Supporting Information

Optimization of the Central Core of Indolinone-Acetic Acid based CRTH2 (DP2) Receptor Antagonists

Stefano Crosignani, Catherine Jorand-Lebrun, Patrick Page, Gordon Campbell, Véronique Colovray, Marc Missotten, Yves Humbert, Christophe Cleva, Jean-François Arrighi, Marilène Gaudet, Zoe Johnson, Pamela Ferro, André Chollet.*

Merck Serono S.A., 9 chemin des Mines, CH-1202 Geneva, Switzerland.

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General

The HPLC data provided were obtained as follows:

Condition A: Column Waters XbridgeTM C8 50 mm x 4.6 mm at a flow of 2 mL/min; 8 min gradient from 0.1 % TFA in H2O to 0.07 % TFA in CH3CN.

Condition B (chiral HPLC): Column Chiralcel OJ-H, 250 x 4.6 mm at a flow of 1 mL/min; eluant 0.1 % formic acid in methanol. UV detection (maxplot) for all conditions.

The MS data provided were obtained using a LC/MS Waters ZMD (ESI)

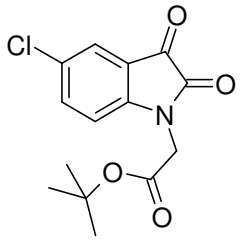
The NMR data were obtained on a Bruker DPX-300MHz.

Preparative HPLC purifications were performed with a mass directed autopurification Fractionlynx from Waters equipped with a Sunfire Prep C18 OBD column 19x100 mm 5 μm, unless otherwise reported. All HPLC purifications were performed with a gradient of ACN/H_2O or ACN/H_2O/HCOOH (0.1%).

Optical rotations were measured using an APP220 Bellingham Stanley Ltd Polarimeter, with a cell length of 1 dm, using a sodium "D" light source (589 nm) at 25 °C.

***tert*-Butyl (5-chloro-2,3-dioxo-2,3-dihydro-1*H*-indol-1-yl)acetate**

(13)

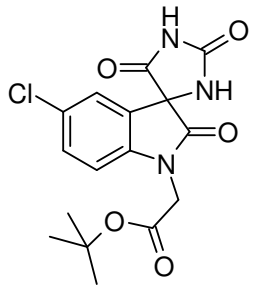


5-Chloroisatin (50.10 g; 275.9 mmol) was dissolved in 520 mL of DMF and the orange solution was cooled in an ice/water-bath. Potassium carbonate (49.57 g; 358.7 mmol) was added in several portions over 45 minutes. Stirring was continued for 1.5 hours at 0 °C, until a thick, difficult to stir solution was obtained. The solution was brought back to RT, then DMF (100 mL) was added and *tert*-butyl bromoacetate (44.8 ml; 304 mmol) was added dropwise, keeping the temperature below 30 °C with a cold water bath. Stirring was continued overnight at RT.

The reaction was quenched by careful addition of water (750mL) of water, upon which precipitation occurred. The solid was filtered and dried under vacuum to give the title compound (53.4 g; 65.5%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.59-7.52 (m, 2 H), 6.73-6.70 (m, 1 H), 4.37 (s, 2 H), 1.44 (s, 9 H). MS(ESI^-): 294.2

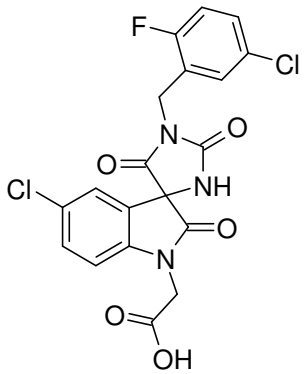
Intermediate 54: *tert*-Butyl (5'-chloro-2,2'5'-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl)acetate (14)



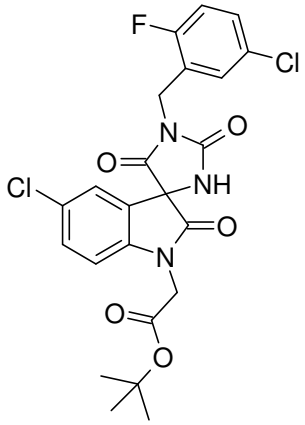
tert-Butyl (5-chloro-2,3-dioxo-2,3-dihydro-1*H*-indol-1-yl)acetate (**13**, 27.11 g; 91.67 mmol), potassium cyanide (7.94 g; 122 mmol) and ammonium carbonate (70.47 g; 733.4 mmol) were suspended in ethanol/water (600 mL; 2:1). The light orange suspension was stirred at reflux for 1.5 h, then the purple solution was poured into 1.5 L of ice-water and acidified to pH = 5 with acetic acid (initial pH = 11). The suspension was kept at 0-5 °C for approx. 1 h, then the precipitate was filtered off, washed with cold water (3 * 150 mL) and dried under high vacuum overnight to yield the title compound (27.88 g; 92%) as a purple solid which was used for the next steps without further purification.

