Supporting Information

Title: Discovery of 6α-Ethyl-23(S)-methyl-cholic Acid (S-EMCA, INT-777) as a Potent and Selective Agonist for the TGR5 Receptor, a Novel Target for Diabesity.

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I. Chemistry

IA. Synthesis and characterization (elemental analysis, mp, NMR, HLPC traces) of 6ECA (21), *S*- and *R*-EMCA (15, 16)

General Methods

Melting points were determined with a Buchi 535 electrothermal apparatus and are uncorrected. NMR spectra were obtained with a Bruker AC 200 MHz or 400 MHZ spectrometer and the chemical shifts are reported in parts per million (ppm). The abbreviations used are as follows: s, singlet; bs, broad singlet; d, doublet; dd, double doublet; m, multiplet. Flash and medium pressure liquid chromatography was performed using Merck silica gel 60 (0.040-0.063 mm) and a LOBAR Lichroprep RP-18 (40-63 μm), respectively. TLC were carried out on pre-coated TLC plates with silica gel 60 F-254 (Merck). Spots were visualized by staining and worming with phosphomolybdate reagent (5% solution in EtOH). All reaction were carried out under a nitrogen atmosphere. Purity of the new compounds was >95% according to the combustion analysis (C,H,N) and HPLC traces.

*Methyl 3α,7,12α-trimethylsilyloxy-5β-cholan-6-en-24-oate (**11**)*

To a solution of diisopropylamine (142mL, 1.01 mol) in dry THF (550 mL) was added dropwise a solution of *n*-Butyllithium (380 mL, 2.5 M in hexane) at –78 °C under nitrogen atmosphere. After 30 min, trimethylchlorosilane (172 mL, 1,35 mol) was added, and the resulting mixture was reacted for additional 20 min. A solution of methyl 3α-hydroxy-7-keto-5β-cholan-24-oate (57 g, 0.135 mol) in dry THF (350 mL) was added dropwise in 40 min. The system was kept to –78 °C for an additional 45 min, and then triethylamine (348 mL, 2.43 mol) was added. After 1 h the reaction mixture was allowed to warm to -20 °C, treated with aqueous saturated solution of $NaHCO_3$ (250 mL), and brought up to room temperature in 3 h. The organic phase was separated,

and aqueous phase was extracted with ethyl acetate (3 x 150 mL). The combined organic phases were washed several times with saturated solution of $NaHCO_3$, water, and brine. After drying over anhydrous Na_2SO_4, the residue was evaporated under vacuum affording to a 97 g of crude compound.

^{1}H-NMR (Acetone-d_6, 400 MHz) δ: 0.55 (s, 3H, 18-CH_3), 0.70 (s, 3H, 19-CH_3), 3.40-3.50 (m, 4H, 3C*H* + COOCH_3), 3.95 (m, 1H, 12-C*H*), 4.55 (dd, 1H, 6-C*H*).

*Methyl-3α,12a-dihydroxy-6-ethylidene-7-keto-5β-cholan-24-oate (**12**)*

To a cooled (-60 °C) and stirred solution of acetaldehyde (17.5 mL, 0.312 mol) and methyl 3α,7,12α-tritrimethylsyliloxy-5β-chol-6-en-24-oate (**11**) (97 g) in dry CH_2Cl_2 (200 mL) was added dropwise a solution of $BF_3{\cdot}OEt_2$ (63 mL, 0.500 mol) in 165 mL of CH_2Cl_2. The reaction mixture was stirred for 2h 30' min at -60 °C and allowed to warm to room temperature. The mixture was quenched with a saturated aqueous solution of $NaHCO_3$ and extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried (Na_2SO_4) and concentrated under *vacuum*. The crude residue was dissolved in dichloromethane (450 mL), treated with HCl (3N, 200mL), and stirred at 0 °C for 1 h. The reaction mixture was quenched with a saturated aqueous solution of $NaHCO_3$ (250 mL) and extracted with CH_2Cl_2. The combined organic phases were dried over Na_2SO_4 anhydrous, filtered and evaporated under reduced pressure. The crude residue was used for the next step without further purifications (85% calculated by NMR analysis).

^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.65 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 3.50-3.65 (m, 4H, 3C*H* + COOCH_3), 4.0 (m, 1H, 12-C*H*), 6.15 (q, 1H, *J*= 7.12, 6-CC*H*CH_2).

