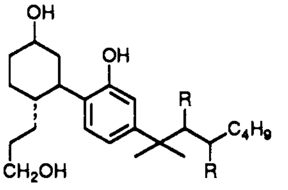
A Novel Probe for the Cannabinoid Receptor

William A. Devane,[*,†] Aviva Breuer,[†] Tzviel Sheskin,[†] Torbjörn U. C. Järbe,[‡,§] Moris S. Eisen,[†] and Raphael Mechoulam[*,†]

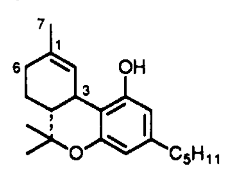
Department of Natural Products, Hebrew University, Medical Faculty, Jerusalem 91120, Israel, and Department of Psychology, University of Uppsala, Uppsala, Sweden. *Received November 6, 1991*

The 1,1-dimethylheptyl (DMH) homologue of 7-hydroxy-Δ^6-tetrahydrocannabinol (3) is the most potent cannabimimetic substance reported so far. Hydrogenation of 3 leads to a mixture of the epimers of 5′-(1,1-dimethylheptyl)-7-hydroxyhexahydrocannabinol or to either the equatorial (7) or to the axial epimer (8), depending on the catalysts and conditions used. Compound 7 discriminates for Δ^1-THC (2) in pigeons (ED_{50} = 0.002 mg/kg, after 4.5 h), at the potency level of 3, and binds to the cannabinoid receptor with a K_D of 45 pM, considerably lower than the K_i of 180 pM measured for compound 3 and the K_i of 2.0 nM measured for CP-55940 (1), a widely employed ligand. Tritiated 7 was used as a novel probe for the cannabinoid receptor.

A cannabinoid receptor was recently identified in the brain,[1] followed shortly by reports on its distribution and localization,[2] on its primary structure,[3] on the cloning of the gene involved in its formation,[3] and on the nucleotide sequence of the human receptor cDNA.[4] The radiolabeled probe used in most of these investigations was [^{3}H]-1, which, in its nontritiated form (CP-55940), has been shown to have a cannabinoid pharmacological profile.[5a] Structurally, however, 1 is only marginally related to the natural Δ^1-tetrahydrocannabinol (Δ^1-THC) (2). An additional probe, with a typical THC-type cannabinoid structure, a K_D in the picomolar range, and high stereospecificity, with respect to both pharmacological activity and binding, may help further exploration of this exciting new area of research.[5b]



1 (CP-55940): R = H
[^{3}H]-1: R = ^{3}H



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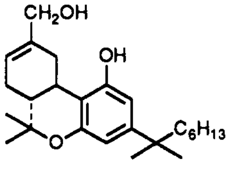
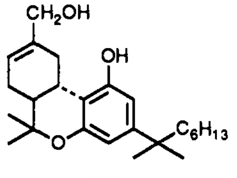
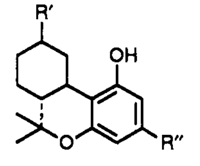


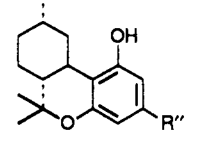
3, HU-210



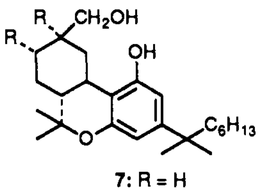
4, HU-211



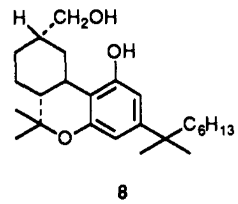
5a: R′ = CH_3, R″ = C_5H_{11}
b: R′ = CH_2OH, R″ = C_5H_{11}
c: R′ = CH_3, R″ = DMH



6a: R′ = CH_3, R″ = C_5H_{11}
b: R′ = CH_2OH, R″ = C_5H_{11}
c: R′ = CH_3, R″ = DMH



7: R = H
[^{3}H]-7: R = ^{3}H



8

DMH = 1,1-dimethylheptyl

Compound 3 (HU-210) is apparently the most potent cannabimimetic prepared so far.[6] In numerous pharmacological tests, in various animal species, 3 has been shown

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[*] Authors to whom correspondence should be addressed.

