

Supporting Information

Magnolia extract, magnolol and metabolites: activation of cannabinoid CB₂ receptors and blockade of the related GPR55

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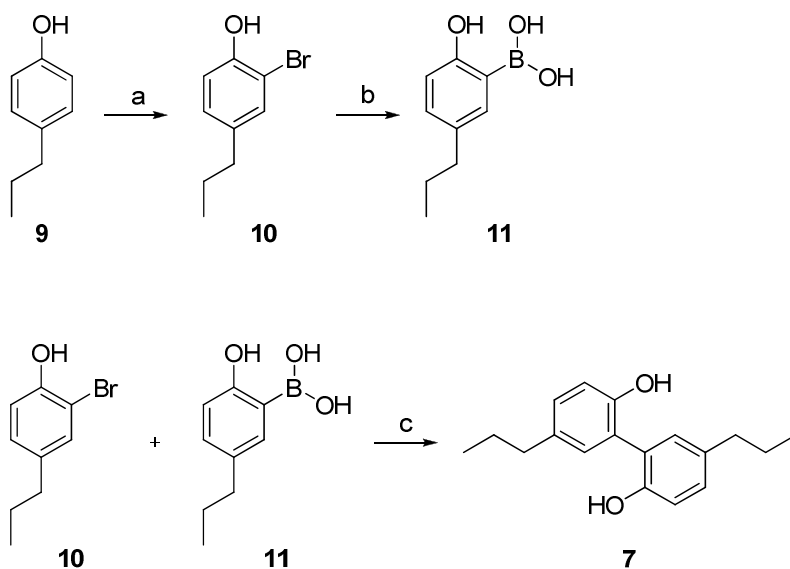
Table of Contents

Page	Topic
S2	Chemical synthesis of compounds
S6	Experimental section
S7	Chemistry: analytical data of compounds
S9	Figure 1. LC/ESI-MS spectrum of 4
S10	Figure 2. LC/ESI-MS spectrum of 7
S11	Figure 3. LC/ESI-MS spectrum of 8
S12	Figure 4. LC/ESI-MS spectrum of 11
S13	Figure 5. Analytical data including HPLC chromatogram of M-extract
S14	Radioligand binding studies at CB ₁ and CB ₂ receptors
S14	cAMP accumulation assays
S15	β-arrestin assays
S16	Figure 6. Radioligand binding assays of <i>trans</i> -isomagnolol at CB receptors
S17	Figure 7. Effects of honokiol and M-extract on CB receptor-mediated cAMP accumulation
S17	Figure 8. Interaction of magnolia-extract with GPR55

- S18 Figure 9. Interaction of CP55,940 with GPR55
S18 Fatty acid amide hydrolyse (FAAH) assay
S20 Monoacylglycerol lipase (MAGL) assay
S21 References

Synthesis of tetrahydromagnolol (**7**) (Scheme 1)^a

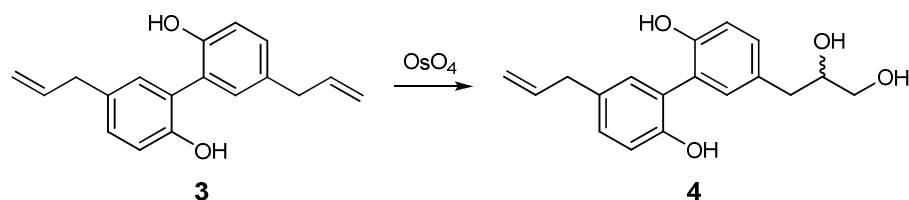
2-Bromo-4-propylphenol (**10**) was synthesized starting from 4-propylphenol (**9**) according to published procedures.¹ Boronic acid (**11**) was obtained by treatment of **10** with butyllithium and subsequent reaction with B(OCH₃)₃ in diethylether, followed by acidic hydrolysis.² The coupling of (**10**) with (**11**) to afford tetrahydromagnolol (**7**) was performed according to the Suzuki-Miyaura cross-coupling reaction procedure.³



^a**Reagents, Conditions:** (a) Br₂, NaHCO₃, CHCl₃, 0°C, 80% yield. (b) three steps (1) butyllithium, Et₂O, -78°C; (2) B(OCH₃)₃, Et₂O, -78°C to rt; (3) HCl, Et₂O, 50% yield. (c) Pd(PPh₃)₄, Na₂CO₃, toluene, EtOH, H₂O, 100°C, 25% yield.

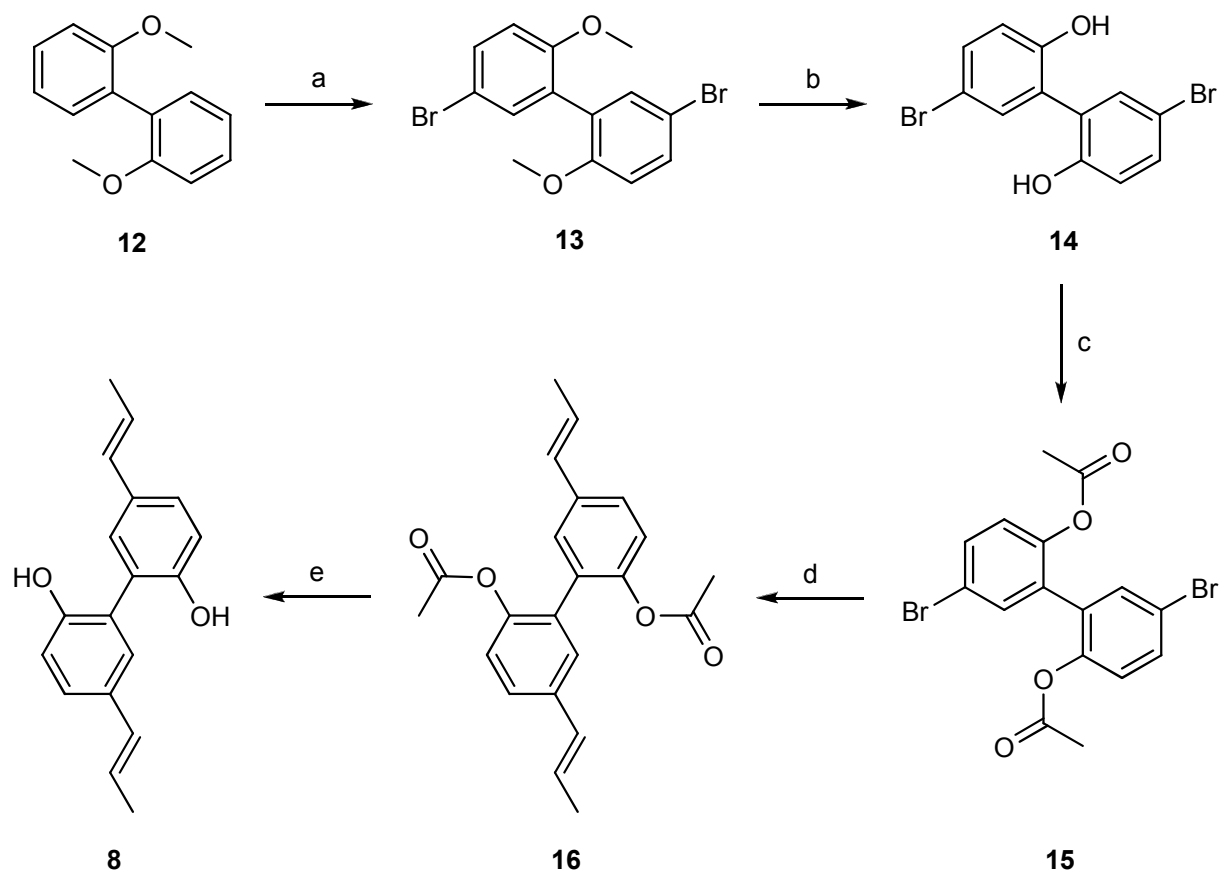
Chemical Synthesis of 8,9-dihydroxydihydromagnolol (4) (Scheme 2)^a

Magnolol (0.8 mmol) was dissolved in a solution of 5 mL of water and 2 mL of acetone in a round-bottom flask. Osmium tetroxide (0.4 mmol) was added to the mixture as a solution in *tert*.butanol (2.5%). The mixture was stirred for 24 h at room temperature. Preparative HPLC work-up (methanol : water, 60 : 40) yielded the desired product.^{4,5}

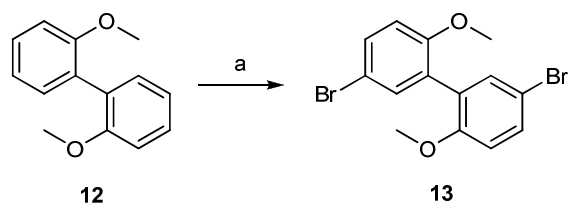


^a**Reagents, Conditions:** OsO₄, *tert*-BuOH, acetone, water, 24 h, 20%.