*3α,7α,12α-Trihydroxy-6α-ethyl-5β-cholan-24-oic acid (6-ECA, **14**)*

A solution of methyl (Z)-3α,12α-dihydroxy-6-ethylidene-7-cheto-5β-cholan-24-oate (**12**) (5.0 g, 11.61 mmol) in glacial acetic acid/HCl (250 mL/12.5 mL, v/v) was hydrogenised in presence of platinum oxide (0.5 g) at 32 psi for 20 h. The catalyst was filtered off and the filtrate was

concentrated. The residue was taken into a mixture of water (200 mL) and ethyl acetate (150 mL) and neutralized with an aqueous saturated solution of $NaHCO_3$. The separated aqueous layer was extracted with ethyl acetate (3 x 150 mL). The combined organic phases were washed with brine, dried (Na_2SO_4) and evaporated under reduced pressure.

The crude product was hydrolysed overnight using a methanol solution of sodium hydroxide (10%, 500 mL). The mixture was then concentrated under vacuum, diluted with water, acidified with HCl 11 N and extracted with ethyl acetate (3 x 150 mL). The collected organic phases were washed with brine, dried over Na_2SO_4 anhydrous and evaporated under reduced pressure.

The residual was dissolved in a solution of tetrahydrofuran/water (500 mL, 4/1, v/v) and treated with sodium borohydride ($NaBH_4$) overnight at room temperature. After evaporation of the solvents the residue was diluted with water, acidified with HCl 3 N and extracted with chloroform/methanol 9/1 (3 x 150 mL). The combined organic phases were washed with brine, dried over Na_2SO_4 anhydrous and evaporated under reduced pressure. The crude residue was purified by flash chromatography on silica gel using dichloromethane/ methanol (7/3, v/v) as eluent, to afford 3.17 g (7.55 mmol, 55%) of 6-ECA (**14**).

mp: 96-97 °C.

Elemental Analysis: Calcd for $C_{26}H_{44}O_5$: C, 71.05; H, 10.16. Found: C, 71.52, H, 10.63.

^{1}H-NMR ($CDCl_3$+CD_3OD, 400 MHz) δ: 0.65 (s, 3H, 18-C*H*$_3$), 0.84-0.87 (m, 6H, 19-C*H*$_3$ + CH_2CH_3), 0.95 (d, 3H, J = 5.9, 21-C*H*$_3$), 3.33 (m, 1H, 3-C*H*), 3.30-3.0 (bs, 3H, 3 x O*H*), 3.66 (s, 1H, 7-C*H*), 3.92 (s, 1H, 12-C*H*). ^{13}C-NMR ($CDCl_3$+CD_3OD, 100.3 MHz) δ: 11.53, 12.36, 17.08, 20.62, 22.08, 22.66, 23.08, 26.61, 27.42, 28.06, 29.08, 30.91, 33.29, 35.07, 35.52, 35.37, 39.92, 41.34, 41.64, 45.09, 46.37, 46.80, 70.69, 71.93, 72.93, 177.36.

*Methyl 3α,7α,12α-tetrahydropyranyloxy-6α-ethyl-5β-cholan-24-oate (**15**)*

6-ECA (3.17 g, 7.55 mmol) was dissolved in 200 ml of methanol and treated *p*-toluensulfonic acid (145 mg, 0.755 mmol) overnight at room temperature. The reaction mixture was concentrated, the residue taken into H_2O and extracted with AcOEt (3 x 100 ml). The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4, and concentrated under

reduced pressure. To a stirred solution of the methyl ester thus formed **14** (7.55 mmol) and *p*-toluensulfonic acid (0.146 g, 0.76 mmol) in dioxane (65 ml), 3,4-dihydro-2*H*-piran (20.7 ml, 226.5 mmol) was added dropwise in 4 hours and, monitoring the reaction by TLC (petroleum ether/AcOEt 8:2) the resulted mixture was strirred for further 2 hours at room temperature. The reaction was quenched with H_2O (70 ml) and extracted with AcOEt (3 x 50 ml). The reunited organic layer were washed with brine (100 ml), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by flash chromatography using petroleum ether/AcOEt to obtain 3.76 g of **15** (71%) as mixture of diasteroisomers and as pale yellow oil.