[†] Department of Natural Products, Hebrew University.

[‡] University of Uppsala.

[§] Present address: Hahnemann University, Mental Health Sciences, Division Addiction Research & Treatment, Mail Stop 403, Broad and Vine, Philadelphia, PA 19120-1192.

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to be 100–800 times more potent than Δ^1-THC;[6b-d] in drug discrimination in pigeons, 3 was 80 times more potent than Δ^1-THC.[7] It competes fully for the specific binding of [^{3}H]-1 to membranes from rat brain in heterologous displacement studies (K_i = 234 pM).[8] Compound 4 (HU-211), the (+)-(3*S*,4*S*)-enantiomer of 3, was several thousand times less potent than 3 in several in vivo pharmacological tests;[6b,c,7] the potency ratio of 3 to 4 for binding to the cannabinoid receptor is ca. 1500.[8] Hence 3 seemed to represent a suitable candidate for labeling.

We have previously shown that hydrogenation of Δ^1- or Δ^6-THC leads to cannabimimetically active products, the C-1 equatorial epimer (**5a**) being more active than the axial one (**6a**).[9] 7-Hydroxyhexahydrocannabinols **5b** and **6b** have also been prepared.[10] Again, the equatorial epimer (**5b**) was considerably more active in discrimination tests than the axial epimer (**6b**) and was ca. 3–17 times more active than Δ^1-THC (depending on the animal used—pigeon or rat—and on the timing of measurement).[10] On this basis we assumed that hydrogenation (or tritiation in the labeling experiments) of 3 would lead to pharmacologically very active compounds, binding well to the receptor. Indeed hydrogenation of 3 over platinum oxide (Adams catalyst) in ethanol at room temperature for 4 h (3.4 atm) led to a nearly 1:1 mixture (by GC) of the C-1 epimers (7 and 8) (combined yield 52%) in addition to hydrogenolyzed material (**5c** and **6c**), which was easily separated from the mixture of epimers by column or thin-layer chromatography (TLC). It should be pointed out that on TLC 7 and 8 have the same R_f values, but are well-separated on gas chromatography (GC).

On the assumption that epimer 7 would be considerably more potent than 8 (see above), we looked into hydrogenations with reagents which lead to stereospecific reductions. Reduction (in ethanol, 140 °C, 48 h, 17 atm) catalyzed with a 1:1 mixture of Wilkinson's catalyst (tris(triphenylphosphine)rhodium chloride) and Kagan's catalyst [2,3-*O*-isopropylidene-2,3-dihydroxy-1,4-bis(diphenylphosphino)butane, (+)-enantiomer] (+-DIOP)[11] gave no hydrogenolyzed products and led to the desired epimer (7) with 93% regiospecificity (GC). On crystallization, 7, mp 80–82 °C, was obtained in a 62% yield. The use of the (−)-enantiomer of Kagan's reagent did not lead to axial epimer 8, but also gave mostly the equatorial one, 7. Axial epimer 8 was obtained by reduction (in ethanol, 100 °C, 34 atm, 48 h) with the (−)-binaphtyl-based phosphine (BINAP)–Ru(II) dicarboxylate hydrogenation catalyst described by Noyori's group.[12] Traces of hydrogenolyzed products were observed. The ratio of the axial (8) to the equatorial epimer (7) was 85:15. Pure 8 could be obtained on crystallization.

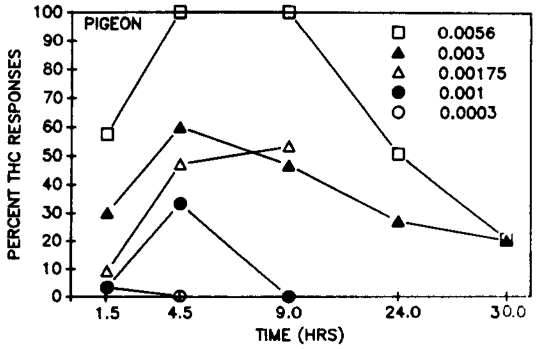

Figure 1. Substitution test results with 7 (in mg/kg) for pigeons trained to discriminate between 0.56 mg/kg of Δ^1-THC and vehicle (5% propylene glycol, 2% Tween-80, and 93% physiologic saline, v/v). *Y*-axis, percentage responding to drug position; *X*-axis, time in hours elapsed until testing after a single injection of the drug dosage. The data points are based on one observation per animal and test interval (n = 5). All administrations were im, and the volume was 1 mL/kg.

