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ACTIVATION OF THE FREE FATTY ACID RECEPTOR GPR40 BY (+)-CURCUPHENOL AND RELATED SYNTHETIC COMPOUNDS

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Keywords: GPR40; *Baccharis genistelloides*; (+)-Curcuphenol; (+)-Curcuphenol-methyl-ether, (+)-Dihydrocurcuphenol; 1-Bisabolone; 1-Bisabolol; Globulol; Bacchopetiolone

ABSTRACT

The bio-guided fractionation of an ethanol extract of *Baccharis genistelloides* (L) Baker, a plant that traditionally is used to treat diabetes, yielded (+)-curcuphenol (**1**) as agonist of GPR40, a cell surface receptor for free fatty acids. This paper describes the agonistic activity of **1** as well as of a series of semi-synthetic and synthetic analogues.

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INTRODUCTION

GPR40 is a cell surface receptor for free fatty acids (FFAs) that belong to the family of G-protein coupled receptors (GPCRs)[1,2,3]. The identification of this novel receptor provided an explanation for many physiological effects attributed to free fatty acids, which previously have been regarded mainly as energy sources and as cell membraneconstituents[2]. GPR40 is activated by medium to long chain FFAs at concentrations corresponding to their physiological plasma levels. Besides FFAs, GPR40 is also activated by anti-diabetic compounds of the thiazolidinedione class such as, rosiglitazone (**2**)[3], MCC-555 (**3**)[4] and by the experimental anti-obesity drug MEDICA 16 (**4**)[5] (Figure 1). The activation of GPR40 by these drugs implies a connection to type IIdiabetes, which was confirmed by the action of GPR40 on insulin secretion from pancreatic β-cells in rodents6 and in humans[7]. A recent knock-out mouse model of GPR40[8] further supports the importance of this receptor as a putative novel target for anti-diabetic therapies. While deletion of the GPR40 gene resulted in protection against several diabetes type II symptoms, including hyperinsulinemia, hepatic steatosis, hypertriglyceridemia, increased hepatic glucose output, hyperglycemia and glucose intolerance, the specific over expression of GPR40 by β-cell was reported to induce diabetes, in mouse.9 On account of GPR40 emerging as a putative therapeutic target the discovery of novel GPR40-interacting scaffolds from natural sources becomesmore important**.**