^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 11.47 (bs, 1 H), 8.80 (bs, 1 H), 7.71 (m, 1 H), 7.58-7.55 (m, 1 H), 7.21-7.18 (m, 1 H), 4.51 (s, 2 H), 1.40 (s, 9 H). LC/MS: (ES^-): 364.3. HPLC (Method A) 80 %; R_t 3.48 min.

[5'-chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid (11b)



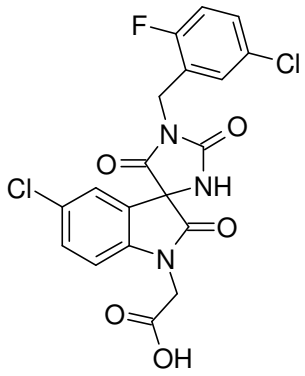
*Step 1: tert-butyl [5'-chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'H)-yl]*acetate **(15b)**



To a solution of *tert*-butyl (5'-chloro-2,2'5'-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl)acetate (**14**, 7.90 g, 21.59 mmol) in *N,N*-dimethylformamide (80 ml) potassium carbonate (3.58 g, 25.91 mmol) was added portionwise at 0-5 °C. Stirring was continued at 0-5 °C for 45 minutes and additional 90 minutes at ambient temperature. The suspension was treated with 5-chloro-2-fluorobenzyl bromide and stirred for one hour at ambient temperature. The solid was filtered, the filtrate was partitioned between water (400 ml) and *tert*-butyl methyl ether (200 ml) and the product was extracted with *tert*-butyl methyl ether (3 x 150 ml). The combined extracts were washed with water (400 ml) and dried ($MgSO_4$) to give a pink residue. Recrystallization from toluene yielded the title compound (6.47 g, 59 %) as slightly pink solid

^{1}H NMR (DMSO-d_6) δ 9.12 (s, br, 1 H), 7.48 (d, *J*= 2.3 Hz, 1 H), 7.32 (dd, *J*= 8.3 Hz, *J*= 2.3 Hz, 1 H), 7.25-7.21 (m, 1 H), 7.13-7.07 (m, 2 H), 6.97 (d, *J*= 8.3 Hz, 1 H), 4.67 (s, 2 H), 4.54 (s, 2 H), 1.40 (s, 9 H). MS(ESI$^+$): 508.2. HPLC (Condition A): Rt 5.09 min (HPLC purity 96%).

*Step 2: [5'-chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'H)-yl]acetic acid **(11b)***

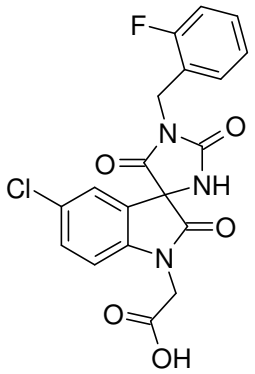


An ice-cold solution of *tert*-butyl [5'-chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetate (**15b**, 8.86 g; 15.5 mmol) in DCM (170 ml) was treated with trifluoroacetic acid (23 ml). After stirring for 4 hours at RT the solvents were evaporated. Toluene was added twice and evaporated to give the title compound as a beige solid (6.48; 93%)

^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13,30 (bs, 1H), 9.34 (s, 1H), 7.68 (d, *J*= 2.0 Hz, 1H), 7.53 (d, *J*= 8.5 Hz, *J*= 2.2 Hz, 1H), 7.48-7.42 (m, 1H), 7.34-7.28 (m, 2H), 7.21 (d, *J*= 8.5 Hz, 1H), 4.68 (s, 2H), 4.58 (d, *J*= 17.9 Hz, 1H), 4.53 (d, *J*= 17.9 Hz, 1H). MS(ESI$^+$): 452.1; MS(ESI$^-$): 454.4; HPLC (Condition A): Rt 3.98 min (HPLC purity 99.6%).

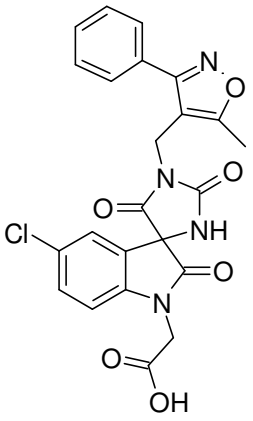
The following products were prepared according to the same two-steps protocol described for **11b**:

[5'-chloro-1-(2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid (11a)



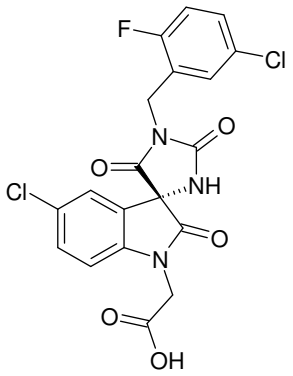
^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.3 (bs, 1H), 9.31 (s, 1H), 7.67 (d, *J*= 2.2 Hz, 1H), 7.53 (dd, *J*= 8.5 Hz, *J*= 2.2 Hz, 1H), 7.41-7.18 (m, 5H), 4.68 (s, 2H), 4.55 (s, 2H). MS(ESI$^+$): 418. HPLC (Condition A): Rt 3.68 min (HPLC purity 97.2%).

