# A fluorescent probe to unravel functional features of cannabinoid receptor $CB_1$ in blood and tonsil immune system cells

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**Supplementary Information**
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**1. Supplementary Figures S1-S5**

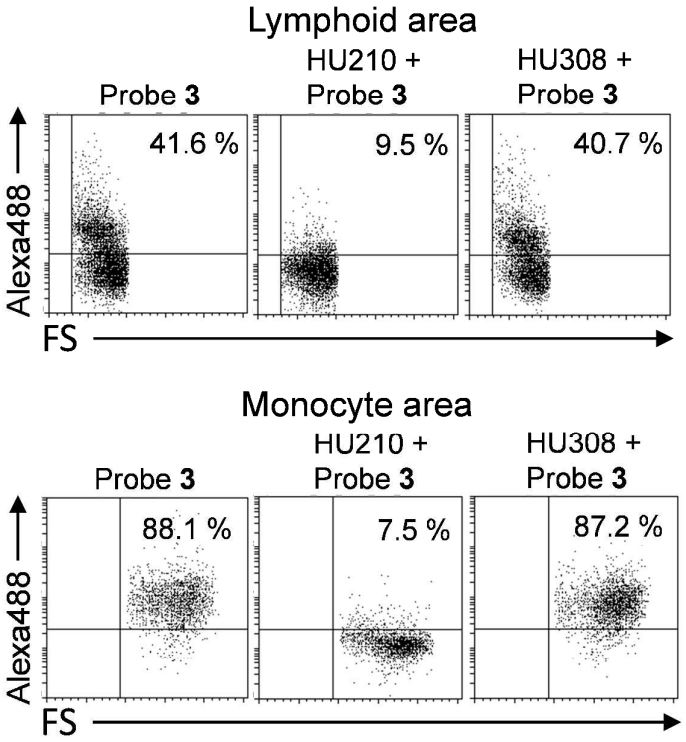



**Figure S1**. Representative flow cytometry dot plots after staining of PBMC with HU210-Alexa488 probe **3** (1 μM) alone or in the presence of an excess of unlabeled HU210 (50 μM) or HU308 (50 μM) after gating in the lymphoid (up) and monocyte (down) areas. The percentages of cells stained positive for probe **3** in each assayed condition are displayed inside the plots.

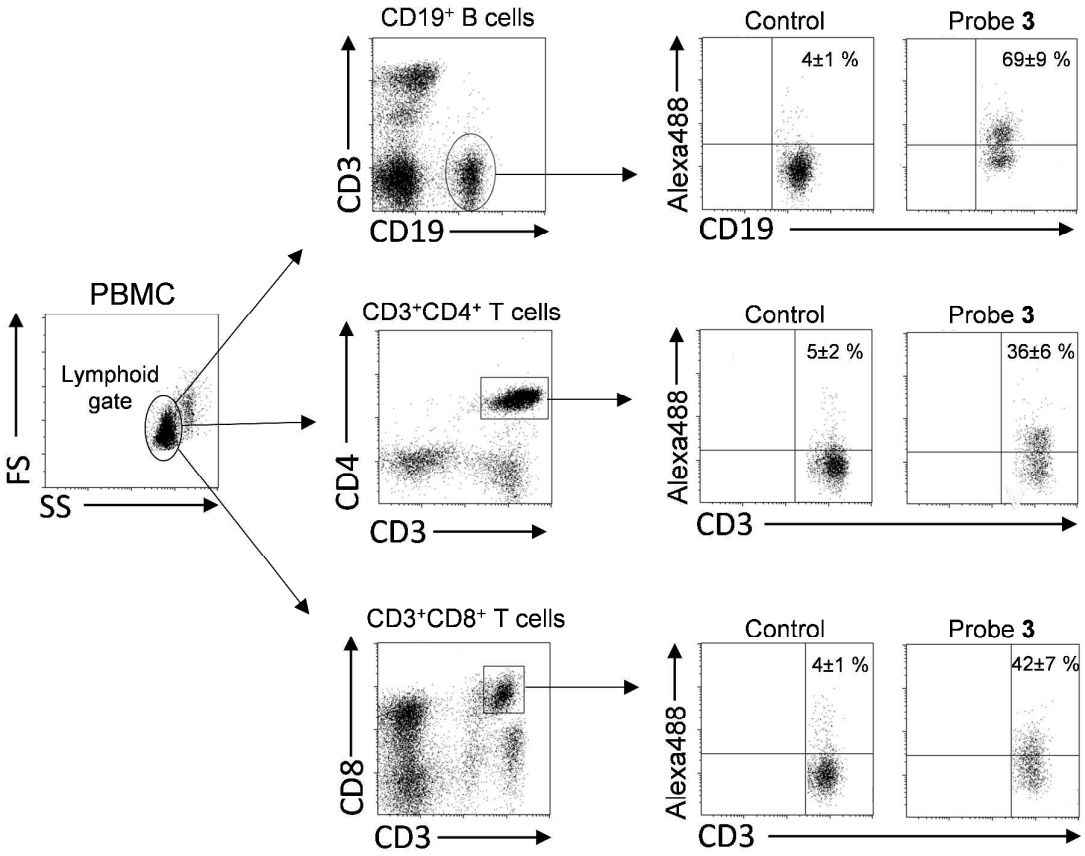



**Figure S2.** Multiplexed flow cytometry in PBMC using HU210-Alexa488 probe **3** in combination with anti-human lineage specific markers for B cells and T cell subsets. Representative flow cytometry dot plots and the gating strategy employed to identify and quantify $CB_1$-expressing B cells, T helper and cytotoxic T cell in freshly isolated PBMC are shown. The percentages of $CD19^+$ B cells, $CD3^+CD4^+$ T helper cells and $CD3^+CD8^+$ cytotoxic T cells stained positive for control or probe **3** (1 μM) are displayed inside the plots. Data represent means with SEMs of 7 independent experiments.

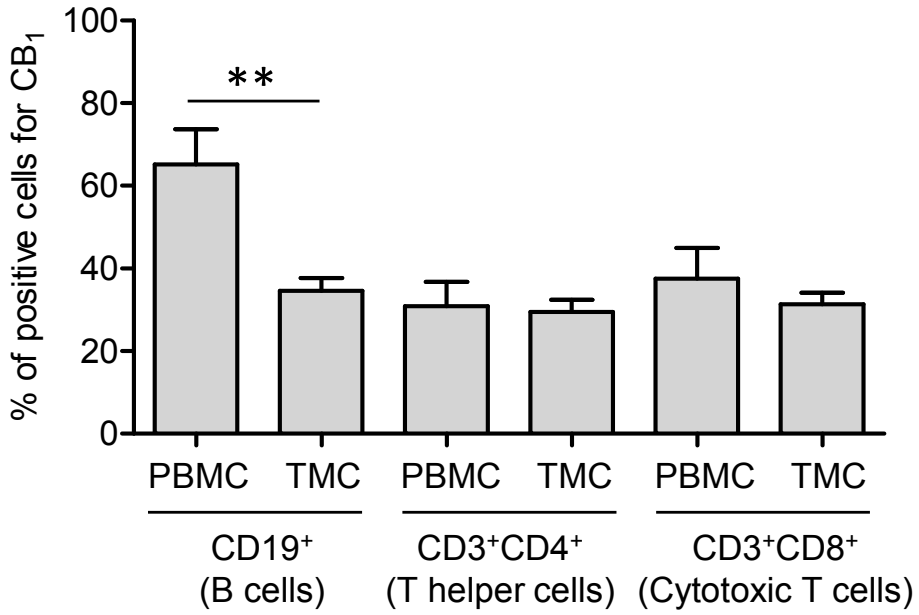



**Figure S3**. Comparison of the percentage of $CB_1$-expressing $CD19^+$ B cells, $CD3^+CD4^+$ T helper cells and $CD3^+CD8^+$ cytotoxic T cells detected with probe **3** in peripheral blood mononuclear cells (PBMC) and tonsil mononuclear cells (TMC).

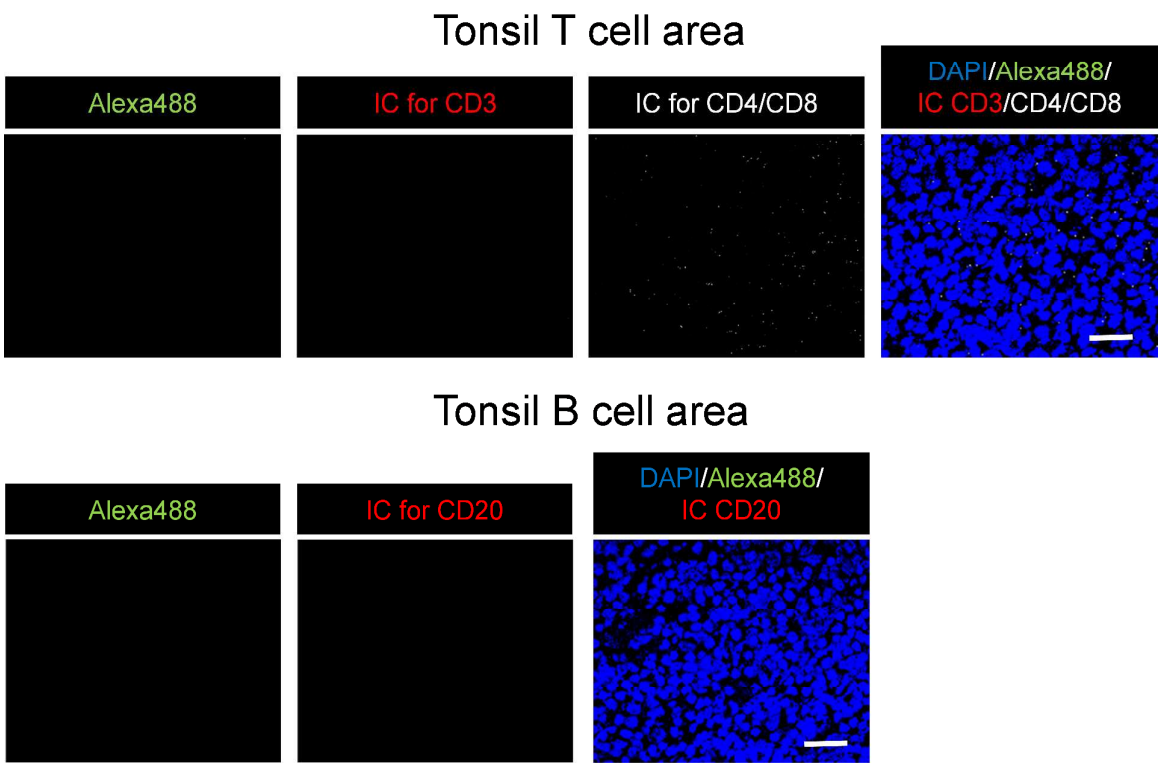



**Figure S4**. Tonsil sections stained with Alexa Fluor 488 alkyne (green) in combination with proper matched isotype controls for the anti-human CD3 (red) and anti-human CD4 or CD8 (white) for the T cell areas and with isotype control for anti-human CD20 (red) for the B cell areas. White bars, 10 μm. DAPI (blue), 4',6-diamidine-2-phenylindole dihydrochloride, to visualize nuclei was also included in all the showed controls.