^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 0.65 (3H, m, 18-CH_3), 0.89-1.21 (9H, m, 19-CH_3 + 21-CH_3 + CH_2CH_3), 3.30-3.55 (4H, m, 2 x OCH_2), 3.60-3.64 (2H, m, 7-C*H* + 12-C*H*), 3.66 (3H, s, COOCH_3), 3.75-3.90 (3H, m, 3-CH + OCH_2), 4.52-4.91 (3H, m, 3 x OC*H*(CH_2)O).

3α,7α,12α-Trihydroxy-6α-ethyl-23-methyl-5β-cholan-24-oic acid (S- and R-EMCA, ***8****,* ***9****):*

To a solution of diisopropylamine (0.56 ml, 4.026 mmol) in freshly distilled THF (15 ml) cooled at -78°C and under N_2 atmosphere, ^{n}BuLi 2.5N in hexane (1.53 ml, 3.840 mmol) was added dropwise. The reaction was stirred at -78°C for 30' and then a solution of **15** (350 mg, 0.601 mmol) dissolved in freshly distilled THF (7 ml) was added dropwise, and the reaction was stirred at -78° for 90'. Iodomethane (0.56 ml, 9.015 mmol) was then added dropwise, and the solution was stirred at -78°C for 60' and slowly warmed to room temperature overnight. The mixture was then concentrated under reduced pressure, and the resulting residue was diluted with H_2O (30 ml) and extracted with AcOEt (3x30 ml). The reunited organic layers were then washed with brine (30 ml), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was then treated with a solution of MeOH/HCl 37% (20 ml, 20:1 vol/vol) at 45° for 8 h. The mixture was then concentrated under reduced pressure, and the resulting residue was diluted with H_2O (30 ml) and extracted with AcOEt (3x30 ml). The combined organic layers were washed with brine (100 ml), dried over anhydrous Na_2SO_4, and concentrate under reduced pressure. The resulting residue was refluxed with a solution of NaOH 10% in MeOH (15 ml) at 45 °C for 24 h.

The mixture was then concentrated under reduced pressure, and the resulting residue was diluted with H_2O (20 ml), washed with $^{i}Pr_2O$ (3x15 ml), acidify with HCl 3 N, and finally extracted with $CHCl_3$ (3x20 ml). The organic layers was washed with brine (100 ml), dried over anhydrous Na_2SO_4, and concentrate under reduced pressure. The resulting residue was purified by medium pressure chromatography (column: "RP-18 Lobar B", MeOH/H_2O from 5:5 to 6:4, 50 psi) to give **8** (111 mg, 53%) and **9** (63 mg, 30%) as white pure solids.

S-EMCA (8). mp: 150-152 °C. Elemental Analysis: Calcd for $C_{27}H_{46}O_5$: C, 71.96; H, 10.29. Found: C, 72.04, H, 10.90.

^{1}H-NMR ($CDCl_3$+CD_3OD, 400 MHz) δ: 0.61 (s, 3H, , 18-C*H*$_3$), 0.82-0.85 (m, 6H, 19-C*H*$_3$ + CH_2C*H*$_3$), 0.95 (m, 3H, 21-C*H*$_3$), 1.10 (d, 3H, J = 6.8, 23-C*H*$_3$), 2.51 (m, 1H, 23-C*H*), 3.28 (m, 1H, 3-C*H*), 3.62 (m, 1H, 7-C*H*), 3.90 (m, 1H, 12-C*H*). ^{13}C-NMR ($CDCl_3$+CD_3OD, 100.3 MHz) δ: 11.43, 12.29, 17.33, 18.83, 22.04, 22.60, 23.05, 26.56, 27.37, 28.01, 29.79, 33.20, 34.46, 35.30, 35.36, 37.02, 39.83, 40.65, 41.38, 41.62, 45.09, 46.35, 47.62, 70.62, 71.74, 72.92, 179.87.

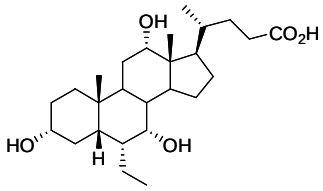
***R*-EMCA (9).** mp: 136-138 °C. Elemental Analysis: Calcd for $C_{27}H_{46}O_5$: C, 71.96; H, 10.29. Found: C, 72.12, H, 10.98.

