New Cannabidiol Derivatives: Synthesis, Binding to Cannabinoid Receptor, and Evaluation of Their Antiinflammatory Activity

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Cannabidiol (CBD) and cannabidiol dimethyl hephtyl (CBD-DMH) were hydrogenated to give four different epimers. The new derivatives were evaluated for their ability to modulate the production of reactive oxygen intermediates (ROI), nitric oxide (NO), and tumor necrosis factor (TNF-α) by murine macrophages, and for their binding to the cannabinoid receptor (CB_1). Surprisingly, we found that these derivatives exhibit good binding to CB_1. In addition hydrogenated CBD and CBD-DMH demonstrate bioactivities different from their original compounds.

Introduction

One of the most controversial issues in the debate over whether to legalize the use of phytochemical products of the plant *Cannabis sativa* L., marijuana and hashish, is its ability to alleviate the pain and some of the debilitating symptoms associated with a number of human diseases. Among the isolated compounds unique to cannabis,[1,2] there are two important ones: the cannabinoids Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) (**1**). Δ^9-THC is best known for its psychotropic activity but also has therapeutic potential that affects the function of various immunoactive cells.[3−6] Although CBD is the most abundant nonpsychotropic plant cannabinoid, it has received considerably less attention than Δ^9-THC. There is, however, a substantial body of literature on the in vitro and in vivo therapeutic effects of CBD.[7,8] In addition to its immunomodulating and antiinflammatory properties, CBD has been reported to exhibit anticonvulsive,[9] antianxiety,[10] and antipyschotic[11,12] activity and function as an efficient neuroprotective antioxidant.[13] The in vitro suppressive effect of CBD on down-modulating the release of tumor necrosis factor (TNF), interleukin 1 (IL-1), and interferon (IFN)-γ from peripheral blood cells has also been reported.[14,15] CBD has demonstrated activity in ameliorating collagen-induced arthritis in mice[8] and has been shown to suppress T-cell responses and the production of TNF and IFN-γ.[8] Unlike Δ^9-THC and its analogues, which exert their action by binding to the cannabinoid receptors, CB_1[16] and CB_2,[17] CBD does not bind to the known cannabinoid receptors and hence has no psychotropic activity.

In view of the potential therapeutic properties of CBD and its measured low toxicity, there is considerable interest in synthesizing new CBD derivatives and evaluating their pharmacological and clinical effects. One such synthetic derivative, cannabidiol dimethyl hephtyl (CBD-DMH) (**2**), has been shown to induce apoptosis in a human acute myeloid leukemia (AML) HL-60 cell line.[18] An additional novel synthetic cannabinoid acid, CBD-DMH-7-oic acid (HU-320), demonstrated strong antiinflammatory and immunosuppressive properties in murine collagen-induced arthritis.[19]

In this paper, the synthesis and evaluation of hydrogenated CBD and CBD-DMH are described. It was hypothesized that hydrogenation of CBD and CBD-DMH would produce compounds considerably more potent than their original counterparts. To prove this hypothesis, we synthesized derivatives of these compounds which were partially or completely hydrogenated at one or two double bonds. It is well-known that macrophages play an important role in inflammatory and immune responses and have been shown to be the major source of inflammatory mediators and cytokines and are used to study antiinflammatory agents. On the basis of the known antiinflammatory effect of CBD and CBD-DMH, we evaluated the derivative compounds for their ability to suppress the production of reactive oxygen intermediates (ROI), nitric oxide (NO), and TNF-α by activated macrophages in in vitro studies.