[5'-chloro-1-[(5-methyl-3-phenylisoxazol-4-yl)methyl]-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid (11c)



^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.2 (bs, 1H), 9.19 (s, 1H), 7.67-7.64 (m, 2H), 7.51-7.46 (m, 4H), 7.30 (d, *J*= 2.1 Hz, 1H), 7.16 (d, *J*= 8.5 Hz, 1H), 4.63-4.45 (m, 4H), 2.46 (s, 3H). MS(ESI$^+$): 481.2. HPLC (Condition A): Rt 3.78 min (HPLC purity 96.3%).

(+)-(*S*)-[5'-Chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2*'H*)-yl]acetic acid ((*S*)-11b)



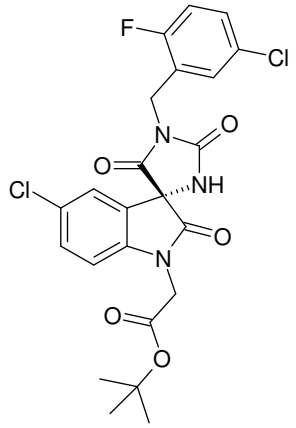
The two enantiomers of [5'-Chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2*'H*)-yl]acetic acid **11b**, were separated by chromatography on a Daicel OJ 20 microns stationary phase, using methanol (containing 0.05% TFA) as eluant, with a flow of 300 mL/min. The compound was fed at a concentration of 20 mg/mL. Each run lasted 12 minutes, and the retention times for the two enantiomers were respectively 6.57 min and 9.9 min (selectivity 1.6).

Of the two enantiomers obtained, the first eluting enantiomer showed the better activity on DP2. While the absolute configuration for the enantiomers of **11b** could not be determined due to the difficulty to obtain crystals suitable for X-ray diffraction, the absolute configuration

of the most potent (on CRTH2) enantiomr **(+)-11b** was deduced by analogy with known **(*R*)-6a** and **(*R*)-6b** to be of (*S*) configuration.

^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13,30 (bs, 1H), 9.34 (s, 1H), 7.68 (d, *J*= 2.0 Hz, 1H), 7.53 (d, *J*= 2.2 Hz, *J*= 8.5 Hz, 1H), 7.48-7.42 (m, 1H), 7.34-7.28 (m, 2H), 7.21 (d, *J*= 8.5 Hz, 1H), 4.68 (s, 2H), 4.58 (d, *J*= 17.9 Hz, 1H), 4.53 (d, *J*= 17.9 Hz, 1H). MS (ESI-): 450.1. HPLC (Condition A): Rt 3.79 min (HPLC purity 99.1%). Chiral HPLC (Condition B) Rt 7.44 min (HPLC purity 99.1%). αD= + 49.1 ± 5.6 (c= 0.71 g/100 mL, MeOH).

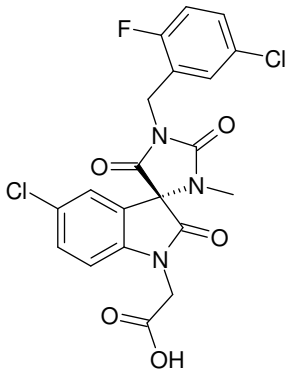
(+)-(*S*)-*tert*-butyl [5'-chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetate ((*S*)-15b)



A solution of (+)-[5'-Chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid (**(*S*)-11b**; 1.00 g; 2.21 mmol) in THF (10 ml) was treated with a solution of *tert*-butyl *N,N'*-diisopropylimidocarbamate (3.54 g; 17.7 mmol) in DCM (2 mL). The reaction mixture was stirred overnight at RT, then left to stand for 1 hour. The white solid was filtered off and washed with DCM, then the solvents were evaporated under vacuum. The residue was purified by flash column chromatography, eluting with cyclohexane containing increasing amounts of EtOAc to give the title compound as a white solid (683 mg, 61%).

^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 9.33 (s, 1H), 7.68 (d, *J*= 2.0 Hz, 1H), 7.54 (d, *J*= 2.2 Hz, *J*= 8.5 Hz, 1H), 7.47-7.42 (m, 1H), 7.34-7.28 (m, 2H), 7.18 (d, *J*= 8.5 Hz, 1H), 4.68 (s, 2H), 4.56 (s, 2H), 1.40 (s, 9H). HPLC (Condition A): Rt 5.13 min (HPLC purity 99.1%). αD= + 64.4 ± 5.6 (c= 0.71 g/100 mL, MeOH).

