Synthesis and Pharmacological Comparison of Dimethylheptyl and Pentyl Analogs of Anandamide

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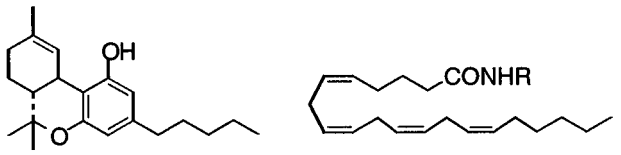
(Dimethylheptyl)anandamide [(16,16-dimethyldocosa-*cis*-5,8,11,14-tetraenoyl)ethanolamine] (**17a**) and its amide analogs were synthesized by Wittig coupling of a ylide derived from a fragment of arachidonic acid. These amides were compared to the endogenous cannabinoid receptor ligand arachidonylethanolamide (anandamide, **2a**) and its amide analogs in pharmacological assays for potential enhancement of cannabimimetic activities. The receptor affinity to rat brain membranes of the dimethylheptyl (DMH) analogs increased by an order of magnitude in most comparisons to the corresponding anandamides in displacement assays versus the cannabinoid agonist [^{3}H]CP 55,940 or antagonist [^{3}H]SR141716A, for which rank order differences in affinity were observed. An order of magnitude enhancement of potency with comparable or higher efficacy in behavioral assays in the mouse tetrad of tests of cannabinoid activity was observed in **17a** versus **2a**. In contrast, no enhancement in potency for the pentyl to DMH side chain exchange was seen in the mouse vas deferens assay. The data indicate a structural equivalence between classical plant cannabinoids and **2a** as well as different receptor−ligand interactions that characterize multiple receptor sites or binding modes.

Introduction

The functional and mechanistic equivalence of the plant cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC, **1**) and the endogenous cannabinoid arachidonylethanolamide (anandamide, **2a**) is paralleled by a three-dimensional structural equivalence. This correspondence of structure and function between two distinct structure types suggests that the design of analogs of the relatively unexplored anandamides can be profitably guided by the identification of structurally equivalent sectors of the two compounds in conjunction with the SAR of Δ^9-THC and the effects of modifications in the latter series. The binding equivalence of anandamide to classical (plant) cannabinoids is implicit from the use of a cannabinoid ligand binding assay to guide its isolation from porcine brain; **2a** and **1** have comparable K_i values.[1] Further, the spectra of pharmacological methods that have characterized THCs have shown similar responses with anandamide. Thus, anandamide inhibits the electrically evoked twitch response in the mouse isolated vas deferens and binds to rat brain receptors.[1] Cells transfected with either rat or human expression plasmids for the cannabinoid binding receptor show specific receptor binding of anandamide as well as exhibiting inhibition of the production of cAMP via the G-protein-coupled receptor similar to the activity of THC.[2,3] The brain region specificity for anandamide-mediated inhibition of cAMP production was the same in the rat as for the cannabinoid agonist WIN 55,212-2.[4] Also, the inhibition of voltage-gated calcium channel currents in N18 neuroblastoma cells by anandamide was similar to but lower than that produced by WIN 55,212-2.[5] In vivo in the mouse model for cannabinoid activity, anandamide induced ring immobility (catalepsy), depressed open field locomotion, and caused hypothermia and antinociception typical of psychotropic THCs, supporting the conclusion that anandamide is an endogenous cannabimimetic.[6−9]



1 **2**

NHR

a) NH-CH_2CH_2OH
b) NH-$CH(CH_3)$-CH_2OH (R)
c) NH-CH_2-$CH(CH_3)OH$ (S)
d) NH-$CH_2CH_2OCH_3$
e) NH-$CH_2CH_2CH_3$

The rationale for using the SAR of the archetypical cannabinoid Δ^9-THC to guide modifications of anandamide is based on the correlation of the three-dimensional structures of the two compounds. There is both chemical and computational evidence that anandamide adopts a bent shape that mimics Δ^9-THC. Chemical evidence comes from the highly regiospecific, intramolecular self-epoxidation of arachidonyl peracid which affords an epoxide only at the most terminal Δ^{14} double bond.[10] In order for this internal epoxidation to occur, the peracid would need to adopt a bent "J"-like conformation. The reported formation of the single epoxide indicates that the 16-membered ring conformation for the arachidonyl peracid is energetically quite favorable

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compared to those conformations that would involve the more proximate double bonds. Further chemical evidence of favored bent conformations of arachidonyl structures is the observation that the two ends of the arachidonic acid chain can be coupled to form a 21-atom lactone ring from the pentyl terminus hydroxy analog 20-hydroxyarachidonic acid active ester.[11] The unusually high rate of cyclization and yield (90%) for such a large ring indicates the low-energy expenditure required to bring the reacting sites together. Both of these findings support a bent conformation.

Molecular dynamics studies of arachidonic acid by Rich[12] and Corey[11] found bent conformations with ends of the molecule in close proximity to be accessible and favored geometries (<3 kcal higher energy than the global minimum). Molecular dynamics studies of anandamide by Thomas[13] have found such bent geometries to be significant contributors to the population of low-energy conformations. Thus, computational evidence is in good agreement with chemical evidence for arachidonyl compounds favoring bent conformations.

Importantly, Thomas has shown that favored anandamide conformations can be superimposed upon that of Δ^9-THC with a high degree of overlap and with functionally important regions and electrostatic potentials coinciding.[13] Included in this overlap is the alignment of the pentyl chains of both structures. This would suggest that replacement of the pentyl side chain of anandamide with the 1,1-dimethylheptyl (DMH) side chain might reasonably be expected to result in an enhancement of activity similar to that seen in classical and non-classical cannabinoids. Such an exchange has been associated with enhanced activity in the range of 100-fold for other classes of cannabinoids.[14−18]

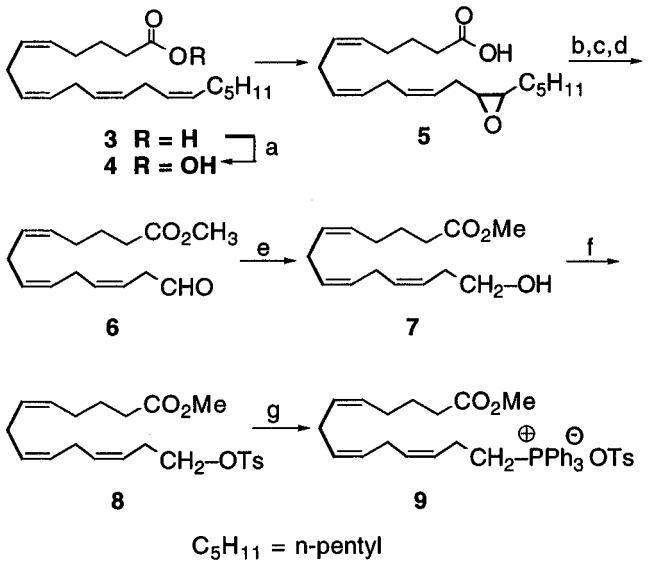
In order to test the validity of the computational correspondence of Δ^9-THC with anandamide and to determine the role of the side chain in anandamide activity as well as to develop anandamides of enhanced activity that could potentially afford biochemical tools to aid in the understanding of the new neurochemical system characterized by the cannabinoid receptor,[19−21] DMH-anandamide analogs of selected arachidonyl (pentyl) amides[13,22−28] were synthesized and evaluated in pharmacological assays.

