Conformationally Constrained Fatty Acid Ethanolamides as Cannabinoid and Vanilloid Receptor Probes

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To investigate if certain acylethanolamides bind to both cannabinoid (CB_1 and CB_2) and vanilloid TRPV1 receptors because of their conformational flexibility, we introduced a methylene lock on their ethanolamine "head", thereby generating a cyclopropane ring with two stereogenic centers and chiral *cis/trans* diastereomers with different topology of presentation to binding sites. After resolution by chiral-phase HPLC, diastereo- and enantiopure arachidonoyl-, oleoyl-, and palmitoylcyclopropanolamides were tested in assays of CB_1, CB_2, and TRPV1 activity. Diastereodifferentiation between pairs of *cis*−*trans* isomers was observed only for TRPV1 activity, with poor enantiodifferentiation. Methylenation introduced (i) CB_1 receptor affinity in oleoylethanolamide while increasing in a diastereoselective way its activity at TRPV1 and (ii) strong diastereoselective activity at TRPV1, but not cannabinoid, receptors in the otherwise inactive palmitoylethanolamide. These results show that the *N*-alkyl group of acylethanolamides has a different role in their interaction with cannabinoid and vanilloid receptors and that acylcyclopropanolamides qualify as CB_1/TRPV1 "hybrids" of potential therapeutic utility.

Introduction

The discovery of specific receptors for Δ^9-tetrahydrocannabinol (**1**, THC[a]), the psychotropic agent of hemp (*Cannabis sativa* L.), and for capsaicin (**2a**), the pungent principle of chilli pepper (*Capsicum annuum* L.), sparked intense research activity aimed at evaluating the possibility of developing therapeutic drugs targeting these receptors for a host of pathological conditions that include pain, inflammation, emesis, obesity, neurodegeneration, and cancer.[1−3] Endogenous agonists for cannabinoid and capsaicin receptors [known as "endocannabinoids" and "endovanilloids", respectively[4,5] (Figure 1)] were also discovered from the pool of fatty acid conjugates of biogenic amines.[6−12] While the plant-derived ligands show little promiscuity in their targets, endocannabinoids like arachidonoylethanolamide (**3a**, anandamide) and arachidonoyldopamide (**4**, NADA) also activate TRPV1 receptors and hence behave as endovanilloids. Conversely, certain fatty acid vanillamides (capsaicinoids) like arachidonoylvanillamide (**2b**, arvanil)[13] and phenylcetyl)ricinoleoylvanillamide (**2c**, PhAR)[14] also target CB_1 and CB_2 cannabinoid receptors. This promiscuity, together with the occurrence of a functional cross-talk between TRPV1 and CB_1,[15−17] and their colocalization in several nervous tissues[18,19] suggest that the two systems are closely intertwined in terms of function and regulation and that synthetic dual CB_1/TRPV1 agonists have the potential to correct dysregulation of both systems better than agents individually targeting either TRPV1 or cannabinoid receptors.[2,3]

Endocannabinoids and endovanilloids are modular compounds in which a polar ethanolamine or dopamine head is conjugated to a fatty acid. At the level of endogenous ligands, TRPV1 channels can accept both mono- and polyunsaturated acyl moieties[12,20] while cannabinoid receptors are more selective and prefer polyunsaturated acyl moieties. Since cannabinoid and vanilloid receptors are structurally unrelated, as they belong to the families of G-protein-coupled receptors and of transient receptor potential channels, respectively, it seems reasonable to assume that the binding sites of endocannabinoids and endovanilloids are different, and that the promiscuous behavior of these ligands is due to their conformational flexibility, which makes it possible for them to fit into distinct binding regions of their target proteins. To test this hypothesis, we have focused on the ethanolamide polar head of anandamide (**3a**), the archetypal hybrid TRPV1−CB_1 ligand, and locked the relative orientation of its hydroxyl and amido functions by inserting a methylene bridge between the two methylene carbons. A similar maneuver was carried out also on oleoylethanolamide (**3b**, OEA), another endogenous TRPV1 ligand with little or no activity at cannabinoid receptors, and palmityoletanolamide (**3c**, PEA), an ethanolamide lacking direct activity at either receptors. By insertion of a methylene lock, the polar head becomes chiral, making it possible also to address the issue of chiral discrimination in the binding of these compounds. In fact, while endocannabinoids and endovanilloids are achiral (Figure 1), THC and

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[a] Abbreviations: AEA, arachidonoylethanolamide; CC, column chromatography; AcOH, acetic acid; DCM, dichloromethane; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMAP, 4-dimethylaminopyridine; EDCI, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; EtOAc, ethyl acetate; EtOH, ethanol; Et_2Zn, diethylzinc; HPLC, high performance liquid chromatography; NADA, *N*-arachidonoyldopamine; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; TBAF, tetrabutylammonium fluoride; TBDMS, *tert*-butyldimethylsilyl; THC, Δ^9-tetrahydrocannabinol; TRPV1, transient receptor potential vanilloid type-1.

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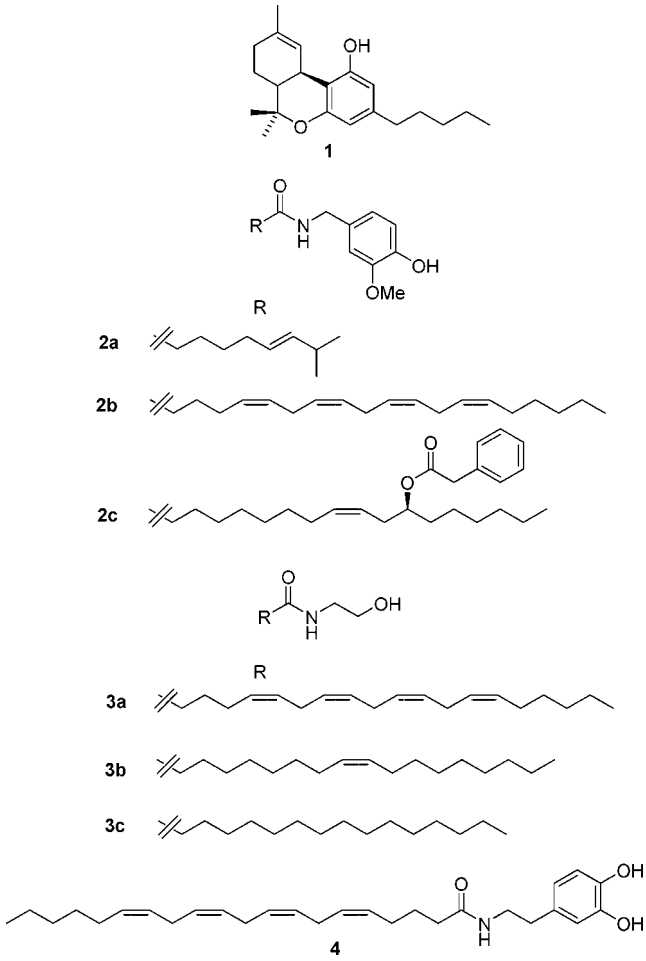


Figure 1. Chemical structures of plant-derived, endogenous, and synthetic agonists of cannabinoid and TRPV1 receptors.

several potent synthetic CB_1 and CB_2 ligands interact with cannabinoid receptors in an enantioselective way.[1,21]

Results

Synthesis of the Racemic Cyclopropanolamides (±)-9a−c and (±)-10a−c. Racemic cyclopropanolamides were prepared by the stereoselective Simmons−Smith cyclopropanation[22] of the *E*/*Z* diastereomeric silyl enol ethers **6a** and **6b**, obtained as an easily separable mixture by enolization and silyl trapping of the known *N*-phtaloylaminoaldehyde **5** (see Experimental Section). Removal of the phtaloyl moiety by hydrazinolysis afforded a silyl protected cyclopropanolamine that was not isolated but directly acylated with the DCC-DMAP protocol and next desilylated (Figure 2).

