# Supporting Information

## Novel selective orally active CRTH2 antagonists for allergic inflammation developed from in silico derived hits

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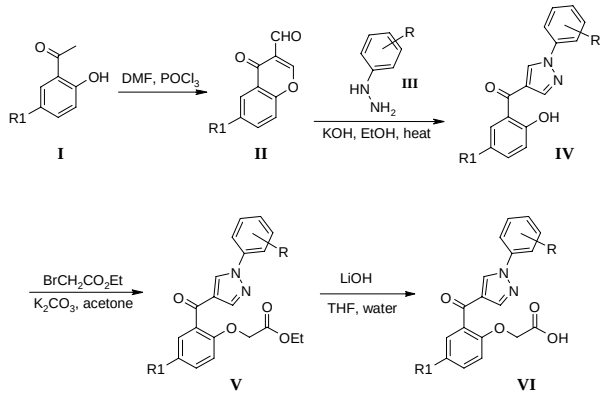
# Synthetic procedures and characterization of compounds

**General comments.** Microwave chemistry was performed in an Emrys™ Optimizer instrument from Personal Chemistry (now Biotage). The instrument automatically regulates the supplied microwave energy to provide the set temperature for the given period of time. NMR spectra were obtained on a Bruker Avance AMX 300 MHz instrument. LC/MS was performed on an Agilent 1100-series instrument. LC/MS methods are as follows: LC/MS-pos: Column: XTerra MS C18; Flow: 1.0 mL/min; Gradient: 0-5 min: 15-100% MeCN in water, 5-7½ min: 100% MeCN; Modifier: 5 mM ammonium formate; MS-ionisation mode: API-ES (pos.). LC/MS-neg: Column: XTerra MS C18; Flow: 1.0 mL/min; Gradient: 0-5 min: 15-100% MeCN in water, 5-7½ min: 100% MeCN; Modifier: 5 mM ammonium formate; MS-ionisation mode: API-ES (neg.).

**General procedures according to Scheme 1.**




**Condensation of 3-formylchromone (II) with arylhydrazine (III).** To 3-formylchromone **II** (1.0 mmol), (prepared according to Högberg et al)[1] and arylhydrazine **III** (1.0 mmol) in ethanol (3.0 mL), in a reaction tube, was added 4.0 M aq. KOH (1.0 mL, 4.0 mmol). The tube was sealed and heated by exposure to microwave radiation at 120 $^{o}$C for 7 min. Following cooling, 3% HCl was carefully added to acidify the mixture to a pH <1; the mixture was allowed to precipitated. The precipitate was filtered off and washed with a small amount of ethanol. The product **IV** was used directly or purified by recrystallisation from ethanol or by flash chromatography ($SiO_2$, EtOAc:heptane, 1:8 to 1:5).

**Alkylation of phenol (IV).** To the phenol **IV** (0.5 mmol) in acetone (1 mL) was added ethyl bromoacetate (85 mg, 0.5 mmol) and $K_2CO_3$ (75 mg, 0.54 mmol), and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was then concentrated *in vacuo* and the residue was partitioned between water and ethyl acetate. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated. The product **V** was used directly or purified by recrystallization from MeOH or by flash chromatography ($SiO_2$, EtOAc:heptane, 1:7 to 1:5).

**Hydrolysis of ester (V).** The ester **V** (0.10 mmol) in THF (0.5 mL) was added $LiOH{\cdot}H_2O$ (6.3 mg, 0.15 mmol) in water (0.5 mL). The reaction was stirred at room temperature for >2 h, before 3% HCl was added to acidify the mixture to a pH <1, and the mixture was extracted with $CH_2Cl_2$. The organic phase was dried ($MgSO_4$) and concentrated to give the product **VI**.

**2-(1-Phenyl-1H-pyrazole-4-carbonyl)phenoxyacetic acid (16)**. Reaction of (2-hydroxyphenyl)-(1-phenyl-1H-pyrazol-4-yl)ketone (264 mg, 1.0 mmol) and ethyl bromoacetate according to general procedure gave 235 mg (67%) of ethyl 2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate as white solid: $^{1}$H NMR ($CDCl_3$): δ 1.26 (t, J = 7.1 Hz, 3H), 4.24 (q, J = 7.1 Hz, 2H), 4.67 (s, 2H), 6.84 (d, J = 8.3 Hz, 1H), 7.10 (ts, J = 7.4, 0.8 Hz, 1Hz), 7.29-7.36 (m, 1H), 7.41-7.53 (m, 4H), 7.72-7.78 (m, 2H), 8.18 (s, 1H), 8.58 (s, 1H); $^{13}$C

NMR ($CDCl_3$): δ 14.3, 61.7, 65.4, 112.2, 118.8, 119.3, 119.7, 120.0, 122.1, 125.8, 127.6, 129.7, 129.9, 130.1, 130.6, 131.8, 132.1, 136.3, 139.7, 142.8, 155.0, 168.9, 188.7.

