Supporting Information

4-aminopyridyl-based CYP51 inhibitors as anti-*Trypanosoma cruzi* drug leads with improved pharmacokinetic profile and *in vivo* potency

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Table S1. Tissue distribution of compounds (in μM) administered p.o. in HPβCD

##	Time, h	Plasma	Brain	Muscle	Intestine	Fat	Heart	Lung	Liver
5	2	0.49	0.10	0.31	0.09	0.17	1.22	13.84	10.53
	8	0.31	0.16	0.33	2.14	0.65	0.94	32.18	5.88
6	2	1.39	0.10	0.63	107.5	0.08	4.19	13.02	31.85
	8	1.53	1.35	4.03	11.51	2.82	10.38	21.41	50.84
7	2	1.18	0.23	0.51	0.09	0.17	4.08	9.62	20.83
	8	1.68	1.41	4.18	11.87	5.69	9.25	15.53	38.23
11	2	0.11	17.0	0.06	184.9	0.11	0.52	1.51	10.75
	8	0.06	0.02	0.06	3.74	0.32	0.31	1.28	6.93
12	2	0.01	0.14	1.03	72.05	0.12	3.87	14.45	31.22
	8	1.67	0.58	3.75	89.15	1.43	10.16	15.84	47.72

Formulation: 50mg/kg in 10 mg/ml suspension in 20% HPβCD

Table S2. Tissue distribution of compounds (in μM) administered p.o. in Kolliphor

##	Time, h	Plasma	Brain	Muscle	Intestine	Fat	Heart	Lung	Liver
5	2	10.3	2.67	6.37	152.35	18.17	23.68	54.21	80.96
	8	5.6	3.47	9.41	53.79	21.19	17.31	38.32	4937
7	2	8.9	0.7	3.4	55.0	5.9	8.5	30.8	42.8
	8	7.7	2.6	4.44	52.61	18.60	20.46	40.87	60.83
12	2	4.37	1.35	11.52	140.49	12.76	17.61	63.48	97.95
	8	2.08	1.26	3.72	45.19	13.84	12.62	20.72	46.11

Formulation: 50mg/kg in 10 mg/ml suspension in 20% Kolliphor

Table S3. X-ray data collection and refinement statistics

Protein	*Tc*CYP51	*TcCYP51*	*Tc*CYP51
PDB ID	**4C0C**	**4UQH**	**4BMM**
Small molecule ID	**WVH (Compound 12)**	**25S (Compound 11)**	**TU1 (Compound 1)**
Data collection			
Space group	$P6_322$	$P6_322$	C2
Cell dimensions			
a, *b*, *c* (Å)	128.5, 128.5, 116.7	128.4, 128.4, 116.6	272.5, 66.5, 122.2
α, β, γ (°)	90.0, 90.0, 120.0	90.0, 90.0, 120.0	90.0, 110.7, 90.0
Molecules in AU	1	1	4
Wavelength	1.11587	1.11587	1.11587
Resolution (Å)	2.04	2.43	2.84
R_{sym} or R_{merge} (%)	9.2 (123.9)[1]	16.9 (199.4)	9.1 (71.9)
$I / \sigma I$	14.6 (1 .5)	11.7 (1.5)	7.0 (1.5)
Completeness (%)	99.9 (99.2)	100.0 (100.0)	99.9 (99.9)
Redundancy	11.0 (7.1)	12.8 (13.1)	3.8 (3.8)
Crystallization conditions	0.4 M ammonium sulfate 0.1 M Bis-Tris, pH 5.0 19% PEG 3350	0.3 M ammonium sulfate 0.1 M Bis-Tris, pH 5.5 19% PEG 3350	0.1 M ammonium acetate 0.1 M Bis-Tris, pH 5.5 17% PEG 10000
Refinement			
No. reflections	34827	20808	46238
R_{work} / R_{free} (%)	19.9/25.4	19.4/24.7	18.9/29.0
No. atoms			
Protein	3540	3509	13726
Heme	43	43	172
Inhibitor	44	44	152
Solvent	125	54	38
Mean B value	43.5	52.2	79.9
B-factors			
Protein	43.8	52.8	80.4
Heme	28.3	32.5	57.3
Inhibitor	37.7	44.1	64.3
Solvent	44.6	44.9	67.5
R.m.s deviations			
Bond lengths (Å)	0.018	0.013	0.010
Bond angles (°)	1.963	1.594	1.570

[1]Values in parentheses are for highest-resolution.

FIGURE S1

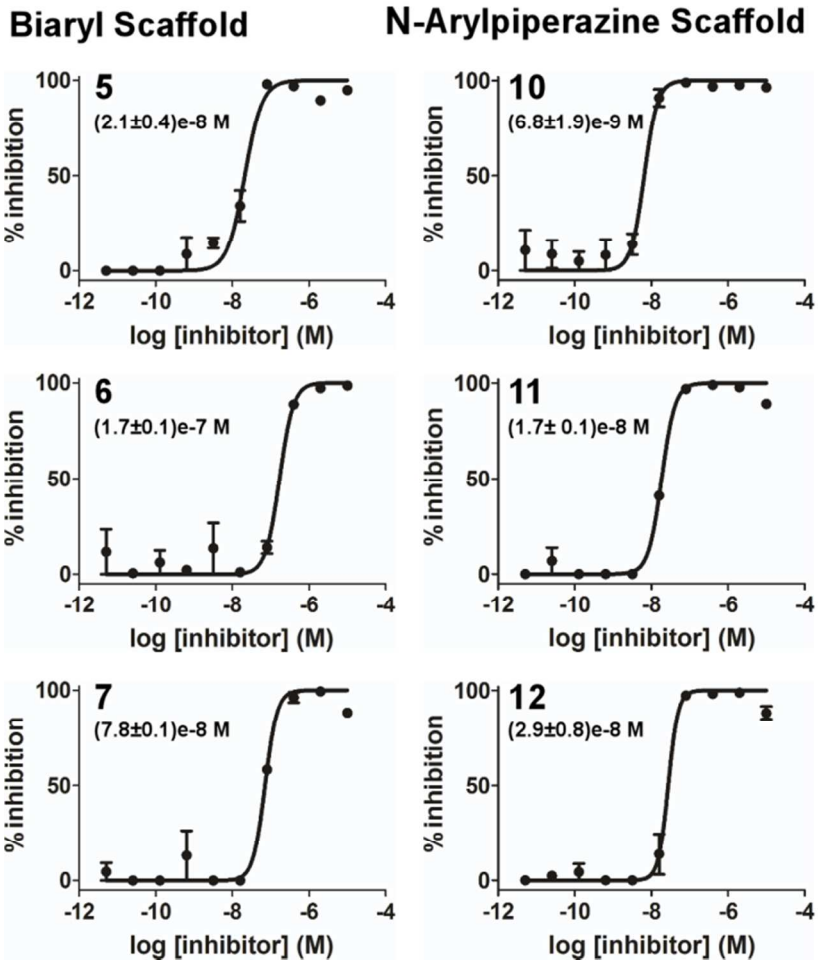


Figure S1. Dose-response curves in the image-based *T. cruzi* cell-based assay.