Results and Discussion

Chemistry. The synthesis of the novel compounds is outlined in Scheme 1. Partial hydrogenation of **1** (Scheme 1) gave a mixture of H_2-CBD epimers **3** (C-1 position) and **4** (C-8 position), with **4** being the predominant epimer (86% by gas chromatography mass spectrometry [GC-MS]). The mixture of epimers was easily separated and characterized by GC-MS. Similarly, partial hydrogenation of CBD-DMH gave compound **6** as the major epimer (83% by GC-MS), with small amounts of **5** being obtained. Under strict purification conditions, compounds **4** and **6** were obtained with 95% chemical purity (GC-MS). Compounds **7** (H_4-CBD) and **8** (H_4-CBD-DMH) were obtained by full hydrogenation at the C-1 and C-8 positions.

The stereochemistry of the C-1 and C-8 epimers was determined by NMR spectroscopy. The differences in the NMR spectra between epimers **4** and **6** and compounds **1** and **2** were the chemical shifts of the protons at position C-9. The signals at position C-9 of compounds **1** and **2** were shifted upfield from δ 4.65 for compounds **4** and **6**, while the signals for the proton at position C-2 remained the same at δ 5.53. For compounds **7** and **8**, the differences were evident at all positions, C-2, and C-9, with higher chemical shift values. Since compounds **4** and **6** were the main compounds, with only small amounts of compounds **3** and **5** being obtained, the evaluation of H_2-CBD

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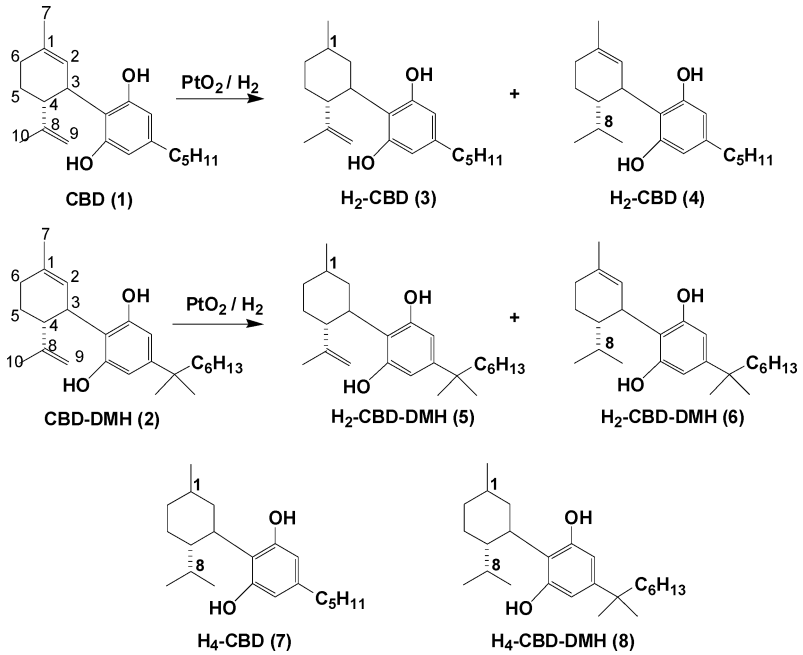
Scheme 1. Chemical Structure of Cannabidiol Derivatives[a]



[a] CBD **1** = cannabidiol; CBD-DMH **2** = dimethylheotyl (DMH) homologue of CBD; H_2-CBD **4** = partially hydrogenated CBD at positions 1 or 8; H_2-CBD-DMH **6** = partially hydrogenated CBD-DMH at positions 1 or 8; H_4-CBD **7** = fully hydrogenated CBD at positions 1 and 8; H_4-CBD-DMH **8** = fully hydrogenated CBD-DMH at positions 1 and 8.

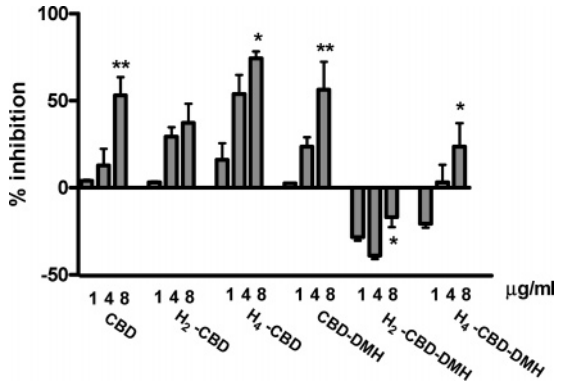


Figure 1. Generation of ROI by RAW 264.7 macrophages. Bars show the mean±SD from three pooled experiments. *P* values were calculated using one-way ANOVA with Newman−Keuls posttest. * = $P < 0.01$; ** = $P < 0.001$; versus control.

