Machine-assisted synthesis of modulators of the histone reader BRD9 using flow methods of chemistry and frontal affinity chromatography

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Supplementary Information

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A- Chemistry

General Information

^{1}H NMR spectra were recorded on a Bruker DPX-400 (400 MHz) or a Bruker Avance-500 (500 MHz) with dual cryoprobe using the deuterated solvent as internal deuterium lock. Chemical shift data are given in units δ in ppm relative to residual protic solvent. The multiplicity of a signal is indicated as: br - broad, s - singlet, d - doublet, t - triplet, q - quartet, m - multiplet, dd - doublet of doublets, dt - doublet of triplets, etc. Coupling constants (*J*) are recorded to the nearest 0.1 Hz.

^{13}C NMR spectra were recorded on a Bruker DPX-400 (100 MHz) or a Bruker Avance-500 (125 MHz) with dual cryoprobe spectrometer with broadband proton decoupling and using the deuterated solvent as the internal deuterium lock. Chemical shift data are given in units δ in ppm relative to residual protic solvent.

Infrared spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer using universal ATR sampling accessories. Letters in parentheses refer to relative absorbance with respect to the most intense peak: w – weak (< 40%), m – medium (40 – 75%), s – strong (> 75%).

High resolution mass spectrometry (HRMS) within ±5 ppm was carried out either on a Waters Micromass LCT Premier spectrometer using time of flight with positive or negative electrospray ionisation, or by Mr Paul Skelton (Department of Chemistry, University of Cambridge) on a Bruker BioApex 47e FTICR spectrometer using positive ESI or EI at 70 eV. All reported values are within ±5 ppm of the calculated value.

Elemental composition microanalysis was performed by the Microanalytical Laboratories at the Department of Chemistry, University of Cambridge and results are reported to one decimal place.

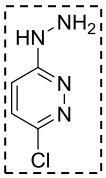
Yields refer to recrystallized material or material purified by HPLC and spectroscopically pure, unless otherwise stated.

Toluene, tetrahydrofuran, acetonitrile and dichloromethane were purchased from Fisher Scientific and distilled before use. HPLC grade methanol was purchased from Fisher Scientific, and absolute ethanol from Sigma-Aldrich and were used without further purification. Commercially available starting materials were purchased from Sigma-Aldrich, Alfa Aesar or Fluorochem and used without further purification.

Synthetic procedures and characterisation

3-chloro-6-hydrazino pyridazine 2

A solution of 3,6-dichloropyridazine 1 (300 mg, 2 mmol) and hydrazine monohydrate (0.11 mL, 1.1 eq) in 8 mL ethanol was heated in a sealed vial under microwave irradiation for 1 h at 100 °C. The resulting mixture was dried ($MgSO_4$), filtered, and the solvents were evaporated. The title compound 2 was obtained as light brown solid (quantitative yield).



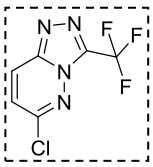
1H NMR (400 MHz, MeOD) : δ /ppm 7,54 (1H, s), 7,42 (1H, d, J = 12 Hz), 7,21 (1H, d, J = 12 Hz), 2,05 (2H, d, *J* = 20 Hz)

IR ν /cm^{-1} = 3252.1 (w), 3138.6 (w), 2889.2 (m), 2709.9 (m), 2594.3 (m), 1994.0 (w), 1604.4 (w), 1563.8 (w), 1497.7 (m), 1418.2 (w), 1253.8 (w), 1121.0 (m), 1093.2 (m), 967.0 (m)

LCMS: Rt 0.28 min m/z (ESI$^+$) 145 (^{35}Cl, [M + H]$^+$, 100%), 147 (^{37}Cl, [M + H]$^+$, 33%).

6-chloro-3-trifluoromethyl-[1,2,4]triazolo[4,3-b]pyridazine 3

A mixture of 3,6-dichloro-3-hydrazinylpyridazin-4-amine (50 mg, 0.4 mmol) and trifluoroacetic acid (1 mL) was heated in a sealed vial under microwave irradiation at 100 °C for 3 h. Water was added to the reaction mixture and the reaction was extracted twice with ethyl acetate. The solvent was removed and the crude was purified by column chromatography (Biotage,.ethyl acetate/hexane gradient). 6-Chloro-3-trifluoromethyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (76 mg, 0.34 mmol, 85 %) was collected as a pale pink crystalline solid (needles).



^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 8.27 (1H, d, *J* = 10 Hz), 7.38 (1H, d, *J* = 10 Hz).

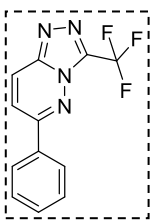
^{13}C NMR (100 MHz, $CDCl_3$) δ/ppm = 151.52, 144.41, 139.66 (q, *J* = 42 Hz), 126.46, 124.60, 117.87 (q, *J* = 269 Hz)

IR (thin film) ν /cm^{-1} = 3080.5 (w), 1530.4 (m), 1511.1 (w), 1459.7 (m), 1421.6 (w), 1358.5 (w), 1332.4 (m), 1289.3 (w), 1221.3 (m), 1180.7 (m), 1163.6 (s), 1135.9 (s), 1113.2 (s), 1051.8 (m), 1014.9 (s), 947.7 (m), 829.7 (m), 782.3 (w), 760.0 (m), 715.8 (m)

HRMS calcd. for $C_6H_3N_4F_3$[^{35}Cl] [M + H]$^+$ 222.9998 found 222.9992 Δ = -2.7 ppm.