Hydrolysis of ethyl 2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate according to general procedure and purification by column chromatography ($SiO_2$, EtOAc:heptane, 1:1) gave 15 mg (56%) of the title compound as white solid: LC/MS-neg: Rt 2.90 min, m/z 321.0 $[M-H]^-$; $^1H$ NMR ($CDCl_3$): δ 4.83 (s, 2H), 7.10 (d, J = 8.3 Hz, 1H), 7.20 (t, J = 7.5 Hz, 1H), 7.35-7.42 (m, 1H), 7.46-7.53 (m, 2H), 7.54-7.61 (m, 1H), 7.66 (dd, J = 7.5, 1.5 Hz, 1H), 7.70-7.75 (m, 2H); $^{13}C$ NMR ($CDCl_3$): δ 67.7, 115.5, 120.1, 122.8, 124.7, 128.3, 128.6, 129.9, 131.0, 131.9, 134.0, 139.3, 143.5, 156.6, 170.4, 189.3.

**4-Fluoro-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetic acid (17).** Reaction of 4-fluoro-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenol (282 mg) and ethyl bromoacetate according to general procedure gave 335 mg (91%) of ethyl 4-fluoro-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate as a yellow solid: LC/MS-neg: Rt 3.16, m/z 339.0 $[M-H]^-$; $^1H$ NMR ($CDCl_3$): δ 1.26 (t, J = 7.0 Hz, 3H), 4.23 (t, J = 7.1 Hz, 2H), 4.63 (s, 2H), 6.81 (dd, J = 9.0, 4.0 Hz, 1H), 7.13 (ddd, J = 8.9, 4.5, 3.0 Hz, 1H), 7.21 (dd, J = 8.1, 3.2 Hz, 1H), 7.31-7.37 (m, 1H), 7.24-7.50 (m, 2H), 7.72-7.78 (m, 2H), 8.18 (s, 1H), 8.58 (s, 1H); $^{13}C$ NMR ($CDCl_3$): δ 14.3, 61.8, 66.1, 114.0 (d, $J_{CF}$ = 7.6 Hz), 116.8 (d, $J_{CF}$ = 24.5 Hz), 118.3 (d, $J_{CF}$ = 23.5 Hz), 119.7, 125.2, 127.7, 129.7, 131.8, 139.6, 142.8, 151.1 (d, $J_{CF}$ = 2.2 Hz), 157.6 (d, $J_{CF}$ = 241.1 Hz), 168.7, 187.l.

Hydrolysis of ethyl 4-fluoro-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate (31 mg) according to general procedure gave 30 mg (100%) of the title compound as a pale yellow solid: LC/MS-neg: Rt 3.16, m/z 339.0 $[M-H]^-$;$^1H$ NMR ($CDCl_3$): δ 4.60 (s, 2H), 6.90 (dd, J = 9.2 Hz, 1H), 7.08-7.17 (m, 1H), 7.21 (dd, J = 7.9, 3.0 Hz, 1H), 7.28-7.35 (m, 1H), 7.38-7.46 (m, 2H), 7.65 (d, J = 7.5 Hz, 2H), 8.06 (s, 1H), 8.44 (s, 1H).

**4-Chloro-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetic acid (18).** Prepared from (2-hydroxy-5-chlorophenyl)-(1-phenyl-1H-pyrazol-4-yl)-methanone and ethyl bromoacetate according to general procedures: LC/MS-pos: Rt 3.08 min, m/z 356.6 $[M + 1]^+$; $^1H$ NMR ($CDCl_3$): δ 4.75 (s, 2H), 6.95 (d, J = 8.9 Hz, 1H), 7.33-7.41 (m, 1H), 7.44-7.52 (m, 3H), 7.55 (d, J = 2.6 Hz, 1H), 7.69-7.75 (m, 2H), 8.18 (s, 1H), 8.52 (s, 1H).

**Ethyl 4-bromo-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate (26).** Reaction of (5-bromo-2-hydroxy-phenyl)-(1-phenyl-1H-pyrazol-4-yl)ketone and ethyl bromoacetate according to general procedure gave 215 mg (100%) white crystals. The product was used without further purification: LC/MS-pos: Rt 6.15 min, m/z 429 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 1.26 (t, J = 7.1 Hz, 3H), 4.24 (q, J = 7.1 Hz, 2H), 4.64 (s, 2H), 6.72 (d, J = 8.9 Hz, 1H), 7.34 (m, 1H), 7.46 (m, 2H), 7.53 (dd, J = 8.9, 2.5 Hz, 1H), 7.59 (d, J = 2.5 Hz, 1H), 7.73-7.78 (m, 2H), 8.17 (s, 1H), 8.58 (s, 1H); $^{13}C$ NMR ($CDCl_3$): δ 14.3, 61.9, 65.5, 114.0, 114.5, 119.8, 125.3, 127.7, 129.8, 131.8, 132.3, 132.7, 134.6, 139.6, 142.7, 154.1, 168.5, 186.9.