The synthesis of the preferred 1,1-DMH-anandamide and analogs and their binding to rat brain membranes containing the cannabinoid receptor have been presented in preliminary studies[29] as have that data for other branched side chain anandamide analogs.[30] This paper presents the details of our study with expanded pharmacological characterization.

Chemistry

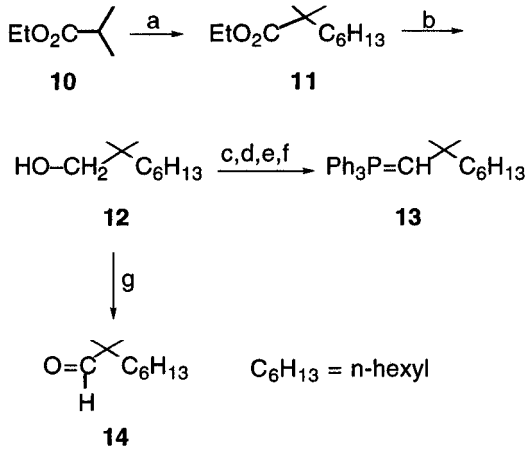
Using the conformationally driven, regiospecific, internal self-epoxidation of arachidonic acid (**3**) to functionalize the terminal Δ^{14} double bond, an entry point for the cleavage of the pentyl side chain can be realized. This affords the corresponding aldehyde **6**[31] (Scheme 1) following the method of Corey[10] and Falck[32] for a potential Wittig coupling to a DMH side chain ylide (**13**, Scheme 2) targeting the DMH arachidonic acid ester (**16**, Scheme 3). To avoid the dangers of working with neat, anhydrous hydrogen peroxide, especially for scale up reactions, alternative conditions were examined for the formation of the arachidonyl peracid (**4**). Thus,

Scheme 1[a]



[a] (a) $CO(im)_2$, H_2O_2; (b) $CO(im)_2$, MeOH; (c) aq $HClO_4$; (d) $Pb(OAc)_4$; (e) $NaBH_4$; (f) TsCl, Pyr; (g) CH_3CN, Ph_3P.

Scheme 2[a]

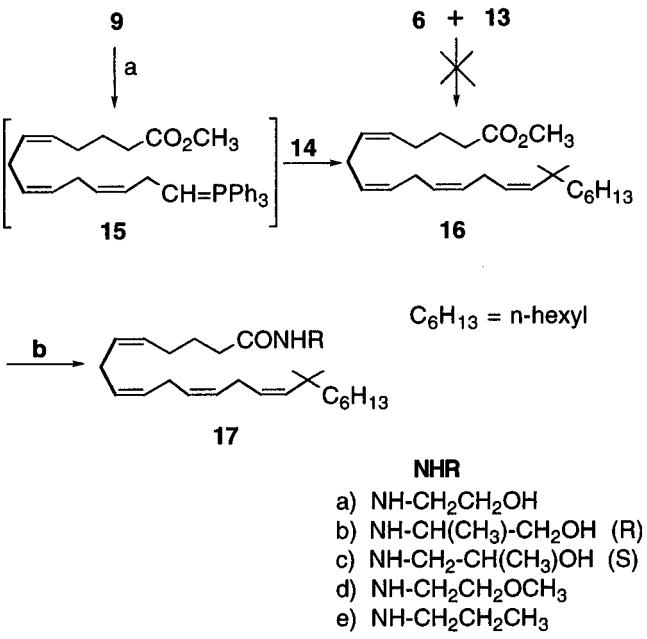


[a] (a) (1) LDA, THF/HMPA, (2) Br-C_6H_{13}; (b) $LiAlH_4$; (c) TsCl, Pyr, (d) NaI, HMPA, H_2O; (e) Ph_3P; (f) nBuLi, THF/HMPA; (g) PCC, CH_2Cl_2.

arachidonic acid (**3**) was treated with carbonyldiimidazole and the resulting arachidonylimidazolide, which exhibited reasonable stability in water,[33] was reacted with mixtures of 50% aqueous H_2O_2 and THF to afford a mixture of 14,15-epoxy arachidonic acid (**5**) and **3**. The epoxide **5** formed from internal self-epoxidation of the terminal Δ^{14} double bond of **4**, while **3** derived from degradation of **4** by the imidazole by product. Attempted trapping of the imidazole by potassium bisulfate[10] or maleic acid[34] had only marginal benefit. Etheral hydrogen peroxide with $KHSO_4$ provided the best combination of safety, convenience, and minimization of diacyl peroxide formation to afford a mixture of **3** and **5**. These were esterified with carbonyldiimidazole/methanol and chromatographed. The resulting epoxy ester was ring-opened to the diol and cleaved to the unstable aldehyde **6**. The neopentyl-like ylide (**13**) was prepared in the straightforward manner outlined in Scheme 2.

In practice, the conditions[35] required to couple the hindered ylide **13** were too stringent for the labile arachidonyl aldehyde **6** to survive the process, and decomposition of **6** was observed.

Scheme 3[a]



[a] (a) nBuLi, THF/HMPA; (b) NaCN, MeOH, H_2NR.

Reversing the ylide and the aldehyde functionalities between the tetradecatriene and the DMH components afforded a successful coupling pair. Thus, the aldehyde **6** was reduced to **7** with $NaBH_4$, tosylated to **8**, and heated with triphenylphosphine to provide the phosphonium salt **9** (Scheme 1). Wittig coupling of the arachidonyl ylide **15**, from *n*-butyllithium deprotonation of **9**, with 2,2-dimethyloctanal **14**, from pyridinium chlorochromate oxidation of the alcohol **12** (Scheme 2), afforded the corresponding tetraene ester **16** (Scheme 3).

Although the Wittig coupling of unstabilized ylides, as employed here, typically yields an olefin with *cis* geometry[36,37] and the reverse sense coupling to the arachidonyl aldehyde **6** with an *n*-hexyl ylide gave arachidonic acid (ie *cis* geometry),[32] a rigorous proof of structure (geometry) was required for the key intermediate **16**. The resonances of the 14,15-vinyl protons were upfield shifted from the overlapping resonances of the protons on the remaining, less sterically distorted double bonds and exhibited a coupling constant with one another of 11.9 Hz, which was inconclusive since *cis* coupling is about 10 Hz and *trans* coupling is about 16 Hz. The Δ^{14} geometry was proven by NOE NMR spectroscopy on **16**. Thus, an NOE interaction of the 13-CH_2 with the geminal methyls on C-16 was observed, indicating their close proximity through space which is only possible on the 14,15-*cis*-olefin. Additionally, each of these two groups exhibited an NOE interaction with only one of the Δ^{14} vinyl protons in contrast to the dual interactions expected for a *trans*-olefin. Together, these observations provide the sufficient and necessary data to identify the terminal double bond as having the desired *cis* geometry of the arachidonyl system.

A direct conversion of the ester to DMH-anandamide (**17a**) was achieved by cyanide-catalyzed amidation[38] with ethanolamine. Similar treatment of **16** with other amines provided a series of analogs for comparison of their activities to their pentyl counterparts. The latter were prepared from arachidonic acid by its conversion to arachidonyl chloride[1] followed by treatment with the same amines.

