(−)-11-Hydroxy-7′-isothiocyanato-1′,1′-dimethylheptyl-Δ⁸-THC: A Novel, High-Affinity Irreversible Probe for the Cannabinoid Receptor in the Brain

Yan Guo,† Vasiliki Abadji,† Kelley L. Morse,† Donna J. Fournier,† Xiuyan Li,† and Alexandros Makriyannis*,†,‡

School of Pharmacy and Institute of Materials Science, University of Connecticut, Storrs, Connecticut 06269

Received July 11, 1994

Introduction. Since the identification of the cannabinoid receptor[1] and its characterization as a G-protein-coupled receptor,[2] significant progress has been made in the field of cannabinoid research. A clearer picture with regard to the molecular features required for cannabimimetic activity within the cannabinoid structure is, thus, emerging.[3] Furthermore, the effects of cannabinoids on membranes have received a great deal of attention.[4] On the receptor front, complimentary DNA from human[5] and rat[6] DNA libraries of this protein, which appears to be associated with the behavioral and pharmacological effects of cannabinoids, has been cloned, sequenced, expressed, and characterized. Although hitherto reports have dealt with studies on the cannabinoid receptor and its mRNA almost exclusively in brain and CNS-associated tissue, new evidence suggests the presence of cannabinoid receptor in testis,[7] leukocytes,[8] and spleen cells.[9] More recently, the cloning of a new peripheral cannabinoid receptor, expressed in macrophages in the marginal zone of the spleen has been reported, providing the first evidence for the existence of receptor subtypes.[10] On another front, efforts aimed at elucidating the physiological role of the cannabinoid receptor culminated in the isolation and identification of arachidonylethanolamide as a putative endogenous ligand.[11]

Despite the above advances, there are still important issues concerning the cannabinoid receptor that remain to be resolved. Although the receptor has been solubilized and shown to retain activity in detergent solution,[12] it has not yet been isolated in pure form, and there is still no information available concerning the tertiary structure of the primary cannabinoid binding site or the nature of the specific amino acid residues that comprise it.

We have sought to develop high-affinity ligands in an effort to address the issues of receptor multiplicity, receptor isolation and identification of its binding site(s). Our first successful attempt was the recently reported photoaffinity label, (−)-5′-azido-Δ⁸-tetrahydrocannabinol.[13] This compound was shown to bind to the cannabinoid receptor in rat forebrain membranes with an IC_{50} of 31 nM. Furthermore, its ability to irreversibly label the receptor was demonstrated in photoirradiation experiments. Preequilibration of the photoaffinity probe with rat forebrain membranes, followed by UV irradiation, inhibited specific binding of the tritiated cannabinoid probe [³H]CP-55940 to the membranes. The usefulness of such a ligand has already been demonstrated in mouse brain preparations and in cultured S49 mouse lymphoma cells where photolabeling experiments using radiolabeled (−)-2-iodo-5′-azido-Δ⁸-THC revealed several ligand−protein adducts, one of which was thought to be that of the cannabinoid receptor.[14]

In a different approach, Richardson et al. have synthesized racemic, isothiocyanate-containing analogs of the nonclassical cannabinoid CP-55244 with the intent of using them as affinity ligands.[15] The resulting ligands exhibited the same affinity for the receptor as the parent molecule. However, to date, there is no report on the ability of these ligands to irreversibly bind to the cannabinoid receptor.

In this communication we report the design, synthesis, and biochemical characterization of (−)-11-hydroxy-(7′-isothiocyanato-1′,1′-dimethylheptyl-Δ⁸-THC (**1**), the first irreversible, classical cannabinoid affinity label for the receptor.[16] The design of this ligand was based on the attachment of an isothiocyanate moiety to the ω-position of the alkyl side chain of (−)-11-hydroxy-1′,1′-dimethylheptyl-Δ⁸-THC, a highly selective ligand for cannabinoid receptors.[17] The isothiocyanate group was chosen because it is inert in water, but capable of nucleophilic reactions with amino, imidazoyl, and sulfhydryl groups on biological macromolecules under physiological conditions.[18] Furthermore, isothiocyanate-containing ligands have been extensively employed as tools for the study and characterization of a series of receptors including the opioid,[19,20] NMDA,[21] σ[22] and benzodiazepine[23] receptors.