**4-Bromo-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetic acid (19).** Hydrolysis of ethyl 4-bromo-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate (31 mg, 0.072 mmol) according to general procedure gave 28.4 mg (99%) white solid: LC/MS-pos: Rt 3.14 min, , m/z 401; $^1H$ NMR ($CDCl_3$): δ 3.51 (s, 1H), 4.80 (s, 1H), 6.97 (d, J = 8.7 Hz, 1H), 7.38-7.44 (m, 1H), 7.49-7.55 (m, 2H), 7.64-7.69 (m, 1H), 7.72-7.77 (m, 3H), 8.19 (s, 1H), 8.52 (s, 1H); $^{13}C$ NMR ($CDCl_3$): δ 67.2, 115.1, 116.6, 120.1, 124.5, 128.4, 130.0, 131.9, 133.3, 136.2, 139.2, 143.3, 155.2, 169.9, 187.6; Anal. ($C_{18}H_{13}BrN_2O_4$) C, H, N.

**4-Methyl-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetic acid (20).** Prepared from (2-hydroxy-5-methylphenyl)-(1-phenyl-1H-pyrazol-4-yl)-methanone and ethyl bromoacetate according to general procedures: LC/MS-pos: Rt 3.08 min, m/z 356.6 $[M + 1]^+$;,$^1H$ NMR ($CDCl_3$): δ 2.37 (s, 3H), 4.78 (s, 2H), 6.97 (d, J = 9.0 Hz, 1H), 7.33-7.51 (m, 5H), 7.73 (d, J = 6 Hz, 2H), 8.16 (s, 1H), 8.51 (s, 1H), 11.21 (br s, 1H)

**4-Methoxy-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetic acid (21).** Prepared from 6-bromo-3-formylchromone, 2,4-dichlorophenylhydrazine and ethyl bromoacetate according to general procedures:

LC/MS-neg: Rt 2.46 min, m/z 350.9 $[M-H]^-$; $^1H$ NMR ($CDCl_3$): δ 3.81 (s, 3H), 4.77 (s, 2H), 7.02-7.16 (m, 3H), 7.41 (d, J = 7.5 Hz, 1H), 7.50 (t, J = 8.1 Hz, 2H), 7.73 (d, J = 8.5 Hz, 2H), 8.17 (s, 1H), 8.52 (s, 1H).

**4-Nitro-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetic acid (22).** Prepared from 6-nitro-3-formylchromone, 2-ethylphenylhydrazine and ethyl bromoacetate according to general procedures: LC/MS-pos: Rt 2.05 min, m/z 367.8 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 4.83 (s, 2H), 7.01 (d, J = 9.0 Hz, 1H), 7.34-7.42 (m, 1H), 7.44-7.53 (m, 1H), 7.65-7.72 (m, 1H), 8.26 (s, 1H), 8.34 (dd, J = 9.0, 2.6 Hz, 1H), 8.41 (d, J = 2.6 Hz, 1H), 8.53 (s, 1H).

**4-Bromo-2-[1-(4-methoxyphenyl)-1H-pyrazole-4-carbonyl]phenoxyacetic acid (27).** Reaction of (5-bromo-2-hydroxyphenyl)-[1-(4-methoxyphenyl)-1H-pyrazol-4-yl]ketone and ethyl bromoacetate according to general procedure gave ethyl 4-bromo-2-[1-(4-methoxyphenyl)-1H-pyrazole-4-carbonyl]phenoxyacetate: LC/MS-pos: Rt 4.59 min, m/z 458.4/460.4 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 1.26 (t, J = 7.1 Hz, 3H), 3.84 (s, 3H), 4.23 (q, J = 7.1 Hz, 2H), 4.62 (s, 2H), 6.72 (d, J = 8.9 Hz, 1H), 6.97 (d, J = 8.7 Hz, 2H), 7.52 (dd, J = 8.9, 2.6 Hz, 1H), 7.58 (d, J = 2.5 Hz, 1H), 7.64 (d, J = 8.9 Hz, 2H), 8.13 (s, 1H), 8.46 (s, 1H); $^{13}C$ NMR ($CDCl_3$): δ 14.3, 55.8, 61.9, 65.6, 114.1, 114.4, 114.8, 121.4, 125.0, 131.6, 132.4, 132.6, 133.2, 134.5, 142.5, 154.1, 159.2, 168.5, 186.9.

Hydrolysis of ethyl 4-bromo-2-[1-(4-methoxyphenyl)-1H-pyrazole-4-carbonyl]phenoxyacetate (23 mg) according to general procedure to give 21 mg (97%) of title compound as a white foam: LC/MS-neg: Rt 5.76 min, m/z 428.9 $[M – H]^-$; $^1H$ NMR ($CDCl_3$): δ 3.86 (s, 3H), 4.77 (s, 2H), 6.94 (d, J = 8.9 Hz, 1H), 6.99 (d, J = 9.2 Hz, 2H), 7.59-7.66 (m, 3H), 7.71 (d, J = 2.4 Hz, 1H), 8.14 (d, J = 0.6 Hz, 1H), 8.40 (d, J = 0.6 Hz, 1H) $^{13}C$ NMR ($CDCl_3$): δ 55.9, 67.2, 115.0, 115.l, 116.6, 121.8, 124.2, 130.7, 131.8, 132.7, 133.2, 136.1, 143.2, 155.2, 159.7, 169.9, 187.6.