FIGURE S2

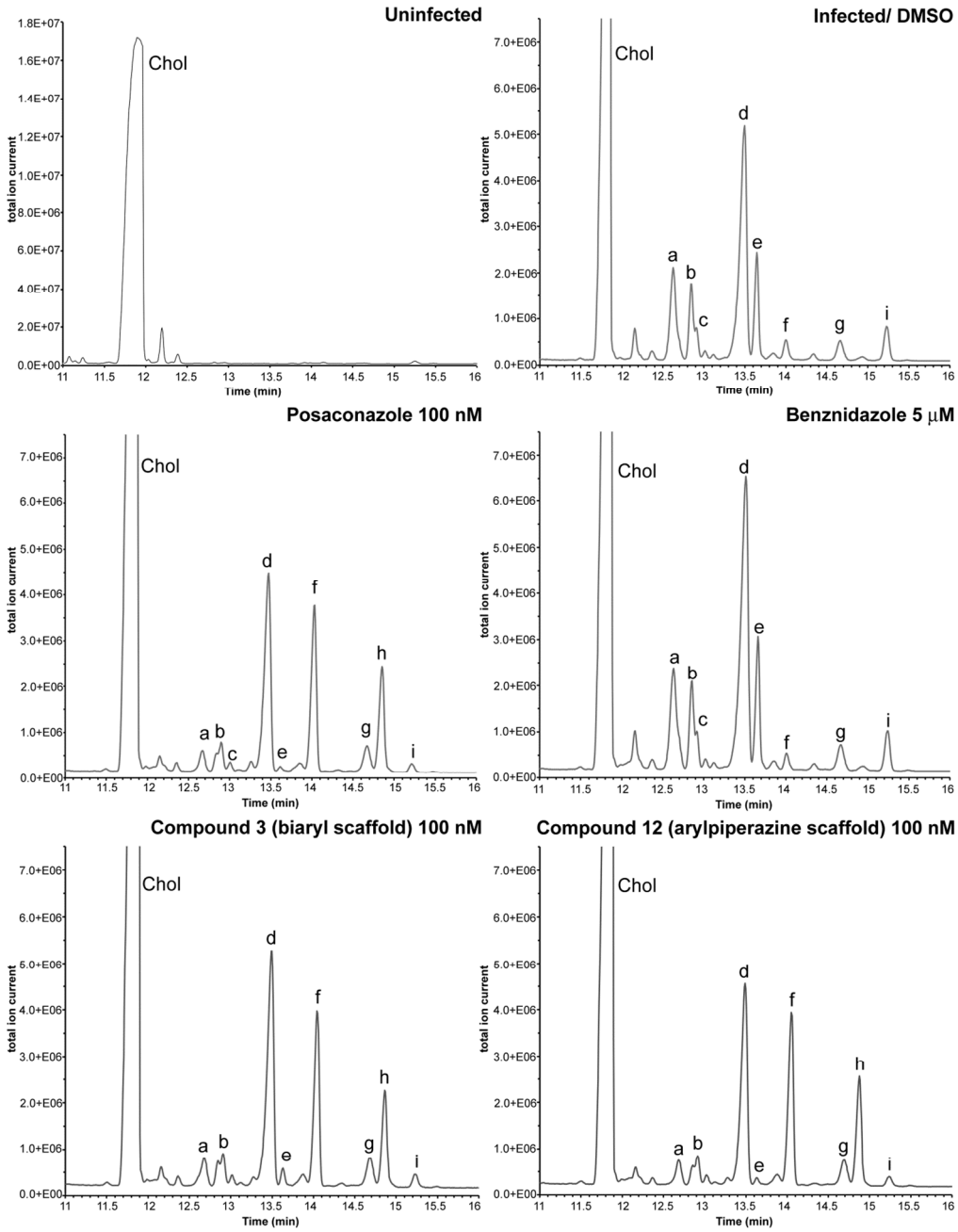


Figure S2. **Sterol profile of *T. cruzi* amastigotes infecting mouse myoblasts analyzed by GC-MS.** C2C12 uninfected mouse myoblasts display a high peak of cholesterol. *T. cruzi* infected cultures also show parasite-specific lipids (peaks **a-i**) corresponding to chromatographic peaks labeled as:

a- cholesta-7,24-dien-3β-ol, $[M]^{\bullet+}$ = m/z 454, RT=12.51-12.74;

b- cholesta-8,24-diene-3β-ol (zymosterol); m/z = 470; RT = 12.81 – 12.86 min;

c- 24-methyl-7-en-cholesta-en-3β-ol; m/z = 472; RT = 12.91 – 12.93 min;

d- ergosta-7,24-diene-3-β-ol (episterol); m/z = 470; RT = 13.2 – 13.5 min;

e- ergosta-8,24-diene-3-β-ol (fecosterol); m/z = 470; RT = 13.5 – 13.7 min;

f- lanosterol : m/z 498; RT = 13.95 – 14.06 min;

g- 4-methylepisterol; m/z = 484; RT = 14.55 – 14.75 min;

h- eburicol; m/z = 512, RT = 14.8 – 14.86 min;

i- 24-ethyl-7,24(24')-en-cholesta-diene-3β-ol; M/z = 484; RT = 15.14 -15.30 min.

Treatment of cultures was performed as indicated in each panel. DMSO (vehicle) and benznidazole were used as negative controls. Posaconazole, validated CYP51 inhibitor, was used as positive control. Treatment of cultures with posaconazole and compounds **3** and **12** resulted in accumulation of lanosterol (**f**) and eburicol (**h**) CYP51 substrates, and decline in episterol (**d**) and fecosterol (**e**) end products of the *T. cruzi* sterol biosynthesis pathway, as well as in 14-demethylated intermediates, (**a**) and (**b**), indicating the inhibition of CYP51.

