H_{23}BrN_4O_2S_2+H]$ 543.0524; found 543.0543.

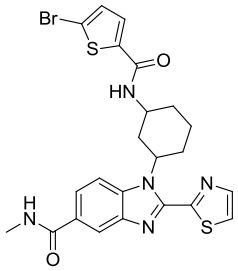
cis 1-3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-methyl-2-(2-methylthiazol-4-yl)-1H-benzo[d]imidazole-5-carboxamide (8h).



To a solution of compound **7** (241 mg, 0.5 mmol) in 1,4-dioxane (10mL) and water (1 mL) was added 2-methylthiazole-4-carbaldehyde (95 mg, 0.750 mmol) and sodium dithionite (261 mg, 1.500 mmol). The resultant was heated at 85 °C for 24 h, then cooled to room temperature. The reaction was concentrated in vacuo, and the residue was treated with water and ethyl acetate (20 mL). The off-white solid formed was filtered off and washed with diisopropyl ether to give, after drying, the title compound **8h** as a off white powder (160 mg, 57.3 % yield). MS (ESI) *m/z* = 559.8 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.48 (m, 2H), 8.26 (s, 1H), 8.19 (s, 1H), 7.83

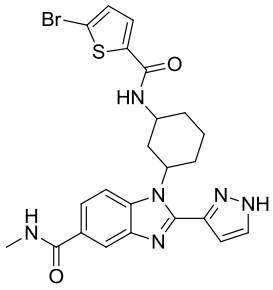
(m, 2H), 7.6 (d, *J* = 3.99 Hz, 1H), 7.27 (d, *J* = 3.99 Hz, 1H), 5.47 (m, 1H), 3.91 (m, 1H), 2.82 (m, 6H), 2.47 - 2.17 (m, 2H), 2.10 (d, *J* = 10.82 Hz, 1H), 1.94 (d, *J* = 7.59 Hz, 3H), 1.5 (m, 2H).

cis 1-3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-methyl-2-(thiazol-2-yl)-1H-benzo[d]imidazole-5-carboxamide (8i).



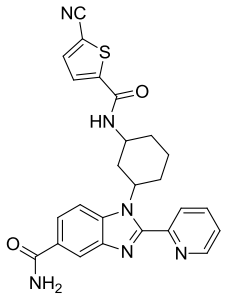
The title compound was prepared analogously to the synthesis of **8h** as a cream powder in 25.7% yield. MS (ESI) *m/z* = 545.8 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.52 (m, 2H), 8.25 (s, 1H), 8.17 (d, *J* = 3.23 Hz, 1H), 8.04 (d, *J* = 3.23 Hz, 1H), 7.89 (q, *J* = 8.73 Hz, 2H), 7.60 (d, *J* = 3.99 Hz, 1H), 7.27 (d, *J* = 3.99 Hz, 1H), 6.11 (br s, 1H), 3.97 (br s, 1H), 2.83 (d, *J* = 4.37 Hz, 3H), 2.22 - 2.48 (m, 2H), 2.15 (d, *J* = 11.58 Hz, 1H), 1.96 (d, *J* = 8.54 Hz, 3H), 1.53 (m, 2H).

cis 1-3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-methyl-2-(1H-pyrazol-3-yl)-1H-benzo[d]imidazole-5-carboxamide (8j).



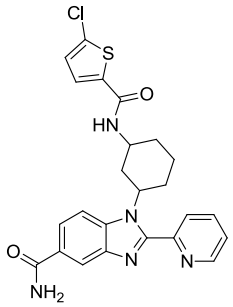
The title compound was prepared analogously to the synthesis of **8h** as a yellow solid in 70.2% yield. MS (ESI) *m/z* = 528.9 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.55 (d, *J* = 6.64 Hz, 2H), 8.21 (br s, 1H), 8.07 (br s, 1H), 7.98 (d, *J* = 8.54 Hz, 1H), 7.88 (d, *J* = 8.16 Hz, 1H), 7.60 (d, *J* = 3.99 Hz, 1H), 7.27 (d, *J* = 3.99 Hz, 1H), 7.04 (br s, 1H), 5.76 (br s, 1H), 3.95 (br s, 1H), 2.84 (d, *J* = 3.99 Hz, 3H), 2.25 - 2.47 (m, 2H), 2.18 (m, 1H), 1.95 (m, 3H), 1.52 (m, 2H).

cis 1-3-(5-cyanothiophene-2-carboxamido)cyclohexyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (9Aa).



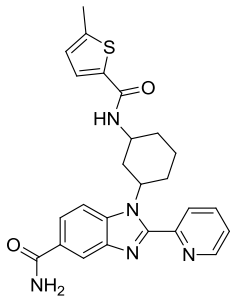
Using 5-cyanothiophene-2-carboxylic acid instead of 5-bromothiophene-2-carboxylic acid, the title compound was prepared analogously to the synthesis of **8b** as a white solid. MS (ESI) *m/z* $[M+1]^+$ 471.2; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81-8.77 (m, 2H), 8.30 (s, 1H), 8.18 (d, 1H, *J* = 6.8 Hz), 8.03 (m, 2H), 7.95 (d, 1H, *J* = 4.0 Hz), 7.88 (s, 2H), 7.82 (d, 1H, *J* = 3.6 Hz), 7.56 (m, 1H), 7.32 (m, 1H), 5.55 (m, 1H), 3.89 (m, 1H), 2.31(m, 2H), 2.26 (m, 1H), 1.91 (m, 3H), 1.49 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{25}H_{22}N_6O_2S + H]$ 471.1603; found 471.1608.

cis 1-(3-(5-chlorothiophene-2-carboxamido)cyclohexyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (9Ab).