6-phenyl-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-b]pyridazine 4

6-Chloro-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-b]pyridazine (60 mg, 0,27 mmol) and phenylboronic acid (1.5 equiv.) were dissolved in a mixture of toluene / EtOH (3:1, 3 mL) and saturated aqueous Na_2CO_3 (0.8 mL). Tetrakis triphenylphosphine palladium (11 mg, 3 mol%) was then added and the resulting mixture was heated in a sealed vial under microwave irradiation for 2 h at 120 °C. After cooling to room temperature, the organic phase was washed water (3 x 3 mL). The solvents were removed under reduced pressure and the product was purified by column chromatography (eluent: EtOAc/MeOH from 100:0 to 90:10). The title compound 4 was obtained as a pale yellow solid (17 mg, 24 % yield).



^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm 8.30 (1H, d, *J* = 12 Hz), 8.03 (2H, m), 7.79 (1H, d, *J* = 12 Hz), 7.60 (3H, m)

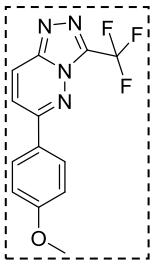
IR ν /cm^{-1} = 2920.8 (w), 2852.1 (w), 1739.7 (w), 1548.0 (w), 1515.2 (w), 1476.4 (w), 1443.9 (w), 1375.5 (w), 1335.6 (w), 1300.7 (w), 1279.0 (w), 1205.4 (w), 1174.9 (m), 1143.8 (m), 1060.3 (w), 1044.8 (w), 1013.3 (m), 925.9 (w), 842.6 (w), 835.8 (w), 793.3 (w), 775.4 (w), 763.0 (w), 747.2 (w), 732.2 (w), 715.5 (w), 686.9 (m).

LC-MS: Rt 4.74 min m/z (ESI$^+$) 265 ([M + H]$^+$).

HRMS calcd. for $C_{12}H_8N_4F_3$ [M + H]$^+$ 265.0701 found 265.0713 Δ = 4.5 ppm.

6-(4-methoxyphenyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-b]pyridazine 5

6-Chloro-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-b]pyridazine (60 mg, 0.27 mmol) and 4-methoxyphenylboronic acid (1.5 equiv.) were dissolved in a mixture of toluene / EtOH (3:1, 3 mL) and saturated aqueous Na_2CO_3 (0.8 mL). Tetrakis triphenylphosphine palladium (11 mg, 3 mol%) was then added and the resulting mixture was heated in a sealed vial under microwave irradiation for 2 h at 120 °C. After cooling to room temperature, the organic phase was washed with water (3 x 3 mL). The solvents were removed under reduced pressure and the product was purified by column chromatography (eluent: EtOAc/MeOH from 100:0 to 90:10). The title compound 4 was obtained as a yellow solid (35 mg, 44 % yield).



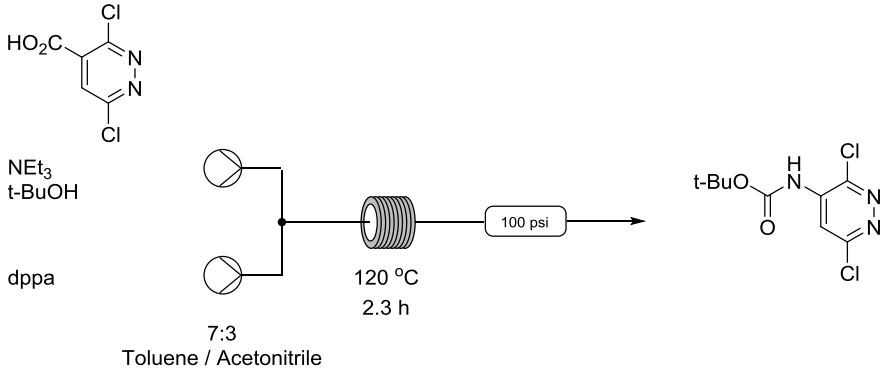
^{1}H NMR (400 MHz, $CDCl_3$): δ/ppm 8.24 (1H, d, *J* = 12 Hz), 7.99 (2H, d, *J* = 8 Hz), 7.74 (1H, d, *J* = 8 Hz), 7.07 (2H, d, *J* = 8 Hz), 3.91 (3H, s).

IR ν /cm-1 = 2922.9 (w), 1604.4 (w), 1555.5 (w), 1494.2 (m), 1471.6 (m), 1409.5 (w), 1379.1 (w), 1357.1 (w), 1338.4 (w), 1303.0 (m), 1274.6 (m), 1249.8 (m), 1207.5 (m), 1181.3 (m), 1142.1 (m), 1062.9 (m), 1040.0 (m), 1018.5 (m), 905.5 (w), 822.5 (m), 809.1 (m), 781.1 (m), 761.8 (m), 718.1 (w).

LCMS: Rt 4.78 min m/z (ESI$^+$) 295 ([M + H]$^+$)

HRMS calcd. for $C_{13}H_9N_4OF_3$ [M + H]$^+$ 295.0807 found 295.0807 Δ = 0.0 ppm.

tert-butyl (3,6-dichloropyridazin-4-yl)carbamate 7



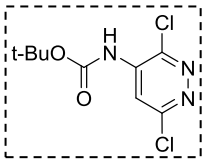
Solution A: 3,6-Dichloropyridazine-4-carboxylic acid (8.0 g, 40 mmol), triethylamine (11.6 mL, 80 mmol, distilled over $CaCl_2$ before use) and *tert*-butanol (18.5 g, 250 mmol) in 7:3 Toluene / Acetonitrile (125 mL).

Solution B: Diphenyl phosphoryl azide (13.5 mL, 75 mmol) in 7:3 Toluene / Acetonitrile (125 mL).

Reactor: A Vapourtec R2+/R4 flow synthesis platform, equipped with two acid-resistant pumps and a nitrogen manifold, available from Vapourtec Ltd.

The reagents were stored in sealed flasks, pressurised to 1 bar under a nitrogen atmosphere, and each stream was drawn through a pump at 0.179 mL/min. The streams are combined (via 1/16" OD, 1 mm ID PFA tubing) in a T-piece (ETFE, 1/16" bore) and then heated at 120 °C in a continuous flow coil (2 × 25 mL inner volume, 2.5 mm ID, stainless steel) before passing through a 100 psi back-pressure regulator.

