SUPPORTING INFORMATION

Discovery of Bi-phenyl Piperazines as Novel and Long Acting Muscarinic Acetylcholine Receptor (mAChR) Antagonists.

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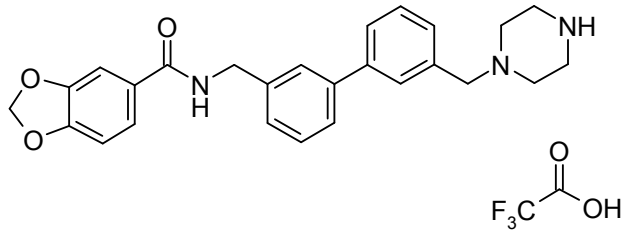
General Procedures:

HPLC data were acquired from an Agilent 1100 Series system with UV detector set to at 254 nm. Samples were injected (3 μL) onto a Waters Sunfire 3.0 x 50 mm, 5.0 μM, C18 column maintained at 40 °C. A linear gradient from 10% to 100% B (MeCN + 0.05% TFA) in 2.5 min was followed by pumping 100% B for another 1.7 minutes with A being H_2O + 0.05% TFA. The flow rate was 1.2 mL/min. Mass spectra (MS) data were acquired in positive ion mode using an Agilent 6110 single quadrupole mass spectrometer with an electrospray ionization (ESI) source. High-resolution (positive ion) mass spectra (HRMS) were acquired using a Micromass Q-Tof 2 hybrid quadrupole time-of-flight mass spectrometer equipped with a Z-spray interface or a Bruker Daltonics 7T FTICR-MS equipped with an Apollo ESI interface. Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra were recorded at 400 MHz or 600 MHz, chemical shifts are reported in ppm (δ).

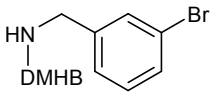
Synthesis of 1

***N*-{[3'-(1-piperazinylmethyl)-3-biphenylyl]methyl}-1,3-benzodioxole-5-carboxamide trifluoroacetate (1)**



1

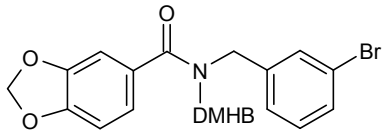
DMHB resin-bound 3-bromo-benzylamine



To a 250 mL shaker vessel was added 2,6-dimethoxy-4-polystyrenebenzyloxy-benzaldehyde (DMHB resin) (10 g, 1.5 mmol/g, 15 mmol) and 150 mL of 1-methyl-2-pyrrolidinone (NMP). 3-Bromo-benzylamine HCl salt (17 g, 75 mmol), diisopropylethylamine (DIEA) (13 mL, 75 mmol), acetic acid (HOAc) (15 mL), and

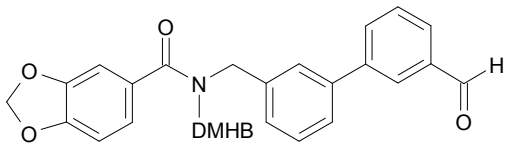
$Na(OAc)_3BH$ (19.1 g, 90 mmol) were then added. The resulting mixture was shaken at rt overnight, and was then washed with NMP (150 mL x 2), dichloromethane (DCM) (150 mL x 2), MeOH (150 mL x 2) and DCM (150 mL x 2). The resulting resin was dried in a vacuum oven at 35 °C overnight to yield DMHB resin-bound 3-bromo-benzylamine (15 mmol, loading 100%).

DMHB resin-bound *N*-[(3-bromophenyl)methyl]-1,3-benzodioxole-5-carboxamide



To DMHB resin-bound 3-bromo-benzylamine [2 g, 1.2 mmol/g (theoretical loading), 2.4 mmol] in DCE/DMF (1:1, 80 mL) was added piperonylic acid (4.0 g, 24 mmol) and DIC (3.7 mL, 24 mmol). The mixture was shaken at rt for overnight and was then washed with DMF (100 mL x 2), DCM (100 mL x 2), MeOH (100 mL x 2) and DCM (100 mL x 2). The resulting resin was dried in a vacuum oven at 35 °C overnight to yield DMHB resin-bound *N*-[(3-bromophenyl)methyl]-1,3-benzodioxole-5-carboxamide (2.4 mmol). An analytical amount of the resin was cleaved with 20% TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of MeOH. MS (ES+) 334 $[M+H]^+$.

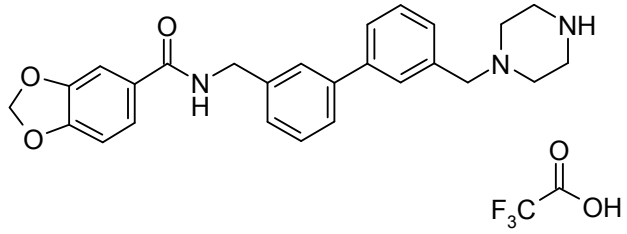
DMHB resin-bound *N*-[(3'-formyl-3-biphenylyl)methyl]-1,3-benzodioxole-5-carboxamide



To DMHB resin-bound *N*-[(3-bromophenyl)methyl]-1,3-benzodioxole-5-carboxamide [3.03 g, 1.0 mmol/g (theoretical loading), 3.03 mmol] in 76 mL of DME was added 3-formylphenyl boronic acid (1.36 g, 9.09 mmol), 2 M K_2CO_3 aqueous solution (4.5 mL, 9.09 mmol), and $Pd(PPh_3)_4$ (0.18 g, 0.15 mmol). After purging with argon for 5-10 min, the mixture was heated at 80 °C under argon for 10 h. The resulting resin was washed with THF (100 mL x 2), THF:H_2O (1:1, 100 mL x 2), H_2O

(100 mL x 2), THF:H_2O (1:1, 100 mL x 2), THF (100 mL x 2), DCM (100 mL x 2), and dried in a vacuum oven at 35 °C overnight. An analytical amount of the resin was cleaved with 20% TFA in DCM for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ES+) 360 $[M+H]^+$.

N-{[3'-(1-piperazinylmethyl)-3-biphenylyl]methyl}-1,3-benzodioxole-5-carboxamide trifluoroacetate (**1**)



1

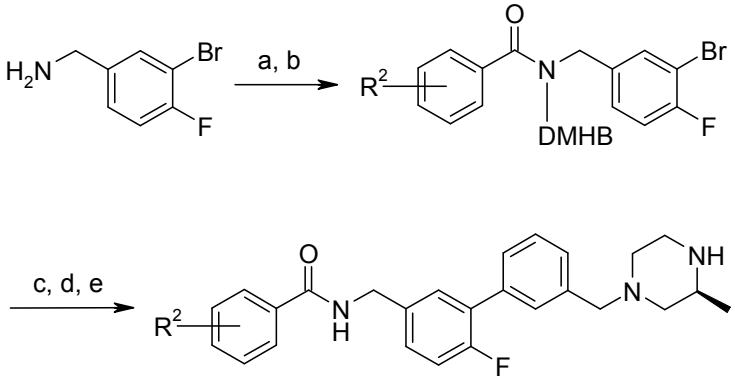
To a mixture of DMHB resin-bound *N*-[(3'-formyl-3-biphenylyl)methyl]-1,3-benzodioxole-5-carboxamide (400 mg, 0.99 mmol/g, 0.40 mmol) in 17 mL of DCE was added Na_2SO_4 (0.24 g, 1.7 mmol) and 1,1-dimethylethyl 1-piperazinecarboxylate (0.32 g, 1.7 mmol). After shaking for 10 min, $Na(OAc)_3BH$ (0.43 g, 2.04 mmol) was added. After shaking at rt for overnight, the resin was washed with THF (100 mL x 2), THF:H_2O (1:1, 100 mL x 2), H_2O (100 mL x 2), THF:H_2O (1:1, 100 mL x 2), THF (100 mL x 2), DCM (100 mL x 2) and dried in a vacuum oven at 35 °C overnight. The resulting resin was cleaved with 8 mL of 20% TFA in DCE for 30 min and treated again with 8 mL of 20% TFA in DCE for 30 min. The combined cleavage solution was concentrated *in vacuo*. The residue was dissolved in DMSO and purified using a Gilson semi-preparative HPLC system with a YMC ODS-A (C-18) column 50 mm by 20 mm ID, eluting with 10% B to 90% B in 3.2 min, hold for 1 min where A = H_2O (0.1% trifluoroacetic acid) and B = CH_3CN (0.1% trifluoroacetic acid) pumped at 25 mL/min, to produce *N*-{[3'-(1-piperazinylmethyl)-3-biphenylyl]methyl}-1,3-benzodioxole-5-carboxamide trifluoroacetate **1** as a white powder (100 mg, 58% over 5 steps).