Chemistry.[24] (−)-11-Hydroxy-7′-isothiocyanato-1′,1′-dimethylheptyl-Δ⁸-THC (**1**) was synthesized from (−)-11-hydroxy-7′-azido-1′,1′-dimethylheptyl-Δ⁸-THC (**2**), which was in turn obtained from (−)-11-hydroxy-7′-bromo-1′,1′-dimethylheptyl-Δ⁸-THC (**3**). The latter was synthesized[16] by the condensation of 4-hydroxymyrtenyl pivalate (**4**) with 7-methyl-7-(3,5-dihydroxyphenyl)-1-bromooctane (**5**), followed by reduction of the pivalate (**6**), Scheme 1. Compound **4** was obtained following the method by Mechoulam et al.,[27] while **5** was prepared using a method developed in our laboratory.[25] Refluxing of **3** with tetramethylguanidinium azide in chloroform afforded the 7′-azido analog (**2**) in 92% yield after purification, which when treated with carbon disulfide and triphenylphosphine in THF at room temperature afforded (−)-11-hydroxy-7′-isothiocyanato-1′,1′-dimethylheptyl-Δ⁸-THC (**1**) in 73% yield after purification[26,27] (Scheme 1). The optical purity of **1** was determined by means of its Mosher's ester.

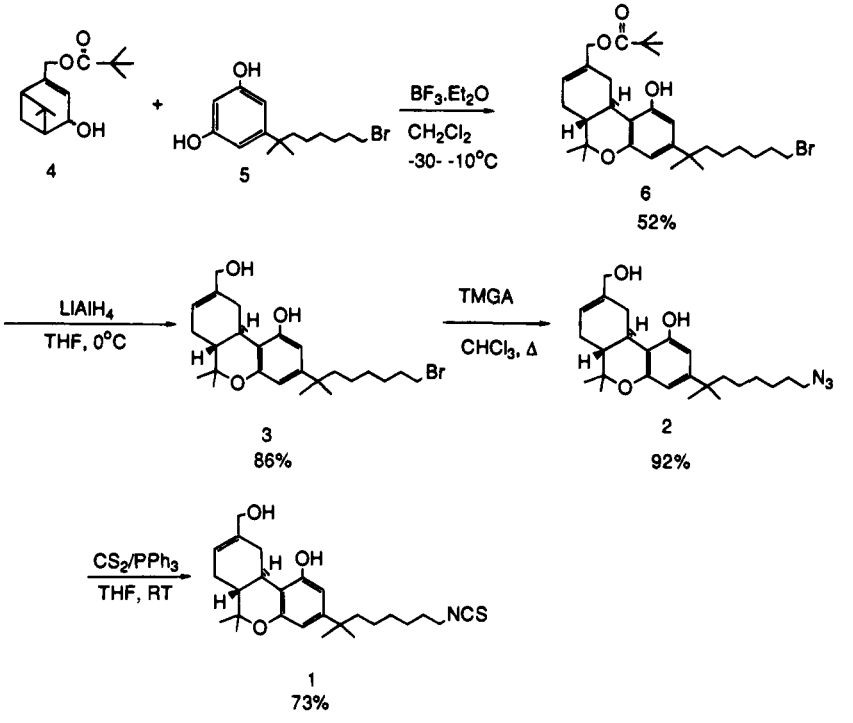
Receptor Binding Studies.[28] Rat forebrain membranes were used to assess the affinity of (−)-11-hydroxy-7′-isothiocyanato-1′,1′-dimethylheptyl-Δ⁸-THC for cannabinoid binding sites.[1] For this purpose a filtration assay was employed in which specific binding of the tritiated ligand CP-55940 to cannabinoid binding site(s) was displaced by increasing concentrations of the affinity ligand.[1,13] Under these conditions the apparent IC_{50} of **1** was calculated to be 1.6 ± 0.3 nM (Figure 2).