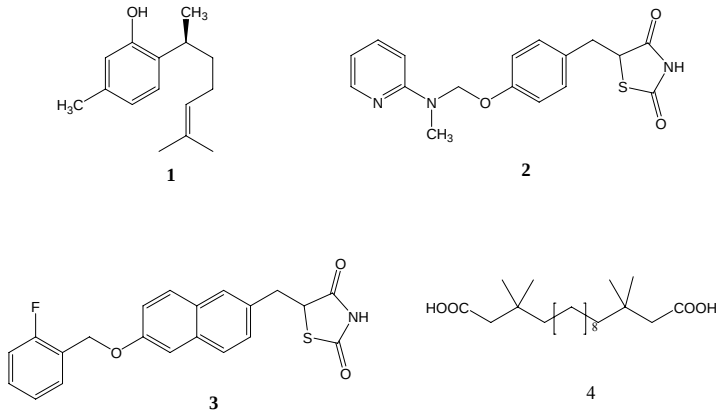

***Fig. 1** Agonists of the receptor GPR40*

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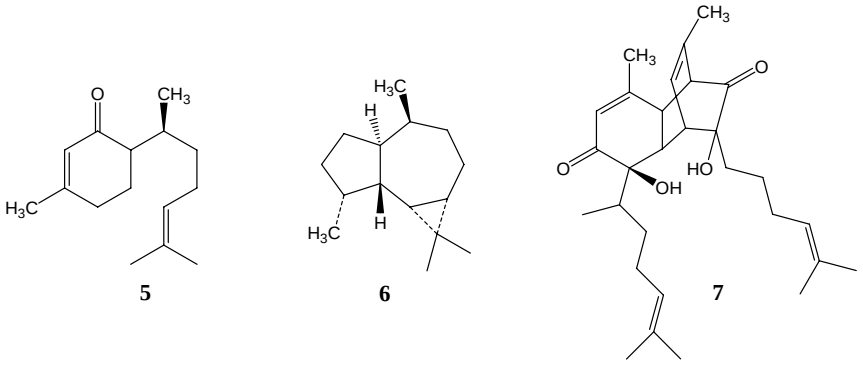
The study of the secondary metabolites from plants used in traditional folk medicine has become an established way to identify novel lead structures in the search for new compounds for the treatment of diseases. In an ongoing study of Bolivian traditional medicine, we decided to search for new agonists of the receptor GPR40 from plants that have been used to treat diabetes. Besides bioassay-guided fractionation, isolation and characterization of active metabolites, chemical modifications of the active molecules and analogues was also planned. A main purpose of our research is to improve our understanding of the molecular basis for the biological activity of natural products. In this paper, we are focusing on the GPR40 receptor and on the structure-activity relationship studies (SAR) of the isolated compounds and their analogues.

RESULTS, DISCUSSION

Baccharis genistelloides (L) Baker, is a small shrub widely distributed in the valleys of Cochabamba, and the infusion of the plant is used by the community of Apillapampa in Bolivia to treat diabetes.10 An ethanol extract of the aerial parts of *B. genistelloides* showed an agonist effect on the receptor GPR40 in the assay developed by Kotarsky *et al*[11] which consists of the stimulation of two kind of cells constructed from HeLa cell lines, GPR40 receptor cells (reporter cells expressing the recombinant receptor) and reporter cells not expressing the receptor HFF11 (negative control). Stimulation of the receptor leads to the activation of a transcription site that initiates production of the firefly luciferase, which is used as a reporter enzyme. This process can be detected by measuring the cells luminescence. The more activated receptor, the more luminescence and the higher values are detected by the luminometer apparatus. The glucose lowering effect in animals of an extract of this plant was also reported previously[12], as well as its anti-arthritic effect[13], gastric cytoprotection properties[14] and antiviral activity[11]. Bioassay-guided fractionation led us to the isolation of (+)-curcuphenol (**1**) as the metabolite responsible for the effect on GPR40. Compound **1** was first isolated from the sponge *Didiscus flavus* and was reported to be cytotoxic towards the tumor cell lines P-388 murine leukemia, A-549 (lung), HCT-8 (colon), and MDAMB (mammary)[15], this compound also inhibits the growth of the fungus *Candida albicans*[16]. (-)-Curcuphenol, the enantiomer, has been reported from the gorgonian soft coral *Pseudopterogorgia rigida*17 and the terrestrial plant *Lasiantheaea podocephala*[18,19]. This is the first report of its agonistic effect on GPR40.
Together with **1**, (-)-1-bisabolone (**5**) [20, 21], (+)-globulol (**6**) [22, 23] and the dimeric sesquiterpene (-)-bacchopetiolone (**7**) [24] were also isolated from the plant extract (Figure 2).



5 **6** **7**

Fig. 2 *Secondary metabolites isolated from Baccharis genistelloides*

The semi-synthetic known analogues (+)-1-bisabolol (**8**) [25, 26, 27], (+)-curcuphenol methyl ether (**9**) [28, 29,30,31] and (+)-dihydrocurcuphenol (**10**)[32] were synthesized. Reduction of the ketone group of **5** with NaBH4 afforded **8**, methylation of the hydroxyl group in **1** with MeI in the presence of K_2CO_3 produced **9** and hydrogenation of the double bond in **1** led to **10** (Figure 3). Compound **9** failed to activate the receptor GPR40, indicating that the hydroxyl group is important for the activity. Compound **5** was also inactive, but compound **8** was active underlining the importance of the hydroxyl group. However, the aromatic ring of **1** is apparently not critical for the activity, as demonstrated by **8**. The fact that **10** is as active as **1** indicates that the double bond in the chain is not essential. Concentration-activity curves were measured for the active compounds **1**, **8** and **10** (Figure 4). From this curve it can be observed that **1** and **10** show similar potency and efficiency, while **8** shows higher efficiency but is less potency.

