Supplemental data:

GPR40-mediated Gα12 activation by allosteric full agonists highly efficacious at potentiating glucose-stimulated insulin secretion in human islets

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Supplemental data legends:

Supplemental Figure 1: Arrestin recruitment to hGPR40. Compound A showed similar efficacy as AM-1638 and fasiglifam was a partial agonist with about 50% efficacy compared to Compound A. Data presented are representative of three independent experiments performed in triplicate for each compound. Data are represented as averages ± S.D.

Supplemental Figure 2: A., B. and C. Bioluminescence resonance energy transfer (BRET)-based biosensor assays were used to directly monitor G protein activation following GPR40 agonist treatment. A. Gα11 sensor. Compound A and AM-1638 were full agonists at the Gα11 pathway with similar efficacy as α-linolenic acid. Fasiglifam was a partial agonist with about ~40% efficacy compared to Compound A. Symbols represent the mean ± S.E.M from two independent experiments combined. B. Gα12 sensor. Ghrelin induced Gα12 activation in ghrelin receptor-transfected cells, consistent with previous reports in the literature (Evron *et al.*, 2014; Sivertsen *et al.*, 2011). Symbols represent the mean ± S.E.M from two independent experiments performed in duplicates. C. Gα12 and Gα13 sensors. Compound A and AM-1638 induced coupling to both Gα12 and Gα13. Baseline was subtracted and symbols represent the mean ± S.E.M from two independent experiments combined.

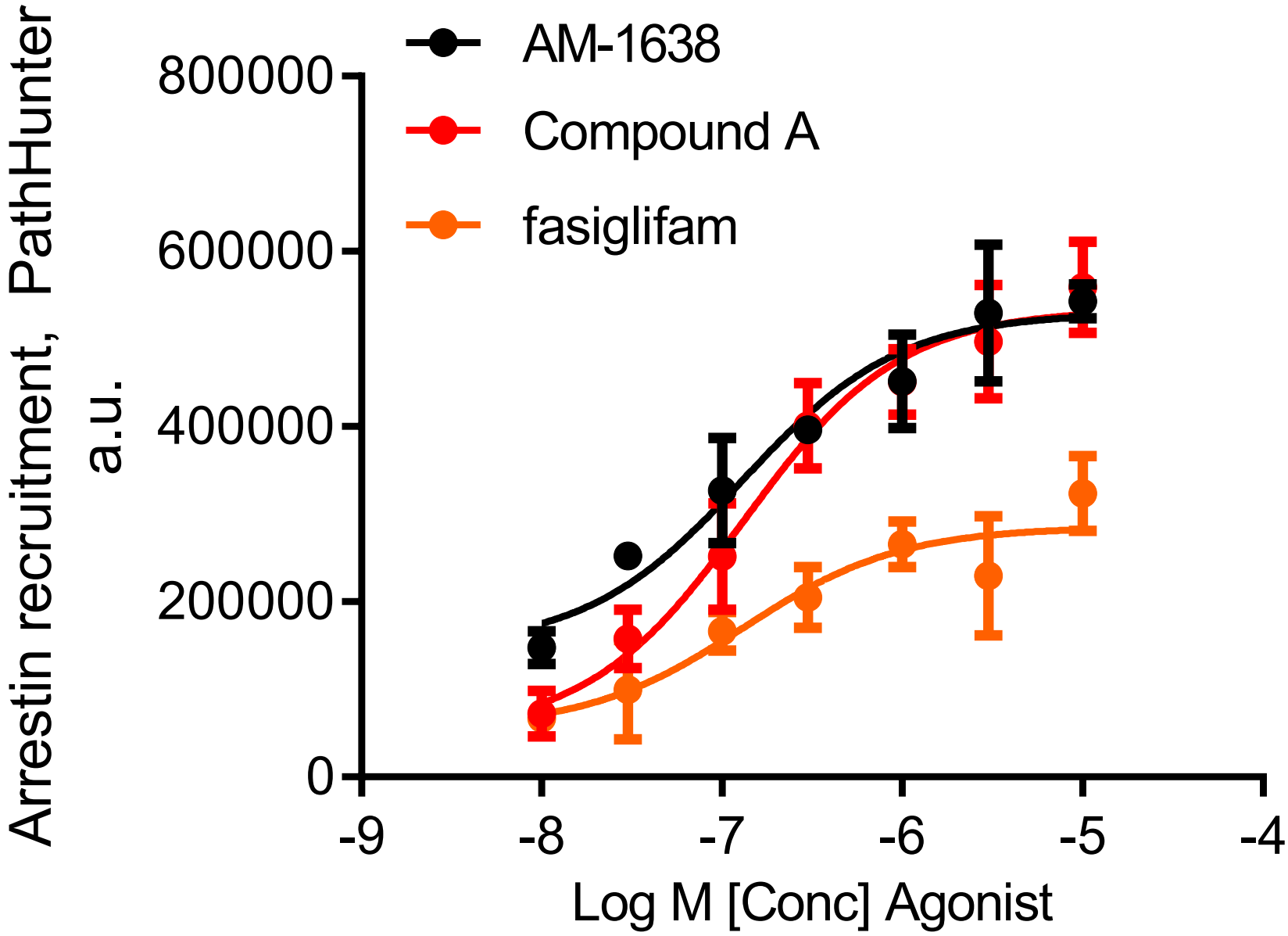
Supplemental Figure 3: A. and B. Bioluminescence resonance energy transfer (BRET)-based