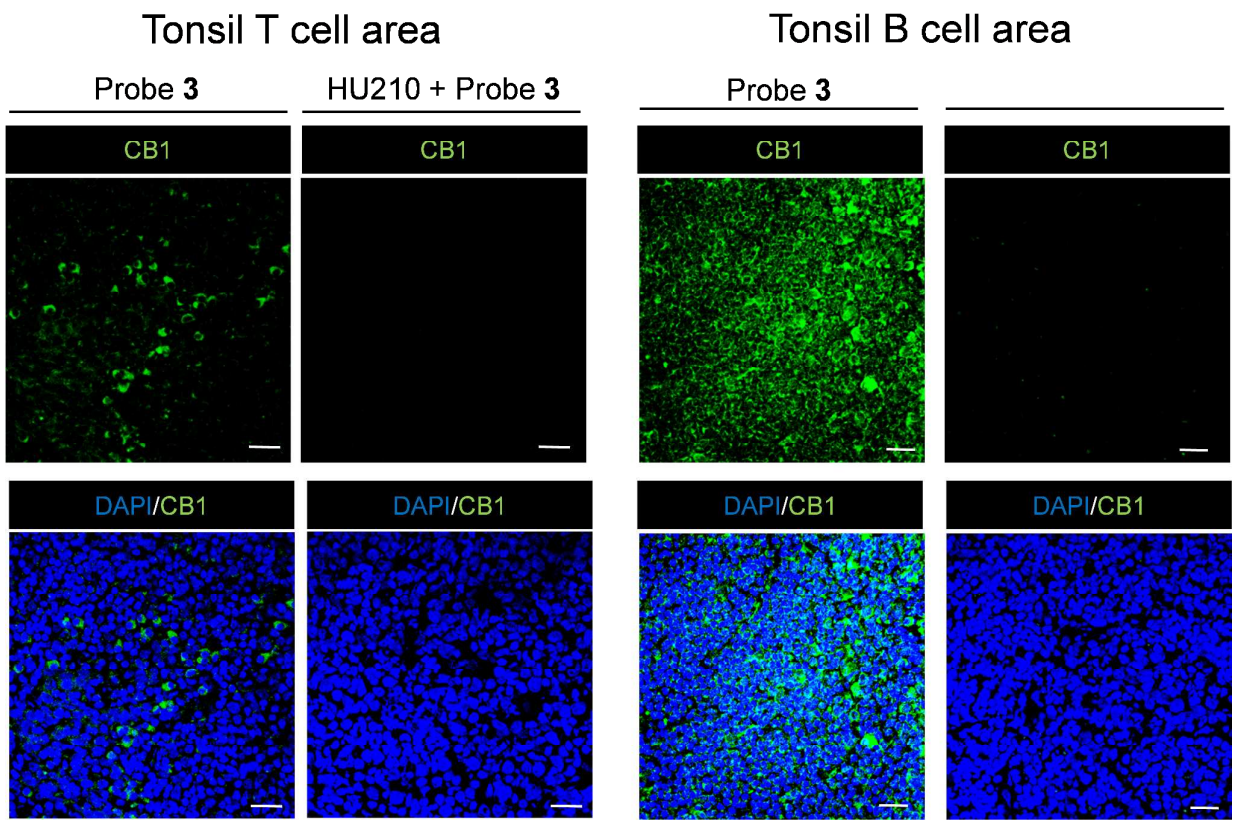



**Figure S5**. Tonsil sections stained with HU210-Alexa488 probe **3** (50 nM) alone or in the presence of an excess of unlabeled HU210 (5 μM). T and B cells areas are displayed. White bars, 10 μm. DAPI (blue), 4',6-diamidine-2-phenylindole dihydrochloride to visualize nuclei was also included in all the shown controls.

## 2. Materials and Methods

### 2.1. Chemistry

#### 2.1.1. General Experimental Details

Unless stated otherwise, starting materials, reagents and solvents were purchased as high-grade commercial products from Abcr, Acros, Scharlab, Sigma-Aldrich, or Thermo Fisher Scientific, and were used without further purification. Tetrahydrofuran (THF) and dichloromethane (DCM) were dried using a Pure SolvTM Micro 100 Liter solvent purification system. Reactions under microwave (MW) irradiation were performed in a Biotage Initiator 2.5 reactor. Reactions were monitored by analytical thin-layer chromatography (TLC) on plates supplied by Merck silica gel plates (Kieselgel 60 F-254) with detection by UV light (254 nm) or 10% phosphomolybdic acid solution in EtOH. Flash chromatography was performed on a Varian 971-FP flash purification system using silica gel cartridges (Varian, particle size 50 μm).

Melting points (mp, uncorrected) were determined on a Stuart Scientific electrothermal apparatus. Optical rotation [α] was measured on an Anton Paar MCP 100 modular circular polarimeter using a sodium lamp (λ=589 nm) with a 1 dm path length; concentrations (c) are given as g/100 mL. Infrared (IR) spectra were measured on a Bruker Tensor 27 instrument equipped with a Specac ATR accessory of 5200-650 $cm^{-1}$ transmission range; frequencies (ν) are expressed in $cm^{-1}$. Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker Avance III 700 MHz ($^{1}H$, 700 MHz; $^{13}C$, 175 MHz), Bruker Avance 500 MHz ($^{1}H$, 500 MHz; $^{13}C$, 125 MHz), or Bruker DPX 300 MHz ($^{1}H$, 300 MHz; $^{13}C$, 75 MHz) instruments at the Universidad Complutense de Madrid (UCM) NMR core facilities. Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (*J*) are in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), qt (quintet), m (multiplet), br (broad), app (apparent). 2D NMR experiments (HMQC and HMBC) of representative compounds were carried out to assign protons and carbons of the new structures. Mass spectrometry (MS) was carried out on a Bruker LC-Esquire spectrometer in electrospray ionization (ESI) mode at the UCM's mass spectrometry core facility. Spectroscopic data of all described compounds were consistent with the proposed structures.

High performance liquid chromatography coupled to mass spectrometry (HPLC-MS) analysis was performed using an Agilent 1200LC-MSD VL instrument. LC separation was achieved with a Zorbax Eclipse XDB-C18 column (5 μm, 4.6 mm x 150 mm) or a Zorbax SB-C3 column (5 μm, 2.1 mm x 50 mm), both together with a guard column (5 μm, 4.6 mm x

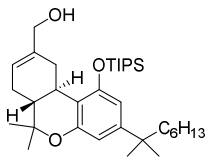
12.5 mm). The gradient mobile phases consisted of A (95:5 water/MeOH) and B (5:95 water/MeOH) with 0.1% ammonium hydroxide and 0.1% formic acid as the solvent modifiers. MS analysis was performed with an ESI source. The capillary voltage was set to 3.0 kV and the fragmentor voltage was set at 72 eV. The drying gas temperature was 350 ºC, the drying gas flow was 10 L/min, and the nebulizer pressure was 20 psi. Spectra were acquired in positive or negative ionization mode from 100 to 1200 m/z and in UV-mode at four different wavelengths (210, 230, 254, and 280 nm). Satisfactory chromatograms were obtained for all tested compounds, which confirmed a purity of at least 95%.

(-)-[(6a*R*,10a*R*)-3-(1,1-Dimethylheptyl)-1-hydroxy-6,6-dimethyl-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-9-yl]methyl pivalate (**4**) and (-)-(6a*R*,10a*R*)-3-(1,1-dimethylheptyl)-9-(hydroxymethyl)-6,6-dimethyl-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]-chromen-1-ol (HU210) were synthesized following procedures previously described and their spectroscopic data are in agreement with those previously reported.[1] Alexa Fluor 488 control [2-(6-amino-3-iminio-4,5-disulfonato-3*H*-xanthen-9-yl)-5-(prop-2-yn-1-ylcarbamoyl)benzoate, bis(triethylammonium) salt] was purchased from Thermo Fisher Scientific (Alexa Fluor™ 488 Alkyne). No unexpected or unusually high safety hazards were encountered in the synthesis of the compounds described in this work.

### 2.1.2. Synthesis and Characterization Data of Compounds 3, 5-10

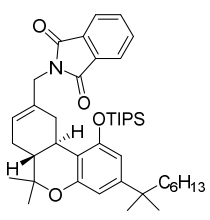
**(-)-{(6a*R*,10a*R*)-3-(1,1-Dimethylheptyl)-6,6-dimethyl-1-[(triisopropylsilyl)oxy]-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-9-yl}-methanol (5).** A solution of **4** (1.31 g, 3.0 mmol), TIPS-Cl (2.0 mL, 9.2 mmol), and imidazole (832 mg, 12 mmol) in anhydrous DMF (18 mL) under an argon atmosphere, was heated at 200 ºC for 45 min under MW irradiation. Once at rt, the solvent was evaporated under reduced pressure and the residue was redissolved in EtOAc, washed with saturated $NaHCO_3$ (aq) and brine. The organic layer was dried ($Na_2SO_4$), filtered, and evaporated under reduced pressure to yield {(6a*R*,10a*R*)-3-(1,1-dimethylheptyl)-6,6-dimethyl-1-[(triisopropylsilyl)oxy]-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]-chromen-9-yl}methyl pivalate (1.82 g, 95%), which was used in the next step without further purification. $^1$H-NMR (300 MHz, $CDCl_3$) δ 0.83 (t, *J*=6.8, 3H, C<u>H</u>$_3$CH$_2$), 1.05-1.30 (m, 47H, $CH_3C_{cyc}$, $C(CH_3)_3$, $2CH_3$, $3CH(CH_3)_2$, $(CH_2)_4$), 1.38 (s, 3H, $CH_3C_{cyc}$), 1.46-1.52 (m, 2H, C<u>H</u>$_2$C(CH$_3$)$_2$), 1.81-1.96 (m, 3H, ½CH$_2$C=, ½C<u>H</u>$_2$CH=, C<u>H</u>C(CH$_3$)$_2$), 2.19-2.27 (m, 1H, ½C<u>H</u>$_2$CH=), 2.57-2.68 (m, 1H, $CHC_{Ar}$), 3.31 (dd, *J*=16.2, 3.9, 1H, ½CH$_2$C=), 4.42-4.51 (m, 2H, $CH_2O$), 5.72 (d, *J*=4.0, 1H, CH=), 6.34 (d, *J*=1.8, 1H, $CH_{Ar}$), 6.39 (d, *J*=1.8, 1H, $CH_{Ar}$).

A solution of the silyl ether synthesized above (1.82 g, 2.9 mmol) in anhydrous THF (50 mL) was added dropwise to a suspension of $LiAlH_4$ (441 mg, 12 mmol) in anhydrous THF (50 mL) at 0 ºC and under an argon atmosphere. The reaction was stirred at that temperature for 2 h and allowed to warm to rt. The mixture was then carefully quenched with water and extracted with $Et_2O$ (2x). The organic extracts were washed with brine, dried ($Na_2SO_4$), filtered, and evaporated under reduced pressure. The residue was purified by chromatography (hexane to hexane/EtOAc, 8:2) to yield alcohol **5** (1.13 g, 72%). $[\alpha]_D^{20}$: -103.2 (c=0.98, chloroform). $R_f$: 0.17 (hexane/EtOAc, 8:2).




IR (ATR) ν 3357, 1565, 1464; $^1$H-NMR (300 MHz, $CDCl_3$) δ 0.84 (t, *J*=6.8, 3H, C<u>H</u>$_3$CH$_2$), 1.05-1.33 (m, 38H, CH$_3$C$_{cyc}$, 2CH$_3$, 3CH(CH$_3$)$_2$, (CH$_2$)$_4$), 1.39 (s, 3H, CH$_3$C$_{cyc}$), 1.40-1.52 (m, 2H, C<u>H</u>$_2$C(CH$_3$)$_2$), 1.79-1.89 (m, 3H, ½CH$_2$C=, ½C<u>H</u>$_2$CH=, C<u>H</u>C(CH$_3$)$_2$), 2.22-2.26 (m, 1H, ½C<u>H</u>$_2$CH=), 2.63 (td, *J*=10.8, 4.4, 1H, CHC$_{Ar}$), 3.36 (dd, *J*=16.8, 2.8, 1H, ½CH$_2$C=), 4.03 (AB system, *J*=12.9, 2H, CH$_2$O), 5.72 (d, *J*=3.7, 1H, CH=), 6.34 (d, *J*=1.8, 1H, CH$_{Ar}$), 6.39 (d, *J*=1.7, 1H, CH$_{Ar}$); $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 13.4 (3CHSi), 14.2 (<u>C</u>H$_3$CH$_2$), 18.2 (3CH(<u>C</u>H$_3$)$_2$), 18.3 (<u>C</u>H$_3$C$_{cyc}$), 22.8, 24.9 (2CH$_2$), 27.6 (<u>C</u>H$_3$C$_{cyc}$), 27.9 (<u>C</u>H$_2$CH=), 28.9, 29.1 (2CH$_3$), 30.2, 32.01 (2CH$_2$), 32.09 (<u>C</u>HC$_{Ar}$), 32.12 (<u>C</u>H$_2$C=), 37.5 (Ar<u>C</u>(CH$_3$)$_2$), 44.8 (<u>C</u>H$_2$C(CH$_3$)$_2$), 45.7 (<u>C</u>HC(CH$_3$)$_2$), 69.9 (CH$_2$O), 76.4 (O<u>C</u>(CH$_3$)$_2$), 108.3, 109.1 (2CH$_{Ar}$), 113.4 (C$_{Ar}$), 120.3 (CH=), 138.7 (C=), 149.4, 154.3, 155.2 (3C$_{Ar}$); MS (ESI) *m/z* 565.4 [M+Na]$^+$.