Using 5-chlorothiophene-2-carboxylic acid instead of 5-bromothiophene-2-carboxylic acid, the title compound was prepared analogously to the synthesis of **8b** as a white solid. MS (ESI) *m/z* $[M+1]^+$ = 480.2 and 482.1; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (d, 1H, *J* = 4.4 Hz), 8.49 (d, 1H, *J* = 8.0 Hz), 8.30 (s, 1H), 8.18 (d, 1H, *J* = 7.6 Hz), 8.05-8.02 (m, 2H), 7.87 (s, 2H), 7.61 (d, 1H, *J* = 3.6 Hz), 7.58-7.55 (m, 1H), 7.32 (br, 1H), 7.14 (d, 1H, *J* = 4.0 Hz), 5.54 (m, 1H), 3.88 (m, 1H), 2.31(m, 2H), 2.13 (m, 1H), 1.89-1.91 (m, 3H), 1.46 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{24}H_{22}ClN_5O_2S + H]$ 480.1261; found 480.1263.

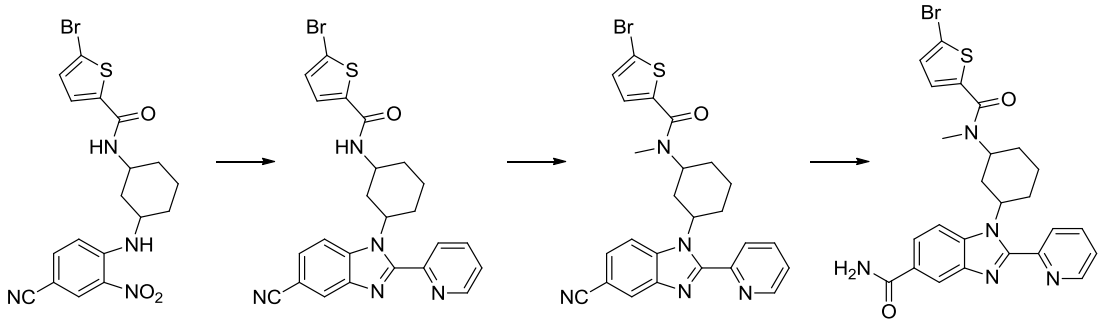
cis 1-(3-(5-methylthiophene-2-carboxamido)cyclohexyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (9Ac).



Using 5-methylthiophene-2-carboxylic acid instead of 5-bromothiophene-2-carboxylic acid, the title compound was prepared analogously to the synthesis of **8b** as a white solid. MS (ESI) *m/z* $[M+1]^+$ = 460.2; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (d, 1H, *J* = 4.8 Hz), 8.30 (s, 1H), 8.25 (d, 1H, *J* = 9.6 Hz), 8.17 (d, 1H, J = 7.6 Hz), 8.04 (m, 2H), 7.89 (s, 2H), 7.60-7.57 (m, 1H), 7.52

(d, 1H, *J* = 3.6 Hz), 7.34 (br, 1H), 6.80 (d, 1H, *J* = 4.0 Hz), 5.52 (m, 1H), 3.86 (m, 1H), 2.30-2.21(m, 2H), 2.13 (m, 1H), 1.96 (m,1H), 1.89 (m, 2H), 1.46 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{25}H_{25}N_5O_2S + H]$ 460.1807; found 460.1808.

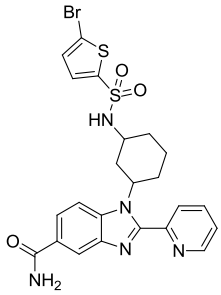
cis 1-(3-(5-bromo-N-methylthiophene-2-carboxamido)cyclohexyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (9Ad).



To a solution of 4-fluoro-3-nitrobenzonitrile (0.717 g, 4.31 mmol) in ethanol (20 mL) was added cis N-(-3-aminocyclohexyl)-5-bromothiophene-2-carboxamide (TFA salt, 1.5 g, 3.60 mmol) and then DIEA (1.307 mL, 7.91 mmol). The reaction mixture was heated at 85 °C for 6 hours and then at room temperature overnight. Ethanol was removed in vacuo then water was added followed by ethyl acetate (200 mL). The yellow solid formed was collected and triturated with diisopropyl ether to afford compound **i** (mixture of cis-isomers) (1.25 g, 77 % yield) as a yellow powder. MS (ESI) *m/z* = 448.8 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.52 (d, *J* = 1.90 Hz, 1H), 8.43 (d, *J* = 7.59 Hz, 1H), 8.23 (d, *J* = 7.97 Hz, 1H), 7.85 (dd, *J* = 9.11, 1.71 Hz, 1H), 7.62 (d, *J* = 3.99 Hz, 1H), 7.22 - 7.39 (m, 2H), 3.90 (br s, 2H), 2.18 (m, 1H), 1.98 (d, *J* = 9.87 Hz, 1H), 1.74 - 1.92 (m, 2H), 1.20 - 1.59 (m, 4 H). Using compound **i** and picolinaldehyde as starting material, compound **ii** (mixture of cis-isomers) was prepared analogouly to **8h** as a cream solid in 43.4% yield. MS (ESI) *m/z* = 507.9 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ

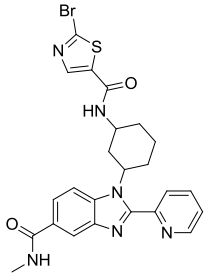
8.81 (d, *J* = 4.18 Hz, 1H), 8.50 (d, *J* = 7.78 Hz, 1H), 8.32 (d, *J* = 1.14 Hz, 1H), 8.21 (d, *J* = 7.97 Hz, 1H), 7.98 - 8.14 (m, 2H), 7.74 (dd, *J* = 8.64, 1.42 Hz, 1H), 7.53 - 7.68 (m, 2H), 7.27 (d, *J* = 3.99 Hz, 1H), 5.58 (t, *J* = 12.24 Hz, 1H), 3.90 (br s, 1H), 2.40 (q, *J* = 12.08 Hz, 1H), 2.27 (d, *J* = 8.92 Hz, 1H), 2.16 (m, 1H), 1.82 - 2.08 (m, 3H), 1.35 - 1.61 (m, 2H). To a solution of compound **ii** (950 mg, 1.876 mmol) in DMF (20 mL) was added sodium hydride (90 mg, 3.75 mmol) and then iodomethane (293 mg, 2.064 mmol). The reaction mixture was stirred at room temperature for 4 h and then concentrated under reduced pressure. The residue was partitioned between water and ethyl acetate (20 mL). The organic layer was washed twice with water, then dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was triturated with diisopropyl ether/CH_2Cl_2, and the resulting precipitate was filtered and dried to give compound **iii** (mixture of cis-isomers) as a off-white solid (910 mg, 93 % yield). MS (ESI) *m/z* = 521.9 $(M+H)^+$. A mixture of compound **iii** (894 mg, 1.718 mmol) in sulfuric acid (2000 mg, 20.39 mmol) and water (37.1 mg, 2.061 mmol) was stirred at room temperature for 1 h and then diluted with water and ethyl acetate (20 mL). The mixture was neutralised with NaOH (1N) . The organic layer was washed twice with water, then dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column eluted with CH_2Cl_2 /MeOH (95/5, v/v) to afford the desired compound **9Ad** (mixture of cis-isomers) (200 mg, 21.62 % yield) as a white solid. MS (ESI) *m/z* = 540.0 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 8.74 (d, *J* = 4.36 Hz, 1H), 8.28 (m, 2H), 7.92 (m, 2H), 7.76 (d, *J* = 8.54 Hz, 1H), 7.44 (ddd, *J* = 7.54, 4.89, 1.04 Hz, 1H), 7.14 (d, *J* = 3.80 Hz, 1H), 7.03 (d, *J* = 3.80 Hz, 1H), 5.77 (m, 1H), 4.54 (br s, 1H), 3.15 (s, 3H), 2.60 (q, *J* = 12.08 Hz, 1H), 2.3 (m, 2H), 2.11 (m, 2H), 1.93 (m, 1H), 1.48 - 1.85 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{25}H_{24}BrN_5O_2S+H]$ 538.0912; found 538.0934.

cis 1,3-(5-bromothiophene-2-sulfonamido)cyclohexyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (9Ae).



Using 5-bromothiophene-2-sulfonyl chloride instead of 5-bromothiophene-2-carboxylic acid, the title compound was prepared analogouly to **8b** in 43.3% yield (13.4 mg). MS (ESI) *m/z* $[M+1]^+$ = 560.1 and 562.1; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72 (d, 1H, J = 4.4 Hz), 8.29 (s, 1H), 8.19 (d, 1H, J = 7.2 Hz), 8.16 (d, 1H, J = 8.0 Hz), 8.04 (m, 2H), 7.85 (s, 2H), 7.58 (m, 1H), 7.45 (d, 1H, J = 4.0 Hz), 7.33 (br, 1H), 7.30 (d, 1H, J = 4.4), 5.34 (m, 1H), 3.23 (m, 1H), 2.26-2.21 (m, 2H), 2.03 (m, 1H), 1.83-1.79 (m, 3H), 1.41 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{23}H_{22}BrN_5O_3S_2 + H]$ 560.0426; found 560.0423.

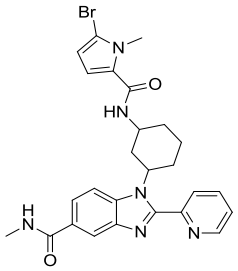
cis-2-bromo-N-(3-(5-(methylcarbamoyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazol-1-yl)cyclohexyl)thiazole-5-carboxamide (9Ba).



Using 2-bromothiazole-5-carboxylic acid instead of 5-bromothiophene-2-carboxylic acid, the title compound was prepared analogously to **8b** in 32% yield (63 mg). MS (ESI) *m/z* = 540.8

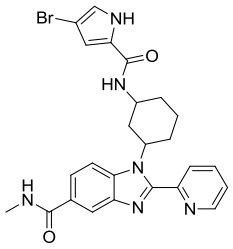
$(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.79 (d, *J* = 4.18 Hz, 1H), 8.73 (d, *J* = 7.78 Hz, 1H), 8.49 (d, *J* = 4.55 Hz, 1H), 8.27 (s, 1H), 8.2 (m, 2H), 8.05 (td, *J* = 7.73 Hz, 1.61 Hz, 1 H), 7.87 (m, 2H), 7.58 (td, *J* = 6.17, 0.95 Hz, 1H), 5.56 (t, *J* = 12.15 Hz, 1H), 3.9 (m, 1H), 2.83 (d, *J* = 4.37 Hz, 3H), 2.23 - 2.47 (m, 2H), 2.17 (d, *J* = 11.20 Hz, 1H), 1.83 - 2.06 (m, 3H), 1.57 (m, 2H); HRMS $(M+H)^+$ calcd for [$C_{24}H_{23}BrN_6O_2S$+H] 539.0865; found 539.0856.

cis 1-(3-(5-bromo-1-methyl-1H-pyrrole-2-carboxamido)cyclohexyl)-N-methyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (9Bb).