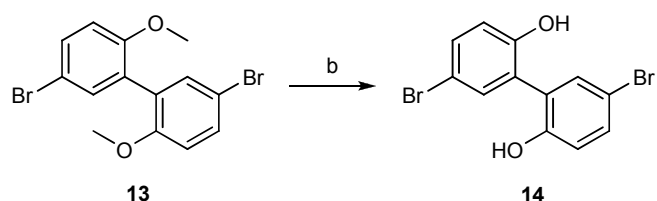
Synthesis of *trans*-isomagnolol (8) (Scheme 3)^a



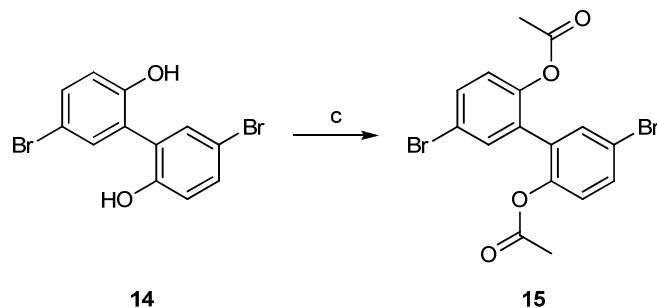
^a**Reagents, Conditions:** (a) *N*-bromosuccinimide, dimethylformamide, rt, 12 h, 80% yield. (b) CH₂Cl₂, BBr₃, -78°C to rt, 75% yield. (c) acetic anhydride, 120 °C, 2 h, 95% yield. (d) Pd(PPh₃)₄, CsF, dioxane, water, 85% yield. (e) NaHCO₃, water, methanol, 90% yield.



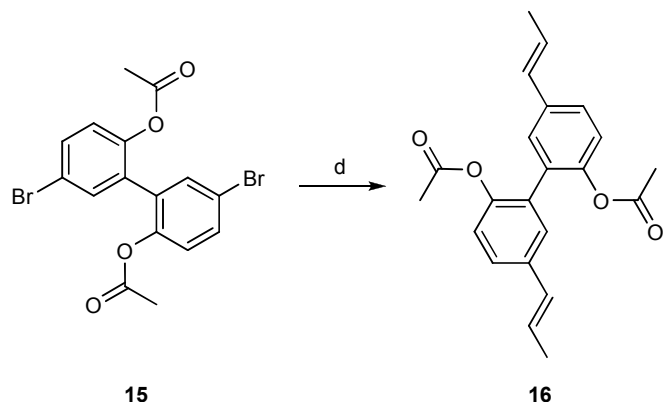
2,2'-Dimethoxybiphenyl (**12**) (1.8 g, 8.34 mmol) was dissolved in 20 mL of anhydrous dimethylformamide, and *N*-bromosuccinimide (3.12 g, 17.5 mmol) was added. The solution was stirred for 12 h at r.t. Water (25 mL) was added and the aqueous layer was extracted three times with 30 mL of dichloromethane each. The combined extract was washed with brine, dried over sodium sulfate and evaporated under reduced pressure. The crude product was purified by chromatography (petroleum ether : EtOAc, 9 : 1) to give **13** in 80% yield.⁶



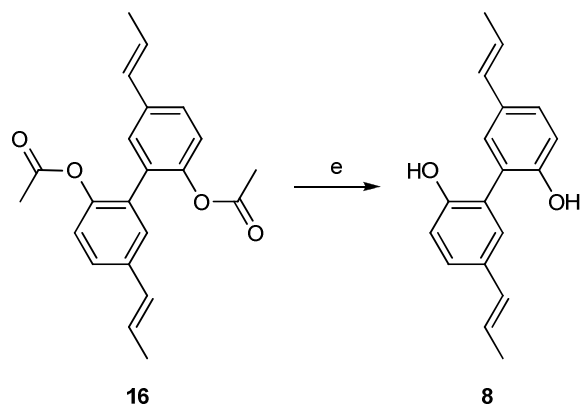
Compound **13** (5.18 g, 14 mmol) was dissolved in anhydrous dichloromethane (75 mL) under an argon atmosphere and cooled to -78 °C. While the solution was constantly stirred, boron tribromide (15 mL, 1 M in hexane) was added dropwise. The resulting solution was kept at -78 °C for 1.5 h and then allowed to warm to 0 °C. Water (120 mL) was added slowly while the solution was vigorously stirred. The aqueous layer was separated and extracted three times with dichloromethane (120 mL portions). The combined extract was washed with brine, dried over sodium sulfate and evaporated under reduced pressure. The crude product was purified by chromatography (petroleum ether : EtOAc, 9 : 1) to give **14** in 75% yield.⁶



Compound **14** (3 g, 8.8 mmol) was added to acetic anhydride (20 mL). Upon stirring, the solution was heated to reflux (120 °C) for 2 h. Then the reaction mixture was cooled to 0 °C and water (40 mL) was added. The resulting white aqueous suspension was extracted three times with ethyl acetate (70 mL portions). The combined extract was washed with brine, dried over sodium sulfate and evaporated under reduced pressure. The crude product was purified by chromatography (petroleum ether : EtOAc, 9 : 1) to give **15** in 95% yield.



A mixture of **15** (500 mg, 1.17 mmol), *trans*-propenylboronic acid (405 mg, 4.7 mmol), CsF (750 mg, 5 mmol) and Pd(PPh₃)₄ in a pressure tube under an argon atmosphere was treated with a mixture of dioxane and water (17 mL : 3 ml). The resulting mixture was treated for 5 min with bubbling argon to remove oxygen and then heated in the sealed tube for 8 h at 80 °C. Then the mixture was diluted with water (60 mL) and extracted three times with ethyl acetate (70 mL portions). The combined extract was washed with brine, dried over sodium sulfate and evaporated under reduced pressure. The crude product was purified by chromatography (petroleum ether : EtOAc, 9 : 1) to give **16** in 85% yield.⁷



Compound **16** (350 mg, 1 mmol) was treated with methanol (10 mL), water (5 mL) and saturated NaHCO₃ solution (10 mL). The resulting solution was heated to reflux for 1 h. Then the solution was diluted with water (20 mL). The solution was extracted three times with dichloromethane (30 mL portions). The combined extract was evaporated under reduced pressure. The crude product was purified by HPLC to give **8** in 90% yield.⁸

Experimental Section

Chemistry

Magnolol and honokiol were obtained from Sigma-Aldrich and used without further purification. Magnolia extract from the bark of *Magnolia officinalis* was obtained from CORTEX Scientific Botanicals, Ojai, USA. The amount of magnolol and honokiol in the extract was 17.9% and 22.8%, respectively, determined by HPLC analysis. As extraction solvent aqueous ethanol (90%) was used. 4-Propylphenol (**9**) was obtained from Sigma-Aldrich. 2,2'-Dimethoxybiphenyl (**12**) was obtained from Alfa Aesar. All compounds were used without further purification.

All commercially available reagents were obtained from various producers (Acros, Aldrich, Fluka, Merck, Sigma) and used without further purification. Solvents were used without additional purification or drying, unless otherwise noted. The reactions were monitored by thin layer chromatography (TLC) using aluminum sheets with silica gel 60 F₂₅₄ (Merck).

The purity of the compounds was controlled by dissolving 1 mg/mL of compound in methanol containing 2 mM ammonium acetate. A sample of 10 µl was injected into an HPLC instrument (Agilent 1100) using a Macherey Nagel EC50/2 Nucleodur C18 Gravity 3µ column. Elution was performed with a gradient of water : methanol (containing 2 mM ammonium acetate) from 90 : 10 to 0 : 100 for 15 min, then methanol 100% for 15 min, at a flow rate of 250 µl /min. UV absorption was detected from 190 - 900 nm using a diode array detector. The purity of all tested compounds was >90%.

Mass spectra were recorded on an API 2000 (Applied Biosystems, Darmstadt, Germany) mass spectrometer (turbo ion spray ion source) coupled with a Waters HPLC system (Agilent 1100) using a Phenomenex Luna 3µ C18 column.

HPLC workup was accomplished on a Knauer System equipped with a 250 x 20 mm reversed phase column (Eurospher 100 – 10 C18). Elution was performed with different gradients of water : methanol and monitored with a Knauer Smartline 2600 diode array detector. As wavelength of 254 nm was chosen for the collection of the compounds.

Melting points were determined with a Büchi Melting Point B-545 and are uncorrected.

¹H- and ¹³C-NMR spectra were performed on a Bruker Avance 500 MHz spectrometer. Shifts are given in ppm relative to the remaining protons of the deuterated solvents used as internal standard (¹H, ¹³C).

