Head Group Analogs of Arachidonylethanolamide, the Endogenous Cannabinoid Ligand

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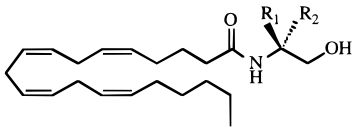
Several analogs of an endogenous cannabimimetic, arachidonylethanolamide (anandamide), were synthesized to study the structural requirements of the ethanolamide head group. CB1 receptor affinities of the analogs were evaluated by a standard receptor binding assay using tritiated CP-55,940 as the radioligand and compared to anandamide which was shown to have a K_i of 78 nM. Replacement of the amide carbonyl oxygen by a sulfur atom had a detrimental effect on the CB1 affinity. The thio analogs of both anandamide and (*R*)-methanandamide showed very weak affinity for CB1. The secondary nature of the amidic nitrogen was also shown to be important for affinity, indicating a possible hydrogen-bonding interaction between the amide NH and the receptor. Introduction of a phenolic moiety in the head group resulted in the loss of receptor affinity except when a methylene spacer was introduced between the amidic nitrogen and the phenol. A select group of analogs were also tested for their affinity for the CB2 receptor using a mouse spleen preparation and were found to possess low affinities for the CB2 sites. Notably, anandamide and (*R*)-methanandamide demonstrated high selectivity for the CB1 receptor. Overall, the data presented here show that structural requirements of the head group of anandamide are rather stringent.

Introduction

An endogenous ligand for the cannabinoid receptor, arachidonylethanolamide (anandamide), has been shown to have biochemical and pharmacological profiles similar to those of Δ^9-THC. It was thus shown to bind competitively to the cannabinoid receptor (CB1),[1,2] inhibit calcium currents as a partial agonist in N18 neuroblastoma cells,[3] inhibit forskolin-stimulated adenylase cyclase activity,[4] and electrically evoked twitch response of the mouse *vas deferens*.[5,6] Anandamide was also shown[7−10] to produce the four characteristic *in vivo* effects of cannabimimetics, namely, analgesia, hypothermia, hypoactivity, and catalepsy. In general, anandamide was found to be somewhat less potent than Δ^9-THC in these and other[11] tests. Despite having a rapid onset of action, the magnitude and duration of action of andanamide was relatively short, presumably because of its rapid hydrolysis by an amidohydrolase, anandamide amidase.[12,13] This amidase has been shown to be sensitive to serine protease inhibitors (e.g., phenylmethanesulfonyl fluoride, PMSF), and these are generally included in structure−activity relationship (SAR) studies where amidase-catalyzed anandamide hydrolysis might be a complicating factor.[14]

Our initial efforts in designing metabolically stable anandamide analogs culminated in the synthesis of (*R*)-methanandamide (**2**).[15] (*R*)-Methanandamide showed approximately 4-fold higher binding affinity for CB1 than did anandamide in the presence of PMSF. In the absence of PMSF the affinity of (*R*)-methanandamide was virtually unchanged, while anandamide showed a 30-fold decrease in affinity. Other structure−activity relationships of anandamide analogs have been recently reported. Pinto et al.[16] found that an analog containing the 3-hydroxypropyl amide head group had marginally higher affinity than that of anandamide for the CB1 receptor. The authors also found that the arachidonyl-*n*-propyl amide, which has a methyl group replacing the hydroxyl group of anandamide, has approximately 3-fold higher receptor affinity than anandamide. The ester analogs (methyl, ethyl, propyl, etc.) of arachidonic acid were shown to have little or no affinity for the CB1 receptor. Adams and co-workers[17,18] found that substitution of the anandamide hydroxyl group for a fluoro group gave a high-affinity anandamide analog. Also, introduction of a methyl group in a position α to the carbonyl gave analogs that were not only metabolically stable but also had high affinity.[17,18]

In this paper, we report the synthesis and receptor binding affinity of several different analogs of anandamide to further elucidate the structure activity relationships of the ethanolamide head group. The CB1 receptor binding affinities of these analogs were determined using rat forebrain membrane preparations with [^{3}H]-CP-55,940 as the radioligand.