Resolution of the Racemic Arachidonoyl- and Oleoylcyclopropanolamides (±)-9a,b and (±)-10a,b and Assignment of the Absolute Configuration of the Enatiomerically Pure Cyclopropanolamides. The four racemates were resolved by preparative chiral HPLC chromatography. Two distinct chromatographic stationary phases (i.e., a Chirex-3020, Phenomenex, column containing a stationary phase made with either *S*-*tert*-leucine or *S*-valine and a *R*-1-α-naphthylethylamine urea linkage) had to be employed to separate the *cis* and the *trans* racemates. In all cases, an isocratic elution mode was employed. The absolute configuration of the enantiomerically pure cyclopropanolamides (shown in Figure 3) was established by application of a modified Mosher method[23] (Figure 4).

Affinity of the Cyclopropanolamides for Cannabinoid Receptors. The two diastereomeric arachidonoylcyclopropanolamides *rac*-**9a** and *rac*-**10a** did not exhibit affinity for both cannabinoid receptors higher than that of anandamide, their affinity at CB_1 receptors being always higher than that at CB_2 receptors. No difference between the *trans* and *cis* diastereomers was observed for CB_1 receptors, while the *cis* diastereomer *rac*-**10a** was slightly more active than its *trans* diastereomer *rac*-**9a** at the CB_2 receptor (Table 1). After chiral phase HPLC separation, however, while the **13a** and **14a** enantiomers exhibited similar affinity for CB_1 or CB_2 receptors, the **12a** enantiomer showed slightly higher affinity than its **11a** enantiomer for both receptors (Table 2). In sharp difference with OEA, the oleoylcyclopropanolamides *rac*-**9b** and *rac*-**10b** could bind with reasonably good affinity both cannabinoid receptors (Table 1). The diastereoisomeric oleoylcyclopropanolamides *rac*-**9b** and *rac*-**10b** exhibited all similar affinities for CB_1 or CB_2 receptors, although within the enantiopure compounds **11b**−**14b**, a trend for a higher affinity toward CB_1 receptors was observed (Table 2). Finally, the diasteroemeric palmitoylcyclopropanolamides *rac*-**9c** and *rac*-**10c** exhibited no affinity for cannabinoid receptors and therefore were not further resolved (Table 1).

Functional Activity of *N*-Arachidonoylcyclopropanolamides at CB_1 Receptors. Two of the compounds with the highest affinity at CB_1 receptors, i.e., compounds **11a** and **12a**, were also tested for their ability to stimulate [^{35}S]GTPγS binding to mouse brain membranes. In this assay, agonists, by activating the coupling of G-protein-coupled receptors to the α-subunits of the G-protein, will stimulate the binding of [^{35}S]GTPγS to cell membranes containing the respective receptors. Inverse agonists will do the opposite, and neutral antagonist will not exert any effect on [^{35}S]GTPγS binding. In our case, both **11a** and **12a** behaved as partial agonists (EC_{50} = 2.55 and 0.78 μM and E_{max} = 47.2% and 39.8%, respectively, compared to the CB_1/CB_2 full agonist WIN55,212-2, E_{max} = 100%), albeit with potency and efficacy lower than that of anandamide tested under the same conditions (EC_{50} = 0.36 μM and E_{max} = 68.6%) (Figure 5).

Activity of Cyclopropanolamides at TRPV1 Receptors. Within the two racemic *cis*/*trans* arachidonoylcyclopropanolamides, only the *trans*-diastereomer *rac*-**9a** retained an activity similar to or even higher than that of anandamide at elevating intracellular Ca^{2+} in HEK-293 cells stably transfected with the human recombinant TRPV1 channel. Conversely, the *cis*-diastereoisomer *rac*-**10a** was significantly less potent than anandamide (Table 1). A further significant, although less dramatic, dissection of the pharmacological activity was also observed with the two enatiopure *cis*- enantiomers **13a** and **14a**, but little enantiodifferentiation was observed within the more active *trans*-enantiomers **11a** and **12a**. Thus, the **13a** enantiomer was 3-fold more potent than the **14a** enantiomer (Table 2 and Figure 6). Similar, although not identical, results were obtained with the cyclopropanolamides of oleic acid. In this case, the *trans*-diastereoisomer *rac*-**9b** was more than 50-fold more potent than OEA, while the *cis*-diastereoisomer *rac*-**10b** was equipotent with OEA (Table 1). Furthermore, the two *trans*-enantiomers **11b** and **12b**, just like their corresponding *cis*-enantiomers **13b** and **14b**, exhibited similar potency, although the former was 10-fold more potent than the latter (Table 2 and Figure 6). However, although the enantiomers **13b** and **14b** exhibited similar potency, the former was still more efficacious than the latter. Finally, despite the almost total lack of direct activity of palmitoylethanolamide at TRPV1 receptors, the *trans*-palmi-

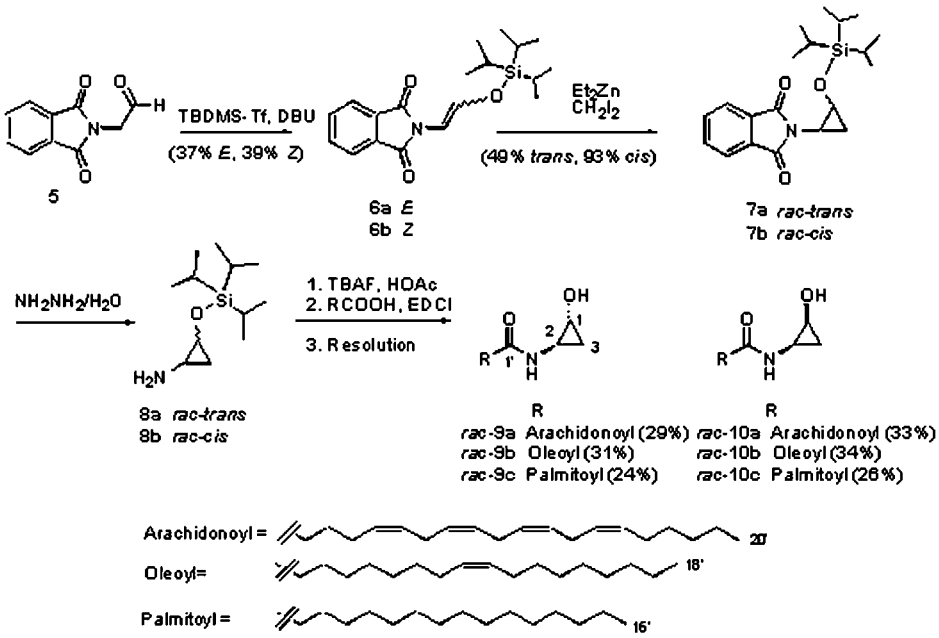


Figure 2. Synthesis of the racemic cyclopropanolamides (±)-**9a**−**c** and (±)-**10a**−**c**.

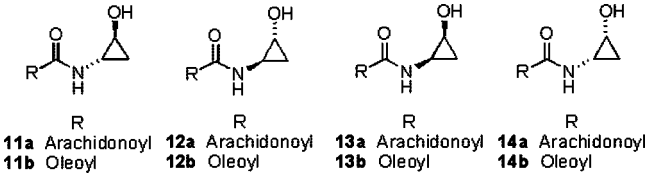


Figure 3. Absolute configuration of the enantiopure *N*-acylcyclopropanolamides resolved by chiral preparative HPLC.

toylcyclopropanolamide *rac*-**9c** was as potent as capsaicin (although less efficacious) at elevating intracellular Ca^{2+} via these channels in HEK-293 cells, whereas its *cis*-diastereoisomer *rac*-**10c** was almost inactive (Table 1).