Table I. Discrimination for Cannabinoids in Pigeons:[a] Median Dose (mg/kg)–Effect Estimates, ED_{50}[b]

time,[c] h	Δ^1-THC (2)	(−)-7-OH-Δ^6-THC-DMH (3)[b,d]	(−)-7-OH-hexahydro cannabinol-DMH (7)[b,e]
1.5	0.16 (0.91)	0.004 (0.94)	0.005 (0.99)
4.5	0.25 (0.94)	0.002 (0.99)	0.002 (0.97)
9.0	1.21 (0.70)	0.002 (0.99)	0.003 (0.91)

[a] For the drug discrimination training and testing, see the Experimental Section and refs 7 and 16. [b] ED_{50} values according to regression analysis; the correlation coefficients for the fits (r) are presented within parentheses (see ref 13b). [c] Time from injection until testing. [d] Included for comparison; the data are published from ref 6b and 7. [e] Five trained animals per dose.

The stereochemistry at C-1 of epimers 7 and 8 was determined by comparison of their NMR spectral data with those reported[10a] for the pentyl homologues **5b** (C-7 hydroxymethyl moiety equatorial) and **6b** (C-7 hydroxymethyl moiety axial). The most conspicuous differences in the NMR spectra of **5b** and **6b** are the chemical shifts of the two C-7 protons at δ 3.48 (d, J = 6 Hz) and 3.70 (m), respectively, and the shift of the 7-carbinyl carbon of the acetates of **5b** and **6b** (δ 68.38 and 65.53, respectively).[10a] The NMR spectra of the dimethylheptyl homologues 7 and 8 are closely related to those of **5b** and **6b**: the C-7 protons are at δ 3.52 (d, J = 6.3 Hz) and 3.80 (m), respectively: the 7-carbinyl carbon of the acetates of 7 and 8 are at δ 69.262 and 66.478, respectively. The close correlations of the

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spectra indicate that 7 has the stereochemistry of **5b** (namely C-7 equatorial) and 8 that of **6b** (namely C-7 axial).

While the above described conditions for the preparation of 7 are facile and reproducible, they are not applicable for the synthesis of tritium-labeled 7. For safety reasons, work under high pressure with tritium is not desirable. In addition, in initial experiments we found that ethanol is not a good solvent for the tritiation experiments, as it led to [^{3}H]-7 with low specific activity, possibly due to proton exchange. We report now that hydrogenation (and tritiation) can be done using Wilkinson's catalyst alone, in ethyl acetate, at 120 °C, with hydrogen introduced at atmospheric pressure. Although, considerable hydrogenolysis was observed, the hydrogenolyzed compounds were found to be much less polar than 7 or 8 and were easily removed by chromatography (on column or TLC). Under these conditions 7 was obtained with 95% chemical purity (GC). On tritiation, under identical conditions, the tritiated product was purified by TLC. Only one band (>99%) was observed on TLC with UV detection as well as on radiochromatography. The specific activity of [^{3}H]-7 was 54 Ci/mmol. This material was subsequently used in our binding experiments.

Generalization tests with 7 were performed with pigeons trained to discriminate between 0.56 mg/kg Δ^1-THC and vehicle. The data, presented in Figure 1, are the average percentage of pecking responses emitted toward the Δ^1-THC-appropriate key out of the total number of key pecking responses performed during a test probe. The generalization was dose responsive, the maximum effect occurring 4.5 h after the administration of 7 (Table I). For the intervals of 1.5, 4.5, and 9 h after administration, the median dose effect (ED$_{50}$) estimates, according to regression analysis, were, respectively, 0.005 (r = 0.99), 0.002 (r = 0.97), and 0.003 mg/kg (r = 0.91). Tests conducted 24 and 30 h after the administration of the two higher doses of the test compound indicated a dose-responsive decline of the THC-like effects. The ED$_{50}$ for Δ^1-THC at the 1.5-, 4.5-, and 9-h time intervals were 0.16, 0.25, and 1.21 mg/kg, respectively. A comparison of the present results with those previously reported indicates that 7 and its precursor 3 are equipotent in drug discrimination tets in pigeons (Table I).