^{1}H NMR (600 MHz, DMSO-*d*6) δ ppm 8.95 (t, *J*=5.7 Hz, 1 H), 8.56 (br s, 2 H), 7.63 - 7.55 (m, 3 H), 7.53 (d, *J*=7.6 Hz, 1 H), 7.51 – 7.41 (m, 4 H), 7.38 – 7.29 (m, 2 H), 7.00 (d, , *J*=7.9 Hz, 1 H), 6.10 (s, 2 H), 4.52 (d, , *J*=5.7 Hz, 2 H), 3.71 (m, 2 H), 3.13 (m, 4 H), 2.67 (m, 4 H).

HPLC: 100%, RT 2.02 min. HRMS (ESI+) calcd for [C26H27N3O3 + H]+: 430.2125. Found: 430.2122.

Scheme 1.

Syntheses of 4a, 4b, and 4c

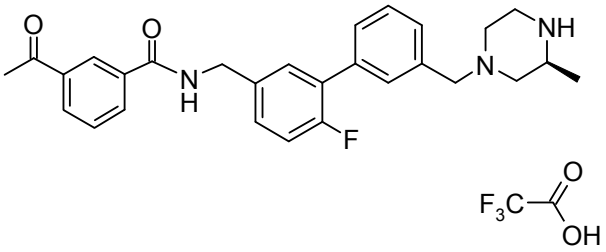


4a, 4b, 4c

(a) 2,6-dimethoxy-4-polystyrenebenzyloxybenzaldehyde (DMHB-resin), $Na(OAc)_3BH$, DIEA, 10% of HOAc in NMP, rt; (b) various benzoic acids, DIC, DCE/DMF 1:1, rt; (c) 3-formylphenyl boronic acid, $Pd(PPh_3)_4$, 2 M Cs_2CO_3, DME, 80 °C; (d) (*S*)-2-methylpiperazine, $Na(OAc)_3BH$, Na_2SO_4, DCE, rt; (e) 50% of TFA in DCE, rt.

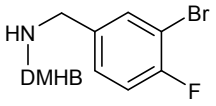
Synthesis of 4a

3-acetyl-*N*-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl] benzamide trifluoroacetate (4a)



4a

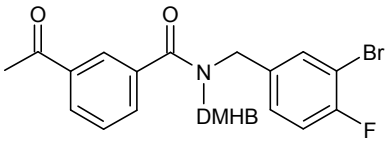
DMHB resin bound 3-bromo-4-fluoro-benzylamine (**4a-1**, step a)



4a-1

To a mixture of DMHB resin (10 g, 1.5 mmol/g loading, 15 mmol) in N-methyl pyrrolidine (NMP, 150 mL), was added 3-bromo-4-fluoro-benzylamine (15.3 g, 75 mmol), acetic acid (15 mL, 1% v/v), and sodium triacetoxyborohydride (19.1 g, 90 mmol). The mixture was shaken at rt overnight and was then washed with NMP (200 mL x 2), dichloromethane (DCM) (200 mL x 2), MeOH (200 mL x 2) and DCM (200 mL x 2). The resulting resin was dried in a vacuum oven at 20 °C overnight to yield DMHB resin bound 3-bromo-4-fluoro-benzylamine **4a-1** (15 mmol).

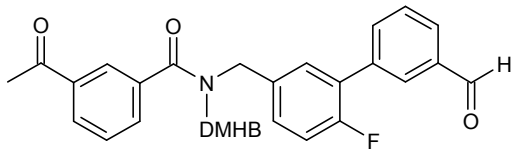
DMHB resin-bound 3-acetyl-*N*-[(3-bromo-4-fluorophenyl)methyl]benzamide (**4a-2**, step b)



4a-2

To a mixture of above resin-bound 3-bromo-4-fluoro-benzylamine **4a-1** (200 mg, 1.17 mmol/g theoretical loading), 0.23 mmol) in DCE/DMF (1:1, 8 mL) was added 3-acetyl benzoic acid (378 mg, 2.3 mmol) and DIC (0.36 mL, 2.3 mmol). The mixture was shaken at rt for overnight and then washed with DMF (20 mL x 2), DCM (20 mL x 2), MeOH (20 mL x 2) and DCM (20 mL x 2). The resulting resin was dried in a vacuum oven at 20 °C overnight to yield DMHB resin-bound 3-acetyl-*N*-[(3-bromo-4-fluorophenyl)methyl]benzamide **4a-2** (0.23 mmol). An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI) 350 [M+H]+.

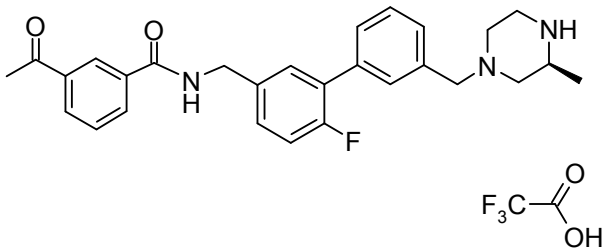
DMHB resin-bound 3-acetyl-*N*-[(6-fluoro-3'-formyl-3-biphenylyl)methyl]benzamide (**4a-3**, step c)



4a-3

To DMHB resin-bound 3-acetyl-*N*-[(3-bromo-4-fluorophenyl)methyl]benzamide **4a-2** (200 mg, 1.00 mmol/g, 0.20 mmol) in 5 mL DME was added 3-formylphenyl boronic acid (90 mg, 0.60 mmol), 2 M Cs_2CO_3 aqueous solution (0.3 mL, 0.60 mmol), and $Pd(PPh_3)_4$ (46 mg, 0.04 mmol). After being purged with argon for 5-10 min, the mixture was heated at 80 °C under argon for 16 h. The resulting resin was washed with THF (20 mL x 2), THF:H_2O (1:1, 20 mL x 2), H_2O (20 mL x 2), THF:H_2O (1:1, 20 mL x 2), THF (20 mL x 2), DCM (20 mL x 2), and dried in a vacuum oven at 20 °C overnight to yield DMHB resin-bound 3-acetyl-*N*-[(6-fluoro-3'-formyl-3-biphenylyl)methyl]benzamide **4a-3** (0.20 mmol). An analytical amount of the resin was cleaved with 50% of TFA in DCM for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 376 [M+H]+.