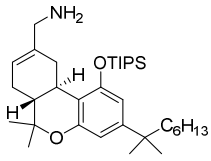
**(-)-2-({(6a*R*,10a*R*)-3-(1,1-Dimethylheptyl)-6,6-dimethyl-1-[(triisopropylsilyl)oxy]-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-9-yl}methyl)-1*H*-isoindole-1,3(2*H*)-dione (6).** To a solution of alcohol **5** (300 mg, 0.55 mmol), phthalimide (130 mg, 0.88 mmol), and $PPh_3$ (216 mg, 0.83 mmol) in anhydrous THF (5 mL) under an argon atmosphere, diethyl azodicarboxylate (0.4 mL, 0.88 mmol, 40% in toluene) was added dropwise and the mixture was stirred at rt for 16 h. $Et_2O$ was then added, and the reaction was washed with water. The organic layer was dried ($Na_2SO_4$), filtered, and evaporated under reduced pressure. The crude was purified by chromatography (hexane to hexane/EtOAc, 8:2) to yield phthalimide **6** (343 mg, 93%). $[\alpha]_D^{20}$: -130.0 (c=0.46, chloroform). $R_f$: 0.29 (hexane/EtOAc, 9:1).




IR (ATR) ν 1774, 1716, 1612, 1565, 1464; $^1$H-NMR (300 MHz, $CDCl_3$) δ 0.83 (t, *J*=6.8, 3H, C<u>H</u>$_3$CH$_2$), 1.00-1.27 (m, 38H, CH$_3$C$_{cyc}$, 2CH$_3$, 3CH(CH$_3$)$_2$, (CH$_2$)$_4$), 1.35 (s, 3H, CH$_3$C$_{cyc}$), 1.42-1.50 (m, 2H, C<u>H</u>$_2$C(CH$_3$)$_2$), 1.81-1.90 (m, 3H, ½CH$_2$C=, ½C<u>H</u>$_2$CH=, C<u>H</u>C(CH$_3$)$_2$), 2.16-

2.21 (m, 1H, ½C<u>H</u>$_2$CH=), 2.65 (td, *J*=10.9, 4.4, 1H, CHC$_{Ar}$), 3.33 (dd, *J*=15.6, 3.9, 1H, ½CH$_2$C=), 4.21 (m, 2H, CH$_2$N), 5.62 (d, *J*=3.6, 1H, CH=), 6.31 (d, *J*=1.4, 1H, CH$_{HU}$), 6.36 (d, *J*=1.4, 1H, CH$_{HU}$), 7.70-7.73 (m, 2H, 2CH$_{Phth}$), 7.83-7.86 (m, 2H, 2CH$_{Phth}$); $^{13}$C-NMR (75 MHz $CDCl_3$) δ 13.2 (3CHSi), 14.2 (<u>C</u>H$_3$CH$_2$), 18.17 (3CH(<u>C</u>H$_3$)$_2$), 18.21 (<u>C</u>H$_3$C$_{cyc}$), 22.8, 24.9 (2CH$_2$), 27.6 (<u>C</u>H$_3$C$_{cyc}$), 27.9 (<u>C</u>H$_2$CH=), 28.9, 29.2 (2CH$_3$), 30.2, 32.0 (2CH$_2$), 32.1 (<u>C</u>HC$_{Ar}$), 32.9 (<u>C</u>H$_2$C=), 37.5 (Ar<u>C</u>(CH$_3$)$_2$), 43.4 (CH$_2$N), 44.8 (<u>C</u>H$_2$C(CH$_3$)$_2$), 45.4 (<u>C</u>HC(CH$_3$)$_2$), 76.4 (O<u>C</u>(CH$_3$)$_2$), 108.2, 109.0 (2CH$_{HU}$), 113.2 (C$_{HU}$), 122.3 (CH=), 123.4 (2CH$_{Phth}$), 132.3 (2C$_{Phth}$), 133.3 (C=), 134.0 (2CH$_{Phth}$), 149.3, 154.3, 155.0 (3C$_{HU}$), 168.2 (2CO); MS (ESI) *m/z* 672.2 [M+H]$^+$.

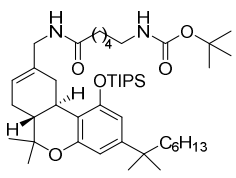
**(-)-{(6a*R*,10a*R*)-3-(1,1-Dimethylheptyl)-6,6-dimethyl-1-[(triisopropylsilyl)oxy]-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-9-yl}methylamine (7).** To a solution of **6** (100 mg, 0.15 mmol) in EtOH (3 mL) under an argon atmosphere, hydrazine monohydrate (30 μL, 0.44 mmol) was added and the mixture was stirred at reflux for 2 h. Once cooled to rt, a mixture of HCl/water 1:1 (0.3 mL) was added, and the reaction was refluxed for 1 h, and stirred at rt for 16 h. Then, toluene was added and the mixture was filtered off and washed with EtOH. The filtrate was washed with 5% $NaHCO_3$ (aq). The organic layer was dried ($Na_2SO_4$), filtered, and evaporated under reduced pressure. The crude was purified by chromatography (DCM to DCM/MeOH, 8:2) to yield amine **7** (78 mg, 96%). $[\alpha]_D^{20}$: -0.27 (c=0.18, chloroform). *R*$_f$: 0.20 (DCM/MeOH, 95:5).




IR (ATR) ν 2957, 2928, 1614, 1566, 1465, 1383; $^1$H-NMR (300 MHz, $CDCl_3$) δ 0.83 (t, *J*=6.8, 3H, C<u>H</u>$_3$CH$_2$), 1.05-1.32 (m, 38H, CH$_3$C$_{cyc}$, 2CH$_3$, 3CH(CH$_3$)$_2$, (CH$_2$)$_4$), 1.38 (s, 3H, CH$_3$C$_{cyc}$), 1.46-1.51 (m, 2H, C<u>H</u>$_2$C(CH$_3$)$_2$), 1.81-1.90 (m, 5H, ½CH$_2$C=, ½C<u>H</u>$_2$CH=, C<u>H</u>C(CH$_3$)$_2$, NH$_2$), 2.17-2.26 (m, 1H, ½C<u>H</u>$_2$CH=), 2.62 (td, *J*=10.9, 4.2, 1H, CHC$_{Ar}$), 3.22 (m, 2H, CH$_2$N), 3.28 (dd, *J*=15.9, 4.3, 1H, ½CH$_2$C=), 5.63 (d, *J*=4.3, 1H, CH=), 6.34 (d, *J*=1.8, 1H, CH$_{Ar}$), 6.39 (d, *J*=1.8, 1H, CH$_{Ar}$); $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 13.4 (3CHSi), 14.2 (<u>C</u>H$_3$CH$_2$), 18.2 (3CH(<u>C</u>H$_3$)$_2$), 18.3 (<u>C</u>H$_3$C$_{cyc}$), 22.8, 24.9 (2CH$_2$), 27.6 (<u>C</u>H$_3$C$_{cyc}$), 27.9 (<u>C</u>H$_2$CH=), 28.9, 29.1 (2CH$_3$), 30.2, 32.0 (2CH$_2$), 32.2 (<u>C</u>HC$_{Ar}$), 33.2 (<u>C</u>H$_2$C=), 37.5 (Ar<u>C</u>(CH$_3$)$_2$), 44.8 (<u>C</u>H$_2$C(CH$_3$)$_2$), 45.8 (<u>C</u>HC(CH$_3$)$_2$), 48.0 (CH$_2$N), 76.4 (O<u>C</u>(CH$_3$)$_2$), 108.3, 109.0 (2CH$_{Ar}$), 113.5 (C$_{Ar}$), 118.3 (CH=), 140.3 (C=), 149.3, 154.3, 155.1 (3C$_{Ar}$); HRMS (ESI) *m/z* calcd for [M+H]$^+$ $C_{34}H_{60}NO_2Si$: 542.4393; found: 542.4388.

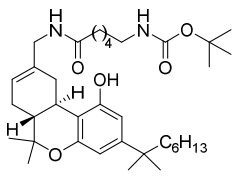
**(-)-*tert*-Butyl {6-[({(6a*R*,10a*R*)-3-(1,1-dimethylheptyl)-6,6-dimethyl-1-[(triisopropylsilyl)oxy]-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-9-yl}methyl)-**

**amino]-6-oxohexyl}carbamate (8).** To a solution of 6-[(*tert*-butoxycarbonyl)amino]hexanoic acid (77 mg, 0.33 mmol) in dry DCM (0.7 mL), *N,N'*-dicyclohexylcarbodiimide (125 mg, 0.60 mmol) was added under an argon atmosphere, and the mixture was stirred at rt for 10 min. Then, a solution of amine **7** (164 mg, 0.30 mmol) in dry DCM (2 mL) was added, and the mixture was stirred at rt for 16 h. The reaction mixture was filtered, washing with EtOAc. The filtrate was washed with water, dried ($Na_2SO_4$), filtered, and evaporated under reduced pressure. The crude was purified by chromatography (DCM to DCM/EtOAc, 9:1) to give compound **8** (169 mg, 74%). Mp: 53 ºC. $[\alpha]_D^{20}$: -87.9 (c=0.96, chloroform). $R_f$: 0.25 (DCM/EtOAc, 9:1).



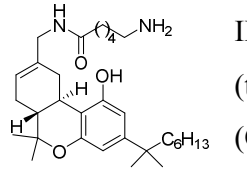
IR (ATR, ν): 3319, 1692, 1649, 1564, 1463; $^1$H-NMR (300 MHz, $CDCl_3$) δ 0.84 (t, *J*=6.8, 3H, C<u>H</u>$_3$CH$_2$), 1.05-1.71 (m, 61H, C(CH$_3$)$_3$, 3CH(CH$_3$)$_2$, CHC(CH$_3$)$_2$, CH$_2$C(CH$_3$)$_2$, (CH$_2$)$_4$, (CH$_2$)$_3$, ½CH$_2$C=, ½C<u>H</u>$_2$CH=), 2.15-2.23 (m, 3H, ½C<u>H</u>$_2$CH=, CH$_2$CO), 2.64 (td, *J*=10.8, 4.4, 1H, CHC$_{Ar}$), 3.07-3.13 (m, 2H, C<u>H</u>$_2$NBoc), 3.28 (dd, *J*=16.1, 3.7, 1H, ½CH$_2$C=), 3.79 (d, *J*=16.1, 2H, CH$_2$N$_{HU}$), 4.53 (br s, 1H, NH), 5.43 (t, *J*=4.9, 1H, NH), 5.60 (d, *J*=4.5, 1H, CH=), 6.34 (d, *J*=1.4, 1H, CH$_{Ar}$), 6.38 (d, *J*=1.6, 1H, CH$_{Ar}$); $^{13}$C-NMR (75 MHz, $CDCl_3$): δ 13.4 (3CHSi), 14.2 (<u>C</u>H$_3$CH$_2$), 18.3 (<u>C</u>H$_3$C$_{cyc}$, 3CH(<u>C</u>H$_3$)$_2$), 22.8, 24.9, 25.5, 26.6 (4CH$_2$), 27.6 (<u>C</u>H$_3$C$_{cyc}$), 28.0 (<u>C</u>H$_2$CH=), 28.6 (C(<u>C</u>H$_3$)$_3$), 28.9, 29.2 (2CH$_3$), 30.2, 31.1, 32.0 (3CH$_2$), 32.2 (<u>C</u>HC$_{Ar}$), 33.4 (<u>C</u>H$_2$C=), 34.1 (<u>C</u>H$_2$NBoc), 36.8 (<u>C</u>H$_2$CO), 37.6 (Ar<u>C</u>(CH$_3$)$_2$), 44.8 (<u>C</u>H$_2$C(CH$_3$)$_2$), 45.1 (CH$_2$N$_{HU}$), 45.6 (<u>C</u>HC(CH$_3$)$_2$), 76.4 (O<u>C</u>(CH$_3$)$_2$), 77.4 (<u>C</u>(CH$_3$)$_3$), 108.4, 109.1 (2CH$_{Ar}$), 113.2 (C$_{Ar}$), 121.2 (CH=), 135.4 (C=), 149.5, 154.3, 155.1 (3C$_{Ar}$), 156.1 (NCOO), 172.7 (CON); MS (ESI) *m/z* 753.6 [M-H]$^-$.