FIGURE S3

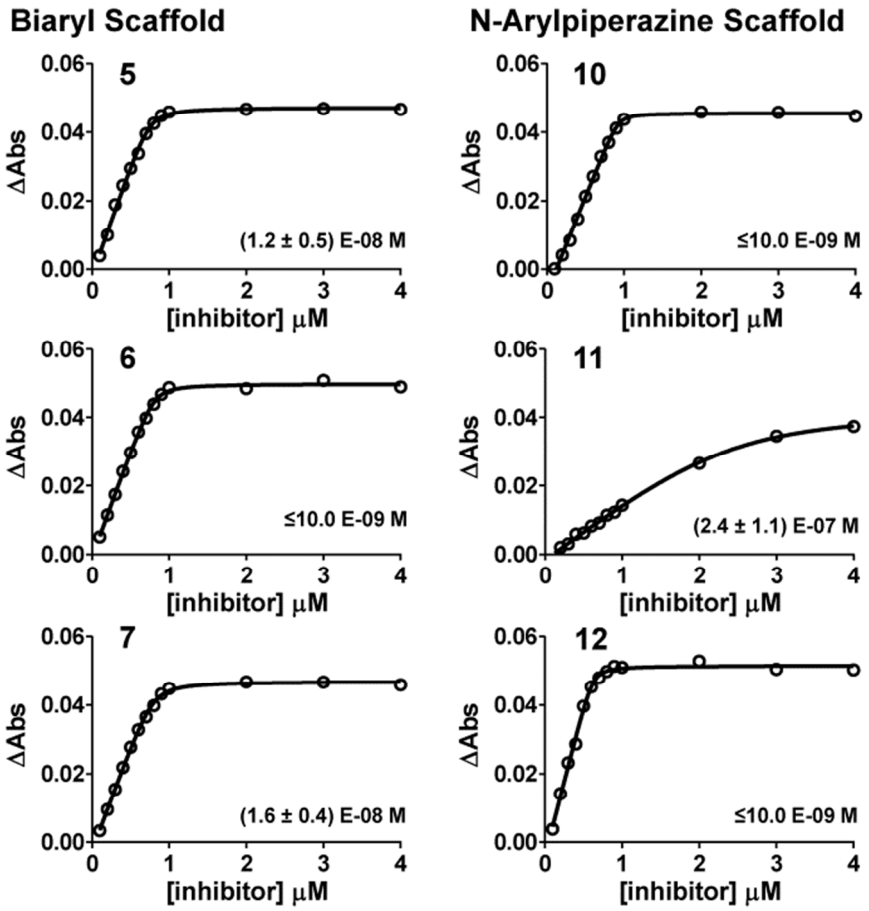


Figure S3. UV-vis titration curves. Apparent K_D values were determined spectrophotometrically, using 1 μM *Tc*CYP51. *ΔAbs* – absorbance shift between 425 nm and 390 nm in difference spectra – is plotted against inhibitor concentration. Linear stoichiometric saturation of the CYP51 active site reaching plateau at 1:1 molar ratio suggests binding affinity estimate equal or below a hundredth of enzyme concentration.

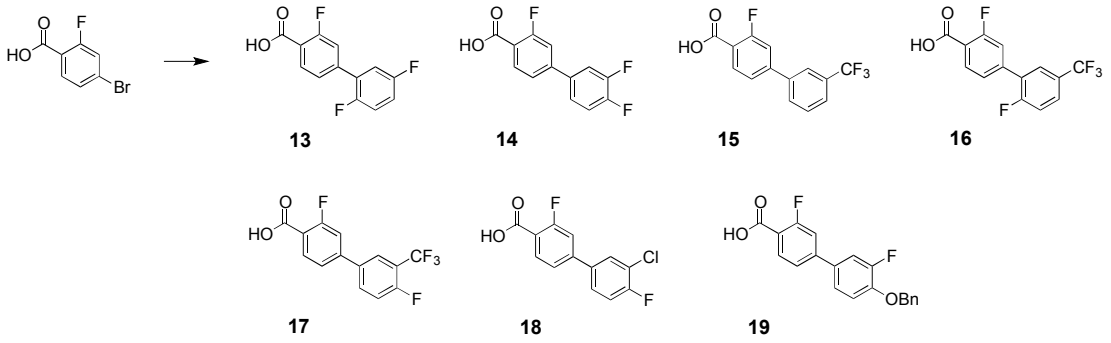
Chemistry, General Methods. All reaction solvents were purified before use. Dichloromethane, tetrahydrofuran, dimethylformamide and toluene were purified by passing through a column of activated A-1 alumina. All other reagents purchased from commercial suppliers were used as received. All reactions sensitive to moisture or oxygen were conducted under an argon atmosphere using flame-dried (under vacuum) or oven-dried (overnight) glassware. Removal of solvents was accomplished by using a rotary evaporator under reduced pressure in a water bath below 30 ^{o}C, followed by exposure to high vacuum using a vacuum pump. Microwave assisted reactions were performed using a Biotage® Initiator microwave reactor.

Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon (^{13}C) NMR spectra were recorded on a commercially available NMR spectrometer at 400 MHz and 100 MHz, respectively. The proton signal for non-deuterated solvent (δ 7.26 for $CHCl_3$ or δ 2.50 for DMSO) was used as an internal reference for ^{1}H NMR chemical shifts. Coupling constants (*J*) are reported in Hertz (Hz). ^{13}C chemical shifts are reported relative to the δ 77.16 resonance of $CDCl_3$ or the δ 39.52 resonance of DMSO-d_6.

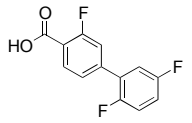
Optical rotations were measured using a quartz cell with 1 mL capacity and a 10 cm path length.

