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Stereochemical Selectivity of Methanandamides for the CB1 and CB2 Cannabinoid Receptors and Their Metabolic Stability

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Abstract—Several chiral, analogues of the endogenous cannabinoid receptor ligand, arachidonylethanolamide (anandamide), methylated at the 2,1′ and 2′ positions using asymmetric synthesis were evaluated in order to study (a) stereoselectivity of binding to CB1 and CB2 cannabinoid receptors; and (b) metabolic stability with regard to anandamide amidase. Enantiomerically pure 2-methyl arachidonic acids were synthesized through diastereoselective methylation of the respective chiral 2-oxazolidinone enolate derivatives and CB1 and CB2 receptor affinities of the resulting chiral anandamides were evaluated using a standard receptor binding assay. Introduction of a single 2-methyl group increased affinity for CB1, led to limited enantioselectivity and only modestly improved metabolic stability. However, a high degree of enantio- and diastereoselectivity was observed for the 2,1′-dimethyl analogues. (*R*)-*N*-(1-methyl-2-hydroxyethyl)-2-(*R*)-methyl-arachidonamide (**4**) exhibited the highest CB1 receptor affinity in this series with a K_i of 7.42 nM, an at least 10-fold improvement on anandamide ($K_i = 78.2$ nM). The introduction of two methyl groups at the 2-position of anandamide led to no change in affinity for CB1 but somewhat enhanced metabolic stability. Conversely, chiral headgroup methylation in the 2-*gem*-dimethyl series led to chiral analogues possessing a wide range of CB1 affinities. Of these the (*S*)-2,2,2′-trimethyl analogue (**12**) had the highest affinity for CB1 almost equal to that of anandamide. In agreement with our previous anandamide structure–activity relationship work, the analogues in this study showed high selectivity for the CB1 receptor over CB2. The results are evaluated in terms of stereochemical factors affecting the ligand's affinity for CB1 using receptor-essential volume mapping as an aid. Based on the results, a partial CB1 receptor site model is proposed, that bears two hydrophobic pockets capable of accommodating 1′- and 2-methyl groups

Introduction

In 1992, arachidonylethanolamide (anandamide) was isolated from porcine brain and considered the first, putative endogenous cannabinoid ligand.[1] Since then, experimental evidence has supported the postulate that this ligand plays an important role in cannabinoid biology. Anandamide was, thus, shown to have inhibitory effects in N-type calcium-currents through a pertussis toxin-sensitive pathway in N18 neuroblastoma cells,[2] forskolin-stimulated adenylate cyclase activity[3,4] and electrically evoked twitch response of the mouse vas deferens.[5,6] In vivo, it was shown[7–11] to produce the four characteristic effects of cannabimimetics, namely, analgesia, hypothermia, hypoactivity and catalepsy. Anandamide's pharmacological profile was generally found[9] to parallel that of Δ^9-THC. However, the endogenous ligand is somewhat less potent, with a rapid onset and shorter duration of action,[9,10] presumably due to metabolic instability which is attributed to the presence of anandamide amidase, an enzyme possessing amidohydrolase activity.[12–15] This enzyme was shown to be colocalized in the same brain regions as the CB1 cannabinoid receptor.[14] The hydrolytic breakdown of anandamide can be prevented in vitro by phenylmethanesulfonyl fluoride (PMSF), a general serine protease inhibitor.[12,13] For this purpose, PMSF is included in the binding assay in structure–activity relationship (SAR) studies of anandamide analogues where amidase-catalyzed anandamide hydrolysis might be a complicating factor.[16] In addition to anandamide, two other unsaturated fatty acid ethanolamides, dihomo-γ-linolenolethanolamide and docosatetraenoylethanolamide, were isolated from porcine brain and shown to possess

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cannabimimetic properties.[17] Recently, a system responsible for the reuptake of anandamide was partially characterized[18] and the ligand's structural requirements for binding and transport were identified.[19] Although the physiological role of anandamide is not yet fully understood, recent evidence suggests that this cannabinoid receptor ligand, at least in the dorsal stratium, acts as an autacoid neuromodulator countering D_2-activation.[20,21]

Several SAR studies[22–34] of anandamides have already identified some of the stereoelectronic requirements for interactions with the CB1 receptor. Most of the published work has addressed the SAR of the head group and more limited work has focused on structural modifications of the hydrophobic tail. It was thus shown that, in close analogy with the classical cannabinoids, replacement of the terminal five carbon atom chain of anandamide by a dimethylheptyl chain, resulted in analogues possessing higher affinity for CB1.[22,23] Substitution of the arachidonyl chain with other fatty acid chains having fewer than 20 carbon atoms or three double bonds or with *trans* or non ω-6 configuration, leads to dramatic loss of affinity.[4,24,29,30,34] In another study, anandamide was oxidized by three different lipoxygenases resulting in monohydroxy products at different positions.[32] Only the product of 11-lipoxygenase showed receptor affinity comparable to that of anandamide. The CB1 SAR of the anandamide head group can be summarized as follows: (a) phenyl or other cyclic groups are not tolerated in that position;[27] (b) the terminal hydroxyl group can be replaced with fluoro,[24,26,30,33] chloro,[30] vinyl,[30] or a methyl[25] group with an increase in affinity while the *O*-methyl analogue had diminished affinity;[24,25] (c) a three carbon chain is the optimal head group length for the *N*-hydroxyalkyl or the *N*-alkyl derivatives;[4,25] and (d) generally, tertiary amides lack affinity for CB1 with the only known exception of *N*,*N*-di-hydroxyethyl analogue.[27]

Our initial efforts to develop metabolically stable anandamide analogues showed that this can be accomplished by sterically hindering the scissile amide bond through the introduction of α-methyl groups. Of the ensuing analogues, (*R*)-(+)-arachidonyl-1′-hydroxy-2′-propylamide, or (*R*)-methanandamide (AM356) was the most successful exhibiting a 4-fold higher affinity for CB1 than anandamide, remarkable hydrolytic stability[28,35] and high in vivo potency.[10,28]

As an extension of our work that led to the discovery of (*R*)-methanandamide we undertook a more comprehensive attempt to study the enantio- and diastereoselectivity of a family of 2-methylated anandamides with regard to their affinities for the cannabinoid receptors and their enzymatic stability. In view of the fact that 2-methylation of anandamide is well tolerated in terms of its CB1 affinity,[26,33,34] we felt that we had good chance of being successful. The results have allowed us to propose a partial pharmacophoric model for the CB1 receptor with respect to anandamide binding. The work was also motivated by earlier observations according to which introduction of methyl substitution in neurotransmitters, endogenous ligands and prototypical drug molecules has served as an excellent vehicle for studying stereochemical recognition in ligand–receptor interactions, as well as a means of increasing hydrolytic stability. Subsequently, the stereochemical implications accompanying methyl group substitution in anandamide are examined using molecular modeling as an aid.