(+)-(*S*)-[5'-chloro-1-(5-chloro-2-fluorobenzyl)-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid ((*S*)-17a)



Step 1: (+)-tert-butyl [5'-chloro-1-(5-chloro-2-fluorobenzyl)-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'H)-yl]acetate

A solution of (+)-*tert*-butyl [5'-chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetate (**(*S*)-15b**; 2500 mg; 0.91 mmol), iodomethane (63 µl; 1.0 mmol) and K_2CO_3 (253 mg; 1.83 mmol) in DMF (10 mL) was stirred for 3h. The reaction mixture was diluted with water and extracted with EtOAc three times. The combined organic phases were then washed with brine, dried over magnesium sulfate, filtered and concentrated to give the Title compound as a white foam.

MS (ESI+): 539.4. HPLC (Condition A): Rt 5.79 min (HPLC purity 99.7%).

Step 2: (+)-[5'-chloro-1-(5-chloro-2-fluorobenzyl)-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'H)-yl]acetic acid

A solution of (+)-*tert*-butyl [5'-chloro-1-(5-chloro-2-fluorobenzyl)-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetate (422 mg; 0.81 mmol) in DCM (15 ml) and TFA (3 ml) was stirred under nitrogen atmosphere for 3 hours.

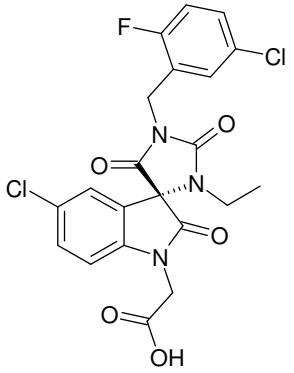
The solvents were removed under vacuum, the residue was redissolved in DCM and washed with water then brine. The organic phase was dried over $MgSO_4$, filtered and concentrated. The oily solid was redissolved in EtOAc and precipitated by addition of cyclohexane. The solid was filtered and dried under vacuum, then redissolved in DCM. The solvent was removed under vacuum to give the Title compound as a white powder.

^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.4 (brs, 1H), 7.77 (d, *J*= 2.2 Hz, 1H), 7.56 (dd, *J*= 8.8, *J*= 2.5 Hz, 1H), 7.41 (m, 1H), 7.32 (m, 2H), 7.27 (d, *J*= 8.5 Hz, 1H), 4.69 (s, 2H), 4.58 (m, 2H), 2.63 (s, 3H). MS (ESI-): 464.1. HPLC (Condition A): Rt 4.30 min (HPLC purity 100%). Chiral HPLC (Condition B): Rt 5.67 min (HPLC purity 99.7%). CHN analysis:

[C20H14N3O5Cl2F - 0.5 H20] Calculated: C 50.54%,H 3.18%,N 8.84%; Found: C 50.56%,H 2.94%,N 8.69%. αD= + 152.6 ± 23.5 (c= 0.71 g/100 mL, MeOH).

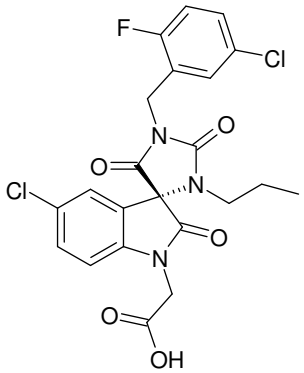
The following products were prepared according to the same two-steps protocol described for **(*S*)-17a**:

[(+)-(*S*)-5'-chloro-1-(5-chloro-2-fluorobenzyl)-3-ethyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid ((*S*)-17b)



^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.4 (brs, 1H), 7.80 (d, *J*= 2.2 Hz, 1H), 7.58 (dd, *J*= 8.5, *J*= 2.2 Hz, 1H), 7.45 (m, 1H), 7.34-7.27 (m, 3H), 4.71 (s, 2H), 4.63 (d, *J*= 17.9 Hz, 1H), 4.58 (d, *J*= 17.9 Hz, 1H), 3.30 (m, 1H), 3.13 (m, 1H), 0.91 (t, *J*= 7.2 Hz, 3H). MS (ESI-): 478.2. HPLC (Condition A): Rt 4.24 min (HPLC purity 95.8%). αD = + 97.1 ± 19.4 (C=0.20 g/100ml, MeOH)

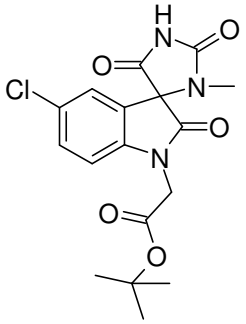
[((+)-(*S*)-5'-chloro-1-(5-chloro-2-fluorobenzyl)-2,2',5-trioxo-3-propylspiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid ((*S*)-17c)



^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.4 (brs, 1H), 7.78 (d, *J*= 2.2 Hz, 1H), 7.57 (dd, *J*= 8.5, *J*= 2.2 Hz, 1H), 7.45 (m, 1H), 7.34-7.26 (m, 3H), 4.72 (s, 2H), 4.60 (d, *J*= 17.9 Hz, 1H), 4.57 (d, *J*= 17.9 Hz, 1H), 3.20 (m, 1H), 3.04 (m, 1H), 1.28 (m, 2H), 0.73 (t, *J*= 7.3 Hz, 3H).