Receptor Binding Studies

The binding affinity of the series of pentyl and DMH-anandamide compounds (**2a**−**e** and **17a**−**e**, respectively) to cannabinoid binding sites was determined using a rat whole brain membrane preparation in a filtration assay as previously described.[19] Phenylmethanesulfonyl fluoride (PMSF) was employed in the assay to inhibit hydrolysis of the amides.[4,39] Increasing concentrations of the compounds were used to displace the specific binding of the potent nonclassical cannabinoid [^{3}H]CP 55,940[19] and also that of the cannabinoid antagonist [^{3}H]SR141716A.[40,41] The K_i values are given in Table 1. The general trends were that in all DMH analogs the binding affinities increased from 2- to 18-fold versus the corresponding pentyl compound. The K_i values for both series **2** and **17** versus [^{3}H]CP 55,940 was 1−2 orders of magnitude higher than those versus [^{3}H]SR141716A and not in the same rank order of affinity. The rank order of affinity in the DMH series was the same as that in the pentyl series when displacing the DMH ligand [^{3}H]CP 55,940, but not when displacing [^{3}H]SR141716A.

Mouse Vas Deferens Assay

All the compounds described in this paper were found to share the ability of cannabinoid CB1 receptor agonists to inhibit electrically evoked contractions of the mouse vas deferens (MVD),[42,43] each log concentration response curve being approximately sigmoid in shape (r^2 = 0.942−0.999). The results obtained are summarized in Table 1. As found previously,[27,44] methanandamide (compound **2b**) was more potent than anandamide (compound **2a**). Like anandamide, it behaved as a full agonist, producing complete inhibition of the twitch response at maximal concentrations. Replacement of the pentyl side chain of these two compounds with a dimethylheptyl side chain to form compounds **17a** and **17b** did not lead to any detectable increase in potency. Indeed, both dimethylheptyl compounds produced less than 100% inhibition of the twitch response at maximal concentrations, indicating them to be partial agonists. Compounds **2c**, **17c**, **2d**, **17d**, **2e**, and **17e** also behaved as partial agonists. Compounds **2c** and **17c** produced similar maximal degrees of inhibition, as did compounds **2d** and **17d** and compounds **2e** and **17e**. This comparison would suggest that, for each of these pairs of compounds, a change from pentyl to dimethylheptyl does not significantly alter efficacy. The potencies of compounds **2c** and **17c** were similar, as were the potencies of compounds **2e** and **17e**. However, compound **17d** was significantly less potent than its pentyl analog, compound **2d**.

Behavioral Evaluations in Mice

DMH-anandamide and its amide analogs were evaluated in the mouse model of cannabinoid activity. This tetrad of tests includes inhibition of spontaneous activity (SA), antinociception as measured by the tail-flick response (TF), hypothermia as changes in rectal temperature (RT), and ring immobility (RI).[45] DMH-anandamide (**17a**) was more potent than anandamide (**2a**) in producing hypoactivity (12 times), antinociception (4

Table 1

	affinity for rat brain membrane cannabinoid receptors[a]				mouse tetrad[b]			
	$K_i \pm$ SEM (nM)		twitch inhibition in MVD		ED_{50} (mg/kg)/(%MPE)[e]			
compd	vs[^{3}H]SR	vs[^{3}H]CP	EC_{50} (nM)[c]	E_{max} (%)[d]	SA[b]	TF[b]	RT[b]	RI[b]
2a	282 ± 42	25 ± 8	52.7[f] (40.3−68.9)	100	17.9[g]	6.20[g]	26.5[g]	19.1[g]
2b	585 ± 170	22 ± 5	10.1 (4.2−24.5)	100				
2c	2890 ± 1450	57 ± 5	36.1 (28.0−46.5)	71.7 ± 5.6				
2d	466 ± 119	44 ± 7	3.0 (1.4−6.4)	34.0 ± 4.9				
2e	238 ± 133	2.6 ± 1.4	10.9 (5.4−21.8)	51.2 ± 9.2				
17a	153 ± 25	1.9 ± 0.6	59.7 (25.4−140.2)	68.3 ± 18.2	1.5 (90)	1.7 (91)	2.9 (−3.5)	1.0 (74)
17b	220 ± 93	1.7 ± 0.5	60.6 (35.3−104.0)	65.2 ± 11.4	1.1 (95)	1.0 (100)	6.0 (−2.8)	2.4 (85)
17c	161 ± 51	5.0 ± 0.6	28.3 (16.7−47.9)	67.2 ± 10.4	2.6 (90)	4.8 (100)	5.0 (−2.0)	2.5 (80)
17d	54 ± 13	3.9 ± 0.4	36.0 (23.1−56.1)	44.6 ± 6.7	4.0 (98)	13.3 (100)	50[h] (−1.5)	5.7 (86)
17e	31 ± 16	1.5 ± 0.2	11.7 (8.2−16.7)	65.3 ± 6.0	1.2	6.3	50[i] (−1.5)	50[i] (81)

[a] $n = 3-5$. [b] The pharmacological measures included inhibition of spontanious activity (SA), antinociception as measured by the tail flick response (TF), hypothermia as changes in rectal temperature (RT), and ring immobility (RI). [c] EC_{50} in mouse vas deferens (MVD) with 95% confidence limits in brackets ($n = 6-12$ different vas deferentia). [d] Maximal degree of inhibition of electrically evoked contractions in percent ± range of 95% confidence limits. [e] Maximum possible effect in percent or degrees Celsius. [f] Reference 9. [g] Reference 8. [h] Effects were not dose responsive and 50 mg/kg produced only a decrease of 1.5 °C. [i] Not tested at lower doses; not an ED_{50}.

times), hypothermia (9 times), and ring immobility (19 times). **17a** was also more efficacious in the ring immobility test (74%) than either **1** or **2a** (60%). All the DMH analogs were more potent than **2a** in tests of SA with comparable efficacy to that of **2a** (90%). High efficacy was maintained in the TF assay, but with **17d−e** being of lesser or comparable potency. In hypothermia tests, all of the DMH analogs were only partial agonists with maximal effects in the range of −1.5 to −3.5 °C, comparable to that of **2a** (−3.0 °C) and in contrast to that of **1** (−6.0 °C); **17d,e** were not dose responsive and 50 mg/kg produced only a 1.5 °C temperature drop. **17a−d** were more potent and more efficacious than was **2a** in the RI test. While significantly enhancing the pharmacological activity of the anandamides, the DMH side chain produced a less dramatic effect than the same change in the classical THCs in the range of 10−75-fold.[18]

Discussion and Conclusions

Substitution of a DMH for a pentyl side chain in anandamide and its amide analogs confers higher binding affinity in both [^{3}H]CP 55,940 and [^{3}H]-SR141716A displacement assays (typically an order of magnitude) and a similar enhancement of activity in the in vivo tetrad of tests in mice for the pentyl- and DMH-anandamides **2a** and **17a** that is consistent with the same substitution in the classical cannabinoids. These observations are in agreement with the interpretation of a computational overlay of **1** with **2a** that indicates that their respective pentyl side chains serve equivalent roles in the binding to the receptor and is supportive of the use of this model to guide future structure modifications. The same rank order of affinities of the pentyl and the DMH-anandamides in the displacement of [^{3}H]CP 55,940 supports an interpretation that the anandamides bind to the same site or sites within a receptor or to the same population of receptors as does [^{3}H]CP 55,940. This population could be CB1 and CB2, for which [^{3}H]CP 55,940 shows equal affinity, and both have been implicated to be present in the brain.[46] The different rank order of affinities seen in the displacement of [^{3}H]SR141716A versus [^{3}H]CP 55,-940, especially in the DMH series, suggests differences either in binding modes, receptor sites, or population of receptors for the SR and CP ligands; the latter being consistent with the CB1 versus CB2 selectivity of SR141716A. While substantial, the degree of binding and in vivo enhancements were on the lower end of the range of the 1−2 orders of magnitude of enhancement seen in classical cannabinoids.

