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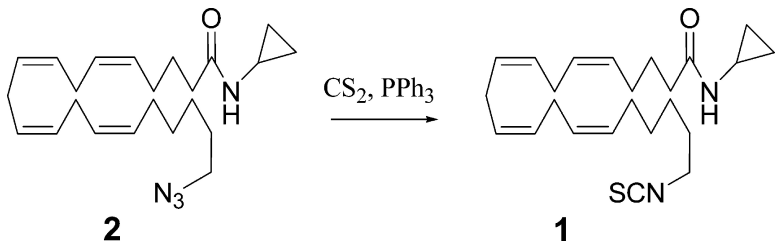


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# High Affinity Electrophilic and Photoactivatable Covalent Endocannabinoid Probes for the CB1 Receptor

Chen Li, Wei Xu, Subramanian K. Vadivel, Pusheng Fan, and Alexandros Makriyannis*

*Center for Drug Discovery, Northeastern University, 116 Mugar Hall, 360 Huntington Avenue, Boston, Massachusetts 02115*

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We have designed and synthesized the first two high affinity covalent anandamide probes for the CB1 receptor by introducing either an electrophilic isothiocyanato or a photoactivatable azido group at the terminal carbon of the arachidonic acid moiety. The headgroup of these anandamide analogues was optimized by using a cyclopropylamide substituent to impart optimal CB1 affinity. Both 20-isothiocyanato-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**1**, AM3677) and 20-azido-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**2**, AM3661) exhibited high selectivities for the CB1 receptor with $K_i$ values of 1.3 and 0.9 nM, respectively. Using suitable experimental conditions, both ligands were shown to covalently label the CB1 receptor with high efficiency. These two covalent probes for the endocannabinoid CB1 binding site open the door for exploring the ligand binding motifs involved in the activation of the CB1 receptor by its endogenous ligand, anandamide.

## Introduction

The main psychoactive ingredient of marijuana, (−)-$\Delta^9$-tetrahydrocannabinol, produces most of its pharmacological effects by interacting with cannabinoid receptors (CBs). To date, two subtypes of the CB receptor, CB1 and CB2, have been identified,[1−3] both belonging to the G-protein-coupled receptor (GPCR) superfamily, which characteristically has seven transmembrane-spanning helical domains connected by intervening intracellular and extracellular loops. An important development in cannabinoid research was the identification of *N*-arachidonoylethanolamine (anandamide, AEA) as an endogenous cannabinoid that was shown to possess biochemical and pharmacological profiles similar to those of cannabinoid agonists.[4−7] The endogenous cannabinoid system[8] is primarily responsible for all of the effects mediated by cannabinoids and is involved in the regulation of a wide variety of physiological functions such as antinociception, brain development, memory, retrograde neuronal communication, control of movement, cardiovascular and immune regulation, and cellular proliferation.[9] Small molecules capable of modulating the endocannabinoid system can be of potential value as therapeutic agents for the treatment of diverse pathologies,[10] including neurodegenerative disorders, nociceptive conditions, obesity, and malignant tumors.

The structure−activity relationship (SAR) of anandamide has been explored through the design and synthesis of a considerable number of anandamide analogues that helped to establish structural requirements for ligand−receptor interactions.[11−17] Many of these ligands exhibit superior binding affinities and potencies than anandamide. Notwithstanding the substantial SAR data, there is only sketchy direct information available regarding the binding motif of the endogenous ligands with the CB1 receptor. Such information is critical for understanding the structural requirements involved in ligand−receptor binding and is of great value in the design of novel, more selective, and more potent drug candidates.

The structural analysis of GPCRs is severely limited by the difficulties associated with their purification and crystallization. An alternative method currently pursued in our laboratory for obtaining such information is based on the identification of key amino acid residues associated with the receptor's binding domain.[18] This experimental approach involves the development of high affinity selective ligands capable of forming a covalent bond with an amino acid residue within the receptor binding site or immediately adjacent to it. The identity of the labeled amino acid residue is then revealed through purification of the covalently linked ligand−receptor complex, followed by digestion of the entire protein, and analysis of the individual peptide fragments using sequencing or mass spectral analysis.

Receptor covalent probes can be designed by incorporating potentially reactive groups into high affinity reversible prototypic ligands followed by suitable structural modifications, if necessary, to ensure that the novel ligands maintain a high affinity for the binding site. Over the past decade, our laboratory has been successful in designing a number of such covalent probes, both photoactivatable and electrophilic.[18−21] Photoactivatable ligands carry chemically less reactive groups, including azides, diazirines, and α-ketodiazo groups, which can be transformed into highly reactive nitrenes or carbenes upon UV irradiation.[22] The ensuing reactive intermediates are then capable of forming covalent bonds with amino acid residues situated in their immediate vicinity. Alternatively, electrophilic affinity ligands incorporate intrinsically reactive groups such as isothiocyanates, Michael acceptors, haloacetamides, aldol esters, or nitrogen mustards, which can react with an amino acid residue carrying a nucleophilic

* Corresponding author. Tel.: (617) 373-4200. Fax: (617) 373-7493. E-mail: a.makriyannis@neu.edu.