Discussion and Conclusions

Conformational constraining is a popular strategy to identify the active conformation of flexible ligands, and this approach has been successfully applied in various fields of medicinal chemistry, including peptidomimetic structures.[24] In ethanolamides, rotation around the sp^3 carbon−carbon bond of the polar head interconverts hydrogen-bonded *syn* and non-hydrogen-bonded *anti* rotamers (Figure 7). The H-bonded *syn* rotamers are expected to prevail in a relatively apolar binding pocket, whereas their fitting into a cavity rich with H-bonding elements would favor the *anti*-conformation, which can establish more H-bonding contacts with a biological surface. The observation that the presence of the hydroxyl is redundant for the cannabinoid receptor activity of anandamide but is critical for its activation of TRPV1 channels[25] suggests that different binding modes are involved for the two receptors. To address this issue, we have introduced a methylene bridge around the central bond of the ethanolamine moiety, thereby transforming fleeting *syn*/*anti* rotamers into noninterconverting diastereomeric *cis*/*trans* isomers (Figure 7, parts A and B, respectively). Our results on the conformationally constrained analogues of anandamide fully confirm this. Thus, the much higher TRPV1-stimulating activity of the *trans*-isomers of methylenandandamide compared to their *cis* isomers suggests that TRPV1 prefers to bind to the *anti*-conformation of anandamide. By contrast, the very similar cannabinoid receptor affinity of the *cis* and *trans* isomers suggests that the terminal oxymethylene is not involved in the binding to CB_1 and CB_2, an observation that is in full accordance with the lack of requirement of the primary hydroxyl of anandamide for its binding to these receptors.[26] In fact, the *N*-alkyl group of anandamide might simply act as a conformational bias to maintain the amide carbonyl and the acidic N−H amide bond in the reciprocal geometry necessary for optimal binding to cannabinoid receptors.[26]

Methylenation of OEA yielded surprising results, since it strongly increased the affinity for CB_1 and CB_2 receptors, which is otherwise marginal in the parent compound, while also potentiating, in a diastereoselective way, the efficacy and potency at TRPV1. A similar potentiation of the vanilloid activity was also observed in PEA. In all cases, the *trans* isomers were more active at TRPV1 than the *cis* isomers, confirming the findings with anandamide.

In all cases, little enantiodifferentiation was observed within ligands, both for the binding to cannabinoid receptors and for TRPV1 functional activity. However, some statistically significant enantioselectivity in terms of either potency or efficacy at TRPV1 channel activity was observed within the less active *cis* diastereoisomers of both arachidonoyl- and oleoylcyclopropanolamides, in both cases with enantiomer **13** (Figure 3) exhibiting the highest activity.

The present observations have several potentially important and previously unsuspected implications. First of all, we have shown for the first time that also TRPV1 receptors, like other receptors, can "sense" configurational differences in their agonists. This observation is likely to open new studies on the molecular organization of the TRPV1 binding site and, in particular, on the role of specific amino acid residues in the recognition of anandamide. This compound binds to TRPV1 at the same site as capsaicin,[27] and our observations should assist in the identification of the residues that recognize the ethanolamine "head" of anandamide and OEA.[28] Second, we have demonstrated that a reduction of the flexibility of the ethanolamine "head" of an unsaturated and a monounsaturated acylethanolamide can dramatically improve their otherwise weak activity at TRPV1 receptors, provided that the cyclopropyl group has a *trans*-configuration. This chemical modification has such

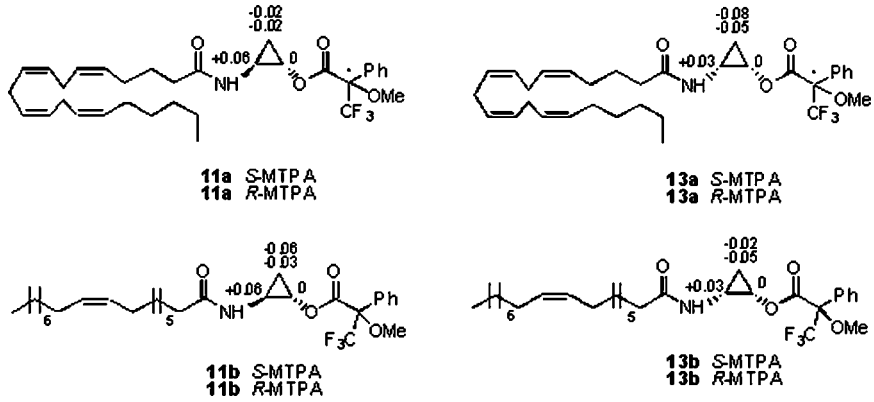
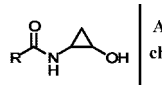


Figure 4. $\Delta\delta_{(S-R)}$ observed for the MTPA esters of **11a**, **13a**, **11b**, and **13b**.

Table 1. Effects of the Racemic Mixtures of the Two Diastereoisomers of Each *N*-Acylcyclopropanolamide Synthesized Here on Cannabinoid and TRPV1 Receptors[a]

	Acyl chain	K_i at hCB_1 (μM)	K_i at hCB_2 (μM)	EC_{50} at hTRPV1 (μM)
AEA	-	0.011±0.003	0.168±0.002	0.20±0.05
***rac*-10a**	C20:4	0.027±0.003	0.15±0.02	1.90±0.21
***rac*-9a**	C20:4	0.032±0.002	0.24±0.09	0.05±0.01
OEA	-	1.10±0.20	>10	3.65±0.80
***rac*-10b**	C18:1	0.60±0.19	0.34±0.24	3.70±0.70
***rac*-9b**	C18:1	0.57±0.17	0.38±0.09	0.06±0.01
PEA		>5	>5	>10
***rac*-10c**	C16:0	>10	>10	26.4±7.1
***rac*-9c**	C16:0	>10	>10	0.04±0.01
Capsaicin	-	>10	>10	0.020±0.003

[a] Affinity of compounds for human recombinant CB_1 and CB_2 receptors was evaluated using membranes from HEK-293 cells transfected with the respective cDNAs and [^{3}H]CP55,940 as the high affinity ligand and WIN 55212-2 as the heterologous competitor for nonspecific binding. K_i values are calculated by applying the Cheng−Prusoff equation to the IC_{50} values for the displacement of the bound radioligand by increasing concentrations of the test compound. Data are reported as mean values ± SEM of at least $n = 3$ experiments. The functional activity of compounds at human recombinant TRPV1 receptors was evaluated by measuring the effect on intracellular Ca^{2+} in HEK-293 cells stably transfected with the cDNA encoding for the receptor. Data are the mean values ± SEM of at least $n = 3$ experiments. The effects of standard compounds is also shown for comparison. The data on OEA and PEA affinity for cannabinoid receptors and functional activity at TRPV1 receptors are in agreement with previously published data.[28,40,41]

a dramatic effect that the *trans*-diastereoisomers of these compounds and of anandamide, which already exhibits activity at TRPV1 receptors, become as potent and, in most cases, as efficacious as capsaicin. These compounds represent the first potent TRPV1 agonists that lack a phenolic (vanillyl or catecholic) group in their polar head. The effect of cyclopropanation on TRPV1 activity was also evident in the activity of the methylene derivatives of PEA, which represent the first example of TRPV1 agonists with unfunctionalized linear acyl chain. With OEA, but not with anandamide, the introduction of the cyclopropyl group also enhanced the affinity for CB_2 and, especially, CB_1 receptors, although in a nondiastereoselective way. This was somewhat surprising, since, although the endocannabinoids are achiral, enantiodifferentiation in binding to cannabinoid receptors was shown in optically active anandamide[29] and 2-arachidonoylglycerol[30] analogues.