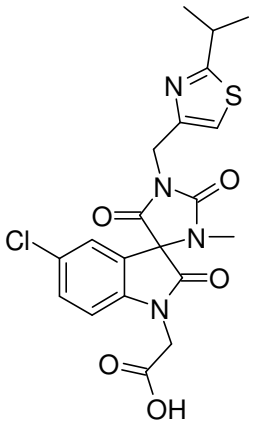
MS (ESI-): 492.3. HPLC (Condition A): Rt 4.46 min (HPLC purity 95.8%). αD = + 48.2 ± 19.3 (C=0.20 g/100ml, MeOH).

tert-butyl (5'-chloro-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl)acetate (18)



A cooled (0 °C) solution of *tert*-butyl (5'-chloro-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl)acetate (**14**; 3.05 g; 8.34 mmol) in THF (50.00 ml) was treated dropwise over 20 minutes with a solution of lithium bis(trimethylsilyl)amide (18.0 ml; 1.00 M; 18.0 mmol) in THF. The reaction solution was stirred for 1 h then iodomethane (0.60 ml; 9.6 mmol) was added dropwise. The reaction solution was then allowed to warm to rt. After stirring for 6 h the reaction mixture was carefully poured into 1M HCl and extracted with EtOAc, the organic phase was dried over $MgSO_4$, filtered and concentrated to give a residue which was then purified by flash column chromatography, eluting with cyclohexane containing increasing amounts of EtOAc to give the title compound as a yellow solid.
^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 11.63 (bs, 1H), 7.75 (d, *J*= 2.2 Hz, 1H), 7.55 (dd, *J*= 8.5, *J*= 2.2 Hz, 1H), 7.22 (d, *J*= 8.5 Hz, 1H), 4.66-4.48 (m, 2H), 2.57 (s, 3H), 1.39 (s, 9H). MS (ESI-): 378.3. HPLC (Condition A): Rt 3.73 min (HPLC purity 78.8%).

[5'-chloro-1-[(2-isopropyl-1,3-thiazol-4-yl)methyl]-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid (20a)



*Step 1: tert-butyl [5'-chloro-1-[(2-isopropyl-1,3-thiazol-4-yl)methyl]-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'H)-yl]acetate (**19a**)*

A solution of 4-(chloromethyl)-2-isopropylthiazole (97 mg; 0.55 mmol) in DMF (0.5 ml) was treated with a suspension of *tert*-butyl (5'-chloro-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl)acetate (**18**; 95 mg; 0.25 mmol), sodium hydrogen carbonate (94 mg; 1.1 mmol) and potassium iodide (10 mg; 0.06 mmol) in DMF (3.00 ml). The reaction mixture was heated at 80 °C for 16 h then cooled and concentrated under vacuum. The residue was dissolved in EtOAc and washed with a sat. NH4Cl solution, then the organic phase was dried on $MgSO_4$, filtered and concentrated to give a residue which was purified by flash column chromatography, eluting with cyclohexane containing increasing amounts of EtOAc to give the title compound as a white solid.

^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 7.68 (d, *J*= 2,.2 Hz, 1H), 7.60 (dd, *J*= 8.5, *J*= 2.2 Hz, 1H), 7.30-7.25 (m, 2H), 4.73 (s, 2H), 4.63 (d, *J*= 17.9 Hz, 1H), 4.59 (d, *J*= 17.9 Hz, 1H), 3.25 (m, 1H), 2.67 (s, 3H), 1.39 (s, 9H), 1.33 (d, *J*= 6.9 Hz, 3H), 1.31 (d, *J*= 6.9 Hz, 3H). MS (ESI+): 519.2. HPLC (Condition A): Rt 5.02 min (HPLC purity 96.7%).

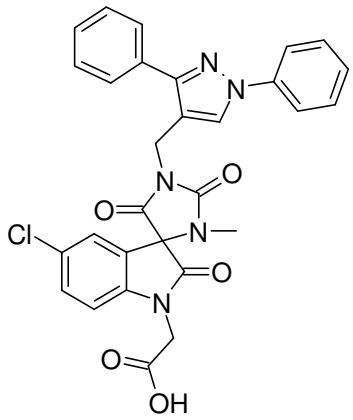
Step 2: [5'-chloro-1-[(2-isopropyl-1,3-thiazol-4-yl)methyl]-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'H)-yl]acetic acid

A solution of *tert*-butyl [5'-chloro-1-[(2-isopropyl-1,3-thiazol-4-yl)methyl]-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetate (**19a**; 41 mg; 0.08 mmol) in HCl in Dioxane (4 N, 5 ml) was stirred for 16 h then concentrated. The residue was redissolved in a mixture of DCM/Et_2O and concentrated then dried under vacuum to give a yellow solid (39 mg, quant.)