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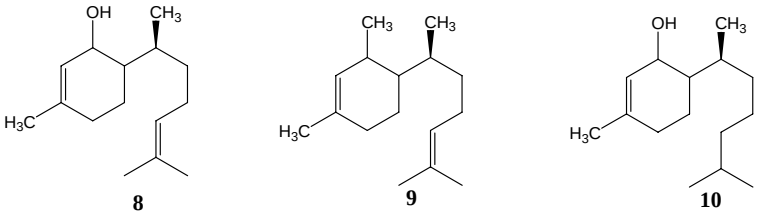


8 **9** **10**

Fig. 3 *Semi-synthetic derivates*

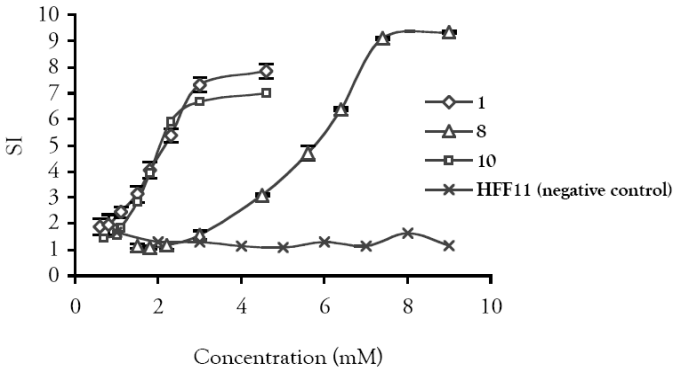
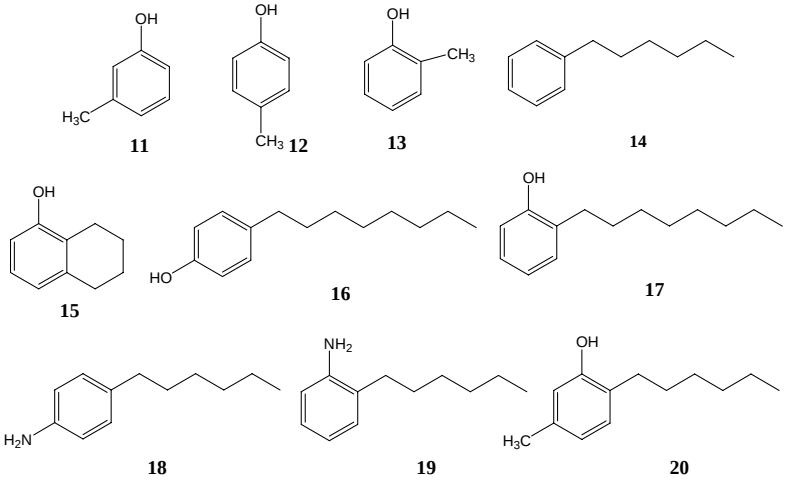


Fig. 4 *Concentration-activity curves for compounds **1**, **8** and **10***

With the purpose of establishing which structural features of these compounds are important for the activation of GPR40, *m*-cresol (**11**), *p*-cresol (**12**), *o*-cresol (**13**), hexylbenzene (**14**), 5,6,7,8-tetrahydronaphtol (**15**), *o*-octylphenol (**16**), *p*-octylphenol (**17**), *o*-hexylaniline (**18**), *p*-hexylaniline (**19**) and 6-hexyl-3-methylphenol (**20**) were assayed (Figure 5).



11 **12** **13** **14** **15** **16** **17** **18** **19** **20**

Fig.5 *Synthetic analogues*

The cresols (**11**-**13**) were inactive, but surprisingly **14** showed a weak activity. The bicyclic phenol **15** was inactive in spite of the hydroxyl group, indicating that a flexible alkyl chain is important. Thus, **16** and **17** were synthesized to compare the effect of a longer chain and the position of the hydroxyl group. As expected, **16** was inactive while **17** was even more efficient than **1**.