The output was collected and the solvent removed. The residue was extracted into toluene (2 × 250 mL) from 0.1 M aqueous citric acid (50 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated. The resultant mixture was purified by column chromatography (50 g KP-NH column, available from Biotage), using a solvent system of Ethyl Acetate / Hexane (0 – 20% EtOAc over 20 column volumes, followed by 20% EtOAc for 5 C.V.) to afford the title compound as a white solid (8.5 g, 39%).



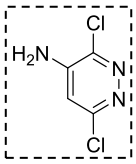
^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 8.3 (s, 1H), 1.5 (s, 9H), 7.2 (br. s, 1H).

^{13}C NMR (100 MHz, $CDCl_3$) δ/ppm = 154.53, 146.26, 143.72, 108.49 (CH).

HRMS calcd. for $C_9H_{12}O_2N_3[^{35}Cl]_2$ $[M + H]^+$ 264.0301 found 264.0292 Δ = -3.3 ppm.

3,6-Dichloropyridazin-4-amine 8

tert-Butyl (3,6-dichloropyridazin-4-yl)carbamate (10 g, 37.8 mmol) was dissolved in dichloromethane (40 mL). 4 M HCl in dioxane (125 mL) was added, with rapid formation of a precipitate. The reaction mixture was allowed to stir at 25 °C for 1 day and then the solid was collected by filtration and resuspended in DCM (100 mL). 2 M NH_3 in methanol (50 mL) was added and stirred at 25 °C for 30 minutes. The solid product was collected by filtration, washing with DCM and then dried *in vacuo*. 3,6-Dichloropyridazin-4-amine (6.2 g, quant.) was collected as a white solid.



^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm = 7.1 (br. s, 2H), 6.8 (s, 1H).

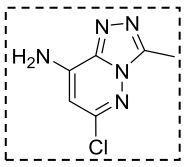
^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm = 154.53, 146.26, 143.72, 108.49 (CH).

HRMS calcd. for $C_4H_4N_3[^{35}Cl]_2$ $[M + H]^+$ 163.9782 found 163.9783 Δ = 0.6 ppm.

6-Chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine 9

A mixture of 3,6-dichloropyridazin-4-amine 8 (3.0 g, 18.2 mmol), hydrazine hydrate (27 mL) and water (8 mL) was heated in two portions in sealed vials under microwave irradiation at 100 °C for 3 h. The reaction mixture was allowed to cool to room temperature, and the resulting slurry collected by filtration, washing on the filter with water (10 mL). 3,6-Dichloro-3-hydrazinylpyridazin-4-amine (2.2 g, 13.6 mmol, 75 %) was collected as a white solid and used without further purification.

A mixture of 3,6-dichloro-3-hydrazinylpyridazin-4-amine (1.2 g, 7.4 mmol) and acetic acid (20 mL) was heated in two portions in sealed vials under microwave irradiation at 120 °C for 5 h. The solvent was removed and the combined residue recrystallized from 10% ethanol in water. 6-Chloro-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-amine (1.35 g, 7 mmol, 79 %) was collected as a pale yellow crystalline solid (needles).



^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm = 7.85 (s, 2H), 6.09 (s, 1H), 2.48 (d, J = 1.8 Hz, 3H).

^{13}C NMR (100 MHz, DMSO-d_6) δ/ppm = 149.77, 147.16, 144.31, 139.79, 94.01 (CH), 9.90 (CH_3).

HRMS calcd. for $C_6H_7N_5[^{35}Cl]$ $[M + H]^+$ 184.0390 found 184.0392 Δ = 1.1 ppm.

Elemental analysis cald. for $C_6H_6N_5Cl$, H_2O (C, H, N, Cl) C 35.74, H 4.00, N 34.74, Cl 17.58; found C 35.51, H 3.91, N 34.54, Cl 17.92.

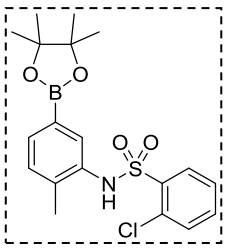
General Procedure A for sulfonamide formation

2-Methyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline, aryl sulfonyl chloride (1.1 equiv), and pyridine (0.5 mL, 2 equiv.) in ethanol (4 mL) were stirred at 25 °C for 18 h. The solvent was removed and the solid product partitioned between ethyl acetate (5 mL) and water (5 mL). The organic phase was dried ($MgSO_4$), concentrated and recrystallized from ethanol.

2-Chloro-*N*-(2-methyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)benzenesulfonamide 11

(3-Amino-4-methylphenyl)boronic acid 10 (0.50 g, 3.3 mmol) and pinacol (0.43 g, 3.6 mmol) in acetonitrile (5 mL) was stirred at 25 °C for 18 h. The solvent was removed and the resulting solid used without purification.

Compound 11 was prepared according to General Procedure A on 3.3 mmol scale. Recrystallisation from ethanol (two rounds) afforded the title compound as a colourless crystalline solid (914 mg, 68%).



^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 7.99 (dd, J = 8.0, 1.6 Hz, 1H), 7.58 – 7.41 (m, 4H), 7.32 (td, J = 7.6, 1.4 Hz, 1H), 7.13 (d, J = 7.4 Hz, 1H), 6.72 (s, 1H), 2.29 (s, 3H), 1.27 (s, 12H).

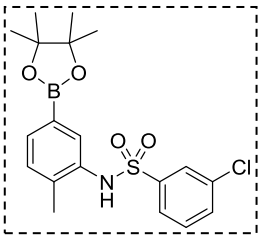
^{13}C NMR (100 MHz, $CDCl_3$) δ/ppm = 137.35, 136.01, 133.98 (CH), 133.68, 132.92 (CH), 132.07 (CH), 131.75 (CH), 131.67, 130.69 (CH), 130.34 (CH), 127.19 (CH), 83.91, 24.97 (CH_3), 18.27 (CH_3).