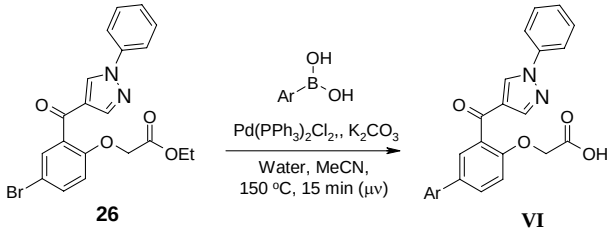
**4-Bromo-2-[1-(4-chlorophenyl)-1H-pyrazole-4-carbonyl]phenoxyacetic acid (28).** Prepared from 6-bromo-3-formylchromone, 4-chlorophenylhydrazine and ethyl bromoacetate according to general procedures: LC/MS-pos: Rt 3.53 min, m/z 436.4 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 4.74 (s, 2H), 6.88 (d, J = 8.9 Hz, 1H), 7.44 (d, J = 8.5 Hz, 2H), 7.61 (dd, J = 8.9, 2.4 Hz, 1H), 7.63-7.70 (m, 3H), 8.17 (s, 1H), 8.49 (s, 1H); $^{13}C$ NMR ($CDCl_3$): δ 66.4, 114.9, 115.7, 121.2, 124.9, 130.0, 130.9, 131.9, 133.1, 133.9, 135.9, 137.7, 143.3, 154.7, 170.8, 187.4.

**4-Bromo-2-[1-(4-bromophenyl)-1H-pyrazole-4-carbonyl]phenoxyacetic acid (29).** Prepared from 6-bromo-3-formylchromone, 4-bromophenylhydrazine and ethyl bromoacetate according to general procedures: LC/MS-pos: Rt 3.75 min, m/z 480.3 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 4.75 (s, 2H), 6.89 (d, J = 8.9 Hz, 1H), 7.59-7.65 (m, 5H), 7.66-7.69 (m, 1H), 8.16 (s, 1H), 8.50 (s, 1H); $^{13}C$ NMR ($CDCl_3$): δ 66.5, 115.0, 115.9, 121.5, 121.8, 124.9, 130.8, 131.8, 133.0, 133.1, 135.9, 138.2, 143.4, 154.8, 170.6, 187.4.

**4-Bromo-2-[1-(3-bromophenyl)-1H-pyrazole-4-carbonyl]phenoxyacetic acid (30).** Prepared from 6-bromo-3-formylchromone, 3-bromophenylhydrazine and ethyl bromoacetate according to general procedures: LC/MS-pos: Rt 4.29 min, m/z 480.5 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 4.79 (s, 2H), 6.95 (d, J = 9.0 Hz, 1H), 7.28-7.40 (m, 1H), 7.53 (d, J = 9 Hz, 1H), 7.65 -7.73 (m, 3H), 7.96 (s, 1H), 8.18 (s, 1H); 8.51 (s, 1H).

**4-Bromo-2-[1-(2-bromophenyl)-1H-pyrazole-4-carbonyl]phenoxyacetic acid (31).** Prepared from 6-bromo-3-formylchromone, 2-bromophenylhydrazine and ethyl bromoacetate according to general procedures: LC/MS-pos: Rt 3.38 min, m/z 480.6 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 4.75 (s, 2H), 6.91 (d, *J* = 9.0 Hz, 1H), 7.34-7.64 (m, 6H), 7.73-7.74 (m, 2H), 8.22 (s, 1H), 8.36 (s, 1H).

**General procedure for Suzuki coupling/ester hydrolysis**




**26** **VI**

To Ethyl 4-bromo-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate (**26)**, 0.20 mmol) in MeCN (0.4 mL, degassed) with 1.0 M $Na_2CO_3$ (0.4 mL, degassed) was added aryl boronic acid (0.21 mmol) and $Pd(PPh_3)_2Cl_2$ (9 mg, 0.01 mmol). The reaction mixture was degassed by allowing argon to bubble through the mixture for ½ min before the reaction vessel was sealed and the mixture heated by exposure to microwave radiation (150 $^oC$, 300 s). Upon cooling, 3% HCl was then added to acidify the mixture to a pH <1. The organics were then extracted with $CH_2Cl_2$. The extract was filtered through celite and concentrated, and the residue was purified by solid-phase extraction (pre-packed 1 g SAX columns), or by flash chromatography.