Compounds **18** and **19** were prepared with the aim to evaluate the importance of the hydroxyl group, and once more the active compound was found to be the *ortho* derivative. Conversely, **19** was considerably less efficient than **17**, which should reflect the superiority of the hydroxyl group over the amine for the interaction with the receptor. Compound **20** is an analogue of **1** that was synthesized with the intention of determining if the methyl group in the

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lateral chain of **1** is important for the activity and was shown to be less efficient than **1**, but slightly more potent. Consequently, the methyl group in the lateral chain of **1** seems to be important for the activity. **17** showed the highest efficacy but lacks potency, while **20** is more potent but less efficient. Concentration-activity curves for **14**, **17**, **19** and **20** are shown in Figure 6 and EC50 values for these compounds are summarized in Table 1.

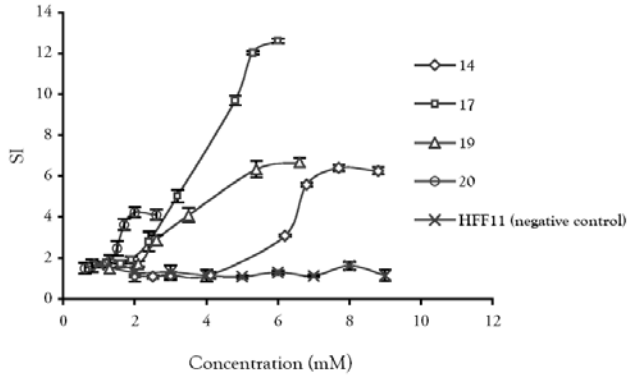

Fig. 6 *Concentration vs activity curves for* ***14***, ***17***, ***19*** *and* ***20***

Table 1*. EC50 values of the active compounds*

Compound	EC50 (mM)
Curcuphenol (**1**)	1.85
Dihydrocurcuphenol (**9**)	2.28
Bisabolol (**8**)	5.80
Hexylbenzene (**14**)	6.22
o-octylphenol (**17**)	3.70
o-aniline (**19**)	3.24
6-hexyl-3-methylt-phenol (**20**)	1.64

CONCLUSIONS

(+)-Curcuphenol (**1**), isolated from *Baccharis genistelloides*, 1-bisabolol (**8**) and (+)- dihydrocurcuphenol (7) activate the receptor GPR40, but at rather high concentrations. Due to the limited number of compounds assayed, the conclusions concerning structure-activity relationships (SAR) that can be made are limited. First, it is not clear if an aromatic ring is necessary, as both, hexyl-benzene (**16)** and (+)-1-bisabolol (**8)** are active. Second, a hydrogen bond donating function on the ring seems to contribute to the compounds efficiency as demonstrated by *o*-octylphenol (**17**), which is the most active and *o*-aniline (**19**) derivative. The presence of an alkyl side chain, next to the hydrogen bond donating function, is also important for activity. The *m*-methyl group in **1** does not seem to play an important role for the efficacy, as demonstrated by the low activity of the 2-hexyl-5-methylphenol (**20**).

EXPERIMENTAL SECTION

1. General

Baccharis genistelloides (Less) Baker, was collected in the province Carrasco by the in Cochabamba, Bolivia in October 2003 a voucher specimen (RB-1102) was authenticated by Rosario Barco, botanist at Martin Cardenas National Herbarium, where it is deposited. *m*-Cresol (**8**), *p*-cresol (**9**), *o*-cresol (**10**), hexyl phenyl (**11**) were purchased from Aldrich Chemical and the 5,6,7,8-tetrahydronaphthol (**12**) was purchased from Acros. The NMR spectra were recorded in a Bruker DRX300 spectrometer at 300 MHz (^{1}H) and at 75 MHz (^{13}C), or a Bruker DRX400 spectrometer (at 400/100 MHz). The chemical shifts (δ) are reported in parts per million relative to chloroform (7.26 ppm for CHCl3 and 77.0 ppm for CDCl3). EIMS spectra (H3PO4 for calibration and as internal