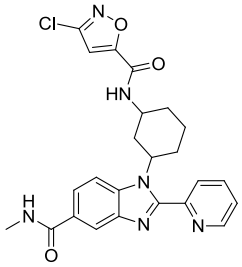
Using 5-bromo-1-methyl-1H-pyrrole-2-carboxylic acid instead of 5-bromothiophene-2-carboxylic acid, the title compound was prepared analogously to **8b** as a cream solid in 30.7% yield. MS (ESI) *m/z* = 536.9 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.80 (d, *J* = 4.18 Hz, 1H), 8.48 (d, *J* = 4.36 Hz, 1H), 8.22 (m, 2H), 8.04 (m, 2H), 7.87 (m, 2H), 7.58 (m, 1H), 6.82 (d, *J* = 3.99 Hz, 1H), 6.22 (d, *J* = 3.99 Hz, 1H), 5.57 (br. s., 1H), 3.87 (m, 1H), 3.8 (s, 3H), 2.84 (d, *J* = 4.18 Hz, 3H), 2.43 (m, 1H), 2.26 (d, *J* = 10.06 Hz, 1H), 2.10 (d, *J* = 11.20 Hz, 1H), 1.99- 1.89 (m, 3H), 1.46 (m, 2H). HRMS $(M+H)^+$ calcd for [$C_{26}H_{27}BrN_6O_2$+H], 535.1457; found 535.1448.

cis-1-(3-(4-bromo-1H-pyrrole-2-carboxamido)cyclohexyl)-N-methyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (9Bc).



Using 4-bromo-1H-pyrrole-2-carboxylic acid instead of 5-bromothiophene-2-carboxylic acid, the title compound was prepared analogously to **8b** as white solid in 7.4% yield. MS (ESI) *m/z* = 523.0 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.79 (br s, 1H), 8.80 (br s, 1H), 8.49 (br s, 1H), 8.26 (br s, 1H), 8.20 (d, *J* = 7.78 Hz, 1H), 8.04 (m, 2H), 7.89 (m, 2H), 7.59 (br s, 1H), 6.95 (br s, 1H), 6.86 (br s, 1H), 5.55 (br s, 1H), 3.90 (br s, 1H), 2.84 (br s, 3H), 2.41-2.13 (m, 3H), 1.92 (m, 3H), 1.46 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{25}H_{25}BrN_6O_2+H]$ 521.1301; found 521.1252.

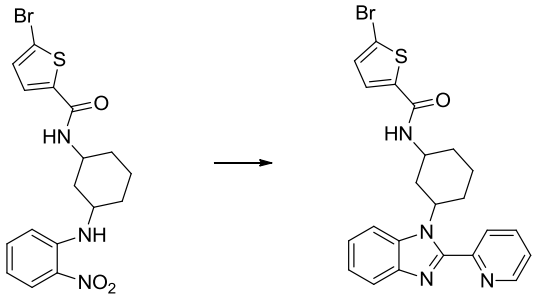
cis-3-chloro-N-(3-(5-(methylcarbamoyl)-2-(pyridin-2-yl)-1H-benzo[d]imidazol-1-yl)cyclohexyl)isoxazole-5-carboxamide (9Bd).



Using 3-chloroisoxazole-5-carboxylic acid instead of 5-bromothiophene-2-carboxylic acid, the title compound was prepared analogously to **8b** as white solid in 66.4% yield. MS (ESI) *m/z* = 479.1 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.07 (d, *J* = 7.97 Hz, 1H), 8.79 (d, *J* = 4.36 Hz,1H), 8.48 (d, *J* = 4.36 Hz, 1H), 8.27 (s, 1H), 8.20 (d, *J* = 7.97 Hz, 1H), 8.04 (td, *J* = 7.69, 1.52 Hz, 1H), 7.87 (m, 2H), 7.58 (dd, *J* = 6.93, 5.41 Hz, 1H), 7.36 (s, 1H), 5.57 (t, *J* = 11.96 Hz, 1H),

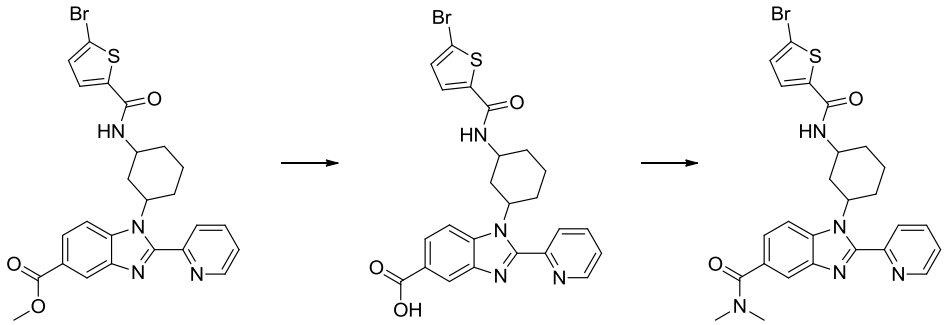
3.97 (br s, 1H), 2.84 (d, *J* = 4.37 Hz, 3H), 2.56 (m, 1H), 2.19 - 2.36 (m, 1H), 2.15 (m, 1H), 1.99-1.9 (m, 3H), 1.38 - 1.65 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{24}H_{23}ClN_6O_3+H]$, 479.1598; found 479.1573.

cis 5-bromo-N-(3-(2-(pyridin-2-yl)-1H-benzo[d]imidazol-1-yl)cyclohexyl)thiophene-2-carboxamide (10a).