The lack of an increase in potency in the MVD assay upon substitution with the DMH side chain is in contrast to the above results. While extensive comparisons of DMH vs pentyl THC analogs in the MVD assay have not been conducted, it has been shown that HU-210 (11-OH-Δ^8-THC-DMH) is significantly (40-fold) more potent than **1** with EC_{50} values of 0.15 and 6.3 nM, respectively.[42] If this is a general trend in classical cannabinoids, then these conformationally mobile DMH-anandamides interact with the MVD receptor in a different manner than the conformationally more rigid classical THCs and possibly even than the pentylanandamides **2a,b**. Indeed, the behavior of **17a−e** as partial agonists suggests altered binding modes or sites within the receptor, assuming that the receptor populations are the same in the MVD as in the rat brain membrane preparation and the mouse CNS. The substantial increase in potency with comparable or higher efficacy of **17a** versus **2a** in the mouse tetrad of tests, however, leaves open the possibility of different receptor populations in the two assays. In this regard, there was a recent report of a second cannabinoid receptor that inhibits the twitch response in the MVD but which is not antagonized by SR141716A and hence not the CB1 receptor.[47] This could account for the MVD results if

this receptor is different from those found in the brain and does not respond to the DMH side chain in the same way as the CB1 receptor.

Finally, the steric strain induced on the terminal *cis* Δ^{14} double bond by the sterically demanding DMH side chain, as evidenced by the upfield shift of the associated vinyl protons in the NMR spectrum, could reasonably induce conformational differences, compared to the pentyl analogs, in this relatively flexible ligand that would be responsible for altered binding modes and hence altered potency and efficacy.

Experimental Section

Chemistry. ^{1}H NMR spectra were recorded on a Bruker AM-250 MHz or AMX-500 MHz (as noted) spectrometer; ^{13}C NMR spectra were recorded in $CDCl_3$ on the Bruker AMX-500 spectrometer operating at 125.77 MHz. The chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) in $CDCl_3$. High-resolution mass spectra (HRMS) were obtained on a VG ZAB-E mass spectrometer via direct probe in the electron impact mode with a 70 ev ionization voltage. Compounds were shown to be homogeneous by HPLC employing two diverse elution solvent mixtures on a Waters dual pump chromatograph operating at 2.0 mL/min with a model 484 tunable absorbance detector, Waters reversed phase C18 RCM 8 mm × 10 cm column, UV detection at 214 nm; eluant a for **17a**−**e**, 90% MeOH−10% H_2O (0.05% HOAc); eluant b, 85% MeOH−15% H_2O (0.05% HOAc); or eluant c, 95% CH_3CN−5% H_2O. TLC on Baker Si254F silica gel plates employing UV and phosphomolybdic acid−ceric sulfate spray detection similarly showed the homogeneity of the compounds. The two eluants that determined TLC homogeneity of the target compounds were 2% MeOH−$CHCl_3$ and EtOAc−hexane−HOAc-H_2O, 100:50:20:100 (organic phase). Other eluants are described in the experimental procedures.

14,15-Epoxyeicosa-*cis*-5,8,11-trienoic Acid (5). 1,1′-Carbonyldiimidazole (2.26 g, 14.0 mmol) in 21 mL of dry CH_2Cl_2 (filtered through basic Al_2O_3) was treated with arachidonic acid (4.4 mL; 13.3 mmol) under argon with stirring at ambient temperature. After 40 min, the resulting arachidonyl imidazolide solution was added dropwise over 5 min to etheral H_2O_2 (4.56 M, 135 mL, 560 mmol) at 0 °C. $KHSO_4$ (26 g, 190 mmol) was added to the reaction when half the imidazolide had been added. The mixture was stirred for an additional 5 min, filtered, washed with water (1×) and brine (3×), and dried over Na_2SO_4. Evaporation afforded 3.8 g of a mixture of arachidonic acid and the title epoxide in a 1:1 area ratio (HPLC, eluant b) estimated as a 3:7 molar ratio correcting for the extinction coefficients. TLC (30% EtOAc-Hex) marginally resolves the components.

Etheral Hydrogen Peroxide. Commercial aqueous 50% hydrogen peroxide (300 mL) was extracted with diethyl ether (3 × 100 mL), and the combined organic phases were dried over Na_2SO_4 and filtered to yield 270 mL of solution. Titration[34] indicated 4.56 M H_2O_2.

Methyl 14,15-Epoxyeicosa-*cis*-5,8,11-trienoate. The epoxide containing ca. 30% arachidonic acid (7.6 g) was added to 1,1′-carbonyldiimidazole (5.0 g, 30.8 mmol) in 100 mL of dry CH_2Cl_2 with stirring under argon at ambient temperature (monitored by TLC, 50% EtOAc-Hex). After stirring for 30 min, MeOH (4.8 mL, 118 mmol) was added, and the reaction was stirred for 4 h at ambient temperature (monitored by TLC, 30% EtOAc-Hex). The volatiles were evaporated in vacuo and the residue partitioned between ether and water (3× with ether). The combined ether layers were washed with brine (3×) and dried over Na_2SO_4. The residue upon evaporation in vacuo (9.2 g) was chromatographed on silica gel (450 g) eluting with 10% EtOAc−hexane to afford 1.97 g of methyl arachidonate (24%) and 4.85 g of the title epoxy ester (55% based on starting arachidonic acid): ^{1}H NMR ($CDCl_3$) δ 5.47 (m, 2H, 11,12-vinyl H), 5.36 (m, 4H, 5,6,8,9-vinyl H), 3.64 (s, 3H, OCH_3), 2.92 (m, 2H, 13-CH_2), 2.79 (m, 4H, 7,10-CH_2), 2.3 (br m, 2H, 14,15-CH), 2.30 (t, 2H, J = 7.4 Hz, 2-CH_2), 2.08 (m, 2H, 4-CH_2), 1.68 (p, 2H, J = 7.4 Hz, 3-CH_2), 1.51 (br m, 2H, 16-CH_2), 1.31 (br m, 6H, 17−19-CH_2), 0.87 (m, 3H, 20-CH_3).

Methyl 14,15-Dihydroxyeicosa-*cis*-5,8,11-trienoate. To the epoxy ester (4.84 g, 14.5 mmol) at 0 °C was added a cold solution of 10% aqueous $HClO_4$−THF (40:105 mL). The ice bath was removed and the reaction stirred at ambient temperature until it appeared to be complete (4 h) by HPLC (eluant b). The reaction was partitioned between diethyl ether and water. The organic phase was washed with aqueous $NaHCO_3$ and then brine. Drying over Na_2SO_4 and chromatography on silica gel 60 (250 g) eluting with ether−hexane 2:1 afforded 3.48 g (68%) of the title compound; ^{1}H NMR ($CDCl_3$) δ 5.51 (m, 2H, 11,12-vinyl H), 5.36 (m, 4H, 5,6,8,9-vinyl H), 3.66 (s, 3H, OCH_3), 3.46 (m, 2H, 14,15-CHO), 2.82 (m, 4H, 7,10-CH_2), 2.32 (t, overlap, 4H, J = 7.4 Hz, 2,13-CH_2), 2.20 (br m, 2H, OH), 2.09 (m, 2H, 4-CH_2), 1.69 (p, 2H, J = 7.4 Hz, 3-CH_2), 1.48 (br m, 2H, 16-CH_2), 1.30 (br m, 6H, 17−19-CH_2), 0.89 (m, 3H, 20-CH_3).