**3-(1-Phenyl-1H-pyrazole-4-carbonyl)biphenyl-4-yloxyacetic acid (23).** Prepared from ethyl 4-bromo-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate and phenylboronic acid according to the general coupling procedure: LC/MS-pos: Rt 1.05 min, m/z 398.6 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 4.86 (s, 2H), 7.17 (d, J = 8.6 Hz, 1H), 7.35-7.57 (m, 8H), 7.70-7.75 (m, 2H), 7.78 (dd, J = 8.6, 2.3 Hz, 1H), 7.84 (d, J = 2.3 Hz, 1H), 8.20 (s, 1H), 8.54 (s, 1H); $^{13}C$ NMR ($CDCl_3$): δ 67.7, 115.8, 120.1, 124.8, 127.1, 128.0, 128.3, 129.1, 129.3, 129.4, 129.9, 132.3, 136.2, 139.3, 139.3, 143.5, 155.8, 170.4, 189.3.

**[3',5'-Difluoro-3-(1-phenyl-1H-pyrazole-4-carbonyl)biphenyl-4-yloxy]acetic acid (25).** Prepared from ethyl 4-bromo-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate and 3,5-difluorophenylboronic acid according to the general coupling procedure: LC/MS-pos: Rt 3.03 min, m/z 434.5 $[M + H]^+$; $^1H$ NMR ($CDCl_3$): δ 4.85 (s, 2H), 6.81 (td, J = 8.9, 2.3 Hz, 1H), 7.07 (d, J = 8.3 Hz, 2H), 7.14 (d, J = 8.9 Hz, 1H), 7.41 (d, J = 6.6 Hz, 1H), 7.48 (s, 1H), 7.52 (d, J = 8.5 Hz, 1H), 7.66-7.78 (m, 4H), 8.23 (s, 1H), 8.57 (s, 1H)

**[4'-Chloro-3-(1-phenyl-1H-pyrazole-4-carbonyl)biphenyl-4-yloxy]acetic acid (24).** Prepared from ethyl 4-bromo-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetate and 4-chlorophenylboronic acid according to the general coupling procedure: LC/MS-pos: Rt 3.08 min, m/z 432.5 $[M + H]^+$.

# Purity determinations

**Purity determined by two independent HPLC systems.**

**HPLC-A**: Column: XTerra MS C18; Flow: 1.0 mL/min; Gradient: 0-5 min: 15-100% MeCN in water, 5-7½ min: 100% MeCN; Modifier: 5 mM ammonium formate. System used for LC/MC experiments.

**HPLC-B**: Additional HPLC analysis of DMSO stock solutions done during revision of manuscript using: Waters Alliance 2695, Waters2996 PDA detector, Compound concentration 100µM, Injection volume 10 µL, Kromasil 5µ C18 150x2.1 mm, Flow 0.4 mL/min, Solvent gradient with 50 mM ammonium acetate (A) and acetonitrile (B) starting with 100% A and reaching 100% B after 9 minutes with total duration for 15 minutes, the detector was set to 254 nm.

| No | HPLC-A | HPLC-B |
|---|---|---|
| **16** | 100% | 99% |
| **17** | 100% | 94% |
| **18** | 100% | 97% |
| **19** | 100% | 99% |
| **20** | 94% | 98% |
| **21** | 100% | 97% |
| **22** | 100% | 81% |
| **23** | 90% | 87% |
| **24** | 97% | 94% |
| **25** | 90% | 92% |
| **26** | 100% | 96% |
| **27** | 96% | 97% |
| **28** | 100% | 98% |
| **29** | 99% | 98% |
| **30** | 93% | 98% |
| **31** | 100% | 99% |

Compounds in chemical libraries from third party vendors have about 90% as purity criterion.

**4-Bromo-2-(1-phenyl-1H-pyrazole-4-carbonyl)phenoxyacetic acid (19).** Anal. ($C_{18}H_{13}BrN_2O_4$)

| Elemental analysis | Calcd. | Found | |
|---|---|---|---|
| C | **53.89 %** | **53.76%** | **53.74%** |
| H | **3.27 %** | **3.25%** | **3.21 %** |
| N | **6.98 %** | **6.85%** | **6.84 %** |

# Biological assays

**Generation/origin of the cDNA Constructs.** The coding sequence of human CRTH2 (genbank accession no NM_004778) was amplified by PCR from a human hippocampus cDNA library and inserted into the pcDNA3.1(+) expression vector (invitrogen) via 5' Hind*III* and 3' EcoR*I*. To generate the CRTH2-*Renilla luciferase* (CRTH2-Rluc) fusion protein, the coding sequences of CRTH2 without a STOP codon and RLuc were amplified, fused in frame by PCR and subcloned into the pcDNA3.1(+)Zeo expression vector (invitrogen). Human β-arrestin2 (β-arr2) N-terminally tagged with GFP² (GFP²/β-arr2) and Renilla luciferase were purchased from BioSignal Packard Inc, Montreal, Canada). The β-arr2 mutant (β-arr2, R393E, R395E) incapable of interacting with the endocytic machinery has been described previously[2]. The use of the GFP²-β-arr2, R393E, R395E mutant for Bioluminescence Resonance Energy Transfer (BRET) requires a license from 7TM Pharma. The sequence identity of the constructs was verified by restriction endonuclease digests and sequencing in both directions on an ABI Prism (Applied Biosystems, Foster City, CA).