Receptor Inactivation.[29] Having exhibited a high affinity for the cannabinoid receptor, the ligand was subsequently evaluated for its ability to irreversibly label the receptor. As depicted in Figure 2, preequili-

* Address correspondence to this author at: School of Pharmacy, U-92, University of Connecticut, Storrs, CT 06269.
† School of Pharmacy.
‡ Institute of Materials Science.

0022-2623/94/1837-3867$04.50/0 © 1994 American Chemical Society

Scheme 1. Synthesis of (−)-11-Hydroxy-7′-isothiocyanato-1′,1′-dimethylheptyl-Δ^8-tetrahydrocannabinol



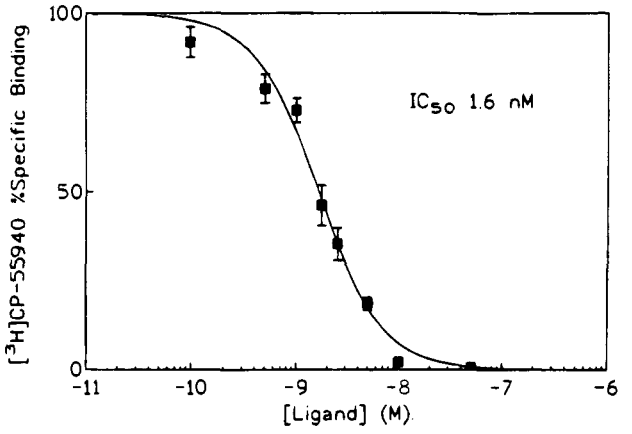


Figure 1. Equilibrium binding of (−)-11-hydroxy-7′-isothiocyanato-1′,1′-dimethylheptyl-Δ^8-THC to the cannabinoid receptor in rat forebrain membranes. The data shown represent the average of duplicates from three different experiments.

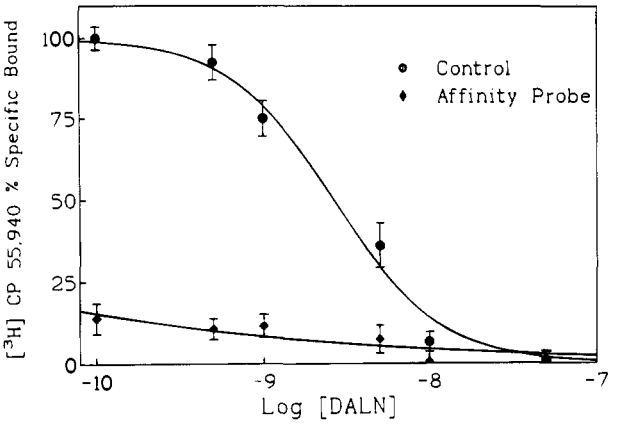


Figure 2. Displacement of [³H]CP-55940 specific binding by desacetyllevonantradol (DALN) from rat forebrain membranes preequilibrated in the absence (J) or presence of 10 nM (−)-11-hydroxy-7′-NCS-1′,1′-diemethylheptyl-Δ^8-THC (F) is presented as mean ± SEM (n = 6).