Results

Chemistry

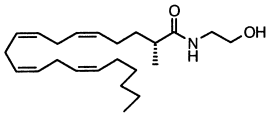
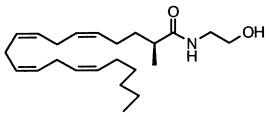
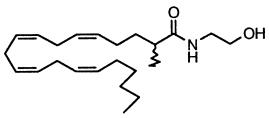
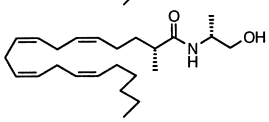
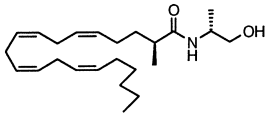
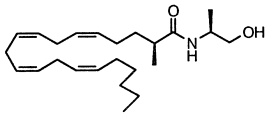
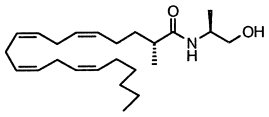
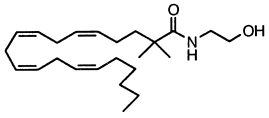
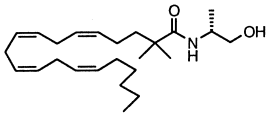
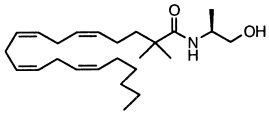
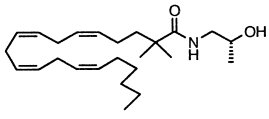
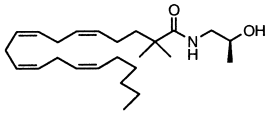
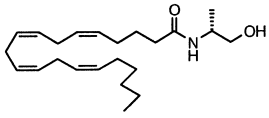
Anandamide analogues **1**–**7** (Table 1) were prepared by reaction of the appropriate amino alcohols with either (*R*)-, (*S*)-, or racemic 2-methyl arachidonic acid chlorides which in turn were obtained from the corresponding acids by treatment with oxalyl chloride in the presence of dimethylformamide.[28,30] Optically pure 2-methyl-arachidonic acids were obtained by asymmetric methylation of arachidonic acid which was derivatized with 4-benzyl-2-oxazolidinone, a chiral auxiliary, first introduced by Evans[36–40] (Scheme 1, method A). Initially, arachidonic acid was coupled with lithiated benzyl-oxazolidinones through its acid chloride. Subsequently, the resulting *N*-arachidonyl-4-benzyl-2-oxazolidinones were transformed into their chiral enolates with sodium bis(trimethylsilyl)amide at −78 °C in THF, which in turn were methylated with a high degree of diastereoselectivity (d.e. was determined to be between 90 and 94% by GC–MS analysis).

There is strong precedence[37,40] that this reaction proceeds through the *Z*-sodium-enolate with the benzyl group blocking one face of the nearly planar ring system. Therefore, alkylation occurs predominately *anti* to the benzyl group.

Purification by silica gel column chromatography led to optically pure *N*-arachidonyl-4-benzyl-2-oxazolidinones. As with earlier reports[36–40] involving similar asymmetric synthesis, the absolute configuration at the 2-position was assigned by analogy, based on the expected direction of the methylation (*anti* to the benzyl group). Unlike the earlier work by Ryan et al.,[33] in which the 2-methyl arachidonic acids were obtained by chiral derivatization-resolution, the chiroselective method described here allows us to assign the absolute configuration of the resulting chiral methyl anandamides, by analogy. Finally, the oxazolidinone chiral auxiliary was removed hydrolytically ($LiOH/H_2O/THF$) at 0 °C. Absence of racemization in this reaction was demonstrated by recoupling the resulting methylated arachidonic acid with optically pure 4-benzyl-2-oxazolidinone, where a single reaction product was obtained.

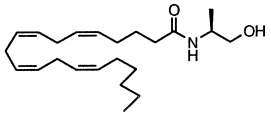
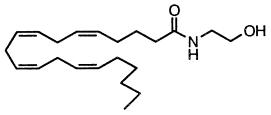
The effectiveness of the above method was validated by resynthesizing diastereomers **4**–**7** via an alternative route (Scheme 1, method B) involving the coupling of racemic 2-methyl-arachidonic acid with enantiomerically pure 2-amino-1-propanol. The resulting diastereomeric mixture was easily resolvable by silica gel column chromatography. In this resolution, the higher

Table 1. Affinities (K_i) of anandamide analogues for the cannabinoid receptors CB1 and CB2[a]

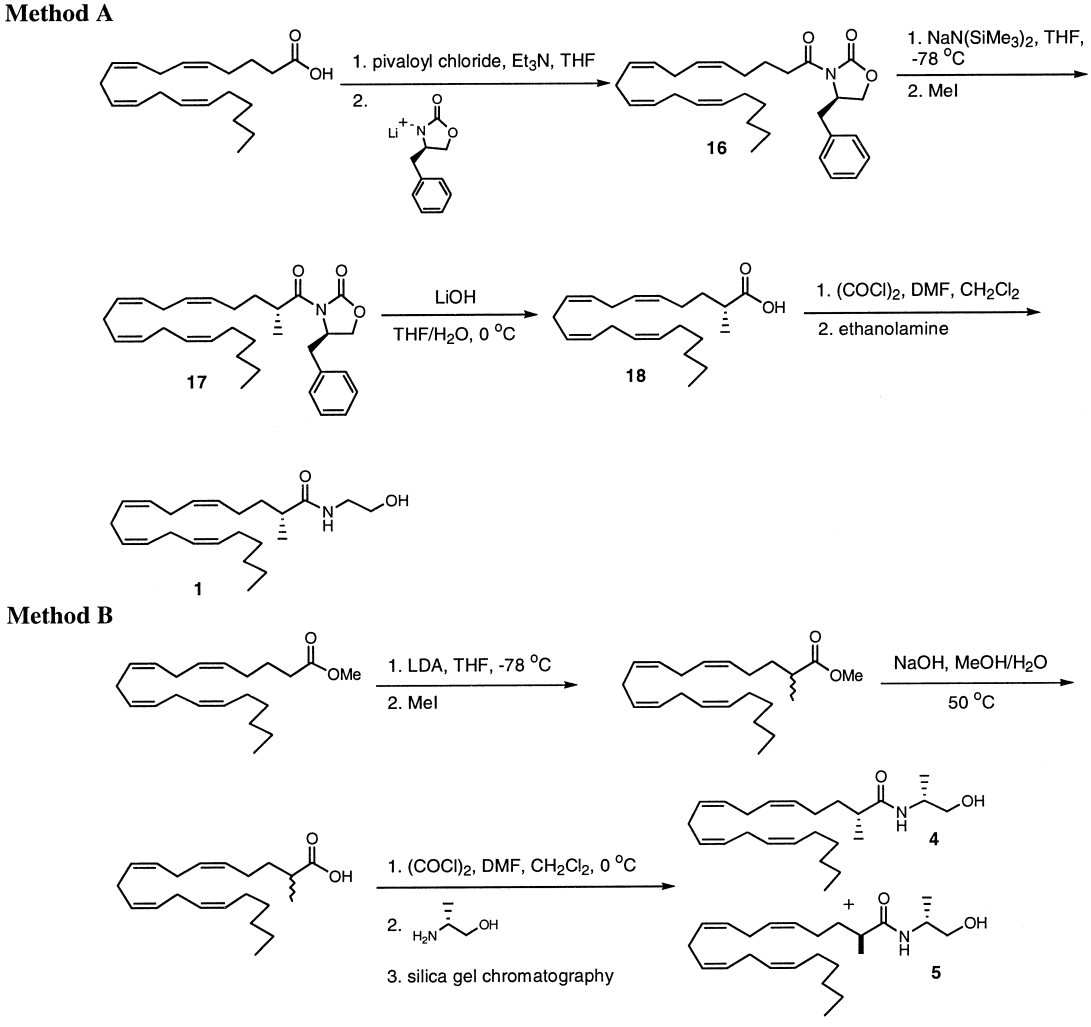
Compound		Affinity for the Cannabinoid receptors (K_i in nM)		
		CB1		CB2
		with PMSF	without PMSF	
1		54.1±5.2	457±32	4905±621
2		35.3±4.3	3941±311	4259±524
3		45.8±6.2	1865±255	3062±548
4		32.5±5.1*53±15** 7.42±0.86	137±20** 40.4±3.9	1952±212
5		185±12	211±29	4876±381
6		389±72	1132±122	2876±381
7		233±69	211±36	4695±646
8		72.2±6.3 25.5±2.8* 47±3**	150±14	$>10\times10^3$
9		220±21	41±3** 257±28	$>10\times10^3$
10		31.1±1.0* 876±92	1661±183	$>10\times10^3$
11		191.4±24.5* 1288±133	1794±194	$>10\times10^3$
12		153.9±30.0* 107±18	273±50	421±38
13		46.6±2.2* 20.6±1.6	28.3±3	868±132

(*continued on next page*)

Table 1 (*continued*)

	Compound	Affinity for the Cannabinoid receptors (K_i in nM)		
		CB1		CB2
		with PMSF	without PMSF	
14		173±46	268±101	8216±904
15		78.2±1.6	1180	1926±4.5

[a]CB1 affinities were determined using rat brain membranes and 0.8 nM [^{3}H] CP-55,940 as the tritiated ligand essentially as previously described. By running the assay both with and without PMSF the metabolic stability of the analogues is assessed. Mouse spleen was used as source of CB2 receptor. Data was analyzed using non-linear regression analysis. K_i values were obtained from a minimum of two independent experiments run in duplicate. The 95% confidence limits are in parentheses. K_i values previously reported in the literature are also included: * indicates data from ref 34 and ** from ref 26. (*R*)-methanandamide (**13**), (*S*)-methanandamide (**14**) and anandamide (**15**) have been studied in previous published papers[27,28] and are included in this table for the purpose of comparison.



