Communications to the Editor

5'-Azido-Δ^8-THC: A Novel Photoaffinity Label for the Cannabinoid Receptor

Various preparations of the plant *Cannabis sativa* L. have been used since ancient times for their behavioral and pharmacological properties.[1] More recently it has been demonstrated that the active plant constituents, known as cannabinoids, produce a variety of effects including bronchodilation, increased heart rate, reduced intraocular pressure, analgesia, antiemesis, alteration in body temperature as well as anticonvulsant and psychotropic activities.[2]

Although the pharmacological and biochemical properties of cannabinoids have been studied extensively,[3] their molecular mechanism of action is not yet well understood. The high lipophilicity[3] of these molecules, together with their reported effects on the thermotropic[4,5] and dynamic[6-8] properties of model and natural membranes, suggests that at least some of the cannabinoid actions could be related to their abilities to interact with the lipid components of the cellular membrane and thus to indirectly affect the function of integral membrane proteins.

Recent evidence supports the hypothesis that cannabinoids also produce some of their effects by interacting with specific protein sites in synaptosomal preparations[9] and mammalian brains.[9,10] This THC binding protein was shown to be a G-protein coupled receptor whose activation leads to the inhibition of adenylate cyclase activity in a dose dependent and stereoselective manner.[11,12] This

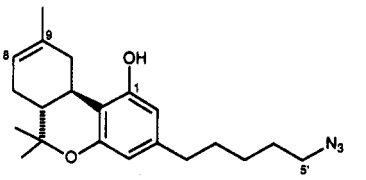
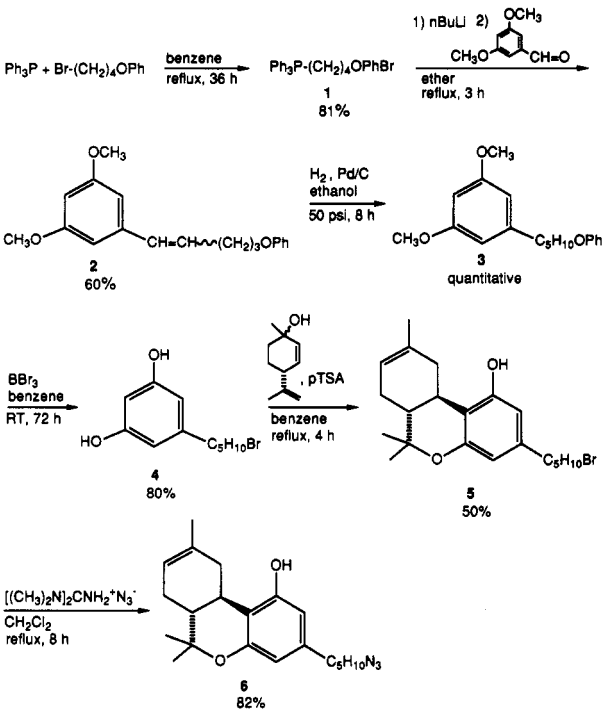


Figure 1. (−)-5'-N_3-Δ^8-THC.

Scheme I. Synthesis of (−)-5'-N_3-Δ^8-THC



receptor was shown to be more responsive to the psychoactive cannabinoids than the nonpsychoactive derivatives.[13] More recently, the cannabinoid receptor was cloned from rat[14] and human[15] cDNA libraries. The cDNA thus obtained when injected into CHO-K_1 cells led to the expression of the cannabinoid G-protein coupled receptor.[14] Furthermore, the human cannabinoid receptor gene was shown to be localized to chromosome 6q14-q15.[16] Using

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0022-2623/92/1835-3076$03.00/0

Table I. Biological Evaluation of (-)-5′-N_3-Δ^8-THC

analog	potency and maximum effect in mice[a]			
	locomotion (inhibition)	hypothermia (Δ temperature)	catalepsy (immobility)	antinociception[b] MPE
(-)-Δ^8-THC	1.9	15.5	5.2	1.5
	[1.3–2.9]	[6.1–3.9]	[3.6–7.7]	[0.6–4.1]
	(79%)	(-5.9 °C)	(58%)	(100%)
(-)-5′-N_3-Δ^8-THC	0.2	0.8	0.8	0.1
	[0.11–0.23]	[0.3–2.6]	[0.4–1.6]	[0.03–0.47]
	(73%)	(-3.8 °C)	(57%)	(100%)