**(-)-*tert*-Butyl [6-({[(6a*R*,10a*R*)-3-(1,1-dimethylheptyl)-1-hydroxy-6,6-dimethyl-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-9-yl]methyl}amino)-6-oxohexyl]-carbamate (9).** To a solution of silyl-protected phenol **8** (62 mg, 83 μmol) in anhydrous THF (4 mL) at 0 ºC and under an argon atmosphere, a 1 M solution of TBAF (110 μL) in anhydrous THF was added, and the mixture was stirred at this temperature for 3 h. EtOAc and water were added, and the organic layer was separated, washed with brine, dried ($Na_2SO_4$), filtered, and evaporated under reduced pressure. The crude was purified by chromatography (DCM to DCM/EtOAc, 9:1) to give compound 9 (40 mg, 81%). Mp: 83-85 ºC. $[\alpha]_D^{20}$: -101.5 (c=0.54, chloroform). $R_f$: 0.35 (DCM/MeOH, 95:5).



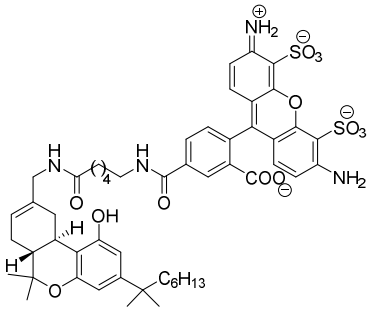
IR (ATR) ν 3308, 1686, 1649, 1575, 1523, 1457; $^{1}$H-NMR (300 MHz, $CDCl_3$) δ 0.84 (t, *J*=6.8, 3H, C<u>H</u>$_3$CH$_2$), 1.04-1.86 (m, 40H, C(CH$_3$)$_3$, CHC(CH$_3$)$_2$, CH$_2$C(CH$_3$)$_2$, (CH$_2$)$_4$, (CH$_2$)$_3$, ½CH$_2$C=, ½C<u>H</u>$_2$CH=), 2.17-2.24 (m, 3H, ½C<u>H</u>$_2$CH=, CH$_2$CO), 2.68 (td, *J*=10.9, 4.6, 1H, CHC$_{Ar}$), 3.15 (q, *J*=6.8, 2H, C<u>H</u>$_2$NBoc), 3.48 (d, *J*=17.2, 1H, ½CH$_2$C=), 3.69 (d, *J*=13.2, 1H, ½CH$_2$N$_{HU}$), 3.99 (dd, *J*=13.8, 7.3, 1H, ½CH$_2$N$_{HU}$), 4.68 (t, *J*=5.4, 1H, NH), 5.47 (br s, 1H, NH), 5.68 (d, *J*=4.3, 1H, CH=), 6.32 (d, *J*=1.7, 1H, CH$_{Ar}$), 6.44 (m, 1H, CH$_{Ar}$), 7.80 (br s, 1H, OH); $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 14.2 (<u>C</u>H$_3$CH$_2$), 18.6 (<u>C</u>H$_3$C$_{cyc}$), 22.8, 24.8, 25.5, 26.1 (4CH$_2$), 27.8 (<u>C</u>H$_3$C$_{cyc}$), 28.0 (<u>C</u>H$_2$CH=), 28.6 (C(<u>C</u>H$_3$)$_3$), 28.7, 29.0 (2CH$_3$), 30.2 (2CH$_2$), 31.7 (<u>C</u>HC$_{Ar}$), 31.8 (<u>C</u>H$_2$C=), 32.0 (CH$_2$), 36.8 (<u>C</u>H$_2$CO), 37.5 (Ar<u>C</u>(CH$_3$)$_2$), 40.5 (<u>C</u>H$_2$NBoc), 44.7 (<u>C</u>H$_2$C(CH$_3$)$_2$), 45.2 (<u>C</u>HC(CH$_3$)$_2$), 45.7 (CH$_2$N$_{HU}$), 76.4 (O<u>C</u>(CH$_3$)$_2$), 77.2 (<u>C</u>(CH$_3$)$_3$), 105.6, 107.0 (2CH$_{Ar}$), 109.7 (C$_{Ar}$), 123.5 (CH=), 135.7 (C=), 150.0, 154.3, 156.1 (3C$_{Ar}$), 156.8 (NCOO), 172.9 (CON); MS (ESI) *m/z* 599.4 [M+H]$^+$.

**(-)-6-Amino-*N*-{[(6a*R*,10a*R*)-3-(1,1-dimethylheptyl)-1-hydroxy-6,6-dimethyl-6a,7,10,10a-tetrahydro-6*H*-benzo[*c*]chromen-9-yl]methyl}hexanamide (10).** Compound **9** (32 mg, 53 μmol) was treated with a mixture of TFA/DCM 1:1 (0.1 mL) under an argon atmosphere, and the mixture was stirred at rt for 1 h. The solvent was evaporated under reduced pressure and the residue was redissolved in DCM, washed with saturated $NaHCO_3$ (aq) and brine. The organic layer was dried ($Na_2SO_4$), filtered, and evaporated under reduced pressure to yield amine **10** (22 mg, 84%), which was used in the next step without further purification. $[\alpha]_D^{20}$: -145.1 (c=0.46, chloroform). *R<sub>f</sub>*: 0.10 (DCM/MeOH, 95:5).



IR (ATR) ν 3302, 1646, 1568, 1449; $^{1}$H-NMR (300 MHz, $CDCl_3$) δ 0.84 (t, *J*=6.7, 3H, C<u>H</u>$_3$CH$_2$), 1.06-1.82 (m, 31H, CHC(CH$_3$)$_2$, CH$_2$C(CH$_3$)$_2$, (CH$_2$)$_4$, (CH$_2$)$_3$, ½CH$_2$C=, ½C<u>H</u>$_2$CH=), 2.08-2.21 (m, 2H, CH$_2$CO), 2.31-2.37 (m, 1H, ½C<u>H</u>$_2$CH=), 2.62 (td, *J*=10.8, 4.5, 1H, CHC$_{Ar}$), 2.58-2.65 (m, 2H, C<u>H</u>$_2$NH$_2$), 3.54 (dd, *J*=14.0, 3.2, 1H, ½C<u>H</u>$_2$NH), 3.60 (dd, *J*=17.7, 3.6, 1H, ½CH$_2$C=), 4.19 (dd, *J*=13.9, 8.3, 1H, ½C<u>H</u>$_2$NH), 5.42-5.45 (m, 1H, NH), 5.62 (d, *J*=3.6, 1H, CH=), 6.12 (d, *J*=1.6, 1H, CH$_{Ar}$), 6.28 (d, *J*=1.5, 1H, CH$_{Ar}$); $^{13}$C-NMR (75 MHz, $CDCl_3$) δ 14.2 (<u>C</u>H$_3$CH$_2$), 18.5 (<u>C</u>H$_3$C$_{cyc}$), 22.8, 24.8, 25.6, 26.2 (4CH$_2$), 27.7 (<u>C</u>H$_3$C$_{cyc}$), 28.0 (<u>C</u>H$_2$CH=), 28.8, 29.0 (2CH$_3$), 30.2, 31.2, 31.4 (3CH$_2$), 31.7 (<u>C</u>HC$_{Ar}$), 32.0 (<u>C</u>H$_2$C=), 36.5 (<u>C</u>H$_2$CO), 37.3 (Ar<u>C</u>(CH$_3$)$_2$), 41.6 (CH$_2$NH$_2$), 44.7 (<u>C</u>H$_2$C(CH$_3$)$_2$), 45.2 (<u>C</u>HC(CH$_3$)$_2$), 45.4 (CH$_2$NH), 76.4 (O<u>C</u>(CH$_3$)$_2$), 105.1, 106.3 (2CH$_{Ar}$), 110.2 (C$_{Ar}$), 123.2 (CH=), 136.3 (C=), 149.7, 154.3, 156.6 (3C$_{Ar}$), 172.8 (CON); MS (ESI) *m/z* 499.3 [M+H]$^+$.

**HU210-Alexa488 probe 3.** To a solution of Alexa Fluor 488 TFP ester (12.5 mg, 18 μmol) in anhydrous DMF (0.2 mL) protected from light and under an argon atmosphere, a solution of amine **10** (11.7 mg, 23 μmol) in dry DCM (0.26 mL) was added and the resulting mixture was stirred at rt for 30 min. The solvent was evaporated under reduced pressure and the crude was purified by chromatography, DCM to DCM/MeOH/ammonia, 1:1:0.02) to yield probe **3** (12 mg, 66%).



$^1$H-NMR (700 MHz, DMSO-$d_6$) δ 0.81 (m, 3H, C$\underline{H}_3$CH$_2$), 1.01-1.80 (m, 31H, CHC(CH$_3$)$_2$, CH$_2$C(CH$_3$)$_2$, (CH$_2$)$_4$, (CH$_2$)$_3$, ½CH$_2$C=, ½C$\underline{H}_2$CH=), 2.11 (m, 3H, CH$_2$CO, ½C$\underline{H}_2$CH=), 2.50 (m, 1H, CHC$_{Ar}$), 3.60 (m, 3H, ½C$\underline{H}_2$NH, C$\underline{H}_2$NH), 4.45 (m, 1H, ½C$\underline{H}_2$NH), 5.51 (m, 1H, CH=), 6.12 (m, 1H, CH$_{Ar}$), 6.30 (m, 1H, CH$_{Ar}$), 7.09 (m, 4H, 3CH$_{Ar}$, NH), 7.40 (m, 2H, CH$_{Ar}$, NH), 7.86 (m, 1H, NH), 8.24 (m, 1H, CH$_{Ar}$), 8.54 (m, 1H, NH), 8.83 (m, 1H, CH$_{Ar}$), 9.17 (m, 1H, CH$_{Ar}$); $^{13}$C-NMR (75 MHz, DMSO-$d_6$) δ 14.0 (C$\underline{H}_3$CH$_2$), 18.3 (C$\underline{H}_3$C$_{cyc}$), 22.1, 24.2, 25.2, 26.3 (4CH$_2$), 27.4 (C$\underline{H}_3$C$_{cyc}$), 28.69 (C$\underline{H}_2$CH=), 28.73, 28.9 (2CH$_3$), 29.4, 31.0, 31.2 (3CH$_2$), 32.2 (C$\underline{H}$C$_{Ar}$), 35.4 (C$\underline{H}_2$C=), 36.9 (C$\underline{H}_2$CO), 39.3 (CH$_2$NH, ArC$\underline{C}$(CH$_3$)$_2$), 43.8, 43.9 (CH$_2$NH, C$\underline{H}_2$C(CH$_3$)$_2$), 44.9 (C$\underline{H}$C(CH$_3$)$_2$), 75.8 (OC$\underline{C}$(CH$_3$)$_2$), 99.5, 104.9, 105.5, 109.5, 114.1, 129.6, 135.8, 136.0, 148.7, 153.8, 156.3 (CH$_{Ar}$, C$_{Ar}$), 164.5 (COO), 172.8 (2CON); HRMS (ESI, *m/z*): calcd for [M]$^-$ C$_{52}$H$_{62}$N$_4$O$_{13}$S$_2$: 1013.3688; found: 1013.3682.