Scheme 1. Synthesis of chiral methylated anandamide analogues.

R_f value diastereomer was the *R,R* analogue (**4**) and the more polar one was the *S,R* (**5**) as revealed by comparison with the corresponding products obtained through method A. Diastereomers **6** and **7** were also similarly synthesized by coupling the above acid with (*S*)-(+)-2-amino-1-propanol. All four diastereomers obtained through this method were chromatographically, spectroscopically and optically identical with those obtained through Method A. In Method B, the head group of (*R*)-(and (*S*)-methanandamide serves in itself as a chiral auxiliary in the resolution of α-methylated arachidonic acids. Unfortunately, this method is very limited, suitable only for the synthesis of the 2,1′ dimethyl analogues. In the β-methylated headgroup series, obtained by reaction of (±)-2-methyl-arachidonic acid with 1-amino-2-propanol (data not shown), the stereochemical communication between chiral methyl groups is lost and the resulting diastereomeric mixture is not easily resolvable.

The 2,2-dimethyl analogues (**8**–**12**) were synthesized, as previously,[26,34] by coupling of the appropriate amino-alcohols with 2,2-dimethyl arachidonic acid, which in turn was synthesized by repeating the first step of method 2, followed by hydrolysis.

CB1 and CB2 receptor binding studies[41,42]

Prior to assaying for CB1 receptor affinity, the synaptosomal membranes were treated with 50 μM PMSF,[27,30] a general serine protease inhibitor, to protect the analogues from the hydrolytic activity of anandamide amidase. CB1 receptor binding assays were also conducted in the absence of PMSF to assess the metabolic stability of the analogues by comparing the K_i's obtained in the presence and absence of PMSF.[27,30] For CB2 receptor affinity, mouse spleen membranes were used without PMSF, since anandamide amidase is not present in this preparation in significant amounts.[43] The K_i values of the novel analogues for the CB1 and CB2 are presented in Table 1, where (*R*)-methanandamide (**13**), (*S*)-methanandamide (**14**) and anandamide (**15**) are included for the purpose of comparison.

Computer molecular modeling

Computational studies on select analogues (**1**, **2**, **4**–**7**, **13**–**15**) were carried out using the TRIPOS Sybyl 6.5 molecular modeling package with the Tripos empirical forcefield,[44] on an SGI Octane R10,000 workstation. Among these analogues, **1**, **2**, **4**, **13**, and **15** were of high affinity for CB1 ('active') whereas **5**–**7**, and **14** were of low affinity ('inactive'). The most active analogue, **4**, was used as the starting point. The initial structure was built using standard bond lengths and angles from the Sybyl molecular modeling package. Atomic charges were calculated for all molecules by the semi-empirical method (MOPAC/AM1),[45] and potential energies were minimized by using the Tripos force field in two steps, first using the steepest descent method for 100 iterations and then the conjugate gradient method until the maximum derivative was less than 0.001 kcal mol^{-1} $Å^{-1}$. The conformation of **4** was determined by the Sybyl random search method while constraining the C5–C20 part of the molecule (torsion force: 100 kcal rad^{-2}). Within the searched low energy conformations, the conformer shown in Figure 2 was selected as a preferred low energy conformation for **4**. Similarly, the corresponding conformers for the other compounds were chosen as low energy conformers. All other compounds were built on the basis of **4** with corresponding modifications. All conformers were energy minimized again until the energy differences between successive iterations were less than 0.001 kcal mol^{-1} $Å^{-1}$. C4 and the amide oxygen and nitrogen atoms were used as three superposition points for the RMS alignment of the analogues. Based on these superpositions, receptor volume maps[46] were generated using the Sybyl multiple-contour tool. The methods used are described in more detail elsewhere.[47]

Discussion

Affinities for CB1 and CB2

In an earlier study involving the synthesis of chiral 1′ and 2′ monomethyl head group substituted anandamides, we demonstrated that the (*R*)-(+)-arachidonyl-1′-hydroxy-2′-propylamide [(*R*)-methanandamide] exhibits the highest CB1 affinity and remarkable enzymatic stability.[28] Furthermore, other reports have shown that certain enantiomeric or diastereomeric mixtures of 2-methylated anandamides possess higher CB1 affinities than anandamide.[26,34] Two enantiomerically pure fluoro analogues of 2-methylarachidonylethylamides[33] were prepared and found to exhibit high affinity but no enantioselectivity in their binding with CB1. We now report the synthesis and evaluation of a series of non-fluorine containing anandamide analogues in which chiral methyl groups are introduced systematically at the head group as well as at the 2-position of the arachidonyl moiety. No effort was made to introduce alkyl groups larger than methyl at any of these positions since such substitutions have been shown to significantly lower the CB1 affinity.[26,34]

Our results show that a chiral methyl group at the 2-position of arachidonic acid leads to anandamide analogues (**1**–**3**) with moderately improved affinities for CB1 when compared to anandamide (**15**) but without significant stereoselectivity. Lack of enantioselectivity at that position was also demonstrated for the fluorinated analogues reported by Ryan et al.[33] The (*S*)-isomer (**2**) exhibits slightly higher affinity, more than twice that of the parent anandamide. However, introduction of a second chiral methyl group at the ethanolamido head group is accompanied with a major degree of enantio- and diastereoselectivity with regard to CB1 binding. Thus, the four analogues (**4**–**7**) encompassing two enantiomeric pairs exhibit an approximately 50-fold range of affinities for CB1. Of these, the 2(*R*),1′(*R*)-dimethyl analogue (**4**) had the highest affinity (K_i: 7.42 nM), 52-fold higher than its enantiomer 2(*S*),1′(*S*)-dimethyl anandamide (**6**) and an 11-fold improvement over the parent anandamide (**15**). The other pair of

enantiomers, 2(*S*),1′(*R*)-and 2(*R*),1′(*S*)-dimethyl (**5** and **7**) had very similar affinities for CB1 with K_i values in between those of the other enantiomeric pair.

As discussed in more detail in the following section, the low level of enantioselectivity observed with the 2-methyl analogues suggests that the steric requirements for that position are not as strict as for the 1′ position. Although it is well known[26,27,34] that alkyl groups larger than methyl, at either of these positions, are detrimental to CB1 affinity, it was shown[26,34] that a 2-*gem*-dimethyl group can be well tolerated. To further substantiate and validate our pharmacophoric model, we synthesized the previously reported 2,2-dimethyl analogue[26,34] (**8**) as well as its trimethyl congeners[34] (**9**–**12**) in which a third methyl group is introduced at the ethanolamido head group. The CB1 binding data reported here are in general agreement with those from earlier reports[26,34] although there are some differences in the individual K_i values. The results show that geminal dimethyl substitution alpha to the carbonyl group maintains essentially the same affinity for CB1, in support of our pharmacophoric model. However, introduction of a third methyl group at the ethanolamine moiety leads to reduction in affinity for CB1. Of the four trimethyl compounds, the 2,2,2′-(*S*)-trimethyl analogue (**12**) had the highest affinity for CB1, slightly less than that of anandamide, followed by the 2,2,1′(*R*)-trimethyl (**9**) with a K_i almost 3 times that of anandamide. The other two analogues (**10** and **11**) exhibited considerably lower affinities as Sheskin et al.[34] have also reported. The results from this series reveal that the SAR due to methyl substitution at different positions of anandamide is not always additive. However, some general trends can be observed. Thus, in the trimethyl anandamide series, 1′-methyl substitution at the head group leads to a considerable reduction in affinity when compared to the 1′-monomethyl and the 2,1′-dimethyl series where the presence of the 1′-(*R*)-methyl group leads to substantial improvement in affinity for CB1. Similarly, 2′-methyl substitution in the trimethyl series also leads to reduced affinity for CB1 unlike the monomethyl series where the 2′-(*R*)-methyl analogue has higher affinity than anandamide. However, the effect of 2′-methyl substitution in the 2,2,1′- and 2,2,2′-trimethyl analogues is less striking than that due to 1′-methyl substitution.