3-acetyl-*N*-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl] benzamide trifluoroacetate (**4a**, steps d, e)



4a

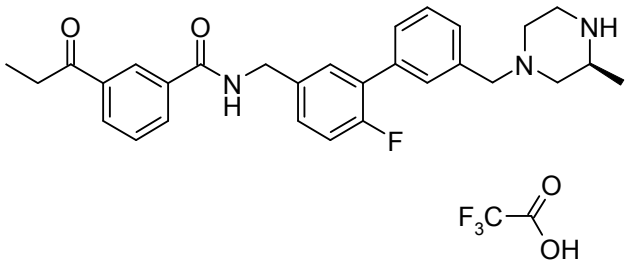
To DMHB resin-bound 3-acetyl-*N*-[(6-fluoro-3'-formyl-3-biphenylyl)methyl]benzamide **4a-3** (200 mg, 0.97 mmol/g, 0.194 mmol) in 17 mL of DCE was added Na_2SO_4 (138 mg, 0.97 mmol) and (*S*)-2-methylpiperazine (97 mg, 0.97 mmol). After shaking for 10 min, $Na(OAc)_3BH$ (246 mg, 1.16 mmol) was added. After being shaken at rt for overnight, the resin was washed with THF (20 mL x 2),

THF:H_2O (1:1, 20 mL x 2), H_2O (20 mL x 2), THF:H_2O (1:1, 20 mL x 2), THF (20 mL x 2), DCM (20 mL x 2) and dried in a vacuum oven at 20 °C overnight. The resulting resin was cleaved with 5 mL of 50% of TFA in DCE for 30 min and treated again with 5 mL of 50% of TFA in DCE for 30 min. The combined cleavage solution was concentrated *in vacuo*. The residue was dissolved in DMSO and purified using a Gilson semi-preparative HPLC system with a YMC ODS-A (C-18) column 50 mm by 30 mm ID, eluting with 10% B to 90% B in 3.2 min, hold for 1 min where A = H_2O (0.1% trifluoroacetic acid) and B = CH_3CN (0.1% trifluoroacetic acid) pumped at 25 mL/min, to produce 3-acetyl-*N*-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl] benzamide trifluoroacetate **4a** as a white powder (51 mg, 46% over 4 steps). MS (ESI): 460 [M+H]+.

^{1}H NMR (600 MHz, DMSO-*d*6) δ ppm 9.31 (t, *J*=5.7 Hz, 1 H), 8.81 (br s, 1 H), 8.44 (s, 1 H), 8.36 (br s, 1 H), 8.13 (dd, *J*=7.9, 7.9 Hz, 2 H), 7.64 (dd, *J*=7.7, 7.7 Hz, 1 H), 7.57– 7.42 (m, 4 H), 7.42 – 7.33 (m, 2 H), 7.29 (m, 1 H), 4.55 (d, *J*=5.7 Hz, 2 H), 3.68 (m, 2 H), 3.28 (m, 2 H), 3.02 (m, 1 H), 2.92 (m, 2 H), 2.63 (s, 3 H), 2.31 (m, 1 H), 2.13 (m, 1 H), 1.16 (d, *J*=6.4 Hz, 3 H). HPLC: 100%, RT 1.64 min. HRMS (ESI+) calcd for [C28H30N3O2 + H]+: 460.2395. Found: 460.2395.

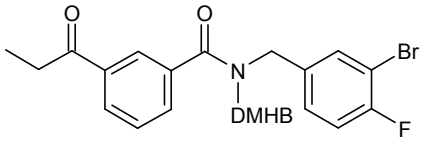
Synthesis of 4b

<u>*N*-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl]-3-propanoyl benzamide trifluoroacetate (4b)</u>



4b

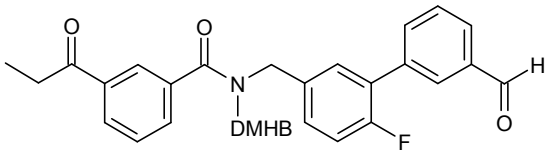
<u>*N*-[(3-bromo-4-fluorophenyl)methyl]-3-propanoylbenzamide (**4b-2**, step b)</u>



4b-2

Preparation is exactly analogous to the preparation of **4a-2**, starting with resin bound 3-bromo-4-fluoro-benzylamine **4a-1** and substituting 3-propanoylbenzoic acid for 3-acetyl benzoic acid. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 364 [M+H]+.

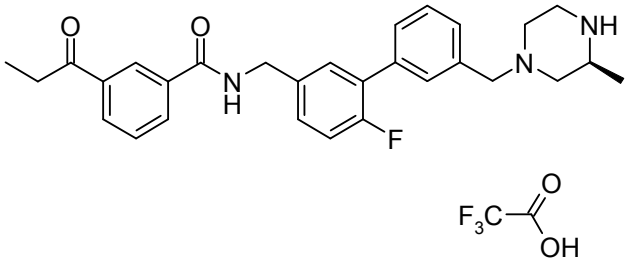
N-[(6-fluoro-3'-formyl-3-biphenylyl)methyl]-3-propanoylbenzamide (**4b-3**, step c)



4b-3

Preparation is exactly analogous to the preparation of **4a-3**, substituting **4b-2** for **4a-2**. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 390 [M+H]+.

N-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl]-3-propanoyl benzamide trifluoroacetate (**4b,** steps d, e)



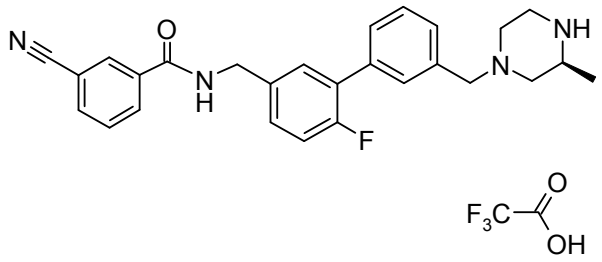
4b

Preparation is exactly analogous to the preparation of **4a**, substituting **4b-3** for **4a-3** (56%).

^{1}H NMR (600 MHz, DMSO-*d*6) δ ppm 9.30 (t, *J*=5.7 Hz, 1 H), 8.78 (br s, 1 H), 8.45 (s, 1 H), 8.33 (br s, 1 H), 8.12 (dd, *J*=1.7, 7.7 Hz, 2 H), 7.64 (dd, *J*=7.7, 7.7 Hz, 1 H), 7.56 – 7.42 (m, 4 H), 7.42 – 7.33 (m, 2 H), 7.29 (m, 1 H), 4.54 (d, *J*=5.7 Hz, 2 H), 3.65 (m, 2 H), 3.26 (m, 2 H), 3.10 (q, *J*=7.18 Hz, 2 H), 3.01 (m, 1 H), 2.90 (m, 2 H), 2.29 (m, 1 H), 2.11 (m, 1 H), 1.15 (d, *J*=6.4 Hz, 3 H), 1.10 (t, *J*=7.18 Hz, 3 H). HPLC: 100%, RT 2.20 min. HRMS (ESI+) calcd for [C29H32FN3O2 + H]+: 474.2551. Found: 474.2552.

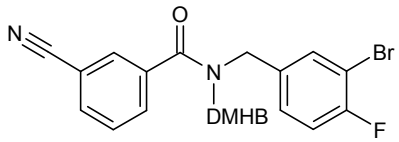
Synthesis of 4c

3-cyano-*N*-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl] benzamide trifluoroacetate (4c)



4c

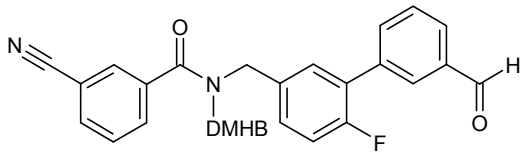
DMHB resin-bound 3-cyano-*N*-[(3-bromo-4-fluorophenyl)methyl]benzamide (**4c-2**, step b)



4c-2

Preparation is exactly analogous to the preparation of **4a-2 st**arting with resin bound 3-bromo-4-fluoro-benzylamine **4a-1** and substituting 3-cyano benzoic acid for 3-acetyl benzoic acid. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 333 [M+H]+.

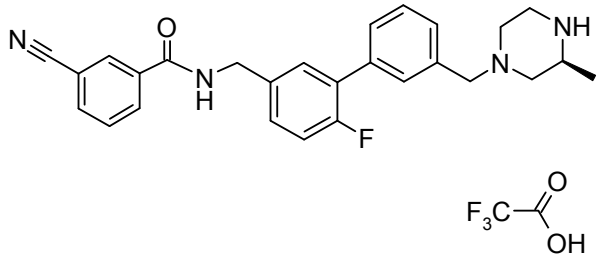
DMHB resin-bound 3-cyano-*N*-[(6-fluoro-3'-formyl-3-biphenylyl)methyl]benzamide (**4c-3**, step c)



4c-3

Preparation is exactly analogous to the preparation of **4a-3**, substituting **4c-2** for **4a-2**. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 359 [M+H]+.