biosensor assays were used to directly monitor G protein activation following GPR40 agonist treatment in presence or absence of PTX. A. Gαq sensor. Gαq activation induced by Compound A, α-linolenic acid and fasiglifam was not significantly affected by PTX. Data from 3 independent experiments were combined and normalized on α-linolenic acid maximal response in the absence of PTX. Symbols represent the mean ± S.E.M from 3 independent experiments. B. Gαi2 sensor. PTX treatment completely abolished Compound A-induced Gαi2 activation. Data from 3 independent experiments were combined and normalized on Compound A-induced maximal response in the absence of PTX. Symbols represent the mean ± S.E.M from 3 independent experiments. C., D. and E. IP1 production induced by GPR40 agonists in the low expressing CHO-K1 cell line stably expressing hGPR40 in the presence or absence of PTX. Data presented are representative of three independent experiments performed in triplicates for each compound. Data are normalized on AM-1638-induced maximal response in the absence of PTX and represented as averages ± S.D. C. and D. The efficacy of Compound A (C) and AM-1638 (D) at inducing IP1 production was not affected by PTX treatment but the potency of both compounds was slightly reduced (3.5 ± 0.4-fold and 2.4 ± 0.1-fold, respectively). E. Fasiglifam-induced IP1 response was almost completely abolished by PTX treatment.

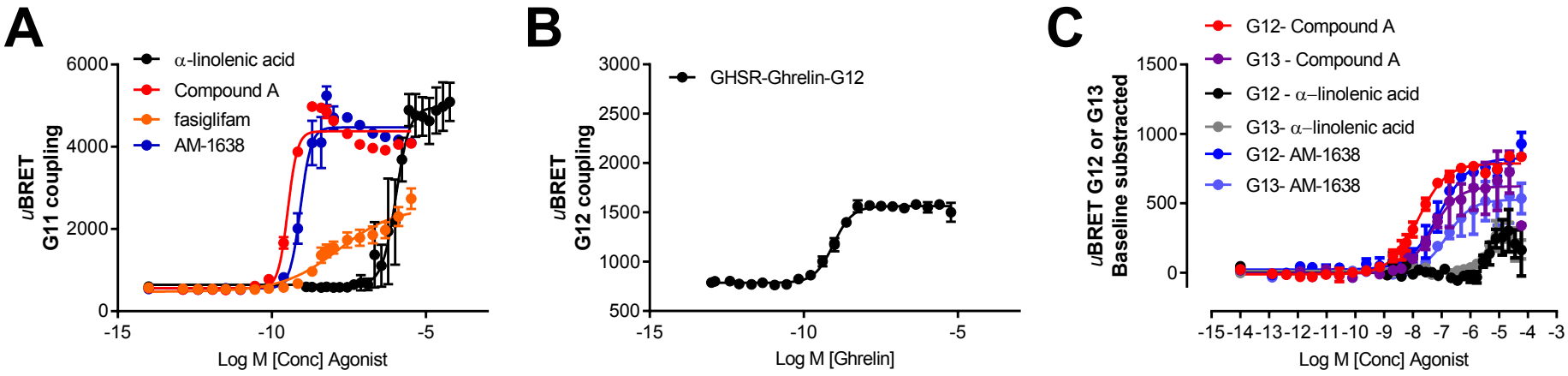
Supplemental Figure 4: A. Competition binding experiment using 50 nM [^{3}H]-Compound A. Both AM-1638 and Compound A completely displaced [^{3}H]-Compound A binding. Fasiglifam had a positive cooperative effect on the binding of [^{3}H]-Compound A, which was consistent with data previously reported in the literature and the allosteric nature of this compound. Data from three independent experiments performed in triplicates for each compound were normalized and combined. Data represented as averages ± S.D. B. Competition binding experiment using 10 nM [^{3}H]-AM-1638. Both AM-1638 and Compound A completely displaced [^{3}H]-AM-1638 binding. Data presented are representative of three independent experiments performed in triplicate for each compound. Data were normalized and represented as averages ± S.D.