[a] Potencies expressed as the ED_{50} (mg/kg) with 95% confidence limits in brackets and efficacies indicated in parentheses were determined as indicated in the text.[32,34,44] [b] Antinociception measured in terms of the maximum possible effect (MPE) on latency to tail-flick.

autoradiography[10,17] and positron emission tomography[18,19] the distribution of cannabinoid binding sites in the brain of mammals was examined. These studies demonstrated high receptor density in the basal ganglia, cerebellum, frontal cerebral cortex, and hippocampus. To date, the cannabinoid receptor has not yet been isolated and no information is available on the amino acid residues involved in the binding of the ligands. Attempts to develop affinity labels for this receptor[20] included the use of isothiocyanate derivatives of nonclassical cannabinoids[21] or photoactivatable groups attached to classical cannabinoid analogs.[22] This communication reports on the first successful cannabinoid receptor affinity label. The availability of this novel photoaffinity receptor probe constitutes an important development in cannabinoid research and should facilitate the full characterization of this receptor.

Photoaffinity labeling of a receptor involves the use of ligands carrying photoactivatable groups which covalently attach to reactive residues at or in the vicinity of the binding site, after equilibration and photoirradiation.[23,24] This methodology has been used for studying a number of receptors including those for PCP,[25] muscarine,[26] catecholamines,[27,28] serotonin,[29] and retinal[30] and has provided useful information on their distribution and molecular weights or amino acid residues at or near the active site.

We now introduce a photoaffinity label, namely (-)-5′-azido-Δ^8-tetrahydrocannabinol (5′-azido-THC, Figure 1), which was used to covalently label the cannabinoid receptor for the first time. We present the synthesis of this receptor probe, its in vivo and in vitro evaluation as well as the receptor labeling experiments.

Although (-)-Δ^9-tetrahydrocannabinol (Δ^9-THC) is the more commonly used and naturally more abundant cannabinoid, we chose instead its pharmacologically nearly equipotent (-)-Δ^8-THC isomer[31] as the parent compound, because of its greater chemical stability. Also, (-)-Δ^8-THC has a very similar affinity[32] for the cannabinoid receptor as (-)-Δ^9-THC. The photoactivatable group was introduced at the terminal side chain carbon of the parent compound on the basis of previous findings that bulky substituents in that position result in an increase in cannabimimetic activity and affinity for the receptor.[31,33,34] Furthermore, introduction of the azido group optimizes the side chain length as reported earlier.[34,35] Even though the nitrene generated after photoirradiation could undergo intramolecular rearrangement to the imine,[36–38] we had

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hoped that the affinity of the probe for the cannabinoid receptor would be high enough to allow for a fast reaction with the active site.

Chemistry.[39] 5′-N_3-(−)-Δ^8-THC was synthesized from (−)-5′-Br-Δ^8-THC, which in turn was obtained following a modification of the method described in the literature[40] (Scheme I). Thus, 4-phenoxybutyl bromide and triphenylphosphine were refluxed in benzene for 36 h to yield triphenyl(4-phenoxybutyl)phosphonium bromide (1) in 81% yield. Conversion of 1 to the ylide using *n*-butyllithium and refluxing with 3,5-dimethoxybenzaldehyde in ether for 3 h gave 1-(3,5-dimethoxyphenyl)-5-phenoxy-1-pentene (2) as a 2:1 mixture of *E*:*Z* isomers, which was purified by column chromatography in 60% yield. Subsequently, hydrogenation over Pd on carbon (10%) under 50 psi of hydrogen gas resulted in quantitative reduction of the double bond to yield 1-(3,5-dimethoxyphenyl)-5-phenoxypentane (3). Deprotection of the methoxy groups and substitution of the phenoxy moiety by bromide to give 5′-bromo olivetol 4 was achieved in 80% yield after purification, by treating 3 with boron tribromide in benzene at 25 °C for 72 h. Coupling of 4 with (+)-*cis*/*trans*-*p*-mentha-2,8-dien-1-ol[41] by refluxing in benzene in the presence of *p*-toluenesulfonic acid for 4 h gave (−)-5′-Br-Δ^8-THC (5) in 50% yield after purification. The desired product (−)-5′-N_3-Δ^8-THC[42] (6) was finally obtained in 82% yield, by treating 5 with tetramethylguanidinium azide in refluxing methylene chloride for 8 h.[43]