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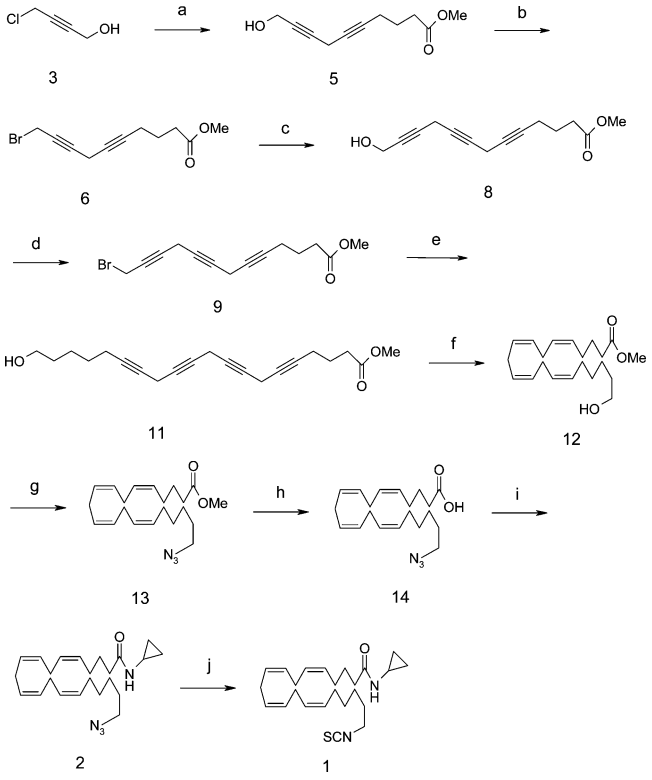
**Scheme 1**[a]



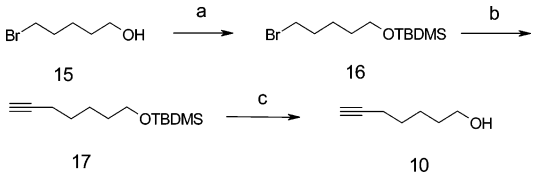

[a] Reagents and conditions: (a) hex-5-ynoic acid methyl ester (**4**), CuI, NaI, $K_2CO_3$, DMF, rt, 85%; (b) $PPh_3$, $CBr_4$, 0 °C, 85%; (c) prop-2-yn-1-ol (**7**), CuI, NaI, $K_2CO_3$, DMF, rt, 80%; (d) $PPh_3$, $CBr_4$, 0 °C, 82%; (e) hept-6-yn-1-ol (**10**), CuI, NaI, $K_2CO_3$, DMF, rt, 85%; (f) $H_2$, Lindlar's catalyst, $Et_2O$/THF, rt, 83%; (g) $Zn(N_3)_2$·2Py, DIAD, $PPh_3$, toluene, 0 °C to room temperature, 87%; (h) 1 M LiOH, THF, rt, 88%; (i) oxalyl chloride/DMF, cyclopropylamine, $CH_2Cl_2$, 0 °C to room temperature, 90%; and (j) $CS_2$, $PPh_3$, THF, 85%.

group, such as imidazolyl, sulfhydryl, amino, or hydroxyl groups, which are at or near the binding site.[23]

Here, we report the design, synthesis, and biochemical characterization of the first two high affinity covalent probes for the anandamide binding site on the CB1 receptor, 20-isothiocyanato-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**1**, AM3677) and 20-azido-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**2**, AM3661). The design of these ligands was based on the attachment of azido or isothiocyanato moieties to the terminal arachidonoyl carbon of (5*Z*,8*Z*,11*Z*,14*Z*)-eicosa-5,8,11,-14-tetraenoic acid cyclopropylamide (*N*-arachidonoylcyclopropylamine, ACPA), a high affinity and selective ligand for the CB1 receptor.[24] The availability of endocannabinoid covalent probes for the CB1 receptor should prove to be useful for obtaining direct information about the structural features involved in the activation of the CB1 receptor by its endogenous ligand, anandamide.

**Chemistry.** Both anandamide analogues, **1** and **2**, were synthesized starting with cross-coupling of the easily accessible 4-chlorobut-2-yn-1-ol (**3**),[25] which was prepared from commercially available but-2-yne-1,4-diol and hex-5-ynoic acid methyl ester (**4**) in the presence of copper(I) iodide (Scheme 1).[26] Subsequent bromination of 10-hydroxy-deca-5,8-diynoic acid methyl ester (**5**) with $CBr_4$ in the presence of $PPh_3$ gave 10-bromo-deca-5,8-diynoic acid methyl ester (**6**) in 85% yield. Coupling of

**Scheme 2**[a]




[a] Reagents and conditions: (a) TBDMSCl, imidazole, DMF, 0 °C to room temperature, 90%; (b) sodium acetylide, DMF, 0 °C to room temperature, 85%; and (c) TBAF, THF, rt, 85%.

**6** with prop-2-yn-1-ol (**7**) in the presence of CuI, NaI, and $K_2CO_3$ followed by bromination of **8** with $CBr_4$/$PPh_3$ afforded 13-bromo-trideca-5,8,11-triynoic acid methyl ester (**9**). Coupling of **9** with hept-6-yn-1-ol (**10**) under the same conditions described for **5** gave 20-hydroxy-eicosa-5,8,11,14-tetraynoic acid methyl ester (**11**). Hydrogenation of **11** using Lindlar's catalyst in the presence of quinoline gave (5*Z*,8*Z*,11*Z*,14*Z*)-20-hydroxy-eicosa-5,8,11,14-tetraenoic acid methyl ester (**12**) in 83% yield as the all cis product in high isomeric purity.[27] 20-Azido-eicosa-5,8,11,14-tetraenoic acid (**14**) was obtained by converting the hydroxyl group of **12** to an azido group using zinc azide under the Mitsunobu reaction[28] conditions followed by hydrolysis of the methyl ester of **13** with LiOH. The desired photoactivatable covalent probe (5*Z*,8*Z*,11*Z*,14*Z*)-20-azido-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**2**) was prepared from acid **14** via the corresponding acid chloride and cyclopropylamine in 90% yield. The electrophilic covalent probe, (5*Z*,8*Z*,11*Z*,-14*Z*)-20-isothiocyanato-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**1**), was obtained in 85% yield by treatment of azide **2** with $CS_2$/$PPh_3$ in THF.[29]

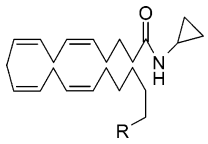
The key intermediate hept-6-yn-1-ol (**10**) was synthesized from 5-bromo-pentan-1-ol (**15**) (Scheme 2). Protection of the hydroxyl group of **15** followed by coupling with sodium acetylide gave 1-(*t*-butyldimethylsilyloxy)-hept-6-yne (**17**). Deprotection of **17** with TBAF in THF afforded **10** in 85% yield.