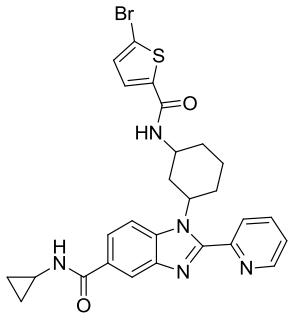
Using cis 5-bromo-N-3-((2-nitrophenyl)amino)cyclohexyl)thiophene-2-carboxamide and picolinaldehyde as starting material, the title compound was prepared analogously to **8f** in 16.6% yield (40 mg) as a yellow solid. MS (ESI) *m/z* = 483.0 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.78 (d, *J* = 4.37 Hz, 1H), 8.50 (d, *J* = 7.78 Hz, 1H), 8.19 (d, *J* = 7.78 Hz, 1H), 8.03 (t, *J* = 7.02 Hz, 1H), 7.85 (d, *J* = 7.78 Hz, 1H), 7.75 (d, *J* = 7.40 Hz, 1H), 7.57 (m, 2H), 7.3 (m, 3H), 5.58 (m, 1H), 3.89 (br s, 1H), 2.38 - 2.47 (m, 1H), 2.30 (d, *J* = 8.92 Hz, 1H), 2.12 (d, *J* = 11.39 Hz, 1H), 1.94 (m, 3H), 1.48 (m, H). HRMS $(M+H)^+$ calcd for $[C_{23}H_{21}BrN_4OS+H]$ 481.0698; found 481.0633.

cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N,N-dimethyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (10b).



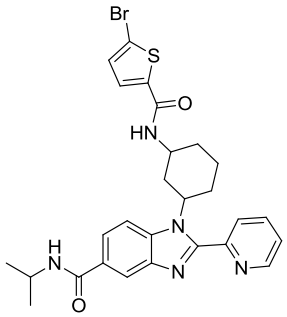
Using methyl 4-fluoro-3-nitrobenzoate as a starting material (instead of 4-fluoro-N-methyl-3-nitrobenzamide converted from 4-fluoro-3-nitrobenzoic acid), the corresponding methyl ester (compound **iv**) was prepared analogously to **8b** in 30% yield (400 mg). Saponification of the methyl ester afforded the corresponding acid **v** as a crude sample. MS (ESI) *m/z* $[M+1]^+$ = 524.9 and 526.9. The above crude acid (60 mg, 0.114 mmol) was added to a solution of HATU (43.4 mg, 0.114 mmol), DIEA (40 uL, 0.228 mmol), and dimethylamine (HCl salt, 13.97 mg, 0.171 mmol) in DMF (2 mL) and stirred at room temperature for 1h. The reaction was condensed *in vacuo* and the residue was purified by HPLC to afford th title compound **10b** (mixture of two cis-isomers) in 15.6% yield (11.9 mg). MS (ESI) *m/z* $[M+1]^+$ = 552.2 and 554.2; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (d, 1H, *J* = 4.4), 8.47 (d, 1H, *J* = 8.0 Hz), 8.17 (d, 1H, *J* = 8.0 Hz), 8.03 (t, 1H, *J* = 7.2 Hz), 7.89 (d, 1H, *J* = 8.2 Hz), 7.76 (s, 1H), 7.57 (m, 2H), 7.37 (d, 1H, *J* = 8.0 Hz), 7.24 (d, 1H, *J* = 4.0), 5.55 (m, 1H), 3.90-3.80 (m, 1H), 2.98 (s, 6H), 235-2.25 (m, 2H), 2.10-2.18 (m, 1H), 1.94-2.00 (m, 1H), 1.86-1.94 (m, 2H), 1.52-1.41 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{26}H_{26}BrN_5O_2S + H]$ 552.1069; found 552.1074.

cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-cyclopropyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (10c).



Using cyclopropylamine instead of dimethylamine, the title compound was prepared analogouly to **10b** in 14.7% yield (12.7 mg). MS (ESI) *m/z* $[M+1]^+$ = 564.3 and 566.3; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (d, 1H, *J* = 5.2), 8.45-8.49 (m, 2H), 8.25 (s, 1H), 8.17 (d, 1H, *J* = 8.0), 8.04 (t, 1H, *J* = 7.2 Hz), 7.87 (d, 1H, *J* = 8.4 Hz), 7.83-7.81 (m, 1H), 7.61-7.55 (m, 2H), 7.25 (d, 1H, *J* = 4.4), 5.60-5.49 (m, 1H), 4.00 – 3.80 (m, 1H), 2.91-2.82 (m, 1H), 2.44-2.33 (m, 1H), 2.32-2.20 (m, 1H), 2.18-2.08 (m, 1H), 2.00-1.82 (m, 3H), 1.53-1.40 (m, 2H), 0.73-0.66 (m, 2H), 0.64-0.55 (m, 2H). HRMS $(M+H)^+$ calcd for $[C_{27}H_{26}BrN_5O_2S + H]$ 564.1069; found 564.1071.

cis 1-(3-(5-bromothiophene-2-carboxamido)cyclohexyl)-N-isopropyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (10d).