1: $R_1=R_2=H$ Anandamide
2: $R_1=H$; $R_2=CH_3$ *(R)*-Methanandamide

Since this study was first initiated, a second cannabinoid receptor (CB2) was discovered[19,20] and shown to be present exclusively outside the central nervous system. The SAR of this receptor appears to differ from that of CB1.[20,21] For this purpose we also examined a

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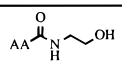
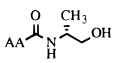
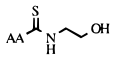
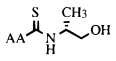
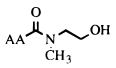
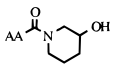
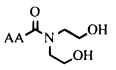
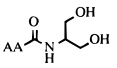
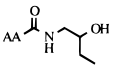
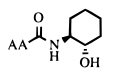
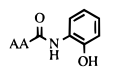
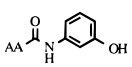
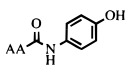
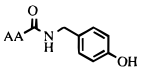
Table 1. Affinities (K_i) of Anandamide and Its Congeners for the Brain Cannabinoid Receptor[a]

Compound	Structure	K_i, nM[b]
anandamide (**1**)		78±2 (1926)
2		20±1.6 (815)
3		1380±140 (6300)
4		2200±195
5		2460±160
6		1690±128
7		174±8
8		363±20
9		203±5
10		>3000
11		1660±111 (2100)
12		1560±120 (3100)
13		1760±142 (1.3×10^4)
14		217±3 (1.1×10^4)

[a] Affinity of anandamide and its analogs for the cannabinoid receptor was determined using rat brain membranes and [^{3}H]CP 55,940 as previously described.[15] K_i values were obtained from three independent experiments run in duplicate and are expressed as the mean ± standard error. [b] K_i values in parentheses are for the mouse spleen CB2 receptor.

select group of the above analogs for their CB2 receptor affinities.

Chemistry

The head group analogs of anandamide presented in Table 1 were synthesized from arachidonic acid chloride[15] by treatment with the appropriate, commercially available amino alcohols (or aminophenols). In the case of analog **14**, the requisite 4-hydroxybenzylamine was prepared by reduction of the THP ether of commercially available 4-cyanophenol followed by reaction of the resulting amine with arachidonic acid chloride. Deprotection of the hydroxyl group under acidic conditions afforded **14**. Thioanandamide (**3**) and (*R*)-thiomethanandamide (**4**) were prepared from anandamide and methanadamide, respectively, using Lawesson's reagent.[22]

Receptor Binding Studies

Prior to assaying the newly synthesized analogs for CB1 affinity, rat forebrain synaptosomal membranes were treated with 50 μM phenylmethanesulfonyl fluoride (PMSF) as described elsewhere.[15] Some of the analogs were also tested for CB2 receptor affinity using mouse spleen membranes as the source of CB2 receptor.[23] Anandamide amidase is not present in this preparation,[23] and therefore pretreatment with PMSF is not necessary. A well-described displacement assay was used to determine the affinity of the synthesized ligands for the CB1 and CB2 receptors.[24,25] A minimum of three independent experiments conducted in duplicate were used to define the IC_{50} for each compound. IC_{50} values obtained by nonlinear least squares fitting of the data were converted to K_i values using the Cheng−Prusoff equation[26] with a K_d for CP-55,940 of 0.85 nM.