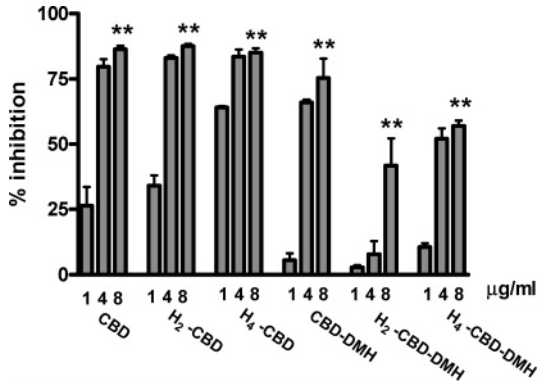


Figure 2. Generation of NO by thioglycollate-elicited macrophages. Bars show the mean ± SD from three pooled experiments. *P* values were calculated using one-way ANOVA with Newman−Keuls posttest. * = $P < 0.01$; ** = $P < 0.001$; versus control.

and H_2-CBD-DMH were performed only with compounds **4** and **6**. Purity above 95% for compounds **4**, **6**, **7**, and **8** was confirmed by HPLC and by GC-MS.

Biological Activity. Generation of ROI. Generation of ROI by RAW 264.7 macrophages was suppressed following incubation with both **1** and **2** at concentrations of 4 and 8 μg/mL (Figure 1). An increase in suppression was seen following hydrogenation of **1**; where only 12% suppression was detected following incubation with 4 μg/mL **1**, the suppression by **4** and **7** increased to 30% and 60% respectively. Similarly, an increase in suppression from 50% to 75% was detected when cells were incubated with compounds **1** and **7**, respectively. A different phenomenon was detected when hydrogenated derivatives of CBD-DMH were employed. A significant decrease in ROI suppressive effect was seen upon hydrogenation of the molecule (Figure 1); thus, compound **6** did not inhibit ROI at all at 4 and 8 μg/mL concentrations, where compound **8** showed only partial inhibition of 25% at 8 μg/mL, compared to 60% by compound **2** ($p < 0.001$).

Nitric Acid Production. As can be seen in Figure 2, generation of NO by LPS-activated pentomeal macrophages was highly suppressed following incubation with 4 and 8 μg/mL **1** and its hydrogen derivatives. An increase in the suppressive effect was seen following treatment of macrophages with 1 μg/mL H_4-CBD compared to 1 μL compound **1**. An opposite effect was seen when CBD-DMH was compared to its derivatives. Compound **2** at concentrations of 4 and 8 μg/mL had a significantly higher suppressive effect on NO generation than both its hydrogen derivatives ($p<0.001$).

TNF-α Production. TNF-α production was suppressed following incubation of LPS-activated peritoneal macrophages with 8 μg/mL **1** or **2** (Figure 3). At 4 μg/mL, **4** and **7** inhibition of TNF-α production was significantly higher than that of **1**. Following hydrogenation of **2**, reduction of the suppressive effect was detected (Figure 3). Whereas 8 μg/mL of compound **2** suppressed TNF-α production by 73%, compounds **6** and **8** lost this capacity ($p < 0.001$).