The ability of [^{3}H]-7 to bind to the cannabinoid receptor was assessed in a synaptosomal membrane preparation derived from rat whole brain, without brain stem, using a centrifugation assay. Homologous displacement studies indicate that 7 is highly potent, binding to the cannabinoid receptor with a K_d of 45 pM (Figure 2). The binding capacity for these experiments was also high, 4.41 ± 0.08 pmol/mg of protein, in agreement with the abundance of cannabinoid receptors previously detected using [^{3}H]CP-55940 and different assay conditions.[1,2] The ability of other cannabinoids to displace [^{3}H]-7 binding was also assessed in heterologous displacement studies. Compound 3 (HU-210) exhibited a K_i of 181 pM, while its (+)-enantiomer, compound 4 (HU-211), was 2500-fold less potent, with a K_i of 466 nM. The axial epimer, compound 8, was also potent, with a K_i of 190 pM. Δ^1-THC was able to fully compete with [^{3}H]-7 for binding to the cannabinoid receptor, but was 1000-fold less potent, with a K_i of 46 nM. The observed relative potencies of these compounds were similar to those reported in other in vitro[1,2,8] and in vivo[6a,d,7] tests of cannabimimetic activity, although some differences are apparent. Thus, while in the discrimination tests 3 and 7 are essentially equivalent in activity, in the binding experiments 7 is ca. 4 times more potent than 3. Compound 7 is ca. 1000 times more potent than Δ^1-THC on binding, but only 125 times more potent in the in vivo experiment. Such differences are not unexpected, as binding and in vivo pharmacological data are not directly comparable.

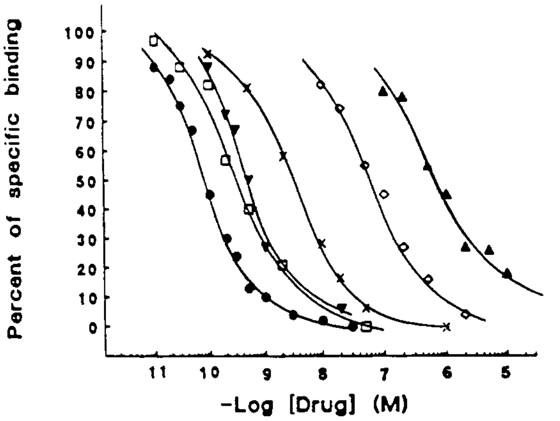

Figure 2. Competitive inhibition of [^{3}H]-7 by cannabinoid compounds: ●, 7, K_i = 45 ± 7 pM; □, 3, K_i = 181 ± 23 pM; ▼, 8, K_i = 190 ± 16 pM; ×, 1, CP-55,940, K_i = 2.0 ± 0.5 nM; ◇, 2, Δ^1-THC, K_i = 46 ± 3 nM; ▲, 4, HU-211, K_i = 466 ± 16 nM. The tritiated material (38–48 pM) was incubated with whole brain synaptosomal membranes (2.4–3.8 μg) for 90 min at 30 °C with either the indicated concentrations of drug or the vehicle alone. The data were normalized to 100% of specific binding, which was determined with 50 nM unlabeled 7. Data points represent the average of triplicate determinations from single experiments. K_i values (mean ± SE, n = 3) were determined using the LIGAND program.

As mentioned above CP-55940 (1) has been widely used for binding (or displacement) on the cannabinoid receptor.[1-3] We have compared the binding potency of CP-55940 with that of 7 (Figure 2); the latter compound is more potent than CP-55940, which, under our conditions, has a K_i of 2.0 nM. Compound 7 is thus apparently the most active cannabinoid reported to bind to the cannabinoid receptor.

The results presented above indicate that 7, a potent cannabimimetic compound, which is easily obtained in a tritiated form, can serve as a novel, alternative probe for the cannabinoid receptor.