**Generation of the human CRTH2 receptor-Renilla Luciferase fusion protein expressing HEK293 cell line and culturing.** The CRTH2-Rluc fusion protein cDNA in pcDNA3.1(+)Zeo was transfected into a HEK293 cell clone stably expressing the $GFP^2$-β-arr2, R393E, R395E mutant under G418 (Gibco #11811) selection. Single clones were expanded under G418 and Zeocin (Invitrogen) selection and tested for [$^3$H]PGD2 binding and PGD2-induced BRET. One clone (hereafter referred to as CRTH2-HEK293 cells) was expanded further for generation of frozen cell stocks and used as described below in whole cell binding and functional BRET assays. CRTH2-HEK293 cells were maintained in Minimum Essential medium (MEM) supplemented with 10% (v/v) heat inactivated fetal calf serum (HIFCS), 2mM Glutamax$^{TM}$-I, 1% non essential amino acids (NEAA), 1% sodium pyruvate, 10 µg/ml gentamicin, 0.5 mg/mL G418 and 0.1 mg/mL Zeocin..

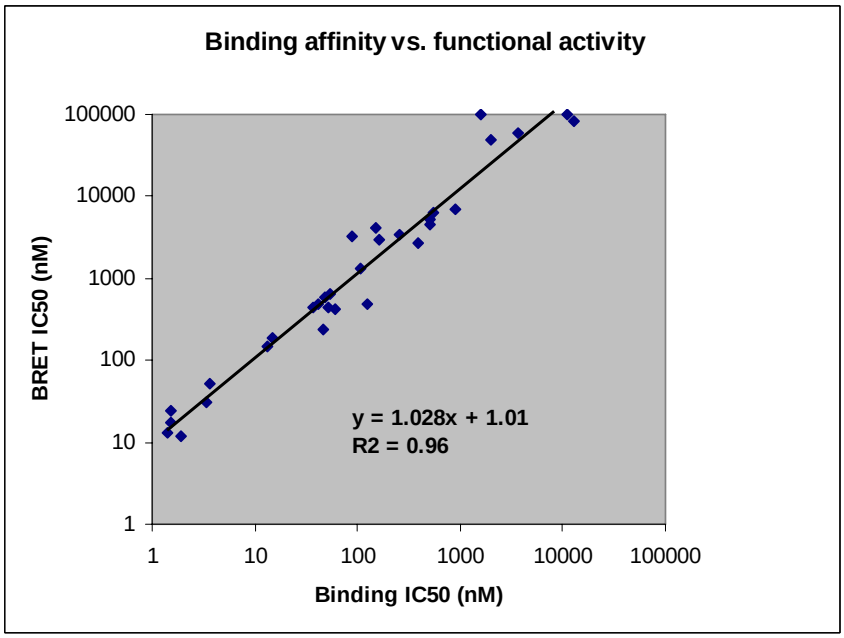
**Binding assay.** CRTH2-HEK293 cells were seeded into white 96well plates (Costar #3917) at a density of 30.000 cells/well. Competition binding experiments on whole cells were performed about 18-24 h later using 1.2 nM [3H]PGD2 (Amersham #TRK734, 166 Ci/mmol) in binding buffer consisting of HBSS (GIBCO #14025) supplemented with 10 mM HEPES (Bie&Berntsen A/S, Denmark). Competing compounds were diluted in DMSO and the DMSO concentration kept constant at 1% (v/v) of the final 200 µL incubation volume. Total and nonspecific binding were determined in the absence and presence of 10 µM PGD2. Binding reactions were conducted for 3 h at 4°C and terminated by 2 washes (100 µl each) with ice-cold binding buffer. Radioactivity was determined by liquid scintillation counting in a TopCount (Packard) following overnight incubation of the washed cells in Microscint 20 (PerkinElmer).

**BRET assay.** An improved functional **B**ioluminescence **R**esonance **E**nergy **T**ransfer ($BRET^2$) assay were performed on CRTH2-HEK293 essentially as described previously[2] Prior to the assay cells were detached with trypsin and re-suspended in Dulbecco's PBS with 1000 mg/L L-Glucose (D-PBS, Gibco #14287) at a density of $1x10^6$ cells/mL. 180 µL of cell suspension was transferred to each well in a 96-well microplate (white OptiPlate-96, PerkinElmer). Antagonists were diluted in DMSO and preincubated with the cell suspension using a shaking table for 5 min. The final DMSO concentration was kept constant at 1% in the assay. PGD2 at was then added to each well to elicit about 75-80% of the maximal agonist efficacy ($1x10^{-7}$ mol/L) and cells were further incubated for 5 minutes. After the incubation period, the 96-well microplate was placed in the Mithras LB 940 instrument (BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany). 10 µL/well DeepBlueC coelenterazine (PerkinElmer) diluted to 100 µM in a 30% EtOH / 70% D-PBS solution was injected to one well at a time. Five seconds after the injection, the light output from the well was measured sequentially at 400 nm and 515 nm. The BRET signal (mBRET ratio) was calculated by the ratio of the fluorescence emitted by $GFP^2$-β-arr2, R393E, R395E (515 nm) over the light emitted by the receptor-Rluc (400 nm): mBRET = em(515 nm) / em(400 nm) x 1000. The use of the $GFP^2$-β-arr2, R393E, R395E mutant for BRET requires a license from 7TM Pharma.