Binding to the Central Cannabinoid Receptor (CB_1). The four tested cannabidiol analogues had weak (compound **4**),

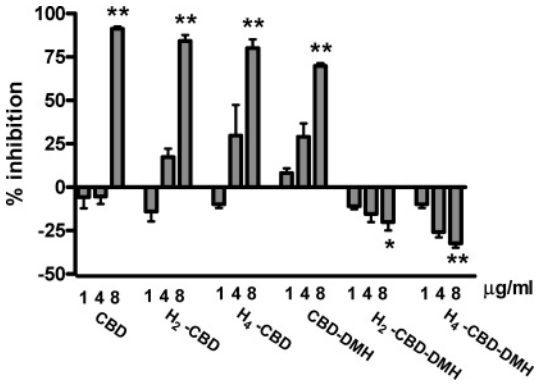


Figure 3. TNF-α production. Bars show the mean ± SD from three pooled experiments. *P* values were calculated using one-way ANOVA with Newman−Keuls posttest. * = $P < 0.01$; ** = $P < 0.001$; versus control.

moderate (compounds **7** and **6**), to strong (compound **8**) affinity to the CB_1 receptor. While **4** bound very weakly to the CB_1 cannabinoid receptor with K_i higher than 1 μM, **7** is ca. 10 times more potent with a K_i of 145 ± 5 nM. In the dimethylheptyl series, **6** binds with a K_i of 124 ± 2 nM while **8** has demonstrated a strong affinity for the cannabinoid CB_1 receptor, 7 times more potent, with a K_i of 17 ± 2 nM. Among the fullly hydrogenated compounds, **8** is ca. 7 times more potent than **7**.

Discussion

It has previously been shown that hydrogenation of the 1,1-dimethylheptyl (DMH) homologue of 7-hydroxy-Δ^8-tetrahydrocannabinol (HU-210), a potent cannabimimetic derivative of THC, leads to a cannabimimetically very active product (HU-243).[21] It was also recently reported that enantiomeric cannabidiol derivatives, (+)-CBD and (+)-CBD-DMH, in contrast to compounds in the (−) series, bind to the CB_1 receptor in the low nanomolar region.[22−24] On this basis, and on the reported antiinflammatory properties of cannabidiol,[8,13,15,25] it was hypothesized that hydrogenation of compounds **1** and **2** would lead to pharmacologically active compounds. The synthesis of hydrogenated **1** has already been published by Gaoni and Mechoulam;[26] however, no work was published on the synthesis of hydrogenated **2**. To this end, new compounds, analogues of **1** and **2**, were synthesized and evaluated for their antiinflammatory action and for their binding to the cannabinoid receptor (CB_1). The ability of these novel cannabidiol analogues to bind to the cannabinoid receptor (CB_1) was examined in a synaptosomal membrane preparation derived from rat brain, using a centrifugation binding assay. In contrast, the binding results from (−)-**1** and its **2** homologue (both with $K_i > 10$ μM), we found that all but one of the hydrogenated (−)-**1** and (−)-**2** homologues tested exhibited much higher affinity for CB_1 receptors. Compound **4** binds to the central cannabinoid receptor with a $K_i > 1$ μM, **7** with a K_i of 145 nM, and in the dimethylheptyl series, **6** binds with a K_i of 124 nM; **8** was the most active in binding to the central cannabinoid receptor with a K_i of 17 nM.

One of the known properties of compound **1** is induction of antiinflammatory effects based on its ability to modulate the release of antiinflammatory or pro-inflammatory mediators.[8] The results presented above indicate that addition of hydrogenated **1** and **2** in vitro to macrophages modulated the release of key inflammatory mediators, such as ROIs, NO, and TNF. In the past few years, ROI and NO have been recognized as important messengers in diverse pathophysiological functions, including neuronal transmission, vascular relaxation, immune modulation, and cytotoxicity against tumor cells.[27] Because of the pivotal role of NO and TNF-α in the inflammatory activities of macrophages, significant effort has been focused on developing therapeutic agents that modulate NO and TNF-α production.[28] Modification of compounds **1** and **2** to make them more efficient could sustain a viable strategy in medical science development of antiinflammatory drugs.