Analytical thin layer chromatography (TLC) was performed using glass plates precoated with a 0.25-mm thickness of silica gel. The TLC plates were visualized with UV light. Column chromatography was performed using a Biotage® Isolera flash purification system using Biotage® SNAP HP-SIL cartridge (30 μm silica, 10 g to 100 g size). Unless noted otherwise, all compounds isolated by flash chromatography were sufficiently pure by ^{1}H NMR analysis for use in subsequent reactions. Polar compounds were purified using preparative high performance liquid chromatography (HPLC) using SunFire column (30 mm × 250 mm) with a linear gradient elution ranging from 10% to 100% of CH_3CN/CH_3OH (1/1) in H_2O (containing 0.1% TFA) at 60 mL/min flow rate.

The purity of all final compounds (typically ≥96%) was assayed at 254 nm wavelength by using analytical HPLC (Varian 1100 series) on a reverse phase ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm). A linear gradient elution ranging from 2% to 98% CH_3CN and H_2O (containing 0.1% TFA and 1% CH_3CN) at 1.5 mL/min was used. Compounds were lyophilized before dissolution in DMSO to give 10 mM stock solutions for use in biochemical and cell-based assays.

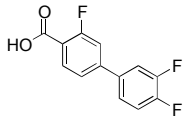


General procedure synthesis of biphenyl-4-carboxylic acids **13**, **14**, **15**, **16**, **17**, **18**, and **19**. A reaction mixture of 4-bromo-2-fluorobenzoic acid (ca. 0.10 g, 0.46 mmol), arylboronic acid (1.1 eq), $Pd_2(dba)_3$ (3 mol%), PCy_3 (6 mol %), and K_3PO_4 (2 M, 1 mL) in dioxane (4 mL) was stirred under microwave heating (100 °C) for 1 h. The palladium catalyst was removed by filtration through a pad of Celite, which was subsequently washed with ethyl acetate. The filtrate was acidified with 2N HCl (aq). The product mixture was diluted with ethyl acetate (30 mL) and washed with water (10 mL × 2) and brine (10 mL × 2). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting crude product was purified by flash chromatography to afford the 3-fluoro-biphenyl-4-carboxylic acids in ca. 90% yield.



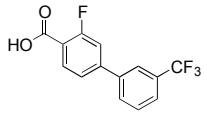
13

*2',3,5'-Trifluoro-[1,1'-biphenyl]-4-carboxylic acid (**13**).* The general procedure was followed (except reaction temperature (120 ^{o}C) and microwave reaction time (2 h)) using (2,5-difluorophenyl)boronic acid to provide **13** as a white solid (89%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.35 (s, 1H), 7.96 (t, J = 7.9 Hz, 1H), 7.54 (dddd, J = 11.5, 8.1, 3.2, 1.7 Hz, 3H), 7.42 (ddd, J = 10.1, 9.1, 4.6 Hz, 1H), 7.38 – 7.30 (m, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.67, 164.63, 162.23, 159.67, 159.54, 157.15, 157.12, 156.45, 154.01, 140.05, 139.96, 132.13, 127.32, 124.91, 124.87, 124.84, 118.99, 118.89, 118.15, 118.06, 117.90, 117.81, 117.40, 117.36, 117.25, 117.19, 117.16, 117.13, 117.10, 117.01, 116.94, 116.91; MS (ESI) *m/z* 251.0 $[M-H]^-$.



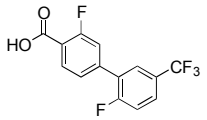
14

*3,3',4'-Trifluoro-[1,1'-biphenyl]-4-carboxylic acid (**14**).* The general procedure was followed using (3,4-difluorophenyl)boronic acid to provide **14** as a white solid (78%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.27 (s, 1H), 7.98 – 7.86 (m, 2H), 7.74 – 7.60 (m, 3H), 7.54 (dt, J = 10.6, 8.5 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.73, 164.69, 162.81, 160.25, 151.17, 151.08, 151.05, 150.96, 148.71, 148.64, 148.59, 148.52, 144.01, 143.93, 135.19, 135.14, 135.09, 132.52, 124.07, 124.04, 124.01, 123.97, 122.53, 122.50, 118.38, 118.27, 118.18, 118.01, 116.41, 116.23, 115.19, 114.95; MS (ESI) *m/z* 251.0 $[M-H]^-$.



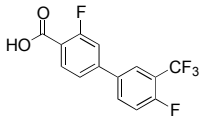
15

*3-Fluoro-3'-(trifluoromethyl)-[1,1'-biphenyl]-4-carboxylic acid (**15**).* The general procedure was followed using (3-(trifluoromethyl)phenyl)boronic acid to provide **15** as a white solid (84%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.34 (s, 1H), 8.08 (d, J = 7.6 Hz, 2H), 7.96 (t, J = 8.0 Hz, 1H), 7.86 – 7.59 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.77, 164.74, 162.86, 160.30, 144.65, 144.57, 138.70, 132.61, 131.18, 130.44, 130.22, 130.13, 129.81, 129.49, 128.15, 125.44, 125.41, 125.37, 125.33, 123.70, 123.66, 123.62, 123.59, 122.87, 122.83, 122.74, 120.02, 118.66, 118.56, 115.53, 115.30; MS (ESI) *m/z* 283.0 [M-H]$^{-}$.



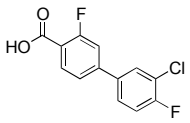
16

*2',3-Difluoro-5'-(trifluoromethyl)-[1,1'-biphenyl]-4-carboxylic acid (**16**)*. The general procedure was followed using (2-fluoro-5-(trifluoromethyl)phenyl)boronic acid to provide **16** as a white solid (93%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.40 (s, 1H), 8.06 – 7.93 (m, 2H), 7.89 (ddd, J = 8.6, 4.4, 2.4 Hz, 1H), 7.68 – 7.59 (m, 2H), 7.56 (dt, J = 8.1, 1.7 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.67, 164.64, 162.29, 162.21, 159.77, 159.65, 139.51, 139.42, 132.15, 128.27, 128.23, 128.20, 128.16, 128.10, 128.05, 127.19, 127.05, 126.21, 126.18, 125.89, 125.85, 125.11, 125.07, 125.03, 122.32, 119.26, 119.16, 117.79, 117.67, 117.64, 117.55, 117.43, 117.40; MS (ESI) *m/z* 301.1 [M-H]$^{-}$.