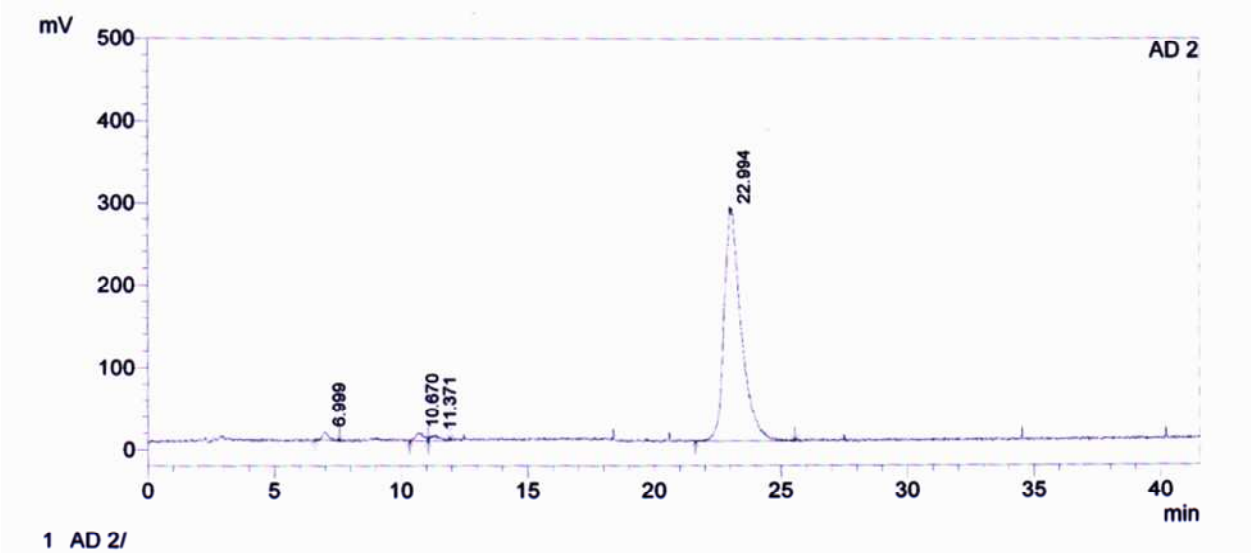
^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 0.56 (s, 3H, 18-C*H*$_3$), 0.75-0.78 (m, 6H, 19-C*H*$_3$ + CH_2C*H*$_3$), 0.84 (m, 3H, 21-C*H*$_3$), 0.93 (m, 3H, 23-C*H*$_3$), 2.27 (m, 1H, 23-C*H*), 3.14 (m, 1H, 3-C*H*), 3.45 (m, 1H, 7-C*H*), 3.78 (m, 1H, 12-C*H*). ^{13}C-NMR (DMSO-d_6, 100.3 MHz) δ: 12.02, 12.69, 16.73, 17.23, 22.50, 23.16, 23.23, 26.85, 28.08, 28.83, 29.71, 30.34, 33.63, 33.92, 35.18, 35.74, 40.53, 41.66, 41.82, 45.66, 46.35, 47.38, 49.01, 69.09, 71.28, 71.68, 179.78.

IB. HPLC Traces of Target Compounds (8, 9 ,14)

The HPLC analyses were carried out on a Shimadzu (Kyoto, Japan) Class-VP equipped with a EZ Start chromatography data software, a LC-10 AT_{VP} pump, a SCL-$10A_{VP}$ system controller, a FCV-$10AL_{VP}$ low pressure gradient formation unit, a DGU-14A on-line degasser and a Rheodyne 7725i injector (Rheodyne, Cotati, CA, USA) with a 20 μL stainless steel loop. A PL-ELS 2100 Ice (Polymer Laboratories Varian, Inc., Amherst, MA, USA) was utilized as the evaporative light scattering detector (ELSD). A SS420X (Scientific Software, Inc., Pleasanton, CA, USA) interface allowed the analog-to-digital conversion of the output signal from the ELSD. An Ultra Aqueous C18 (Restek, Bellefonte, PA, USA) 250 x 4.6 mm i.d., 5 μm, 100Å polar end-capped analytical column was used after previous conditioning by passing through the column the selected mobile phase at a 1.0 mL/min flow rate for at least 30 min. The column temperature was controlled through a Grace (Sedriano, Italy) heather/chiller (Model 7956R) thermostat. The adopted chromatographic condition for the analysis of all compounds were: eluent composition, H_2O/MeCN (Sigma-Aldrich, Milano, Italy) - 50/50 (v,v) buffered with NH_4AcO (Carlo Erba, Milano, Italy) at the total concentration of 5.0 mM and pH* equal to 6.0 obtained after adding concentrated AcOH (Sigma-Aldrich, Milano, Italy); eluent flow rate: 1.0 mL/min; column temperature, 25 °C. The HPLC-grade water was obtained from a tandem Milli-Ro/Milli-Q apparatus (Millipore, Bedford, MA, USA). The mobile phase was always filltered through a 0.22 μm Millipore filter (Bedford, MA, USA) and then degassed with 20 min sonication. The adopted ELSD conditions for the analysis of all compounds were: nebulization temperature, 30 °C; evaporation temperature, 50 °C; gain factor, 2; gas flow rate (air), 1.5 L/min. The injected analyte were always solubilized in the filtered mobile phase.