3-cyano-*N*-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl] benzamide trifluoroacetate (**4c**, steps d and e)



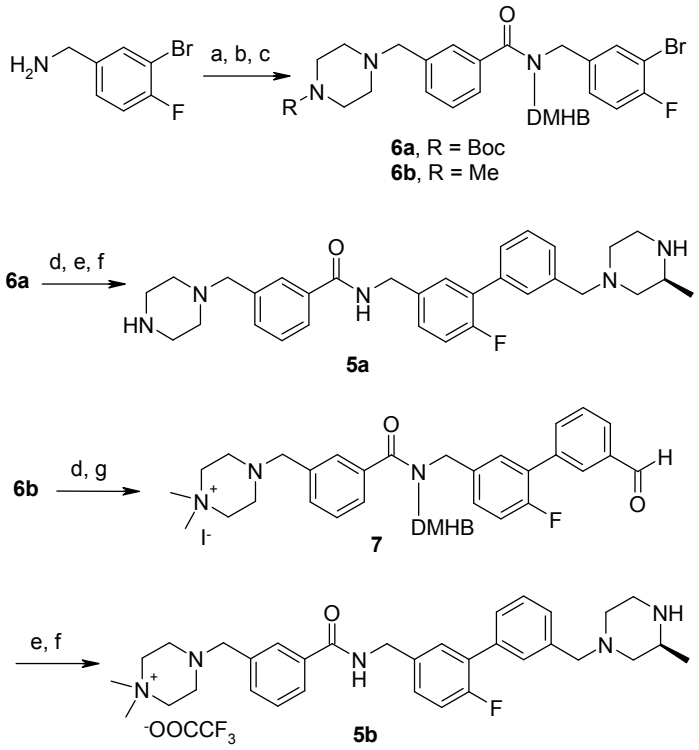
4c

Preparation is exactly analogous to the preparation of **4a**, substituting **4c-3** for **4a-3** (63%).

^{1}H NMR (600 MHz, DMSO-*d*6) δ ppm 9.29 (t, *J*=5.7 Hz, 1 H), 8.79 (br s, 1 H), 8.41 – 8.26 (m, 2 H), 8.19 (d, *J*=7.9 Hz, 1 H), 8.03 (d, *J*=7.6 Hz, 1 H), 7.71 (dd, *J*=7.9, 7.9 Hz, 1 H), 7.55 – 7.42 (m, 4 H), 7.42 – 7.33 (m, 2 H), 7.29 (m, 1 H), 4.53 (d, *J*=5.7 Hz, 2 H), 3.66 (m, 2 H), 3.27 (m, 2 H), 3.02 (m, 1 H), 2.91 (m, 2 H), 2.30 (m, 1 H), 2.11 (m, 1 H), 1.16 (d, *J*=6.4 Hz, 3 H). HPLC: 100%, RT 1.66 min. HRMS (ESI+) calcd for [C27H27FN4O + H]+: 443.2242. Found: 443.2242.

Scheme 2.

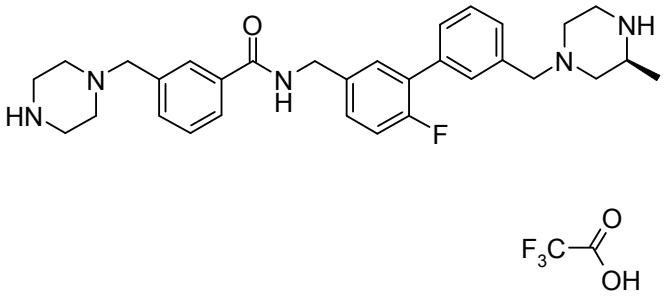
Syntheses of **5a** and **5b**



(a) 2,6-dimethoxy-4-polystyrenebenzyloxybenzaldehyde (DMHB-resin), $Na(OAc)_3BH$, DIEA, 10% of HOAc in NMP, rt; (b) 3-formyl benzoic acid, DIC, DCE/DMF 1:1, rt; (c) *N*-Boc piperazine or *N*-methyl piperazine, $Na(OAc)_3BH$, Na_2SO_4, DCE, rt; (d) 3-formylphenyl boronic acid, $Pd(PPh_3)_4$, 2 M Cs_2CO_3, DME, 80 °C; (e) (2*S*)-2-methylpiperazine, $Na(OAc)_3BH$, Na_2SO_4, DCE, rt; (f) 50% of TFA in DCE, rt; (g) MeI, CH_3CN, rt.

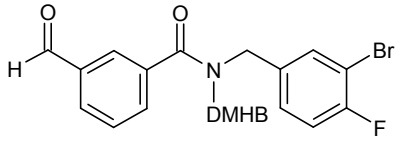
Synthesis of 5a

***N*-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl]-3-(1-piperazinylmethyl)benzamide bistrifluoroacetate (5a)**



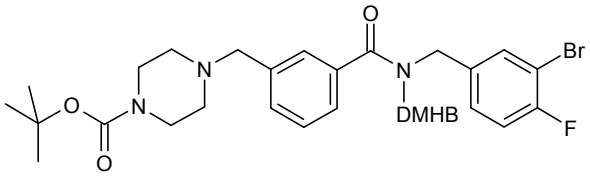
5a

DMHB resin-bound *N*-[(3-bromo-4-fluorophenyl)methyl]-3-formylbenzamide (step b)



To a mixture of DMHB resin-bound 3-bromo-4-fluoro-benzylamine (intermediate **4a-1** from Synthesis of 4a) (200 mg, 1.17 mmol/g (theoretical loading), 0.23 mmol) in DCE/DMF (1:1, 8 mL) was added 3-formylbenzoic acid (345 mg, 2.3 mmol) and DIC (0.36 mL, 2.3 mmol). The mixture was shaken at rt for overnight and was then washed with DMF (20 mL x 2), DCM (20 mL x 2), MeOH (20 mL x 2) and DCM (20 mL x 2). The resulting resin was dried in vacuum oven at 20 °C for overnight to yield DMHB resin-bound *N*-[(3-bromo-4-fluorophenyl)methyl]-3-formylbenzamide (0.23 mmol). An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 336 [M+H]+.

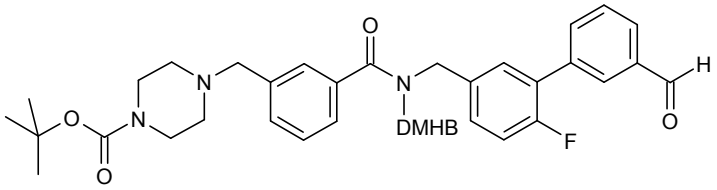
DMHB resin-bound 1,1-dimethylethyl 4-{[3-({[(3-bromo-4-fluorophenyl)methyl]amino} carbonyl)phenyl]methyl}-1-piperazinecarboxylate (**6a,** step c)



6a

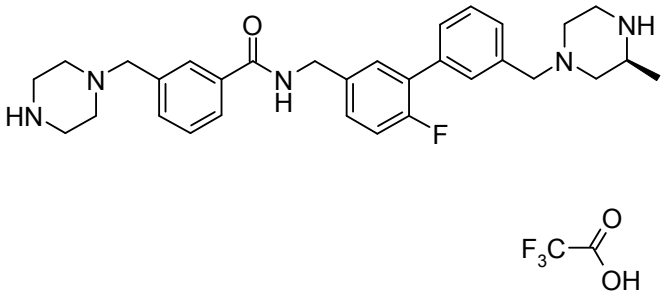
To the above obtained DMHB resin-bound *N*-[(3-bromo-4-fluorophenyl)methyl]-3-formylbenzamide (200 mg, 0.86 mmol/g, 0.172 mmol) in 17 mL of DCE was added Na_2SO_4 (122 mg, 0.86 mmol) and N-boc piperazine (160 mg, 0.86 mmol). After shaking for 10 min, $Na(OAc)_3BH$ (218 mg, 1.03 mmol) was added. After being shaken at rt for overnight, the resin was washed with THF (20 mL x 2), THF:H_2O (1:1, 20 mL x 2), H_2O (20 mL x 2), THF:H_2O (1:1, 20 mL x 2), THF (20 mL x 2), DCM (20 mL x 2) and dried in a vacuum oven at 20 °C overnight. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 406 [M+H-Boc]+.