HRMS calcd. for $C_{19}H_{23}[^{11}B][^{35}Cl]NO_4S$ $[M + H]^+$ 408.1208 found 408.1199 Δ = -2.2 ppm.

3-Chloro-*N*-(2-methyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)benzenesulfonamide 12

(3-Amino-4-methylphenyl)boronic acid 10 (0.50 g, 3.3 mmol) and pinacol (0.43 g, 3.6 mmol) in acetonitrile (5 mL) was stirred at 25 °C for 18 h. The solvent was removed and the resulting solid used without purification.

Compound 12 was prepared according to General Procedure A on 3.3 mmol scale. Recrystallisation from ethanol (two rounds) afforded the title compound as a colourless crystalline solid (741 mg, 55%).



^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 7.73 (t, *J* = 1.9 Hz, 1H), 7.64 – 7.47 (m, 4H), 7.37 (t, *J* = 7.9 Hz, 1H), 7.15 (d, *J* = 7.5 Hz, 1H), 6.22 (s, 1H), 2.10 (s, 3H), 1.32 (s, 12H).

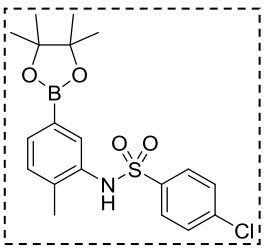
^{13}C NMR (100 MHz, $CDCl_3$) δ/ppm = 141.36, 136.70, 135.29, 133.59 (CH), 133.46, 133.11 (CH), 132.19 (CH), 130.74 (CH), 130.35 (CH), 127.64 (CH), 125.58 (CH), 84.05, 25.00 (CH_3), 18.14.

HRMS calcd. for $C_{19}H_{23}[^{11}B][^{35}Cl]NO_4S$ $[M + H]^+$ 408.1208 found 408.1202 Δ = -1.5 ppm.

4-Chloro-*N*-(2-methyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)benzenesulfonamide 13

(3-Amino-4-methylphenyl)boronic acid 10 (0.50 g, 3.3 mmol) and pinacol (0.43 g, 3.6 mmol) in acetonitrile (5 mL) was stirred at 25 °C for 18 h. The solvent was removed and the resulting solid used without purification.

The compound 13 was prepared according General Procedure A on 2.7 mmol scale. Recrystallisation from ethanol (two rounds) afforded the title compound as a colourless crystalline solid (852 mg, 77%).



^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 7.67 – 7.58 (m, 2H), 7.58 – 7.51 (m, 1H), 7.49 – 7.44 (m, 1H), 7.44 – 7.35 (m, 2H), 7.15 (d, *J* = 7.5 Hz, 1H), 6.14 (s, 1H), 2.09 (s, 3H), 1.32 (s, 12H).

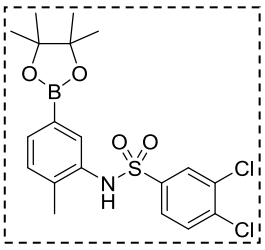
^{13}C NMR (100 MHz, $CDCl_3$) δ/ppm 139.54, 138.10, 136.94, 133.58 (CH), 133.52, 132.47 (CH), 130.73 (CH), 129.33 (CH), 128.96 (CH), 84.05, 24.99 (CH_3), 18.16.

HRMS calcd. for $C_{19}H_{23}[^{11}B][^{35}Cl]NO_4S$ $[M + H]^+$ 408.1208 found 408.1198 Δ = -2.5 ppm.

3,4-Dichloro-*N*-(2-methyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)benzenesulfonamide 14

(3-Amino-4-methylphenyl)boronic acid 10 (0.50 g, 3.3 mmol) and pinacol (0.43 g, 3.6 mmol) in acetonitrile (5 mL) was stirred at 25 °C for 18 h. The solvent was removed and the resulting solid used without purification.

Prepared according General Procedure A on 3.3 mmol scale. Recrystallisation from ethanol (two rounds) afforded the title compound as a colourless crystalline solid (922 mg, 63%).



^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm 7.81 (s, 1H), 7.57 (d, *J* = 7.5, 1H), 7.51 (m, 2H), 7.40 (s, 1H), 7.18 (d, *J* = 7.5 Hz, 1H), 6.29 (s, 1H), 2.17 (s, 3H), 1.31 (s, 12H).

^{13}C NMR (100 MHz, $CDCl_3$) δ/ppm 139.28, 137.86, 137.23, 133.83 (CH), 133.69, 133.18, 132.35 (CH), 131.11 (CH), 130.91 (CH), 129.56 (CH), 126.57 (CH), 126.36, 84.09, 24.99, (CH_3) 18.29 (CH_3).

HRMS calcd. for $C_{19}H_{24}[^{11}B][^{35}Cl]_2NO_4S$ $[M + H]^+$ 442.0818 found 442.0806 Δ = -2.7 ppm.

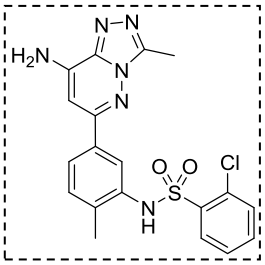
General Procedure B for Aryl Coupling

3,6-dichloro-3-hydrazinylpyridazin-4-amine 9 (0.1 mmol), boronic ester (1.1 equiv.), potassium phosphate (4 equiv.) and [1,1'-Bis(di-tert-butylphosphino)ferrocene]dichloropalladium(II) (10 mol%) were combined with 7:3 *n*-Butanol / Water (2 mL) in a sealed vial. The solvent was degassed under vacuum by three freeze-thaw cycles, and the vial then backfilled with N_2. The reaction mixture was heated to 100 °C for 4 h under microwave irradiation. The solvent was removed, the remaining crude mixture was dissolved in 1:1 MeOH / water and purified by preparative HPLC under the following conditions on a Supelcosil ABZ+PLUS column (25 cm x 21.2 mm, 12 µm particle size):

Time	Methanol % in water
0	30
40	80
45	80
50	30
55	30

N-(5-(8-Amino-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)-2-methylphenyl)-2-chlorobenzenesulfonamide 15

The compound 15 was prepared according to General Procedure B above. Purification by HPLC afforded the title compound as a white solid (15 mg, 35%).