Supplemental Table 1. A one-site competition binding model was used to fit the data from Supplemental Figure 4. Competition binding experiments using either 50 nM [^{3}H]-Compound A or 10 nM [^{3}H]-AM-1638: the Ki values and robustness of the fit obtained are represented in this table.

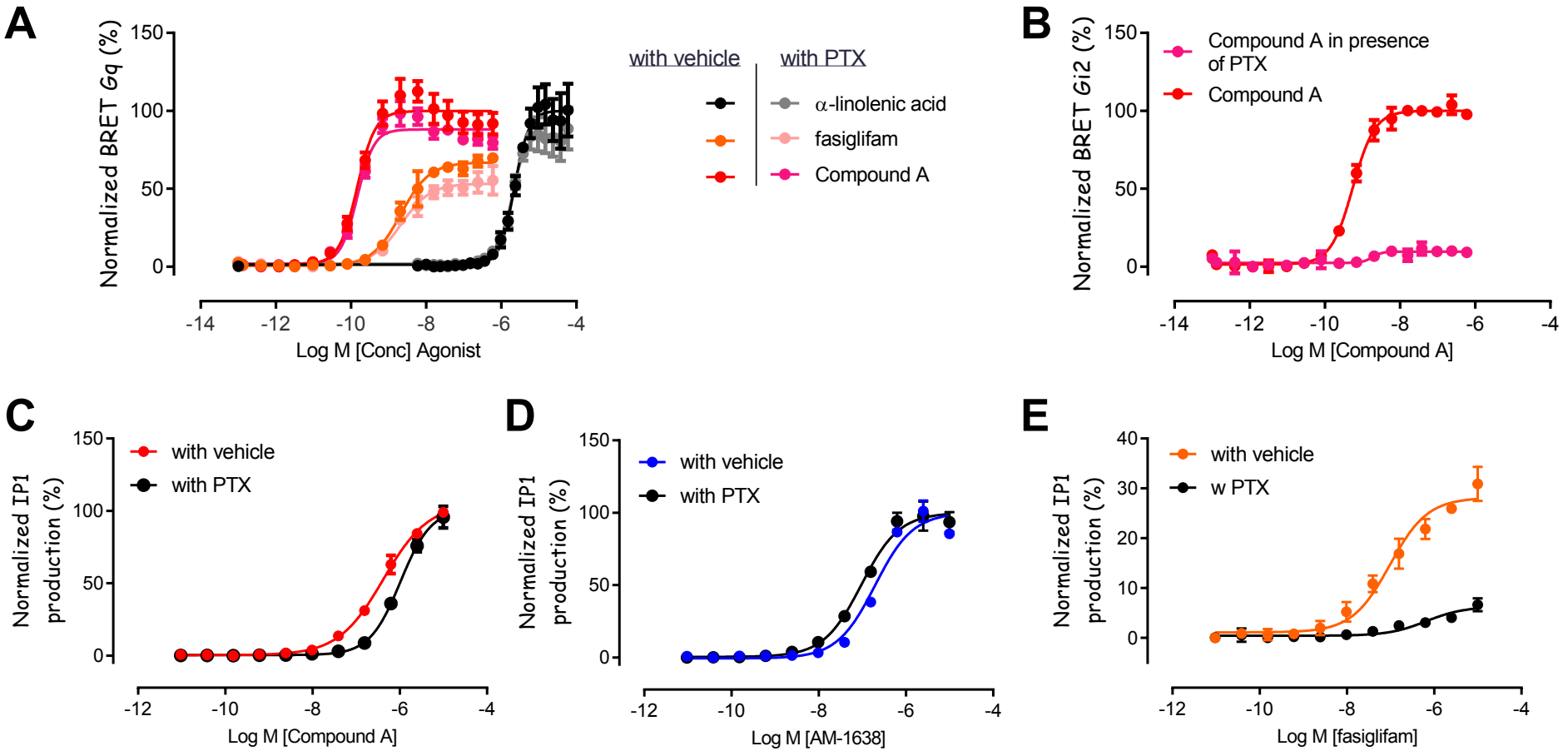
Supplemental Methods: Synthesis of Compound A. Synthesis of (S)-3-Cyclopropyl-3-(3-(((1r,4S)-4-(2-fluoro-5-methoxyphenyl)cyclohexyl)methoxy)phenyl) propanoic acid (Compound A).