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standard) were recorded with a macromass Q-tof Micro spectrometer and ESIMS spectra were recorded with a JEOL SX 102 spectrometer. Organic extracts were dried over MgSO4. All Chromatography was performed on 60 Å 35-70 µm Matrex silica gel (Grace Amicon). The luciferase assay was carried out following the methodology developed by Kotarsky *et al*[11]. All the assays were carried out on 96-well plate (12 columns and 8 rows). In this assay, two kinds of cells were used. Reporter cells not expressing the receptor (HFF11) approximately 180000 per well, as a negative control and reporter cells expressing the recombinant receptor GPR40, approximately 270000 cells per well. The cells (HFF11 and GPR40) were seeded by duplicate in each half of the 96-well plate. After three days of incubation at 37°C, the compounds in stock solution of 0.1 mg/mL in DMSO are diluted to 1:50 and 1:250 with PBS and then added to the cells. After 6 hours stimulation, the medium is removed and lysis buffer are added to each well. The plate is stored until analysis, usually overnight at –20 °C. Before reading the plates, luciferase (luciferase assay kit biothema, Sweden) is added. Stimulation of the receptor leads to the activation of a transcription site that initiates production of the firefly luciferase, used as a reporter enzyme. This process can be detected by measuring the cells luminescence. The more activated receptor, the more luminescence and the higher values are detected by the luminometer apparatus. For an active compound on the receptor GPR40 values of stimulation index (SI) above two are considered positive. All points represent measurements carried out in triplicate. The data were stadistically analysed using the GraphPad PRISM software package.

2. Chemistry

2.1 (+)-Curcuphenol (**1**), *(-)-1-bisabolone* (**5**), *(-)-globulol* (**8***) and bacchopetiolone* (**7**).

The dried aerial parts of *Baccharis genistelloides* (L) Baker were extracted with ethanol during 3 days. After evaporation of the solvent, the ethanol extract was fractionated by column chromatography using the following solvents: petroleum ether, dichloromethane and methanol, to obtain fractions of different polarity. From this fraction compounds **1**, **5**, **8** and **7** were isolated and characterize and spectral data were identical in all respects to those previously reported[16-21].

2.1 1-Bisabolol (**5**)

A solution of 1-bisabolone (**2**) (24 mg, 0.11 mmol) in 20 mL of anhydrous ether, was cooled to 0°C and LiAlH4 (6 mg, 0.16 mmol) was added. The mixture was then stirred for 4 hrs after which the reaction was quenched by the slow addition of 3 mL of methanol. The mixture was filtered through a pad of celite, the solvent was evaporated, and the residue purified by preparative thin layer chromatography using petroleum ether:EtOAc (4:1) to afford (15 mg, 61%) of 1-bisabolol as a colorless oil. 1H NMR δ 5.39 (1H, br s); 5.12 (1H, t, *J* = 7 Hz); 4.03 (1H, d, *J* = 7.9); 1.99 (2H, m); 1.91 (2H, m); 1.68 (6H, br s); 1.63 (1H, m); 1.31 (4H, m), 0.96 (3H, d, *J* = 6.8 Hz). 13C NMR δ 137.4, 125.7, 124.8, 69.2, 46.5, 35.5, 31.0, 30.5, 26.1, 25.7, 23.1, 20.8, 17.7, 14.4. HRESIMS: m/z 245.1908 calcd for C15H26O + Na, 245.1881.