bration of rat forebrain membranes with 10 nM of **1**, followed by three washes to remove excess, unbound ligand, lowered by 80% the [³H]CP-55940 specific binding to the cannabinoid receptor. By contrast, membranes treated exactly as above, but in the absence of the affinity label retained their ability to bind cannabinoid ligands, as evidenced by the displacement of [³H]-CP-55940 specific binding to the receptor by desacetyllevonantradol. When the membranes were pretreated with 100 nM of the affinity probe, virtually all cannabinoid binding was depleted (data not shown). In a control experiment not shown here, membranes were equilibrated with the parent compound (−)-11-hydroxy-1′,1′-dimethylheptyl-Δ^8-THC as described above. The membranes were subsequently washed as before and found to exhibit characteristic cannabinoid binding activity comparable to that of control membranes. In a time-dependent study, when the membranes were incubated for different periods of time with 20 nM of affinity ligand (over ten fold its apparent IC_{50}), complete labeling of the receptor was achieved after only 5 min incubation, indicating a rapid reaction between the ligand and the receptor. To better demonstrate a time dependence for receptor labeling by the affinity ligand, we carried out the same experiment using much reduced concentrations of ligand, 0.5 and 2 nM, with incubation periods between 5 and 60 min (Figure 3). At 0.5 nM of **1**, approximately 10% and 25% labeling was observed after 5 and 15 min incubation, respectively. After 30 min incubation the maximum amount of receptor labeling of 50% was observed at this ligand concentration. When the membranes were incubated with 2 nM of **1**, 50% labeling is achieved after 5 min incubation, with maximum labeling of 70% obtained after 60 min. This time-dependent receptor labeling does not necessarily reflect the true kinetic behavior of the covalent reaction between **1** and the cannabinoid receptor since the incubation time does not represent the true reaction time. In this regard, our experimental conditions involved the termination of this reaction by repeatedly

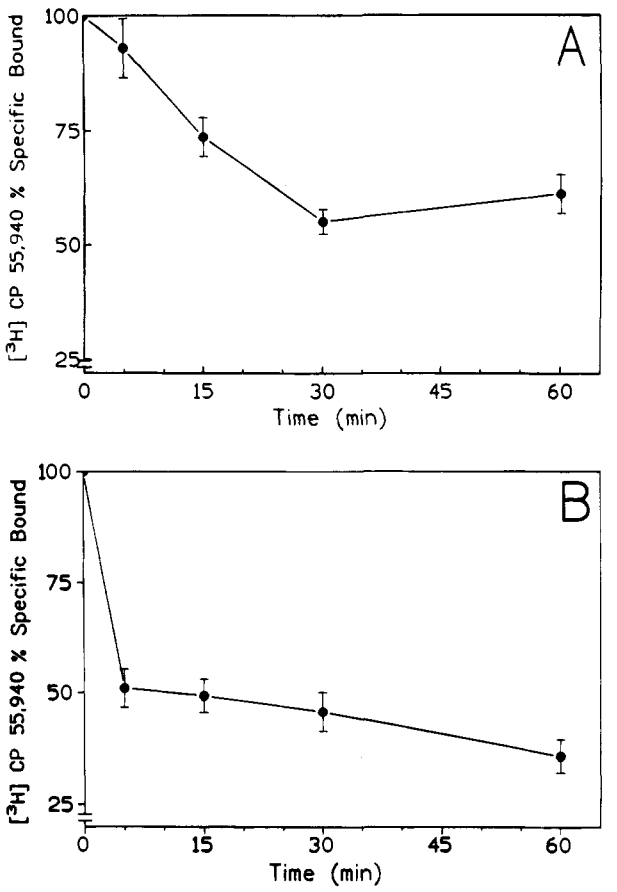


Figure 3. Time course cannabinoid receptor labeling with (−)-11-hydroxy-7′-NCS-1′,1′-dimethylheptyl-Δ^8-THC. Rat forebrain membranes were preincubated with 0.5 nM (A) and 2 nM (B) (−)-11-hydroxy-7′-NCS-1′,1′-dimethylheptyl-Δ^8-THC for 5, 15, 30, and 60 min at 30 °C, then washed three times with 1 mg/mL BSA in 25 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA. The final pellet was resuspended in the working buffer to its original protein concentration and subjected to the competitive binding assay. Heterologous displacement of [^{3}H]CP-55940 by DALN is presented as the mean ± SEM (n = 6). Data shown are representative of three independent experiments.

pelleting and washing the membrane preparation, after the incubation period was completed, in order to remove any unreacted ligand. However, it is very likely that during this procedure, which is approximately 30 min long, not all of the ligand is immediately removed from the receptor's environment at the end of each incubation period and that the receptor-affinity ligand reaction continues after the initial incubation, although at a progressively decreasing rate. Such a possibility would account for the apparent fast initial rate of receptor labeling in our experiments. The observation that full receptor labeling is not observed at lower concentrations even if the incubation period is increased is probably the reflection of the slow inactivation of the probe through nonspecific reactions. Notwithstanding the above experimental limitations, our data clearly demonstrate that a time-dependent receptor labeling does occur.