Pharmacological and Biochemical Evaluation. 5′-Azido-THC was evaluated in vivo and compared to the parent compound (−)-Δ^8-THC for cannabimimetic activity. Both analogs were tested in male ICR (Institute of Cancer Research) mice (Harlan, Frederick, MD) following intravenous injection, for their abilities to produce sedation (decreased locomotor activity) and catalepsy (induction of ring immobility) as measures of drug-induced behavioral effects.[32,34,44] They were also evaluated in the same mice for their abilities to produce hypothermia and antinociceptive activity in the mouse tail-flick assay, as previously described.[32,34,44]

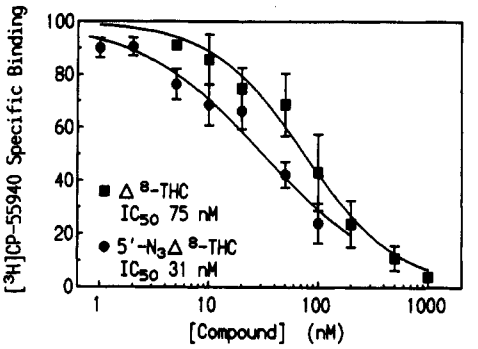

Figure 2. Binding of (−)-Δ^8-THC and (−)-5′-N_3-Δ^8-THC to the cannabinoid receptor in rat forebrain membranes. The data shown are means ± SEM from three to five experiments, respectively.

The results obtained are shown in Table I. Dose–response data were analyzed statistically by ANOVA, and the potency (ED_{50} value) and efficacy (maximum effect) determined as described previously.[32,34,44] The maximum effects given represent percent inhibition (of locomotor activity), °C (of hypothermia), percent immobility (of catalepsy) and percent MPE (maximum possible effect of latency of antinociception procedure). The data indicate that 5′-azido-THC is 6.5 times (catalepsy) and 19 times (hypothermia) more potent (on a milligram/kilogram basis) than the parent compound in these pharmacological tests.

5′-Azido-THC was also evaluated for its affinity for cannabinoid binding sites using membrane preparations obtained from rat forebrains. The experiment consists of incubating the membrane preparations with the cannabinoid radioligand [^{3}H]CP-55,940 (Dupont-NEN) plus varying concentrations of 5′-azido-THC or (−)-Δ^8-THC in the appropriate buffer containing no bovine serum albumin in order to minimize exposure of the photolabel to nonprotein amines.[45] Nonspecific binding was calculated using desacetyllevonatradol.[9] The K_i for binding of 5′-azido-THC to the cannabinoid receptor was calculated to be 19 ± 6 nM (mean ± SEM, n = 5 experiments). This is a 2-fold increase in affinity for the receptor when com-

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(39) ^{1}H NMR spectra were recorded on a Bruker WP-200SY 200 MHz spectrometer using tetramethylsilane as an internal reference. Elemental analysis for compound 6 is within ±0.4% of the calculated values. Its mass spectrum is a chemical ionization (CI) spectrum and was obtained on a Finigan 4500 GC/MS spectrometer using a direct exposure probe and ammonia as the carrier gas.

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(41) (+)-cis/trans-p-mentha-2,8-dien-1-ol was supplied by Firmenich Inc., Princeton, NJ.

(42) Colorless gum; ^{1}H NMR (200 MHz, $CDCl_3$) δ (TMS) 1.1 (s, 3 H, 6α-CH_3), 1.37 (s, 3 H, 6β-CH_3), 1.7 (s, 3 H, 11-CH_3), 2.42 (t, 2 H, J = 6.8 Hz, 1′-CH_2), 2.7 (dt, 1 H, H_{10a}), 3.12–3.28 (m, 3 H, $H_{10\alpha}$ and 5′-CH_2), 5.42 (s, 1 H, H_3), 6.1 (s, 1 H, H_2), 6.26 (s, 1 H, H_4). Anal. ($C_{21}H_{29}N_3O_2$) C, H, N; MS m/z 356 (MH^+).