Using isoproylamine instead of dimethylamine, the title compound was prepared analogouly to **10b** in 15.7% yield (12.2 mg). MS (ESI) *m/z* $[M+1]^+$ = 568.2; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.79 (d, 1H, *J* = 4.7), 8.50 (d, 1H, *J* = 3.9), 8.31 (s, 1H), 8.26 (d, 1H, *J* = 7.9), 8.17 (d, 1H, *J* = 7.9), 8.10-8.02 (m, 1H), 7.91-7.82 (m, 2H), 7.60-7.54 (m, 2H), 7.26 (d, 1H, *J* = 4.3), 5.60-5.49

(m, 1H), 4.17-4.05 (m, 1H), 3.90-3.75 (m, 1H), 2.48-2.35 (m, 1H), 2.34-2.20 (m, 1H), 2.18-2.09 (m, 1H), 2.00-1.84 (m, 3H), 1.54-1.40 (m, 2H), 1.18 (d, 6H, J = 6.6). HRMS $(M+H)^+$ calcd for $[C_{27}H_{28}BrN_5O_2S + H]$ 566.1225; found 566.1229.

BCATm fluorescent assay. All reagents were purchased from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK) unless otherwise stated. Assay buffer was 50 mM HEPES (pH 7.5), 50 mM NaCl and 1 mM CHAPS. Horseradish Peroxidase was initially diluted to 500 units/ml in water. 4-methyl-2-oxovalerate was initially diluted to 10 mM in assay buffer. L-Leucine and α-ketogluterate were both initially diluted to 100 mM in 50 mM HEPES (pH 7.5) with pyridoxal phosphate (PLP) initially diluted to 10 mM in 50 mM HEPES (pH 7.5). Amplex red (Invitrogen, Paisley, UK) was initially diluted to 20 mM in DMSO. BCATm and L-GOx protein were cloned, expressed, and isolated in house (GSK, Stevenage, UK) (references: (1) Arima, J.; Tamura, T.; Kusakabe, H.; Ashiuchi, M.; Yagi, T.; Tanaka, H.; Inagaki, K. Recombinant Expression, Biochemical Characterization and Stabilization through Proteolysis of an L-Glutamate Oxidase from *Streptomyces sp.* X-119-6. *J. Biochem*. **2003**, *134*, 805-812. (2) Conway, M. E.; Hutson, S. M. Mammalian Branched-Chain Aminotransferases. *Methods Enzymol*. **2000**, *324*, 356-356). The assay monitors the production of L-glutamate from branch chain amino-acids and α-ketoglutarate through the coupling of hBCATm activity to two additional enzymes, L-Glutamate Oxidase (L-GOx) and Horseradish Peroxidase (HRP). L-GOx catabolises L-glutamate to generate α-Ketoglutarate and hydrogen peroxide, the later being utilised by HRP and leading to the formation of fluorescent resorufin from the redox sensitive dye Amplex Red. The BCATm fluorescent assay was carried out in low volume 384-well plates (Greiner Bio-one, Stonehouse, UK) at a final volume of 10 μL per well. Test compounds were

added to plates as 50 nL solution in DMSO using an Echo 555 acoustic dispenser (Labcyte, Sunnyvale, CA) prior to the addition of assay components. Additionally, 50 nL DMSO or positive control compound was included in two columns each to give 100% activity and 100% inhibition controls, respectively. Single-concentration testing was at 10 μM compound concentration. For pIC_{50} determination, compounds were tested using an 11-point, three-fold dilution series from either 625 μM or 6.25 μM prepared using a Biomek FX (Beckman Coulter, Wycombe, UK). To these compound plates, 4 μL of an enzyme-PLP solution containing 20 nM BCATm and 40 nM PLP in assay buffer was added. Following this, 4 μL of a coupling solution containing 3 mM L-Leucine, 0.5 mM α-ketogluterate, 10 units/mL HRP and 80 μM Amplex red in assay buffer was added to initiate the reaction. The coupling solution was incubated on a roller at room temperature in a 15 mL tube with 1 mL Agarose immobilised Catalase (Sigma-Aldrich Ltd.) per 10 mL of coupling solution to 'scrub' the coupling solution prior to addition of Amplex Red and remove background levels of hydrogen peroxide. After a 10 minute incubation, 2 μL of 100 mM 4-methyl-2-oxovalerate was added to stop the reaction. Final assay concentrations were 10 nM BCATm, 20 nM PLP, 5 units per mL HRP, 1.5 mM L-Leucine, 0.25 mM alpha-ketoglutarate and 40 μM Amplex Red. All additions were performed using a Multidrop Combi (Thermo Fisher Scientific, Waltham, MA). Plates were transferred to an EnVision reader (PerkinElmer) (excitation filter 525/20 nm; emission filter 598/25 nm).