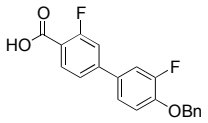
17

*3,4'-Difluoro-3'-(trifluoromethyl)-[1,1'-biphenyl]-4-carboxylic acid (**17**)*. The general procedure was followed using (4-fluoro-3-(trifluoromethyl)phenyl)boronic acid to provide **17** as a white solid (90%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.33 (s, 1H), 8.20 – 8.06 (m, 2H), 7.94 (t, J = 8.0 Hz, 1H), 7.76 (dd, J = 12.3, 1.8 Hz, 1H), 7.68 (dd, J = 8.2, 1.8 Hz, 1H), 7.63 (dd, J = 10.6, 8.7 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.73, 164.70, 162.79, 160.41, 160.24, 157.87, 143.71, 143.62, 134.73, 134.70, 133.95, 133.86, 132.56, 125.95, 125.90, 125.86, 123.85, 122.82, 122.78, 121.14, 118.60, 118.49, 118.07, 117.86, 117.50, 117.37, 117.17, 117.05, 115.52, 115.28; MS (ESI) *m/z* 301.1 $[M-H]^-$.



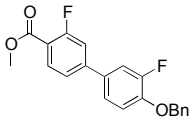
18

*3'-Chloro-3,4'-difluoro-[1,1'-biphenyl]-4-carboxylic acid (**18**)*. The general procedure was followed using (3-chloro-4-fluorophenyl)boronic acid to provide **18** as a white solid (83%), which included as by-products, 3''-chloro-3,4',4''-trifluoro-[1,1':3',1''-terphenyl]-4-carboxylic acid (**18a**) and 3'''-chloro-3,4',4'',4'''-tetrafluoro-[1,1':3',1'':3'',1'''-quaterphenyl]-4-carboxylic acid (**18b**) (10/7/2 = **18**/**18a**/**18b** by LCMS analysis); ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.28 (s, 1H), 8.03 (dd, J = 7.1, 2.4 Hz, 1H), 7.93 (t, J = 8.0 Hz, 1H), 7.80 (ddd, J = 8.7, 4.6, 2.4 Hz, 1H), 7.71 (dd, J = 12.4, 1.8 Hz, 1H), 7.65 (dd, J = 8.2, 1.8 Hz, 1H), 7.53 (t, J = 8.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.72, 164.69, 162.78, 160.23, 158.82, 156.35, 143.86, 143.77, 135.43, 135.39, 132.51, 132.43, 129.22, 127.86, 127.79, 122.60, 122.57, 120.44, 120.26, 118.38, 118.27, 117.56, 117.35, 115.26, 115.02; MS (ESI) *m/z* 267.1, 361.2, and 455.2 $[M-H]^-$.



19

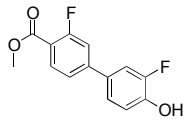
*4'-(Benzyloxy)-3,3'-difluoro-[1,1'-biphenyl]-4-carboxylic acid (**19**).* The general procedure was followed using (4-(benzyloxy)-3-fluorophenyl)boronic acid to provide **19** as a white solid (98%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.16 (s, 1H), 7.90 (t, J = 8.0 Hz, 1H), 7.74 (dd, J = 12.8, 2.3 Hz, 1H), 7.69 – 7.56 (m, 3H), 7.52 – 7.45 (m, 2H), 7.45 – 7.39 (m, 2H), 7.38 – 7.30 (m, 2H), 5.26 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.79, 164.75, 162.93, 160.37, 153.24, 150.81, 146.82, 146.71, 144.80, 144.72, 136.36, 132.46, 130.65, 130.57, 128.52, 128.11, 127.81, 123.32, 123.29, 121.95, 121.92, 117.55, 117.45, 115.70, 114.76, 114.57, 114.49, 114.26, 70.25; MS (ESI) *m/z* 339.1 $[M-H]^-$.



20

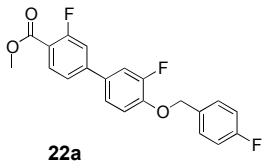
*Methyl 4'-(benzyloxy)-3,3'-difluoro-[1,1'-biphenyl]-4-carboxylate (**20**).* A solution of **19** (3.17 g, 9.3 mmol) in H_2SO_4/methanol (2/20 mL) was stirred at 70 ^{o}C for 5 h. The reaction mixture was cooled to ambient temperature, and was neutralized by addition of saturated $NaHCO_3$ at 0 ^{o}C. Ethyl acetate (100 mL) was added to the crude mixture, separated and washed with water (50 mL × 2) and brine (50 mL × 2). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography to provide **20** as a white solid (3.01 g, 8.5 mmol, 91%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (t, J = 7.9 Hz, 1H), 7.49 – 7.44 (m, 2H), 7.43 – 7.33 (m, 5H), 7.32 – 7.26 (m, 2H), 7.08 (t, J = 8.5 Hz, 1H), 5.20 (s, 2H), 3.95 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.85, 164.81, 163.71, 161.13, 154.38, 151.92, 147.48, 147.37, 146.30, 146.28, 146.21, 136.31, 132.84, 132.18,

132.12, 128.83, 128.40, 127.55, 123.06, 123.02, 122.07, 122.04, 117.09, 117.00, 116.01, 115.99, 115.27, 115.07, 115.05, 114.81, 71.50, 52.46; MS (ESI) *m/z* 377.2 $[M+Na]^+$.



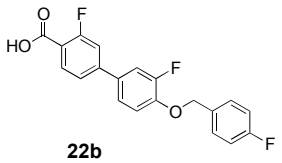
21

*Methyl 3,3'-difluoro-4'-hydroxy-[1,1'-biphenyl]-4-carboxylate (**21**).* To a solution of **20** (1.48 g, 4.2 mmol) in methanol/acetone (20/20 mL) was added 10% Pd/C (62.1 mg) at room temperature. Air was removed from the flask using a vacuum pump, then hydrogen gas was introduced using a balloon. The reaction mixture was stirred for 1 h, and the flask was evacuated under vacuum and refilled with hydrogen gas. This procedure was repeated three times, then the reaction mixture was stirred overnight at ambient temperature. Palladium on carbon was removed by filtration of the mixture through a Celite pad. The filtrate was collected and evaporated to give the crude product, which was purified by flash chromatography to afford the product **21** as a white solid (1.01 g, 3.8 mmol, 92%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.28 (s, 1H), 7.89 (t, J = 8.0 Hz, 1H), 7.73 – 7.54 (m, 3H), 7.51 – 7.43 (m, 1H), 7.14 – 6.97 (m, 1H), 3.85 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.82, 163.78, 162.83, 160.27, 152.52, 150.12, 146.06, 145.94, 145.73, 145.63, 132.26, 128.70, 128.64, 123.41, 123.38, 121.81, 121.78, 118.20, 118.17, 115.93, 115.82, 114.89, 114.70, 114.22, 113.98, 52.25; MS (ESI) *m/z* 287.1 $[M+Na]^+$.