^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.3 (bs, 1H), 7.68 (d, *J*= 2.2 Hz, 1H), 7.58 (dd, *J*= 8.5, *J*= 2.2 Hz, 1H), 7.31 (d, *J*= 8.5 Hz, 1H), 7.28 (s, 1H), 4.73 (s, 2H), 4.63 (d, *J*= 17.8 Hz, 1H), 4.59 (d, *J*= 17.8 Hz, 1H), 3.27 (sep, *J*= 6.9 Hz, 1H), 2.66 (s, 3H), 1.32 (d, *J*= 6.9 Hz, 3H), 1.31 (d, *J*= 6.9 Hz, 3H). MS (ESI-): 461.1. HPLC (Condition A): Rt 3.80 min (HPLC purity 93.8%).

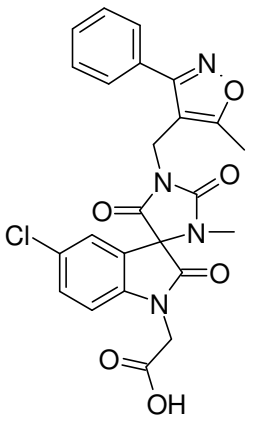
The following products were prepared according to the same two-steps protocol described for **20a**:

[5'-chloro-1-[(1,3-diphenyl-1H-pyrazol-4-yl)methyl]-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid (20b)



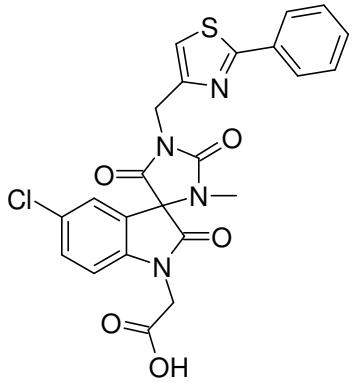
^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 8.36 (s, 1H), 7.85 (d, *J*= 7.9 Hz, 2H), 7.77 (d, *J*= 7.2 Hz, 2H), 7.66 (s, 1H), 7.60-7.24 (m, 8H), 4.90-4.70 (m, 2H), 4.69-4.50 (m, 2H), 2.66 (s, 3H). MS (ESI-): 554.2. HPLC (Condition A): Rt 4.61 min (HPLC purity 97.8%).

[5'-chloro-3-methyl-1-[(5-methyl-3-phenylisoxazol-4-yl)methyl]-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2'*H*)-yl]acetic acid (20c)



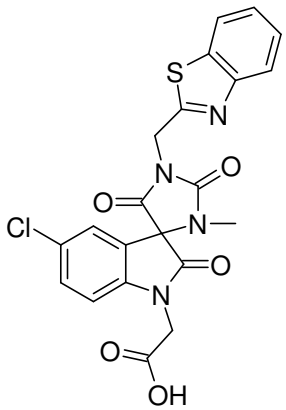
^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.3 (bs, 1H), 7.69-7.62 (m, 2H), 7.57-7.42 (m, 5H), 7.25 (d, *J*= 8.4 Hz, 1H), 4.66-4.44 (m, 4H), 2.57 (s, 3H), 2.46 (s, 3H). MS (ESI-): 493.3. HPLC (Condition A): Rt 3.92 min (HPLC purity 88.4%).

[5'-chloro-3-methyl-2,2',5-trioxo-1-[(2-phenyl-1,3-thiazol-4-yl)methyl]spiro[imidazolidine-4,3'-indol]-1'(2*'H*)-yl]acetic acid (20d)



^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.4 (bs, 1H), 7.96-7.89 (m, 2H), 7.68 (d, *J*= 2.2 Hz, 1H), 7.58 (dd, *J*= 8.5, *J*= 2.2 Hz, 1H), 7.55-7.47 (m, 4H), 7.30 (d, *J*= 8.5 Hz, 1H), 4.83 (s, 2H), 4.64 (d, *J*= 17.9 Hz, 1H), 4.56 (d, *J*= 17.9 Hz, 1H), 2.68 (s, 3H). MS (ESI-): 495.2. HPLC (Condition A): Rt 4.12 min (HPLC purity 97.1%).

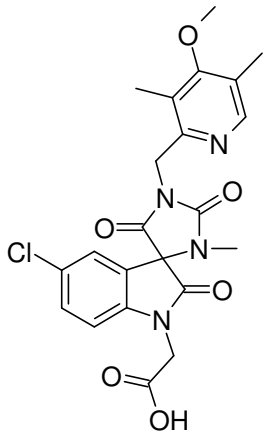
[1-(1,3-benzothiazol-2-ylmethyl)-5'-chloro-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2*'H*)-yl]acetic acid (20 e)



^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.40 (bs, 1H), 8.13 (d, *J*= 7.3 Hz, 1H), 8.01 (d, *J*= 7.7 Hz, 1H), 7.78 (d, *J*= 2.2 Hz, 1H), 7.64-7.42 (m, 3H), 7.31 (d, *J*= 8.5 Hz, 1H), 5.17 (s, 2H),

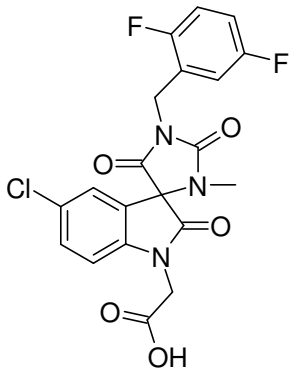
4.63 (d, *J*= 17.7 Hz, 1H), 4.55 (d, *J*= 17.7 Hz, 1H), 2.70 (s, 3H). MS (ESI-): 469.2. HPLC (Condition A): Rt 3.81 min (HPLC purity 96.5%).