DMHB resin-bound 1,1-dimethylethyl 4-{[3-({[(6-fluoro-3'-formyl-3-biphenylyl)methyl]amino} carbonyl)phenyl]methyl}-1-piperazinecarboxylate (step d)



Preparation is exactly analogous to the preparation of **4a-3** in Synthesis of 4a, substituting **6a** for **4a-3**. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 432 [M+H-Boc]+.

N-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl]-3-(1-piperazinylmethyl)benzamide bistrifluoroacetate (**5a,** steps e and f)



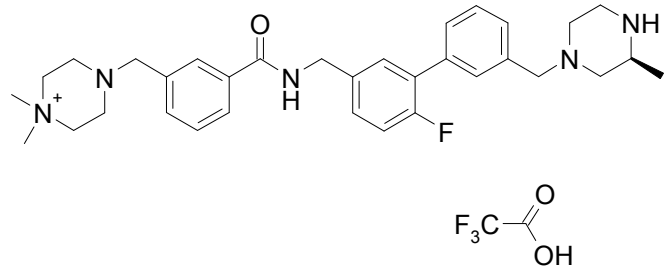
5a

Preparation is exactly analogous to the preparation of **4a** in Scheme 1, substituting the above obtained DMHB resin-bound 1,1-dimethylethyl 4-{[3-({[(6-fluoro-3'-formyl-3-biphenylyl) methyl]amino} carbonyl)phenyl]methyl}-1-piperazinecarboxylate for **4a-3** (61%).

^{1}H NMR (600 MHz, DMSO-*d*6) δ ppm 9.11 (t, *J*=6.0 Hz, 1 H), 8.87 (br s, 1 H), 8.60 (br s, 2 H), 8.41 (br s, 1 H), 7.90 – 7.76 (m, 2 H), 7.59 - 7.42 (m, 6 H), 7.42 – 7.33 (m, 2 H), 7.28 (m, 1 H), 4.51 (d, *J*=6.0 Hz, 2 H), 3.67 (m, 4 H), 3.28 (m, 2 H), 3.11 (m, 4 H), 3.01 (m, 1 H), 2.93 (m, 2 H), 2.61 (m, 4 H), 2.33 (m, 1 H), 2.15 (m, 1 H), 1.16 (d, *J*=6.8 Hz, 3 H). HPLC: 100%, RT 0.46 min. HRMS (ESI+) calcd for [C31H38FN5O + H]+: 516.3133. Found: 516.3134.

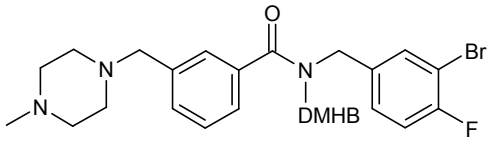
Synthesis of 5b

4-{[3-({[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl]amino} carbonyl)phenyl]methyl}-1,1-dimethylpiperazin-1-ium bistrifluoroacetate (5b)



5b

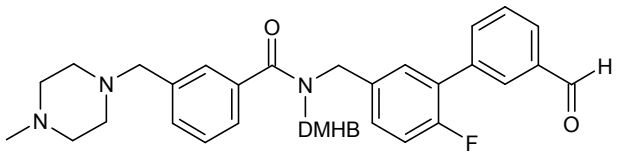
DMHB resin-bound *N*-[(3-bromo-4-fluorophenyl)methyl]-3-[(4-methyl-1-piperazinyl) methyl]benzamide (**6b**, step c)



6b

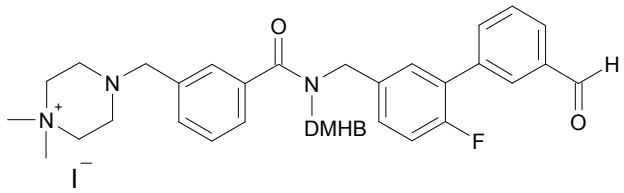
6b is prepared from DMHB resin-bound *N*-[(3-bromo-4-fluorophenyl)methyl]-3-formylbenzamide (obtained in step b in synthesis of 5a above) using the same procedure as the preparation of **6a**, by substituting N-methyl piperazine for N-boc piperazine. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 420 [M+H]+.

DMHB resin-bound *N*-[(6-fluoro-3'-formyl-3-biphenylyl)methyl]-3-[(4-methyl-1-piperazinyl) methyl]benzamide (step d)



Preparation is exactly analogous to the preparation of **4a-3** in Scheme 1, substituting **6b** for **4a-3**. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 446 [M+H]+.

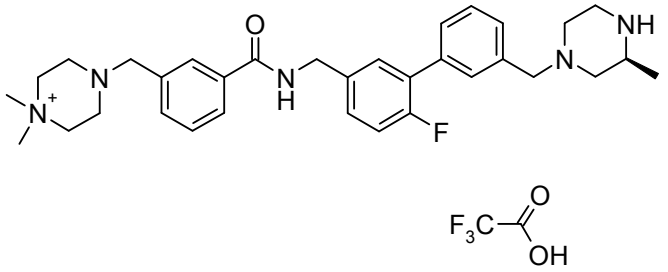
DMHB resin-bound 4-{[3-({[(6-fluoro-3'-formyl-3-biphenylyl)methyl]amino}carbonyl)phenyl] methyl}-1,1-dimethylpiperazin-1-ium iodide (**7**, step g)



7

The above obtained DMHB resin-bound *N*-[(6-fluoro-3'-formyl-3-biphenylyl)methyl]-3-[(4-methyl-1-piperazinyl) methyl]benzamide (200 mg, 0.91 mmol/g, 0.182 mmol) was mixed with CH_3CN (8 mL) and methyl iodide (0.113 mL, 1.82 mmol) was added. After being shaken at rt for overnight, the resin was washed with CH_3CN (20 mL x 2), (DCM, MeOH, 20 mL each wash) x 3, DCM (20 mL x 2) and dried in a vacuum oven at 20 °C overnight. An analytical amount of the resin was cleaved with 50% of TFA in DCE for 10 min. The resulting solution was concentrated *in vacuo* and dissolved in 0.5 mL of CH_3CN. MS (ESI): 460 [M]+.

4-{[3-({[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl]amino} carbonyl)phenyl]methyl}-1,1-dimethylpiperazin-1-ium bistrifluoroacetate (**5b**, steps e and f)



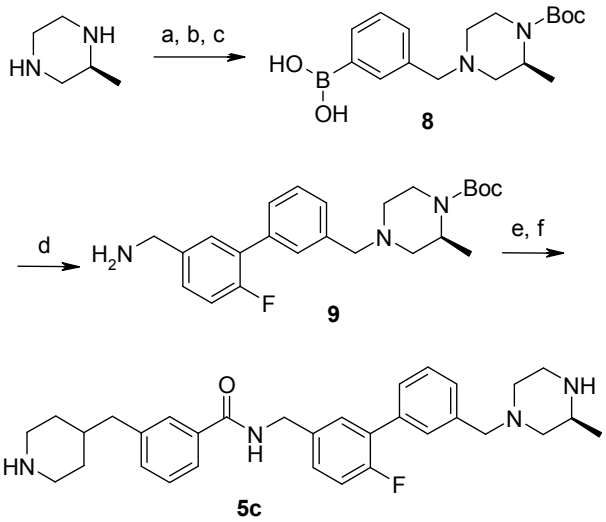
5b

Preparation is exactly analogous to the preparation of **4a** in Scheme 1, substituting **7** for **4a-3** (54%).