Methyl 14-Oxotetradeca-*cis*-5,8,11-trienoate (6). The diol ester (140 mg, 0.40 mmol) in dry CH_2Cl_2 (1 mL filtered through basic Al_2O_3) was cooled to −24 °C and treated with lead tetraacetate (0.42 mmol/1 mL CH_2Cl_2) and stirred for 30 min. The residue after evaporation was chromatographed on silica gel with 5% EtOAc−hexane to afford 40 mg (40%) of the unstable aldehyde: ^{1}H NMR ($CDCl_3$) δ 9.68 (t, 1H, J = 1.9 Hz, CHO), 5.61 (m, 2H, 11,12-vinyl H), 5.38 (m, 4H, 5,6,8,9-vinyl H), 3.67 (s, 3H, OCH_3), 3.24 (d, 2H, J = 6.6 Hz, 13-CH_2), 2.80 (m, 4H, 7,10-CH_2), 2.33 (t, 2H, J = 7.4 Hz, 2-CH_2), 2.08 (m, 2H, 4-CH_2), 1.71 (p, 2H, J = 7.4 Hz, 3-CH_2).

Methyl 14-Hydroxytetradeca-*cis*-5,8,11-trienoate (7). To a solution of the diol ester (3.48 g, 9.89 mmol) in dry CH_2Cl_2 (40 mL) under nitrogen at −20 °C was added lead tetraacetate (4.85 g 95%, 10.4 mmol) with stirring. The resulting slurry was stirred at −20 °C for 20 min and 0 °C for 30 min. Ethylene glycol (0.04 mL) was added to quench the excess lead tetraacetate (starch iodide paper). After stirring for 45 min at 0 °C the reaction was filtered through silica gel 60 (50 g) topped with Celite eluting with CH_2Cl_2. The solvent was evaporated in vacuo and traces of residual acetic acid were removed azeotropically with toluene. The slightly yellow oil was dissolved in methanol (40 mL), cooled in an ice bath, and treated with $NaBH_4$ (1.5 g, 40 mmol) with stirring. Stirring overnight at ambient temperature left some aldehyde unreacted, and a further 350 mg $NaBH_4$ was added at 0 °C and stirred for 3 h. The reaction was quenched with 1 N HCl to pH 3 and the methanol evaporated in vacuo. The aqueous layer was extracted with CH_2Cl_2 and then ether, and the combined organic layers were washed with aqueous $NaHCO_3$ and then brine and dried over Na_2SO_4. Chromatography on silica gel 60 (100 g) eluting with 50% ether−hexane afforded 1.93 g (73%) of the title compound: ^{1}H NMR ($CDCl_3$) δ 5.50 (m, 1H, vinyl H), 5.35 (m, 5H, vinyl H), 3.64 (s, overlap, 3H, OCH_3), 3.63 (t, J = 6.6 Hz, 14-CH_2-O-), 2.81 (m, 4H, 7,10-CH_2), 2.33 (m, 2H, 13-CH_2), 2.31 (t, overlap, 2H, J = 7.4 Hz, 2-CH_2), 2.09 (m, 2H, 4-CH_2), 1.77 (br s, 1H, OH), 1.71 (p, 2H, J = 7.4 Hz, 3-CH_2).

Methyl 14-(*p*-Tolylsulfonyl)tetradeca-*cis*-5,8,11-trienoate (8). The 14-hydroxy ester (1.17 g, 4.64 mmol) and anhydrous pyridine (0.75 mL, 9.69 mmol) in $CHCl_3$ (4.6 mL) was cooled to 0 °C and treated with *p*-toluenesulfonyl chloride (1.33 g, 6.98 mmol) and refrigerated overnight. The reaction was diluted with ether and washed with 2 N HCl, aqueous $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and combined with a similar preparation from 2.06 mmol of the hydroxy ester and chromatographed on silica gel 60 eluting with 30% ether in hexane to yield 2.3 g (84%) of the title tosylate; ^{1}H NMR ($CDCl_3$) δ 7.77 (d, 2H, J = 8.3 Hz, ArH_2), 7.32 (d, 2H, J = 8.3 Hz, ArH'_2), 5.43 (m, 1H, vinyl H), 5.31 (m, 5H, vinyl H), 4.01 (t, 2H, J = 6.9 Hz, 14-CH_2-O-Ts), 3.64 (s, 3H, OCH_3), 2.74 (m, 4H, 7,10-CH_2), 2.43 (s, overlap, 3H, Ar-CH_3), 2.42 (m, 2H, 13-CH_2), 2.30 (t, 2H, J = 7.5 Hz, 2-CH_2), 2.07 (m, 2H, 4-CH_2), 1.68 (p, 2H, J = 7.4 Hz, 3-CH_2).

Methyl 14-(Triphenylphosphoniumyl)tetradeca-*cis*-5,8,11-trienoate *p*-Toluenesulfonate (9). A solution of the tosyl ester (1.63 g, 4.01 mmol) and triphenylphosphine (1.15

g, 4.38 mmol) in dry acetonitrile (1.5 mL) was heated at 88 °C in a sealed Reacti-vial with stirring until TLC (ethyl ether−hexane, 3:7) analysis of an aliquot showed complete consumption of the starting tosylate (4 days). Evaporation of the acetonitrile in vacuo, addition of a minimal amount of CH_2Cl_2, and trituration with ethyl ether gave 2.1 g (78%) of the title compound as a red oil after high-vacuum drying. A subsequent, larger scale reaction under atmospheric pressure reflux (98 °C) and a petroleum ether trituration gave a 96% yield of comparable material: ^{1}H NMR ($CDCl_3$) δ 7.67 (m, 17H, PPh_3 + ArH_2), 6.96 (d, 2H, J = 7.9 Hz, ArH'_2), 5.41 (m, 1H, vinyl H), 5.22 (m, 5H, vinyl H), 3.57 (s, 3H, OCH_3), 3.54 (m, 2H, 14-CH_2), 2.55, 2.46 (t, t, 4H, J = 6.4, 7.1 Hz, 7,10-CH_2), 2.32 (br m, 2H, 13-CH_2), 2.22 (s, overlap, 3H, Ar-CH_3), 2.21 (m, 2H, 2-CH_2), 1.97 (m, 2H, 4-CH_2), 1.60 (p, 2H, J = 7.4 Hz, 3-CH_2).