### 2.2. Binding assays

Membranes from HEK-293-EBNA cells expressing the hCB$_1$ or hCB$_2$ receptors were purchased from PerkinElmer and conserved at -80 ºC in packaging buffer for subsequent use. Competitive inhibition assays were performed according to standard procedures, briefly detailed below.[2,3]

Cell membranes (8 mg/mL for CB$_1$ and 4 mg/mL for CB$_2$) were homogenized at 0 ºC using a glass dounce homogenizer in assay buffer (50 mM Tris·HCl, 2.5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM MgCl$_2$ and 0.5 mg/mL bovine serum albumin (BSA), pH=7.4 for CB$_1$; or 50 mM Tris·HCl, 2.5 mM ethyleneglycoltetraacetic acid (EGTA), 5 mM MgCl$_2$ and 1 mg/mL fatty acid free BSA, pH=7.4 for CB$_2$) at 1:500 dilution. Fractions of 500 μL of the membrane suspension were incubated in 96-multiwell plates (Optiplate), which had been previously silanized with Sigmacote (Sigma) to prevent adsorption of the compounds, at 30 ºC for 90 min with [$^3$H]-CP55940 at a concentration of 0.4 nM for CB$_1$ and

0.53 nM for $CB_2$, respectively (144 Ci/mmol, PerkinElmer), in the absence or presence of different concentrations of the compound under study (ranging from $10^{-5}$ to $10^{-11}$ M), in a final volume of 550 μL. Nonspecific binding was determined by radioligand binding in the presence of a saturating concentration of 10 μM (R)-(+)-WIN552122 (Tocris), and represented less than 15% of total binding.

For all binding assays, competing drug and nonspecific, total, and radioligand bindings were defined in triplicate. Incubation was terminated by rapid vacuum filtration through Wallac Filtermat A filters (PerkinElmer), presoaked in polyethylenimine (0.05%), using a FilterMate Unifilter 96-Harvester (PerkinElmer). The filters were then washed 9 times with 500 μL/well of ice-cold assay buffer and air-dried. Then, a MeltiLex solid scintillator sheet (PerkinElmer) was immediately melted onto the filter and the radioactivity bound to the filter was quantified by scintillation spectrometry, using a Microbeta TopCount instrument (PerkinElmer). The data were analyzed by an iterative curve-fitting procedure using GraphPad Prism program and $K_i$ values were calculated from the IC50 values using the Cheng-Prusoff equation[4] and are expressed as the average and standard error obtained from two to four independent experiments carried out in triplicate.

### 2.3. Biological methods

**Isolation of peripheral blood mononuclear cells and tonsil mononuclear cells.** Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation from heparinized peripheral venous blood. Tonsil mononuclear cells (TMC) were isolated from tonsil tissues following exactly the same protocol previously described by Palomares et al.[5] RPMI 1640 supplemented with 5% heat-inactivated FCS (Amimed), antibiotics (penicillin, streptomycin, kanamycin from Life Technologies; amphotericine B from Bristol-Myers Squibb; and ciprofloxacin from Fluka), MEM vitamin, L-glutamine, nonessential amino acids and sodium pyruvate (Life Technologies) was used for isolation of TMC and cell cultures. The same medium above described without amphotericine B and ciprofloxacin and supplemented with 10% FCS was used for PBMC isolation and cell cultures.

**Flow cytometry.** PBMC, TMC or HEK293T cell line as a negative control for cells that do not express CBRs constitutively (0.25-0.52x$10^6$ cells) were incubated with Alexa Fluor 488 alkyne or probe **3** at a final concentration of 1 μM in PBS for 30 min at rt with shaking. After that, cells were washed with PBS and fixed with 0.1% paraformaldehyde (PFA). Staining

with probe **3** in the presence of an excess of the unlabeled $CB_1/CB_2$ agonist HU210 (50 μM) or $CB_2$ selective agonist HU308 (50 μM) were also performed. For co-staining with monoclonal antibodies (mAbs) specific for human lineage specific markers for B and T cell subsets, PBMC and TMC were first incubated with the corresponding mAbs in PBS for 15 min at rt with shaking, washed with PBS, and then stained with probe **3** or Alexa Fluor 488 alkyne as above described. The following anti-human mAbs were used for flow cytometry: Alexa Fluor 647-conjugated anti-CD3 (Biolegend, IgG2a mouse, clone Hit3a); PC5.5-conjugated anti-CD4 (eBioscience, IgG1 mouse, clone SK3), Alexa Fluor 700-conjugated CD8 (eBioscience, IgG2a mouse, clone 53-6.7), PE-conjugated antiCD14 (Beckman Coulter, IgG2a mouse, clone RMO52), and Cy7-conjugated anti-CD19 (Biolegend, IgG1 mouse, clone HIB19). Flow cytometric analysis was performed using a 10 color Galios flow cytometer (Beckman Coulter, Brea, USA) and further by Weasel v2.5 or Kaluza Flow cytometry software (Beckman Coulter).

**Visualization of $CB_1$ in PBMC and HEK293T cells by confocal microscopy.** PBMC or HEK293T cells were suspended in PBS, centrifuged onto glass slides (cytospin, 200 rpm, 2 min) and fixed in 4% PFA for 7 min. Then, cells were washed twice with PBS and incubated with probe **3** 0.05 μM or Alexa Fluor 488 alkyne 0.05 μM (for the nonspecific fluorescence) in PBS for 30 min. Then, samples were rinsed twice with PBS and treated with 1% PFA and mounted with ProLong Gold antifade reagent with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) for nuclei staining. Nonspecific fluorescence was determined in the presence of 5 μM HU210. Images were acquired and analyzed by using the confocal microscope LMS 780 Zeiss (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). All assay and control samples were imaged under the same microscope conditions and the images shown are representative of two independent experiments. There was no significant background in any of the assayed control conditions.

**Purification of cells by magnetic and flow cytometry cell sorting**

Monocytes and lymphocytes (T and B cells) were isolated from PBMC with anti-human CD14 microbeads or with anti-human CD3 and CD19 microbeads, respectively, by positive selection in autoMACS (Miltenyi-Biotec) according to manufacturer's recommendations. Purity was confirmed by flow cytometry with lineage-specific markers mAbs. PBMC was stained with probe **3** and the positive and negative fractions separated by flow cytometry cell

sorting with FACSAria II (Becton Dickinson). Purified cells were employed for total RNA isolation to determine $CB_1$-specific gene expression levels by quantitative PCR.

**Cell culture.** PBMC and TMC were seeded at a density of $2x10^5$ cells/200 μL of the specific complete RPMI medium in flat-bottomed 96-well plates with the cannabinoid agonist WIN552122 or HU210 (10μM) or in medium alone for 18 h and the expression of $CB_1$ was monitored using fluorescent probe **3** by flow cytometry as above described. For functional experiments, TMC were stimulated with the following combination of mAbs to T cell surface molecules to induce T cell proliferation: anti-CD2 (clone 4B2 and 6G4; 0.5 μg/ml), anti-CD3 (clone OKT3; 0.5 μg/ml), and anti-CD28 mAb (clone B7G5; 0.5 μg/ml; all from Sanquin) and cultured in complete RPMI medium alone or in the presence of different concentrations of the synthetic cannabinoid agonist HU210 or HU308 for 5 days. Proliferative responses were measured by adding 1μCi [$^3$H]-thymidine during the last 8 hours of culture. At day 5, cell free supernatants were collected for cytokine determinations by cytometric bead arrays (BioRad) and cell pellets were used to isolate total RNA for gene expression analysis by quantitative PCR.

**RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR.** Total RNA was isolated using the RNeasy mini kit (Qiagen). Reverse transcription was performed with the Revert Aid M-MuLV Reverse Transcriptase (Fermentas) using random hexamer primers according to the manufacturers protocol. Gene expression was analyzed by quantitative CFX384 Real-Time PCR instrument (Biorad). The sequences of the employed primer pairs were: Elongation factor 1α (EF1α) (fwd, CTGAACCATCCAAT; rev, GCCGTGTGGCAATCCAAT); IFN-γ (fwd, TCTCGGAAACGATGAAATATACAAGTTAT; rev, GTAACAGCCAAGAGAACCCAAAA), IL-10 (fwd, GTGATGCCCCAAGCTGAGA; rev, CACGGCCTTGCTCTTGTTTT), IL-13 (fwd, GCCCTGGAATCCCTGATCA; rev, GCTCAGCATCCTCTGGGTCTT); IL-5 (fwd, GACCTTGGCACTGCTTTCTACTC; rev, TGTACAGGAACAGGAATCCTCAGA); IL-17 (fwd, CCATCCCCAGTTGATTGGAA; rev, CTCAGCAGCAGTAGCAGTGACA); and $CB_1$ (fwd, AAGGTGACATGGCATCCAAAT: rev, AGGACGAGAGAGACTTGTTGTAA). PCR conditions were 10 min at 95 ºC followed by 40 cycles of 15 s at 90 ºC and 1 min at 60 ºC. Relative quantification was performed using the comparative ΔΔCT method.[6]

**Immunohistochemistry.** Palatine tonsil tissues were embedded in Tisue-Tek (Sakura Finetek), frozen in liquid nitrogen-cooled 2-methylbutane (Fluka), and stored at -80 ºC until cryosections were cut using a HM 500 OM microtome (Mikrotom). Paraformaldehyde-fixed cryosections (7 μm) were sequentially stained with the following Abs. First, the slides were incubated with anti-human CD3 rat or the corresponding isotype control (Serotec), or anti-human CD20 rabbit IgG (Abcam) or the corresponding isotype control (Dako) for 45 min. After washing with PBS-Tween, CD3- or CD20-binding Abs were detected by using Alexa Fluor 633-conjugated goat anti-rat IgG (CD3) or goat anti-rabbit (CD20) (Invitrogen), by incubation for 30 min. The sections were washed with PBS-Tween and a blocking step with rat or rabbit IgG mAb was included. Then, the sections were incubated with anti-human CD4 (Biolegend), CD8 (Biolegend) or CD123 (eBioscience) -all IgG1 mouse- or the corresponding isotype controls and these Abs were detected by using Alexa Fluor 546-conjugated goat anti-mouse. The slides were washed with PBS-Tween and PBS and incubated with probe **3** (50 nM) or Alexa Fluor 488 alkyne (50 nM). After washing with PBS, the sections were treated with 1% PFA, washed with PBS and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) for nuclei demonstration. Nonspecific fluorescence was determined by co-labelling in the presence of 5 μM HU210. Images were acquired and analyzed using the confocal microscope LMS 780 Zeiss (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The general histology of palatine tonsils was demonstrated by staining of the sections with hematoxylin and eosin (H&E).