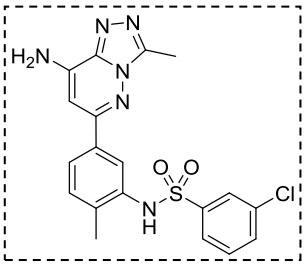
^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 8.07 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.79 (d, J = 1.8 Hz, 1H), 7.64 – 7.44 (m, 3H), 7.36 (t, *J* = 7.4 Hz, 1.4 Hz, 1H), 7.25 (d, *J* = 7.4 Hz, 1H), 6.98 (br. s, 1H), 6.41 (s, 1H), 5.57 (br. s, 2H), 2.78 (s, 3H), 2.34 (s, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ/ppm = 154.16, 148.03, 141.15, 140.02, 136.97, 134.84, 134.70, 134.24 (CH), 132.92, 131.82 (CH), 131.82 (CH), 131.54, 131.49 (CH), 127.29 (CH), 124.45 (CH), 121.18 (CH), 93.69 (CH), 17.69 (CH_3), 10.00 (CH_3).

HRMS calcd. for $C_{19}H_{18}N_6O_2S[^{35}Cl]$ $[M + H]^+$ 429.0900 found 429.0901 Δ = 0.2 ppm.

N-(5-(8-Amino-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)-2-methylphenyl)-3-chlorobenzenesulfonamide 16

The compound 16 was prepared according to General Procedure B above. Purification by HPLC afforded the title compound as a white solid (1 mg, 4%).



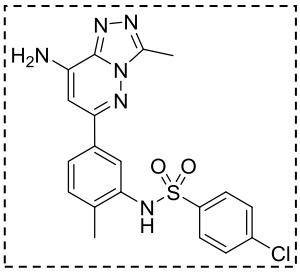
^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 7.85 (d, *J* = 1.9 Hz, 1H), 7.81 (t, *J* = 1.9 Hz, 1H), 7.73 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.55 (d, *J* = 8.2 Hz, 1H), 7.40 (t, *J* = 8.0 Hz, 1H), 7.26 (d, *J* = 8.2 Hz, 1H), 6.48 (s, 1H), 6.41 (br. s, 1H), 5.43 (br. s, 2H), 2.81 (s, 3H), 2.10 (s, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ/ppm = 153.99, 148.13, 141.23, 141.16, 140.02, 135.33, 135.07, 134.42, 133.62, 133.28 (CH), 131.47 (CH), 130.44 (CH), 127.25 (CH), 125.29 (CH), 125.22 (CH), 123.27 (CH), 93.72 (CH), 17.50 (CH3), 10.00 (CH_3).

HRMS calcd. for $C_{19}H_{18}N_6O_2S[^{35}Cl]$ $[M + H]^+$ 429.0900 found 429.0909 Δ = 2.1 ppm.

N-(5-(8-Amino-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)-2-methylphenyl)-4-chlorobenzenesulfonamide 17

The compound 17 was prepared according to the General Procedure B above. Purification by HPLC afforded the title compound as a white solid (12 mg, 32%).



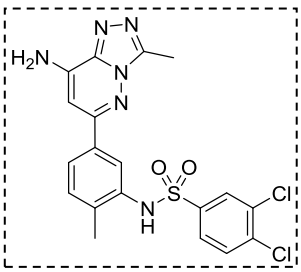
^{1}H NMR (500 MHz, DMSO-d_6) δ/ppm = 9.81 (br. s, 1H), 7.73 – 7.52 (m, 7H), 7.46 (br. s, 2H), 7.27 (d, J = 8.0 Hz, 1H), 6.45 (s, 1H), 2.61 (s, 3H), 2.06 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ/ppm = 153.35, 146.70, 142.85, 140.12, 139.86, 137.24, 135.62, 134.08, 131.10 (CH), 129.29 (CH), 128.50 (CH), 123.69 (CH), 91.45 (CH), 17.66 (CH_3), 9.53 (CH_3).

HRMS calcd. for $C_{19}H_{18}[^{35}Cl]N_6O_2S$ $[M + H]^+$ 429.0900 found 429.0894 Δ = -1.4 ppm.

N-(5-(8-Amino-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)-2-methylphenyl)-3,4-dichlorobenzenesulfonamide 18

The compound 18 was prepared according to the General Procedure B above Purification by HPLC afforded the title compound as a white solid (9 mg, 13%).



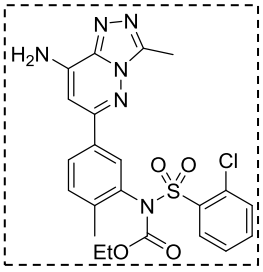
^{1}H NMR (400 MHz, DMSO-d_6) δ/ppm = 10.00 (br. s, 1H), 7.84 (d, *J* = 2.1 Hz, 1H), 7.75 (d, *J* = 8.3 Hz, 1H), 7.64 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.54 (d, *J* = 1.9 Hz, 1H), 7.41 (br. s, 1H), 7.46 – 7.33 (br. s, 2H), 7.19 (d, *J* = 7.8 Hz, 1H), 6.44 (s, 1H), 2.60 (s, 3H), 2.10 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6) δ/ppm = 154.13, 146.67, 142.74, 139.97, 134.49, 133.97, 133.82, 131.47, 131.19 (CH), 130.58 (CH), 128.19 (CH), 126.67 (CH), 91.73 (CH), 18.23 (CH_3), 9.58 (CH_3).