(43) (−)-5′-Azido-Δ^8-tetrahydrocannabinol. A solution of (−)-5′-Br-Δ^8-THC (500 mg, 1.27 mmol) in methylene chloride (5 mL) was added dropwise to a solution of tetramethylguanidinium azide (287 mg, 1.82 mmol) in methylene chloride (5 mL) at 0 °C. The reaction mixture was then refluxed for 8 h and the solvent was removed using a stream of nitrogen. The residue was extracted with ethyl ether and the extract was purified by column chromatography using silica gel and ethyl ether/petroleum ether (1:5) as an eluent, to yield 368 mg (81%) of the title compound.

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(45) Cannabinoid binding to rat brain membranes: membranes were prepared from rat forebrain and receptor binding assays were performed by a modification of the method described in prior reports.[9] Briefly, 30 μg of membranes was incubated in a buffer containing 20 mM potassium diethyl malonate, pH 7.4, 3 mM $MgCl_2$, 0.01% β-cyclodextrin, and 100 fmol/mL [^{3}H]CP-55,940 (0.2 or 1 mL). These reagents were used in lieu of Tris buffer and BSA, in order to minimize exposure of the photolabel to nonprotein amines. Nonspecific binding was calculated from tubes containing 100 nM desacetyllevanantrol. Assays were incubated at 30 °C for 1 h in Regisil-treated Eppendorf tubes and terminated either by centrifugation at 23000g for 10 min or by filtration through GF/C filters immediately after addition of 200 μL of 50 mg/mL BSA. The bound ligand was counted in a Beckman liquid scintillation counter. Data were calculated by nonlinear regression analysis using Graphpad Inplot. Data from three to five experiments were combined for Figure 2 and were analyzed by nonlinear regression analysis using Graphad Inplot. K_i values from each individual experiment were calculated from IC_{50} values using the assumptions of Cheng and Prusoff (*Biochem. Pharmacol.* **1973**, *22*, 3099–3102).

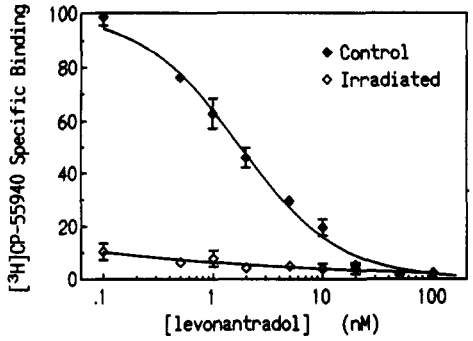

Figure 3. Photoirradiation of (−)-5′-N_3-Δ^8-THC with forebrain membranes inhibits specific binding of [^{3}H]CP-55,940 to the cannabinoid receptor. Heterologous displacement by levonantradol is shown for membranes irradiated in the absence (◆) or after equilibration with 5′-azido-THC (◇). The data shown are the mean ± SD of triplicate determinations from a single representative experiment.

pared with (−)-Δ^8-THC (K_i = 35 ± 11 nM, mean ± SEM, n = 3 experiments) (Figure 2).