Methyl 16,16-Dimethyldocosa-*cis*-5,8,11,14-tetraenoate (16). A solution of the phosphonium salt (1.46 g, 2.18 mmol) in anhydrous THF (20 mL) under argon was cooled to −73 °C and treated with *n*-BuLi (1.4 mL 1.56 M in hexanes, 2.18 mmol) with stirring. The resulting deep red solution was stirred at −73 °C for 30 min when 2,2-dimethyl-1-octanal (360 mg, 2.3 mmol) was added. Stirring for 1 h at −73 °C and then 1.5 h at −73 to −30 °C was followed by quenching with pH 7 phosphate buffer and extraction with ethyl ether (3×). The combined organic layers were washed with brine and dried over Na_2SO_4. The residual oily solids upon evaporation of the volatiles in vacuo were triturated with ethyl ether, refrigerated, and filtered to remove triphenylphosphine oxide. Evaporation of the ether and chromatography of the residual oil (0.96 g) on silica gel 60 with CH_2Cl_2 afforded 463 mg of a mixture which was readily separated by chromatography on a size B Merck Prepak C18 reverse phase column eluting with 95% methanol−water to yield 196 mg (24%) of the title compound: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.36 (m, 6H, 5,6,8,9,11,12-vinyl-H), 5.24 (d, 1H, J = 12 Hz, 15-vinyl-H), 5.16 (dt, 1H, J = 12, 7.3 Hz, 14-vinyl-H), 3.66 (s, 3H, OCH_3), 2.93 (dt, 2H, J = 6.4, 0.6 Hz, 13-CH_2), 2.81 (m, 4H, 7,10-CH_2), 2.32 (t, 2H, J = 7.5 Hz, 2-CH_2), 2.10 (m, 2H, 4-CH_2), 1.70 (p, 2H, J = 7.4 Hz, 3-CH_2), 1.28 (m, 10H, 17−21-CH_2), 1.09 (s, 6H, *gem*-Me_2), 0.87 (t, 3H, J = 6.8 Hz, 22-CH_3); HRMS calcd for $C_{25}H_{42}O_2$ 374.3185, found 374.3188; ^{13}C NMR δ 174.0, 139.2, 129.0, 128.96, 128.89, 128.22, 128.20, 127.9, 127.0, 51.4, 44.3, 36.4, 33.5, 31.9, 30.2, 29.0, 26.8, 26.6, 25.7, 25.6, 24.8 (overlapped), 22.7, 14.1.

Ethyl 2,2-Dimethyloctanoate (11). Ethyl isobutyrate (11.7 mL, 87.6 mmol) was added to a commercial solution of lithium diisopropylamide (2.0 M in heptane−THF−ethylbenzene) (49.5 mL, 99 mmol) in anhydrous THF (86 mL, distilled from Na/benzophenone) at −74 °C with stirring. After 30 min, 1-bromohexane (13.5 mL, 96.2 mmol) in HMPA (16.5 mL) was added, and the reaction was stirred for 10 min at −74 °C and then allowed to warm slowly to room temperature over approximately 2 h. The reaction was quenched with water (5 mL), stirred briefly, and treated with solid Na_2SO_4 followed by filtration and evaporation in vacuo. The oily mixture was chromatographed on silica gel (400 g) eluting with ethyl ether to afford 17.4 g (99%) of the title compound: ^{1}H NMR ($CDCl_3$) δ 4.11 (q, 2H, J = 7.1 Hz, OCH_2), 1.48 (m, 2H, 3-CH_2), 1.23 (m, 11H, 4−7-CH_2, OCH_2CH_3), 1.15 (s, 6H, *gem*-Me_2), 0.87 (t, 3H, J = 6.4 Hz, 8-CH_3).

2,2-Dimethyloctan-1-ol (12). A solution of $LiAlH_4$ in THF (45 mL, 1.0 M, 45 mmol) was added to a ice-bath-cooled solution of ethyl 2,2-dimethyloctanoate (17.4 g, 87 mmol) in anhydrous ethyl ether (100 mL) with stirring. After the vigorous reaction subsided, the cooling bath was removed and stirring was continued overnight at ambient temperature. The reaction was cooled in an ice bath and quenched by sequential addition of water (5 mL), 15% NaOH (5 mL), and water (15 mL) with stirring. After 45 min, the slurry was filtered, washing the solids with ethyl ether. The aqueous phase was separated, and the ether solution was dried over Na_2SO_4. The residue obtained upon evaporation in vacuo was chromatographed on silica gel (200 g) eluting with CH_2Cl_2 affording 12.5 g (93%) of the title alcohol: ^{1}H NMR ($CDCl_3$) δ 4.11 (s, 2H, OCH_2), 1.61 (br, 1H, OH), 1.22 (br "d", 10H, 3−7-CH_2), 0.85, 0.83 (m, s, overlap, 9H, 8-CH_3, *gem*-Me_2).

2,2-Dimethyloctanal (14). A solution of 2,2-dimethyloctan-1-ol (3.4 g, 21.5 mmol) in dry CH_2Cl_2 (30 mL) was added to pyridinium chlorochromate (7.16 g, 33.2 mmol) in dry CH_2Cl_2 (30 mL) at ambient temperature with stirring. After stirring for 3 h, the completed reaction was diluted with ethyl ether (75 mL) which was filtered through a plug of Florisil followed by 300 mL of ether washes of the tarry residue from the flask. The resulting pale yellow liquid from evaporation of the ether in vacuo was chromatographed on silica gel 60 (100 g) eluting with 20% EtOAc−hexane affording 2.9 g (86%) of the title aldehyde: ^{1}H NMR ($CDCl_3$) δ 9.41 (s, 1H, CHO), 1.42 (m, 2H, 3-CH_2), 1.23 (br m, 8H, 4−7-CH_2), 1.01 (s, 6H, *gem*-Me_2), 0.85 (m, 3H, 8-CH_3).

(16,16-Dimethyldocosa-*cis*-5,8,11,14-tetraenoyl)ethanolamine (17a). A solution of the corresponding ester (199 mg, 0.53 mmol), NaCN (2.6 mg, 0.053 mmol), and ethanolamine (0.34 mL, 5.5 mmol) in methanol (1 mL) in a sealed Reacti-vial was heated in a hot block at 50 °C overnight. The completed reaction was diluted with ethyl ether and washed consecutively with 1 N HCl, aqueous $NaHCO_3$, and brine. Drying over Na_2SO_4, evaporation of the solvent, and chromatography on silica gel 60 (10 g) with 75% EtOAc−hexane afforded 175 mg (82%) of the title (dimethylheptyl)anandamide analog: ^{1}H NMR ($CDCl_3$) δ 5.96 (br s, 1H, NH), 5.34 (m, 6H, 5,6,8,9,11,12-vinyl-H), 5.21 (m, 2H, 14,15-vinyl-H), 3.71 (br, 2H, OCH_2), 3.42 (q, 2H, J = 5.2 Hz, N-CH_2), 2.92 (t, 2H, J = 5.8 Hz, 13-CH_2), 2.82 (m, 4H, 7,10-CH_2), 2.21 (t, 2H, J = 7.7 Hz, 2-CH_2), 2.11 (m, 2H, 4-CH_2), 1.71 (p, 2H, J = 7.5 Hz, 3-CH_2), 1.25 (m, 10H, 17−21-CH_2), 1.08 (s, 6H, *gem*-Me_2), 0.87 (m, 3H, 22-CH_3); HRMS calcd for $C_{26}H_{45}NO_2$: 403.3450, found 403.3453; ^{13}C NMR δ 174.1, 139.2, 129.10, 129.08, 128.9, 128.3, 128.2, 127.9, 127.0, 62.7, 44.4, 42.5, 36.4, 36.0, 31.9, 30.2, 29.0, 26.8, 26.7, 25.74, 25.67, 25.5, 24.8, 22.7, 14.1. HPLC, eluant a, 10.1 min (98%); eluant c, 9.3 min (∼98%).