HRMS calcd. for $C_{19}H_{17}[^{35}Cl]_2N_6O_2S$ $[M + H]^+$ 463.0511 found 463.0505 Δ = -1.3 ppm.

Ethyl (6-(3-((2-chlorophenyl)sulfonamido)-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate 19

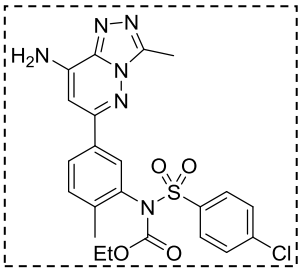
To a solution of *N*-(5-(8-amino-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)-2-methylphenyl)-2-chlorobenzenesulfonamide (1 mg), *N*,*N*-dimethylaminopyridine (3 mg) and triethylamine (8 µL) in tetrahydrofuran (5 mL) under an N_2 atmosphere was added a solution of ethyl chloroformate in tetrahydrofuran (0.1 M, 0.4 mL) in two portions. The mixture was stirred at room temperature for 20 minutes. Aqueous citric acid (0.1 M, 5 mL) was added, and the mixture concentrated under reduced pressure. The residue was dissolved in 1:1 methanol / water and purified by HPLC to afford the title compound (< 1 mg).



^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 8.43 (d, *J* = 8.1, 1.9 Hz, 1H), 8.10 (d, *J* = 1.9 Hz, 1H), 7.88 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.66 – 7.56 (m, 2H), 7.56 – 7.49 (m, 1H), 7.45 (d, *J* = 8.0 Hz, 1H), 6.52 (s, 1H), 5.45 (s, 2H), 4.25 – 4.04 (m, 2H), 2.83 (s, 3H), 2.49 (s, 3H), 1.13 (t, *J* = 7.1 Hz, 3H).

Ethyl (6-(3-((4-chlorophenyl)sulfonamido)-4-methylphenyl)-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-8-yl)carbamate 20

To a solution of *N*-(5-(8-amino-3-methyl-[1,2,4]triazolo[4,3-b]pyridazin-6-yl)-2-methylphenyl)-4-chlorobenzenesulfonamide (10 mg) and triethylamine (10 µL) in tetrahydrofuran (10 mL) was added a solution of ethyl chloroformate in tetrahydrofuran (0.1 M, 0.5 mL). The mixture was stirred at room temperature for 5 minutes. Aqueous citric acid (0.1 M, 5 mL) was added, and the mixture concentrated under reduced pressure. The residue was extracted into ethyl acetate (2 x 10 mL), and the combined organic phases were dried ($MgSO_4$), filtered, and concentrated under reduced pressure to afford the title compound as a white solid (11 mg, 95%).



^{1}H NMR (400 MHz, $CDCl_3$) δ/ppm = 8.06 (d, *J* = 8.7 Hz, 2H), 7.89 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.65 (d, *J* = 1.9 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 2H), 7.45 (d, *J* = 8.0 Hz, 1H), 6.49 (s, 1H), 5.50 (s, 2H), 4.27 – 4.06 (m, 2H), 2.82 (s, 3H), 2.34 (s, 3H), 1.18 (t, J = 7.1 Hz, 3H).

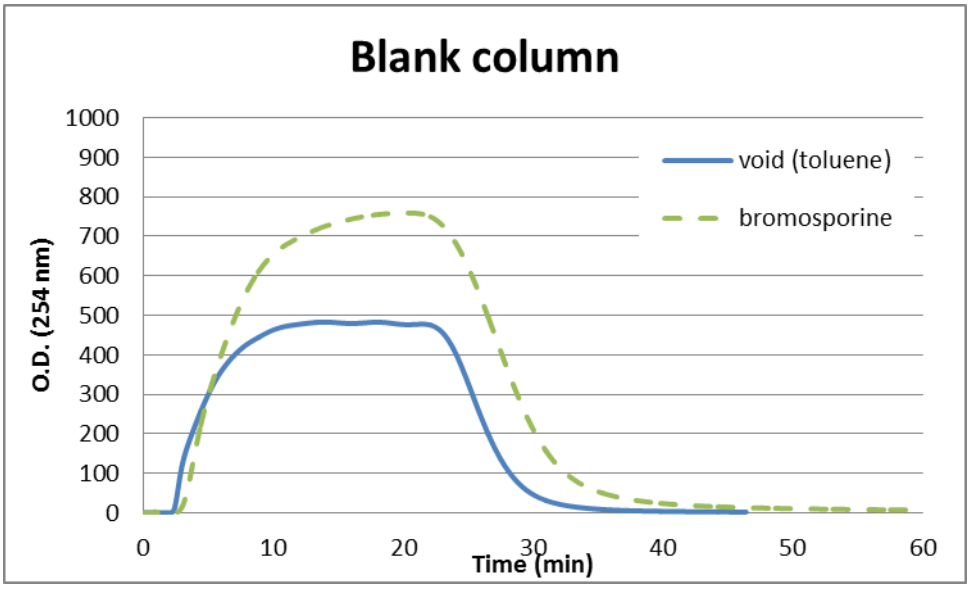
^{13}C NMR (126 MHz, $CDCl_3$) δ/ppm = 153.78, 151.77, 148.10, 141.24, 140.81, 140.42, 139.99, 137.51, 135.61, 134.88, 131.71 (CH), 130.72 (CH), 129.19 (CH), 128.06 (CH), 127.88 (CH), 93.66 (CH), 63.87 (CH_2), 29.69 (CH_3), 18.11 (CH_3), 14.12 (CH_3), 10.01 (CH_3).

HRMS calcd. for $C_{22}H_{22}[^{35}Cl]N_6O_4S$ $[M + H]^+$ 501.1112 found 501.1133 Δ = 4.2 ppm.