All the analogues in this series exhibit a high degree of selectivity for CB1 compared to CB2 with selectivity ratios ranging from 4- to at least 260-fold. This finding is in agreement with previous SAR studies[27,31] in which anandamide and its analogues were found to have low affinities for CB2. The above results may argue that anandamide is not the preferred endogenous ligand for CB2. However, the characterization of a CB2 selective endogenous ligand remains elusive, notwithstanding a report attributing this role to palmitylethanolamide.[48]

Molecular modeling of CB1 binding stereochemical requirements

It is clear from the above results that the stereoselective introduction of methyl groups at or near the anandamide head group produces striking effects on CB1 affinities and leads to chiral analogues covering a 175-fold range of K_i values. The causes for the above-mentioned steric effects can be 2-fold. First, the presence of methyl groups can lead to variations in the conformational properties of the anandamide head groups which can, in turn, play a role in the ligand's interactions with its site of action. Thus, if the preferred conformation of the ligand resembles the pharmacophoric conformation of anandamide at CB1, its ability to engage in a productive interaction with receptor is enhanced. Conversely, a conformation differing from the pharmacophoric one may lead to reduced affinity. The second reason for the broad spectrum of affinities of methylated anandamides for CB1 may be related to whether individual methyl groups can interact favorably with the receptor site by 'fitting' into a putative 'hydrophobic pocket', thus resulting in enhanced affinity. Alternatively, methyl substitution may be responsible for unfavorable interactions with the binding site due to steric constraints thus reducing the ligand's affinity for the receptor. Therefore, the introduction of two or three methyl substituents in anandamide may lead to a combination of favorable and unfavorable steric interactions at the CB1 site, depending on the positions of individual methyl groups and the absolute configurations of the methyl substituted carbons.

Examination of the binding data for the methylated anandamides described here reveals that the variation in affinities for CB1 cannot be solely attributed to the conformational properties of the ligands. The wide differences in affinities between the enantiomeric pairs of ligands (e.g., **4** and **6**) which are expected to have identical rotamer distributions, are congruent with such a conclusion. Ligand conformational analyses which were simplified by restricting our computation to the ethanolamido head group and the first five carbon atoms of the arachidonyl tail, further support this interpretation. In this study, several conformational minima were identified for each compound, all within 1 Kcal/mol of potential energy difference between them. Of these, we chose the fully extended conformers for superposition (Fig. 1). Based on the known anandamide SAR, we

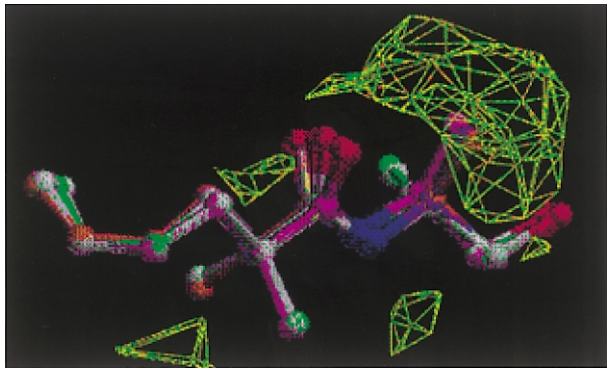


Figure 1. CB1 receptor essential volume map as calculated by subtracting the sum of molecular volumes of the aligned 'inactive' analogues (**5**, **6**, **7**, and **14**), from the sum of molecular volumes of the aligned 'active' ones (**1**, **2**, **4**, **13**, and **15**). By extrapolation, the yellow contours correspond to areas occupied by structural features of the receptor.

could assume that all the analogues in this study interact with the CB1 receptor following the same binding motif, which at least in the head group region, is dictated by the anchoring of three principal pharmacophoric groups: the terminal hydroxyl, the amide proton and the carbonyl oxygen. Based on this model, the main difference between the receptor-bound analogues is the direction in which the methyl groups are projecting out of the anandamide backbone. Therefore, the differences in affinities can be attributed, at least in part, to the relative topologies of the methyl groups of the anandamide analogues while interacting with the CB1 receptor site.

To gain more insight on the interactions of methylated chiral anandamides with CB1, we employed molecular modeling methods which allowed us to explore the regio- and stereochemical requirements of methyl group substituents for binding with CB1. The receptor-excluded and receptor-essential volume concepts[46,47] were used to develop a visual model of the receptor site region that accommodates the head group moiety of anandamide.

According to this approach, it is assumed that a family of active ligands adopt similar shapes and occupy similar volumes at the receptor site, while inactive ligands occupy different spaces which may negatively affect their binding to the receptor. Our newly synthesized, chiral 2-monomethyl analogues along with *R*-, *S*-methanandamide and anandamide were used for the construction of the model. More specifically, analogues **1**, **2**, **4**, **13**, and **15** of high affinity for CB1 ('active') and analogues **5**, **6**, **7**, and **14** of low affinity ('inactive') were superimposed using the C5 and the amide nitrogen and oxygen as superimposition points. The sum of the van der Waals volumes of the 'inactive' analogues deviating from the sum of volumes of the 'active' ones is illustrated by the yellow contours in Figure 1. According to the 'receptor essential volume' approach, these yellow contours can be extrapolated as areas occupied by structural features of the receptor site. Occupancy of these areas by ligand molecules is hindered by negative steric interactions leading to lower binding affinities. The sum of the van der Waals volumes of the 'active' analogues representing the free space within the receptor region that accommodates the anandamide head group is depicted in Figure 2 with the green cloud (receptor-excluded volume map). The yellow colored clouds represent the volumes occupied by the methyl groups of the most active analogue, **4**. These methyl groups are presumably interacting with putative small hydrophobic pockets in the CB1 receptor site whose outer circumscription are partially outlined by the yellow contours of Figure 1. These pockets can be accessed simultaneously by analogue **4**, which bears both methyl groups in the appropriate relative configurations leading to an optimal interaction with the receptor site. In contrast, the prefered conformers for analogues **5**–**7** place their methyl group(s) beyond these pharmacophoric pockets resulting in negative steric interactions with CB1 and subsequently lower affinities. Based on this pictorial model, the pocket that accommodates methyl group(s) at the 2 position is larger than that of the 1′ position. This postulate explains the low CB1 enantioselectivity for the 2-monomethyl analogues (**1**–**2**). The 2-dimethyl congeners (**8**–**12**) were synthesized in order to test and validate our model. Indeed, in support of our hypothesis, we found the 2-*gem*-dimethyl group to be well tolerated (**8**). However, the simultaneous presence of a third methyl group in the ethanolamido moiety (analogues **9**–**12**) leads to unfavorable steric interactions and lower CB1 affinities.

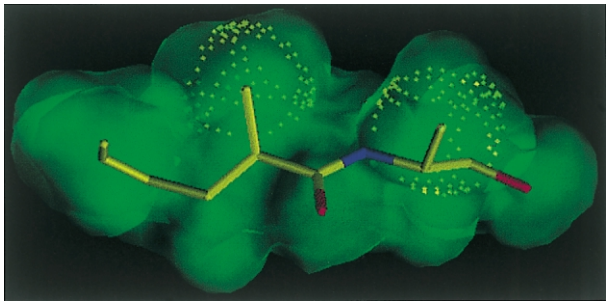


Figure 2. CB1 receptor excluded volume map as calculated by adding the molecular volumes of the aligned 'active' analogues (**1**, **2**, **4**, **13**, and **15**). The yellow colored clouds represent the volumes occupied by the methyl groups of the most active analogue, **4**.