**Materials.** White 96well Optiplates and DeepBlueC™ were obtained from Packard BioScience, Montreal, Canada. Tissue culture media and reagents were purchased from the GIBCO invitrogen corporation (Breda, Netherlands). PGD2 was from Cayman and [$^3$H]PGD2 from NEN. All other laboratory reagents were from Sigma (St. Louis, MO), unless explicitly specified.

**Calculations.** The $IC_{50}$'s in competition binding experiments and BRET assays was determined by applying the following "four parameter logistic equation" by non-linear regression (GraphPad prism 4.0, San Diego): Y = Bottom + (Top-Bottom)/(1+10^((Log$IC_{50}$-X)*HillSlope)). For competition binding assays Y denotes specific binding, X denotes the logarithm of antagonist concentration, Top denotes the total [$^3$H]PGD2 binding, Bottom denotes the non-specific [$^3$H]PGD2 binding, and Hillslope the slope factor. For BRET assays Y denotes the mBRET response, X denotes the logarithm of antagonist concentration, Top denotes the 75-80% of the maximal PGD2 induced mBRET response that is depressed to the Bottom by antagonist, and Hillslope the slope factor. $IC_{50}$ values generated by competing a PGD2 concentration that elicits about 75-80% of the maximal agonist efficacy with antagonists closely match the true affinity of antagonists determined by the analysis according to Arunlakshana & Schild[3].

**Correlation of receptor affinity with functional activity.** The $IC_{50}$ for the binding affinity is on average ten-fold lower than for the BRET functional activity as shown in the graph based on all 31 compounds investigated. A good correlation between the results returned by the two methods ($r^2$=0.96) and a slope close to unity is seen.




**Receptor profiling** of compound **19** was conducted by CEREP according to protocols available via http://www.cerep.fr/Cerep/Users/index.asp. : A1 (h), A2A (h), alpha 1 (non-select.), alpha 2 (non-select.), beta 1 (h), AT1 (h), AT2 (h), BZD (central), B2 (h), CCKA (h) (CCK1), C5a (h), ChemR23, D1 (h), D2S (h), DP (DP1), ETA (h), fMLP (h), GABA (non-select.), NMDA, GIP, H1 (h), H2 (h), LTD4 (h), MCH1 (h), MC4 (h), M (non-selective), NK1 (h), Y (non-selective), Nicotinic (neuronal), Opiate (non-selective), ORL1 (h) (NOP), PAF (h), PCP, TXA2/PGH2 (h) (TP), 5-HT (non-selective), sigma (non-select.), sst (non-

selective), Glucocorticoid (h), V1a (h), NE transporter (h), DA transporter (h), 5-HT transporter (h), PLA2, 12-LO, 15-LO, COX-1, COX-2 were inhibited less than 30% at 10 µM**.**

**ADME profiling** of compound **19** detailed in Table 5 was conducted by CEREP according to protocols available via http://www.cerep.fr/Cerep/Users/index.asp.

## OVA mouse study protocols

### Peritonitis model[5]

**Animals.** Male mice Balb/cByJ@ICo (Charles River Laboratories– France) weighing 20 to 25 g were housed in a temperature (19.5-24.5°C) and relative humidity (45-65%) controlled room with a 12-h light/dark cycle, with ad libitum access to filtered tap-water and standard pelleted laboratory chow (SAFE, France) throughout the study.

**Allergen sensitization and challenge protocol.** On day 0, each mouse was weighed and then immunized subcutaneously (s.c) with 0.2 mL of ovalbumin (A5503 Sigma, France) solution at 0.5 mg/mL (equivalent to 100 µg of ovalbumin). On day 7, each mouse was again weighed and immunized subcutaneously (s.c) with 0.2 mL of ovalbumin solution at 0.5 mg/mL (equivalent to 100 µg). Ovalbumin challenge: On day 14 following weighing, each mouse was injected intraperitoneally (i.p.) with 0.5 mL of ovalbumin solution at 2 µg/mL (equivalent to 1 µg of ovalbumin) 1 hour after oral administration of the test substances or hydrocortisone or vehicle.

**Collection of abdominal fluid.** 24 hours after ovalbumin challenge, each mouse was injected intraperitoneally with 5 mL of sterile PBS containing 3 mM EDTA under isoflurane anaesthesia. After gentle massaging of the abdomen (at least 30 seconds), the total lavage fluid was aspirated with a syringe from the each abdomen.