Results and Discussion

The K_i values for the analogs synthesized are presented in Table 1. The rationale for replacing the ethanolamide head group of anandamide with a thioethanolamido group was to produce anandamide analogs with higher metabolic stability without diminishing the receptor affinity. However we found that such a substitution results in a drastic reduction of the CB1 receptor affinity. Thus, both thioanandamide (**3**) and (*R*)-thiomethanandamide (**4**) showed very low affinity for CB1 (1380 and 2200 nM, respectively). A second approach toward higher metabolic stability was to sterically restrict access to the amido group by replacing the secondary amido nitrogen in anandamide with a tertiary amido nitrogen. Of the three analogs synthesized (**5**, **6**, and **7**), **5** and **6** showed low affinity for the CB1 receptor (2460 and 1690 nM, respectively), a greater than 5-fold decrease in affinity relative to anandamide. Compound **7** (174 nM), however, had an affinity approximately half that of anandamide. These results indicate a binding role for the amide NH group of anandamide, possibly via hydrogen bonding with the receptor. This hydrogen-bonding hypothesis is supported by the increased affinity of analog **7** relative to **5** and **6**, where compound **7** has the potential hydrogen-bonding capability restored by the inclusion of the hydroxyethyl substituent. This data is also consistent with the lack of affinity in the esters of anandamide reported by Pinto et al.[16] Analog **8** containing a hydroxymethyl group in the 1′-position gave a K_i value of 363 nM. This 16-fold drop in affinity when compared to (*R*)-methanandamide (**3**) (K_i = 20 nM) indicates a strict steric requirement in this position. Introduction of an ethyl group in the 2′-position gave an analog (**9**) with moderate receptor affinity (203 nM), although about 3 times weaker than that of anandamide. This compares to the butyl derivative reported by Pinto et al.,[16] which has 50% lower affinity than anandamide. We have also synthesized a rigid cyclic analog **10** which showed very weak receptor affinity (>3000 nM). Both **9** and **10** were racemates. The SAR of all the optically pure enantiomers of these compounds is currently under investigation.

From the SAR of classical and nonclassical cannabinods it was thought until recently that the phenolic hydroxyl is essential for cannabinoid activity.[27−29] We

therefore decided to introduce this group in the ethanolamide head group of anandamide. Four such analogs **11**, **12**, **13**, and **14** were prepared, and their K_i values determined. Out of these four analogs, three (**11**, **12**, and **13**) containing *ortho*, *meta*, and *para* phenolic hydroxy groups showed equally weak affinity for the receptor (K_i = 1660, 1560, and 1760 nM, respectively). However, analog **14** containing a methylene spacer between the amide nitrogen and the phenol had a K_i approximately 8 times lower (217 nM). The K_is for these four compounds are in good agreement with the values recently reported by Edgemond et al.[30] for *N*-[2-(4-hydroxyphenyl)ethyl]arachidonamide (600 nM) and *N*-(2-hydroxyphenyl)arachidonamide (**11**) (2200 nM). Consideration of these results indicate that a 4-hydroxybenzyl group could be optimal for activity.

Analogs **1**, **2**, **3**, **11**, **12**, **13**, and **14** were also evaluated for their affinity toward the recently discovered CB2 receptor using a mouse spleen preparation. As can be seen from the K_i values presented in Table 1, these analogs showed rather weak CB2 receptor affinity. The most interesting results were with anandamide and (*R*)-methanandamide which demonstrated remarkable selectivity for the CB1 receptor. Munro et al.[20] described a 30-fold selectivity for anandamide in human CB1 over CB2. These results differ from those obtained by Felder et al.[21] using expressed human CB2 receptors in which anandamide was shown to have a similar affinity for CB2 but a much lower affinity for CB1 when compared with our data, suggesting perhaps a very different SAR for mouse and human CB2 receptors. This would not be unreasonable since the two receptors have only 67% homology in their amino acid sequences.[20] No anandamide analog showed greater affinity for CB2 than CB1; indeed the phenolic and benzylic analogs all had K_is greater than 3000 nM.