Metabolic stability

In earlier publications,[28,35] we had reported on the remarkable stability of (*R*)-methanandamide (**13**) with regard to enzymatic activity by anandamide amidase. The introduction of a methyl group at the 1′-position of the head group of anandamide is governed by considerable stereoselectivity since the *S*-enantiomer is more susceptible to amidase activity than its enantiomer. Unlike previous reports,[26,33] our present results indicate that this metabolic stability imparted by the presence of a methyl group alpha to the amide nitrogen is only partially duplicated by the introduction of a methyl group alpha to the amido carbonyl. Thus, the respective two α-methyl enantiomers **1** and **2** are less metabolically stable than (*R*)-methanandamide with the *R*-isomer exhibiting the greater stability. These findings were confirmed (unpublished results) by examining these analogues as substrates of the anandamide amidase on an HPLC assay developed in our laboratory.[49] The combined presence of the above two chiral 1′,2 methyl groups does not increase to any significant extent the metabolic stability of anandamide although it leads to some interesting observations. For example, the 2(*R*)- and 1′(*R*)-methyl analogues are the most metabolically stable chiral isomers within each enantiomeric pair. This would lead to the prediction that the dimethyl anandamide analogues incorporating the above two chiral centers [i.e., 2*R*, 1*R* (**4**)] must also possess the highest degree of metabolic stability. However, contrary to our expectation, the results of this study find analogues 2*S*, 1′*R* (**5**) and 2*R*, 1*S* (**7**) to be the most enzymatically stable dimethyl anandamides. This indicates that the substrate-enzyme interactions cannot be always predicted based on 'additive SARs' but involve more complex contributions to the binding and/or catalysis at the enzyme's active site.

Finally, it appears that the incorporation of *gem*-dimethyl substituents at the 2-position of the arachidonic acid moiety leads only to partial metabolic stability for the respective anandamides. Our results clearly indicate that metabolic stability with regard to anandamide amidase is optimally accomplished by a stereospecific *R*-methyl substitution at the carbon α to the amide nitrogen and is superior to substitution at the α-position to the carbonyl group. This may appear to be a somewhat surprising steric effect in view of the fact that the 1′-methyl group is two bonds removed from the carbonyl site of catalytic action when compared to the 2-methyl group which is only one bond away from that site. A possible explanation for this observation is that α-amidomethyl protons are separated by five bonds from the carbonyl oxygen and are then subject to the Newman's 'rule of six'[50] which describes steric crowding during the hydrolysis of carbonyl esters, while the α-arachidonyl methyl hydrogens are only four bonds away from the carbonyl oxygen, leading to less effective steric crowding at the hydrolysable carbonyl group. According to the 'rule of six', the greater the number of atoms at the six position, the greater the steric effect. This rule explains, for example, why the ratio of the rates of hydrolysis for ethyl acetate and ethyl isobutyrate is 8.5 whereas the ratio of the rates of hydrolysis for methyl benzoates and isopropyl benzoate is 20.[50]

Conclusions

Asymmetric methylation at the 2-position of anandamide leads to analogues with only moderately improved affinity for the CB1 receptor regardless of absolute configuration and provides only modest protection against anandamide amidase. However, when a second methyl group is present at the ethanolamido head group a high degree of enantio- and diastereoselectivity is observed. The diastereomer with the highest affinity (**4**) incorporates two methyl groups with an optimal absolute configuration (*R*,*R*) suggesting the presence of two respective small hydrophobic pockets at the receptor site. A *gem*-dimethyl group at the 2-position is well tolerated in one of these pockets (the larger one) but results in negative steric interactions when a third methyl is introduced at the head group. The above results support a CB1 receptor site model which incorporates two pockets capable of accommodating methyl groups in the 1′- and 2-positions of the molecule with the 2-methyl pocket being significantly larger. Regarding stability towards anandamide amidase imparted by methyl group substitution, our present results confirm that (*R*)-1′-methylation leads to the most stable analogues.

Experimental

Chemistry

^{1}H NMR spectra were recorded either on a Bruker DMX 500 MHz or a Bruker WP-200SY 200 MHz spectrometer using TMS as the internal standard. All chemical shifts are reported in ppm. Specific rotations were determined using a Perkin-Elmer 241 polarimeter within either a 1.00 or 0.01 dm cell. Satisfactory elemental analyses was obtained for all the analogues synthesized and are within ±0.4% except for compounds **1** (0.42%), **5** (0.66%), and **8** (0.43%). The optically pure amino alcohols were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Arachidonic acid and methyl ester were purchased from SIGMA Chemical Company and Nu-Chek-Prep. Inc.

(*R*)-*N*-arachidonyl-4-benzyl-2-oxazolidinone (16). To a stirring solution of 500 mg (1.64 mmol) of arachidonic acid and 274.5 μL (1.97 mmol) of triethylamine in 9 mL of anhydrous THF at −78 °C, 285.3 μL (1.97 mmol) of pivaloyl chloride was added. A white suspension was formed and stirring was continued for 10 min at −78 °C and 30 min at 0 °C. The mixture was recooled to −78 °C and a −78 °C solution of lithiated (*R*)-(+)-4-benzyl-oxazolidinone was cannulated. The metallated oxazolidinone was prepared by addition of 1.23 mL (1.97 mmol, 1.6 M in THF) of *n*-BuLi in a solution of 348.5 mg (1.97 mmol) of (*R*)-(+)-4-benzyl-oxazolidinone in 7 mL of THF at −78 °C. The reaction mixture was stirred for 3 h while raising slowly the temperature to 0 °C and then for 30 min at 0 °C. The reaction was then quenched by the addition of 10 mL of saturated aqueous solution of ammonium chloride and extracted from ether (3×30 mL). The combined organic phases were washed with water, dried over anhydrous magnesium sulfate and stripped of volatiles. Purification with silica gel column chromatography (eluent CH_2Cl_2/petroleum ether) yielded 692.1 mg (88%) of the title compound as a colorless oil: R_f 0.51 (CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ?7.38–7.18 (m, 5H, aromatic-Hs), 5.49–5.29 (m, 8H, vinylic-Hs), 4.72–4.60 (m, 1H, C4′-H), 4.24–4.13 (m, 2H, C5′-Hs), 3.30 (dd, 1H, J = 3.2 Hz, 13.3 Hz, CH*H*-Ph), 3.05–2.92 (m, 2H, C2-H), 2.89–2.69 (m, 7H, double allylic Hs and C*H*H-Ph), 2.20–2.13 (m, 2H, C4-Hs), 2.10–2.00 (m, 2H, C16-Hs), 1.80 (quintet, 2H, J = 7.3 Hz, C3-Hs) 1.42–1.23 (m, 6H, C17,18,19-Hs), 0.88 (t, 3H, J = 6.8 Hz, C20-Hs); $[\alpha]^{28}$ −37.7° (neat). Anal. calcd for $C_{30}H_{41}O_3N$: C, 77.71; H, 8.91; N, 3.02. Found: C, 77.36; H, 9.19; N, 2.91.