BCATm cellular assay. Differentiated primary human adipocytes (Zenbio) were challenged overnight using compounds dissolved in HBSS (Gibco 1g/L glucose) complemented with Hepes 10mM, L-Serine 50μM and L-Leucine 150 μM. Next day cell supernatants were subjected to amino acid determinations using HPLC analysis essentially as previously described [Henderson,

J.W., Ricker, R.D., Bidlingmeyer, B.A., Woodward, C. Rapid, Accurate, Sensitive, and Reproducible HPLC Analysis of Amino Acids. (1999) Agilent Technical Note, 5980-1193E]. Briefly, the method is based on automated, online derivatization using o-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids, using an Eclipse Plus C18 3×10mm 3.5µm column to perform reversed phase HPLC (all chemicals and hardware from Agilent Technologies, Inc.). Percent inhibition was calculated based on leucine concentration remaining relative to vehicle treated cells.

In vivo experimental methods. Male, C57Black/6J mice (from Charles River Laboratories, L'Arbresle, France) were used in all experiments. Animals were housed under controlled conditions of temperature and humidity (%), in a 12 h light/12 h dark cycle and provided with food and water ad libitum. Oral administration of vehicle (0.5% HPMC K100 + 0.1% Tween 80 pH=10.0) or indicated doses of compound as suspensions were typically administered after 5 hours fasting. Then 30 min or 6 hours after compounds administration, each animal was challenged with a mix of amino acids 3g AA/20mL/kg mice. Amino acid mix was prepared in 0.5%HPMC/0.1%Tween pH7 and compose of L-Cysteine 114 mg, L-Histidine 114 mg, L-Isoleucine 468 mg, L-Leucine 939 mg, L-Lysine 486 mg, L-Methionine 39 mg, L-Phenylalanine 75 mg, L-Threonine 264 mg, L-Tryptophan 15 mg, L-Tyrosine 21 mg, and L-Valine 468 mg (all amino acids from Sigma). One hour after amino acid challenge, blood samples were taken by intra-cardiac puncture. Sera were collected and frozen at –20 °C until use. Sera amino acid analyses were performed by HPLC as described in the in vitro cell assay method.

BCATm/PLP protein production and crystallisation.

Human branched-chain amino acid aminotransferase mitochondrial (BCATm) (residues 28-392) deleted of the N-terminal mitochondria signal peptide (residues 1-27) was cloned into Kanamycin resistant pET28a, via NdeI/XhoI restriction endonuclease sites with an N-terminal 6His-Thrombin cleavage site. The protein was expressed in inclusion bodies using BL21 Star (DE3) *E.coli* (Invitrogen). The purification of holo BCATm used a modified version of the method described by Conway and Huston. The frozen cell pellet was defrosted in buffer (25 mM HEPES, 500 mM NaCl, 4M Urea, 1 mM DTT, 20 mM Imidazole, Protease Inhibitor Cocktail III(MERCK)) containing 0.1 mM Pyridoxal phosphate, 0.2 mg/mL lysozyme and 10U/ml Benzonase (MERCK) and mixed for 30 mins at room temp. The cell suspension was sonicated and the cell debris removed by centrifugation at 100,000g for 90mins. The supernatant was loaded onto a 5 ml HisTrap HP column(GE Healthcare), washed with 10Bv of buffer before running a gradient to 0 M Urea in buffer over 10 Bv at 5 m/min. The column was then eluted using a series of Imidazole steps 25 mM, 50 mM, 100 mM, 200 mM and 500 mM 10Bv each in buffer at 5 mL/min. The BCATm containing fractions (100 mM Imidazole) were pooled and concentrated (Amicon Ultra 15 ml, 30kDa MWCO, Millipore) and further purified using Hi Prep 26/70 Superdex200 prep grade column (GE Healthcare). The dimeric BCAT was pooled and buffer exchanged using a Hi Prep Desalting column (GE Healthcare) into thrombin cleavage buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 2 mM CaCl) and incubated for 2 hours at room temp with 10U per ml of High Purity Thrombin (LeeBio) after cleavage DTT was added to 1 mM. The thrombin was removed by purification on Butyl Sepharose High Performance(GE Healthcare), the cleaved BCATm was made up to 1.5M $(NH_4)_2SO_4$ and loaded onto the column, the BCATm was eluted using a gradient to 0 M $(NH_4)_2SO_4$ over 20Bv. The BCATm was pooled and buffer exchanged (Hi Prep Desalting) into storage buffer (25 mM HEPES pH7.5, 25 mM

NaCl, 20 mM DTT, 20 mM EDTA, 2.5% glycerol), concentrated to 10 mg/ml for crystallography. Crystals of BCATm were grown by hanging drop vapour diffusion at 20°C with microseeding using MDL Morpheus™ screen condition B2 supplemented with 10 mM DTT in a protein to microseed solution of drop ratio of 1:1. The Hampton Research seed-bead method was used to generate microseeds. The crystals had a yellow hue suggestive of bound PLP cofactor despite none being added during purification or crystallization.