Conclusions

Several new head group analogs of anandamide, the putative endogenous cannabinoid, were synthesized and evaluated for their affinity for the cannabinoid receptors. Substitution of the amide O with S, seemingly a minor variation, resulted in the loss of binding affinity. Analogs in which the ethanolamide head group is part of a carbocyclic ring showed weak affinity for the cannabinoid receptor. From the data presented here it appears that the amide NH is involved in hydrogen bonding with the receptor. Furthermore, our results suggest that the stereochemical requirements for the anandamide head group are quite strict and that the receptor site will tolerate only small changes in the bulk of this group. Introduction of a phenolic hydroxyl in the head group had a detrimental effect on activity except when a methylene spacer is present between the amide nitrogen and the phenol. It is apparent from the CB2 affinities reported here that anandamide analogs, in general, show selectivity for the CB1 receptor.

Experimental Section

General. ^{1}H NMR spectra were recorded on a Bruker WP-200SY 200 MHz spectrometer using TMS as the internal standard. All chemical shifts are reported in ppm. Satisfactory elemental analyses were obtained for all the newly synthesized analogs and are within ±0.4% of the theoretical values. Specific rotations were determined with a Perkin-Elmer 241 polarimeter using a 1.00 dm cell. The optically pure amino alcohols were obtained from Aldrich Chemical Co. (Milwaukee, WI). Anandamide (**1**) and (*R*)-methanandamide (**2**) were prepared as described previously.[15]

***N*-(2-Hydroxyethyl)arachidonylthioamide (3).** To a magnetically stirred solution of 100 mg (0.29 mmol) of arachidonylethanolamide (**1**) in 10 mL of toluene was added 70 mg (0.17 mmol) of Lawesson's reagent[22] at room temperature. The reaction mixture was stirred at 100 °C until TLC analysis showed that the starting material had reacted completely (~5 h). Toluene was evaporated *in vacuo*, and the brownish oily residue was chromatographed on silica gel (eluting first with $CHCl_3$−petroleum ether (50:50) followed by EtOAc−petroleum ether (2.5:97.5)) to give 50 mg (48%) of the title thioamide (**3**) as a yellowish oil with a sulfur odor: R_f 0.30 ($CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.37−5.41 (m, 8H), 4.21 (t, J = 8.45 Hz, 2H), 3.28 (t, J = 8.42 Hz, 2H), 2.79−2.84 (m, 6H), 2.53 (t, J = 7.64 Hz, 2H), 2.01−2.24 (m, 4H), 1.70−1.80 (m, 2H), 1.25−1.38 (m, 6H), 0.89 (t, J = 6.49 Hz, 3H); mass spectrum m/z 363 (M^+); HRMS calcd for $C_{22}H_{37}NOS$ (M^+) m/z 363.2596, found m/z 363.2590. Anal. ($C_{22}H_{37}NOS$) C, H, N.

***N*-(2-Hydroxy-1(*R*)-methylethyl)arachidonylthioamide (4).** The title compound was prepared from 120 mg (0.33 mmol) of (*R*)-methanandamide (**2**) and 80.5 mg (0.20 mmol) of Lawesson's reagent[22] in toluene following the procedure described for compound **3** to give 55 mg (44% yield) of **4** as an oil: $[\alpha]^{25}_D$ = +14.56° (c = 1, $CHCl_3$); R_f 0.47 ($CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.27−5.46 (m, 8H), 4.43−4.61 (m, 1H), 3.35−3.53 (m, 1H), 2.29−2.94 (m, 7H), 2.50 (t, J = 7.53 Hz, 2H), 2.01−2.18 (m, 4H), 1.64−1.79 (m, 2H), 1.18−1.36 (m, 9H), 0.89 (t, J = 6.25 Hz, 3H); mass spectrum m/z 377 (M^+); HRMS calcd for $C_{23}H_{39}NOS$ (M^+) m/z 377.2752, found m/z 377.2759. Anal. ($C_{23}H_{39}NOS$) C, H, N.