^{1}H NMR (600 MHz, DMSO-*d*6) δ ppm 9.11 (t, *J*=5.7 Hz, 1 H), 8.83 (br s, 1 H), 8.37 (br s, 1 H), 7.87 – 7.74 (m, 2 H), 7.54 - 7.41 (m, 6 H), 7.40 – 7.32 (m, 2 H), 7.28 (m, 1 H), 4.51 (d, *J*=5.7 Hz, 2 H), 3.71 – 3.61 (m, 4 H), 3.38 (m, 4 H), 3.25 (m, 2 H), 3.11 (s, 6 H), 3.01 (m, 1 H), 2.88 (m, 2 H), 2.71 (m, 4 H), 2.28 (m, 1 H), 2.09 (m, 1 H), 1.16 (d, *J*=6.4 Hz, 3 H). HPLC: 100%, RT 1.39 min. HRMS (ESI+) calcd for [C33H43FN5O]+: 544.3452. Found: 544.3452.

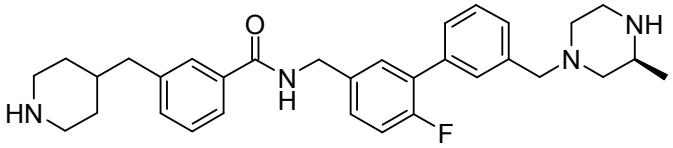
Scheme 3.

Synthesis of **5c**



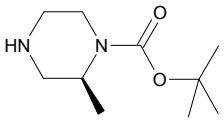
(a) *n*-BuLi, TBDMSCl, $(Boc)_2O$, THF, rt; (b) 3-bromobenzaldehyde, $Na(OAc)_3BH$, DCM, rt; (c) *n*-BuLi, $B(OMe)_3$, THF, -78 °C - rt; (d) [(3-bromo-4-fluorophenyl)methyl]amine hydrochloride, $Pd(PPh_3)_4$, 2 M K_2CO_3, dioxane, H_2O, 150 °C; (e) 3-[(1-Boc-piperidin-4-yl)methyl]benzoic acid, EDC, HOBt, DIEA, $CHCl_3$, rt; (f) TFA, DCM, 0 °C – rt.

<u>*N*-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl]-3-(4-piperidinylmethyl)benzamide (5c)</u>



5c

<u>1,1-dimethylethyl (2*S*)-2-methyl-1-piperazinecarboxylate (step a)</u>

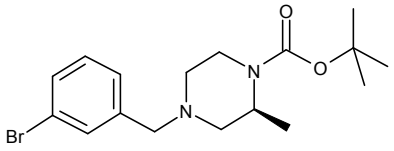


A solution of (S)-2-methyl piperazine (2 g, 20 mmol) in THF (200 mL) was mixed with *n*-BuLi (25 mL, 1.6 M in hexanes, 40 mmol) at rt. The solution was stirred for 30 min before TBDMSCl (3.04 g, 20 mmol) was added. The mixture was stirred for an additional hour and $(Boc)_2O$ (5.2 g, 24 mmol) was added to the solution. The resulting mixture was stirred for another hour and diluted with H_2O (50 mL).

The organic layer was separated, washed with brine (50 mL), dried over Na_2SO_4 and concentrated under vacuum. Flash chromatography on silica (5% MeOH / 2% NH_4OH / 93% CH_2Cl_2) provided the title compound as a yellow oil (3.7 g, 93%).

MS (ESI): 201 [M+H]+. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 4.2 (m, 1 H). 3.8 (m, 1 H), 3.0 (m, 3 H), 2.85 (m, 1 H), 2.7 (m, 1 H), 2.1 (m, 1 H), 1.49 (s, 9 H), 1.26 (d, *J*=6.8 Hz, 3 H).

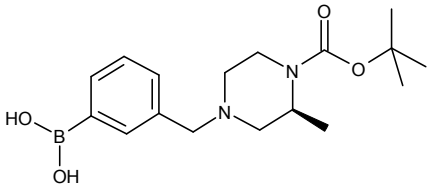
1,1-dimethylethyl (2*S*)-4-[(3-bromophenyl)methyl]-2-methyl-1-piperazine carboxylate (step b)



A solution of 1,1-dimethylethyl (2*S*)-2-methyl-1-piperazinecarboxylate (100 mg, 0.5 mmol) in CH_2Cl_2 (5 mL) was mixed with 3-bromo benzaldehyde (0.06 mL, 0.5 mmol) and $Na(OAc)_3BH$ (0.16 g, 0.75 mmol). The resulting mixture was stirred for 12 hours, diluted with dichloromethane (30 mL) and washed with brine (50 mL). The organic layer was collected, dried over Na_2SO_4 and concentrated under vacuum. The crude material was further purified by flash chromatography to give the title compound (150 mg, 81%).

MS (ESI): 369 [M+H]+. HPLC: 100%. ^{1}H NMR (400 MHz, MeOD) δ ppm 7.55 (s, 1 H), 7.4 (m, 1 H), 7.3 (m, 1 H), 7.25 (m, 1 H), 4.88 (m, 1 H), 4.2 (m, 1 H), 3.8 (m, 1 H), 3.5 (m, 1 H), 3.4 (m, 1 H), 3.3 (s, 2 H), 3.1 (m, 1 H), 2.8 (m, 1 H), 2.6 (m, 1 H), 2.1 (m, 1 H), 2.0 (m, 1 H), 1.47 (s, 9 H), 1.26 (d, *J*=6.8 Hz, 3 H).

{3-[((3*S*)-4-{[(1,1-dimethylethyl)oxy]carbonyl}-3-methyl-1-piperazinyl)methyl]phenyl} boronic acid (**8**, step c)

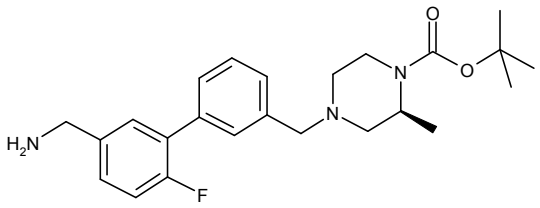


8

A solution of 1,1-dimethylethyl (2S)-4-[(3-bromophenyl)methyl]-2-methyl-1-piperazine carboxylate (1.8 g, 4.9 mmol) in THF (4.9 mL) was mixed with *n*-BuLi (3.7 mL, 1.6 M in Hexane, 5.9 mmol) at -78 °C and stirred for 30 min before $B(OMe)_3$ (2.2 mL, 19.6 mmol) was added. After addition, the resulting solution was allowed to warm to room temperature over 2 hours. The mixture was then mixed with saturated aqueous NH_4Cl solution (10 mL), stirred for 25 minutes at room temperature, diluted with H_2O (5 mL) and extracted with Et_2O (2 X 30 mL). The organic layers were collected, dried over Na_2SO_4, filtered and concentrated to afford the crude title compound (1.7 g, quantitative yield).

MS (ESI): 335 [M+H]+. HPLC: 100%. ^{1}H NMR (400 MHz, MeOD) δ ppm 7.59 (s, 1 H), 7.51 (m, 1 H), 7.38 (m, 1 H), 7.33 (m, 1 H), 4.18 (m, 1 H), 3.80 (m, 1 H), 3.56 (m, 1 H), 3.44 (m, 1 H), 3.12 (m, 1 H), 2.82 (m, 1 H), 2.68 (m, 1 H), 2.13 (m, 1 H), 2.00 (m, 1 H), 1.46 (s, 9 H), 1.24 (d, *J*=6.8 Hz, 3 H).