(*R*)-*N*-((*R*)-2-Methyl-arachidonyl)-benzyl-2-oxazolidinone (17). To a −78 °C solution of 1.150 mL (1.15 mmol, 1.1 equiv, 1.0 M in THF) of sodium hexamethyldisilamide, a 0 °C solution of 500 mg (1.05 mmol, 1.0 equiv) of (*R*)-*N*-arachidonyl-4-benzyl-2-oxazolidinone (**16**) in 5 mL of anhydrous THF was added dropwise, via a cannula. The solution was stirred at −78 °C for 30 min and then 326 μL (5.24 mmol, 5 equiv) of iodomethane was added and the reaction mixture was stirred for 4 h at −78 °C. The reaction was quenched by the addition of 8 mL of saturated aqueous solution of ammonium chloride. The product was extracted from diethyl ether (3×30 mL). The combined ether extracts were washed with water and dried over anhyd $MgSO_4$. Purification with silica gel column chromatography (eluent CH_2Cl_2/petroleum ether) yielded 370.6 mg (74%) of the title compound as a colorless oil: R_f 0.72 (CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.38–7.20 (m,

5H, aromatic-Hs), 5.44–5.35 (m, 8H, vinylic Hs), 4.70–4.63 (m, 1H, C4′-H), 4.19–4.14 (m, 2H, C5′-Hs), 3.72 (sext., 1H, $J=6.9$ Hz, C2-Hs), 3.26 (dd, 1H, $J=3.2$ Hz, 13.3 Hz, CH*H*-Ph), 2.90–2.71 (m, 7H, double allylic-Hs and C*H*H-Ph), 2.16–2.00 (m, 4H, C4,16-Hs), 2.00–1.77 (m, 1H, CH*H*CH(CH_3)), 1.50–140 (m, 1H, C*H*HCH(CH_3)), 1.40–1.23 (m, 9H, C17,18,19-Hs, $CH_2CH(CH_3)$), 0.88 (t, 3H, $J=6.8$ Hz, C20-Hs); $[\alpha]^{28}$ −54.3° (neat). Anal. calcd for $C_{31}H_{43}O_3N$: C, 77.94; H, 9.07; N, 2.93. Found: C, 78.28; H, 9.01; N, 2.82.

(*R*)-2-Methyl-arachidonic acid (18). To a stirred solution of 300 mg (0.63 mmol) of (*R*)-*N*-((*R*)-2-methyl-arachidonyl)-benzyl-2-oxazolidinone (**17**) in 5 mL of THF at 0 °C, 3 mL of 1.0 M aqueous solution of lithium hydroxide was added. The reaction mixture was stirred for 6 h at 0 °C and then it was neutralized with saturated aqueous solution of ammonium chloride. The product was extracted from diethyl ether (3×30 mL), washed with water and dried over anhyd $MgSO_4$. Purification with silica gel column chromatography (eluent: CH_2Cl_2/ethyl acetate) afforded 177.5 mg (89%) of the title compound as a colorless oil:R_f 0.76 (10% ethyl acetate in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ?5.40–5.31 (m, 8H, vinylic Hs), 2.84–2.81 (m, 6H, double allylic Hs), 2.49 (sext., 1H, $J=6.9$ Hz, C2-H), 2.14–2.00 (m, 4H, C4,16-Hs), 1.80–1.70 (m, 1H, CH*H*CH(CH_3), 1.56–1.45 (m, 1H, C*H*HCH(CH_3)), 1.38–1.29 (m, 6H, C17,18,19-H), 1.20 (d, 3H, $J=7.2$ Hz, CHHCH(CH_3)), 0.88 (t, 3H, $J=6.8$ Hz, C20-H); $[\alpha]^{28}$ −20.2° (0.3 g/mL CH_2Cl_2). Anal. calcd for $C_{21}H_{34}O_2$: C, 79.19; H, 10.76. Found: C, 79.62; H, 10.90.

(*R*)-*N*-(1-Methyl-2-hydroxyethyl)-2-(*R*)-methyl-arachidonamide (4). To a stirred solution of 50 mg (0.16 mmol) of (*R*)-2-methyl-arachidonic acid (**18**) and 12.4 μL (0.16 mmol) of DMF in 1 mL of dichloromethane at 0 °C, 100 μL (0.20 mmol) of a 2.0 M solution of oxalyl chloride in dichloromethane was added dropwise. The reaction mixture was stirred at 0 °C for 20 min. Then, 62.2 μL (0.80 mmol) of (*R*)-(−)-2-amino-1-propanol was added and stirring was continued for 20 min at ambient temperature. The reaction mixture was diluted with 3 mL of dichloromethane and washed with brine. The organic phase was separated, dried over anhydrous magnesium sulfate and stripped of volatiles in vacuo. Purification by silica gel column chromatography (eluent: CH_2Cl_2/ethyl acetate) yield 47.4 mg (79%) of **4** as a colorless oil: R_f 0.35 (40% CH_2Cl_2 in ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.57 (s, br, 1H, N*H*), 5.40–5.30 (m, 8H, vinylic Hs), 4.10–4.04 (m, 1H, C1-H), 3.67–3.64 (m, 1H, CH*H*OH), 3.54–3.48 (m, 1H, C*H*HOH), 2.84–2.79 (m, 6H, double vinylic-Hs), 2.23–2.18 (m, 1H, C2-H), 2.09–2.03 (m, 4H, C4, 16-Hs), 1.76–1.70 (m, 1H, CH*H*CH(CH_3)), 1.48–1.42 (m, 1H, C*H*HCH(CH_3)), 1.34–1.36 (m, 6H, C17, 18, 19-Hs), 1.18–1.14 (m, 6H, CH(CH_3), NH–CH(CH_3)), 0.88, (t, 3H, $J=6.8$ Hz, C20-Hs); $[\alpha]^{28}$ −4.1° (0.2 g/mL CH_2Cl_2). Anal. calcd for $C_{24}H_{41}O_2N$: C, 76.75; H, 11.00; N, 3.73. Found: C, 77.06; H, 11.24; N, 3.61.

(*S*)-*N*-(1-Methyl-2-hydroxyethyl)-2-(*R*)-methyl-arachidonamide (7). Compound **7** was synthesized as **4** by coupling (*R*)-2-methyl-arachidonic acid (**18**) with (*S*)-(−)-2-amino-1-propanol. (yield: 79%) colorless oil: R_f 0.25 (40% CH_2Cl_2 in ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$) δ?5.57 (s, br, 1H, N*H*), 5.40–5.30 (m, 8H, vinylic-Hs), 4.10–4.04 (m, 1H, C1′-H), 3.67–3.64 (m, 1H, CH*H*OH), 3.54–3.48 (m, 1H, C*H*HOH), 2.84–2.79 (m, 6H, double vinylic Hs), 2.23–2.18 (m, 1H, C2-H), 2.09–2.03 (m, 4H, C4,16-Hs), 1.76–1.70 (m, 1H, CH*H*CH(CH_3)), 1.48–1.42 (m, 1H, C*H*HCH(CH_3)), 1.34–1.36 (m, 6H, C17, 18, 19-Hs), 1.18–1.14 (m, 6H, CH(CH_3), NH–CH(CH_3)), 0.88, (t, 3H, $J=6.8$ Hz, C20-Hs); $[\alpha]^{28}$ +16.2° (0.2 g/mL CH_2Cl_2). Anal. calcd for $C_{24}H_{41}O_2N$: C, 76.75; H, 11.00; N, 3.73. Found: C, 76.50; H, 10.92; N, 3.59.

(*R*)-*N*-(2-Hydroxyethyl)-2-methyl-arachidonamide (1). Compound **1** was synthesized as **4** by coupling (*R*)-2-methyl-arachidonic acid (**18**) with ethanolamine; (yield: 85%) colorless oil: R_f 0.47 (60% ethyl acetate in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.94 (s, br, 1H, N*H*), 5.43–5.31 (m, 8H, vinylic-Hs), 3.73 (t, 2H, $J=4.8$ Hz, C1′-Hs), 3.43 (quart., 2H, $J=5.1$ Hz, C2′-Hs), 2.83–2.80 (m, 6H, double allylic-Hs), 2.24 (sext., 1H, $J=6.9$ Hz, C2-Hs), 2.10–2.03 (m, 4H, C4,16-Hs), 1.77–1.72 (m, 1H, CH*H*CH(CH_3)), 1.50–1.45 (m, 1H, C*H*HCH(CH_3)), 1.37–1.26 (m, 6H, C17, 18, 19-Hs), 1.16 (d, 3H, $J=6.9$ Hz, CH(CH_3)), 0.89 (t, 3H, $J=6.0$ Hz, C20-Hs); $[\alpha]^{28}$ −9.4° (0.1 g/mL CH_2Cl_2). Anal. calcd for $C_{23}H_{39}O_2N$: C, 76.40; H, 10.87; N, 3.87. Found: C, 76.82; H, 10.98; N, 3.66.