B- Frontal Affinity Chromatography

Control experiments with a 'blank' column

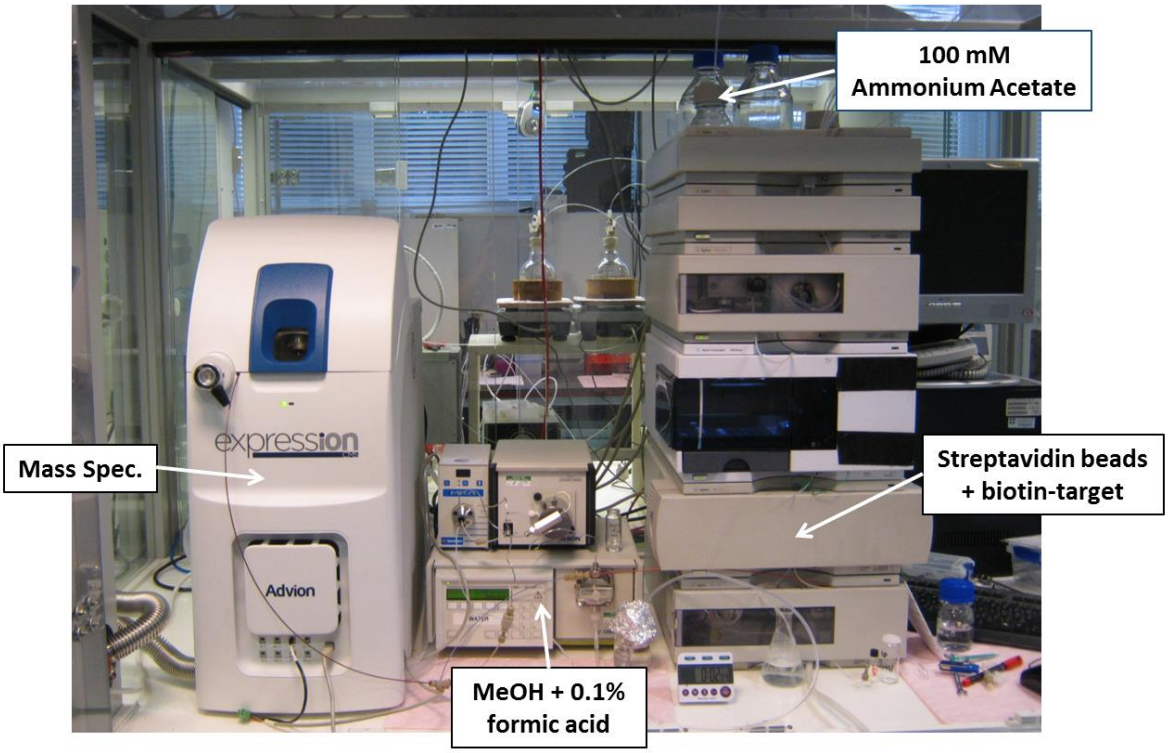
A 15 µL guard column (Kinesis) was packed with streptavidin coated polyacrylate beads (Streptavidin Plus UltraLink Resin, Pierce) and injections of bromosporine (stock solution at a concentration of 50 mM in DMSO was stored at -20°C) and a void marker (toluene in this instance) were carried on a modified Agilent 1100 HPLC system with a 100 µL injection syringe, a 600 µL storage loop; Phosphate buffer Saline (PBS) was used as the eluent. The HPLC system was set to inject sequentially 600 µL of the analyte at 30 µL min^{-1} for 60 min. The 254 nm wavelength was monitored using a Diode Array UV Detector.



In the blank column, the retention volumes for the void marker (toluene, 75 mM) and bromosporine (50 µM) were found to be respectively 125 µL and 172 µL. These volumes are in accordance with the number obtained with a second void marker in in the following section (compound 22, retention volume of 160 µL), showing that bromosporine exhibited minimal non-specific interactions with the solid support.

Toluene could not be employed as a void marker in the following section as unfortunately it did not give any signal when mass spectrometer was used as detection system. Compound 22 could not be used in the UV experiments as it has no UV absorbance.