5-Allyl-5'-(2,3-dihydroxypropyl)biphenyl-2,2'-diol (4)

¹H NMR (500 MHz, DMSO) δ 6.98 – 6.92 (m, CH_{ar}, 4H), 6.81 – 6.75 (m, CH_{ar}, 2H), 5.93 (ddt, *J* = 16.8, 10.0, 6.8 Hz, CH₂=CH, 1H), 5.06 (dd, *J* = 17.0, 2.1 Hz, 1H), 5.00 (dd, *J* = 10.0, 2.2 Hz, 1H), 4.42 (m, OH, 2H), 3.57 (s, ar-CH₂-CH-OH, 1H), 3.35 – 3.22 (m, 6H), 2.66 (dd, *J* = 13.7, 5.1 Hz, ar-CH₂, 1H), 2.44 (dd, *J* = 13.7, 7.4 Hz, ar-CH₂, 1H). ¹³C NMR (126 MHz, DMSO) δ 152.75 (C_{ar}-O), 152.50 (C_{ar}-O), 138.43 (CH=), 132.34 (C_{ar}), 131.49 (C_{ar}), 129.95 (C_{ar}), 129.89 (C_{ar}), 129.05 (C_{ar}), 128.01 (C_{ar}), 126.25 (C_{ar}), 125.62 (C_{ar}), 115.96 (C_{ar}), 115.59 (C_{ar}), 115.38 (CH₂=), 72.89 (CH-O), 65.39 (CH₂-O), 39.13 (ar-CH₂), 38.90 (ar-CH₂). LC/ESI-MS (negative mode) m/z 299 (M-H)⁻ 98.7%.

5,5'-Dipropylbiphenyl-2,2'-diol (7)

¹H NMR (500 MHz, CDCl₃) δ 7.12 (dd, *J* = 8.2, 2.2 Hz, CH_{ar}, 2H), 7.07 (d, *J* = 2.0 Hz, CH_{ar}, 2H), 6.94 (d, *J* = 8.2 Hz, CH_{ar}, 2H), 5.51 (s, *J* = 85.8 Hz, OH, 2H), 2.58 – 2.55 (t, *J* = 7.8 Hz, CH₂, 4H), 1.70 – 1.58 (m, CH₂, 4H), 0.95 (t, *J* = 7.3 Hz, CH₃, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 150.98 (C_{ar}-O), 135.95 (C_{ar}), 131.13 (C_{ar}), 129.92 (C_{ar}), 123.67 (C_{ar}), 116.56 (C_{ar}), 37.32 (ar-CH₂), 24.89 (CH₂), 13.98 (CH₃). LC/ESI-MS (negative mode) m/z 269 (M-H)⁻ 97.6%
M.p. 143°C (lit. m.p.⁹ 144.5°C).

2-Bromo-4-propylphenol (10)

¹H NMR (500 MHz, CDCl₃) δ 7.27 (d, *J* = 2.1 Hz, CH_{ar}, 1H), 7.02 (dd, *J* = 8.3, 2.1 Hz, CH_{ar}, 1H), 6.93 (d, *J* = 8.3 Hz, CH_{ar}, 1H), 5.34 (s, OH, 1H), 2.50 (t, *J* = 7.8 Hz, ar-CH₂, 2H), 1.60 (tq, *J* = 8.0, 8.0, 4.0, 4.0, 4.0 Hz, CH₂, 2H), 0.92 (t, *J* = 7.3 Hz, CH₃, 3H). ¹³C NMR (126 MHz, CDCl₃) δ 150.10 (C_{ar}-O), 136.36 (C_{ar}), 131.49 (C_{ar}), 129.15 (C_{ar}), 115.67 (C_{ar}), 109.81 (C_{ar}), 36.75 (ar-CH₂), 24.51 (CH₂), 13.58 (CH₃). LC/ESI-MS (negative mode) m/z 213 (M-H)⁻ 99.0%.

2-Hydroxy-5-propylphenylboronic acid (11)

The structure was confirmed by LC/ESI-MS (negative mode) m/z 179 (M-H)⁻ 98.62% **M.p.** 148-149°C.

5,5'-Dibromo-2,2'-dimethoxybiphenyl (13)

¹H NMR (500 MHz, CDCl₃) δ 7.43 (dd, *J* = 8.8, 2.5 Hz, CH_{ar}, 2H), 7.33 (d, *J* = 2.5 Hz, CH_{ar}, 2H), 6.84 (d, *J* = 8.8 Hz, CH_{ar}, 2H), 3.76 (s, CH₃, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 156.09 (C_{ar}-O), 133.76 (C_{ar}), 131.60 (C_{ar}), 128.36 (C_{ar}), 112.71 (C_{ar}), 112.47 (C_{ar}), 55.90 (O-CH₃). LC/ESI-MS (positive mode) m/z 357 (M+H-methyl)⁺, (negative mode) m/z 339 (M-H-2methyl)⁻ 99.2% **M.p.** 126-127°C (lit m.p.¹⁰ 129-130°C).

5,5'-Bromobiphenyl-2,2'-diol (14)

¹H NMR (500 MHz, CDCl₃) δ 7.43 (dd, *J* = 8.6, 2.5 Hz, CH_{ar}, 2H), 7.39 (d, *J* = 2.4 Hz, CH_{ar}, 2H), 6.91 (d, *J* = 8.6 Hz, CH_{ar}, 2H), 5.66 (s, OH, 2H). ¹³C NMR (126 MHz, CDCl₃) δ 151.89 (C_{ar}-O), 133.73 (C_{ar}), 132.97 (C_{ar}), 125.01 (C_{ar}), 118.64 (C_{ar}), 113.70 (C_{ar}). LC/ESI-MS (negative mode) m/z 343 (M-H)⁻ 77.4% **M.p.** 187°C (lit. m.p.¹¹ 191-192°C).

5,5'-Dibromo-2,2'-diacetate (15)

¹H NMR (500 MHz, CDCl₃) δ 7.53 (dd, *J* = 8.6, 2.4 Hz, CH_{ar}, 2H), 7.44 (d, *J* = 2.4 Hz, CH_{ar}, 2H), 7.06 (d, *J* = 8.6 Hz, CH_{ar}, 2H), 2.07 (s, CH₃, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 168.79 (O-C=O), 147.06 (C_{ar}-O), 133.72 (C_{ar}), 132.34 (C_{ar}), 131.06 (C_{ar}), 124.40 (C_{ar}), 118.93 (C_{ar}),

20.64 (CH₃). **LC/ESI-MS** (positive mode) 429 (M+H)⁺ 87.9% **M.p.** 105-106°C (lit. m.p.¹⁰ 105-106°C)

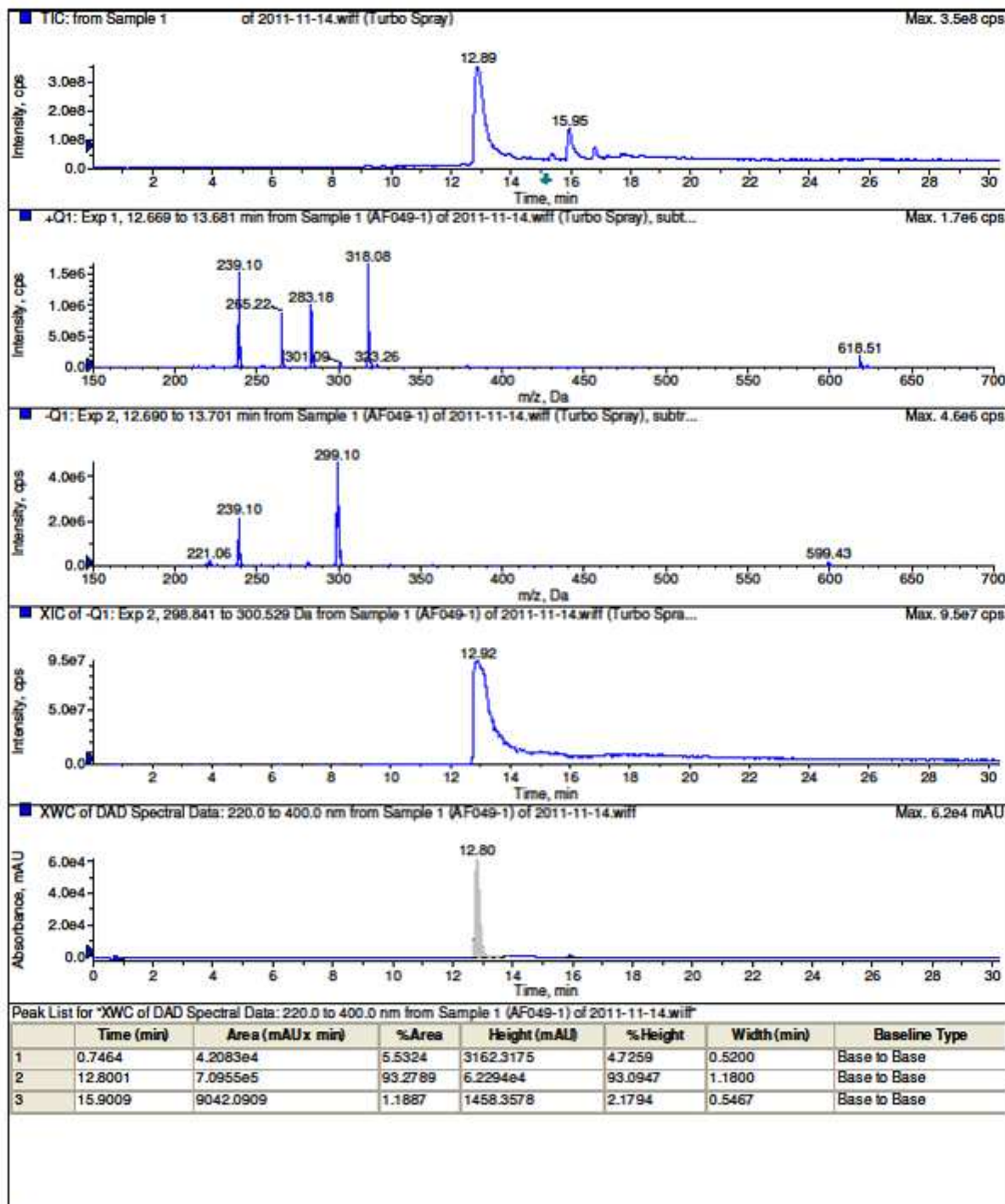
5,5'-Di-((E)-prop-1-enyl)biphenyl-2,2'-diacetate (16)