2.2 (+)-Curcuphenol methyl ether (**6**)

A solution of (**1**) (20 mg, 9.2 mg) in acetone (8 mL), K2CO3 (1 g, 9.2 mmol) and MeI (2 g, 13.8 mmol) were added and the mixture refluxed for 4 hrs. After cooling to r. t. the reaction mixture was diluted with water and extracted with ether. The ether fractions were combined and concentrated to yield the methyl ether derivative (**4**), as a colourless oil. [α]22 D +11° (c 4.3, chloroform); ^{1}H-NMR δ 7.09 (1H, d, *J* = 7.7 Hz); 6.78 (1H, d, *J* = 7.65 Hz); 6.71 (1H, br s); 5.16 (1H, q, *J* = 7 Hz); 3.84 (3H, s); 3.18 (1H, q, *J* = 7 Hz); 2.37 (3H, s); 1.95 (2H, m); 1.71 (3H, s); 1.67 (1H, m); 1.58 (3H, s); 1.55 (1H, m); 1.22 (3H, d, *J* = 7 Hz). ^{13}C-NMR δ (ppm): 156.8, 136.2, 132.8, 131.0, 126.5, 124.9, 121.1, 111.4, 55.3, 37.1, 31.4, 26.3, 25.7, 21.4, 21.1, 17.6. HRESIMS *m/z* 233.1882 calcd for C16H24O + H, 233.1905

2.3 (+)-Dihydrocurcuphenol **(3)**

A solution of (**1**) (20 mg, 9.2 mmol) in ethanol (5 mL) was hydrogenated over Pd/C (10% Pd on C, 2 mg) at atmospheric pressure for 2 hrs. 10 mL of dichloromethane was added and the solution was filtered through a pad of celite. The solvent was evaporated to yield (+)-dihydrocurcuphenol (**7**) as colorless oil (19 mg, 98%). $[\alpha]^{21}$ D +17 c 3.9; ^{1}H-NMR δ (ppm): 7.05 (1H, d, *J* = 7.8 Hz); 6.74 (1H, d, *J* = 7.8 Hz); 6.59 (1H, s), 3.0 (1H, q, *J* = 7 Hz); 1.55 (3H, m); 1.22 (3H, d, J=6.9 Hz); 1.22 (4H, m); 0.86 (3H, d, *J* = 1.8 Hz); 0.83 (3H, d, *J* = 1.8 Hz). ^{13}C- NMR δ (ppm): 153.1, 136.4, 130.4, 126.9, 121.6, 115.9, 39.1, 37.4, 31.9, 27.8, 25.4, 22.6, 21.0, 20.9. HRESIMS *m/z* 221.1935 calcd. For C15H24O + H, 221.1905.

2.4 o-Octylphenol ***(16)*** *and p-octyl-phenol* (**17**)

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A mixture of phenol (300 mg, 3.2 mmol), octyl bromide (135mg, 0.7 mmol) and FeCl3 (2 mg, 0.012 mmol) were heated at 140°C for 4 hrs. After cooling, the mixture was purified by flash column chromatography using petroleum ether : EtOAc (9:1) to afford *p*-octylphenol (30 mg, 22%) and *o*-octyl phenol (12 mg, 8%). 1H and 13C NMR spectra were identical with those already reported[35]. HRESIMS *m/z* 207.1737 cald. for C14H22O + H, 207.1749.

*2.5 o-Hexyl-aniline (***18**) *and p-hexyl-aniline* (**19**)
To a cold mixture (0°C) of HNO3 (500 mg) and H2SO4 (700 mg), hexylbenzene (500 mg, 3 mmol) was added and stirred for 4 hours. Then the mixture was poured into cold water (25 mL) and extracted with ether. The organic phase was dried over MgSO4, and after evaporation of the solvent 450 mg of a mixture of *para* and *ortho* nitrohexylbenzene was obtained. To this mixture, tin (63 mg) and 2 mL of HCl was added and the mixture stirred for 2 hours, after which 20 mL of a saturated solution of NaHCO3 was added, and the mixture extracted with ether. After the organic phase was dried over MgSO4 and the solvent evaporated, the residue was purified by CC using petroleum ether : EtOAc (9.5: 0.5) to afford 50 mg (28%) of the *p*-hexylaniline and 20 mg (11%) of the *o*-hexylaniline. p-Hexylaniline (**18**): ^{1}H-NMR 7.0 (2H, d, *J* = 8 Hz), 6.6 (2H, d, *J* = 8 Hz), 2.51 (2H, t, *J* = 7.5 Hz), 1.64 (2H, m), 1.36 (6H, m), 0.92 (3H, t, *J* = 6.2 Hz). *o*-Hexylaniline (**19**): ^{1}H-NMR δ 7-6.7 (4H, m), 2.51 (2H, t, *J* = 7.5 Hz), 1.64 (2H, m), 1.36 (6H, m), 0.92 (3H, t, *J* = 6.2 Hz). p HRESIMS *m/z* 178.1546 cald for C12H19N + H, 178.1596.