FAC-MS Set-up

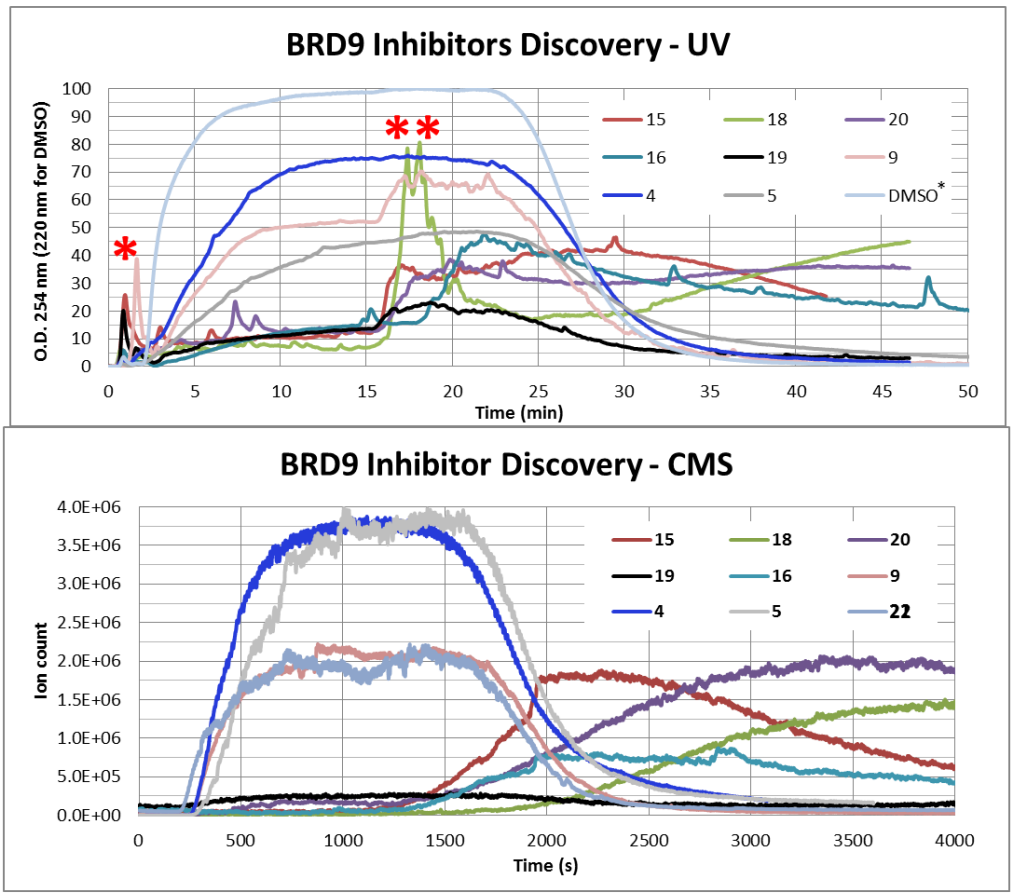


FAC assays were run on a modified Agilent 1100 HPLC system with a 100 µL injection syringe, a 600 µL storage loop and 100 mM ammonium acetate was used as the eluent. An Expression CMS mass spectrometer (Advion) was connected via a T-piece to the HPLC output and to a pump which delivered solution of 0.1% formic acid in methanol (make-up buffer). Stock solutions of compounds were prepared at a concentration of 50 mM in DMSO and stored at -20°C.

The HPLC system was set to inject 600 µL of 4 µM of analyte at 30 µL min^{-1} for 120 min. The make-up buffer (0.1% formic acid in methanol) pump was set at a flow rate of 200 µL min^{-1}. Two wavelengths were simultaneously monitored at 220 nm (DMSO) and 254 nm (analyte) using a Diode Array UV Detector, and the mass detection on the Expression CMS was used in positive mode, with Selective Ion Mode (SIM, span 1, dwell time 500 ms).

UV and MS chromatograms of representative experiments

For each experiment, the wavelength at 220 nm (DMSO), 254 nm (compounds) were recorded by the DAD detector, and selective ion on each compound was followed using an Advion expression Compact Mass Spectrometer (CMS). All compounds were injected at a concentration of 4 µM, with the exception of DMSO). The raw data are shown in the graphs below.



* The UV spectrum of DMSO was recorded at 220 nm since DMSO has no UV signature at 254 nm. Furthermore, the absorbance of DMSO is ten times stronger than the other tested compounds.

The void marker, compound 22, does not have any UV signature but can be easily followed by mass spectrometry. The comparison of UV and MS data clearly showed that a better sensitivity is achieved with the Advion CMS compared to a standard DAD detector even with the dilution factor introduced by the make-up buffer pump. Raw data appear also smoother with the MS system. Indeed, the peaks caused by the injection from the autosampler (*) and the end of the 600 µL injection loop (**) are seen in the UV chromatograms only. These limitations were not encountered in our previous work[1] which made use of a DAD detection system, because the dimensions of the column were bigger (20 nmol of immobilised protein target compared to only 2 nmol here).

C- Thermal shift Assay

Thermal melting experiments were carried out using a Mx3005p Real Time PCR machine (Stratagene) as previously described.[2]

The five synthetic compounds 4, 5, 15, 18 and 20 were screened against eight other bromodomain proteins that are spread across the bromodomain phylogenetic tree.[3] Data obtained using thermal shift assay are presented in the table below.

Bromodomain	**Bromosporine**	**4**	**5**	**15**	**18**	**20**
BRD9 (ΔTm_{obs})[#]	***+8.8***	*-0.6*	*+0.6*	*+2.4*	*+4.3*	*+2.7*
BRD1A (ΔTm_{obs})	+2.5	-1.6	+0.0	+2.3	+3.7	+2.0
BRD4 (ΔTm_{obs})[#]	**+6.6**	-3.5	-1.3	**+6.5**	**+6.7**	+4.3
BRPF1 (ΔTm_{obs})[#]	+0.6	*n.d.*	*n.d.*	+1.0	+5.4	+1.7
BRPF3 (ΔTm_{obs})[#]	+2.1	*n.d.*	*n.d.*	+1.1	+3.1	+1.6
CECR2 (ΔTm_{obs})[#]	**+8.3**	**-7.4**	-0.1	+3.0	+5.6	**-10.9**
CREBBP (ΔTm_{obs})[#]	+3.8	-2.1	-0.9	+4.1	+4.3	+4.6
EP300A (ΔTm_{obs})[#]	*n.d.*	+1.3	+1.3	+3.5	**+8.0**	+4.3
TIF1 (ΔTm_{obs})[#]	+0.9	-1.7	-0.5	+1.3	+1.8	+1.2

[#] Difference in temperature of denaturation of the protein with and without the ligand, data in °C - n.d.: non determined using thermal shift assay.

Compounds 4, 5 and 20 displayed no noteworthy stabilisation of any of the bromodomains tested. Compound 15 on the other hand showed a mild selectivity towards BRD4, while compound 18 exhibited stabilisation of both BRD4 and the transcription factor EP300, a general transcriptional co-activator.

D- References

1 L. Guetzoyan, N. Nikbin, I.R. Baxendale and S.V. Ley, *Chem. Sci.*, 2013, **4**, 764.

2 M. Philpott, J. Yang, T. Tumber, O. Fedorov, S. Uttarkar, P. Filippakopoulos, S. Picaud, T. Keates, I. Felletar, A. Ciulli, S. Knapp, T.D. Heightman, *Mol. BioSyst.,* 2011, **7**, 2899.

3 P. Filippakopoulos, S. Picaud, M. Mangos, T. Keates, J.-P. Lambert, T. Barsyte-Lovejoy, I. Felletar, R. Volkmer, S. Muller, T. Pawson, A.-C. Gingras, C. H. Arrowsmith, S. Knapp, Cell, 2012, 149, 214.