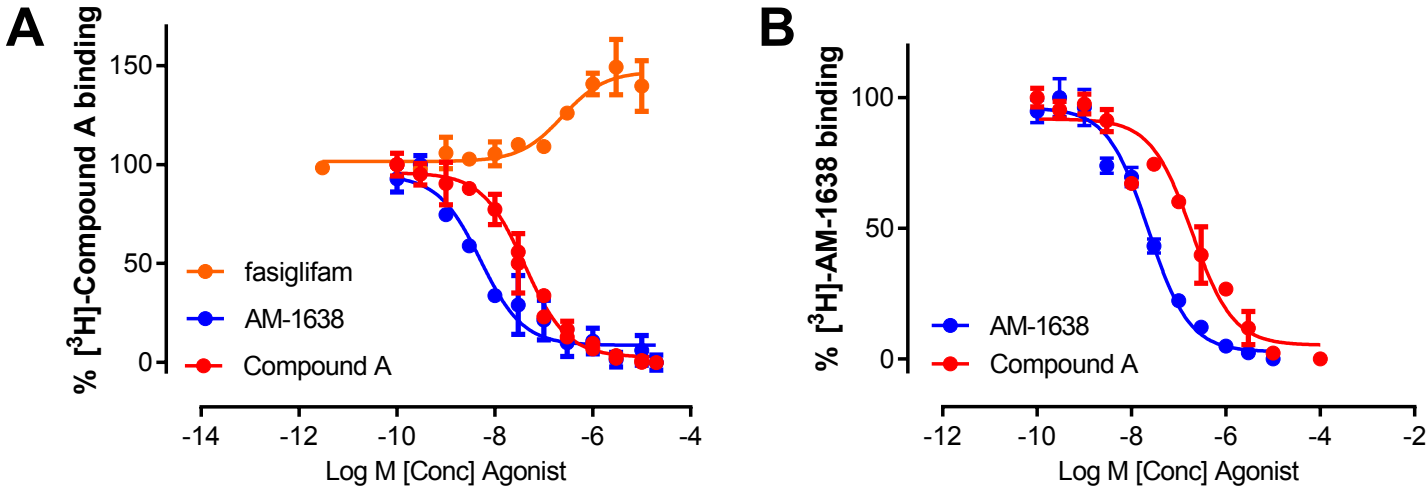
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



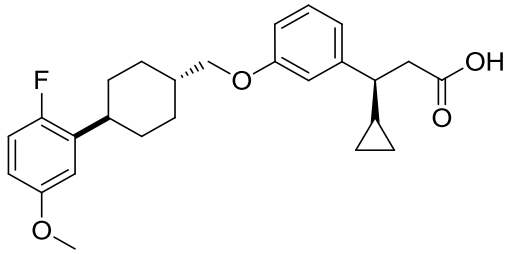
Supplemental Figure 4

SUPPLEMENTAL TABLE 1

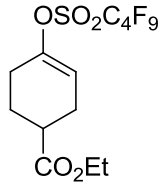
Competition binding experiments using either 50 nM [^{3}H]-Compound A or 10 nM [^{3}H]-AM-1638: A one-site competition binding model was used to fit the data from Supplemental Figure 4. The Ki values and robustness of the fit are represented in the table below:

Compound	*Radioligand*	*Ki (nM ± STDEV)*	R^2
Compound A	[^{3}H]-Compound A	20 ± 4	0.98
AM-1638	[^{3}H]-Compound A	2.5 ± 1.7	0.93
Compound A	[^{3}H]-AM-1638	96 ± 47	0.95
AM-1638	[^{3}H]-AM-1638	11 ± 4	0.98

Supplemental Methods: Synthesis of (S)-3-Cyclopropyl-3-(3-(((1r,4S)-4-(2-fluoro-5-methoxyphenyl)cyclohexyl) methoxy) phenyl) propanoic acid (Compound A)

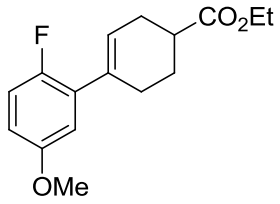


(A) Ethyl 4-(perfluorobutylsulfonyloxy)cyclohex-3-enecarboxylate



To a solution of ethyl 4-oxocyclohexanecarboxylate (615.0 g, 3.613 mol) in THF (1.2 L) at 15 °C under the atmosphere of nitrogen was added 1,1,2,2,3,3,4,4,4-nonafluorobutane-1-sulfonyl fluoride (1256 g, 4.158 mol), followed by addition of THF (1.3 L), a solution of DBU (617.0 g, 4.158 mol) in THF (1.25 L), and THF (1.25 L), sequentially. The reaction was kept at an internal temperature of 25 °C with mild heating overnight. Ice water (2.0 L) was added, followed by addition of water (3.0 L), sodium chloride (150 g) and ethyl acetate (5 L). The resulting mixture was stirred for 30 min. The organic layer was separated and washed with aqueous 4% NaCl (5.0 L). The organic layer was separated and dried over Na_2SO_4 (100 g), filtered, and concentrated under reduced pressure to give the title compound which was used directly in the next step without further purification. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.81 (t, J = 3.1 Hz, 1H), 4.18 (q, J=7.1 Hz, 2H), 2.62 (m, 1H), 2.45 (m, 4H), 2.16 (m, 1H), 2.11-1.87 (m, 1H), 1.28 (t, J=7.1 Hz, 3H).

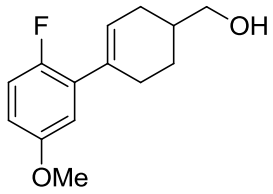
(B) Ethyl 4-(2-fluoro-5-methoxyphenyl)cyclohex-3-enecarboxylate



To a mixture of (2-fluoro-5-methoxyphenyl)boronic acid (200.0 g, 1.173 mol) in 1,4-dioxane (1.0 L) at 20 °C, was added K_3PO_4 (918.6 g, 4.327 mol), followed by dioxane (4.0 L) and water (233.8 g, 13.0 mol) and the flask was evacuated and backfilled with nitrogen gas. Crude ethyl 4-(perfluorobutylsulfonyloxy)cyclohex-3-enecarboxylate (731.2 g, 1.620 mol) and 1,4-dioxane (1.0 L) were added. The flask was evacuated and backfilled with nitrogen gas. $Pd(dppf)Cl_2.CH_2Cl_2$ (72.0 g, 0.087 mol) was added, the flask was evacuated, and backfilled with nitrogen gas. The reaction was heated to 60 °C for 4 h under an inert atmosphere of nitrogen, after which time the reaction was judged completed by LCMS. The resulting solution was cooled to 25 °C and filtered. The filter cake was