(*S*)-N-Arachidonyl-4-benzyl-2-oxazolidinone. (*S*)-*N*-Arachidonyl-4-benzyl-2-oxazolidinone was synthesized as **16** by coupling arachidonic acid (*S*)-(−)-4-benzyl-oxazolidinone; (yield: 82%) colorless oil: R_f 0.51 (CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.38–7.18 (m, 5H, aromatic-Hs), 5.49–5.29 (m, 8H, vinylic-Hs), 4.72–4.60 (m, 1H, C4′-H), 4.24–4.13 (m, 2H, C5′-Hs), 3.30 (dd, 1H, $J=3.2$ Hz, 13.3 Hz, CH*H*-Ph), 3.05–2.92 (m, 2H, C2-Hs), 2.89–2.69 (m, 7H, double allylic-Hs and C*H*H-Ph), 2.20–2.13 (m, 2H, C4-Hs), 2.10–2.00 (m, 2H, C16-Hs), 1.80 (quintet, 2H, $J=7.3$ Hz, C3-Hs) 1.42–1.23 (m, 6H, C17,18,19-Hs), 0.88 (t, 3H, $J=6.8$ Hz, C20-Hs); $[\alpha]^{26}$ +37.9° (neat).

(*S*)-*N*-((*S*)-2-Methyl-arachidonyl)-benzyl-2-oxazolidinone. (*S*)-*N*-((*S*)-2-Methyl-arachidonyl)-benzyl-2-oxazolidinone was synthesized as **17** by methylating (*S*)-*N*-arachidonyl-4-benzyl-2-oxazolidinone. Similar diastereoselectivity was achieved as in preparation of **17;** (yield: 71%); colorless oil:R_f 0.72 (CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.38–7.20 (m, 5H, aromatic-Hs), 5.44–5.35 (m, 8H, vinylic-Hs), 4.70–4.63 (m, 1H, C4′-H), 4.19–4.14 (m, 2H, C5′-Hs), 3.72 (sext, 1H, $J=6.9$ Hz, C2-Hs), 3.26 (dd, 1H, $J=3.2$ Hz, 13.3 Hz, CH*H*-Ph), 2.90–2.71 (m, 7H, double allylic-Hs and C*H*H-Ph), 2.16–2.00 (m, 4H, C4,16-Hs), 2.00–1.77 (m, 1H, CH*H*CH(CH_3)), 1.50–140 (m, 1H, C*H*HCH(CH_3)), 1.40–1.23 (m, 9H, C17, 18, 19-Hs, CHHCH(CH_3)), 0.88 (t, 3H, $J=6.8$ Hz, C20-Hs); $[\alpha]^{26}$ +54.5° (neat).

(*S*)-2-Methyl-arachidonic acid. (*S*)-2-Methyl-arachidonic acid was synthesized as **18** by hydrolysis of (*S*)-*N*-((*R*)-

2-methyl-arachidonyl)-benzyl-2-oxazolidinone; (yield: 86%); colorless oil:R_f 0.76 (10% ethyl acetate in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.40–5.31 (m, 8H, vinylic-Hs), 2.84–2.81 (m, 6H, double allylic-Hs), 2.49 (sext., 1H, J = 6.9 Hz, C2-H), 2.14–2.00 (m, 4H, C4,16-Hs), 1.80–1.70 (m, 1H, CH*H*CH(CH_3), 1.56–1.45 (m, 1H, C*H*HCH(CH_3)), 1.38–1.29 (m, 6H, C17,18,19-H), 1.20 (d, 3H, J = 7.2 Hz, CHHCH(CH_3)), 0.88 (t, 3H, J = 6.8 Hz, C20-Hs); $[\alpha]^{28}$ +20.1° (0.3 g/mL CH_2Cl_2).

(*S*)-*N*-(1-Methyl-2-hydroxyethyl)-2-(*S*)-methyl-arachidonamide (6). Compound **6** was synthesized as **4** by coupling (*S*)-2-methyl-arachidonic acid with (*S*)-(+)-2-amino-1-propanol; (yield: 77%); colorless oil:R_f 0.35 (40% CH_2Cl_2 in ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.57 (s, br, 1H, N*H*), 5.40–5.30 (m, 8H,vinylic-Hs), 4.10–4.04 (m, 1H, C1′-H), 3.67–3.64 (m, 1H, CH*H*OH), 3.54–3.48 (m, 1H, C*H*HOH), 2.84–2.79 (m, 6H, double vinylic-Hs) 2.23–2.18 (m, 1H, C2-H), 2.09–2.03 (m, 4H, C4,16-Hs), 1.76–1.70 (m, 1H, CH*H*CH(CH_3)), 1.48–1.42 (m, 1H, C*H*HCH(CH_3)), 1.34–1.36 (m, 6H, C17,18,19-Hs), 1.18–1.14 (m, 6H, CH(CH_3), NH–CH(CH_3)), 0.88, (t, 3H,J = 6.8 Hz, C20-Hs); $[\alpha]^{28}$ +4.2° (0.2 g/mL CH_2Cl_2). Anal. calcd for $C_{24}H_{41}O_2N$: C, 76.75; H, 11.00; N, 3.73. Found: C, 76.56; H, 11.22; N, 3.63.

(*R*)-*N*-(1-Methyl-2-hydroxyethyl)-2-(*S*)-methyl-arachidonamide (5). Compound **5** was synthesized as **4** by coupling (*S*)-2-methyl-arachidonic acid with (*R*)-(−)-2-amino-1-propanol; (yield: 84%) colorless liquid:R_f 0.25 (40% CH_2Cl_2 in ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.57 (s, br, 1H, N*H*), 5.40–5.30 (m, 8H, vinylic-Hs), 4.10–4.04 (m, 1H, C1′-H), 3.67–3.64 (m, 1H, CH*H*OH), 3.54–3.48 (m, 1H, C*H*HOH), 2.84–2.79 (m, 6H, double vinylic-Hs) 2.23–2.18 (m, 1H, C2-H), 2.09–2.03 (m, 4H, C4,16-Hs), 1.76–1.70 (m, 1H, CH*H*CH(CH_3)), 1.48–1.42 (m, 1H, C*H*HCH(CH_3)), 1.34–1.36 (m, 6H, C17, 18, 19-Hs), 1.18–1.14 (m, 6H, CH(CH_3), NH-CH(CH_3)), 0.88, (t, 3H, J = 6.8 Hz, C20-Hs); $[\alpha]^{28}$ −16.3° (0.2 g/mL CH_2Cl_2). Anal. calcd for $C_{24}H_{41}O_2N$: C, 76.75; H, 11.00; N, 3.73. Found: C, 77.41; H, 11.38; N, 3.34.

(*S*)-*N*-(2-Hydroxyethyl)-2-methyl-arachidonamide (2). Compound **2** was synthesized as **4** by coupling (*S*)-2-methyl-arachidonic acid with ethanolamine; (yield: 89%) colorless oil:R_f 0.47 (40% CH_2Cl_2 in ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.94 (s, br, 1H, N*H*), 5.43–5.31 (m, 8H, vinylic-Hs), 3.73 (t, 2H, J = 4.8 Hz, C1′-H), 3.43 (quart., 2H, J = 5.1 Hz, C2′-H), 2.83–2.80 (m, 6H, double allylic Hs), 2.24 (sext., 1H, J = 6.9 Hz, C2-Hs), 2.10–2.03 (m, 4H, C4,16-Hs), 1.77–1.72 (m, 1H, CH*H*CH(CH_3)), 1.50–1.45 (m, 1H, C*H*HCH(CH_3)), 1.37–1.26 (m, 6H, C17, 18, 19-Hs), 1.16 (d, 3H, J = 6.9 Hz, CH(CH_3)), 0.89 (t 3H, J = 6.0 Hz, C20-Hs); $[\alpha]^{28}$ +9.6° (0.1 g/mL CH_2Cl_2). Anal. calcd for $C_{23}H_{39}O_2N$: C, 76.40; H, 10.87; N, 3.87. Found: C, 76.68; H, 10.79; N, 3.70.