***N*-Methylarachidonylethanolamide (5).** A solution of arachidonic acid (200 mg, 0.66 mmol) and dry *N*,*N*-dimethylformamide (0.06 mL, 0.66 mmol) in 4 mL of dry benzene was cooled in an ice bath, and oxalyl chloride (0.12 mL, 1.32 mmol) was added dropwise under nitrogen. The reaction mixture was stirred at 0−5 °C for an additional hour. Then, a solution of 498 mg (6.6 mmol) of 2-(methylamino)ethanol in benzene was added, and the reaction mixture was stirred at room temperature for 15 min. The reaction mixture was then washed with water and dried ($MgSO_4$), and the solvents were removed by rotary evaporation. The residue was chromatographed on silica gel using 5% MeOH−$CHCl_3$ as eluent to afford 150 mg (63%) of **5** as a colorless oil: R_f 0.25 (5% MeOH−$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.30−5.41 (m, 8H), 3.78 (t, J = 5.0 Hz, 2H), 3.56 (t, J = 4.9 Hz, 2H), 3.06 (s, 3H), 2.79−2.84 (m, 6H), 2.35 (t, J = 7.61 Hz, 2H), 2.01−2.16 (m, 4H), 1.69−1.77 (m, 2H), 1.18−1.30 (m, 6H), 0.89 (t, J = 6.27 Hz, 3H). Anal. ($C_{23}H_{39}NO_2$) C, H, N.

1-(Arachidonylcarbonyl)-3-hydroxypiperidine (6). A solution of arachidonic acid chloride was prepared from 100 mg (0.33 mmol) of arachidonic acid as described under **5** and then a solution of 3-hydroxypiperidine hydrochloride (100 mg, 0.6 mmol) in 2 mL dry pyridine was added portionwise. After being stirred at room temperature for a further 15 min, the mixture was evaporated *in vacuo* at room temperature and the residue was dissolved in 10 mL of methylene chloride. The solution was washed successively with 10% HCl, 10% NaOH, and water. The organic layer was dried ($MgSO_4$) and concentrated by rotary evaporation, and the residue was chromatographed on silica gel using 2:1 diethyl ether−petroleum ether as eluent to afford 94 mg (72%) of the title compound **6**: R_f = 0.15 (5% MeOH−$CHCl_3$); ^{1}H NMR δ ($CDCl_3$) 5.36 (m, 8H), 3.90−3.10 (m, 5H), 2.81 (m, 6H), 2.40 (t, J = 8.16 Hz, 2H), 2.12 (m, 4H), 1.80−1.10 (series of m, 10H), 0.91 (t, J = 6.48 Hz, 3H). Anal. ($C_{25}H_{41}NO_2$) C, H, N.

***N*,*N*-Bis(2-hydroxyethyl)arachidonylamide (7).** A solution of arachidonic acid chloride was prepared from 100 mg (0.33 mmol) of arachidonic acid as described under **5**, and then a solution of diethanolamine (54.6 mg, 0.6 mmol) in 2 mL of dry pyridine was added portionwise. After being stirred at room temperature for a further 15 min, the mixture was evaporated *in vacuo* at room temperature, and the residue was dissolved in 10 mL of methylene chloride. The solution was washed successively with 10% HCl, 10% NaOH, and water.

The organic layer was dried ($MgSO_4$) and concentrated by rotary evaporation, and the residue was chromatographed on silica gel using 2:1 diethyl ether−petroleum ether as eluent to afford 108 mg (85%) of the title compound **7**: $R_f = 0.11$ (10% MeOH−$CHCl_3$); ^{1}H NMR δ ($CDCl_3$) 5.36 (m, 8H), 3.90 (t, J = 8.38 Hz, 2H), 3.78 (t, J = 8.38 Hz, 2H), 3.55 (t, J = 8.38 Hz, 2H), 3.50 (t, J = 8.38 Hz, 2H), 2.80 (m, 6H), 2.41 (t, J = 8.20 Hz, 2H), 2.10 (m, 4H), 1.76 (m, 2H), 1.32 (m, 6H), 0.90 (t, J = 6.50 Hz, 3H). Anal. ($C_{24}H_{41}NO_3$) C, H, N.