## Results and Discussion

**Receptor Binding Studies.** Affinity binding values for **1** and **2** to the CB1 and CB2 receptors were determined according to previously reported procedures.[1,13,15] For the CB1 receptor, binding data were obtained using a rat brain membrane in the presence of phenylmethanesulfonyl fluoride (PMSF),[30,31] a general serine protease inhibitor to protect the tested analogues from the hydrolytic activity of fatty acid amide hydrolase.[14,31−33] CB2 data were obtained using a membrane preparation from mouse spleen known to be rich in CB2. [$^3$H]CP-55,940 was chosen as a competing ligand for the assays as it has high affinity for both CB1 and CB2 receptors and is nonselective. It is one of the most widely used radioligands for characterizing both CB1 and CB2 cannabinoid receptors.[34] SAR, mutation, and computer modeling study results indicate that nonclassical cannbinoids (e.g., CP-55940), classical cannabinoids, and anandamide share some binding motifs, while aminoalkylindole cannabinoids (e.g., WIN55212-2) might have a different binding site.[35]

The affinities of **1** and **2** for the CB1 and CB2 receptors are summarized in Table 1, where we also include data for *N*-arachidonoylcyclopropylamine (ACPA), the parent compound, for comparison. The $K_i$ values of

**Table 1.** Affinity ($K_i$)[a] of **1** and **2** for CB1 and CB2 Receptors (95% Confidence Limits)



| compound | R | CB1 ($K_i$, nM) (PMSF) | CB2 ($K_i$, nM) |
|---|---|---|---|
| **1** | -NCS | 1.3 ± 0.2 | 48.5 ± 13 |
| **2** | -N3 | 0.9 ± 0.2 | 57.6 ± 19 |
| **ACPA** | -H | 2.7 ± 0.4 (2.2)[b] | 157 ± 37 (715)[b] |

[a] CB1 affinities were determined using rat brain membranes and 0.76 nM [$^3$H]CP-55,940 as the radioligand following previously described procedures.[1,13,15] Mouse spleen was used as the source of CB2 receptor. Data were analyzed using nonlinear regression analysis. $K_i$ values were obtained from a minimum of two independent experiments run in duplicate and are expressed as the mean ± standard error. [b] Data from ref 24. ACPA: *N*-arachidonoylcyclopropylamine

**1** and **2** for the CB1 receptor in the presence of PMSF are 1.3 and 0.9 nM, respectively, both ligands exhibiting higher CB1 affinities than ACPA. Our results indicate that substitution of the terminal C-20 carbon with an azido or isothiocyanato group leads to enhanced binding affinities for the CB1 receptor, while both **1** and **2** exhibited over 50-fold selectivities for CB1 versus CB2.

**Irreversible Binding Studies.** The two novel affinity ligands (**1** and **2**) were subsequently evaluated for their abilities to irreversibly label the CB1 receptor through formation of a covalent bond following previously reported procedures.[19−21] To minimize potential hydrolysis of the ligands by endogenous FAAH, the membranes were pretreated with PMSF before the labeling experiments, and excess PMSF was subsequently removed by extensive washing.

**Reaction of Electrophilic Ligand 1 with CB1.** As depicted in Figure 1, equilibration of rat forebrain membranes for 1 h with 3.9 nM **1** (3 times its $K_i$ value of 1.3 nM), followed by the removal of excess unbound ligand, lowered the [$^3$H]CP-55940 specific binding to the CB1 receptor by 23%. When the membranes were equilibrated with 13 and 26 nM **1** (10 and 20 times its $K_i$ value), the [$^3$H]CP-55940 specific binding was lowered by 32 and 58%, respectively. In the control experiments, the membranes were treated according to the same procedures but in the absence of the electrophilic affinity ligand **1** and fully retained their ability to bind the [$^3$H]-CP-55940.

To better understand the receptor labeling characteristics of **1**, we carried out a time-dependent study using a 3.9 nM concentration of **1** and varied the incubation time (Figure 2). Our studies showed that with 15 min of incubation, 15% of the receptor sites was occupied irreversibly. By increasing the incubation time to 30 min, covalent binding increased to 25% with no further increase seen if the incubation time was extended to 60 min. Thus, at the ligand concentration used, covalent binding had reached its plateau at 30 min incubation time. The control experiment was a 60 min incubation with no added **1**. However, we note that the true kinetic behavior of the covalent reaction between **1** and the cannabinoid receptor is not necessarily reflected by this time-dependent receptor labeling since the incubation time does not represent the precise reaction time. According to our experimental conditions,