The production of ROI, NO, and TNF-α by macrophages was investigated to find out whether the hydrogenated **1** and **2** might have immunomodulator properties. We found in our study that these compounds indeed affect NO, TNF-α, and ROI production. An increase in the suppressive effects on ROI, NO, and TNF-α production by macrophages was detected by hydrogenated-**1** derivatives (with an advantage of **7** over **4**), whereas following hydrogenation of **2** an opposite effect, a reduction in the suppressive activities of the molecules, was detected. Both derivatives, **7** and **8**, are strongly bound to the central cannabinoid receptor (CB1), but with an opposite effect on their ability to modulate the release of inflammatory mediators. Because of this behavior, we concluded that the activation of such mediators is not directly through the central cannabinoid receptor (CB1).

Experimental Section

CBD and CBD-DMH were kindly donated by Prof. Raphael Mechoulam. CBD was purified from hashish as previously reported.[20] The purity of the compound was established on the basis of its melting point (66−67 °C), optical rotation ($\alpha_D = -125°$), and single peak on gas chromatography.[20] PtO_2 (Adam's catalyst) was purchased from Sigma (Israel), and ethyl acetate, from Frutarom (Israel).

General Procedure for Preparation of Hydrogenated (−)-CBD and (−)-CBD-DMH. The hydrogenation reactions were performed as follows: CBD or CBD-DMH (100 mg; 0.32 and 0.27 mmol, respectively), PtO_2 (10%, 10 mg), and ethyl acetate (10 mL) were placed in a hydrogenation vessel under 60 psi of hydrogen at room temperature. The reaction mixture was stirred for 4 h for partial hydrogenation or overnight for full hydrogenation. The catalyst was then removed by filtration, and the filtrate was evaporated to dryness. The oil that was obtained was loaded onto a silica gel column (5 g), and the compounds were separated with a solvent system of 5% ether−petroleum ether (TLC eluent, 15% ether−petroleum ether). NMR spectra were recorded on a Varian VXR-500S instrument, at 500 MHz for ^1H. The spectral data of all the novel compounds are given below.

Reduction of (−)-CBD with PtO_2 (Adam's catalyst) To Obtain H_2-CBD (4). ^1H NMR (500 MHz, $CDCl_3$) δ 6.21 (1H, br s, Ar), 6.13 (1H, s, Ar), 5.53 (1H, s, olefin), 3.85−3.88 (1H, m, CH-benzyl), 2.41−2.46 (2H, t, benzyl), 2.12−2.17 (1H, t, CH ring), 1.84 (2H, t, CH_2 allyl), 1.81 (1H, m, C*H*$(CH_3)_2$), 1.77 (3H, s, CH3 allyl), 1.65 (2H, m, CH_2 ring), 1.56 (2H, m), 1.33−1.37 (4H, m), 0.93 (6H, s, CH(C*H*$_3)_2$), 0.84−0.87 (3H, t, terminal CH_3). GC-MS: *m*/*z* (rel int.) 316 [M^+] (15%), 273 (7%), 260 (6%), 246 (25%), 231 (100%), 193 (10%). HR-MS: mass calcd for $C_{21}H_{32}O_2$ 316.2402, found 316.2398.

Reduction of (−)-CBD with PtO_2 (Adam's catalyst) To Obtain H_4-CBD (7). ^1H NMR (500 MHz, $CDCl_3$) δ 6.19 (1H, s, Ar), 6.12 (1H, s, Ar), 3.05 (1H, m, CH-benzyl), 2.39−2.45 (2H, t, benzyl), 2.08 (1H, m, C*H*$(CH_3)_2$), 2.03 (1H, m, CH ring), 1.77 (2H, m), 1.72 (2H, m), 1.60 (2H, m), 1.48 (4H, m), 1.2 (2H, m), 0.90 (3H, m, CH_3), 0.0.84 (6H, m, CH(C*H*$_3)_2$), 0.71−0.76 (3H, t, terminal CH_3). GC-MS: *m*/*z* (rel int.) 318 [M^+] (52%), 275 (5%), 262 (12%), 233 (97%), 193 (100%), 136 (10%). HR-MS: mass calcd for $C_{21}H_{34}O_2$ 318.2559, found 318.2551.