22a

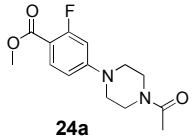
*Methyl 3,3'-difluoro-4'-((4-fluorobenzyl)oxy)-[1,1'-biphenyl]-4-carboxylate (**22a**).* To a solution of **21** (0.155 g, 0.59 mmol) in acetone (10 mL) were added Cs_2CO_3 (0.167 g, 1.2 mmol) and 4-fluorobenzyl bromide (0.3 mL). The reaction mixture was stirred at 70 ^oC for 5h. The reaction mixture was allowed to cool to ambient temperature, then ethyl acetate (20 mL) was added. The organic phase was separated and washed with water (5 mL × 2) and brine (5 mL × 2). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo. The product mixture was purified by flash chromatography to afford the titled product **22a** as a white solid (0.207 g, 0.56 mmol, 95%): ^1H NMR (400 MHz, $CDCl_3$) δ 7.99 (t, J = 7.9 Hz, 1H), 7.50 – 7.41 (m, 2H), 7.38 (dd, J = 3.4, 2.0 Hz, 1H), 7.36 (d, J = 1.9 Hz, 1H), 7.33 – 7.26 (m, 2H), 7.09 (q, J = 8.4 Hz, 3H), 5.16 (s, 2H), 3.96 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.77, 164.73, 163.99, 163.67, 161.54, 161.09, 154.35, 151.89, 147.25, 147.14, 146.15, 146.06, 132.82, 132.34, 132.27, 132.07, 132.03, 129.52, 129.44, 123.04, 123.01, 122.03, 121.99, 117.11, 117.01, 116.04, 116.01, 115.83, 115.62, 115.27, 115.07, 115.00, 114.77, 70.85, 52.41; MS (ESI) *m/z* 395.2 $[M+Na]^+$.



22b

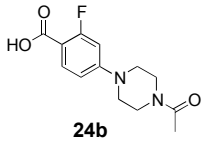
*3,3'-Difluoro-4'-((4-fluorobenzyl)oxy)-[1,1'-biphenyl]-4-carboxylic acid (**22b**).* To a solution of ester **22a** (0.207 g, 0.56 mmol) in methanol/THF (10/10 mL) was added 10% NaOH (10 mL). The reaction mixture was stirred for 2 h at 60 ^oC. After completion of the reaction, the reaction mixture was allowed to cool to ambient temperature, and then 2N HCl was added until a white solid precipitated. The mixture was diluted with ethyl acetate (60 mL) and washed with brine (10 mL × 2). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting crude product was purified by flash chromatography to yield **22b** (0.188 g, 0.53 mmol, 95%) as a white solid: ^1H NMR (400 MHz, DMSO-d_6) δ 13.21 (s, 1H), 7.90 (t, J = 8.0 Hz, 1H), 7.74 (dd, J = 12.8, 2.3 Hz, 1H), 7.71 – 7.57 (m, 3H),

7.58 – 7.49 (m, 2H), 7.36 (t, J = 8.8 Hz, 1H), 7.30 – 7.20 (m, 2H), 5.24 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.78, 164.74, 163.13, 162.91, 160.70, 160.37, 153.23, 150.80, 146.70, 146.59, 144.76, 144.68, 132.61, 132.58, 132.46, 130.72, 130.67, 130.19, 130.10, 123.33, 123.30, 121.96, 121.93, 117.57, 117.47, 115.74, 115.47, 115.26, 114.77, 114.58, 114.50, 114.26, 69.55; MS (ESI) *m/z* 357.3 $[M-H]^-$.



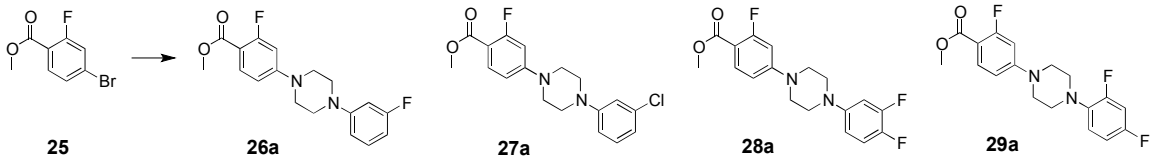
24a

*Methyl 4-(4-acetylpiperazin-1-yl)-2-fluorobenzoate (**24a**).* To a 0 °C suspension of **23** (101 mg, 0.37 mmol) in dry CH_2Cl_2 (5 mL) were slowly added acetic anhydride (0.05 mL) and triethylamine (0.1 mL). The reaction mixture was stirred for 15 min at 0 °C, and then was allowed to warm to ambient temperature and stirred for another 30 min. After the reaction was judged to be complete as monitored by TLC analysis, the solvent was removed by rotary evaporation. Ethyl acetate (30 mL) was added to the residue, the organic phase was separated and washed with saturated aqueous $NaHCO_3$ (5 mL × 2) and brine (5 mL × 2). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo. Purification of the crude product by flash chromatography provided 86 mg (0.31 mmol, 94%) of **24a** as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (t, J = 8.8 Hz, 1H), 6.62 (dd, J = 8.9, 2.5 Hz, 1H), 6.50 (dd, J = 14.4, 2.5 Hz, 1H), 3.87 (s, 3H), 3.76 (dd, J = 6.5, 4.2 Hz, 2H), 3.69 – 3.57 (m, 2H), 3.42 – 3.26 (m, 4H), 2.14 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.22, 165.09, 164.92, 164.88, 162.52, 155.14, 155.03, 133.59, 133.56, 109.60, 109.58, 108.23, 108.13, 101.94, 101.68, 51.97, 47.30, 47.12, 45.66, 40.87, 21.43.