Experimental Section

A. Chemistry. Melting points (mp) were taken in glass capillary tubes with a Thomas-Hoover Uni-Melt apparatus. Infrared spectra (IR) were recorded on a JASCO A-200 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR-300 S instrument, at 300 MHz for ^{1}H and 75.429 MHz for ^{13}C. Rotations were determined on a Perkin-Elmer Model 141 polarimeter in chloroform. The hydrogenations were performed in a stainless steel vessel; the hydrogen was introduced at the pressure indicated before closing the vessel. The microanalyses were performed by the Microanalytical Laboratory of the Hebrew University, and the elemental compositions of the compounds agreed to within ±0.4% of the calculated value. High-resolution mass spectra (HRMS) were recorded on a Varian 711 at the mass spectrometry center at the Technion (Haifa, Israel). Chromatographic separations were performed on silica gel columns (Woelm TSC silica, for dry chromatography, activity III/30 mm, cat. no. 04530). Preparative TLC was performed on Merck silica gel F$_{254}$ (cat no. 5717). Gas chromatography (GC) was performed on a Varian gas chromatograph, Model 3700, under the following conditions: injector, 270 °C: detector, 260 °C; capillary column, fused silica, DB 17; film thickness, 0.25 μm; column dimensions, 30 m × 0.245 mm (J.W. Scientific, cat. no. 122-1732) at 260 °C. For the radiochromatography a Berthold scanner Model LB 2733 was used.

Reduction of 3 with Adams Catalyst. A mixture of 3 (ee > 99%)[6a] (100 mg, 0.26 mmol) and platinum oxide (16 mg) in ethanol (50 mL) under 3.4 atm of hydrogen was stirred at room temperature for 4 h. The catalyst was removed by filtration and the resulting solution was evaporated and separated on preparative TLC (eluent, ether–petroleum ether, 6:4). Two main bands were observed and the compounds present in them were extracted with ether. The *less polar* band was shown to contain a 1:1 mixture (by GC) of two compounds (together 42 mg), presumably **5c** and **6c**. The NMR spectrum of the mixture was essentially identical to that of a 1:1 mixture of the known **5a** and **6a**, except for differences in the 0–2 ppm region due to the different side chains: ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 3 H, w-CH_3), 2.42, 2.68 (2 t, together 1 H, C-3 H), 2.87, 3.05 (2 brd, together 1 H, C-2 eq H), 6.18, 6.36 (2 d, together 2 H, arom); $[\alpha]_D$ −86.4° (*c* 19.5 mg/mL, $CHCl_3$). The absence of signals for the $-CH_2O-$ groups at ca. δ 3.5–4.0 indicated hydrogenolysis. No olefinic proton was observed. These materials were not further investigated. The *more polar* fraction (52 mg, 52%) was shown to be a 1:1 mixture (by GC) of compounds **7** and **8**. Under our GC condition **7** was eluted after 16.2 min and **8** after 18.0 min. On TLC only one spot was observed (R_f 0.25). The NMR spectrum of the mixture was identical to a superposition of the NMR spectra of pure **7** and **8** (see below). Of particular interest are the aromatic region and the region of the C-7 protons. In the aromatic region three peaks are observed, at δ 6.35 (1 H), apparently from both **7** and **8**; at 6.27 (0.5 H), from axial epimer **8**; and at 6.21 (0.5 H), from equatorial epimer **7**. In the $-CH_2O-$ region again three wide peaks are observed, at δ 3.90 (t, 0.5 H) and 3.78 (m, 0.5 H), both from axial epimer **8**, and at δ 3.52 (brd, 1 H) from equatorial epimer **7**.