¹H NMR (500 MHz, CDCl₃) δ 7.32 (dd, *J* = 8.4, 2.2 Hz, CH_{ar}, 2H), 7.24 (d, *J* = 2.4 Hz, CH_{ar}, 2H), 7.06 (d, *J* = 8.4 Hz, CH_{ar}, 2H), 6.42 (m, ar-CH₂, 2H), 6.20 (dq, *J* = 15.7, 6.6 Hz, CH₂, 2H), 2.02 (s, CH₃, 6H), 1.86 (dd, *J* = 6.6, 1.7 Hz, CH₃, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 169.39 (O-C=O, 2C), 146.77 (C_{ar}-O, 2C), 135.86 (ar-C_{ar}, 2C), 130.42 (ar-CH, 2C), 129.82 (C_{ar}, 2C), 128.45 (C_{ar}, 2C), 126.37 (C_{ar}, 2C), 126.12 (CH, 2C), 122.48 (C_{ar}, 2C), 20.71 (O-C-CH₃, 2C), 18.43 (CH₃, 2C). **LC/ESI-MS**: positive mode 351 (M+H)⁺ 84.92%

5,5'-Di-((E)-prop-1-enyl)biphenyl-2,2'-diol (8)

¹H NMR (500 MHz, CDCl₃) δ 7.30 (dd, *J* = 8.4, 2.2 Hz, CH_{ar}, 2H), 7.22 (d, *J* = 2.2 Hz, CH_{ar}, 2H), 6.96 (d, *J* = 8.4 Hz, CH_{ar}, 2H), 6.36 (m, ar-CH, 2H), 6.13 (dq, *J* = 15.7, 6.6 Hz, CH, 2H), 5.46 (s, OH, 2H), 1.87 (dd, *J* = 6.6, 1.7 Hz, CH₃, 6H). ¹³C NMR (126 MHz, CDCl₃) δ 151.84 (C_{ar}-O), 131.79 (C_{ar}), 129.88 (ar-CH), 128.53 (C_{ar}), 127.37 (C_{ar}), 124.47 (C_{ar}), 123.38 (CH), 116.75 (C_{ar}), 18.40 (CH₃). **LC/ESI-MS**: positive mode 267 (M+H)⁺, negative mode 265 (M-H)⁻ 92.5% **M.p.** 138°C

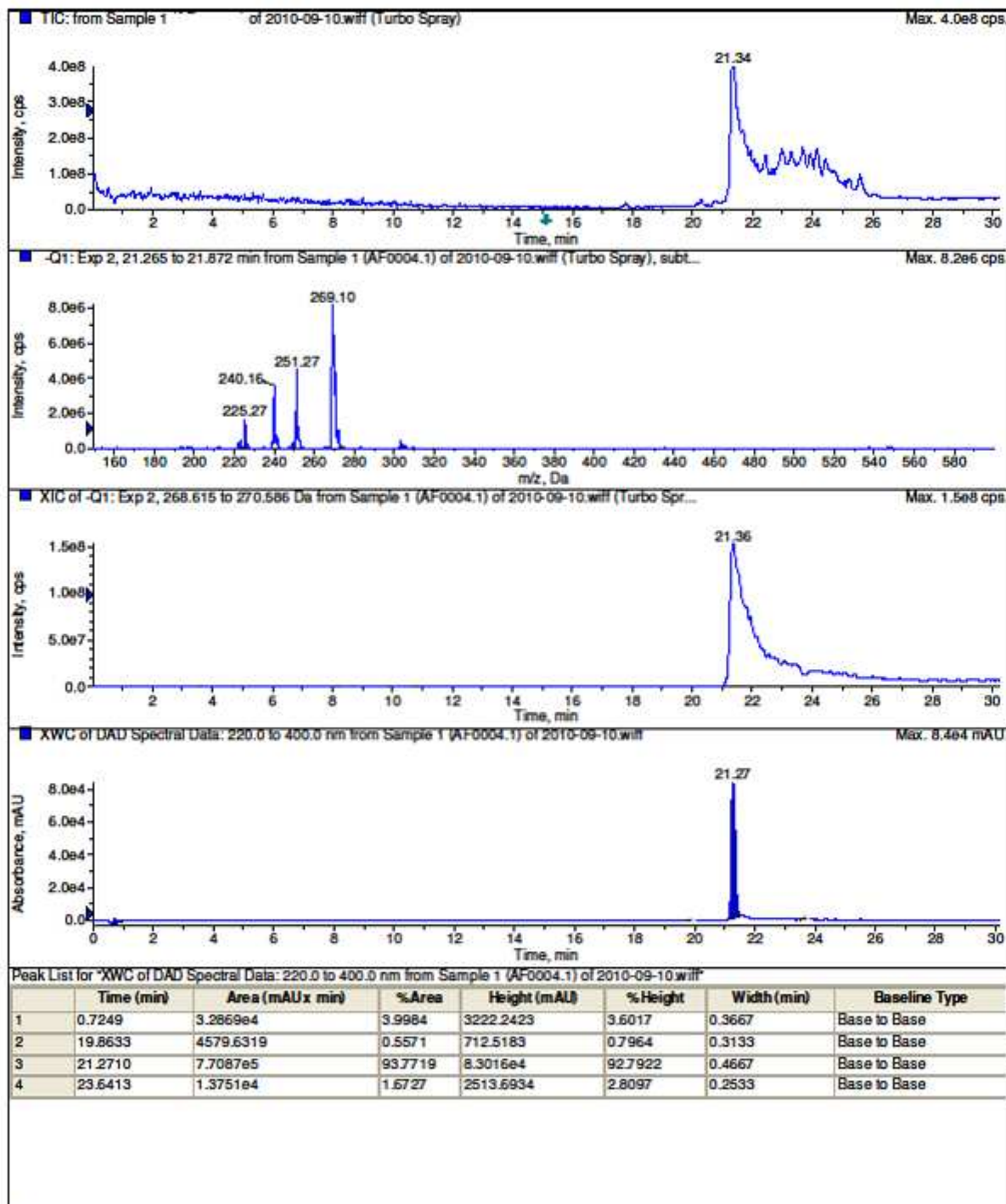
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LC Pump Device: Agilent 1100 binary pump

Figure 1. LC/ESI-MS spectrum of **4** (mass spectrum in the positive and negative mode), HPLC chromatogram (HPLC-DAD measured from 220-400 nm) of **4**, and its purity determined by HPLC-DAD from 220-400 nm (98.7%).

Acq. Date: Friday, September 10, 2010 Polarity/Scan Type: Positive Q1 MS, ...
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 Acq. File: 2010-09-10.wiff



LC Pump Device: Agilent 1100 binary pump

Figure 2. LC/ESI-MS spectrum of **7** (mass spectrum in the negative mode), HPLC chromatogram (HPLC-DAD measured from 220-400 nm) of **7**, and its purity determined by HPLC-DAD from 220-400 nm (97.6%).