24b

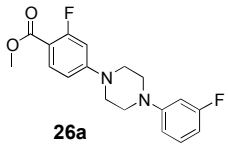
*4-(4-Acetylpiperazin-1-yl)-2-fluorobenzoic acid (**24b**).* A mixture of **24a** (0.345 g, 1.2 mmol) in methanol (5 mL) and 10% NaOH (5 mL) was stirred for 2 h at room temperature. When the reaction was complete as indicated by TLC analysis, the organic solvent was removed by using a rotary evaporator, and 2N HCl was added until a white solid formed. The white solid was filtered, washed with water, and dried by lyophilization to obtain the titled product **24b** (0.115 g, 0.43 mmol, 36%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.50 (s, 1H), 7.71 (t, J = 9.0 Hz, 1H), 6.87 – 6.62 (m, 2H), 3.55 (dd, J = 6.6, 4.0 Hz, 4H), 3.36 (ddd, J = 28.8, 6.7, 4.1 Hz, 4H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.43, 164.86, 164.83, 164.48, 161.95, 154.87, 154.76, 133.17, 133.14, 109.21, 106.90, 106.80, 101.02, 100.75, 46.42, 46.14, 44.86, 21.20; MS (ESI) *m/z* 267.2 $[M+H]^+$.



25 **26a** **27a** **28a** **29a**

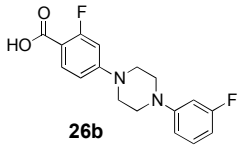
General procedure for the synthesis of methyl 2-fluoro-4-(4-aryl)piperazine-1-yl)benzoates **26a**, **27a**, **28a**, and **29a**. A reaction mixture of **25** (1.0 mmol), 1-arylpiperazine (1.1 mmol), $Pd(OAc)_2$ (0.1 mmol), BINAP (0.2 mmol), and Cs_2CO_3 (1.1 mmol) in dry toluene (5 mL) was stirred at 60 °C for 48 h. after the reaction mixture was allowed to cool to ambient temperature, then the palladium catalyst was removed by filtration through a Celite pad. The product mixture was diluted with ethyl acetate (50 mL) and washed with aqueous $NaHCO_3$ (10 mL × 2) and brine (10 mL × 2). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting crude product was purified by flash

chromatography to yield the appropriate methyl 2-fluoro-4-(4-aryl)piperazine-1-yl)benzoate as a solid in ca. 70% yield.



26a

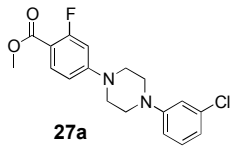
*Methyl 2-fluoro-4-(4-(3-fluorophenyl)piperazin-1-yl)benzoate (**26a**)*. The general N-arylation procedure was followed using 1-(3-fluorophenyl)piperazine to provide **26a** as a white solid (86%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.73 (t, J = 9.0 Hz, 1H), 7.23 (q, J = 7.8 Hz, 1H), 6.90 – 6.72 (m, 4H), 6.57 (td, J = 8.5, 1.9 Hz, 1H), 3.77 (s, 3H), 3.54 – 3.46 (m, 4H), 3.34 – 3.24 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.48, 164.36, 163.87, 163.83, 162.09, 161.82, 155.12, 155.01, 152.39, 152.29, 132.89, 132.86, 130.43, 130.33, 110.89, 109.27, 105.73, 105.62, 105.03, 104.82, 102.00, 101.75, 100.92, 100.66, 51.53, 47.12, 45.99; MS (ESI) *m/z* 355.1 $[M+Na]^+$.



26b

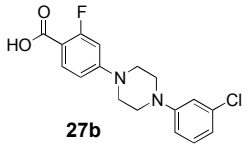
*2-Fluoro-4-(4-(3-fluorophenyl)piperazin-1-yl)benzoic acid (**26b**)*. To a solution of **26a** (0.176 g, 0.53 mmol) in methanol (10 mL) was added 10% NaOH (10 mL), and the reaction mixture was stirred for 2 h at 60 ^{o}C. After the reaction was complete as indicated by TLC analysis, the mixture was cooled to ambient temperature, and 2N HCl was added until a white solid precipitated. The mixture was diluted with ethyl acetate (60 mL) and washed with brine (10 mL × 2). The organic layer was dried over magnesium sulfate, filtered, and concentrated in vacuo. The resulting product was purified by flash chromatography to yield **26b** (0.164 g, 0.52 mmol, 97%) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6)

δ 7.72 (t, J = 8.9 Hz, 1H), 7.25 (q, J = 7.9 Hz, 1H), 6.94 – 6.74 (m, 4H), 6.61 (td, J = 8.6, 2.0 Hz, 1H), 3.50 (dd, J = 6.7, 3.8 Hz, 4H), 3.33 (dd, J = 6.5, 3.9 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.87, 164.84, 164.50, 164.43, 162.04, 161.97, 154.93, 154.82, 152.03, 151.93, 133.19, 133.16, 130.53, 130.43, 111.30, 109.31, 107.06, 106.96, 105.60, 105.39, 102.42, 102.17, 101.14, 100.87, 47.47, 46.05; MS (ESI) *m/z* 317.1 $[M-H]^-$.



27a

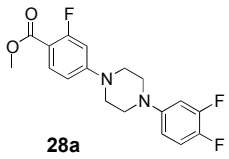
*Methyl 4-(4-(3-chlorophenyl)piperazin-1-yl)-2-fluorobenzoate (**27a**).* The general N-arylation procedure was followed using 1-(3-chlorophenyl)piperazine hydrochloride to provide **27a** as a white solid (51%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (t, J = 8.8 Hz, 1H), 7.19 (t, J = 8.1 Hz, 1H), 6.90 (t, J = 2.2 Hz, 1H), 6.83 (dddd, J = 17.6, 8.4, 2.2, 0.8 Hz, 2H), 6.65 (dd, J = 8.9, 2.5 Hz, 1H), 6.54 (dd, J = 14.5, 2.5 Hz, 1H), 3.87 (s, 3H), 3.53 – 3.41 (m, 4H), 3.38 – 3.23 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.10, 164.94, 164.90, 162.54, 155.28, 155.17, 151.85, 135.12, 133.50, 133.47, 130.28, 119.95, 116.03, 114.17, 109.43, 109.41, 107.74, 107.64, 101.67, 101.40, 51.89, 48.39, 47.00; MS (ESI) *m/z* 349.1 $[M+H]^+$.