Conclusion. Our data demonstrate that the isothiocyanate group, when placed at the tail of the side chain of a high-affinity classical cannabinoid ligand, can covalently attach itself to the active site of the receptor. This, in turn, points to the presence of a thiol, amino, or imidazole amino acid residue at or in the vicinity of the receptor active site.

Acknowledgment. This work was supported by National Institute on Drug Abuse Grants DA-3801, DA-152, and DA-7215. We would like to thank Dr. L. S. Melvin from Pfizer, Inc., for providing us with desacetyllevonantradol.

References

(1) Devane, W. A.; Dysarz, F. A., III; Johnson, R. M.; Melvin, L. S.; Howlett, A. C. Determination and Characterization of a Cannabinoid Receptor in Rat Brain. *Mol. Pharmacol.* **1988**, *34*, 605–613.
(2) Howlett, A. C. Cannabinoid Inhibition of Adenylate Cyclase. *Mol. Pharmacol.* **1985**, *27*, 429–436.
(3) Makriyannis, A.; Rapaka, R. S. The Molecular Basis of Cannabinoid Activity. *Life Sci.* **1990**, *47*, 2173–2184.
(4) Makriyannis, A.; Yang, D. P.; Griffin, R. G.; Das Gupta, S. K. The Perturbation of Model Membranes by (−)-Δ^9-Tetrahydrocannabinol. Studies Using Solid-state ^{2}H- and ^{13}C-NMR. *Biochim. Biophys. Acta* **1990**, *1028*, 31–42.
(5) Gerard, C. M.; Mollereau, C.; Vassart, G.; Parmentier, M. Nucleotide Sequence of Human Cannabinoid Receptor cDNA. *Nucleic Acids Res.* **1990**, *18*, 7142.
(6) Matsuda, L. A.; Lolait, S. J.; Brownstein, M. J.; Young, A. C.; Bonner, T. I. Structure of a Cannabinoid Receptor and Functional Expression of the Cloned cDNA. *Nature* **1990**, *346*, 561–564.
(7) Gerard, C. M.; Mollereau, C.; Vassart, G.; Parmentier, M. Molecular Cloning of a Human Cannabinoid Receptor Which is Also Expressed in Testis. *Biochem. J.* **1991**, *279*, 129–134.
(8) Casellas, P.; Bouaboula, M.; Rinaldi, M.; Carayon, P.; Carillon, C.; Delpech, B.; Shire, D.; LeFur, G. Cannabinoid Receptor Expression in Human Leukocytes. *Eur. J. Biochem.* **1993**, *214*, 173–180.
(9) Kaminski, N. E.; Abood, M. E.; Kessler, F. K.; Martin, B. R.; Schatz, A. R. Identification of a Functionally Relevant Cannabinoid Receptor on Mouse Spleen Cells that is Involved in Cannabinoid-Mediated Immune Modulation. *Mol. Pharmacol.* **1992**, *42*, 736–742.