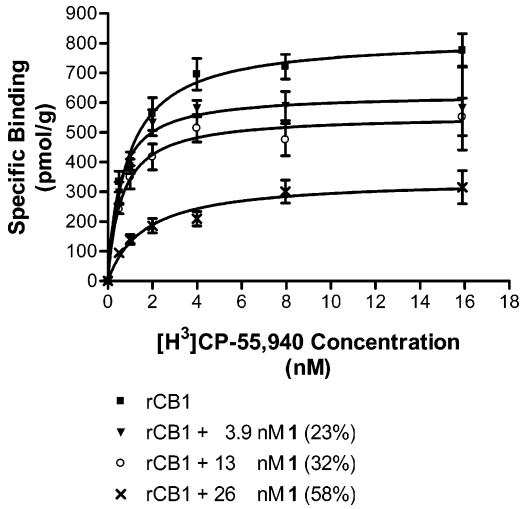



**Figure 1.** 20-Isothiocyanato-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**1**) in rat forebrain membranes inhibits the specific binding of [$^3$H]CP-55940 to the cannabinoid receptor (CB1). The membranes were pretreated with 0.15 mM PMSF for 10 min on ice twice and then were washed 3 times with 0.1% BSA in TME to remove excess PMSF. Heterologous displacements are shown for membranes preequilibrated for 1 h in the absence or presence of 3.9, 13, and 26 nM isothiocyanate **1**. The [$^3$H]CP-55,940 specific bindings were determined by the reported saturation binding method,[36] and the reductions in binding as percentages of control are given in parentheses.

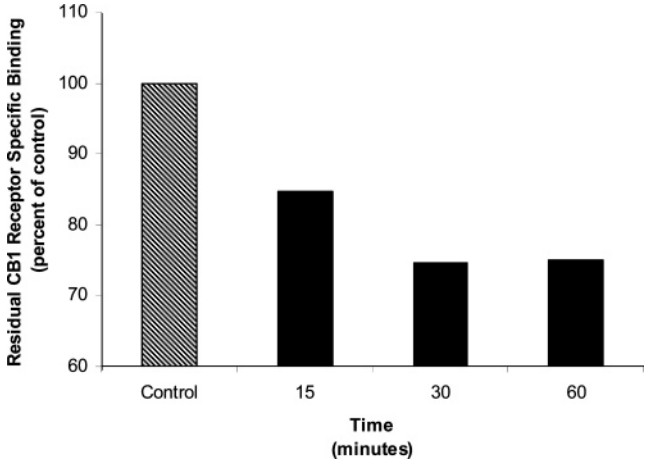



**Figure 2.** Time course for cannabinoid receptor labeling with 20-isothiocyanato-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**1**). The membranes were pretreated with 0.15 mM PMSF for 10 min on ice twice and then were washed 3 times with 0.1% BSA in TME to remove excess PMSF. Then, rat forebrain membranes were preincubated with 3.9 nM isothiocyanate **1** for 15, 30, and 60 min. Membranes in the control experiment were preincubated for 60 min without isothiocyanate **1**. The [$^3$H]CP-55,940 specific bindings were determined by the reported saturation binding method.[36]

the termination of the ligand−receptor covalent reaction is accomplished by centrifugation. Unreacted ligand was removed by repeatedly pelleting and washing the membrane preparation after the incubation period was completed. The observation that full receptor labeling is not observed at lower concentrations, even if the incubation period is increased, is probably a reflection of the slow inactivation of the isothiocyanato group of the probe through nonspecific reactions. In this regard, the isothiocyanate analogue **1** was found to be stable

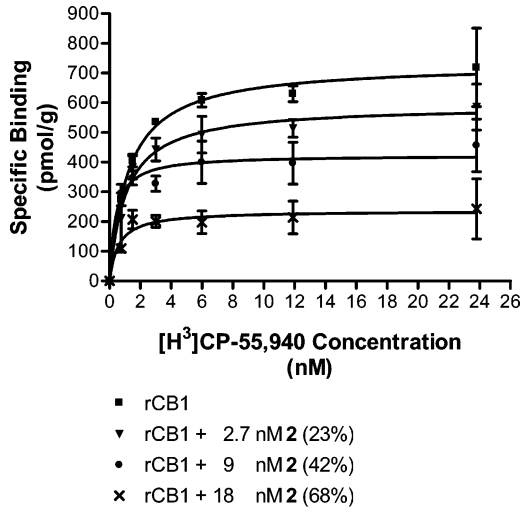



**Figure 3.** Photoirradiation of 20-azido-eicosa-5,8,11,14-tetraenoic acid cyclopropylamide (**2**) in rat forebrain membranes inhibits the specific binding of [$^3$H]CP-55940 to the cannabinoid receptor (CB1). The membranes were pretreated with 0.15 mM PMSF for 10 min on ice twice and then were washed 3 times with 0.1% BSA in TME to remove the excess PMSF. Heterologous displacements are shown for membranes irradiated in the absence or presence of 2.7, 9, and 18 nM azide **2**. The [$^3$H]CP-55,940 specific bindings were determined by the reported saturation binding method,[36] and the reductions in binding as percentages of control are given in parentheses.

when tested under the buffer conditions utilized in our experiments. We thus speculate that its slow inactivation is due to other nucleophiles (e.g., cysteines) present in the membrane preparation. Notwithstanding these experimental limitations, our data clearly demonstrate that time-dependent receptor labeling does occur.

**Reaction of Photoactivatable Ligand 2 with CB1.** Photolabeling experiments with the azidoendocannabinoid **2** initially followed similar procedures as with **1** (Figure 3). Rat forebrain membranes were equilibrated with **2** at a concentration that is 3 times its $K_i$ value for 1 h. After equilibration, membranes were centrifuged and resuspended in ice-cold TME buffer. Then, membranes were photoirradiated with ultraviolet light (254 nm), washed extensively to remove unbound **2**, and then tested for cannabinoid receptor binding. As shown in Figure 3, 23% of the CB1 receptor was irreversibly labeled by **2**. In control experiments, membranes were irradiated in the absence of **2**. Furthermore, using [$^3$H]CP-55,940 to determine the fraction of unoccupied CB1 receptor demonstrated that the equilibration of the membrane preparation with higher concentrations of **2** led to progressively larger percentages of CB1 within the membrane preparation being irreversibly labeled. Thus, when **2** was used at concentrations of 9 and 18 nM, the covalent receptor occupancy was 42 and 68%, respectively (Figure 3). These experiments demonstrate that the azido analogue **2** can be used as an efficient and high-yield photoaffinity probe for covalent labeling of the CB1 cannabinoid receptor.