6α-Ethyl-cholic Acid (14)





PeakTable

AD2 Ch1

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2	10.670	231250	10880	1.680	3.478
3	11.371	166528	7133	1.210	2.281
4	22.994	13219882	284722	96.064	91.029
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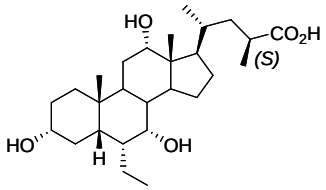
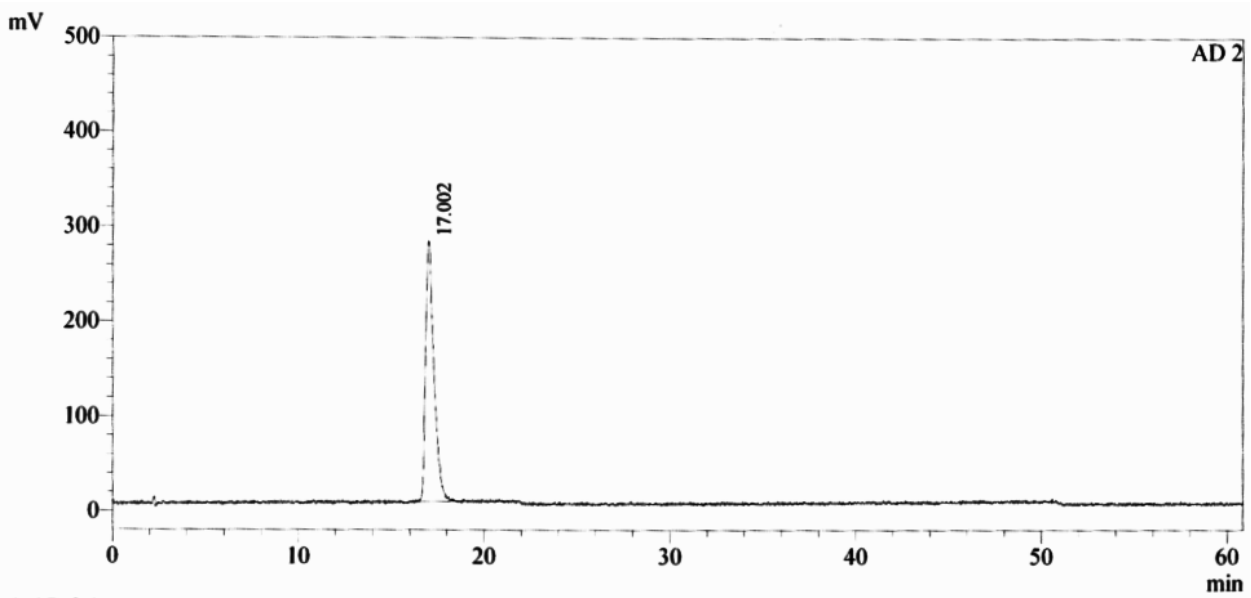
6α-Ethyl-23(*S*)-methyl-cholic Acid (8)