Reduction of 3 with a Mixture of Kagan's and Wilkinson's Catalysts. A solution of **3** (160 mg, 0.415 mmol), (+)-DIOP (Kagan's catalyst) (10.7 mg, 0.02 mmol), and tris(triphenylphosphine)rhodium(I) chloride (Wilkinson's catalyst) (19.8 mg, 0.02 mmol) in ethanol (8 mL) was stirred under 13.6 atm of hydrogen to 140 °C for 48 h. The solution was evaporated to dryness and the residue was purified by preparative TLC. The product (140 mg), which showed one spot on TLC, consisted of **7** and **8** in a ratio of 93:7 (GC). On crystallization from pentane, **7** (100 mg) was obtained: >99% chemical purity (GC), mp 80–82 °C; $[\alpha]_D$ −92° (*c* 10 mg/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 3 H w-CH_3), 2.46 (dt, 1 H, C-3 H), 3.25 (brd, 1 H, C-2 H), 3.52 (d, J = 6.3 Hz, 2 H, C-7 H), 6.21 (d, 1 H, arom), 6.35 (d, 1 H, arom); HRMS calcd for $C_{25}H_{40}O_3$ 388.2978, found 388.3002. Anal. ($C_{25}H_{40}O_3$) C, H. Acetylation of **7** (10 mg) with acetic anhydride (0.1 mL) and pyridine (0.2 mL), followed by the standard workup, gave the diacetate of **7**, an oil which produced one spot on TLC and one peak on GC: ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3 H, $OCOCH_3$), 2.29 (s, 3 H, $OCOCH_3$), 2.36 (t, 1 H, C-3 H), 2.74 (brd, 1 H, C-2 H), 3.86 (m, 1 H, C-7 H), 3.95 (m, 1 H, C-7 H), 6.48 (d, 1 H, arom) and 6.66 (d, 1 H, arom); ^{13}C NMR δ 69.262 (C-7).

Reduction of 3 with Wilkinson's Reagent Alone. Compound **3** (33 mg) was dissolved in ethyl acetate (4 mL), tris(triphenylphosphine)rhodium(I) chloride (6 mg) was added, and the solution was stirred for 24 h, at 120 °C, under hydrogen introduced at atmospheric pressure. The reaction mixture was cooled to room temperature, filtered, and evaporated. It was purified on preparative TLC by elution with ether/petroleum ether (6:4). Two main bands were observed. The less polar band was extracted with ethyl acetate and the solution was evaporated. It consisted of a mixture of **5c** and **6c** (8 mg), as determined by direct comparison (GC). The more polar band was extracted with ethyl acetate, stirred for 20 min, filtered, and evaporated. The residue obtained (20 mg) was a mixture of **7** and **8** in a ratio of 95:5 (GC).

The above reaction conditions were employed at the Radiochemistry Department, Nuclear Research Centre, Rotem Industries Ltd. (Beer Sheva, Israel) for the radiolabeling with tritium. After the yields and level of stereospecificity were first confirmed with unlabeled material, identical conditions were used for tritiation of **3**. Tritiated **7** thus obtained was purified by preparative TLC (ether/petroleum ether, 6:4). The material obtained showed one band (>99%) on UV spectrophotometry and one band on radiochromatography coincident with that of the reference material **7**. The specific activity was 54 Ci/mmol, which was determined by dividing the total radioactivity recorded by the number of millimoles of labeled compound. The amount of labeled compound was determined using UV spectroscopy at the 280-nm absorption band. On the basis of the "cold" experiment we assume that tritiated **7** has a chemical purity of 95%, the rest being mostly tritiated **8**.

Reduction of 3 with Noyori's Catalyst. (*S*)-2,2′-Bis(diphenylphosphino)-1,1′-binaphtyl [(*S*)-BINAP] (Noyori's catalyst) was prepared as described by Ohta et al.[12b] This catalyst (10 mg, 0.012 mmol) was added to **3** (286 mg, 0.74 mmol) in ethanol (6 mL). The solution was stirred under 34 atm of hydrogen at 100 °C for 48 h. After evaporation the mixture was purified by preparative TLC using ether/petroleum ether (6:4). Only traces of **5c** and **6c** were noted. The main product obtained (250 mg, 87%) was shown to consist mainly of axial epimer **8** (ratio of **8** to **7**, 85:15, by GC). On crystallization from pentane, **8** was obtained, >99% chemical purity (GC); mp 95 °C (sintering at 75 °C); $[\alpha]_D$ −62.3° (*c* 8.05 mg/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 3 H, w-CH_3), 2.50 (t, 2 H, C-3 H), 3.15 (brd, 2 H, C-2 H), 3.75 (m, 1 H, C-7 H), 3.90 (m, 1 H, C-7 H), 6.27 and 6.35 (2 d, 2 H, arom); HRMS calcd for $C_{25}H_{40}O_3$ 388.2978, found 388.2970. Anal. ($C_{25}H_{40}O_3$) C, H.