## Conclusions

Novel anandamide analogues **1** and **2**, which can be used as covalent probes for the CB1 receptor, were designed and synthesized. Our data demonstrate that the isothiocyanato or azido groups, when placed at the end of the hydrophobic tail of an anandamide analogue, led to high affinity ligands capable of covalently binding to the receptor. We plan to utilize probes **1** and **2** in more detailed experiments aimed at identifying and characterizing the endocannabinoid binding domain within the CB1 receptor. Such information will assist in the design of improved cannabinoid receptor probes and enhance our understanding of the structural features involved in CB1 activation.

## Experimental Procedures

**General.** Unless otherwise noted, starting materials were obtained from commercial suppliers and were used without further purification. All final compounds were oils. Tetraenoic compounds were very oxygen sensitive. Final compounds were shown to be homogeneous by HPLC employing two diverse elution solvent mixtures. HPLC experiment A was performed using a Waters 510 single pump chromatograph with a Waters 2487 dual $\lambda$ absorbance detector, UV detection at 254 nm, normal phase Altech 5 $\mu$ silica, 4.6 mm $\times$ 25 cm column, eluting with ethyl acetate at 0.75 mL/min. HPLC experiment B was performed on a Waters 717 plus autosampler dual pump chromatograph with a Waters 2487 dual $\lambda$ absorbance detector, UV detection at 204 nm, reversed-phase Ultrasphere ODS, 4.6 mm $\times$ 4.5 cm column, eluting with acetonitrile/8.5% aqueous phosphoric acid, 70:30 at 1.0 mL/min. HPLC experiment C was performed on a Beckman 126 solvent module dual pump chromatograph with a model 168 detector, UV detection at 220 nm, normal phase Phenosphere 5 $\mu$m silica, 10 mm $\times$ 25 cm column, eluting with 2-propanol/hexane, 5:95 at 2.0 mL/min. The final products were fully characterized by high-resolution $^1$H NMR, IR, and HRMS. $^1$H NMR spectra were recorded on either a Bruker DRX 400 or 500 MHz spectrometer using $CDCl_3$ as the solvent with TMS as the internal standard. All chemical shifts are reported in ppm. Multiplicities of the signals are indicated as follows: s for singlet, d for doublet, t for triplet, q for quartet, p (pentet) for quintet, m for multiplet, and any combinations as appropriate. IR spectra were performed on a Perkin-Elmer Spectrum One FT-IR spectrometer. High-resolution mass spectra (HRMS) for all final compounds were obtained on a ZAB-SE mass spectrometer and performed by the School of Chemical Sciences, University of Illinois at Urbana-Champaign, Urbana, IL. TME buffer contains 25 mM Tris base, 5 mM $MgCl_2$, and 1 mM EDTA, and was adjusted to pH 7.4 with HCl. All solvent ratios are by volume.

**10-Hydroxy-deca-5,8-diynoic Acid Methyl Ester (5).** 4-Chloro-but-2-ny-1-ol (**3**)[25] (3.0 g, 28.7 mmol) and hex-5-ynoic acid methyl ester (**4**) (3.62 g, 28.7 mmol) were added to a suspension of CuI (11.0 g, 57.4 mmol), NaI (8.61 g, 57.4 mmol), and $K_2CO_3$ (5.94 g, 43 mmol) in 10 mL of anhydrous DMF under Ar. The mixture was stirred overnight at room temperature and then quenched with saturated aqueous $NH_4Cl$, and the lipophilic products were extracted with $Et_2O$. The combined organic extracts were washed with water and brine, and dried with $Na_2SO_4$. After rotary evaporation of solvents, the residue was chromatographed on silica gel to afford **5** (4.73 g, 85%) as an oil: $R_f$ = 0.51 (petroleum ether/$Et_2O$, 1:2). $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 4.26 (t, $J$ = 2.3 Hz, 2H), 3.68 (s, 3H), 3.18 (p, $J$ = 2.3 Hz, 2H), 2.42 (t, $J$ = 7.5 Hz, 2H), 2.25 (tt, $J$ = 6.9, 2.3 Hz, 2H), 1.81 (p, $J$ = 7.2 Hz, 2H).

**10-Bromo-deca-5,8-diynoic Acid Methyl Ester (6).** A solution of $PPh_3$ (7.03 g, 26.8 mmol) in 15 mL of dry $CH_2Cl_2$ was added dropwise to a stirred solution of **5** (4.73 g, 24.4 mmol) and $CBr_4$ (8.9 g, 26.8 mmol) in 15 mL of dry $CH_2Cl_2$ at 0 °C. Then, the mixture was stirred for another 1.5 h at 0 °C. The solvent was evaporated, and the residue was diluted with $Et_2O$ and filtered through a short pad of Celite. The filtrate was concentrated and then chromatographed on silica gel to provide **6** (5.33 g, 85%) as an oil: $R_f$ = 0.41 (petroleum ether/$Et_2O$, 5:1). $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 3.69 (t, $J$ = 2.3 Hz, 2H), 3.68 (s, 3H), 3.20 (p, $J$ = 2.3 Hz, 2H), 2.42 (t, $J$ = 7.5 Hz, 2H), 2.24 (tt, $J$ = 6.9, 2.3 Hz, 2H), 1.82 (p, $J$ = 7.2 Hz, 2H).