**Determination of total cell number in abdominal fluid**. An aliquot of each sample was diluted (by a factor of 2) in 6% (v/v in water) acetic acid to lyse red blood cells. The total leukocyte count was calculated using a Malassez hemocytometer under microscopy (x 40 objective) (Optical microscope Olympus CK2). The cell number was calculated using a fraction of 1 $mm^3$ from each sample. The total cell number for each sample was calculated using the total quantity of fluid (/cavity x 106 and /mL x 106).

**Determination of differential cells counts in abdominal fluid.** A further aliquot of each sample was diluted (1/40 v/v in PBS) before cytospin centrifugation. A slide of each sample was obtained after centrifugation at 800 RPM for 5 min using Cytospin3®. The cytospin preparations was air-dried and stained in May Grünwald solution for 5 min. Then they were rinsed using distilled water and Giemsa stain was applied for 2 min then the slides was rinsed in distilled water. The number of eosinophils was calculated by multiplying the total cell number times the percentage of eosinophils on the differential count (/cavity x 106 and /mL x 106).

**Treatment with CRTH2 antagonists in vivo**. The test substances or hydrocortisone or vehicle was administered by oral route (p.o.) in a coded and random order with a volume of 10 mL/kg (equivalent to 0.20 mL/20 g).

**Data Analysis**. Software "Sigmastat ™" version 2.0.3, SPSS Science software, Erkrath, GmbH.

**Results**:

| Treatment | | Total abdominal fluid (mL) | Cell number / mL (x $10^6$) | |
|---|---|---|---|---|
| | | | Total cells | Eosinophils |
| Vehicle p.o. | Mean<br>± SEM (n) | 2.4<br>±0.4 (4) | 2.6<br>±0.7 (4) | 0.90<br>±0.21 (4) |
| Hydrocortisone<br>100 mg/kg p.o. | Mean<br>± SEM (n) | 3.0<br>±0.1 (4) | 1.2<br>±0.2 (4) | 0.06 (94% inhib)<br>±0.02 (4) |
| **19**<br>30 mg/kg p.o. | Mean<br>± SEM (n) | 2.6<br>±0.2 (5) | 1.6<br>±0.2 (5) | 0.34 (62% inhib)<br>±0.10 (5) |
| **19**<br>100 mg/kg p.o. | Mean<br>± SEM (n) | 3.0<br>±0.2 (4) | 1.8<br>±0.3 (4) | 0.40 (55% inhib)<br>±0.15 (4) |

**Lung model**[6]

**Animals.** Female BALB/c mice 6 weeks old (MoB A/S; Ry, Denmark) were kept in well-controlled animal housing facilities and fed ad libitum. The study was approved by the Regional Ethics Committee in Malmö-Lund, Sweden.

**Allergen sensitization and challenge protocol.** All groups of mice (n=10 in each group) were immunized i.p with 10 μg OVA (grade V; Sigma, St Louis, Mo) absorbed to alum adjuvant ($Al(OH)_3$; Sigma) at day 0 as described previously [1] . Fourteen days after immunization mice were exposed to aerosolized OVA (1 % wt / vol) 30 minutes daily for 2 days. Control animals received saline challenge. Animals were sacrificed 24 h after the last allergen challenge by pentobarbital i.p and lung tissue was collected and put in Stefanini fixative for histological analysis.

**Treatment with CRTH2 antagonists in vivo.** On days 14 and 15 mice were treated twice daily with **19** 5 mg/kg orally. Control animals received vehicle (PBS 10%Tween80). Drugs were administered 1 hour before each challenge and then once more 4 hours after challenge.

**Staining and quantification of lung tissue eosinophils and mucus-containing cells.** Eosinophils were detected by histochemical visualization of cyanide-resistant eosinophil peroxidase (EPO) activity. In brief, 5 μm cryo sections were incubated for 8 min at room temperature in PBS buffer (pH 7.4) supplemented with 3.3-diaminobenzidine tetrahydrochloride (60 mg / 100 ml; Sigma), 30% $H_20_2$ (0.3 ml / 100 ml), and NaCN (120 mg / 100 ml). Slides were then rinsed in tap water and mounted in Kaisers medium (Merck, Darmstadt, Germany). Eosinophils were identified by their dark brown reaction product and eosinophils were counted around bronchi using a depth of 120 μm from the epithelial basement membrane and expressed as cells/0.1$mm^2$ tissue area as previously described [6]. 5 μm cryo sections were stained with periodic acid-Schiff reagent (PAS) and the total number of PAS-positive cells counted and expressed as cells/mm basement membrane as previously described [7]

**Data Analysis.** Histology analyses were performed and quantified in a blinded manner. For statistical analysis Wilcoxon Rangsumtest was performed using Analyze It™ (Analyse-it software, Ltd. Leeds, UK). Data are expressed as mean ± SEM. A value of $p<0.05$ was considered statistically significant.

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