(*R*)-(16,16-Dimethyldocosa-*cis*-5,8,11,14-tetraenoyl)-1′-hydroxy-2′-propylamine (17b). A methanol solution (1 mL) of the corresponding ester (204 mg, 0.55 mmol), NaCN (2.7 mg, 0.055 mmol), and (*R*)-(−)-2-amino-1-propanol (0.45 mL, 5.8 mmol) heated overnight in a hot block at 50 °C, worked up as for the corresponding ethanolamide, and chromatographed on silica gel 60 (40 g) eluting with 50% EtOAc−hexane afforded some recovered ester and 176 mg (77%) of the title amide: ^{1}H NMR ($CDCl_3$) δ 5.73 (br d, 1H, J = 6.5 Hz, NH), 5.36 (m, 6H, 5,6,8,9,11,12-vinyl-H), 5.21 (m, 2H, 14,15-vinyl-H), 4.04 (m, 1H, N-CH), 3.64 (br m, 1H, OCH), 3.52 (br m, 1H, OCH′), 3.18 (br s, 1H, OH), 2.91 (t, 2H, J = 5.9 Hz, 13-CH_2), 2.79 (m, 4H, 7,10-CH_2), 2.18 (t, 2H, J = 7.6 Hz, 2-CH_2), 2.08 (m, 2H, 4-CH_2), 1.71 (p, 2H, J = 7.4 Hz, 3-CH_2), 1.27 (m, 10H, 17−21-CH_2), 1.15 (d, 3H, J = 6.8 Hz, N-C-CH_3), 1.08 (s, 6H, *gem*-Me_2), 0.86 (m, 3H, 22-CH_3); HRMS calcd for $C_{27}H_{47}NO_2$ 417.3607, found 417.3609; ^{13}C NMR δ carbonyl and quaternary carbon too weak to be seen, 139.2, 129.1 (overlapped), 128.9, 128.3, 128.2, 127.9, 127.0, 67.5, 47.9, 44.4, 36.1, 31.9, 30.2, 29.0, 26.8, 26.7, 25.74, 25.67, 25.5, 24.8, 22.7, 17.1, 14.1; HPLC, eluant a, 10.7 min (99%); eluant c, 9.1 min (∼99%).

(*S*)-(16,16-Dimethyldocosa-*cis*-5,8,11,14-tetraenoyl)-2′-hydroxy-1′-propylamine (17c). A methanol solution (0.5 mL) of the corresponding ester (100 mg, 0.27 mmol), NaCN (1.4 mg, 0.028 mmol), and (S)-(+)-1-amino-2-propanol (0.21 mL, 2.7 mmol) heated 2 days in a hot block at 50 °C resulted in the formation of product along with residual ester (TLC). The reaction was worked up as for the corresponding ethanolamide and chromatographed on silica gel 60 (5 g) eluting with 50% to 75% EtOAc−hexane mixtures to afford ∼20 mg of the recovered ester and 72 mg (65%) of the title amide: ^{1}H NMR ($CDCl_3$) δ 5.99 (br s, 1H, NH), 5.36 (m, 6H, 5,6,8,9,11,12-vinyl-H), 5.18 (m, 2H, 14,15-vinyl-H), 3.89 (m, 1H, O-CH), 3.42 (m, 1H, NCH), 3.10 (m, 1H, NCH′), 2.92 (t, 2H, J = 6.1 Hz, 13-CH_2), 2.81 (m, 4H, 7,10-CH_2), 2.21 (t, 2H, J = 7.6 Hz, 2-CH_2), 2.09 (m, 2H, 4-CH_2), 1.71 (p, 2H, J = 7.5 Hz, 3-CH_2), 1.25 (br m, 10H, 17−21-CH_2), 1.18 (d, 3H, J = 6.3 Hz, O-C-CH_3), 1.08 (s, 6H, *gem*-Me_2), 0.87 (m, 3H, 22-CH_3); HRMS calcd for $C_{27}H_{47}NO_2$ 417.3607, found 417.3609; ^{13}C NMR δ 173.9, 139.1, 129.0 (overlapped), 128.8, 128.2, 128.1, 127.8, 126.9, 67.6, 47.0, 44.3, 36.3, 35.9, 31.8, 30.1, 28.9, 26.7, 26.6, 25.65, 25.58, 25.4, 24.7,

22.6, 21.0, 14.0; HPLC, eluant a, 10.7 min (~97%); eluant c, 8.7 min (99%).

(16,16-Dimethyldocosa-*cis*-5,8,11,14-tetraenoyl)-2′-methoxyethylamine (17d). A methanol solution (0.5 mL) of the corresponding ester (100 mg, 0.27 mmol), NaCN (1.3 mg, 0.026 mmol), and 2-methoxyethylamine (0.24 mL, 2.8 mmol) heated 2 days in a hot block at 50 °C resulted in the formation of product along with residual ester (TLC). The reaction was worked up as for the corresponding ethanolamide and chromatographed on silica gel 60 (5 g) eluting with 40% EtOAc - hexane to afford 40 mg of the recovered ester and 46 mg (41%) of the title amide; ^{1}H NMR ($CDCl_3$) δ 5.79 (br s, 1H, NH), 5.38 (m, 6H, 5,6,8,9,11,12-vinyl-H), 5.21 (m, 2H, 14,15-vinyl-H), 3.44, 3.43 (s & d overlapped, 4H, OCH_2-CH_2-N), 3.36 (s, 3H, OCH_3), 2.92 (t, 2H, J = 6.0 Hz, 13-CH_2), 2.80 (m, 4H, 7,10-CH_2), 2.18 (t, 2H, J = 7.7 Hz, 2-CH_2), 2.12 (m, 2H, 4-CH_2), 1.71 (p, 2H, J = 7.7 Hz, 3-CH_2), 1.25 (m, 10H, 17−21-CH_2), 1.08 (s, 6H, *gem*-Me_2), 0.86 (m, 3H, 22-CH_3); HRMS calcd for $C_{27}H_{47}NO_2$ 417.3607, found 417.3609; ^{13}C NMR δ carbonyl and quaternary carbon too weak to be seen, 139.1, 129.1, 129.0, 128.7, 128.2, 128.1, 127.8, 126.9, 71.2, 58.7, 44.3, 39.1, 36.0, 31.8, 30.1, 28.9, 26.7, 26.6, 25.64, 25.57, 25.4, 24.7, 22.6, 14.0. HPLC eluant a, 12.7 min (96%); eluant c, 11.5 min (~93%).

(16,16-Dimethyldocosa-*cis*-5,8,11,14-tetraenoyl)propylamine (17e). Heating a methanol solution (0.5 mL) of the corresponding ester (100 mg, 0.27 mmol), NaCN (1.5 mg, 0.031 mmol), and *n*-propylamine (0.22 mL, 2.7 mmol) for 2 days in a hot block at 50 °C resulted in the formation of product along with residual ester (TLC). The reaction was worked up as for the corresponding ethanolamide and chromatographed on silica gel 60 (5 g) eluting with 20% EtOAc−hexane to afford 30 mg of the recovered ester and 47 mg (44%) of the title amide: ^{1}H NMR ($CDCl_3$) δ 5.36 (overlap m, 7H, NH, 5,6,8,9,-11,12-vinyl-H), 5.21 (m, 2H, 14,15-vinyl-H), 3.21 (q, 2H, J = 6.7 Hz, N-CH_2), 2.92 (t, 2H, J = 5.9 Hz, 13-CH_2), 2.80 (m, 4H, 7,10-CH_2), 2.16, 2.12 (t, m, 4H, J = 7.6 Hz, 2&4-CH_2), 1.71 (p, 2H, J = 7.4 Hz, 3-CH_2), 1.52 (hx, 2H, J = 7.3 Hz, 2′-CH_2), 1.25 (m, 10H, 17−21-CH_2), 1.08 (s, 6H, *gem*-Me_2), 0.91,0.87 (t, m, 6H, 3′-CH_3, 22-CH_3); HRMS calcd for $C_{27}H_{47}NO$ 401.3658, found 401.3654; ^{13}C NMR δ 172.7, 139.2, 129.2, 129.1, 128.7, 128.3, 128.2, 127.9, 127.0, 44.4, 41.2, 36.4, 36.2, 31.9, 30.2, 29.0, 26.8, 26.7, 25.74, 25.67, 25.6, 24.8, 23.0, 22.7, 14.1, 11.4. HPLC, eluant a, 14.1 min (97%); eluant c, 11.7 min (98%).