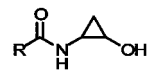
Taken together, our observations qualify conformational constraint as a critical maneuver[31] to map the binding site of ethanolamides to cannabinoid receptors. Finally, our findings might also have important therapeutic implications, since we have identified the enantiomeric *trans*-methylenoleamides **11a** and **12a** as potent CB_1/TRPV1 "hybrid" agonists worth further investigation as potent analgesic, antiemetic, neuroprotective, and antitumor leads.[13,32−35] Compared to arvanil, the best documented CB_1/TRPV1 "hybrid", **11a** and **12a**, are endowed with a better balance between the two types of activity that are instead biased toward TRPV1 for arvanil.[36] Clearly, dual CB_1/TRPV1 agonists are likely to correct dysregulation of endocannabinoid and endovanilloid signaling systems better than agents individually targeting either TRPV1 or CB_1 receptors.[2,3] Possibly for this reason, compounds like arvanil were found to elicit beneficial effects in animal models of pain, neurotoxicity, and emesis that were stronger than those of "single" CB_1 and TRPV1 agonists tested under the same conditions.[32−34] On the other hand, the development of CB_1/TRPV1 "hybrids" should pose less toxicological and/or pharmacokinetic problems than the coadministration of "single" CB_1 and TRPV1 agonists.

In conclusion, our results show that the ethanolamine moiety has a different role in the activation of vanilloid and cannabinoid receptors by their endogenous ligand anandamide. For the first time, a diastereomeric dependence for TRPV1 activation by its ligands has been observed, identifying the *anti*-rotamer of acylethanolamides as their active conformation and obtaining compounds the potency of which rivals that of capsaicinoids. Finally, we have presented evidence that methylenation of ethanolamides can dramatically potentiate the activity of non-arachidonoyl homologues of this family of lipids at TRPV1 and, at least for oleoyl derivatives, also at cannabinoid receptors. Taken together, these findings have potentially important implications for our understanding of the molecular mechanisms involved in the activation of TRPV1 and cannabinoid receptors and for their translation into the development of new therapeutic drugs.

Experimental Section

1. General Synthetic Procedures. For gravity column chromatography (CC), Merck silica gel (70−230 mesh) and Macherey-Nagel aluminum oxide neutral were used. Chiral-phase HPLC was performed on a Knauer apparatus equipped with a refractive index detector. For IR experiments, a Shimadzu DR 8001 spectrophotometer was used. For NMR experiments, Jeol Eclipse (300 and 75 MHz for ^{1}H and ^{13}C, respectively) and Varian INOVA (500

Table 2. Effects of the Two Enantiomers of Each Racemic Mixture of *N*-Arachidonoyl- and *N*-Oleoylcyclopropanolamides on Cannabinoid and TRPV1 Receptors[a]

	Acyl chain	K_i at hCB_1 (μM)	K_i at hCB_2 (μM)	EC_{50} at hTRPV1 (μM)	Max efficacy at TRPV1 (% ionomycin 4μM)
13a	C20:4	0.014±0.001	0.20±0.003	0.61±0.13	76.5±3.2
14a	C20:4	0.020±0.002	0.19±0.004	2.4±0.60	65.8±4.3
11a	C20:4	0.028±0.001	0.29±0.006	0.047±0.01	75.7±2.8
12a	C20:4	0.011±0.003	0.19±0.005	0.023±0.005	80.3±3.9
13b	C18:1	0.70±0.14	2.04±1.22	0.15±0.015	62.5±1.3
14b	C18:1	0.82±0.06	1.63±0.72	0.18±0.02	39.4±1.3
12b	C18:1	0.70±0.41	4.41±2.91	0.013±0.001	72.5±1.1
11b	C18:1	1.40±0.07	2.42±0.92	0.019±0.002	76.2±1.3

[a] Affinity of compounds for human recombinant CB_1 and CB_2 receptors was evaluated using membranes from HEK-293 cells transfected with the respective cDNAs and [^{3}H]CP55,940 as the high affinity ligand and WIN 55212-2 as the heterologous competitor for nonspecific binding. K_i values are calculated by applying the Cheng−Prusoff equation to the IC_{50} values for the displacement of the bound radioligand by increasing concentrations of the test compound. Data are reported as mean values ± SEM of at least $n = 3$ experiments. The functional activity of compounds at human recombinant TRPV1 receptors was evaluated by measuring the effect on intracellular Ca^{2+} in HEK-293 cells stably transfected with the cDNA encoding for the receptor. Data are mean values ± SEM of at least $n = 3$ experiments. Efficacy was evaluated in comparison to the effect on intracellular Ca^{2+} by ionomycin (4 μM).

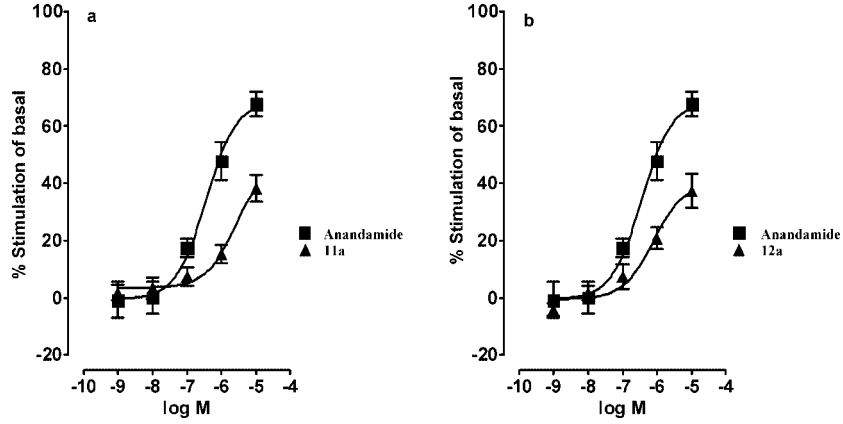


Figure 5. Effect of (a) **11a** ($n = 8$), (b) **12a** ($n = 8$), and (a, b) anandamide ($n = 12$) on the level of [^{35}S]GTPγS binding to mouse brain membranes. Each symbol represents the mean percent increase in [^{35}S]GTPγS binding ± SEM. WIN55,212-2 was taken as the full agonist ($E_{max} = 100\%$). As a reference compound, CP55,940 behaved as a more efficacious ($E_{max} = 83\%$) and potent ($EC_{50} = 10$ nM) agonist in this assay.

and 125 MHz for ^{1}H and ^{13}C, respectively) spectrometers were used and chemical shifts are reported in δ values. Benzene, CH_2Cl_2, and THF by filtration over alumina prior were used directly in the reaction flask. Petroleum ether refers to the fraction boiling between 40 and 60 °C. All reactions were conducted under nitrogen in dry solvents unless stated otherwise. Monitoring by TLC was done on Merck 60 F_{254} (0.25 mm) plates, which were visualized by UV inspection and/or staining with 5% H_2SO_4 in ethanol and heating. Organic phases were dried with Na_2SO_4 before evaporation. ESI MS data were taken on a LCQ Finnigan MAT apparatus, in the positive ion mode. A Knauer HPLC apparatus equipped with refraction index detector was used to assess purity (>95%) of all final products. Chirex-3014 or Chirex-3020 (Phenomenex) columns were used, with elution with EtOAc/*n*-hexane mixtures and 0.7 mL/min as flow rate.