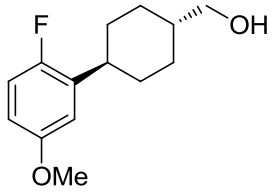
washed with ethyl acetate (1.0 L). To the filtrate was added ethyl acetate (4.0 L) and water (5.0 L) and the mixture was stirred for 30 min. The organic layer was separated, washed with water (5.0 L), dried over Na_2SO_4 and concentrated in vacuo. The residue was dissolved in ethyl acetate (5.0 L), petroleum ether (15.0 L) was added and stirred for 30 min. The precipitate was removed by filtration and washed with a mixture of petroleum ether and ethyl acetate (1.0 L, V: V=5:1). The filtrate was concentrated under reduced pressure to afford the title compound. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.93 (t, J = 3.1 Hz, 1H), 6.72 (m, 2H), 5.94 (m, 1H), 4.17 (q, J = 7.1 Hz, 2H), 3.78 (s, 3H), 2.64 (m, 1H), 2.45 (m, 4H), 2.16 (m,1H), 1.87 (m, 1H), 1.24 (t, J = 7.1 Hz, 3H).

(C) (4-(2-Fluoro-5-methoxyphenyl)cyclohex-3-enyl)methanol



To a suspension of $LiAlH_4$ (55.1 g, 1.452 mol) in anhydrous THF (5.0 L), under a nitrogen atmosphere, cooled to 0-10 °C was added a solution of ethyl 4-(2-fluoro-5-methoxyphenyl)cyclohex-3-enecarboxylate (250. g, 0.899 mol) in anhydrous THF (2.5 L) over a period of 60 min. The reaction mixture was stirred at 0 – 10 °C for 2 h, after which time the reaction was judged complete by LCMS. The reaction was quenched by successive addition of water (55.1 mL), aqueous NaOH (15%, 55.1 mL) and water (165 mL) and the mixture was stirred for 30 min. The precipitate was removed by filtration and the organic phase was concentrated under reduced pressure. The residue obtained was purified by silica gel chromatography using a mixture of heptane and ethyl acetate (10:1 to 5:1 to 3:1) to give the title compound. ^{1}H-NMR (300 MHz, acetone-d_6) δ (ppm): 6.95 (m, 1H), 6.75 (m, 2H), 5.90 (m, 1H), 3.74 (m, 3H), 3.65-3.38 (m, 3H), 2.52-2.15 (m, 3H), 2.00 -1.67 (m, 3H), 1.47-1.22 (m, 1H).

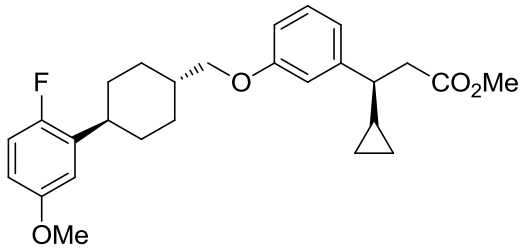
(D) ((1r,4r)-4-(2-Fluoro-5-methoxyphenyl)cyclohexyl)methanol



To a solution of 4-(2-fluoro-5-methoxyphenyl)cyclohex-3-enyl)methanol (50.0 g, 0.212 mol) in DCM (1.0 L), was added $Ir(COD)(Py)(PCy_3)PF_6$ (5.12 g, 6.36 mmol). The reaction was purged with hydrogen gas (3 x) followed by pressurization under a hydrogen gas atmosphere (40 atm) and heating to 30 °C. The reaction was judged complete (LCMS) after 5 h. The mixture was concentrated under reduced pressure. The residue was dissolved in THF (100 mL) and heated to 40 °C to give a clear solution. The solution was gradually cooled to 15 °C, heptane (30 mL) was added and stirred for 2 h. A white solid precipitated from the mixture, then another portion of heptane (500 mL) was added over 30 min and the slurry was stirred for an additional 2 h. The mixture was cooled to 0-5 °C before it was filtered, washed with heptane (50 mL), dried under reduced pressure, to give the title compound (trans:cis > 99:1,

purity 99.2% by HPLC) as a yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.94 (m, 1H), 6.77 (m, 1H), 6.68 (m, 1H), 3.80 (s, 3H), 3.54 (m, 2H), 2.83 (m,1H), 2.01-1.90 (m, 4H), 1.68 -1.47 (m, 3H), 1.25 -1.10 (m, 2H). Mass Spectrum (LCMS, ESI pos.): Calcd. For $C_{14}H_{19}FO_2$: 239.1 $[M-H]^+$; found: 239.4.