1,1-dimethylethyl(2*S*)-4-{[5'-(aminomethyl)-2'-fluoro-3-biphenylyl]methyl}-2-methyl-1-piperazinecarboxylate (**9**, step d)

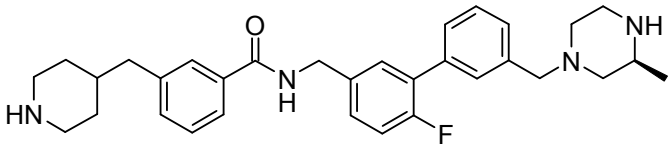


9

To a solution of [(3-bromo-4-fluorophenyl)methyl]amine hydrochloride (1.68 g, 7 mmol) in dioxane/H_2O (10 mL/3.3 mL) was added {3-[((3*S*)-4-{[(1,1-dimethylethyl)oxy]carbonyl}-3-methyl-1-piperazinyl)methyl]phenyl} boronic acid **8** (2.33 g, 7 mmol), K_2CO_3 (4.83, 35 mmol) and $Pd(PPh_3)_4$ (405 mg, 0.35 mmol). The resulting mixture was heated at 150 °C in a pressure vessel for 2 hours, then cooled to rt and diluted with EtOAc (50 mL). The organic layer was collected and the aqueous layer was extracted by EtOAc (30 mL). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The residue was purified by Gilson preparatory HPLC, eluting with acetonitrile/water/0.1%TFA (10/90 to 90/10, v/v,

over 12 min), to give the title compound (1.08 g, 37%). LC/MS: m/z, 414 (M+H), 1.83 min. HPLC: 100%

N-[(6-fluoro-3'-{[(3*S*)-3-methyl-1-piperazinyl]methyl}-3-biphenylyl)methyl]-3-(4-piperidinylmethyl)benzamide (**5c**, steps e and f)



5c

To a solution of 1,1-dimethylethyl(2*S*)-4-{[5'-(aminomethyl)-2'-fluoro-3-biphenylyl] methyl}-2-methyl-1-piperazinecarboxylate **9** (25 mg, 0.06 mmol) in $CHCl_3$ (5 mL) was added 3-[(1-{[(1,1-dimethylethyl)oxy]carbonyl}-4-piperidinyl)methyl]benzoic acid (28.7 mg, 0.09 mmol), EDC (12 mg, 0.06 mmol), HOBt (1 mg, 0.006 mmol) and diisopropyl ethylamine (0.1 mL). The resulting mixture was stirred for 12 hours at rt, concentrated and purified by Combiflash to provide the BOC protected product. The residue was dissolved in CH_2Cl_2 (2 mL) and the resulting solution was mixed with TFA (0.7 mL) at 0 °C. After stirring at ambient temperature for 12 hours, Et_3N (0.1 mL) was added to the reaction mixture at -78 °C. After removal of the solvent under vacuum, the residue was purified by Gilson reverse phase HPLC, eluting with acetonitrile/water/0.1%TFA (10/90 to 70/30, v/v, over 12 min), to give **5c** (20 mg, 93%) as a TFA salt.

MS (ESI): 515 [M+H]+. ^{1}H NMR (400 MHz, MeOD) δ ppm 7.73 (m, 3 H) 7.65 (d, *J*=7.5 Hz, 1 H), 7.55 (m, 3 H), 7.43 (m, 3H), 7.20 (t, *J*=8.5 Hz, 1 H), 4.62 (s, 2 H), 4.30 (s, 2H), 3.60 (m, 4 H), 3.38 (m, 4 H), 3.13 (m, 1 H), 2.98 (m, 3 H), 2.69 (d, *J*=7.1 Hz, 2 H), 1.93 (m, 3 H), 1.45 (m, 2 H), 1.4 (d, *J*=8.6 Hz, 3 H). HPLC: 100%, RT 1.90 min. HRMS (ESI+) calcd for [C32H39FN4O + H]+: 515.3181 Found: 515.3184

M_3, M_2 and M_1 FLIPR Assays

Cell Source:

The human M_1-M_3 receptors were cloned and stably expressed in Chinese Hamster Ovary (CHO) cells. The M_2 receptors were co-expressed with the chimeric G protein, Gqi5.

Method of Culture:

CHO-M_1, CHO-Gqi5-M_2 and CHO-M_3 cells were cultured to confluence at 37 °C in a humidified incubator with 5% CO_2/95% air. CHO-M_1 and CHO-M_3 were cultured in Alpha MEM with nucleosides and L-glutamine and 10% fetal calf serum. Cells expressing the M_2 receptor + Gqi5 were cultured in DMEM/F12 media, supplemented with 200 mg/L G418 (geneticin), and 10% fetal calf serum.

Experimental Protocols:

Cell plating and dye loading

A microtiter plate based calcium mobilization Fluorometric Imaging Plate Reader (FLIPR) assay was used for the functional characterization of compounds against M_1, M_2 (w/Gqi5) and M_3 acetylcholine receptors stably expressed in CHO cells.

On the day prior to assay, cells were plated in 96-well, blackwall, clear bottom plates (Packard View) at a concentration of 40000 cells per well. After 18 to 24 hours, media was aspirated and replaced with 100 μL load media containing Eagles Minimal Essential Medium (EMEM) with Earl's salts and L-Glutamine, 0.1% BSA (Seriologicals Corporation), 4 μM Fluo-4-acetoxymethyl ester fluorescent indicator dye (Fluo-4 AM, Molecular Probes, Eugene, OR) and 2.5 mM probenecid. Cells are then incubated for 1 hour at 37 °C.

The dye containing media was then aspirated off the cells and replaced with identical media without Fluo-4 AM and with 0.1% Gelatin (BSA removed) and 2.5 mM probenecid. Cells were incubated for 10 minutes at 37 °C and then washed 3 times with KRH assay buffer [Krebs Ringer Henseleit (120 mM NaCl, 4.6 mM KCl, 1.03 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.0 mM $CaCl_2$, 1.1 mM $MgCl_2$, 11 mM Glucose, 20 mM HEPES (pH 7.4)) with 0.1% gelatin and 2.5 mM probenecid].

Receptor agonist characterization (EC_{50} determination), compounds tested for agonist potential at muscarinic receptors

To evaluate agonist potential of compounds and acetylcholine (ACh) potency for the M_1, M_2 and M_3 receptors, 100 μL KRH assay buffer with 0.1% gelatin and 2.5 mM probenecid was added to wells of dye loaded and washed cells and plate warmed to 37 °C for 10 minutes before being placed in FLIPR where dye loaded cells are exposed to excitation light (488 nm) from a 6 watt

Argon Laser. The basal emission fluorescence was measured, then the cellular response to a concentration range of ACh or compound (50 μL of 3X in assay buffer), was monitored in FLIPR for 90 seconds and then 50 μL of 400 μM ATP (assay concentration of 100 μM) was added to check cell viability. The EC_{50} is the ACH or compound concentration required to obtain 50% the maximal response.

Calcium mobilization, monitored as change in cytosolic calcium concentration, is measured as change in 516 nm emission fluorescence intensity, the change in intensity being directly related to cytosolic calcium levels. The emitted fluorescence from all 96 wells is measured simultaneously using a cooled CCD camera. Data points are collected every second. Maximal change in emission from each well after simultaneous addition of agonist or compound to each of the 96 wells is then exported to an excel spreadsheet. This data is then transferred to GraphPad Prism Version 4.03 for plotting of response to each treatment condition (ACh or compound).

Receptor antagonist characterization: IC_{50} determination

To evaluate antagonist potency of compounds against the M_1, M_2 and M_3 receptors, 100 μL KRH assay buffer with 0.1% gelatin and 2.5 mM probenecid was added to wells of dye loaded and washed cells followed by 50 μL of 3X compound ($1x10^{-8}$ – $3.3x10^{-5}$ M final in the assay) and plate warmed to 37 °C for 10 minutes before being placed in FLIPR where the dye loaded, compound pretreated cells are exposed to excitation light (488 nm) from a 6 watt Argon Laser. The basal emission fluorescence was measured, then the cellular response to an EC_{80} concentration of ACh (3.3 nM against M_1, 10 nM against M_2 and 1.0 nM against M_3) prepared in KRH assay buffer with 0.1% BSA (no gelatin) was monitored in FLIPR for 90 seconds and then 50 μL of 500 μM ATP (assay concentration of 100 μM) was added to check cell viability. Maximal change in emission from each well after simultaneous addition of ACh to each of the 96 wells is then exported to an excel spreadsheet. This data is then transferred to GraphPad Prism Version 4.03 for plotting of response after each treatment condition (compound pretreatment followed by ACh). The IC_{50} is defined as the compound pretreatment concentration which inhibits 50% of the ACh induced response.