Reduction of (−)-CBD-DMH with PtO_2 (Adam's catalyst) To Obtain H_2-CBD-DMH (6). ^1H NMR (500 MHz, $CDCl_3$) δ 6.20 (1H, s, Ar), 6.14 (1H, s, Ar), 5.53 (1H, s, olefin), 3.82−3.84 (1H, m, CH-benzyl), 2.11 (1H, br t, CH ring),2.05 (2H, m), 1.76 (1H, m, C*H*$(CH_3)_2$), 1.62 (3H, s, CH3 allyl), 1.53 (2H, m), 1.49 (2H,

m), 1.25 (6H, s, $(CH_3)_2$ benzyl), 1.20 (8H, m), 0.87 (6H, m, CH-$(CH_3)_2$), 0.84 (3H, t, terminal CH_3). GC-MS: *m*/*z* (rel int.) 372 [M^+] (17%), 329 (6%), 302 (32%), 287 (100%), 249 (7%), 217 (18%), 164 (7%). HR-MS: mass calcd for $C_{25}H_{40}O_2$ 372.3028, found 372.3015.

Reduction of (−)-CBD-DMH with PtO_2 (Adam's catalyst) To Obtain H_4-CBD-DMH (8). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.22 (1H, s, Ar), 6.16 (1H, s, Ar), 2.42 (1H, t, CH benzyl), 1.98 (1H, m, CH ring), 1.83 (2H, m), 1.80 (1H, m, **CH**-CH_3 ring), 1.78 (2H, m), 1.76 (1H, m, **CH**$(CH_3)_2$), 1.63 (3H, s, CH3), 1.54 (2H, m), 1.50 (2H, m), 1.27 (6H, s, $(CH_3)_2$ benzyl), 1.21 (8H, m), 0.88 (6H, m, CH**(CH**$_3)_2$), 0.86 (3H, t, terminal CH_3). GC-MS: *m*/*z* (rel int.) 374 [M^+] (23%), 289 (100%), 249 (48%), 217 (16%), 164 (43%). HR-MS: mass calcd for $C_{25}H_{42}O_2$ 374.3185, found 374.3175.

ROI Production by Raw 264.7 Macrophages. Raw 264.7 cells were removed using a scrapper, washed, and resuspended in Hanks balanced salt solution (without phenol red). For measurement of chemiluminescence, 0.5 mL of cell suspension (5×10^5 cells) was added to each luminometer tube, together with various doses of cannabinoids tested (dissolved in ethanol and diluted with Hanks). The cells were incubated for 24 hs and then 10 μL of luminol (Sigma) and 30 μL of zymosan (Sigma) were added to the tubes; the chemiluminescence was measured immediately in a luminometer (Biolumate LB 95, Berhold, Wilbad, Germany).[8]

Mass Spectrometry. The samples were analyzed by GC-MS in a Hewlett-Packard G1800 A GCD system with HP-5971 gas chromatograph with an electron ionization detector. Ultralow-bleed 5% phenyl capillary column (28 m × 0.25 mm (i.d.) × 0.25 μm film thickness) based on diphenylmethylsiloxane chemistry (HP-5MS; Agilent Technologies) was used. The instrument setting used was as follows: Temperature programmed from 90 to 280 °C at 30 °C/min; initial time 2.00 min; final time 15.00 min; total time 23.3 min; injection port 250 °C; detector transfer line 280 °C; carrier gas helium, flow rate 1.0 mL/min.