Acetylation of **8** (10 mg) with acetic anhydride (0.1 mL) and pyridine (0.2 mL) followed by the standard workup gave an oil, one spot on TLC and one peak on GC: ^{1}H NMR ($CDCl_3$) δ 2.04 (s, 3 H, $OCOCH_3$), 2.29 (s, 3 H, $OCOCH_3$), 2.48 (t, 1 H, C-3 H), 2.62 (brd, 1 H, C-2 H), 4.02 (m, 1 H, C-7 H), 4.30 (m, 1 H, C-7 H), 6.48 (d, arom H), 6.66 (d, arom H); ^{13}C NMR δ 66.478 (C-7).

B. Pharmacology. Animals. Male white carneaux pigeons (Estuna AB, Sweden and Palmetto Pigeons Plant, Sumter, SC) were used for training and testing. The animals were housed individually under standard laboratory conditions (temperature, 20–22 °C; relative humidity, ca. 50–60%; and 12 h light–dark cycle). The animals were deprived of food to maintain their weight at 80% of their expected free-feeding weights. This deprivation was accomplished through individual rationing of food. Other nutrients such as shellgrit and water were freely available in the home cages.

Drug-Discrimination Training. Five male white carneaux pigeons were trained to discriminate between (−)-Δ^1-THC and vehicle (5% propylene glycol, 2% Tween-80, and 93% physiologic saline (v/v), according to published procedures.[13,14] The training dose was 0.56 mg/kg (1 mL/kg) administered intramuscularly (im) 90 min prior to the onset of the training session. The experimental chambers, adapted after those of Ferster and Skinner,[15] contained two response keys separated by a recess in which grain rewards (4-s access to chicken pellets) could be presented. The weights of the animals were reduced to about 80% of their free-feeding weights. Which key was correct on a given training session depended upon whether Δ^1-THC or vehicle had been administered prior to the session. The schedule of reinforcement used was fixed ratio 15 (FR-15); i.e. the reward was available during 4 s when 15 key-pecking responses had been accumulated on the key appropriate for the administration (drug or no drug). Responses on the inappropriate key were also recorded but had no programmed consequences. The program ended when the pigeons had received 40 rewards or 20 min had elapsed, whichever occurred first. The animals were trained 3 days a week. Suspensions for the DDL training and testing were prepared as described previously.[16]

Drug-Discrimination Testing. Once trained, the animals were tested once a week (Fridays), provided that correct base-line responding was maintained, i.e. no more than 29 pecking responses

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should have been emitted prior to receiving the first reward in the regular training sessions occurring on Mondays and Wednesdays. In testing, the program ended after six rewards had been obtained or 20 min had elapsed since the initiation of the test. Both keys were operable throughout the test probe of six trials and consequently pecking (FR-15) on either of the two keys produced the 4-s access to the hopper filled with chicken pellets. Several intervals, usually occurring 1.5, 4.5, and 9 h after a single administration were examined for each drug dose. During the period between the test intervals the animals were in their home boxes. Test dosages were also given im and were studied in a mixed order.

During the training sessions in the test period the pigeons averaged 95.0% and 95.3% correct initial selections after Δ^1-THC and vehicle administration, respectively ($n = 5$). Data concerning the initial six rewards have been indicated elsewhere.[10b,13,14]