X-ray crystal structure of BCATm/PLP complexed with inhibitor 8b. A holocrystal was transferred to soaking buffer comprising MDL Morpheus™ screen condition B2 supplemented with 20mM DTT and compound 8b (from a stock solution dissolved in DMSO) at the soaking concentration and soaking time given in the X-ray summary table (Table 1). The soaked crystal was harvested in a cryo-loop and plunge-frozen into liquid nitrogen before loading in a puck for mounting with a robotic sample collector. Diffraction data from this single crystal were collected at 100K on an in-house RIGAKU FR-E^{+} SUPERBRIGHT generator/Saturn A200 detector/ACTOR robotic system. Data processing was achieve using XDS (Kabsch, W. Acta Cryst. (2010) **D66**, 125-132) followed by scaling using SCALA (Evans, P.R. Acta Cryst. (2006) **D62**, 72-82) within the CCP4 programming suite (Collaborative Computational Project, Number *4*. "*The CCP4 Suite: Programs for Protein Crystallography*." Acta Cryst. (1994) **D50**, 760-763). The crystal structure was solved by Fourier synthesis using REFMAC (Murshudov, G.N., Vagin, A.A. and Dodson, E.J. Acta Cryst. (1997) **D53**, 240-255) (via CCP4) starting from a previously determined in-house structure. Model-building was performed using COOT (P. Emsley and K. Cowtan Acta Cryst. (2004) **D60**, 2126-2132) and refinement carried out with REFMAC. The

statistics for the data collection and refined co-ordinates are given in Table X (below) and the final crystal structure is deposited in the Protein Data Bank under the accession code 5HNE.

Table 1. X-ray data summary table for 8b

Parameter[a]

Data collection	
Space Group	$P2_12_12_1$
Cell Dimensions	
a,b,c (Å)	69.12,106.48,106.26
a, b, γ (°)	90.00,90.00, 90.00
Resolution (Å)	2.04 (2.16)
Rmerge[b]	0.058 (0.467)
Average I/σI	24.5 (4.5)
Completeness (%)	99.9 (99.8)
Redundancy	6.9 (6.9)
No. Reflections	348243
No. Unique Reflections	50259 (7209)
Refinement	
Resolution (Å)	20.00-2.04
Rwork/Rfree	0.172/0.224
No. Reflections	47623
No. atoms	
Protein (chain A/B)	2916/2864
Cmpd (L1/L2)	34/34
B- factors [Å^2]	
Protein (chain A/B)	32.3/35.1
Cmpd (L1/L2)	29.1/32.8
R.m.s deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.383
Crystal soaking conditions	60mM compound, 12% DMSO, 20mM DTT, 7 days

[a] Data for the highest resolution shell are given in parentheses.

[b] $R_{merge} = \Sigma | I_j - \langle I_j \rangle | / \Sigma \langle I_j \rangle$.

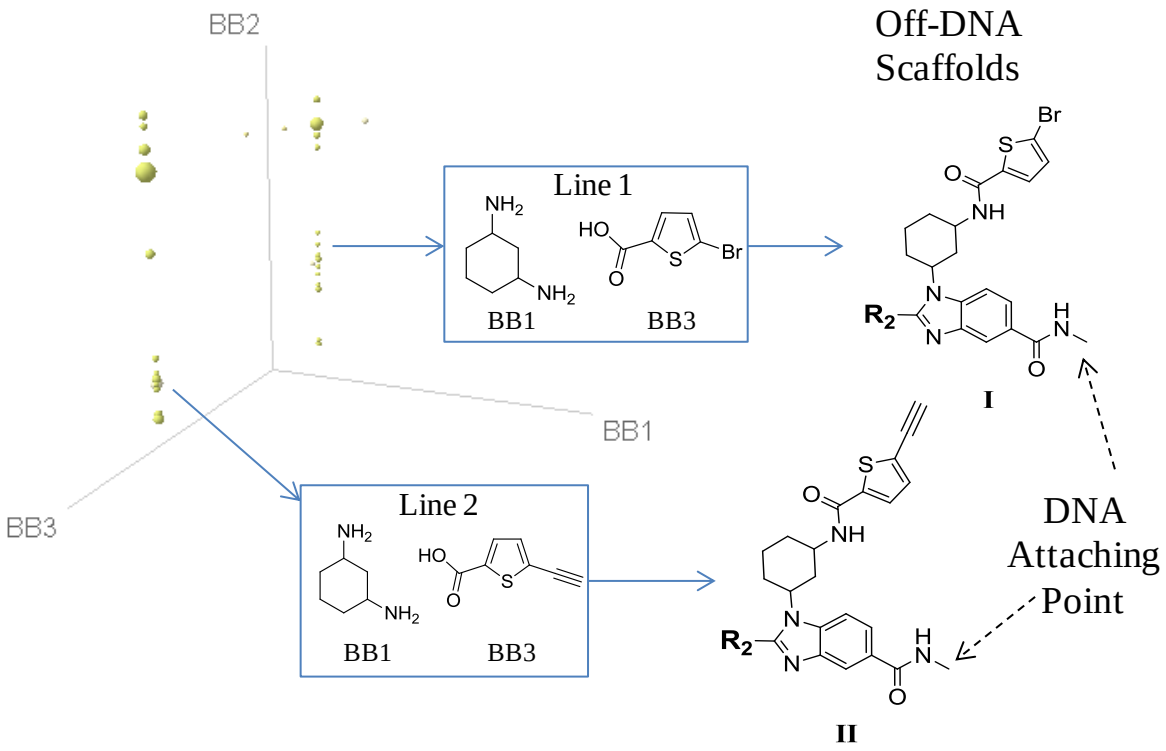


Figure 1. Result of BCATm screen of the DNA encoded benzimidazole library and the generic structures of the two BCATm hit series I and II, where BB1, BB2, and BB3 refer to cycle 1, 2, and 3 building blocks, respectively. Compounds on line 1 and line 2 share common BB1 and BB3 as indicated in their corresponding frames with BB2 as a variable component. The size of a dot is proportional to the DNA tag copy number. Library members with a single copy were removed to simplify visualization. An enlarged Figure 1 can be found in SI section.