[5'-chloro-1-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2*'H*)-yl]acetic acid (20 f)



^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.4 (bs, 1H), 8.15 (s, 1H), 7.63-7.56 (m, 2H), 7.28 (d, *J*= 8.4 Hz, 1H), 4.77 (s, 2H), 4.58 (d, *J*= 17.9 Hz, 1H), 4.53 (d, *J*= 17.9 Hz, 1H), 3.73 (s, 3H), 2.68 (s, 3H), 2.22 (s, 3H), 2.21, (s, 3H). MS (ESI-): 471.2. HPLC (Condition A): Rt 2.58 min (HPLC purity 96.3%).

[5'-chloro-1-(2,5-difluorobenzyl)-3-methyl-2,2',5-trioxospiro[imidazolidine-4,3'-indol]-1'(2*'H*)-yl]acetic acid (20 g)



^{1}H NMR (300MHz, DMSO-d_6) δ [ppm] 13.4 (bs, 1H), 7.79 (d, *J*= 2.2 Hz, 1H), 7.58 (dd, *J*= 8.5, *J*= 2.2 Hz, 1H), 7.37-7.18 (m, 3H), 7.09 (m, 1H), 4.72 (s, 2H), 4.62 (d, *J*= 17.8 Hz, 1H), 4.56 (d, *J*= 17.8 Hz, 1H), 2.65 (s, 3H). MS (ESI-): 448.2. HPLC (Condition A): Rt 3.90 min (HPLC purity 98.9%).

Biological assays

Preparation of hCRTH$_2$-CHO expressing cell membranes

Adherent CHO cells expressing hCRTH2 (Euroscreen, Belgium) were cultured in 225 cm^2 cell culture flasks (Corning, USA) in 30ml of medium. After two rinses of phosphate buffered saline (PBS), cells were harvested in 10ml of PBS containing 1mM EDTA, centrifuged at 500 x g for 5 min at 4°C and frozen at –80°C. The pellet was re-suspended in 50 mM Tris-HCl, pH 7.4, 2mM EDTA, 250mM Sucrose, containing protease inhibitor cocktail tablets, (Complete EDTA-free, Roche, Germany) and incubated 30 min at 4°C. Cells were disrupted by nitrogen cavitation (Parr Instruments, USA) at 4°C (800 p.s.i. for 30 min), and centrifuged at 500 x g for 10min at 4°C. Pellet containing nuclei and cellular debris was discarded and supernatant was centrifuged 60 min at 4°C at 45000 x g. Membrane pellet was re-suspended in storage buffer (10mM HEPES/KOH pH 7.4, 1mM EDTA, 250mM sucrose, protease inhibitor cocktail tablets) using Dounce homogenization and frozen in liquid nitrogen, and stored at –80°C.

Radioligand binding assay

The compounds inhibit the binding of PGD2 to its receptor CRTH2. The inhibitory activity can be investigated by a radioligand binding Scintillation Proximity Assay (SPA) (Sawyer et al., Br. J. Pharmocol 2002, 137, 1163-72). The SPA radioligand binding assay was performed at room temperature in binding buffer (10mM HEPES/KOH pH 7.4, 10mM MnCl2, with protease inhibitor cocktail tablets), containing 1.5nM [^{3}H]PGD2 (Perkin Elmer), 10-50µg/ml of hCRTH$_2$-CHO cell membrane protein and 2mg/ml of Wheat-germ agglutinin Scintillation Proximity Assay beads (RPNQ0001, GE-Healthcare) in a final volume of 100µl in 96 well plates (Corning, USA). Non-specific binding was determined in the presence of 10µM PGD2 (Cayman, USA). Competing Compounds of Formula (I) were diluted in dimethylsulphoxide so that the total volume of dimethylsulfoxide was kept constant at 1% dimethylsulphoxide (Me_2SO). Serial dilutions of 100µM to 100 pM were prepared and 10 µl each of the compounds of Formula (I) stock solutions were added to the binding assay reagents and incubated for 90 min with agitation at room temperature. Binding activity was determined by using a 1450 Micro-beta scintillation counter (Wallac, UK).