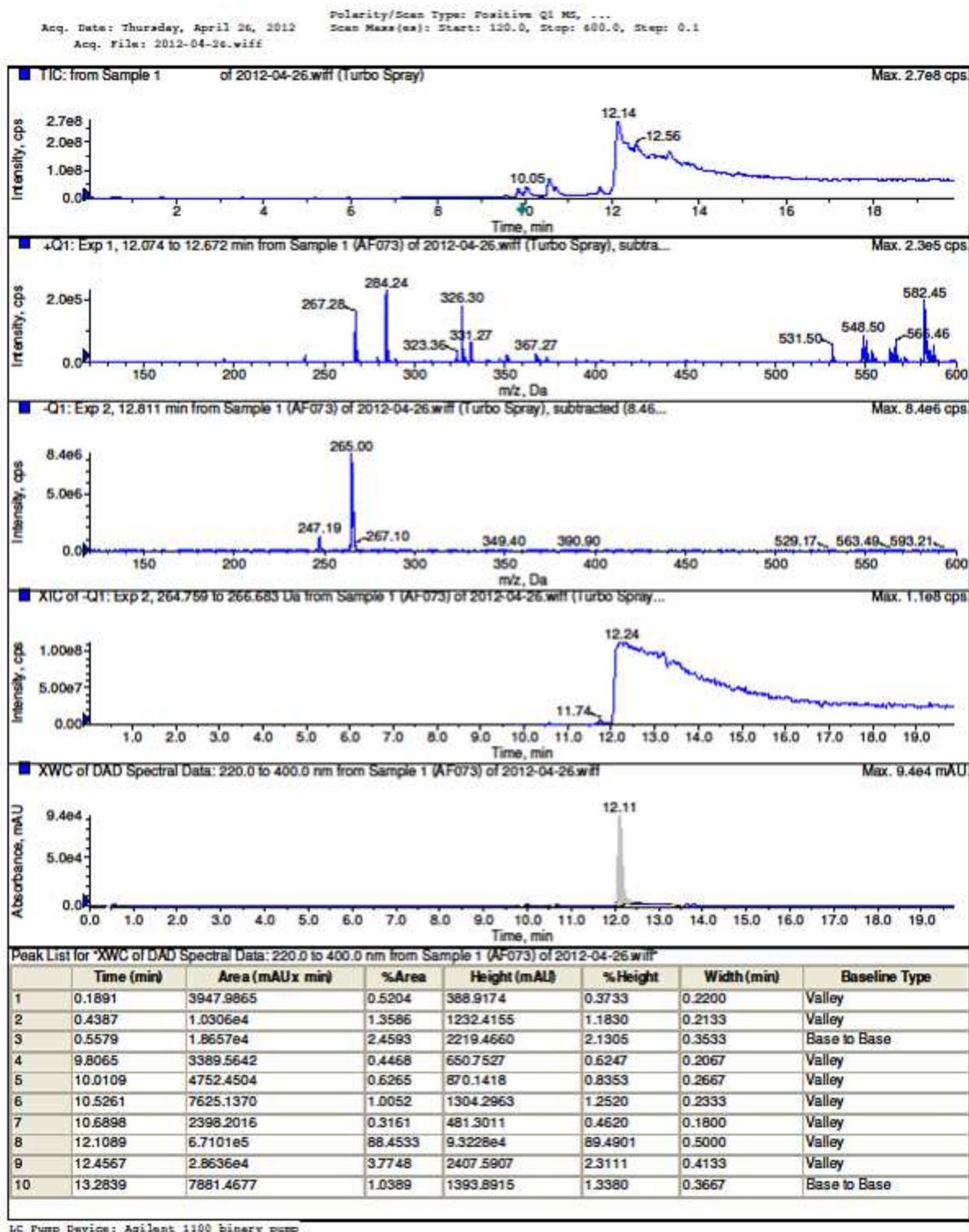


Figure 3. LC/ESI-MS spectrum of **8** (mass spectrum in the positive and negative mode), HPLC chromatogram (HPLC-DAD measured from 220-400 nm) of **8**, and its purity determined by HPLC-DAD from 220-400 nm (92.5%).

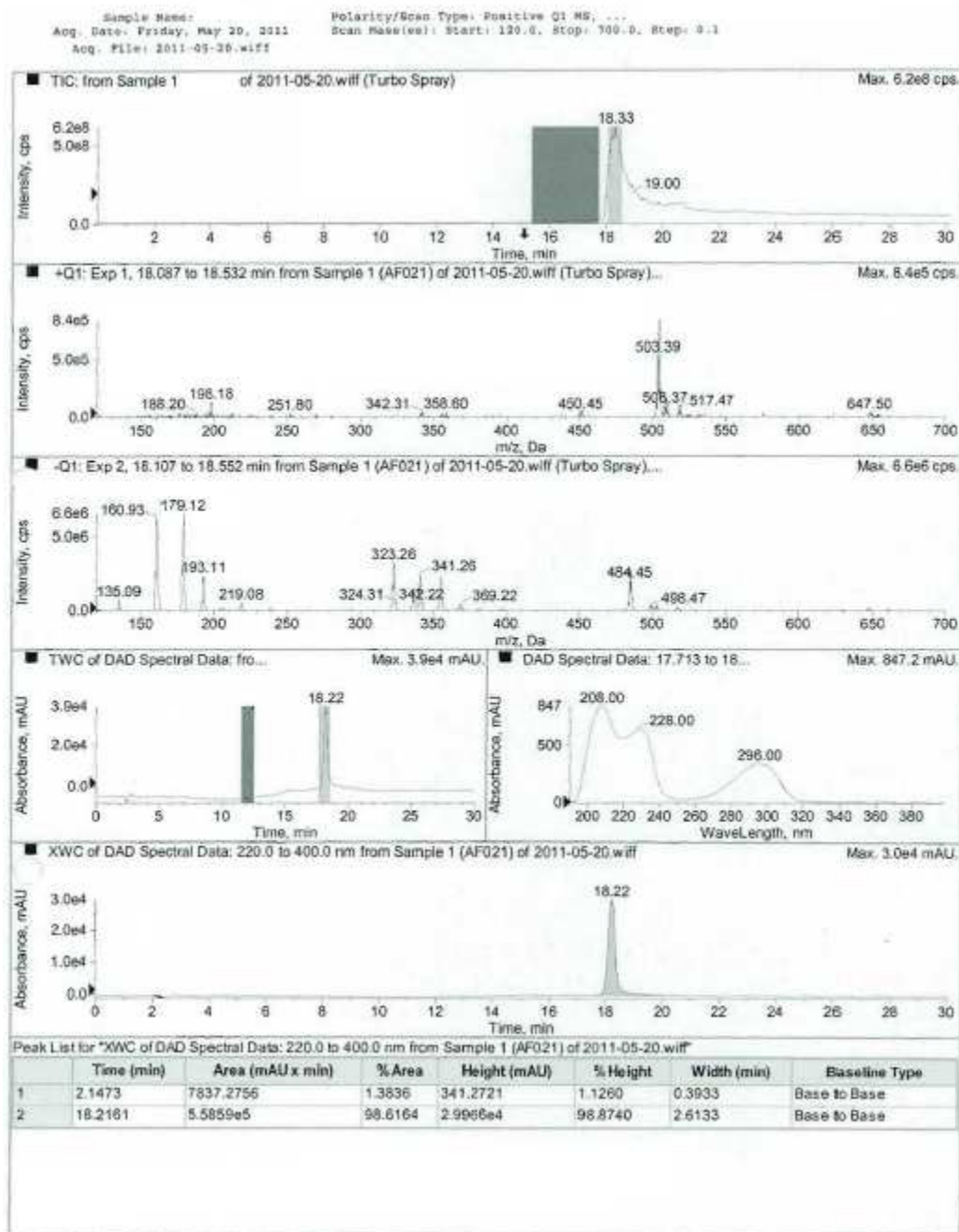
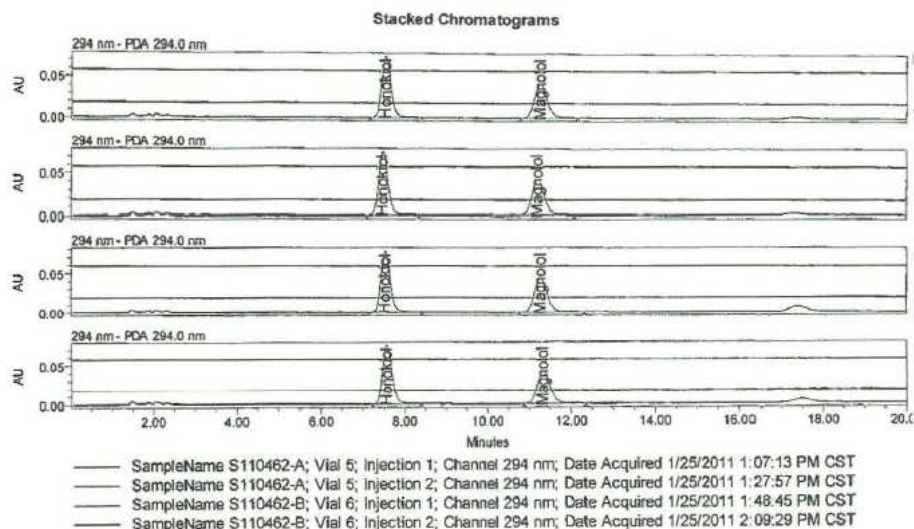


Figure 4. LC/ESI-MS spectrum of **11** (mass spectrum in the positive and negative mode), HPLC chromatogram (HPLC-DAD measured from 220-400 nm) of **8**, and its purity determined by HPLC-DAD from 220-400 nm (98.62%).

MAGNOLIA BARK EXTRACT CERTIFICATE OF ANALYSIS

Batch No.: 009110101
Latin name: *Magnolia officinalis* Rehder & E.H. Wilson
Plant : final extract ratio: 15.7:1
Extraction solvent: 90% ethanol
Other ingredients: Dicalcium phosphate anhydrous (17%), silica (1%)
Production date: 01/06/2011

Item	Results	Test Method
Appearance	Conforms	Visual
Color	Conforms	Visual
Honokiol	22.8%	HPLC
Magnolol	17.9%	HPLC
Loss on drying	1.3%	5 g / 105 °C



Reviewed by: Staci J. Eisner
 Staci J. Eisner (QA Management)

Date: 03/29/2011

Figure 5. Analytical data including HPLC chromatogram (HPLC-PDA measured at 294 nm) of *Magnolia officinalis* extract.