(*E*)-(*Z*)-2-[2-(*tert*-Butyldimethylsilyloxy)ethenyl]isoindoline-1,3-dione (6a and 6b). To a stirred solution of (1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)acetaldeide (**5**) (420 mg, 2.22 mmol) in dry CH_2Cl_2 (7 mL), TBDMS triflate (997 μL, 4.36 mmol, 1.9 mol. equiv) and DBU (1.081 mL, 7.23 mmol, 3.25 mol equiv) were added.[37] The mixture was stirred for 15 min at room temperature and then worked up by the addition of 2 N H_2SO_4 and dilution with CH_2Cl_2. The organic phase was dried and evaporated, and the residue was purified by gravity CC on silica gel (12.5 g, petroleum ether−EtOAc, 95:5, as eluant) to afford 250 mg (37%) of the *E* isomer (**6a**) and 265 mg (39%) of the *Z* isomer (**6b**). Because of their instability, both compounds were used without further separation for the methylenation step. *E*-Isomer (**6a**): yellowish oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (2H, m), 7.67 (2H, m), 7.50 (1H, d, $J =$ 11.3 Hz), 6.40 (1H, d, $J = 11.3$ Hz), 0.95 (9H, s), 0.19 (6H, s). *Z*-Isomer (**6b**): yellowish oil. ^{1}H NMR (300 MHz, CD_3COCD_3): δ 7.86 (4H, m), 6.60 (1H, d, $J = 4.3$ Hz), 5.41 (1H, d, $J = 4.3$ Hz), 0.85 (9H, s), 0.17 (6H, s). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 166.8 (s), 141.0 (d), 133.9 (d), 132.4 (s), 123.3 (d), 99.2 (d), 25.4 (q), −5.3 (q).

(*trans*/*cis*)-2-((±)-2-(*tert*-Butyldimethylsilyloxy)cyclopropyl)-isoindoline-1,3-dione (7a and 7b). To a cooled (ice bath) stirred solution of **6a** (610 mg, 2 mmol) in dry benzene, Et_2Zn (1 M solution in hexane, 30 mL, 30 mmol, 15 mol equiv) and CH_2I_2 (2.2 mL, 30 mmol, 15 mol equiv) were added. The mixture was stirred overnight at 65 °C and then worked up by the addition of 2 N H_2SO_4 and dilution with petroleum ether. The organic phase was washed with saturated $NaHCO_3$, dried, evaporated, and the residue was purified by gravity CC on silica gel (18 g, petroleum ether−EtOAc, 95:5, as eluant) to afford 310 mg (49%) of **7a** as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.79 (2H, m), 7.69 (2H, m), 3.90 (1H, m), 2.79 (1H, m), 1.27−1.19 (2H, m), 0.91 (9H, s), 0.21 (6H, s). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 168.5 (s), 134.0 (d),

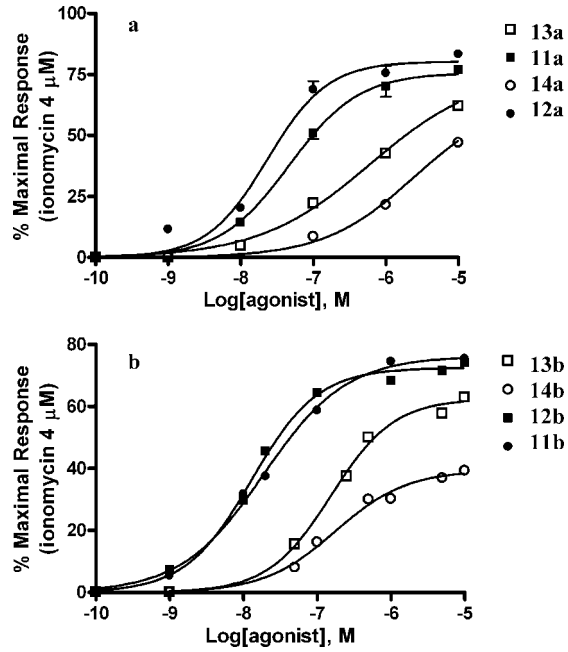


Figure 6. Dose response curves for the four pure enantiomers of (a) *N*-arachidonoylcyclopropanolamides **11a**, **12a**, **13a**, and **14a** and (b) *N*-oleoylcyclopropanolamides **12b**, **11b**, **13b**, and **14b**. Experimental conditions are described in the Experimental Section and in the footnotes to Tables 1 and 2.

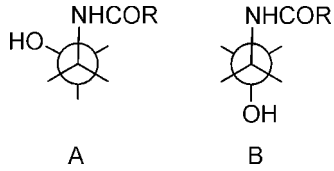


Figure 7. *Syn* (A) and *anti* (B) conformations of *N*-acylethanolamides.

131.8 (s), 123.2 (d), 51.3 (d), 28.8 (d), 25.8 (q), 15.5 (t), −4.7 (q), −5.1 (q). CI-EIMS: *m/z* $[M + H]^+$ 360 $[C_{20}H_{29}NO_3Si + H]^+$. Under similar conditions, **6b** (335 mg, 1.1 mmol) afforded 326 mg (93%) of **7b** as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (2H, m), 7.67 (2H, m), 3.63 (1H, m), 2.61 (1H, m), 1.48 (1H, m), 1.22 (1H, m), 0.66 (9H, s), 0.01 (3H, s) 0.00 (3H, s). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.3 (s), 133.8 (d), 132.0 (s), 122.9 (d), 48.2 (d), 25.4 (q), 25.2 (d), 12.4 (t), −5.1 (q). CI-EIMS: *m/z* $[M + H]^+$ 360 $[C_{20}H_{29}NO_3Si + H]^+$.

(±)-*trans*-*N*-(2-Hydroxycyclopropyl)acylamides 9a−c. Synthesis of 9a as Representative. To a stirred solution of **7a** (422 mg, 1.37 mmol) in CH_2Cl_2 (5 mL) and EtOH (1 mL), $NH_2NH_2 \cdot H_2O$ (440 μL, 8.26 mmol, 6 mol equiv) was added. The mixture was stirred for 4 h at room temperature, and then filtered over a Celite pad. The organic phase was evaporated to afford **8a**, directly used for the acylation step. To this purpose, to a stirred solution of crude **8a** (175 mg, 0.93 mmol) in dry CH_2Cl_2 (2.5 mL), arachidonic acid (305 μL, 0.93 mmol, 1 mol equiv), EDCl (356 mg, 1.86 mmol, 2 mol equiv), and catalyst DMAP were added. The mixture was stirred under nitrogen at room temperature overnight and then worked up by evaporation. The residue was adsorbed on silica gel and filtered by gravity CC on neutral alumina (5 g, petroleum ether−EtOAc, 95:5, as eluant) to afford 330 mg of the protected amide. The latter was dissolved in dry THF (5 mL), and AcOH (40 μL, 1.07 mmol, 1.5 mol equiv) and TBAF (1 M in THF, 1.07 mL, 1.07 mmol, 1.5 mol equiv) were added. The solution was stirred under nitrogen at room temperature for 10 min and then worked up by the addition of brine and dilution with EtOAc. The organic phase was dried, evaporated, and the residue was purified by gravity CC on silica gel (8 g, petroleum ether−EtOAc, 5:5, as eluant) to afford 145 mg (29% from **7a**) of **9a** as a pale-yellow oil. Compounds *rac*-**9b** and *rac*-**9c** were obtained in the same way from **7a**, using oleic and palmitic acid, respectively, in the acylation step (31% and 24% overall yield, respectively).