**13-Hydroxy-trideca-5,8,11-triynoic Acid Methyl Ester (8).** Coupling of **6** (5.33 g, 20.7 mmol) with prop-2-yn-1-ol (**7**) (1.4 g, 24.9 mmol) following the procedure described for compound **5** gave 13-hydroxy-trideca-5,8,11-triynoic acid methyl ester (**8**) (3.84 g, 80%) as a pale yellow oil: $R_f$ = 0.43 (petroleum ether/$Et_2O$, 1:1.5). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 4.25 (t, $J$ = 2.3 Hz, 2H), 3.68 (s, 3H), 3.23 (p, $J$ = 2.3 Hz, 2H), 3.12 (p, $J$ = 2.3 Hz, 2H), 2.43 (t, $J$ = 7.5 Hz, 2H), 2.24 (tt, $J$ = 6.9, 2.3 Hz, 2H), 1.82 (p, $J$ = 7.2 Hz, 2H).

**13-Bromo-trideca-5,8,11-triynoic Acid Methyl Ester (9).** The bromide **9** was prepared from alcohol **8** (3.8 g, 16.6 mmol), $CBr_4$ (6.06 g, 18.3 mmol), and $PPh_3$ (4.8 g, 18.3 mmol) following the procedure described for **6** to give **9** (4.02 g, 82%) as a pale yellow oil: $R_f$ = 0.33 (petroleum ether/$Et_2O$, 5:1). $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 3.91 (t, $J$ = 2.3 Hz, 2H), 3.68 (s, 3H), 3.23 (p, $J$ = 2.3 Hz, 2H), 3.14 (p, $J$ = 2.3 Hz, 2H), 2.43 (t, $J$ = 7.4 Hz, 2H), 2.25 (tt, $J$ = 6.9, 2.3 Hz, 2H), 1.84 (p, $J$ = 7.2 Hz, 2H).

**20-Hydroxy-eicosa-5,8,11,14-tetraynoic Acid Methyl Ester (11).** The coupling of **9** (4.02 g, 13.6 mmol) with hept-6-yn-1-ol (**10**) (1.83 g, 16.3 mmol) following the procedure described for **5** gave **11** (3.78 g, 85%) as a pale yellow oil: $R_f$ = 0.35 (petroleum ether/acetone, 2:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 3.68 (s, 3H), 3.65 (t, $J$ = 6.1 Hz, 2H), 3.14 (m, 6H), 2.43 (t, $J$ = 7.5 Hz, 2H), 2.24 (m, 2H), 2.17 (m, 4H), 1.83 (p, $J$ = 7.2 Hz, 2H), 1.58 (p, $J$ = 7.2 Hz, 2H), 1.54 (p, $J$ = 7.2 Hz, 2H), 1.45 (p, $J$ = 7.2 Hz, 2H).

**(5*Z*,8*Z*,11*Z*,14*Z*)-20-Hydroxy-eicosa-5,8,11,14-tetraenoic Acid Methyl Ester (12).** To a 250 mL flask containing 5.67 g of Lindlar's catalyst was added 20 mL of $Et_2O$. The mixture was saturated with $H_2$ at room temperature, a solution of **11** (3.78 g, 11.6 mmol) and quinoline (200 mg) in 15 mL of THF was added, and a stream of $H_2$ was bubbled through the mixture. The reaction was monitored by $^1H$ NMR. After the hydrogenation was finished, the mixture was filtered, washed with 2 N HCl and brine, and dried with $Na_2SO_4$. The solvent was evaporated, and the residue was chromatographed on silica gel to give **12** (3.21 g, 83%) as a colorless oil: $R_f$ = 0.34 (petroleum ether/acetone, 5:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 5.38 (m, 8H), 3.67 (s, 3H), 3.63 (t, $J$ = 6.1 Hz, 2H), 2.84 (m, 6H), 2.33 (t, $J$ = 7.5 Hz, 2H), 2.10 (m, 4H), 1.71 (p, $J$ = 7.2 Hz, 2H), 1.58 (p, $J$ = 6.8 Hz, 2H), 1.39 (m, 4H).

**(5*Z*,8*Z*,11*Z*,14*Z*)-20-Azido-eicosa-5,8,11,14-tetraenoic Acid Methyl Ester (13).** A zinc azide/bis-pyridine complex[28] (2.36 g, 7.7 mmol) was suspended in a solution of **12** (3.22 g, 9.6 mmol) and $PPh_3$ (5.03 g, 19.2 mmol) in 20 mL of anhydrous toluene. To this stirred mixture at room temperature was added diisopropyl azodicarboxylate (3.88 g, 19.2 mmol) dropwise. Stirring was continued until complete consumption (TLC monitoring) of **12** was observed. The heterogeneous mixture was filtered through a Celite pad, concentrated, and purified by column chromatography to afford pure **13** (3.0 g, 87%) as a colorless oil: $R_f$ = 0.48 (petroleum ether/$Et_2O$, 10:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 5.41 (m, 8H), 3.67 (s, 3H), 3.26 (t, $J$ = 7.5 Hz, 2H), 2.82 (m, 6H), 2.32 (t, $J$ = 7.5 Hz, 2H), 2.10 (m, 4H), 1.71 (p, $J$ = 7.4 Hz, 2H), 1.60 (p, $J$ = 6.9 Hz, 2H), 1.39 (m, 4H); IR (neat) $cm^{-1}$ 2094, 1737.