## 3. References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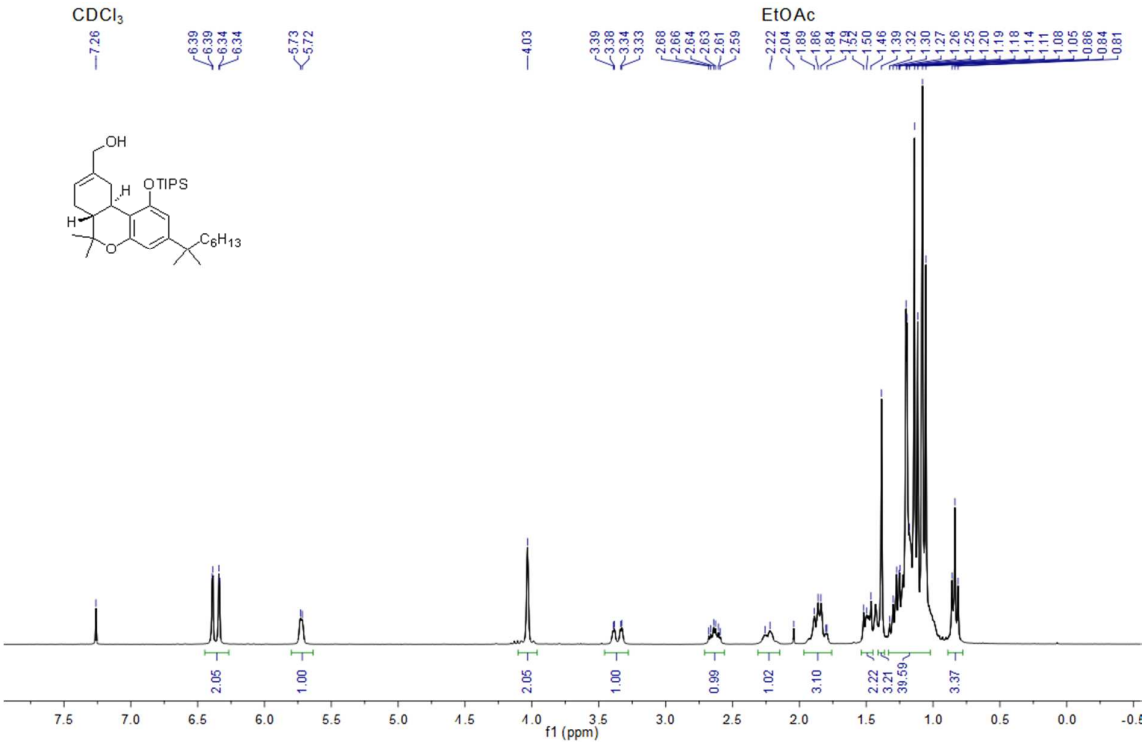
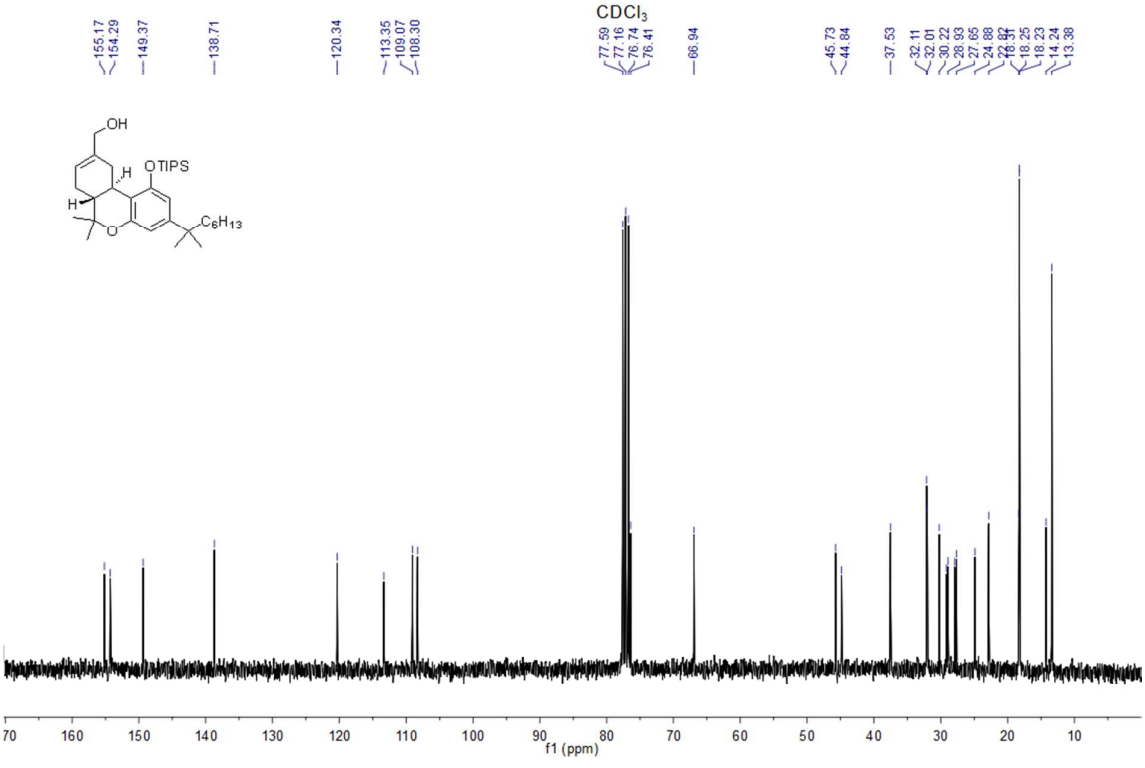
## 4. NMR Spectra

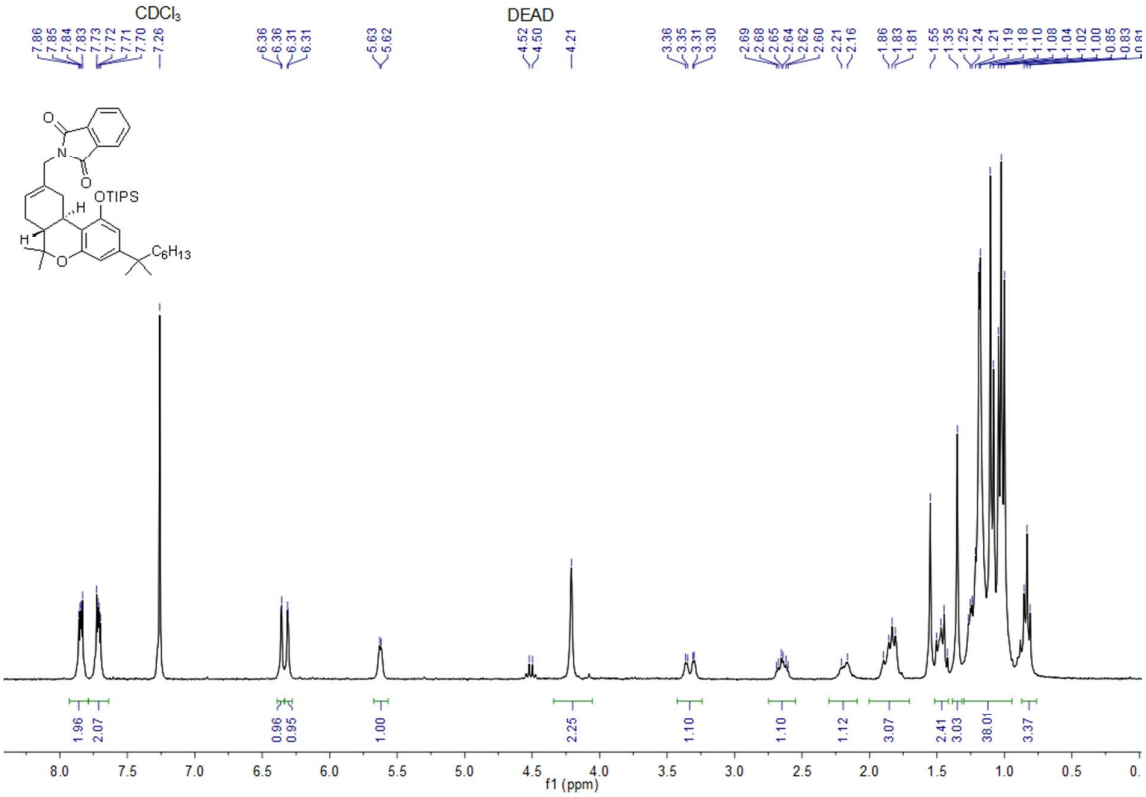
$^{1}$H NMR spectrum for **5** ($CDCl_3$, 300 MHz)



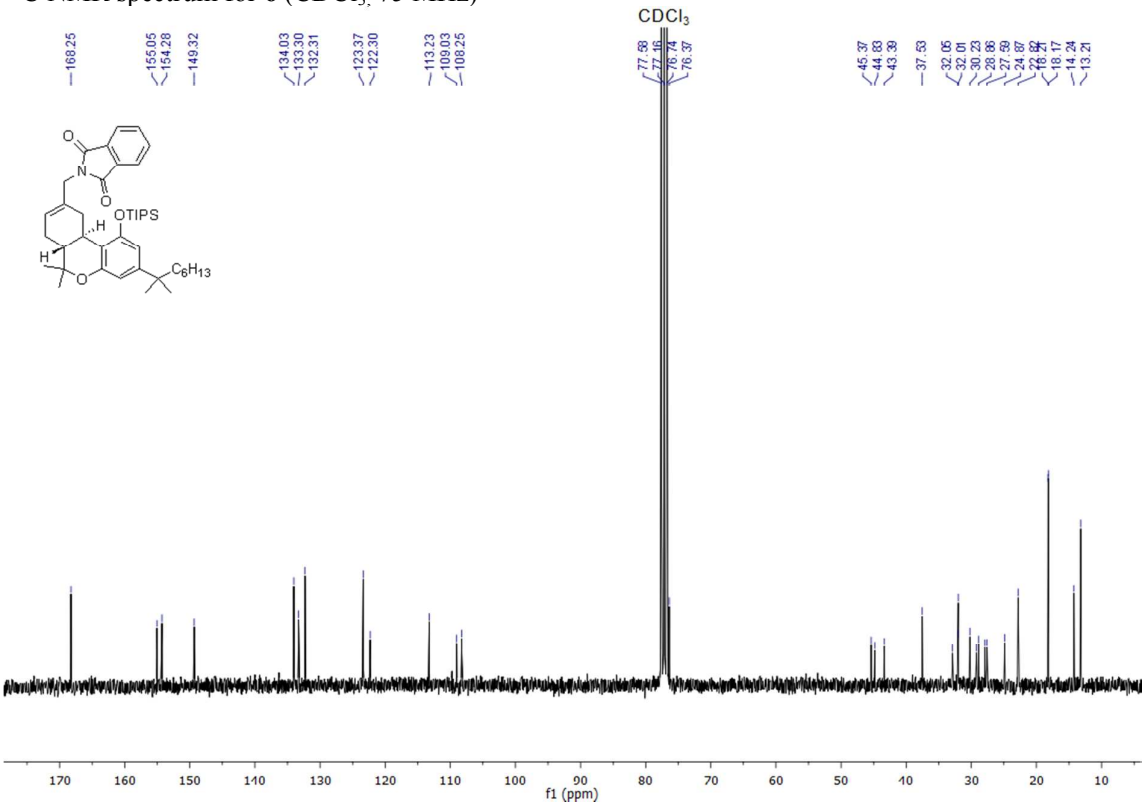

$^{13}$C NMR spectrum for **5** ($CDCl_3$, 75 MHz)



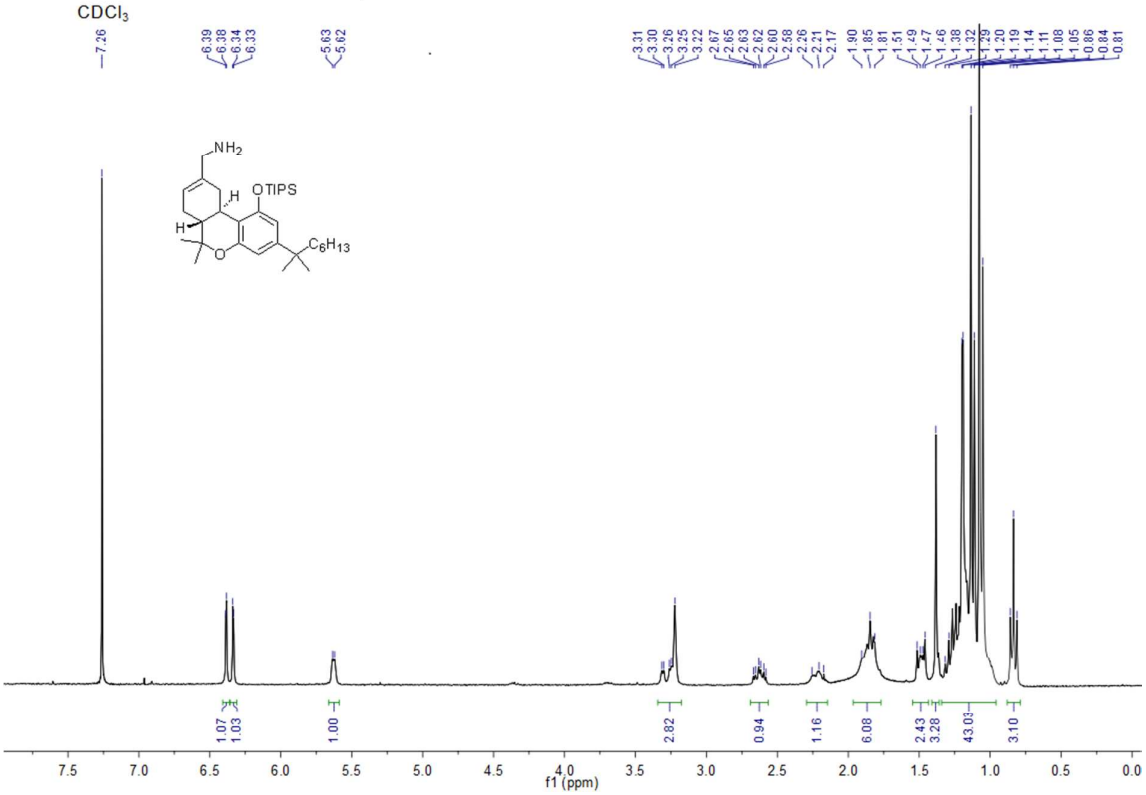
$^{1}$H NMR spectrum for **6** ($CDCl_3$, 300 MHz)