(±)-*trans*-*N*-(2-Hydroxycyclopropyl)palmitamide (9c). Colorless foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.38 (1H, br s, NH), 3.39 (1H, m, H-2), 2.67 (1H, m, H-1), 2.10 (2H, t, J = 7.3 Hz, H-2′a,b,), 1.58 (2H, m, H-3′a,b), 1.24 (24H, br s, H_2-4′a,b to H_2-15′a,b), 1.09 (1H, m, H-3a), 0.87 (3H, t, J = 6.4 Hz, H-16′), 0.76 (1H, m, H-3b). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 174.8 (s), 52.7 (d), 36.5 (t), 31.9 (t), 30.5 (d), 29.7 (t), 29.5 (t), 29.43 (t), 29.40 (t), 29.3 (t), 25.6 (t), 22.7 (t), 15.8 (t), 14.1 (q). CI-EIMS: *m/z* $[M + H]^+$ 312 $[C_{19}H_{37}NO_2 + H]^+$.

(±)-*cis*-*N*-(2-Hydroxycyclopropyl)acylamides 10a−c. Synthesis of 10a as Representative. To a stirred solution of **7b** (392 mg, 1.28 mmol) in CH_2Cl_2 (5 mL) and EtOH (1 mL), $NH_2NH_2 \cdot H_2O$ (410 μL, 7.68 mmol, 6 mol equiv) was added. The mixture was stirred for 3 h at room temperature and then filtered over a pad of Celite. The organic phase was evaporated to afford **8b** that was directly used for the acylation step. To this purpose, to a stirred solution of crude **8b** (160 mg, 0.85 mmol) in dry CH_2Cl_2 (2.5 mL), arachidonic acid (279 μL, 0.85 mmol, 1 mol equiv), EDCl (325 mg, 1.7 mmol, 2 mol equiv), and catalyst DMAP were added. The mixture was stirred under nitrogen at room temperature overnight and then worked up by evaporation. The residue was adsorbed on silica gel and next vacuum-filtered on neutral alumina (5 g, petroleum ether−EtOAc, 95:5, as eluant) to afford 360 mg of the crude protected amide. Under a nitrogen atmosphere, the latter was dissolved in dry THF (5 mL), and AcOH (45 μL, 1.2 mmol, 1.5 mol equiv) and TBAF (1 M in THF, 1.20 mL, 1.12 mmol, 1.5 mol equiv) were then added. The solution was stirred under nitrogen at room temperature for 10 min and next worked up by the addition of brine and dilution with EtOAc. The organic phase was dried, evaporated, and the residue was purified by gravity CC on silica gel (8 g, petroleum ether−EtOAc, 5:5, as eluant) to afford 153 mg (33% from **7b**) of **10a** as a pale-yellow oil. Compounds *rac*-**10b** and *rac*-**10c** were obtained in the same way from **7b**, using oleic and palmitic acid, respectively, in the acylation step (34% and 26% overall yield, respectively).

(±)-(*cis*)-*N*-(2-Hydroxycyclopropyl)palmitamide (7b). Amorphous foam. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.76 (1H, bs, NH), 3.61 (1H, m, H-2), 2.60 (1H, m, H-1), 2.18 (2H, t, J = 7.0 Hz, H-2′), 1.61 (2H, m, H-3′a,b), 1.25 (24H, H-4′a,b to H_2-15′a,b, m), 0.97 (1H, m, H-3a), 0.87 (3H, t, J = 6.4 Hz, H-16′), 0.59 (1H, m, H-3b). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 175.6 (s), 49.0 (d), 36.6 (t), 30.0 (t), 29.7 (t), 29.5 (t), 29.4 (t), 29.3 (t), 27.5 (d), 25.8 (t), 22.7 (t), 14.5 (t), 14.2 (q). CI-EIMS: *m/z* $[M + H]^+$ 312 $[C_{19}H_{37}NO_2 + H]^+$.

Resolution of (±)-9a and (±)-10a by Chiral Phase HPLC. (a) The racemic *trans N*-arachidonoylcyclopropanolamide (±)-**9a** was separated by chiral-phase HPLC with a Chirex-3020 (Phenomenex) column (stationary phase with *S*-*tert*-leucine and *R*-1-α-naphthylethylamine urea linkage). The eluent mixture EtOAc/*n*-hexane, 65:35, was used in isocratic mode with the flow 0.7 mL/min to obtain the enantiomers **11a** (1*S*,2*S*) (t_R = 7.8) and **12a** (1*R*,2*R*) (t_R = 8.5) (see below for the assignment of the absolute configuration).

(1*S*,2*S*)-*N*-Arachidonoylcyclopropanolamide (11a). Light-yellow oil. $[\alpha]_D^{22}$ +5 (*c* 0.3, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.71 (1H, d, J = 5.0 Hz, NH), 5.35 (16H, m, H-5′a,b, H-6′a,b, H-8′a,b, H-9′a,b, H-11′a,b, H-12′a,b, H-14′a,b, H-15′a,b), 3.35 (1H, m, H-2), 2.82 (6H, m, H-7′a,b, H-10′a,b, H-13′a,b), 2.65 (1H, m, H-1), 2.13 (2H, t, J = 6.7 Hz, H-2′a,b), 2.06 (2H, q, J = 6.0 Hz, H-4′a,b), 2.05 (2H, m, H-16′a,b), 1.70 (4H, m, H-3′a,b, H-17′a,b), 1.30 (4H, m, H-18′a,b, H-19′a,b), 1.11 (1H, m, H-3a), 0.88 (3H, t, J = 7.1 Hz, H-20′), 0.73 (1H, m, H-3b). ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.3 (C-1′, s), 130.3 (C-5′, C-15′, d), 127.8 (C-6′, C-8′, C-9′, C-11′, C-12′, C-14′, d), 52.4 (C-2, d), 36.3 (C-2′, t), 32.0 (C-18′, t), 31.6 (C-1, d), 30.0 (C-17′, t), 27.8 (C-16′, t), 27.7 (C-3′, t), 26.7 (C-4′, t), 25.9 (C-3, t), 25.7 (C-7′, C-10′, C-13′, t), 23.1 (C-19′, t), 13.5 (C-20′, q). ESI-MS: *m/z* 382 $[M + Na]^+$.

(1*R*,2*R*)-*N*-Arachidonoylcyclopropanolamide (12a). Light-yellow oil. $[\alpha]_D^{22}$ −5 (*c* 0.3, $CHCl_3$). For ^1H NMR, ^{13}C NMR, and MS data, see those of **11a**.

(b) The racemic *cis*-*N*-arachidonoylcyclopropanolamide (±)-**10a** was resolved by chiral-phase HPLC with a Chirex-3014 (Phenomenex) column (stationary phase with *S*-valine and *R*-1-α-naphthylethylamine urea linkage). The eluent mixture EtOAc/*n*-hexane, 8:2, was used in isocratic mode with the flow 0.7 mL/min to obtain the enantiomers **13a** (1*S*,2*R*) (t_R = 4.1) and **14a** (1*R*,2*S*) (t_R = 5.0) (see below for the assignment of the absolute configuration).