**(5*Z*,8*Z*,11*Z*,14*Z*)-20-Azido-eicosa-5,8,11,14-tetraenoic Acid (14).** To a solution of **13** (1.0 g, 2.8 mmol) in 10 mL of THF was added 1 M LiOH (5.6 mL) at room temperature under Ar. Stirring was continued for 48 h, and then the reaction mixture was acidified with 2 N HCl to pH 5.5, and lipophilic products were extracted with $Et_2O$. The combined organic extracts were washed with water and brine, and dried with $Na_2SO_4$. The solvent was evaporated to give a yellowish oily residue. Chromatography on silica gel gave **14** (0.85 g, 88% yield) as a colorless oil: $R_f$ = 0.39 (petroleum ether/$Et_2O$, 3:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 5.40 (m, 8H), 3.26 (t, $J$ = 7.5 Hz, 2H), 2.84 (m, 6H), 2.37 (t, $J$ = 7.5 Hz, 2H), 2.15 (q, $J$ = 7.0 Hz, 2H), 2.07 (q, $J$ = 7.0 Hz, 2H), 1.72 (p, $J$ = 7.4 Hz, 2H), 1.62 (p, $J$ = 6.9 Hz, 2H), 1.39 (m, 4H); IR (neat) $cm^{-1}$ 2092, 1706.

**5-Bromo-1-(*t*-butyldimethylsilyloxy)-pentane (16).** To a solution of 5-bromo-pentan-1-ol (**15**) (2.0 g, 12 mmol) and imidazole (1.23 g, 18 mmol) in 10 mL of DMF was added 1 M TBDMSCl in THF (15.6 mL, 15.6 mmol) at 0 °C under Ar. Stirring was continued at room temperature overnight. The reaction mixture was diluted with $Et_2O$ and filtered through a short pad of Celite. The filtrate was washed with 1 N HCl, water, and brine, dried with $Na_2SO_4$, and concentrated. The residue was purified by column chromatography to afford **16** (3.03 g, 90%) as a colorless oil: $R_f$ = 0.43 (petroleum ether/$Et_2O$, 50:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 3.61 (t, $J$ = 6.1 Hz, 2H), 3.41 (t, $J$ = 6.7 Hz, 2H), 1.88 (p, $J$ = 6.9 Hz, 2H), 1.53 (m, 4H), 0.89 (s, 9H), 0.05 (s, 6H).

**1-(*t*-Butyldimethylsilyloxy)-hept-6-yne (17).** To a solution of **16** (3.03 g, 10.8 mmol) in 10 mL of DMF was added an 18 wt % slurry of sodium acetylide (4.32 g, 16.2 mmol) in xylene/light mineral oil at 0 °C under Ar. Stirring was continued at room temperature overnight. The reaction mixture was diluted with $Et_2O$ and quenched with water. Then, the reaction mixture was washed with 1 N HCl, water, and brine, dried with $Na_2SO_4$, and concentrated. Chromatography on silica gel gave **17** (2.07 g, 85%) as a colorless oil: $R_f$ = 0.27 (petroleum ether/$Et_2O$, 60:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 3.61 (t, $J$ = 6.3 Hz, 2H), 2.20 (td, $J$ = 7.0, 2.6 Hz, 2H), 1.93 (t, $J$ = 2.6 Hz, 1H), 1.53 (m, 4H), 1.45 (m, 2H), 0.89 (s, 9H), 0.05 (s, 6H).

**Hept-6-yn-1-ol (10).** To a solution of **17** (2.07 g, 9.18 mmol) in 10 mL of THF was added 1 M TBAF in THF (18.4 mL, 18.4 mmol). Stirring was continued for 2 h. The reaction was quenched with MeOH, washed with water and brine, dried with $Na_2SO_4$, and concentrated. The residue was purified by column chromatography to give **10** (0.87 g, 85%) as a colorless oil: $R_f$ = 0.33 (petroleum ether/ethyl acetate, 3:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 3.66 (t, $J$ = 6.3 Hz, 2H), 2.21 (td, $J$ = 6.9, 2.6 Hz, 2H), 1.93 (t, $J$ = 2.6 Hz, 1H), 1.56 (m, 4H), 1.48 (m, 2H), 1.34 (br, 1H).

**(5*Z*,8*Z*,11*Z*,14*Z*)-20-Azido-eicosa-5,8,11,14-tetraenoic Acid Cyclopropylamide (2).** To a solution of acid **14** (0.10 g, 0.29 mmol) and DMF (0.02 mL) in 5 mL of $CH_2Cl_2$ at 0 °C was added 2 M oxalyl chloride in $CH_2Cl_2$ (0.29 mL, 0.58 mmol) in a dropwise manner. The reaction mixture was stirred further at 0 °C for an additional 1.5 h. Then cyclopropylamine (166 mg, 2.9 mmol) was added to the reaction mixture very slowly at 0 °C. Stirring was continued at room temperature overnight. $Et_2O$ was added, and the mixture was washed with water and brine, and dried over $Na_2SO_4$. The solvent was removed to give crude product. Chromatography on silica gel gave pure **2** (100 mg, 90%) as an oil: $R_f$ = 0.37 (petroleum ether/acetone, 3:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 5.62 (br, 1H), 5.39 (m, 8H), 3.26 (t, $J$ = 7.5 Hz, 2H), 2.84 (m, 6H), 2.70 (m, 1H), 2.11 (m, 6H), 1.70 (p, $J$ = 7.4 Hz, 2H), 1.61 (p, $J$ = 6.8 Hz, 2H), 1.40 (m, 4H), 0.76 (m, 2H), 0.47 (m, 2H); IR (neat) $cm^{-1}$ 2092, 1643. HRMS for $C_{23}H_{37}N_4O$ ($MH^+$) 385.2966. Calcd. 385.2967. HPLC A, 5.85 min (99%); HPLC B, 3.15 min (99%).