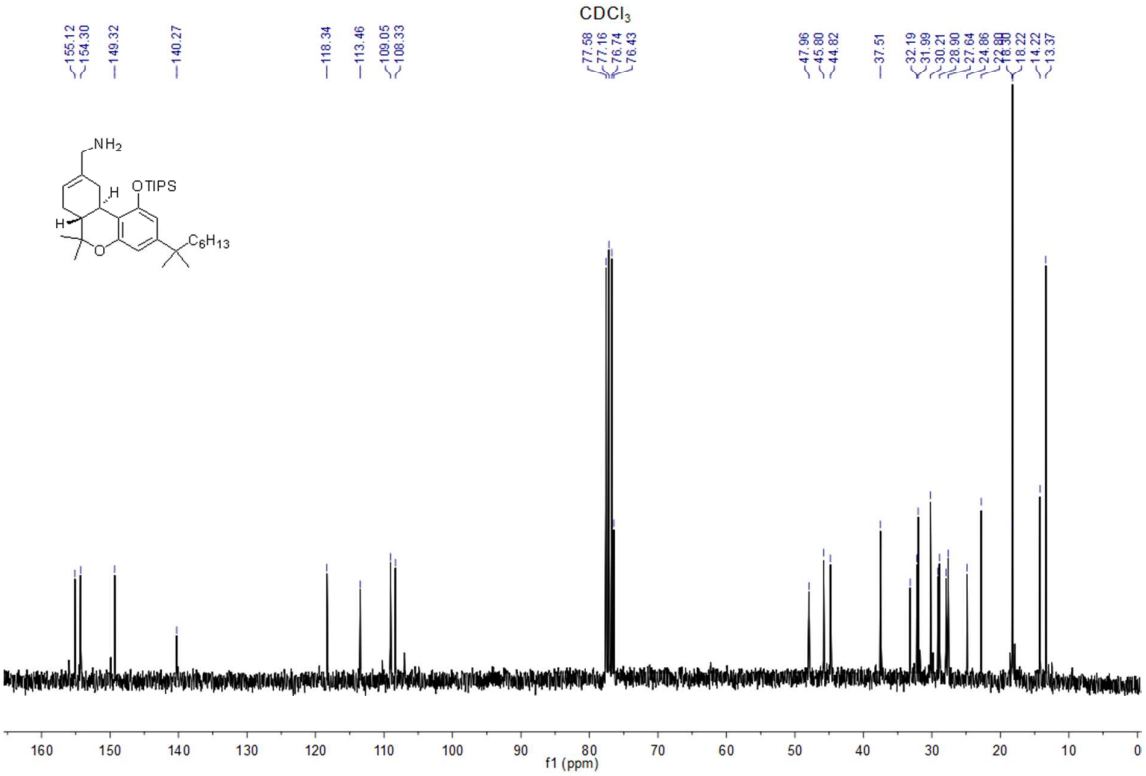

$^{13}$C NMR spectrum for **6** ($CDCl_3$, 75 MHz)



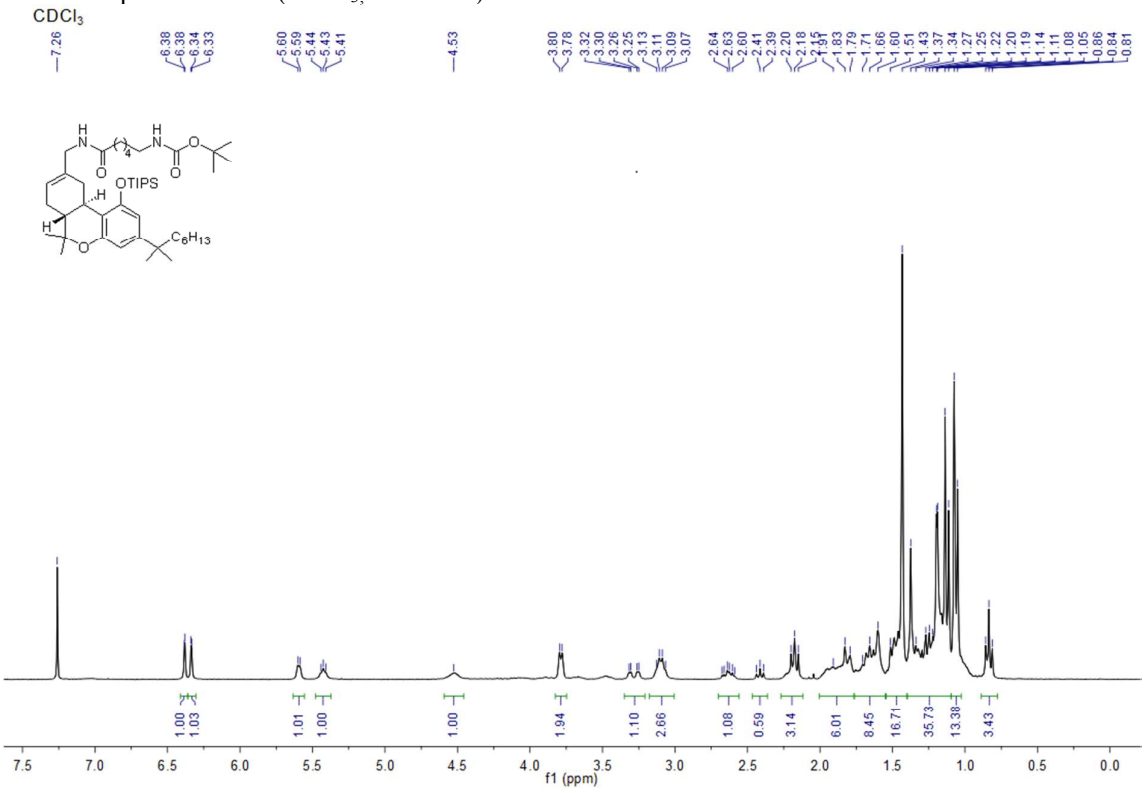
$^{1}$H NMR spectrum for **7** ($CDCl_3$, 300 MHz)



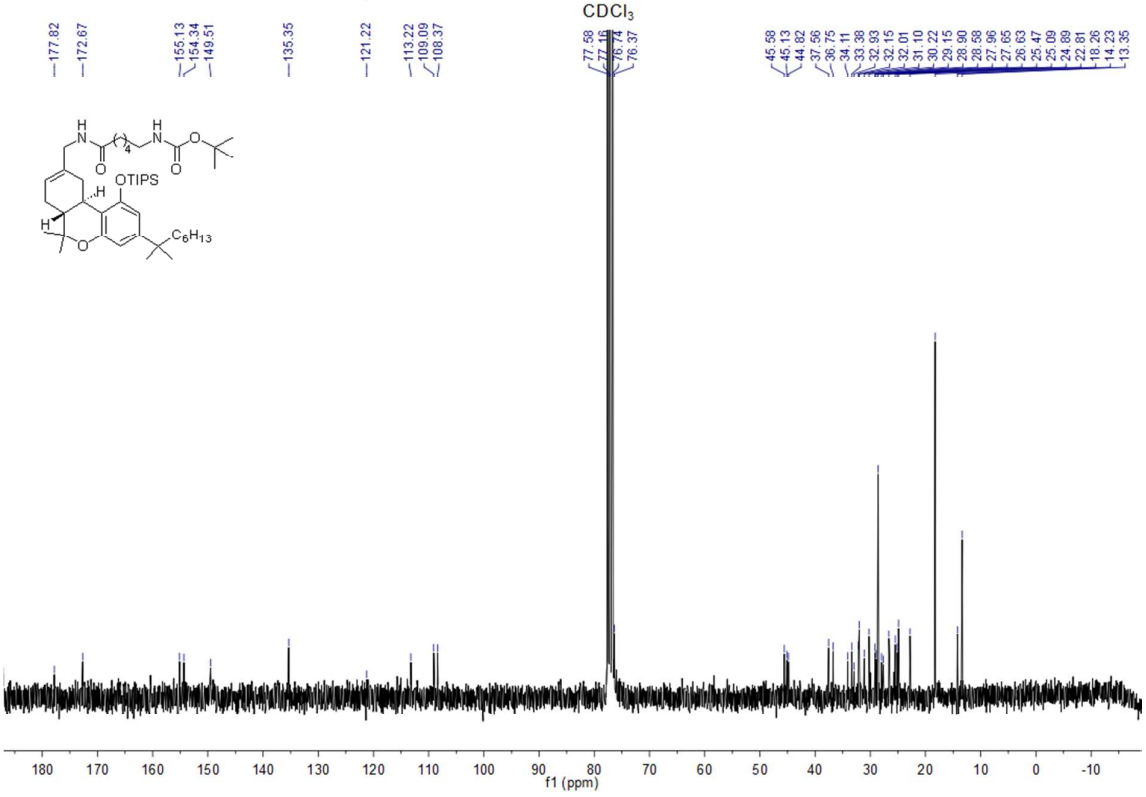

$^{13}$C NMR spectrum for **7** ($CDCl_3$, 75 MHz)



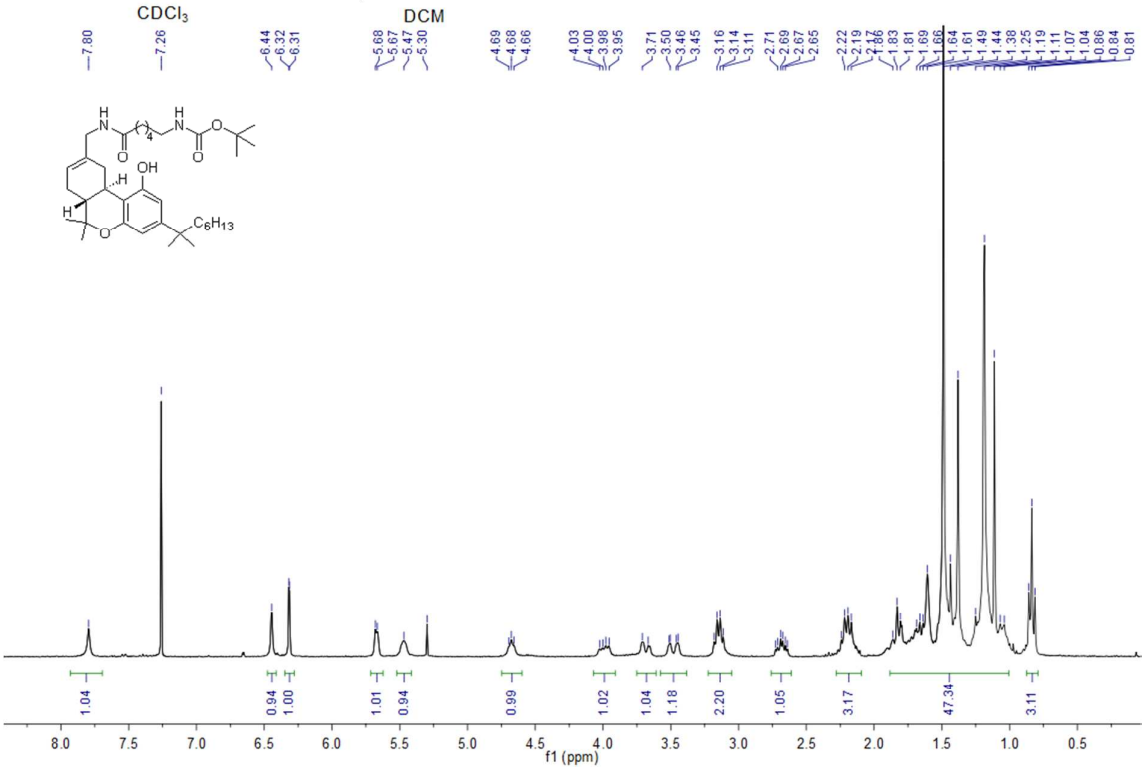
$^{1}$H NMR spectrum for **8** ($CDCl_3$, 300 MHz)