Photoaffinity Labeling.[46] The ability of 5′-azido-THC to inactivate the cannabinoid receptor was tested in photoirradiation experiments. Rat forebrain membranes were equilibrated with a concentration of 5′-azido-THC that was 50 times the K_d in order to ensure that receptor occupancy was greater than 98%. After equilibration, membranes were photoirradiated with shortwave ultraviolet light, washed extensively to remove unbound 5′-azido-THC, and then tested for cannabinoid receptor binding (Figure 3). In control membranes irradiated in the absence of 5′-azido-THC, specific binding of [^{3}H]CP-55,940 to the cannabinoid receptor was displaced by increasing concentrations of levonantradol as shown in Figure 3 or desacetyllevonantradol (experiment not shown). However, in the 5′-azido-THC equilibrated and irradiated membranes, no specific binding of [^{3}H]CP-55,940 was observed. Photoirradiation did not alter the nonspecific component which is observed in the presence of 100 nM desacetyllevonantradol. In experiments not shown, equilibration of membranes with lower concentrations of 5′-azido-THC and/or photoirradiated for shorter periods of time yielded a smaller fraction of receptor population made refractory to specific binding (12% with 50 nM 5′-azido-THC for 5-min exposure; 31% with 500 nM 5′-azido-THC for 5-min UV exposure). The decrease in specific binding to the cannabinoid receptor after photoirradiation with 5′-azido-THC was not due to residual 5′-azido-THC after the washing procedure because supernatants from the last centrifugation wash did not inhibit binding when added to the control assay. Control membranes incubated with Δ^8-THC at 500 nM followed by photoirradiation and the washing procedure exhibited cannabinoid receptor binding activity that was equal to that of control membranes incubated in the absence of any cannabinoid compounds (data not shown). This was expected, in as much as previous studies determining adenylate cyclase activity as the biological endpoint had determined that the activity of Δ^9-THC was reversible after washing of the membranes by sedimentation,[11] demonstrating that, although lipophilic in nature, Δ^9-THC does not undergo an irreversible interaction with the cannabinoid receptor. Exposure to UV light did not damage the membrane preparations because control membranes that were exposed to UV light under these experimental conditions did not exhibit reduced binding. However, because we were measuring only cannabinoid receptor binding activity, these experiments do not preclude the possibility that other membrane proteins may also have been labeled during the photoirradiation of brain membranes with 5′-azido-THC. As an additional control, 5′-azido-THC was photoirradiated in the presence of a 5-fold molar excess of glycine, in an effort to obtain a photoproduct with glycine. When this mixture was subsequently used to photolabel brain membrane preparations as described above, no loss of binding of [^{3}H]CP-55,940 was observed.

The above experiments demonstrate the potential of photoaffinity labeling as a useful method for the isolation and further characterization of the cannabinoid receptor. The usefulness of the photoaffinity label described here was recently demonstrated in two other systems, namely, mouse brain preparations and culture S-49 mouse lymphoma cells.[47] In these systems cannabinoid binding sites were detected using (−)-2-^{125}I-5′-N_3-Δ^8-THC. Currently, we are in the process of developing novel photoaffinity labels having higher affinities for the receptor. We also hope to develop probes which would help in identifying cannabinoid receptor subtypes if such subtypes, in fact, exist.

Acknowledgment. This work was supported by Grants DA-3801,† DA-152,† DA-3690,‡ DA-6312,‡ and DA-03672.§ The assistance of G. H. Wilken and C. Song with ligand binding experiments is appreciated. For the generous supply of chemicals we also thank Firmenich, Inc. for (+)-*cis*/*trans*-*p*-mentha-2,8-dien-1-ol, NIDA for Δ^8-THC, and Pfizer, Inc. for levonantradol and desacetyllevonantradol.

(46) Photoaffinity studies: Rat forebrain membranes (0.5–3 mg) were incubated with 1 μM 5′-azido-THC (previously resuspended in 5% β-cyclodextrin) in 12-well tissue culture plates containing 1 mL of 20 mM potassium diethyl malonate, pH 7.4 and 0.1% β-cyclodextrin. All procedures utilizing 5′-azido-THC were conducted in the dark or under safety light. After equilibration for 1 h at 30 °C, the samples were placed on ice and exposed to shortwave ultraviolet light (UVGL-25 mineralight at 10-cm distance for 20 min). Control membranes were incubated and irradiated in the absence of 5′-azido-THC. The membranes were then placed in microfuge tubes and centrifuged at 23000*g* for 2 min at room temperature. This washing procedure was repeated a minimum of four times, using TME buffer containing 1 mg/mL bovine serum albumin at room temperature, and 5–10 min allowed between centrifugations to allow reversibly bound ligand to dissociate from the membranes. The final membrane resuspension was subsequently assayed for cannabinoid receptor binding using [^{3}H]CP-99,540 as the radioligand.

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Received March 4, 1992