(1*S*,2*R*)-*N*-Arachidonoylcyclopropanolamide (13a). Light-yellow oil. $[\alpha]_D^{22}$ −5 (*c* 0.5, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ 5.80 (1H, d, *J* = 5.0 Hz, NH), 5.37 (8H, m, H-5′, H-6′, H-8′, H-9′, H-11′, H-12′, H-14′, H-15′), 3.62 (1H, m, H-2), 2.82 (6H, m, H-7′a,b, H-10′a,b, H-13′a,b), 2.63 (1H, m, H-1), 2.20 (2H, t, *J* = 6.7 Hz, H-2′a,b), 2.10 (2H, q, *J* = 6.0 Hz, H-4′a,b), 2.07 (2H, m, H-16′a,b), 1.72 (4H, m, H-3′a,b, H-17′a,b), 1.31 (4H, m, H-18′a,b, H-19′a,b), 1.00 (1H, m, H-3a), 0.88 (3H, t, *J* = 7.1 Hz, H-20′), 0.58 (1H, m, H-3b). ^{13}C NMR (125 MHz, $CDCl_3$): δ 175.3 (C-1′, s), 130.3 (C-5′, C-15′, d), 127.8 (C-6′, C-8′, C-9′, C-11′, C-12′, C-14′, d), 49.3 (C-2, d), 36.2 (C-2′, t), 32.0 (C-18′, t), 30.0 (C-17′, t), 28.2 (C-1, d), 27.8 (C-16′, t), 27.7 (C-3′, t), 26.7 (C-4′, t), 26.3 (C-3, t), 25.9 (C-7′, C-10′, C-13′, t), 23.1 (C-19′, t), 13.5 (C-20′, q). ESI-MS: *m*/*z* 382 $[M + Na]^+$.

(1*R*,2*S*)-*N*-Arachidonoylcyclopropanolamide (14a). Light-yellow oil. $[\alpha]_D^{22}$ +5 (*c* 0.8, $CHCl_3$). For ^1H NMR, ^{13}C NMR, and MS data, see those of **13a**.

2. Resolution of (±)-9b and (±)-10b by Chiral Phase HPLC. (a) The racemic *trans*-*N*-oleoylcyclopropanolamide (±)-**9b** was separated by chiral-phase HPLC with a Chirex-3020 (Phenomenex) column. The eluent mixture EtOAc/*n*-hexane, 55:45, was used in isocratic mode with the flow 0.7 mL/min to obtain pure stereoisomers **11b** (1*S*,2*S*) (t_R = 9.5) and **12b** (1*R*,2*R*) (t_R = 8.5) (see below for the assignment of the absolute configuration).

(1*S*,2*S*)-*N*-Oleoylcyclopropanolamide (11b). Gum. $[\alpha]_D^{22}$ +11 (*c* 0.2, $CHCl_3$). ^1H NMR (500 MHz Varian INOVA, $CDCl_3$): δ 5.87 (1H, d, *J* = 5.0 Hz, NH), 5.32 (2H, m, H-9′, H-10′), 3.35 (1H, m, H-2), 2.63 (1H, m, H-1), 2.10 (2H, t, *J* = 6.7 Hz, H-2′a,b), 2.00 (4H, m, H-8′a,b, H-11′a,b), 1.58 (2H, m, H-3′a,b), 1.30 (20H, m, H-4′a,b to H-7′a,b, and H-12′a,b to H-17′a,b), 1.09 (1H, m, H-3a), 0.87 (3H, t, *J* = 7.1 Hz, H-18′), 0.75 (1H, m, H-3b). ^{13}C NMR (125 MHz Varian INOVA, $CDCl_3$): δ 175.2 (C-1′, s), 130.1 (C-9′, C-10′, d), 52.9 (C-2, d), 36.1 (C-2′, t), 32.1 (C-4′ to C-7′, C-12′ to C-16′, t), 30.6 (C-1, d), 29.6 (C-3′, t), 25.7 (C-3, t), 24.7 (C-8′, C-11′, t), 23.0 (C-17′, t), 14.3 (C-18′, q). ESI-MS (LCQ Finnigan MAT, positive ions) *m*/*z* 360 $[M + Na]^+$.

(1*R*,2*R*)-*N*-Oleoylcyclopropanolamide (12b). Amorphous foam, $[\alpha]_D^{22}$ −11 (*c* 0.2, $CHCl_3$). For ^1H NMR, ^{13}C NMR, and MS data, see those of **11b**.

(b) The racemic *cis*-*N*-oleoylcyclopropanolamide **10b** was resolved by chiral-phase HPLC with a Chirex-3014 (Phenomenex) column (see above). The eluent mixture EtOAc/*n*-hexane, 65:35, was used in isocratic mode with the flow 0.7 mL/min to obtain pure stereoisomers **13b** (1*S*,2*R*) (t_R = 3.5) and **14b** (1*R*,2*S*) (t_R = 4.5) (see below for the assignment of the absolute configuration).

(1*S*,2*R*)-*N*-Oleoylcyclopropanolamide (13b). Gum. $[\alpha]_D^{22}$ −4 (*c* 0.2, $CHCl_3$). ^1H NMR (500 MHz Varian INOVA, $CDCl_3$): δ 5.87 (1H, d, *J* = 5.0 Hz, NH), 5.33 (2H, H-9′, H-10′), 3.59 (1H, m, H-2), 2.63 (1H, m, H-1), 2.18 (2H, t, *J* = 6.7 Hz, H-2′a,b), 2.00 (4H, H-8′a.b, H-11′a,b), 1.62 (2H, m, H-3′a,b), 1.30 (20H, m, H-4′a,b to H-7′a,b, and H-12′a,b to H-17′a,b), 1.00 (1H, m, H-3a), 0.87 (3H, t, *J* = 7.1 Hz, H-18′), 0.58 (1H, m., H-3b). ^{13}C NMR (125 MHz Varian INOVA, $CDCl_3$): δ 175.8 (C-1′, s), 130.2 (C-9′, C-10′, d), 49.2 (C-2, d), 36.2 (C-2′, t), 32.1 (C-4′ to C-7′, C-12′ to C-16′, t), 29.6 (C-3′, t), 27.8 (C-1, d), 26.1 (C-3, t), 24.7 (C-8′, C-11′, t), 23.1 (C-17′, t), 14.3 (C-18′, q). ESI-MS: *m*/*z* 360 $[M + Na]^+$.

(1*R*,2*S*)-*N*-Oleoylcyclopropanolamide (14b). Brown powder. $[\alpha]_D^{22}$ +4 (*c* 0.2, $CHCl_3$). For ^1H NMR, ^{13}C NMR, and MS data, see those of **13b**.

3. Determination of the Absolute Configuration of the Cyclopropanolamides of Arachidonic and Oleic Acid. (a) Cyclopropanolamides of Arachidonic Acid (11a, 13a). To a solution of compound **11a** (1.5 mg) in dry pyridine (0.3 mL), an excess of (*R*)- or (*S*)-MTPA chloride was added and the mixture left at room temperature for 12 h under stirring. The reaction mixture was then diluted with ether and washed with H_2O and saturated NaCl aqueous solution. The organic layer was dried over Na_2SO_4, concentrated under reduced pressure to give (*S*)-MTPA ester (1.1 mg) and *R*-MTPA ester (1.2 mg), respectively (Figure 4). (*S*)-MTPA ester of **13a**: colorless oil. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.53 (3H, m, MTPA phenyl), 7.42 (2H, m, MTPA phenyl), 4.40 (1H, m, H-2), 3.61 (3H, s, MTPA-OMe), 3.08 (1H, m, H-1), 1.37 (1H, m, H-3a), 0.91 (1H, m, H-3b). The signals of the arachidonoyl portion are identical to those of **13a**. (*R*)-MTPA ester of **13a**: colorless oil. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.56 (3H, m, MTPA phenyl), 7.42 (2H, m, MTPA phenyl), 4.40 (1H, m, H-2), 3.56 (3H, s, MTPA-OMe), 3.05 (1H, m, H-1), 1.45 (1H, m, H-3a), 0.96 (1H, m, H-3b). The signals of the arachidonoyl portion are identical to those of **13a**. To a solution of compound **11a** (1.2 mg) in dry pyridine (0.3 mL), an excess of (*R*)- or (*S*)-MTPA chloride was added and treated as described above, affording (*S*)-MTPA ester (1.0 mg) and *R*-MTPA (1.0 mg) ester of **11a**, respectively (Figure 4). (*S*)-MTPA ester of **11a**: colorless oil. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.53 (3H, m, MTPA phenyl), 7.42 (2H, m, MTPA phenyl), 4.37 (1H, m, H-2), 3.61 (3H, s, MTPA-OMe), 2.96 (1H, m, H-1), 1.37 (1H, m, H-3a), 1.15 (1H, m, H-3b). The signals of the arachidonoyl portion are identical to those of **11a**. (*R*)-MTPA ester of **11a**: colorless oil. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.56 (3H, m, MTPA phenyl), 7.42 (2H, m, MTPA phenyl), 4.37 (1H, m, H-2), 3.56 (3H, s, MTPA-OMe), 2.90 (1H, m, H-1), 1.39 (1H, m, H-3a), 1.17 (1H, H-3b). The signals of the arachidonoyl portion identical to those of **11a**.