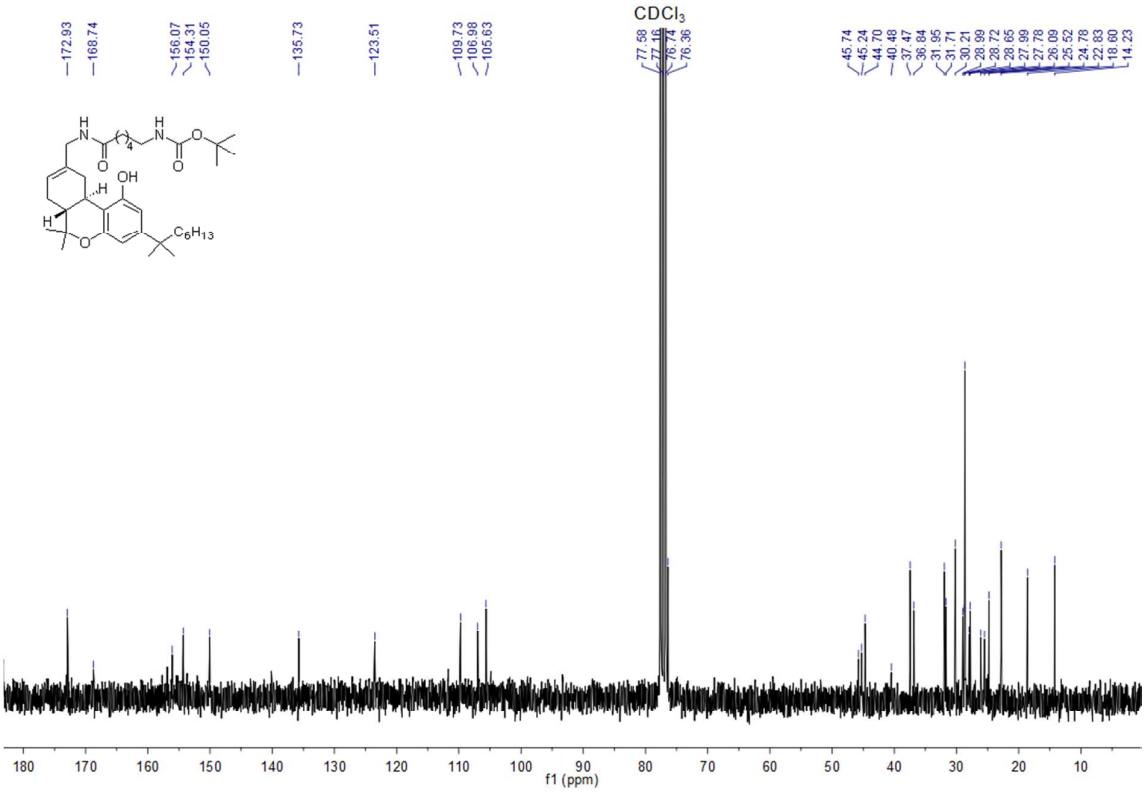

$^{13}$C NMR spectrum for **8** ($CDCl_3$, 75 MHz)



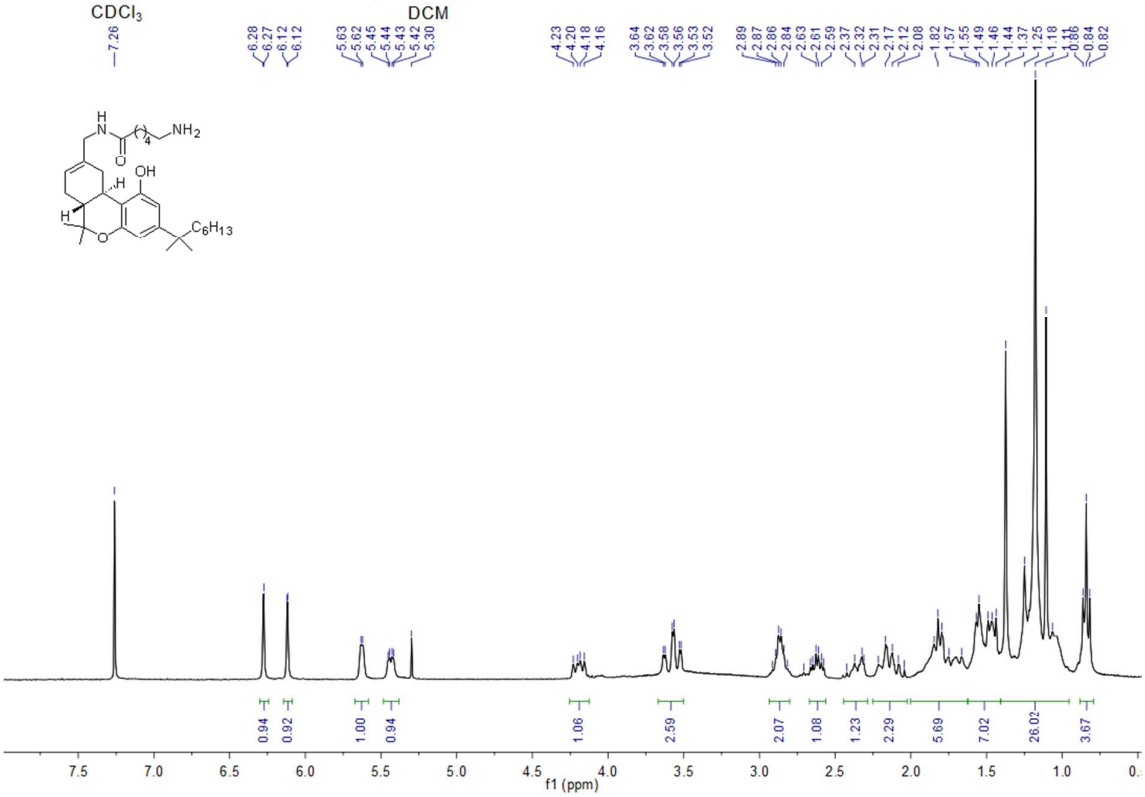
$^{1}$H NMR spectrum for **9** ($CDCl_3$, 300 MHz)



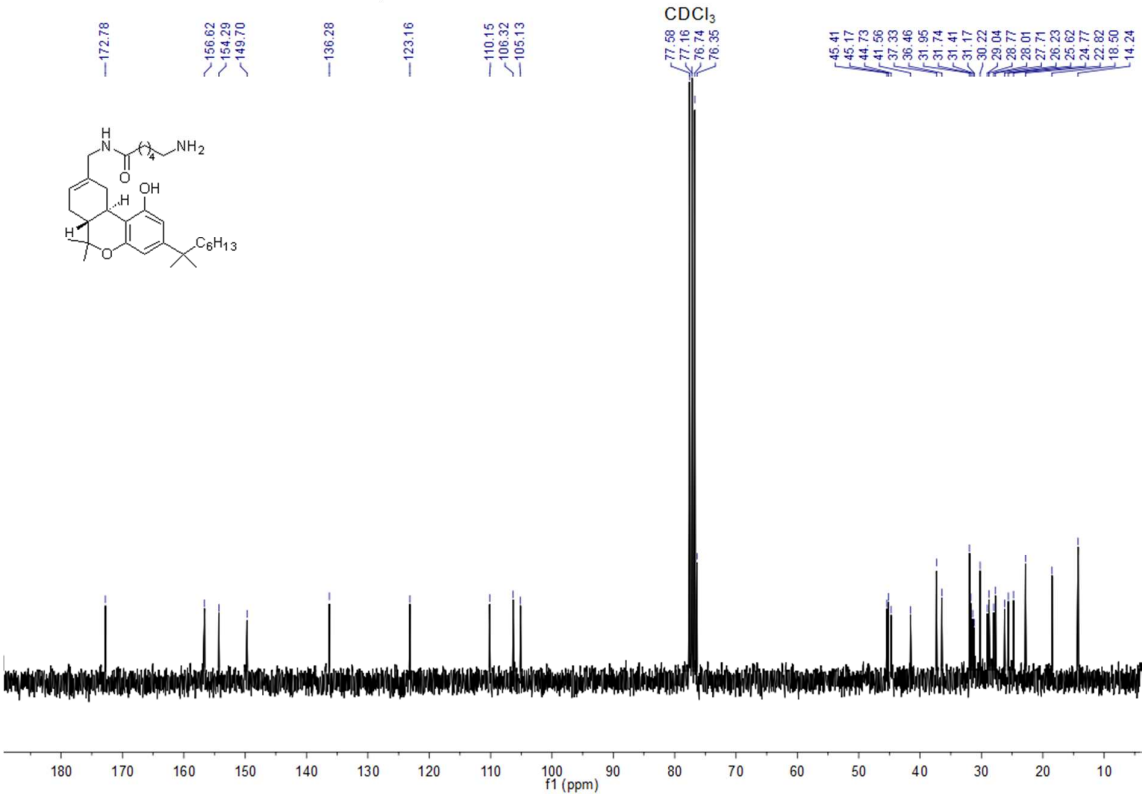

$^{13}$C NMR spectrum for **9** ($CDCl_3$, 75 MHz)



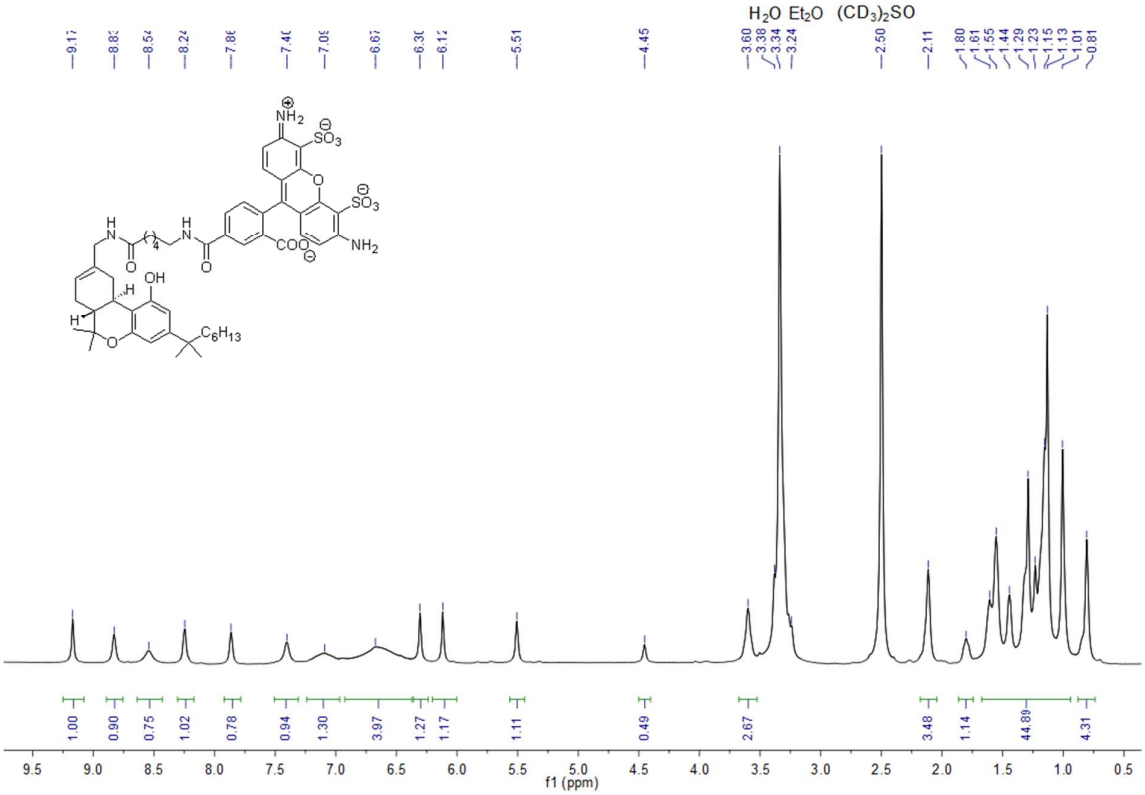
$^{1}$H NMR spectrum for **10** ($CDCl_3$, 300 MHz)




$^{13}$C NMR spectrum for **10** ($CDCl_3$, 75 MHz)



$^{1}$H NMR spectrum for **3** ($(CD_3)_2SO$, 700 MHz)




$^{13}$C NMR spectrum for**3** ($(CD_3)_2SO$, 75 MHz)