**(5*Z*,8*Z*,11*Z*,14*Z*)-20-Isothiocyanato-eicosa-5,8,11,14-tetraenoic Acid Cyclopropylamide (1).** To a solution of **2** (50 mg, 0.13 mmol) in 2 mL of anhydrous THF was added $PPh_3$ (52 mg, 0.2 mmol) in a single portion at room temperature. Then, carbon disulfide (99 mg, 1.3 mmol) was added. The reaction mixture was stirred at room temperature for 48 h and was then concentrated. The residue was purified by column chromatography to give pure **1** (44 mg, 85%) as an oil: $R_f$ = 0.32 (petroleum ether/$Et_2O$, 4:1). $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 5.58 (br s, 1H), 5.40 (m, 8H), 3.51 (t, $J$ = 7.5 Hz, 2H), 2.81 (m, 6H), 2.70 (m, 1H), 2.10 (m, 6H), 1.72 (m, 4H), 1.42 (m, 4H), 0.77 (m, 2H), 0.47 (m, 2H); IR (neat) $cm^{-1}$ 2089, 1643. HRMS for $C_{24}H_{37}N_2OS$ ($MH^+$) 401.2628. Calcd. 401.2627. HPLC B, 2.83 min (98%); HPLC C, 8.09 min (97%).

**Radioligand Binding Assay.** For CB1, rat forebrain membranes were prepared according to the procedure of Dodd et al.[36] The binding of the novel anandamide analogues to the cannabinoid receptor was assessed as previously described,[1,13,15] except that the membranes were pretreated with PMSF. Membranes, previously frozen at −80 °C, were thawed on ice. To the stirred suspension was added 3 volumes of 25 mM Tris-HCl buffer, 5 mM $MgCl_2$, and 1 mM EDTA, pH 7.4 (TME

buffer) containing 150 $\mu$M PMSF (made fresh in 2-propanol as a 100 mM stock). The suspension was incubated at 4 °C, after 15 min, a second addition of PMSF stock brought the concentration to 300 $\mu$M PMSF, and then the mixture was incubated for another 15 min. At the end of the second 15 min incubation, the membranes were pelleted and washed 3 times with TME to remove unreacted PMSF. The pretreated membranes were subsequently used in the binding assay described next. Approximately 30 $\mu$g of PMSF-treated membranes was incubated in a silanized 96-well microtiter plate with TME containing 0.1% essentially fatty acid-free bovine serum albumin (BSA), 0.76 nM [$^3$H]CP-55,940, and various concentrations of anandamide analogues in a final volume of 200 $\mu$L. The binding assay was performed at 30 °C for 1 h with gentle agitation. The resultant material was transferred to Unifilter GF/B filter plates, and unbound ligand was removed using a Packard Filtermate-96 Cell Harvester (Perkin-Elmer Packard, Shelton, CT). Filter plates were washed 4 times with ice-cold wash buffer (50 mM Tris, 5 mM $MgCl_2$ containing 0.5% BSA, pH 7.4). Radioactivity was determined using a Packard TopCount. Data collected from three independent experiments performed with duplicate determinations were normalized between 100 and 0% specific binding for [$^3$H]CP-55,940, determined using buffer and 100 nM CP-55,940, respectively. The normalized data were analyzed using nonlinear regression to yield $IC_{50}$ values. Data from at least two independent experiments performed in duplicate were used to calculate $IC_{50}$ values that were converted to $K_i$ values using the assumptions of Cheng and Prusoff.[37]

For CB2 receptor binding studies, membranes were prepared from frozen mouse spleen essentially according to the procedure of Dodd et al.[36] Silanized centrifuge tubes were used throughout to minimize receptor loss due to absorption. The CB2 binding assay was conducted in the same manner as for CB1.

**Photoaffinity Covalent Labeling.** Rat brain synaptosomal membranes (CB1) were prepared following the previously described procedures with modification.[19] The membranes were pretreated with 0.15 mM PMSF for 10 min on ice twice and then washed 3 times with 0.1% BSA in TME to remove the excess PMSF. The pretreated membranes were incubated with various concentrations of azido ligand **2** at 30 °C for 1 h in the dark, followed by centrifugation at 27200*g* for 4 min. The pellets were resuspended in ice-cold TME containing 0.1% BSA and exposed to UV (254 nm) to activate the ligand. Unbound excess azide **2** was washed out with 1% BSA in TME 3 times by centrifugation, followed by three washes with TME without BSA.

**Electrophilic Covalent Labeling.** Rat brain synaptosomal membranes (CB1) were pretreated with PMSF as described previously and incubated with various concentrations of isothiocyanate ligand **1** at 30 °C for 1 h. Unbound excess **1** was washed out with 1% BSA in TME 3 times by centrifugation, followed by three washes with TME.

**Saturation Binding Assay.** Protein concentrations were determined by using a Bio-Rad protein assay kit. Saturation binding assays were performed in a 96-well format. Membrane pellets were resuspended in TME containing 0.1% BSA (TME-BSA). A total of 25 $\mu$g of protein was added to each assay well. [$^3$H]CP-55,940 was diluted in TME-BSA to yield ligand concentrations ranging from 0.5 to 23.8 nM. Nonspecific binding was assayed in the presence of 4 $\mu$M unlabeled CP-55,940. The assay was performed at 30 °C for 1 h with gentle agitation. The resultant material was transferred to Unifilter GF/B filter plates, and unbound ligand was removed using a Packard Filtermate-96 Cell Harvester (Perkin-Elmer Packard). Filter plates were washed 4 times with ice-cold wash buffer (50 mM Tris, 5 mM $MgCl_2$ containing 0.5% BSA, pH 7.4). Bound radioactivity was quantitated in a Packard TopCount Scintillation Counter. Nonspecific binding was subtracted from the total bound radioactivity to calculate specific binding of [$^3$H]-CP-55,940 (represented as pmol/g protein). All assays were done in triplicate, and data points were presented as the mean. $B_{max}$ and $K_D$ values were calculated by nonlinear regression using GraphPad Prism version 3.03 (one site binding analysis equation $Y = B_{max}X/(K_d + X)$; GraphPad Software, San Diego, CA) on a Windows platform.

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