(±)-2-Methyl-arachidonic acid methyl ester. To a stirred solution of 200 mg (0.63 mmol, 1.0 equiv) arachidonic acid methyl ester in 4 mL of anhyd THF, at −78 °C, 375 μL (0.75 mmol, 1.2 equiv) of a 2.0 M solution of lithium diisopropylamide (LDA) in heptane/THF/benzene was added dropwise. The dark orange-red mixture was stirred for 45 min at −78 °C and then 196 μL of MeI (3.2 mmol, 5 equiv) was added and the reaction mixture was stirred for 2 h at −78 °C. The temperature was raised overnight to ambient while stirring was continued. Another 5 equiv of MeI was added and 20 min later the reaction was quenched with the addition of 3 mL aqueous saturated solution of ammonium chloride. The product was extracted with ether (3×20 mL), washed with water and dried over anhyd $MgSO_4$. Purification with silica gel column chromatography afforded 133.1 mg (64%) of the title compound as a colorless liquid.R_f 0.58 (50% CH_2Cl_2/petroleum ether); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.42–5.27 (m, 8H, vinylic-Hs), 3.67 (s, 3H, COOCH_3), 2.85–2.80 (m, 6H, double vinylic Hs), 2.47 (sext, 1H, J = 7.0 Hz C*H*CH$_3$), 2.08–2.03 (m, 4H, C4, 16-Hs), 1.79–1.72 (m, 1H, CH*H*CH(CH_3)), 1.49–1.42 (m, 1H, C*H*HCH(CH_3)), 1.37–1.15 (m, 6H, C17, 18, 19-Hs), 1.16 (d, 3H, J = 7.0 Hz, CH(CH_3), 0.88 (t, 3H,J = 6.7 Hz, C20-Hs).

(±)-2-Methyl-arachidonic acid. (±)-2-Methyl-arachidonic acid methyl ester (100 mg, 0.30 mmol) was dissolved in 3 mL of a 15% solution of KOH in methanol/water and stirred for 24 h at 50–60 °C. The mixture was then neutralized with 2 N HCl, most of methanol was evaporated and extracted from ether (3×20 mL). Purification with silica gel column chromatography afforded 65.8 mg (69%) of the title compound as a colorless liquid: R_f 0.76 (10% ethyl acetate in CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.40–5.31 (m, 8H, vinylic-Hs), 2.84–2.81 (m, 6H, double allylic-Hs), 2.49 (sext., 1H, J = 6.9 Hz, C2-H), 2.14–2.00 (m, 4H, C4, 16-Hs), 1.80–1.70 (m, 1H, CH*H*CH(CH_3), 1.56–1.45 (m, 1H, C*H*HCH(CH_3)), 1.38–1.29 (m, 6H, C17, 18, 19-Hs), 1.20 (d, 3H, J = 7.2 Hz, CHHCH(CH_3)), 0.88 (t, 3H, J = 6.8 Hz, C20-Hs).

(±)-*N*-(2-Hydroxyethyl)-2-methyl-arachidonamide (3). Compound **3** was synthesized as **4** by coupling (±)-2-methyl-arachidonic acid with ethanolamine; (yield: 86%) colorless oil:R_f 0.47 (40% CH_2Cl_2 in ethyl acetate); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.94 (s, br., 1H, N*H*), 5.43–5.31 (m, 8H, vinylic Hs), 3.73 (t, 2H, J = 4.8 Hz, C1-H), 3.43 (quart., 2H, J = 5.1 Hz, C2-Hs), 2.83–2.80 (m, 6H, double allylic Hs), 2.24 (sext., 1H, J = 6.9, C2-Hs), 2.10–2.03 (m, 4H, C4,16-Hs), 1.77–1.72 (m, 1H, CH*H*CH(CH_3)), 1.50–1.45 (m, 1H, C*H*HCH(CH_3)), 1.37–1.26 (m, 6H, C17, 18, 19-Hs), 1.16 (d, 3H, J = 6.9 Hz, CH(CH_3)), 0.89 (t, 3H, J = 6.0 Hz, C20-Hs). Anal. calcd for $C_{24}H_{41}O_2N$: C, 76.75; H, 11.00; N, 3.73. Found: C, 77.12; H, 11.18; N, 3.64.

Preparation of 4 and 5 by method B. Compounds **4** and **5** were also synthesized by coupling, as described above, (±)-2-methyl-arachidonic acid with (*R*)-(−)-2-amino-1-propanol: To a stirred solution of 100 mg (0.32 mmol) of (±)-2-methyl-arachidonic acid and 24.8 μL (0.32 mmol) of DMF in 2 mL of methylene chloride at 0 °C, 200 μL (0.40 mmol) of 2.0 M solution of oxalyl chloride in methylene chloride was added dropwise. The reaction

mixture was stirred at 0 °C for 20 min. Then 48.8 μL (0.80 mmol) of ethanolamine was added and stirring was continued for 20 min at ambient temperature. The reaction was stopped and the product was extracted from diethyl ether (3×30 mL), dried over anhydrous $MgSO_4$ and stripped of volatiles. TLC indicated two products with R_f 0.35 and 0.25 (40% CH_2Cl_2 in petroleum ether), corresponding to the two diastereomers **4** and **5**. This diastereomeric mixture was separable by silica gel column chromatography (eluent: CH_2Cl_2/ethyl acetate) to afford 46.8 mg (39%) of **4** and 40.8 mg (34%) of **5**. The isolated compounds were found identical to the corresponding products from method A.

Preparation of 6 and 7 by method B. Following the above procedure, analogues **6** and **7** were also synthesized by coupling (±)-2-methyl-arachidonic acid with (*S*)-(+)-2-amino-1-propanol to afford, after resolution by column chromatography 43.2 mg (36%) of **6** and 39.6 mg (33%) of **7**. The isolated compounds were found identical to the corresponding products from method A.

Preparation of compounds 8–12. Compounds **8**–**12** were prepared as in ref 33.

Radioligand binding assay. For CB1, rat forebrain membranes were prepared according to the procedure of Dodd et al.[51] The binding of the novel anandamide analogues to the cannabinoid receptor was assessed as previously described[41,42] except that the membranes were treated with PMSF. Membranes, previously frozen at −80 °C, were thawed on ice. To the stirred suspension were added three volumes of 25 mM Tris–HCl Buffer, 5 mM $MgCl_2$, and 1 mM EDTA, pH 7.4 (TME) containing 150 μM PMSF (made fresh in isopropanol as a 100 mM stock). The suspension was incubated at 4 °C and after 15 min a second addition of PMSF stock brought the concentration to 300 μM of PMSF then incubated for another 15 min. At the end of the second 15 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 30 μg of PMSF-treated membranes were incubated in silanized 96-well microtiter plate 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 anandamide analogues in a final volume of 200 μL. Assays were incubated at 30 °C for 1 h. The samples were filtered using Packard Filtermate 196 and Whatman GF/C Filterplates and washed with wash buffer (TME) containing 0.5% BSA. Radioactivity was detected using MicroScint 20 scintillation cocktail added directly to the dried filterplates, and the filterplates were counted using a Packard Instruments Top-Count. Nonspecific binding was assessed using 100 nM CP-55,940. 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. The normalized data were analyzed using a 4 parameter nonlinear logistic equation to yield IC_{50} values. Data from at least two independent experiments performed in duplicate were used to calculate IC_{50} values, which were converted to K_i values using the assumptions of Cheng and Prusoff.[52]

For CB2 receptor binding studies, membranes were prepared from frozen mouse spleen essentially according to the procedure of Dodd et al.[51] as described elsewhere.[27,30] Silanized centrifuge tubes were used throughout to minimize receptor loss due to adsorption. The CB2 binding assay was conducted in the same manner as for CB1.

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