***N*-[2-Hydroxy-1-(hydroxymethyl)ethyl]arachidonylamide (8).** Arachidonic acid chloride, prepared from 100 mg (0.33 mmol) of arachidonic acid as described under compound **5**, was dissolved in 3 mL of anhydrous methylene chloride. and a solution of 60 mg (0.6 mmol) of 2-amino-1,3-propanediol (serinol) in 2 mL of dry pyridine was added portionwise. After the mixture was stirred at room temperature for a further 15 min, the yellow precipitate formed was filtered off and washed with methylene chloride (10 mL). The combined filtrate and washings were concentrated *in vacuo*, and the residue was dissolved in 10 mL of methylene chloride and washed successively with 10% HCl, 10% NaOH, and water. The organic layer was dried ($MgSO_4$), and the solvents were removed by rotary evaporation. The residue was chromatographed on silica gel using 2% MeOH−$CHCl_3$ as eluent to afford 60 mg (48%) of the title compound **8**: ^{1}H NMR ($CDCl_3$) δ 6.19 (br s, 1H), 5.30 (m, 8H), 3.91−3.99 (m, 1H), 3.82 (d, J = 4.6 Hz, 4H), 2.79-2.84 (m, 6H), 2.15−2.29 (t, J = 7.5 Hz, 2H), 2.00−2.14 (m, 4H), 1.70−1.81 (m, 2H), 1.25−1.39 (m, 6H), 0.89 (t, J = 6.5 Hz, 3H). Anal. ($C_{23}H_{39}NO_3$) C, H, N.

***N*-(2-Hydroxy-2-ethylethyl)arachidonylamide (9).** A solution of arachidonic acid chloride was prepared from 100 mg (0.33 mmol) of arachidonic acid as described under **5**, and then a solution of (±)-1-amino-2-butanol (293 mg, 3.3 mmol) in 2 mL of dry methylene chloride was added. After a further 15 min of stirring at room temperature, the reaction mixture was diluted with 10 mL of methylene chloride, washed successively with 10% HCl and 10% NaOH, and dried ($MgSO_4$). The solvent was removed by rotary evaporation, and the residue was purified by column chromatography using 30% petroleum ether−diethyl ether as eluent to yield 65 mg (52%) of the title compound **9**: ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 6.50 Hz, 3H), 0.96 (t, J = 7.00 Hz, 3H), 1.31 (s, 6H), 1.47 (m, 2H), 1.67−1.80 (m, 2H), 2.03−2.25 (m, 6H), 2.81 (m, 6H), 3.08−3.17 (m, 1H), 3.43−3.62 (m, 2H), 5.30 (m, 8H), 5.90 (br s, 1H). Anal. ($C_{24}H_{41}NO_2$) C, H, N.

***N*-(*trans*-2-Hydroxycyclohexyl)arachidonylamide (10).** A solution of arachidonic acid chloride was prepared from 100 mg (0.33 mmol) of arachidonic acid as described under **5**, and then a solution of 2-aminocyclohexanol hydrochloride (100 mg, 0.6 mmol) in 2 mL of dry pyridine was added portionwise. After being stirred at room temperature for a further 15 min, the mixture was evaporated *in vacuo* at room temperature, and the residue was dissolved in 10 mL of methylene chloride. The solution was washed successively with 10% HCl, 10% NaOH, and water. The organic layer was dried ($MgSO_4$) and concentrated by rotary evaporation, and the residue was chromatographed on silica gel using 2:1 diethyl ether−petroleum ether as eluent to afford 90 mg (68%) of the title compound **10**: ^{1}H NMR ($CDCl_3$) δ 5.30−5.40 (m, 9H), 3.60−3.72 (m, 2H), 3.37−3.34 (m, 1H), 2.79−2.84 (m, 6H), 1.97−2.23 (m, 8H), 1.70−1.81 (m, 4H), 1.25−1.35 (m, 10H), 0.89 (t, J = 6.50 Hz, 3H). Anal. ($C_{24}H_{41}NO_2$) C, H, N.