Single concentration kinetic characterization of compounds: pA_2 determination

Compounds which show IC_{50}'s of < 1.0 μM, are further characterized in a single compound concentration kinetic assay. To confirm antagonist potency of more potent compounds against the M_1, M_2 and M_3 receptors, dye loaded and washed cells (column of 12 wells) were treated with

150 μL of KRH assay buffer with 0.1% gelatin and 2.5 mM probenecid containing vehicle (0.01% DMSO), for control response, or appropriate concentration of antagonist (single concentration for each column determined from IC_{50} value) and incubated for 20 minutes at 37 °C. Buffer was aspirated off and 150 μL of fresh KRH assay buffer with 0.1% gelatin and 2.5 mM probenecid containing vehicle (0.01% DMSO) or appropriate concentration of compound was added and incubated for 10 minutes at 37 °C. Plates were then placed into FLIPR for fluorescent measurements. After determination of basal fluorescence emission, a concentration range of ACh (0.033-100,000 nM for M_1/M_3 and 0.33-1,000,000 nM for M_2) was added to vehicle or compound treated cells to determine the shift of receptor potency in response to ACh in presence of compound. Compound potency at the receptor is determined using the following formula: $pA_2=\log(DR-1) - \log[B]$ where DR is the dose ratio defined as the ratio of equiactive concentration (EC_{50}) of agonist in presence and absence of antagonist and [B] is concentration of antagonist.

Kinetic studies for SCHILD analysis (30 minutes pretreatment) of antagonist: pK_B determination

After aspirating off the dye containing media, the cells were washed 3 times with 100 μL of KRH assay buffer with 0.1% gelatin, and 2.5 mM probenecid. Cells (columns of 12 wells) were treated with 150 μL of KRH assay buffer with 0.1% gelatin and 2.5 mM probenecid containing vehicle (0.01% DMSO), for control response, or appropriate concentration of antagonist (7 concentrations of antagonist). Cells were incubated for 20 minutes at 37 °C. Buffer was aspirated off and 150 μL of fresh buffer with 0.1% gelatin and 2.5 mM probenecid containing vehicle (0.01% DMSO) or appropriate concentration of compound added and incubated for 10 minutes at 37 °C. Plates were placed into FLIPR for fluorescent measurements. A concentration range of ACh is then added to vehicle or compound treated cells to allow a determination of shift of receptor affinity response to ACh in presence of compound. Compound potency and type of receptor interaction is determined using classical Schild analysis.

M_3 Radioligand Binding Assay

The human M_3 receptor was cloned and stably expressed in Chinese Hamster Ovary (CHO) cell lines. Competition for [^{3}H]-N-methyl scopolamine (0.5 nM) binding was performed using crude CHO cell membranes using a Scintillation Proximity Assay (SPA). Atropine was run in every assay as the control.

In the SPA assay membranes were preincubated with wheatgerm agglutinin beads (GE) in 50 mM HEPES buffer (Sigma, St. Louis MO) (pH 7.4) at 4 °C for 30 min, then incubated with 0.5 nM [^{3}H]-N-methyl scopolamine (PerkinElmer) in a 96-well Optiplate (Perkin Elmer), for 2 hr in the presence of vehicle (1% DMSO) or compound (0.01-1000 nM), in 0.2 mL final volume, at room temperature. At the end of the incubation the plates were centrifuged (Beckman CS-6R) for 5 min at 2000 RPM, and counted in a Top Count Microplate Scintillation counter (model A9912 Packard, Meriden CT).

Concentration-response curves for each compound were run using duplicate samples in 3 independent experiments. Specific binding was determined by subtracting non-specific binding (defined in the presence of 0.3 μM Atropine) from total binding. IC_{50} values were estimated from concentration-response curves and used to determine the inhibition constant (K_i) of each inhibitor using the Cheng and Prusoff equation for competitive antagonists. The K_d utilized for the calculation was 0.16 nM for M_3.

$$K_i = \frac{IC_{50}}{[L]/K_d + 1}$$

Membrane Preparation

Cells were harvested by centrifugation at 1000 x g for 10 min at 4 °C. The cell pellet was washed with Phosphate Buffered Saline (PBS) and quick frozen with liquid nitrogen. The pellet was stored at -80 °C until the membrane preparation was made. The frozen pellet was thawed and resuspended in cold hypotonic membrane buffer (40 mM Tris, pH 7.5, 1 mM $MgSO_4$, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2.5 mg/L leupeptin, 0.1 mg/mL aprotinin) and incubated on ice for 5 min. The cell suspension was homogenized in a 40 mL Dounce homogenizer and centrifuged at 2000 rpm at 4 °C for 6 min to remove nuclei and cellular debris. The 2000 rpm pellet was resuspended in homogenization buffer and spun again at 2000 rpm for 6 min. This process was repeated two more times. The combined supernatant was collected and cell membranes were pelleted at 100000 x g for 1 hr at 4 °C. The membrane pellet was resuspended in membrane buffer and aliquots stored at -80 °C. Protein concentration was quantified using the Bio-Rad protein assay reagent.

Methacholine-induced bronchoconstriction model in conscious mice

Male Balb/c mice from Charles River Breeding Laboratories were used and maintained in a barrier-sustained facility. Age-matched mice were used throughout the studies in the weight range from 23-25 grams in groups of 4 mice per group**.** The mice were allowed free access to food and water during the entire length of the experiment. All procedures were performed in accordance with protocols approved by the Animal Care and Use Committee at GlaxoSmithKline Pharmaceuticals (ACUC #02-222) and met or exceeded the standards of the American Association for the Accreditation of Laboratory Animal Care (AAALAC), the United states Department of Health and Human Services and all local and federal animal welfare laws (DHSS #NIH 85-23).

Mice were pretreated at predetermined times before methacholine challenge with vehicle (15 DMSO in 0.9% saline, 50 µl/mouse, i.n.) or test compound (5 or 25µg/mouse, i.n.). After dosing with vehicle or compound, the mice were placed into individual plethysmograph chambers (BUXCO Electronics, Troy, NY, USA) where airway responsiveness (Penh) to a methacholine challenge was measured by whole body plethysmography using a BUXCO plethysmography assembly consisting of unrestrained plethysmograph chambers, a signal analyser and data acquisition hardware and software. The mice were allowed to acclimate in the chambers for 5 min, baseline respiratory values (Penh) were collected for another 5 min and then the mice were challenged by aerosol (DeVilbiss Model 5500D, Utrasonic Nebulizer, Somerset, PA) with methacholine, 30 mg/ml (bronchoconstriction ED_{80} of methacholine in mice) at a flow rate of 1.6 ml/min for 2 min. Respiratory values were measured for 5 min after challenge and the mean Penh value was calculated for that time period. During the entire study period, fresh air was supplied by bias flow pumps to the chambers.

Enhanced pause (Penh) values were calculated by the computer software and used as a measure of airway responsiveness. The algorithm for the Penh calculation is as follows: Penh = [(expiratory time / relaxation time)-1] x (peak expiratory flow / peak inspiratory flow) where relaxation time is the amount of time required for 70% of the tidal volume to be expired. This is an index of airway resistance calculated by software from chamber pressures recorded during the inhalation and exhalation of the mice. Penh measurements were taken at predetermined time points after treatment of mice with compound or vehicle, i.e. 0.25, 5, 24, 48, 72 and 96 h.

The data are expressed as the mean ± SEM of the ratio of the Penh value for each individual drug treated mouse over the mean Penh values for the control group (n=8). GraphPad Prism 3.0 computer software (GraphPad Software Inc., CA) was used to graph data.