PGD2-induced Eosinophil Cell Shape assay in Human Whole Blood

The test compounds were diluted in dimethylsulphoxide so that the total volume of dimethylsulfoxide was kept constant at 2% dimethylsulphoxide (Me2SO). Serial dilutions of 200 µM to 0.09 µM were prepared. Samples of 90 µl of human blood from healthy volunteers (Centre de Transfusion Sanguine de Genève) were pre-incubated in polypropylene Falcon tubes (BD 352063) for 20 minutes in a water bath at 37 °C with 10 µl of diluted compounds. For CRTH2 activation, 100 µl PGD2 (Cayman 12010) at 20 nM was added (10 nM final) to each tube and cells were maintained at 37 °C. Cells treated with PBS were used as a negative control. After 10 minutes, cell activation was stopped with 120 µl Formaldehyde 10% (4% final, Fluka 41650) and cells were rested for 10 minutes at room temperature. Fixed cells were transferred into polypropylene tubes and then treated for 1 hour in a water bath at 37 °C with 2ml of Triton – Surfact-Amps X-100 (Pierce 28314) at 0.166% (0.13% Triton final). After several washes with PBS cells were analyzed by flow cytometry on a FACSCalibur.

***In vivo* Pharmacokinetic Evaluation in Rat and Mouse.**

In order to study the pharmacokinetic (PK) profile of test compounds *in vivo*, Sprague Dawley male rats or C57BL/6 female mice were dosed intravenously or after oral gavage. For both species, test compounds were dosed in solution at 1 mg/kg for i.v. route (10% ethanol, 10% N, N-dimethylacetamide, 30% propylene glycol, 50% water, v/v) and in suspension at 5 mg/kg (0.5% carboxymethylcellulose suspension, containing 0.25% Tween 20 in water) for oral gavage. PK profile in rat was obtained from 3 animals per dosing route and the mouse PK profile was determined using 3 animals at each time point. The volume of administration was 2 mL/kg for i.v. dosing in both species and either 5 mL/kg (rat) or 10 mL/kg (mouse) for oral gavage. Blood samples (100 µL/time point) were collected at 0.083 (5 min), 0.25, 0.5, 1, 4, 7 and 24 hours post-dose for i.v. dosing, and at 0.5, 1, 4, 7 and 24 h for oral dosing, into heparin-Li+ containing tubes. For rats, all blood samples were collected via a catheter in the carotid artery (placed in the artery the day before the experiment), under light isoflurane anesthesia, and stored on ice until centrifugation and plasma isolation. For mouse, blood samples were collected from intracardiac puncture at sacrifice at each time point and processed as described above for the rat. Plasma samples were stored frozen (-20 °C to -70 °C). until analysis. For bioanalysis, samples were processed by protein precipitation (acetonitrile, formic acid 0.1%, addition of 3 volumes) after addition of one internal standard and analysed using a sensitive and selective LC/MS/MS method. An aliquot of the resulting supernatant was subject to LC/MS/MS analysis using a reverse phase column (Waters Xterra,

C8, (3.5 µm particle size, 2.1 x 50 mm) and a short gradient (1 min) from (Solvent A) 85% water, 15% acetonitrile and 0.1% formic acid to (Solvent B) 90% acetonitrile, 10% water and 0.1% formic acid followed by isocratic conditions of Solvent B for 3.5 min at 0.4 mL/min. Column effluent was monitored using a Sciex API 4000 triple quadrupole mass spectrometer with a Turbo V electrospray ion source. Unknown concentrations of test compounds were determined using a calibration curve ranging from 1 to 3000 ng/mL.

Ovalbumin-induced lung eosinophilia in mice

BALB/c mice (6 - 8 weeks old) were immunized with ovalbumin (10 µg i.p) on day 0 and 7. In order to elicit a local inflammatory response in the lung, mice were challenged between days 15 – 17 with a nebulised solution of ovalbumin (10 µg/ml; De Vilibiss Ultraneb 2000, once daily for 30 min during the 3 days). On each separate day between 15 and 17 each animal received via oral gavage the test compound, at t -1 h and t +7 h with respect to OVA exposure at t =0 h. Eight hours after the final OVA challenge, bronchoalveolar lavage (BAL) was then carried out. Total cell numbers in the BAL fluid samples were measured using a haemocytometer. Cytospin smears of the BAL fluid samples were prepared by centrifugation at 1200 rpm for 2 min at room temperature and stained using a DiffQuik stain system (Dade Behring) for differential cell counts.

Data was reported as total and differential number of cells per mL of BALF, mean ± S.E.M. (standard error of the mean). Inter-group deviations were statistically analyzed by a one-way analysis of variance (ANOVA). In the case of significant difference in the mean values among the different levels of treatment, comparisons versus the sham group were carried out using the Dunnett's test. In case the equal variance test fails, a Kruskal-Wallis one-way analysis of variance on ranks followed by a Dunn's test were used. $p < 0.05$ was considered statistically significant.

(*S*)-17a was dosed at 30 mg/kg (oral route, vehicle of carboxymethylcellulose (0.5%w/v) TWEEN 20 (0.25% w/v) in distilled water) and gave a 67%±11% reduction in eosinophils in the BALF compared to vehicle-treated control animals ($P<0.05$).

As reference, dexamethasone (1 mg/kg by ip route) gave a 83%±8% reduction in eosinophils in the BALF compared to vehicle-treated control animals ($P<0.01$).