27b

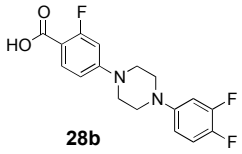
*4-(4-(3-Chlorophenyl)piperazin-1-yl)-2-fluorobenzoic acid (**27b**)*. The procedure for the synthesis of **26b** was followed using **27a** to provide **27b** as a white solid (70%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.51 (s, 1H), 7.72 (t, J = 8.9 Hz, 1H), 7.23 (t, J = 8.1 Hz, 1H), 7.00 (t, J = 2.2 Hz, 1H), 6.94 (dd, J = 8.3, 2.4

Hz, 1H), 6.87 – 6.74 (m, 3H), 3.48 (dd, J = 6.7, 3.8 Hz, 4H), 3.40 – 3.18 (m, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.90, 164.87, 164.52, 161.98, 154.99, 154.88, 151.89, 133.86, 133.19, 130.50, 118.34, 114.70, 113.80, 109.28, 106.96, 106.86, 101.09, 100.83, 47.17, 46.14; MS (ESI) *m/z* 333.1 $[M-H]^-$.



28a

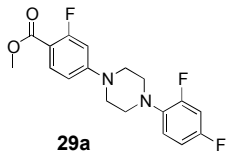
*Methyl 4-(4-(3,4-difluorophenyl)piperazin-1-yl)-2-fluorobenzoate (**28a**)*. The general N-arylation procedure was followed using 1-(3,4-difluorophenyl)piperazine to provide **28a** as a white solid (62%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (t, J = 8.8 Hz, 1H), 7.05 (dt, J = 10.1, 9.1 Hz, 1H), 6.72 (ddd, J = 13.1, 6.8, 3.0 Hz, 1H), 6.66 (dd, J = 8.9, 2.5 Hz, 1H), 6.61 (dtd, J = 9.0, 3.2, 1.5 Hz, 1H), 6.54 (dd, J = 14.4, 2.5 Hz, 1H), 3.87 (s, 3H), 3.52 – 3.40 (m, 4H), 3.28 – 3.15 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.10, 164.93, 164.89, 162.54, 155.31, 155.19, 151.92, 151.79, 149.47, 149.34, 148.10, 148.08, 148.02, 148.00, 145.96, 145.83, 143.56, 143.43, 133.51, 133.47, 117.53, 117.36, 111.95, 111.92, 111.90, 111.87, 109.53, 109.50, 107.84, 107.73, 105.83, 105.63, 101.78, 101.51, 51.89, 49.28, 47.12; MS (ESI) *m/z* 351.2 $[M+H]^+$.



28b

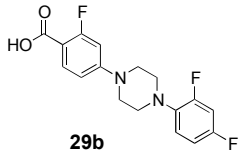
*4-(4-(3,4-Difluorophenyl)piperazin-1-yl)-2-fluorobenzoic acid (**28b**)*. The procedure for the synthesis of **26b** was followed using **28a** to provide **28b** as a white solid (92%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.51 (s, 1H), 7.72 (t, J = 8.9 Hz, 1H), 7.26 (q, J = 9.6 Hz, 1H), 7.05 (ddd, J = 14.2, 7.1, 3.0 Hz, 1H), 6.90 – 6.69 (m, 3H), 3.47 (dd, J = 6.7, 3.9 Hz, 4H), 3.24 (dd, J = 6.5, 4.0 Hz, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.91, 164.88, 164.52, 161.99, 155.01, 154.90, 151.03, 150.90, 148.62, 148.49, 148.23,

148.16, 148.14, 144.20, 144.08, 141.85, 141.72, 133.20, 133.17, 117.42, 117.25, 111.39, 111.37, 111.34, 111.31, 109.37, 107.04, 106.94, 104.76, 104.56, 101.21, 100.94, 47.88, 46.21; MS (ESI) *m/z* 335.2 $[M-H]^-$.



29a

*Methyl 4-(4-(2,4-difluorophenyl)piperazin-1-yl)-2-fluorobenzoate (**29a**)*. The general N-arylation procedure was followed using 1-(2,4-difluorophenyl)piperazine to provide **29a** as a white solid (72%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (t, J = 8.8 Hz, 1H), 6.95 – 6.87 (m, 1H), 6.86 – 6.76 (m, 2H), 6.65 (dd, J = 9.0, 2.5 Hz, 1H), 6.54 (dd, J = 14.6, 2.5 Hz, 1H), 3.86 (s, 3H), 3.49 – 3.41 (m, 4H), 3.13 (dd, J = 6.2, 4.0 Hz, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.09, 164.93, 164.89, 162.53, 159.53, 159.42, 157.11, 157.06, 157.00, 156.95, 155.53, 155.42, 154.59, 154.47, 136.30, 136.26, 136.21, 136.17, 133.44, 133.41, 119.80, 119.76, 119.71, 119.67, 111.02, 110.99, 110.81, 110.78, 109.50, 109.48, 107.70, 107.60, 105.16, 104.92, 104.90, 104.66, 101.75, 101.48, 77.16, 51.84, 50.60, 50.57, 47.43; MS (ESI) *m/z* 351.2 $[M+H]^+$.



29b

*4-(4-(2,4-Difluorophenyl)piperazin-1-yl)-2-fluorobenzoic acid (**29b**)*. The procedure for the synthesis of **26b** was followed using **29a** to provide **29b** as a white solid (94%): ^{1}H NMR (400 MHz, THF-d_8) δ 10.89 (s, 1H), 7.81 (t, J = 8.8 Hz, 1H), 7.07 (td, J = 9.3, 5.8 Hz, 1H), 6.96 (ddd, J = 12.0, 8.8, 2.9 Hz, 1H), 6.92 – 6.83 (m, 1H), 6.77 (dd, J = 8.9, 2.5 Hz, 1H), 6.69 (dd, J = 14.6, 2.4 Hz, 1H), 3.54 – 3.44 (m, 4H), 3.20 –

3.09 (m, 4H). ^{13}C NMR (101 MHz, THF-d_8) δ 166.27, 165.43, 165.39, 163.72, 160.47, 160.36, 158.18, 158.07, 157.95, 156.78, 156.67, 155.71, 155.60, 137.97, 137.93, 137.88, 137.84, 134.56, 134.53, 121.08, 121.03, 120.98, 120.94, 111.78, 111.74, 111.56, 111.53, 110.21, 109.10, 108.99, 105.67, 105.42, 105.16, 102.48, 102.21, 51.69, 51.66, 48.40; MS (ESI) *m/z* 335.2 $[M-H]^-$.