Radioligand binding studies at cannabinoid CB₁ and CB₂ receptors

Competition binding assays were performed using the cannabinoid receptor agonist radioligand [³H](*-cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxy-propyl)cyclohexanol (CP55,940) as described before with some modifications.¹² [³H]CP55,940 was used in a concentration of 0.1 nM and as a source for human CB₁ and CB₂ receptors membrane preparations of Chinese hamster ovary (CHO) cells stably expressing the respective receptor subtype were employed (25 µg of protein per vial for CB₁ assays, and 1 µg of protein per vial for CB₂ receptor assays, respectively). Stock solutions of the test compounds were prepared in dimethyl sulfoxide (DMSO). The final DMSO concentration in the assay was 2.5%. After the addition of 15 µL of test compound in DMSO, 60 µL of [³H]CP55,940, 60 µL of membrane preparation and 465 µL of assay buffer (50 mM tris(hydroxymethyl)aminomethane (TRIS), 3 mM MgCl₂, 0.1% bovine serum albumin (BSA), pH 7.4) were added, and the suspension was incubated for 2 h at room temperature. Total binding was determined by adding DMSO without test compound. Nonspecific binding was determined in the presence of 10 µM of unlabeled CP55,940. Incubation was terminated by rapid filtration through GF/C glass fiber filters presoaked with 0.3% polyethyleneimine, using a Brandel 48-channel cell harvester (Brandel, Gaithersburg, Maryland, USA). Filters were washed three times with ice-cold washing buffer (50 mM TRIS, 0.1% BSA, pH 7.4) and then dried for 1.5 h at 50°C. Radioactivity on the filters was determined in a liquid scintillation counter (TRICARB 2900TR, Packard / Perkin-Elmer) after 6 h of preincubation with 3 mL of scintillation cocktail (LumaSafe plus, Perkin-Elmer). Data were obtained from three independent experiments, performed in duplicates. Data were analyzed using Graph Pad Prism Version 4.02 (San Diego, CA, USA). For the calculation of K_i values the Cheng-Prusoff equation and a K_D value of 2.4 nM ([³H]CP55,940 at hCB₁) and 0.7 nM ([³H]CP55,940 at hCB₂) were used.

cAMP accumulation assays

The measurement of intracellular cAMP concentration was performed as described before with some modifications.¹³ Inhibition of adenylate cyclase activity was determined in CHO cells stably expressing the CB₁ or the CB₂ receptor subtype using a competition binding assay for cAMP. Cells were seeded into a 24-well plate at a density of 200,000 cells per well. After an incubation of 24 h the medium was removed and cells were washed with Hank's buffered saline solution (HBSS) consisting of NaCl (13 mM), HEPES (20 mM), glucose (5.5 mM), KCl (5.4 mM), NaHCO₃ (4.2 mM), CaCl₂ x 2 H₂O (1.25 mM), MgSO₄ (0.8 mM), MgCl₂ (1 mM), KH₂PO₄ (0.44 mM), Na₂HPO₄ (0.34 mM) dissolved in deionized, autoclaved water. After adding 190 µL of HBSS per well cells were incubated for 2 h at 37°C. After this period the phosphodiesterase (PDE) inhibitor Ro-20-1724 (40 µM) dissolved in HBSS, test compound, and forskolin (10 µM), all dissolved in HBSS containing 10% DMSO, were added to each well. The final DMSO amount was 1.9 %. The suspension was incubated for 10 min after the addition of Ro-20-1724, for 5 min after the addition of test compound and for another 15 min after adding forskolin. For testing the antagonistic potency of compound honokiol (**1**) cAMP accumulation was determined in the presence of the agonist CP55,940. Therefore cells were incubated for 20 min with compound **1** before the agonist was added to the assay. cAMP accumulation was stopped by

removing the supernatant from the cell suspension and subsequently lysing the cells with 500 μ L of hot lysis buffer (100°C; 4 mM ethylenediaminetetraacetic acid (EDTA), 0.01% Triton X-100). Aliquots of 50 μ L of cell suspension were transferred to 2.5 mL tubes, and 30 μ L of [³H]cAMP and 40 μ L of cAMP-binding protein were added, followed by 1 h of incubation on ice. The cAMP binding protein was obtained from bovine adrenal cortex as previously described.¹⁴ For determining intracellular cAMP concentrations 50 μ L of various cAMP concentrations were measured instead of cell lysates, to obtain a standard curve. Total binding was determined by adding radioligand and binding protein to lysis buffer, and the background was determined without addition of binding protein. Bound and free radioligand were separated by rapid filtration through GF/B glass fiber filter. Radioactivity on the filters was determined in a liquid scintillation counter (TRICARB 2900TR, Packard / Perkin-Elmer) after 6 h of preincubation with 3 mL of scintillation cocktail (LumaSafe plus, Perkin-Elmer). Data were obtained from three independent experiments, performed in duplicates. Data were analyzed using Graph Pad PRISM Version 4.02 (San Diego, CA, USA).

β -Arrestin translocation assays

Interaction with the GPR55 was investigated by performing β -arrestin assays, based on β -galactosidase enzyme fragment complementation technology (β -arrestin PathHunter™ assay, DiscoverX, Fremont, CA, USA). Therefore CHO cells stably expressing the GPR55 were seeded in a volume of 90 μ L into a 96-well plate and were incubated at a density of 20,000 cells/well in assay medium (Opti-MEM, 2 % fetal calf serum (FCS), 100 U/mL penicillin, 100 μ g/mL Streptomycin, 800 μ g/mL geneticin und 300 μ g/mL hygromycin) for 24 h at 37°C. After preincubation, test compounds were diluted in phosphate buffered saline (PBS buffer) containing 10 % DMSO and 0.1 % BSA and added to the cells in a volume of 10 μ L, followed by an incubation for 90 min at 37°C. For determination of baseline luminescence PBS buffer (containing 10 % DMSO, 0.1 % BSA) in the absence of test compound was used. During the incubation period, the detection reagent was prepared by mixing the chemiluminescent substrate Galacton-Star® (2 mM), with the luminescence enhancer Emerald-II™ and a lysis buffer (10 mM TRIS, 1 mM EDTA, 100 mM NaCl, 5 mM MgCl₂, 1 % Triton-X; pH 8) in a ratio of 1:5:19. After the addition of 50 μ L/well of detection reagent the measurement plate was incubated for further 60 min at room temperature. Finally luminescence was determined in a luminometer (TopCount NXT, Packard / Perkin-Elmer).

For the determination of antagonistic properties of test compounds the assay was performed as described for agonists, except that the test compounds were added to the cells in a volume of 5 μ L/well 60 min prior to addition of the agonist (lysophosphatidylinositol = LPI, 5 μ L/well). Data were obtained from three independent experiments, performed in duplicates.

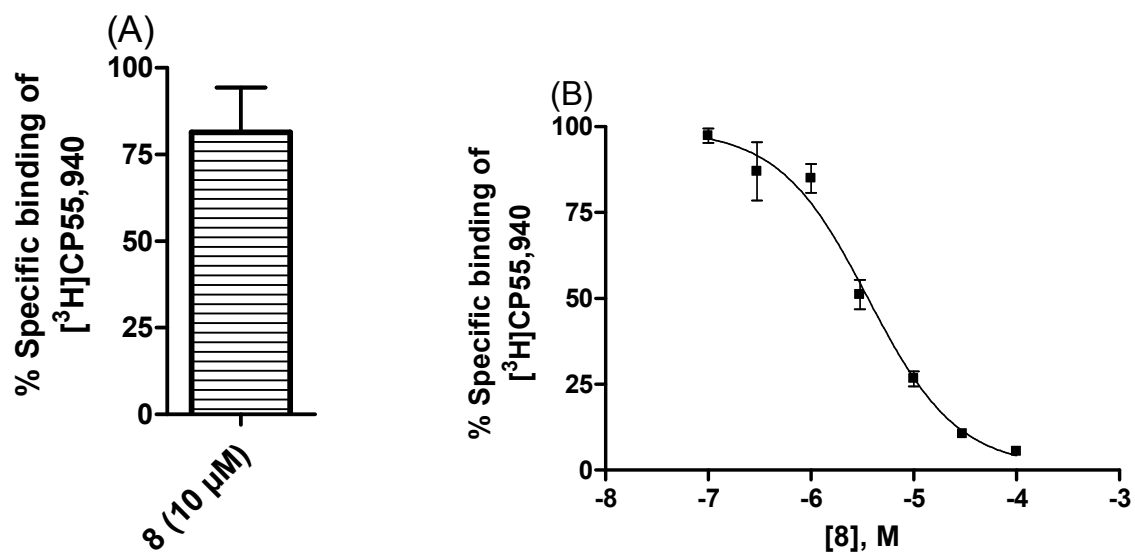


Figure 6. Inhibition of specific [³H]CP55,940 binding by *trans*-isomagnolol (**8**) at CHO cells recombinantly expressing human (A) CB₁ receptor or (B) CB₂ receptor. The results are means ± SEM of three independent experiments, performed in duplicates.