***N*-(2-Hydroxyphenyl)arachidonylamide (11).** A solution of arachidonic acid chloride in dry benzene, prepared from 100 mg (0.33 mmol) of arachidonic acid according to the procedure described under **5**, was cooled in an ice bath, and a solution of 360 mg (3.3 mmol) of 2-aminophenol in 4.5 mL of anhydrous THF was added dropwise. The reaction mixture was stirred at room temperature for a further 15 min and then diluted with 15 mL of methylene chloride and 5 mL of water. The organic layer was separated and dried ($MgSO_4$), and the solvents were removed by rotary evaporation. The residue was purified by column chromatography on silica gel (1:1 diethyl ether−petroleum ether) to afford 120 mg (85%) of the title compound **11**: $R_f = 0.39$ (1:1 diethyl ether−petroleum ether); ^{1}H NMR δ ($CDCl_3$) 8.95 (s, 1H), 8.00 (s, 1H), 7.21−6.79 (m, 4H), 5.35 (m, 8H), 2.80 (m, 6H), 2.43 (t, J = 7.76 Hz, 2H), 2.21−1.99 (m, 4H), 1.80 (m, 2H), 1.34 (m, 6H), 0.88 (t, J = 6.73 Hz, 3H). Anal. ($C_{26}H_{37}NO_2$) C, H, N.

***N*-(3-Hydroxyphenyl)arachidonylamide (12).** The title compound was synthesized from 100 mg (0.33 mmol) of arachidonic acid and 3-aminophenol as described under **11** to afford 86 mg (61%) of compound **12**: ^{1}H NMR (2:1 DMSO−$CDCl_3$) δ 8.70 (s, 1H), 8.04 (s, 1H), 7.32 (s, 1H), 7.14 (m, 1H), 6.67 (m, 1H), 6.46 (m, 1H), 5.36 (m, 8H), 2.80 (m, 6H), 2.39 (t, J = 7.71 Hz, 2H), 2.10 (m, 4H), 1.83 (m, 2H), 1.29 (m, 6H), 0.88 (t, J = 6.75 Hz, 3H). Anal. ($C_{26}H_{37}NO_2$) C, H, N.

***N*-(4-Hydroxyphenyl)arachidonylamide (13).** The title compound was synthesized from 100 mg (0.33 mmol) of arachidonic acid and 4-aminophenol as described under **11** to afford 96 mg (68%) of compound **13**: ^{1}H NMR (2:1 DMSO−$CDCl_3$) δ 9.51 (s, 1H), 9.00 (s, 1H), 7.34 (d, 2H), 6.65 (d, 2H), 5.33 (m, 8H), 2.80 (m, 6H), 2.26 (t, J = 7.50 Hz, 2H), 2.06 (m, 4H), 1.66 (m, 2H), 1.28 (m, 6H), 0.87 (t, J = 6.76 Hz, 3H). Anal. ($C_{26}H_{37}NO_2$) C, H, N.