Biochemical Pharmacology. Synaptosomal Membrane Preparation. Male Sprague–Dawley rats weighing 300–450 g were killed by decapitation and their brains were rapidly removed. The whole brain minus the brain stem was used to make the synaptosomal membrane preparation following the protocol of Dodd et al.[17] with the following modifications. The initial homogenization was performed in 10 vol of 0.32 M sucrose with a 15 mL Kontex glass tissue grinder. All centrifugations were carried out at 4 °C. The initial homogenate was centrifuged at 2000 rpm for 10 min in a Hermle 7320K centrifuge using a swinging bucket rotor (15-mL tubes). The supernatant was then centrifuged for 5 min at 18000 rpm in a Sorvall Type SS-34 rotor using a Sorvall RC-5 centrifuge. The pellet was resuspended in 0.32 M sucrose and layered over a discontinous sucrose gradient (1.2, 0.8, 0.32 M) in 13.2-mL Kontron centrifuge tubes. The gradients were centrifuged for 15 min at 39000 rpm using a Kontron TST-41 rotor in a Kontron Centrikon T-2080 ultracentrifuge. The material at the 1.2 M/0.8 M sucrose interface was collected. After the addition of TME buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA; pH 7.4 at 30 °C) the membranes were centrifuged at 18000 rpm for 5 min using the Sorvall RC-5 centrifuge. The pellet was resuspended in TME buffer and incubated for 30 min at 30 °C. After centrifugation at 10000 rpm for 10 min with the Sorvall RC-5 centrifuge, the pellets were resuspended to a concentration of 1.5–2 mg/mL protein in TME buffer, aliquoted, and stored at −80 °C until use. Protein concentrations were determined by the method of Bradford[18] using bovine γ-globulin as the standard.

Ligand-Binding Assay. Radiolabeled 7 (ratio of 7 to 8, 95:5) was stored at a concentration of 1 μM (54 μCi/mL) in absolute ethanol at −20 °C. Unlabeled drugs were stored as 10 mM and 100 μM ethanol solutions at −20 °C. Following evaporation of the ethanol, drug dilutions were performed serially from 1 μM or 100 μM solutions in a vehicle of 5 mg/mL fatty acid free BSA (Sigma) in water. Due to the partial insolubility of Δ^1-THC at concentrations above 5 μM in 5 mg/mL BSA, 0.01 mL of 20 mM Δ^1-THC in ethanol was added directly to 0.99 mL of 50 mg/mL BSA and further dilutions were made such that the vehicle contained 5 mg/mL BSA and 0.1% (v/v) ethanol. The radioligand was placed in a beaker, the ethanol evaporated, and 0.2 mL of 50 mg/mL BSA was added, followed by 100 mL of TME buffer. This was aliquoted (0.8 mL) to siliconized microfuge tubes (Sigma), followed by the addition of 0.1 mL of drug or vehicle and 0.1 mL of synaptosomal membrane preparation in TME buffer. The final assay volume of 1 mL contained 45 mM Tris-HCl, 2.7 mM MgCl, 0.9 mM EDTA, 0.58 mg BSA, 2.4–3.8 μg of protein of synaptosomal membrane preparation, 38–48 fmol of [^{3}H]-7, and vehicle or drug as indicated. The assays tubes for Δ^1-THC also contained 0.01% (v/v) ethanol.

Tubes were incubated at 30 °C for 90 min and then centrifuged at 13000 rpm for 6 min. Following centrifugation, samples of the supernatant were saved and counted. The remaining supernatant was aspirated off and the tubes were placed inverted on drying pins. After 20 min, the tips of the tubes containing the pelleted membranes were cut off with a heated scalpel blade in a rig designed to ensure consistency. The tips were then placed in an emulsifier cocktail (Packard) and vortexed, and after several hours the radioactivity was determined as DPM. The radioligand adhering to the remaining part of the vial after tip removal was also determined. Nonspecific binding to the microfuge tip was assessed in tubes containing radioligand and protein but which were not centrifuged. This value was subtracted from the total binding of the cut tips to give the total bound to the pelleted membranes. Specific binding was defined as the difference between the total bound to the pelleted membranes in the absence or presence of 50 nM unlabeled 7 and was typically 70–80% of the total bound. For example, in a set of tubes containing 39.8 fmol total of [^{3}H]-7, 4.63 fmol was specifically bound to the pelleted membrane, 1.3 fmol was nonspecifically bound to the pelleted membrane, 0.75 fmol adhered to the centrifuge tube tip, 10 fmol adhered to the remaining part of the centrifuge vial, and 23.1 fmol remained in solution. Assays were performed in triplicate and experiments were repeated three times. K_d and K_i values were determined using the LIGAND program (version 2.3.11, May 1987),[19] and results are given as means ±SE. The bound values used in the calculations represented the total [^{3}H]-7 bound to the pelleted membrane, whereas the total ligand concentration value used included the total [^{3}H]-7 bound to the pelleted membrane, that which adhered to the vial, and that which remained in solution.[20]

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