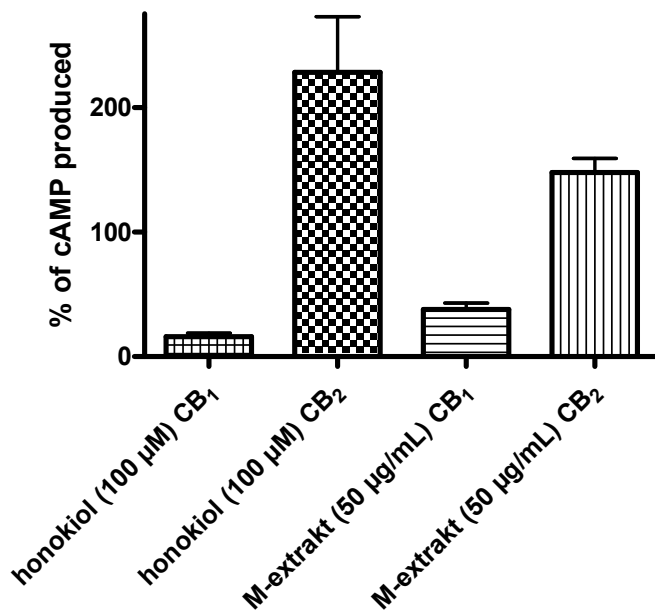


Figure 7. Honokiol (100 µM) and M-extract inhibited forskolin- (10 µM) induced cAMP production in CHO cells transfected with the human CB₁ receptor, while they exhibited inverse agonistic properties at human CB₂ receptors. The results are means ± SEM of three independent experiments, performed in duplicates. The effect of 10 µM forskolin is set at 100%.

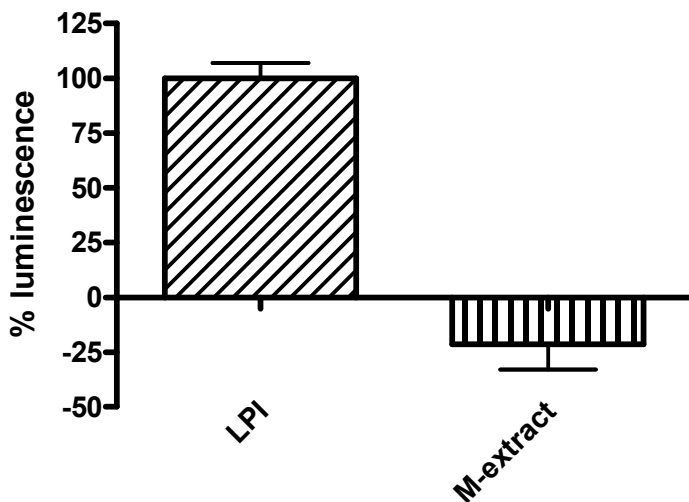


Figure 8. Effect of magnolia extract (M-extract) (50 µg/mL) on LPI-induced β-arrestin recruitment to activated human GPR55 receptors recombinantly expressed in CHO cells. Recruitment of β-arrestin to the receptor was detected by measuring luminescence emission based on a β-galactosidase enzyme fragment complementation assay. The results are the means ± SEM of three independent experiments, performed in duplicates. Data are expressed as percent luminescence related to the effect of LPI (1 µM) set at 100%.

The negative luminescence of the M-extract in β -arrestin translocation assays indicates an inverse agonistic activity at the human GPR55.

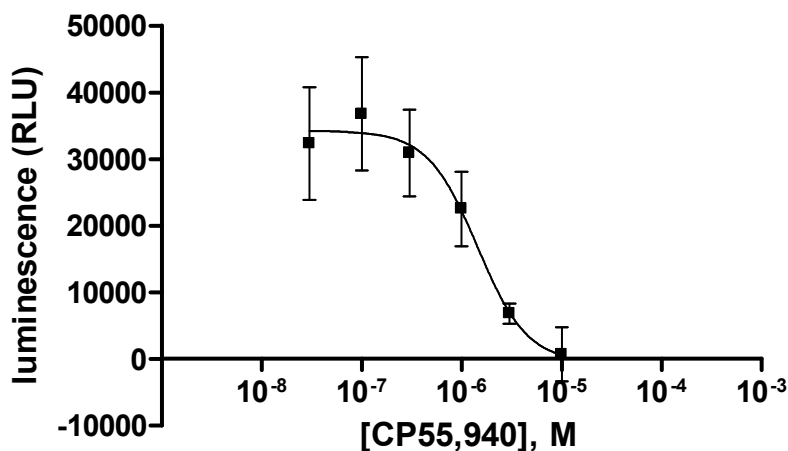


Figure 9. Concentration-dependent inhibition of LPI (1 μ M)-induced β -arrestin recruitment to activated human GPR55 receptors recombinantly expressed in CHO cells by CP55,940. Recruitment of β -arrestin to the receptor was detected by measuring luminescence emission based on a β -galactosidase enzyme fragment complementation assay. The results represent means \pm SEM of three independent experiments, performed in duplicates. IC_{50} : $1.61 \pm 0.47 \mu$ M.

Inhibition of fatty acid amide hydrolyse (FAAH)

Isolation of rat brain microsomes

Two rat brains (female Sprague Dawley rats, Harlan-Winkelmann) were homogenized for 3 min under ice-cooling in a five-fold volume of potassium phosphate buffer (0.1 M, pH 7.4 at 20 °C) containing EDTA (1 mM) using a Potter-Elvehjem homogenizer at 1000 – 1200 rpm. The homogenate was centrifuged at 1000 x g at 4 °C for 10 min and the resulting supernatant was centrifuged again at 10000 x g at 4 °C for 30 min. Finally, the supernatant was centrifuged at 40000 x g at 4 °C for 60 min. The obtained supernatant was discarded. The pellet was resuspended in 2 mL of ice-cold potassium phosphate buffer (0.1 M, pH 7.4) containing EDTA (1 mM) and stored in aliquots at -80 °C. Before each incubation, dependent of the activity of FAAH in the preparation, an aliquot of about 50 μ L was diluted with about 200 to 400 μ L potassium phosphate buffer (0.1 M, pH 7.4) containing EDTA (1 mM) and homogenized (2 x 5 s) at 0 °C with a Branson sonifier B15. Under the conditions applied, in the controls the peak area of the enzyme product 4-pyren-1-ylbutanoic acid amounted to about 70% of the peak area of the internal standard 6-pyren-1-ylhexanoic acid.

Incubation procedure

The substrate *N*-(2-hydroxyethyl)-4-pyren-1-ylbutanamide¹⁵ was dissolved in methanol (2.5 mg/mL). An aliquot of this solution was thoroughly dried under a stream of nitrogen. The residue

was resuspended by intense vortexing and sonication in a sonication bath in such an amount of a solution of 0.2% (m/v) Triton X-100 in phosphate buffered saline (0.01 M, pH 7.4 at 20 °C, prepared from tablets from Sigma-Aldrich, P4417) containing EDTA (1 mM), that the concentration of the substrate was 114 μ M. 88 μ L of the obtained mixture were added to 2 μ L of a DMSO solution of inhibitor or to 2 μ L of DMSO in case of the controls. The mixture was pre-incubated for 10 min at 37 °C. Then the enzymatic reaction was started by adding 10 μ L of rat brain microsome preparation and continued at 37 °C for 60 min. The final incubation volume of 100 μ L contained a pyrenylbutanamide substrate concentration of 100 μ M. The enzyme reaction was terminated by the addition of 200 μ L acetonitrile/methanol (1:1, v/v), which contained the internal standard 6-pyren-1-ylhexanoic acid (0.025 μ g/200 μ L). After cooling in an ice bath for 10 min, the samples were centrifuged at 2000 g at 4 °C for 5 min. Blank incubations in the absence of the enzyme were carried out in parallel.

HPLC Analysis

The HPLC system consisted of a Bischoff HPLC-compact pump model 2250, a Midas Cool Autosampler, a Bischoff Chromatography column oven and a Waters fluorescence detector model 2475. Data analysis was carried out using a McDAC control chromatography software from Bischoff. Separation was achieved on a Nucleosil 100 C18 analytical column (3 mm inside diameter x 125 mm, particle size 3 μ m) (Macherey & Nagel) protected with a Phenomenex C18 guard column (3 mm inside diameter x 4 mm). 50 μ L of each sample were injected into the HPLC system. The mobile phase consisted of methanol/water/trifluoroacetic acid (80:20:0.1, v/v/v). Injector temperature was maintained at 10 °C, oven temperature at 20 °C. The flow rate was 0.4 mL/min. The fluorescence detector was set at an excitation wavelength of 340 nm and emission was monitored at 380 nm. To eliminate the large substrate peak in the chromatogram occurring between 3.0 min and 6.3 min and to protect the photomultiplier of the detector from damage by intense light, from 3.0 – 6.3 min excitation wavelength was set to 400 nm and emission wavelength to 600 nm. After 7.0 min an auto zero was carried out.

For calculation of enzyme inhibition the peak ratio of enzyme product and internal standard obtained in presence of a test compound was compared with the mean level of this peak ratio determined in absence of test compounds (= control tests, n = 3). The IC₅₀-values were calculated with the aid of Probit transformation.