(E) (S)-Methyl 3-cyclopropyl-3-(3-(((1r,4S)-4-(2-fluoro-5-methoxyphenyl)cyclohexyl)methoxy) phenyl)propanoate



To a solution of ((1r,4r)-4-(2-fluoro-5-methoxyphenyl)cyclohexyl)methanol (200.0 g, 0.840 mol) and (S)-methyl 3-cyclopropyl-3-(3-hydroxyphenyl)propanoate (203.0 g, 0.924 mol) in acetonitrile (1.5 L) was added tributylphosphine (255.0 g, 1.260 mol) under a nitrogen atmosphere. The solution was warmed to 80 °C and a solution of diethyl diazene-1,2-dicarboxylate (219.0 g, 1.260 mol) in acetonitrile (0.5 L) was added drop-wise over 1.5 h. The solution was stirred for 1 h and judged complete by LCMS. The mixture was concentrated to about 1.0 L under reduced pressure and ethyl acetate (3.0 L) was added. The organic layer was washed with saturated NaCl (3.0 L) and concentrated under reduced pressure. The residue was purified by chromatography on silica gel with heptane: ethyl acetate (20:1) to give the title compound. ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm); 7.26 (m, 1H), 6.96 (m, 1H), 6.83 (m, 4H), 6.70 (m, 1H), 3.86 (m, 1H), 3.83 (s, 3H), 3.65 (s, 3H), 2.88 (m, 1H), 2.78 (m, 2H), 2.37 (m, 1H), 2.04 (m, 5H), 1.59 (m, 2H), 1.45 (m, 1H), 1.30 (m, 3H), 0.99 (m, 3H), 0.59 (m, 1H), 0.47 (m, 1H), 0.29 (m, 1H), 0.19 (m, 1H). Mass Spectrum (LCMS, ESI pos.): Calcd. For $C_{27}H_{33}FO_4$: 441.2 $[M-H]^+$; found: 441.3.

(F) (S)-3-Cyclopropyl-3-(3-(((1r,4S)-4-(2-fluoro-5-methoxyphenyl)cyclohexyl) methoxy)phenyl)propanoic acid

To a solution of (S)-methyl 3-cyclopropyl-3-(3-(((1r,4S)-4-(2-fluoro-5-methoxyphenyl)cyclohexyl) methoxy) phenyl)propanoate (570.0 g, 1.295 mol) in THF (2.85 L) and methanol (2.85 L) was added a solution of NaOH (259.1 g, 6.478 mol) in water (2.85 L) at 20 °C over 30 min. The reaction mixture was stirred at 30 °C overnight. The mixture was cooled to 20 °C and the pH of the solution was adjusted to 4-5 with 4 N aq. HCl. Ethyl acetate (8.5 L) was added and the resulting mixture was stirred for 20 min. The separated organic layer was washed with 5% NaCl (5.7 L), dried over Na_2SO_4 and concentrated to about 2.8 L. Heptane (5.7 L) was then added and the resulting mixture was concentrated to about 5.7 L. This procedure was repeated twice. Heptane (2.85 L) was then added and the solution was cooled to 10-20 °C with stirring. The precipitate formed was collected by filtration, washed with heptane (2.0 L) and dried under reduced pressure to a constant weight to give the title compound. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm): 10.2 (brs, 1H), 7.25 (m, 1H), 6.94 (m, 1H), 6.82 (m, 4H), 6.68 (m, 1H), 3.82 (m, 2H), 3.80(s, 3H), 2.85 (m, 3H), 2.37 (m, 1H), 2.05 (m, 2H), 1.99 (m, 2H),1.96 (m, 1H),1.54(m, 2H),1.31(m, 2H),1.06(m, 1H),0.61(m, 1H),

0.47(m, 1H), 0.33(m, 1H), 0.21(m, 1H). Mass Spectrum (LCMS, ESI neg.): Calcd. For $C_{26}H_{31}FO_4$: 425.3 $[M-H]^+$; found: 425.3.