(b) Cyclopropanolamides of Oleic Acid (11b, 13b). To a solution of compound **11b** (2.0 mg) in dry pyridine (0.5 mL), an excess of (*R*)- or (*S*)-MTPA chloride was added and treated as described above, affording the (*S*)-MTPA ester (1.6 mg) and *R*-MTPA (1.6 mg) esters of **11b**, respectively (Figure 4). (*S*)-MTPA ester of **11b**: amorphous solid. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.53 (3H, m, MTPA phenyl), 7.42 (2H, m, MTPA phenyl), 4.37 (1H, m, H-2), 3.61 (3H, s, MTPA-OMe), 3.11 (1H, m, H-1), 1.39 (1H, m, H-3a), 0.89 (1H, m, H-3b). The signals of the oleoyl moiety are identical to those of **11b**. (*R*)-MTPA ester of **11b**: amorphous solid. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.56 (3H, m, MTPA phenyl), 7.42 (2H, m, MTPA phenyl), 4.37 (1H, m, H-2), 3.56 (3H, s, MTPA-OMe), 3.08 (1H, m, H-1), 1.44 (1H, m, H-3a), 0.91 (1H, m, H-3b). The signals of the oleoyl moiety are identical to those of **11b**.

To a solution of compound **13b** (1.6 mg) in dry pyridine (0.4 mL), an excess of (*R*)- or (*S*)-MTPA chloride was added, and the solution was treated as above, affording (*S*)-MTPA ester (1.2 mg) and *R*-MTPA ester (1.2 mg) of **13b**, respectively (Figure 4). (*S*)-MTPA ester of **13b**: amorphous solid. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.53 (3H, m, MTPA phenyl), 7.42 (2H, m, MTPA phenyl), 4.40 (1H, m, H-2), 3.61 (3H, s, MTPA-OMe), 2.92 (1H, m, H-1), 1.37 (1H, m, H-3a), 1.10 (1H, m, H-3b). The signals of the oleoyl moiety are identical to those of **13b**. (*R*)-MTPA ester of **13b**: amorphous solid. ^1H NMR ($CDCl_3$, 500 MHz): δ_H 7.56 (3H, MTPA phenyl, m), 7.42 (2H, MTPA phenyl, m), 4.40 (1H, H-2, m), 3.56 (3H, MTPA-OMe, s), 2.86 (1H, H-1, m), 1.40 (1H, H-3a, m), 1.16 (1H, H-3b, m). The signals of the oleoyl moiety were identical to those of **13b**.

4. Cannabinoid CB_1 and CB_2 Receptor Binding Assays. Membranes from HEK-293 cells transfected with the human recombinant CB_1 receptor (B_{max} = 2.5 pmol/mg protein) and human recombinant CB_2 receptor (B_{max} = 4.7 pmol/mg protein) were incubated with $[^3H]$CP-55,940 (0.14 nM, K_d = 0.18 and 0.084 nM, K_d = 0.31 nM, respectively, for CB_1 and CB_2 receptors) as the high affinity ligand and displaced with 10 μM WIN 55212-2 as the heterologous competitor for nonspecific binding (K_i values of 9.2 and 2.1 nM, respectively, for CB_1 and CB_2 receptor). All compounds were tested following the procedure described by the manufacturer (Perkin-

Elmer, Italy). Displacement curves were generated by incubating drugs with [^{3}H]CP-55,940 for 90 min at 30 °C. K_i values were calculated by applying the Cheng−Prusoff equation to the IC_{50} values (obtained by GraphPad) for the displacement of the bound radioligand by increasing concentrations of the test compound. Data are reported as mean values ± SEM of at least $n = 3$ experiments.

5. Cannabinoid CB_1 Receptor Functional Assay. The method used for measuring agonist-stimulated [^{35}S]GTPγS-binding to CB_1 receptors was as described previously.[38] The assays were carried out with mouse brain membranes (10 μg mL^{-1}), preincubated for 30 min at 30 °C with 0.5 U mL^{-1} adenosine deaminase (200 U mg^{-1}) to remove endogenous adenosine, GTPγS binding buffer (50 mM Tris-HCl, 50 mM Tris-Base, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 0.1% BSA) in the presence of 0.1 nM [^{35}S]GTPγS and 30 μM GDP, in a final volume of 500 μL. Binding was initiated by the addition of [^{35}S]GTPγS to the wells. Nonspecific binding was measured in the presence of 30 μM GTPγS. The compounds were incubated in the assay for 60 min at 30 °C. The mixture was terminated by a rapid vacuum filtration method using a washing buffer (50 mM Tris-HCl, 50 mM Tris-Base, 0.1% BSA), and the radioactivity was quantified by liquid scintillation spectrometry. Compounds were stored as a stock solution of 10 mM in DMSO, the vehicle concentration in all assay wells being 0.1% DMSO. Net agonist stimulated [^{35}S]GTPγS-binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist) as detailed elsewhere.[39] Values for EC_{50} and maximal effect (E_{max}) have been calculated by nonlinear regression analysis using the equation for a sigmoid concentration−response curve (GraphPad Prism5.0).

6. TRPV1 Channel Activity Assay. HEK-293 cells stably overexpressing recombinant human TRPV1 cDNA were grown as monolayers in minimum essential medium supplemented with nonessential amino acids, 10% fetal calf serum, and 2 mM glutamine and maintained under O_2/CO_2 (95%/5%) at 37 °C. The effect of the substances on Ca^{2+} influx was determined by using Fluo-4, a selective intracellular fluorescent probe for Ca^{2+}. On the day of the experiment, the cells (50−60000 per well) were loaded for 1 h at 25 °C with Fluo-4 methyl ester (Molecular Probes, Invitrogen), 4 μM in dimethyl sulfoxide containing 0.02% Pluronic (Molecular Probes, Invitrogen), in minimum essential medium without fetal bovine serum. After being loaded, cells were washed twice in Tyrode's buffer, pH 7.4 (NaCl 145 mM, KCl 2.5 mM, $CaCl_2$ 1.5 mM, $MgCl_2$ 1.2 mM, D-glucose 10 mM, HEPES 10 mM, pH 7.4), resuspended in Tyrode's buffer, and transferred (50−60000 cells) to the quartz cuvette of the fluorescence detector (Perkin-Elmer LS50B) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25 °C ($\lambda_{EX} = 488$ nm, $\lambda_{EM} = 516$ nm) before and after the addition of the test compounds at various concentrations. Agonist activity was determined in comparison to the maximum Ca^{2+} influx due to the application of 4 μM ionomycin (Sigma). EC_{50} values were determined as the concentration of test substances required to produce half-maximal increases in $[Ca^{2+}]_i$. All determinations were at least performed in triplicate. Curve fitting (sigmoidal dose−response variable slope) and parameter estimation were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA).

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