Binding Assay. Chemicals. [^{3}H]CP 55,940 (101 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and [^{3}H]SR141716A (22.4 Ci/mmol) was synthesized at Research Triangle Institute (Research Triangle Park, NC).

Preparation of Brain Tissue. Male F344 rats (Charles River Laboratories, Raleigh, NC) weighing 200−225 g were sacrificed. The whole brains were quickly removed and placed into a 55 mL Potter-Elvehjem glass homogenizer tube maintained on ice. The tissue was subjected to the homogenization and centrifugation procedure described previously[19] to yield the final membrane preparation used in the binding assay. Total protein concentration of the resuspended membrane pellet was determined by a dye-binding assay commercially available from Bio-Rad Laboratories (Hercules, CA). Aliquots of the membrane preparation were stored at −70 °C until use.

Competition Assays. The anandamides **2a**−**e** and **17a**−**e** were evaluated for their ability to compete for the binding of [^{3}H]CP 55,940 or [^{3}H]SR141716A. Competing compounds were prepared in buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 3 mM $MgCl_2$, and 0.5% (w/v) BSA (buffer A) at concentrations ranging from 0.01 to 25 000 nM for **17a**−**e** and ranging from 0.4 to 100 000 nM for **2a**−**e**. Tritiated compounds were diluted in buffer A to yield a concentration of 7.2 nM for [^{3}H]CP 55,940 and 20 nM for [^{3}H]SR141716A. Unlabeled drug for determination of nonspecific binding was prepared at a concentration of 100 μM.

The competition assays were conducted in a total volume of 1 mL in silylated glass test tubes. The reaction mixtures (in duplicate) consisted of 100 μL of tritiated drug, 100 μL of unlabeled drug dilution, 100 μL of 300 μM PMSF, and sufficient buffer A such that a total volume of 1 mL was achieved with the addition of brain extract. Duplicate tubes for nonspecific binding and total binding were prepared by adding 100 μL aliquots of the unlabeled compound to be displaced and 100 μL of buffer A, respectively. An aliquot of brain extract equivalent to 150 or 45 μg of protein was added to each tube. The final volume of the reaction mixture was brought to a total of 1 mL by the addition of buffer A. After mixing by vortex, the reaction tubes were incubated at 30 °C for 1 h. After the incubation period was complete, the reaction tubes were processed as follows. A 24-manifold Brandel cell harvester was prepared by priming with approximately 1 L of cold 50 mM Tris-HCl, pH 7.4, containing 0.1% (w/v) BSA (buffer B). Filter paper (Whatman GF/C) pretreated for 1 h in 0.1% polyethylenimine was placed into the cell harvester. At the end of the receptor binding incubation period, the reaction was terminated by vacuum filtration of the reaction mixture. The reaction tubes were then rinsed twice with approximately 4 mL of buffer B. After rinsing, the filter paper was removed and placed into liquid scintillation vials. To each vial was added 1 mL of H_2O and 10 mL of scintillation cocktail. The samples were placed on a shaker for 30 min and then counted in a liquid scintillation counter for a statistically appropriate amount of time.

Calculations. Competition assays were analyzed using the EBDA Ligand software (release 2.0, Biosoft), which calculated and plotted the displacement curve and the Hofstee plot and calculated the K_i for the competing compounds. As was done for the K_d values, K_i values are presented as means ± SEM ($n \geq 3$). Two-tailed *t*-tests were performed in order to statistically compare the K_i values obtained between the two radioligands for all compounds.

In Vitro Pharmacology. Log concentration−response curves were constructed using the mouse isolated vas deferens assay. The measured response was drug-induced inhibition of electrically evoked isometric contractions.[43] The degree of inhibition of evoked contractions has been expressed in percentage terms and was calculated by comparing the amplitude of contractions immediately before drug administration with their amplitude at various times after drug administration. All compounds were mixed with two parts of Tween 80 by weight and dispersed in a 0.9% aqueous solution of NaCl (saline).[42] To determine the goodness of fit of log concentration response curves to a sigmoid shape, correlation coefficients (r^2) were calculated by nonlinear regression analysis using GraphPad InPlot (GraphPad Software, San Diego) ($P < 0.05$). This method was also used to calculate the size of the mean maximal effect of each compound (E_{max}), the mean drug concentration (EC_{50}) producing 50% of E_{max} and the 95% confidence limits of these values. Calculated E_{max} values that did not deviate significantly from 100% were constrained to this value when calculating EC_{50} and r^2 values.

In Vivo Pharmacology. Methods. Drug Preparation and Administration. For binding assays, compounds were prepared as 1 mg/mL stock solutions in absolute ethanol and were stored at −20 °C. For behavioral assays, drugs were dissolved in a 1:1:18 mixture of ethanol, Emulphor (GAF Corporation, Linden, NJ), and saline (0.9% NaCl) and were administered intravenously (iv) in the mouse tail vein in volumes of 0.1 mL/10 g of body weight.

Behavioral Evaluations. Mice were acclimated to the laboratory overnight. Depression of locomotor activity and antinociception, as determined by the tail-flick (TF) response to a heat stimulus,[48] were measured in the same animal. Control tail-flick latencies of 2 to 4 s were measured for each animal with a standard tail-flick apparatus prior to drug or vehicle administration. Four minutes following an iv injection of either vehicle or drug, mice were tested for tail flick response. Immediately thereafter, the mice were placed into individual photocell activity cages (11 × 6.5 in.) for measurement of spontaneous activity (SA). For the next 10 min the total number of beam interruptions in the 16 photocell beams per cage were recorded using a Digiscan animal activity monitor (Omnitech Electronics Inc., Columbus, OH). SA was expressed as percent of control activity, whereas antinociception was expressed as the percent maximum possible effect (% MPE) using a 10 s maximum test latency as described earlier.[48] Each dose tested in the antinociception and hypomotility assays represents one group of animals (six mice per

group). A separate group of animals were used to determine cannabinoid-induced hypothermia and immobility. Prior to vehicle or drug administration, rectal temperature (RT) was determined by a thermistor probe (inserted 25 mm) and a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Four minutes after the iv injection of the drug, the body temperature of the mice was recorded. The difference between pre- and postinjection rectal temperatures was calculated. Immediately after measure of body temperature, the mice were placed on a 5.5 cm ring attached at a height of 16 cm to a ring stand and the amount of time the animals remained motionless during a 5-min period was recorded.[49] The time that each animal remained motionless on the ring was divided by 300 s and multiplied by 100 to obtain a percent ring immobility rating (RI).

Data Analysis. Dose−response relationships were determined for each analog in the pharmacological assays. Percent effect was determined on the basis of the maximal effects that are produced by Δ^9-THC and anandamide in the behavioral assays which are 90% (SA), 100% (TF), and 60% (RI). The percent effect for hypothermia was based upon the maximal effect produced by anandamide (−3.0 °C) rather than produced by Δ^9-THC (−6.0 °C). Antinociception, hypomotility, and immobility data were converted to probit values, and ED_{50}'s were calculated by unweighted least-squares linear regression analysis of the log dose versus the probit values. Analogs that produced dose-responsive effects that failed to exceed 60% effect were classified as partial agonists. Analogs producing effects less than 30% or hypothermia less than 1 °C at the highest dose tested were considered to be inactive.

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