***N*-(4-Hydroxybenzyl)arachidonylamide (14).** A solution of arachidonic acid chloride in dry benzene, prepared from 100 mg (0.33 mmol) of arachidonic acid according to the procedure described under **5**, was cooled in an ice bath, and a solution of 340 mg (1.65 mmol) of the tetrahydropyranyl ether of 4-hydroxybenzylamine in 2.0 mL of anhydrous THF was added dropwise. The reaction mixture was stirred at room temperature for a further 15 min; then 10 mL of saturated $NaHCO_3$ solution and 20 mL of diethyl ether were added, the organic layer was separated, and solvents were removed by rotary evaporation. The residual oil was dissolved in 5 mL of methanol, and the pH of the solution was adjusted to approximately pH 3 by dropwise addition of concentrated hydrochloric acid. The solution was warmed in a water bath to 50 °C for 10 min and then cooled to room temperature, saturated $NaHCO_3$ solution was added to raise the pH to approximately pH 8, and most of the solvents were removed by rotary evaporation. The residue was dissolved in diethyl ether, and the solution was dried ($MgSO_4$). The ether was removed by rotary evaporation. The crude product was purified by column chromatography on silica gel (80:20 diethyl ether−petroleum ether) to afford 110 mg (82%) of the title amide **14**: $R_f = 0.29$ (80:20 diethyl ether−petroleum ether); ^{1}H NMR δ ($CDCl_3$) 7.08 (d, J = 8.50 Hz, 2H), 6.79 (d, J = 8.50 Hz, 2H), 5.95 (t, J = 5.33 Hz, 1H), 5.36 (m, 8H), 4.33 (d, J = 5.56 Hz, 2H), 2.80 (m, 6H), 2.28−1.98 (m, 6H), 1.73 (m, 2H), 1.28 (m, 6H), 0.88 (t, J = 6.78 Hz, 3H). Anal. ($C_{26}H_{37}NO_2$) C, H, N.

Radioligand Binding Assay. For CB1, rat forebrain membranes were prepared according to the procedure of Devane et al.[24] The binding of the novel probes to the cannabinoid receptor was assessed as previously described,[24,25] except that the membranes were treated with PMSF. Membranes, previously frozen at −80 °C, were thawed on ice. To the stirred suspension were added five volumes of 25 mM Tris-HCl Buffer, pH 7.4, 5 mM $MgCl_2$, and 1 mM EDTA (TME) containing 50 μM PMSF (made fresh daily in 2-propanol as a 10 mM stock). After 30 min the membranes were pelleted, the supernatant was discarded, and the pellet was resuspended in five volumes of the PMSF containing buffer. At the end of the second 30 min incubation, the membranes were pelleted and washed three times with TME to remove unreacted PMSF. The treated membranes were subsequently used in the binding assay described below. Approximately 50 μg of PMSF-treated membranes were incubated in silanized culture tubes with TME containing 0.1% essentially fatty acid free bovine serum albumin (BSA), 0.8 nM [^{3}H]CP-55,940, and various concentrations of the anandamide analogs in a final volume of 200 μL. Assays were incubated for 1 h at 30 °C and terminated by the addition of 250 μL of TME containing 5% BSA. The tubes were immediately filtered using GF/C filters on a Brandell M-24 cell harvester. Following four washes with ice-cold wash buffer (20 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 4 mL/well), the filters were collected, shaken for 1 h with 2 mL of 0.1% sodium dodecyl sulfate, and counted in a liquid scintillation counter to determine the amount of bound radioligand. Nonspecific binding was assessed using 250 nM

desacetyllevonantradol. 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 250 nM DALN. The normalized data was analyzed using Inplot (GraphPad Software, San Diego, CA) to yield IC_{50} values. The IC_{50} values from three independent experiments were averaged and converted to K_i values using the assumptions of Cheng and Prusoff.[26]

For CB2 receptor binding studies, membranes were prepared from frozen mouse spleen according to the procedure of Dodd et al.[31] as described elsewhere.[23] Silanized centrifuge tubes were used throughout to minimize receptor loss due to adsorption. The CB2 binding assay was conducted in essentially the same manner as for CB1. The assay differed only in as much as it was conducted in a 96-well microtiter plate, and the samples were filtered using a Packard Filtermate 96 and Whatman GF/C filterplates; 5% BSA was not added to terminate the binding, rather 0.5% BSA was incorporated into the wash buffer. Radioactivity was detected using MicroScint 20 scintillation cocktail added directly to the dried filterplates, and the filterplates were counted using a Packard Instruments 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 0 and 100 nM CP-55,940. The normalized data from three independent experiments were combined and analyzed using using a four-parameter logistic equation to yield IC_{50} values which were converted to K_i. This assay method had previously been shown to give equivalent K_i values to the method described for CB1 binding.[23]

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