(10) Munro, S.; Thomas, K. L.; Abu-Shaar, M. Molecular Characterization of a Peripheral Receptor for Cannabinoids. *Nature* **1993**, *365*, 61–65.
(11) Devane, W. A.; Hanus, L.; Breuer, A.; Pertwee, R. G.; Stevenson, L. A.; Griffin, G.; Gibson, D.; Mandelbaum, A.; Etinger, A.; Mechoulam, R. Isolation and Structure of a Brain Constituent That Binds to the Cannabinoid Receptor. *Science* **1992**, *258*, 1946–1949.
(12) Houston, D. B.; Howlett, A. C. Solubilization of the Cannabinoid Receptor from Rat Brain and Its Functional Interaction with Guanine Nucleotide-Binding Proteins. *Mol. Pharmacol.* **1993**, *43*, 17–22.
(13) Charalambous, A.; Guo, Y.; Houston, D. B.; Howlett, A. C.; Compton, D. R.; Martin, B. R.; Makriyannis, A. 5′-azido-Δ^8-THC: A Novel Photoaffinity Label for the Cannabinoid Receptor. *J. Med. Chem.* **1992**, *35*, 3076–3079.
(14) Burstein, S. H.; Audette, A. A.; Charalambous, A.; Doyle, S. A.; Guo, Y.; Hunter, S. A.; Makriyannis, A. Detection of Cannabinoid Receptors by Photoaffinity Labelling. *Biochem. Biophys. Res. Commun.* **1991**, *176*, 492–497.
(15) Richardson, S.; Mirasadeghi, S.; Lynn, A.; Johnson, M. R.; Melvin, L. S.; Herkenham, M.; Rice, K. C. Synthetic and Biological Studies of Potential Affinity Ligands for the Cannabinoid Receptor Based on CP-55,240. In *Problems of Drug Dependence 1990 Proceeding of the 52nd Annual Scientific Meeting*; NIDA Research Monograph Series No. 105; National Institute on Drug Abuse: 1991; pp 140–146.
(16) Abadji, V.; Guo, Y.; Grzybowska, J.; Makriyannis, A. High Affinity Probes for the Cannabinoid Receptor. College on Problems of Drug Dependence, 55th Annual Scientific Meeting, Toronto, Canada, 1993.
(17) Mechoulam, R.; Lander, N.; Breuer, A.; and Zahalka, J.; Synthesis of the Individual, Pharmacologically Distinct Enantiomers of a Tetrahydrocannabinol Derivative. *Tetrahedron: Asymmetry* **1990**, *1*, 315–319.
(18) Assony, S. J. In *Organic Sulfur Compounds*; Kharasch, N., Ed.; Pergamon Press: New York, 1961; p 326.
(19) Rice, K. C.; Jacobson, A. E.; Burke, T. R., Jr.; Bajwa, B. S.; Streaty, R. A.; Klee, W. A. Irreversible Ligands with High Selectivity toward δ and μ Opiate Receptors. *Science* **1983**, *220*, 314–316.
(20) Portoghese, P. S.; Sultana, M.; Takemori, A. E. Naltrindole 5′-Isothiocyanate: A Nonequilibrium Highly Selective δ Opioid Receptor Antagonist. *J. Med. Chem.* **1990**, *33*, 1547–1548.