PeakTable

AD2 Ch1

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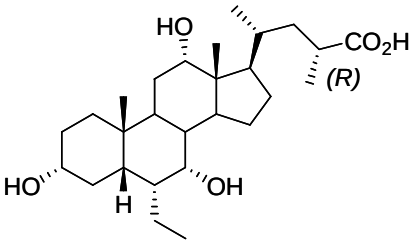
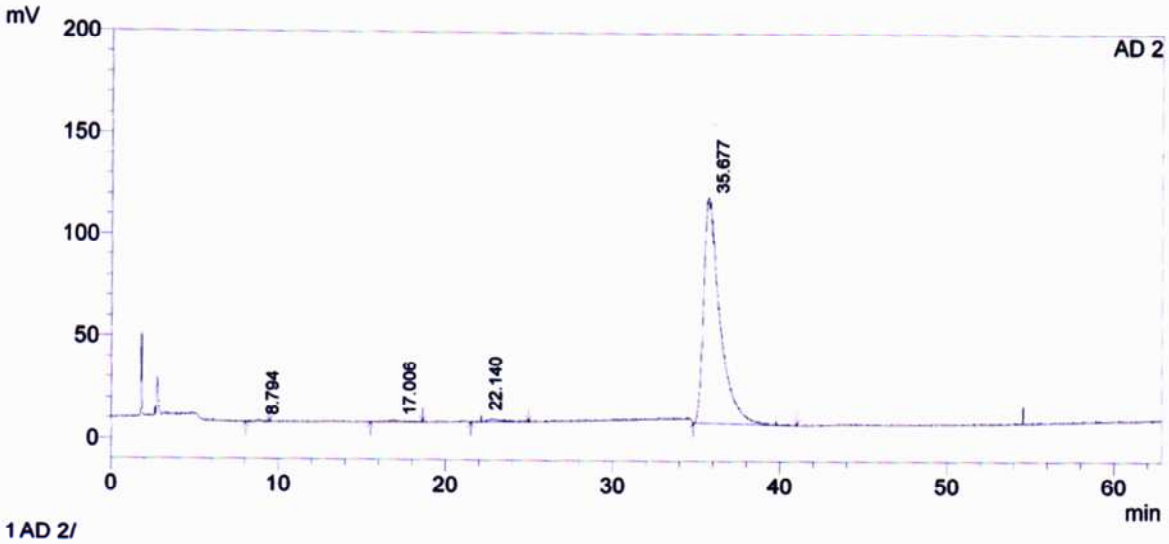
6α-Ethyl-23(*R*)-methyl-cholic Acid (8)





PeakTable

AD2 Ch1

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1	8.794	2036	723	0.027	0.625
2	17.006	12373	673	0.164	0.582
3	22.140	72325	3189	0.960	2.759
4	35.677	7444242	111020	98.848	96.034
Total		7530975	115605	100.000	100.000

II. Biology

IIA. Physicochemical and Biological Properties.

Water solubility, detergency (CMC: critical micellar concentration and surface tension at the CMC), lipophilicity (Octanol/water log P), and albumin binding were measured following previous developed and optimized procedures used for the screening of a large series of natural occurring bile acid and synthetic analogues.

The pharmacokinetics and metabolism were evaluated in bile fistula rat model after iv (femoral) and Id (intraduodensal) infusion of the studied compounds at a dose of 1umol/min/Kg over 1 hour infusion. The effect on bile flow (choleresis) and the recovery of the compounds and the main metabolites were evaluated in bile using HPLC-ES-MS/MS.

IIB. Measurement GLP-1 release *ex vivo*.

The assessment of GLP-1 release from ileal mucosa was performed in 4hr fasted TGR5-Tg mice fed a HF diet for 18 weeks. Mice were sacrificed and an 8 cm section of ileum was collected for organ culture. The ileum was placed in cold HBSS containing 2% horse serum, opened longitudinally and cut into 5-mm-long pieces. These fragments were washed 5 times in HBSS/2% horse serum and then incubated for 10 min in HBSS/2% horse serum containing 1mM 1,4-dithiothreitol at 4°C to remove the excess mucus. After additional washing in HBSS/2% horse serum, tissue pieces were equally distributed in a 24-well plate and incubated in DMEM containing 10% FCS at 37°C. After 4hr stabilization, medium was replaced with medium treated with 0.1% DPP4i (Millipore) and containing either the compound or vehicle (DMSO). After 1hr of treatment, the medium was removed and immediately frozen at -20 °C. Ileal explants were rinsed with cold PBS, then immediately snap-frozen in liquid nitrogen. Protein content was assessed according to Bradford's method. GLP-1 content in culture medium was assayed by ELISA according to manufacturer's instructions (Millipore).