2.6 2-Hexyl-5-methylphenol (**20**)
To Sc(OTf)3 (98 mg, 0.2 mmol) was added m-cresol (100 mg, 1mmol) and acetic acid (1
mmol) in 10 mL of a solution of toluene/nitromethane (10:1) at room temperature. The mixture was stirred for 2 hrs at 100°C and was cooled to room temperature. Water was added to quench the reaction, then 30 mL ether was added. After separation of the organic layer, the aqueous layer was extracted with ether, the combined organic layers were dried over MgSO4 and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel using petroleum ether:EtOAc (9:1) to afford 1-(2-hydroxy-4-methylphenyl)-1-hexanone (80 mg, 40%). This compound was reduced using the Clemmensen method. To a mixture of 400 mg of Zinc (wool) and 30 mg of HgCl2, 8 mL of 40% HCl aq. were added and stirred 5 minutes. The aqueous solution was decantated and the amalgamated zinc covered with 10 mL of a solution of conc. HCl and water (1:1). The 1-(2-hydroxy-4- methylphenyl)-1-hexanone (50 mg, 0.26 mmol) in ethanol was then added immediately. The mixture was then stirred under N2 (12 hr). After that, 30 mL of water was added and the solution was extracted with 20 mL of ether, the organic phase was washed with water and dried over Mg2SO4. After evaporation of the solvent 40 mg (21%)of the desired compound (**16**) was obtained. ^{1}H-NMR δ 7.00 (1H, d, *J* = 7.6 Hz); 6.69 (1H, d, *J* = 7.6 Hz); 6.61 (1H, s); 2.57 (2H, t, *J*1=7.5 Hz and J2=7.9 Hz); 2.28 (3H, s); 1.60 (2H, m); 1.33 (6H, m); 0.89 (3H, t, *J* = 7 Hz). ^{13}C-NMR δ 153.3; 136.8; 129.9; 125.5; 121.3; 115.9; 31.7; 29.9; 29.6; 29.2; 22.6; 20.9, 14.1. HRESIMS *m/z* 193.1556 calcd for C15H20O + H, 193.1592.

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REFERENCES

1. Sawzdargo, M.; George, S. R.; Nguyen, T.; Kolakowski, L. F.; O' Dowd, B. F. *Biochem. Biophys. Res. Commun.*, **1997**, 239, 543-547.
2. Kotarsky, K. L., Nilsson, N. E.; Flodgren, E.; Owman, C.; Olde, B. *Biochem. Biophys. Res. Commun.* **2003**, 301, 406-410.
3. Cantello, B. C.; Cawthorne, M. A.; Cottam, G. P.; Duff, P. T.; Haigh, D.; Hindley, R. M.; Lister, C. A.; Smith, S. A.; Thurlby, P. L. *J. Med. Chem.* **2003**, 37, 3977-3985.
4. Reginato, M. J.; Bailey, S. T.; Krakow, S. L.; Minami, C.; Ishii, S.; Tanaka, H.; Lazar, M. A. *J. Bio. Chem.* **1998**, 273, 32679-84.
5. Russel, J. C.; Shillaber, G.; Bar-Tana, J.; Lau, D. C.; Richardson, M.; Wenzel, L. M.; Graham, S. E.; Dolphin, P. J. *Diabetes*, **1998**, 47, 770-778.