(21) Raftery, M. F.; Mattson, M.; Jacobsen, A. E.; Rice, K. C. A Specific Acylating Agent for the [^{3}H]Phencyclidine Receptors in Rat Brain. *FEBS Lett.* **1985**, *181*, 318–322.
(22) Adams, J. T.; Teal, P. M.; Sonders, A. S.; Tester, B.; Esherick, J. S.; Scherz, M. W.; Keana, J. F. W.; Weber, E. Synthesis and Characterization of an Affinity Label for Brain Receptors to Psychotomimetic Benzomorphans: Differentiation of σ-type and Phencyclidine Receptors. *Eur. J. Pharmacol.* **1987**, *142*, 61–71.
(23) Allen, M. S.; Hagen, T. J.; Trudell, M. L.; Codding, P. W.; Skolnick, P.; Cook, J. M. Synthesis of Novel 3-Substituted β-Carbolines as Benzodiazepine Receptor Ligands: Probing the Benzodiazepine Receptor Pharmacophore. *J. Med. Chem.* **1988**, *31*, 1854–1861.
(24) ^{1}H NMR spectra were recorded on a Brucker WP-200SY MHz spectrometer using tetramethylsilane as an internal reference. Elemental analysis for compound 1 is within ±0.4% of the calculated values. Specific rotation was determined with a Perkin-Elmer 241 polarimeter using a 1.00 dm cell.
(25) Charalambous, A.; Grzybovska, J.; Lin, S.; Banijamali, A. R.; Marciniak, G.; Guo, Y.; Wang, C. J.; Compton, D. R.; Martin, B. R.; Makriyannis, A. Novel Halocannabinoids. Synthesis and Biological Evaluation. Submitted to *J. Med. Chem.*
(26) White solid; mp 47–49 °C; $[\alpha]^{25}_D = -152.64°$; ^{1}H NMR ($CDCl_3$) δ 6.35–6.26 (d, 2H), 5.75–5.73 (d, 1 H), 4.08 (s, 2H), 3.49–3.43 (t, 2H), 1.38 (s, 3H), 1.17 (s, 6H), 1.04 (s, 3H). Anal. ($C_{26}H_{37}NO_3S$) C, H, N.
(27) (−)-11-Hydroxy-7′-isothiocyanato-1′,1′-dimethylheptyl-Δ^8-THC. A solution of 11-hydroxy-7′-bromo-1′,1′-dimethylheptyl-Δ^8-THC (138.2 mg, 0.297 mmol) in 10 mL of dry chloroform was added dropwise to a solution of TMGA (94 mg, 0.594 mmol) in 5 mL of dry chloroform at 0 °C under nitrogen. The resulting mixture was allowed to reach room temperature and refluxed overnight. Subsequently, the solvent was removed under a flow of nitrogen and ethyl ether was added until no more precipitate was formed. The precipitate was filtered out, and the filtrate was dried over sodium sulfate. Removal of the solvent yielded 124.8 mg of crude product which was purified by column chromatography (using 70% ethyl ether in petroleum ether as eluent). The desired product, 117.3 mg (light yellow oil), was obtained in 92% yield. 11-Hydroxy-7′-azido-1′,1′-dimethylheptyl-Δ^8-THC (100 mg, 0.23 mmol) and carbon disulfide (0.4 mL, 6.6 mmol) were dissolved in 10 mL of anhydrous THF. The mixture was stirred at room temperature, and triphenylphosphine (92 mg, 0.35 mmol) was added. After 3 days, the solvent was evaporated under vacuum, and the residue was purified by column chromatography (using 70% ethyl ether in petroleum ether as eluent). After purification 74.4 mg of 1 (white solid) was obtained in 73% yield.
(28) Rat forebrain membranes were prepared according to the procedure of Devane et al.[1] The binding of the novel probes to the cannabinoid receptor was assessed as previously described.[1,13] Briefly, approximately 50 μg of rat forebrain membranes was incubated in 25 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA containing 0.1% bovine serum albumin along with 0.8 nM [^{3}H]CP-55940 and varying concentrations of 1 to a final volume of 0.2 mL. Assays were incubated in Regisil-treated culture tubes for 1 h at 30 °C and terminated by the addition of 0.25 mL of 25 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA containing 5% bovine serum albumin. The assays were immediately filtered on GF/C filters using a Brandell M-24 cell harvester. Following four washes with 25 mM Tris buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, the filters were collected, shaken for 1 h with 2 mL of 0.1% sodium dodecyl sulfate, and counted in a Beckman liquid scintillation counter to determine the bound ligand. Nonspecific binding was assessed from tubes containing 250 nM desacetyllevonantradol. Data were collected from three different experiments performed with duplicate determinations. IC_{50} values were calculated by nonlinear regression analysis using the commercially available program GraphPad InPlot (GraphPad Software, San Diego, CA).
(29) **Receptor Labeling.** Rat forebrain membranes (1–3 mg) in 25 mM Tris buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, were incubated with the appropriate amount of affinity label (initially dissolved in DMSO and resuspended in the working buffer containing 1% BSA) in a Regisil-treated six-well culture plate for 1 h at 30 °C. At the end of the equilibration membranes were pelleted using a bench top microcentrifuge and washed three times with the working buffer containing 0.1% bovine serum albumin. The final pellet was resuspended in the working buffer and assayed for cannabinoid binding activity according to the above procedure.