Under these conditions for the reference inhibitor cyclohexylcarbamoyl acid 3'-carbamoyl-biphenyl-3-ylester (URB 597) (Cayman Chemical) an IC₅₀ value of 0.060 \pm 0.0067 μ M (mean \pm standard deviation, n = 4) and for the reference inhibitor 1-oxazolo[4,5-b]pyridin-2-yl-6-phenylhexan-1-one (PHOP) (Cayman Chemical) an IC₅₀ of 0.0029 \pm 0.00049 μ M (mean \pm standard deviation, n = 4) was measured. These IC₅₀ values vary somewhat from the data recently published for these compounds^{15,16} due to slightly different reaction conditions. While in the first publication the enzyme reaction was performed in Tris buffer for 60 min,¹⁵ in a second publication an incubation time in Tris buffer of only 45 min was used.¹⁶ In the present study, a PBS-buffer was used applying an incubation time of 60 min.

Results

Sample	Concentration	Inhibition of FAAH
Honokiol	10 μ M	not significant
Magnolol	10 μ M	not significant
Dihydroxydihydromagnolol	10 μ M	not significant
Tetrahydromagnolol	10 μ M	not significant
Isomagnolol	10 μ M	not significant
Magnolia extract	50 μ g/mL	not significant

Inhibition of monoacylglycerol lipase (MAGL)

Incubation procedure

An aliquot of a solution of human recombinant MAGL (10 μ g/50 μ L) (Cayman Chemical) was diluted 1:100 with HEPES buffer (50 mM, pH 7.4 at 20 °C) containing 100 mM NaCl, 5 mM MgCl₂, 0.1% (m/v) Triton X-100 and 25% (m/v) glycerol. Applying this enzyme solution, in the controls the peak area of the enzyme product 4-pyren-1-ylbutanoic acid amounted to about 70% - 100% of the peak area of the internal standard 6-pyren-1-ylhexanoic acid.

To 2 μ L of a DMSO solution of inhibitor or to 2 μ L of DMSO in case of the controls was added 91 μ L of HEPES buffer (50 mM, pH 7.0 at 20 °C) containing 1 mM EDTA and 0.2% (m/v) Triton X-100. After addition of 2 μ L of a solution of the substrate 1,3-dihydroxypropan-1-yl 4-pyren-1-ylbutanoate¹⁷ in DMSO (5 mM), the mixture was pre-incubated at 37 °C for exactly 15 min. Then the enzymatic reaction was started by adding of 5 μ L enzyme solution and continued for 45 min at 37 °C. The final incubation volume of 100 μ L contained 100 μ M of the substrate and 10 ng MAGL. The enzyme reaction was terminated by the addition of 200 μ L acetonitrile/methanol (1:1, v/v) spiked with the internal standard 6-pyren-1-ylhexanoic acid (0.10 μ g/200 μ L). After cooling in an ice bath for 10 min, the samples were centrifuged at 2000 x g and 4 °C for 5 min and stored at -20 °C until HPLC analysis. Blank incubations in the absence of the enzyme were carried out in parallel.

HPLC analysis

The HPLC system and the separation conditions were the same as for the measurement of FAAH inhibition, with the exception that only 5 μ L of each sample were injected. For calculation of enzyme inhibition the peak ratio of enzyme product 4-pyren-1-ylbutanoic acid and internal standard 6-pyren-1-ylhexanoic acid obtained in presence of a test compound was compared with the mean level of this peak ratio determined in absence of test compounds (= control tests, n = 3). Under these conditions for the reference inhibitor [4-(5-methoxy-2-oxo-1,3,4-oxadiazol-3-yl)-2-methylphenyl]carbamic acid benzyl ester (CAY10499) (Cayman Chemical) an IC₅₀ values 0.48 \pm 0.019 μ M (mean \pm standard deviation, n = 4) was measured.

Results

Sample	Concentration	Inhibition of MAGL
Honokiol	10 μ M	not significant
Magnolol	10 μ M	not significant
Dihydroxydihydromagnolol	10 μ M	not significant
Tetrahydromagnolol	10 μ M	not significant
Isomagnolol	10 μ M	not significant
Magnolia extract	50 μ g/mL	22 \pm 3 % ^a

^a mean \pm standard deviation (n = 4)

References

1. Ulrich, O.; Pfeiffer, H. P.; Breitmaier, E. Taschenporphyrine mit intramolekularen Liganden. *Chem. Ber.* **1986**, *119*, 3507-3514.
2. Konakahara, T.; Kiran, Y. B.; Okuno, Y.; Ikeda, R.; Sakai, N. An expedient synthesis of ellipticine via Suzuki-Miyaura coupling. *Tetrahedron Lett.* **2010**, *51*, 2335-2338.
3. Suzuki, A.; Miyaura, N. A. New stereospecific cross-coupling by the palladium catalized reaction of 1-Alkenylboranes with 1-Alkenyl or 1-Alkynyl halides. *Tetrahedron Lett.* **1979**, *36*, 3437-3440.
4. Ogino, Y.; Chen, H.; Kwong, H.L.; Sharpless, K.B. On the timing of Hydrolysis/Reoxidation in the Osmium catalyzed asymmetric dihydroxylation of olefins using potassium ferricyanide as the reoxidant. *Tetrahedron Lett.* **1991**, *32*, 3965-3968.
5. vanRheenen, V.; Kelly, R.C.; Cha, D.Y. An improved catalytic OsO₄ oxidation of olefins to E-1,2-glycols using tertiary amine oxides as the oxidant. *Tetrahedron Lett.* **1976**, *23*, 1973-1976.
6. Agharahimi, M.R.; LeBel, N.A. Synthesis of (-)-monoterpenylmagnolol and magnolol. *J. Org. Chem.* **1995**, *60*, 1856-1863.
7. Crosignani, S.; Pretre, A.; Jorand-Lebrun, C.; Fraboulet, G.; Seenisamy, J.; Augustine, J.K.; Missotten, M.; Humbert, Y.; Cleva, C.; Abia, N.; Daff, H.; Schott, O.; Schneider, M.; Burgat-Charvillon, F.; Rivron, D.; Hamerig, I.; Arrighi, J.F.; Gaudet, Marilene, G.; Zimmerli, S.C.; Juillard, P.; Johnson, Z. Discovery of potent, selective and orally bioavailable alkynylphenoxyacetic acid CRTH2 (DP2) receptor antagonists for the treatment of allergic inflammatory diseases. *J. Med. Chem.* **2011**, *54*, 7299-7317.

8. Büchi, G.; Weinreb, S.M. Total syntheses of Aflatoxins M1 and G1 and an Improved Synthesis of Aflatoxin B1. *J. Am. Chem. Soc.* **1940**, *62*, 1963-1967.
9. Sugii, Y., Constituents of the bark of *Magnolia officinalis*, Rhed. et Wils and *Magnolia obovata*, Thumb. *Yakugaku Zasshi* **1930**, *50*, 183-217.
10. Gilman, H.; Swiss, J.; Cheney, L. C. Dibenzofuran. XIX. Derivatives of 2,2'-dihydroxybiphenyl. *J. Am. Chem. Soc.* **1940**, *62*, 1963-1967.
11. Zhang, H. Chemical research in Chinese Universities. **2008**, *24*, 798-804.
12. Behrenswerth, A.; Volz, N.; Torang, J.; Hinz, S.; Bräse, S.; Müller, C. E. Synthesis and pharmacological evaluation of coumarin derivatives as cannabinoid receptor antagonists and inverse agonists. *Bioorg. Med. Chem.* **2009**, *17*, 2842-2851.
13. Drabczynska, A.; Yuzlenko, O.; Köse, M.; Paskaleva, M.; Schiedel, A. C.; Karolak-Wojciechowska, J.; Handzlik, J.; Karcz, T.; Kuder, K.; Müller, C. E.; Kiec-Kononowicz, K. Synthesis and biological activity of tricyclic cycloalkylimidazo-, pyrimido- and diazepinopurinediones. *Eur. J. Med. Chem.* **2011**, *46*, 3590-3607.
14. Nordstedt, C.; Fredholm, B. B. A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal. Biochem.* **1990**, *189*, 231-234.
15. Forster, L.; Schulze Elfringhoff, A.; Lehr, M. High-performance liquid chromatographic assay with fluorescence detection for the evaluation of inhibitors against fatty acid amide hydrolase. *Anal. Bioanal. Chem.* **2009**, *394*, 1679-1685.
16. Forster, L.; Ludwig, J.; Kaptur, M.; Bovens, S.; Schulze Elfringhoff, A.; Holtfrerich, A.; Lehr, M. 1-Indol-1-yl-propan-2-ones and related heterocyclic compounds as dual inhibitors of cytosolic phospholipase A₂a and fatty acid amide hydrolase. *Bioorg. Med. Chem.* **2010**, *18*, 945-952.
17. Holtfrerich, A.; Makharadze, T.; Lehr, M. High-performance liquid chromatography assay with fluorescence detection for the evaluation of inhibitors against human recombinant monoacylglycerol lipase. *Anal. Biochem.* **2010**, *399*, 218-224.