Macrophages. Peritoneal cells were harvested from C57BL/6 female mice 4 days after intraperitoneal injection of 1.5 mL of a 3% thioglycollate medium (Difco). The cells were washed with phosphate-buffered saline, resuspended in Dulbecco's modified Eagle medium (DMEM) suplemented with 5% fetal calf serum (FCS), and plated (1.2×10^5) in 96-microwell flat-bottom plates (Nunc, Roskide, Denmark). Following 2−3 h incubation at 37 °C, the nonadherent cells were removed by intensive rinsing. About 95% of the adherent cells were macrophages.

Raw 264.7 Macrophage Cell Line. Raw 264.7 cells, a monocytic-macrophage cell line derived from BALB/c mice, were obtained from American Type Culture collection (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) suplemented with 5% fetal calf serum (FCS) and sodium pyrovate, glutamine, and antibiotics. For activation, the cells were incubated with LPS (*E. coli* 1 μg/mL for 24 h, Sigma, Israel).

Treatment of Macrophages with Cannabinoids. The cannabinoids were first dissolved in absolute ethanol (1 mg/50−100 μL ethanol), and the solutions were further diluted with DMEM medium. For each cannabinoid compound, various nontoxic concentrations were added to the macrophages, followed by addition of 1 μg/mL of lipopolysaccharide (LPS, *E. coli*, Sigma) for activation. The macrophages were then cultivated in a humid atmosphere with 5% CO_2 for 24 h. The supernatant fluids were harvested and kept at −20 °C until assayed for NO and TNF-α.

Nitric Oxide Determination. NO generation was determined by measuring the nitrite accumulated in the supernatants (100 μL) of the cannabinoid-treated macrophages as follows. An equal volume (100 μL) of Griess reagent (1% sulfanilamide, 0.1% naphthalene diamine HCl, 2% H_3PO_4) was added to each supernatant. Following 10 min of incubation at room temperature, the color production was measured at 550 nm with an ELISA reader. The concentration of nitrite was calculated according to a standard curve.[8]

TNF-α Determination. TNF-α in the supernatants of the cannabinoid-treated (1, 4, or 8 μL/ml) LPS-activated macrophages was determined by ELISA (R&D) with Ab pairs from Biosource (Camarillo, CA). Procedures were carried out following the manufacturer's instructions.

Statistical Treatment of the Data. Data were analyzed using GraphPad Prism version 3.0 software package. One-way analysis of variance (ANOVA) with Newman-Keuls posttest was applied for evaluation of statistical significance of the differences between various treatment groups. *P* values less than or equal to 0.05 were considered significant for all data comparisons.

Preparation of Synaptosomal Membranes. Synaptosomal membranes, used in this assay for CB_1 receptor binding, were prepared from the brains of Sabra male rats (250−300 g) after removal of the brain stem by centrifugation and gradient centrifugation after their homogenization.[20] For CB_2 receptor binding assays, transfected cells were prepared. COS-7 cells were transfected with plasmids containing CB_2 receptor cDNA, and crude membranes were prepared.[17]

Receptor Binding Assays. The high affinity receptor probe,[21] [^{3}H]-HU-243 (Tocris Cookson Ltd., United Kingdom), with a dissociation constant of 45 ± 7 pM for the CB_1 receptor, was incubated with synaptosomal membranes (3−4 μg) for CB_1 assays for 90 min at 30 °C with different concentrations of the assayed CBD derivatives or with the vehicle alone (fatty-acid-free bovine serum albumin at a final concentration of 0.5 mg mL^{-1}). Bound and free radioligands were separated by centrifugation. The data were normalized to 100% of specific binding, which was determined with 50 nM unlabeled HU-243. The results presented are the average of triplicate determination from three independent experiments. The K_i value was determined with the GraphPad Prism (Version 3.02) program which follows the Cheng−Prusoff equation. A sigmoid dose−response (variable slope) built-in equation in this Prism program was used to fit the curves.

Supporting Information Available: Experimental data. This material is available free of charge via the Internet at http://pubs.acs.org.

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