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The anandamide membrane transporter. Structure–activity relationships of anandamide and oleoylethanolamine analogs with phenyl rings in the polar head group region

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Abstract—A new series of anandamide and *N*-oleoylethanolamine analogs, most of which with aromatic moieties in the head group region, has been synthesized and evaluated as inhibitors of anandamide uptake. Some of them efficaciously inhibit the uptake process with K_i values in the low micromolar range (2.4–21.2 μM). Strict structural requisites are needed to observe a significant inhibition and in no case inhibition of fatty acid amidohydrolase overlaps with inhibition of anandamide uptake.

1. Introduction

The endogenous cannabinoid system (ECS)[1] comprises two G-protein coupled receptors, CB_1[2] and CB_2,[3] their endogenous ligands [anandamide (AEA),[4] 2-arachidonoylglycerol (2-AG),[5] 2-arachidonyl glyceryl ether (2-AGE, noladin ether)[6] and *O*-arachidonoylethanolamine (virodhamine)][7] and an endocannabinoid inactivation system. AEA and 2-AG are inactivated via a cellular uptake, facilitated by one or more transporters,[8] followed by enzymatic hydrolysis, which is catalyzed by a fatty acid amide hydrolase (FAAH)[9] and, in the case of 2-AG, also by monoacylglycerol lipases.[10] The putative AEA transporter (AMT) has not yet been cloned and its nature is still controversial,[11,12] although considerable evidence, including selective inhibition by various structural analogs of AEA, points to its existence.[13–22] The well established involvement of ECS in the regulation of a wide series of both central and peripheral processes has stimulated an enormous interest in the potential therapeutic applications of compounds acting on the proteins that constitute the ECS.[23–30] In particular, AEA transport inhibitors could offer a therapeutic approach to a variety of diseases including pain,[31] multiple sclerosis,[32] Huntington's chorea[33] in which elevation of AEA levels would seem to afford more favorable responses and fewer undesirable side-effects than direct activation of CB_1 receptors by agonists.

Studies of structure–activity relationships concerned with the AMT and its substrates/inhibitors represent therefore an area of considerable interest. A large number of modifications on the three potential pharmacophore moieties of AEA, the hydrophobic *cis*-tetraene side chain, the carboxamido group and the polar head group, has been already carried out[34] and resulted in the identification of a number of potent inhibitors such as AM404,[35] some *N*-acylvanillylamides (*N*-AVAMs),[36] VDM11,[37] UCM707,[15] 3-pyridinylarachidonylamide[38] and SKM4451.[39] However, AM404 also activates vanilloid (TRPV1) receptors, inhibits FAAH[37,40] and is not very stable to enzymatic hydrolysis; *N*-AVAMs are also agonists at TRPV1 and CB_1 receptors, while VDM11, although almost inactive at these receptors and at FAAH, is hydrolytically instable.[37] Finally, 3-pyridinylarachidonylamide also has very high affinity for FAAH.[38] The identification of new potent, selective

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and metabolically stable AMT inhibitors appears thus still highly desirable.

The most potent AMT inhibitors so far developed have been obtained by introducing aromatic moieties in the polar head group region. The stabilization of the binding has been tentatively attributed to aromatic stacking interactions between inhibitors and the transporter.[38] In this line and taking also into account changes previously shown to diminish the affinity of aromatic long chain fatty acid amides for TRPV1 receptors and/or FAAH, we have recently designed and synthesized a series of 1′-(4-hydroxybenzyl) analogues of *N*-oleoylethanolamine (OEA) and AEA (OMDM1-4, compounds **1**, **2** of Table 1).[14] OMDM1-4 inhibited to a varied extent the AMT, the oleoyl analogues being 6- to 7-fold more potent than the arachidonoyl counterparts. All compounds exhibited poor affinity for both CB_1 and CB_2 receptors and almost no activity at TRPV1 receptors and at FAAH.

In the present study we have conducted a systematic investigation on the structure–activity relationships of AMT inhibitors related to OMDM1-4 in order to gain further insight into the molecular requisites for the inhibitory action. To this end, a representative series of derivatives of arachidonic and oleic acids with general structure **I** (Fig. 1) has been designed. In these derivatives the influence of factors such as the position of the aromatic ring, its distance from the ethanolamine portion, the presence or not of phenolic or benzylic hydroxy groups and the stereochemistry of the substitution has been analyzed. Furthermore, the oleoyl analogue (**II**, R = oleoyl) of AM404 (**II**, R = arachidonoyl), two homologs of AM404 (**III**) and amides derived from (*S*) and (*R*)-prolinol (**IV**) have been prepared and evaluated.

2. Results and discussion

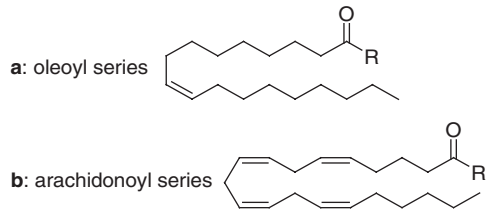
2.1. Chemistry

The synthesis of amides listed in Table 1 has been accomplished by direct condensation between arachidonic or oleic acid and the appropriate amine using 1-hydroxybenzotriazole (HOBt)/*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as the carboxylate activator in 64–87% yields (Fig. 2). The synthesis of the noncommercial amines (*R*) and (*S*)-4-hydroxyphenylglycinol was carried out by reduction of (*R*) and (*S*)-*N*-Boc-4-hydroxyphenylglycine methyl esters with $LiAlH_4$, followed by deprotection with $SOCl_2$/MeOH.

2.2. Biological data

The effect of compounds **1**–**15** on the uptake of [^{14}C]AEA by intact RBL-2H3 cells, where the AMT has been well characterized,[41] is shown in Table 1. For comparative purposes, we have also included the results previously obtained with OMDM1-4[14] and AM404 (compound **12b**).[37]

Table 1. Effect of compounds **1**–**15** on [^{14}C]AEA uptake by RBL-2H3 cells[a]



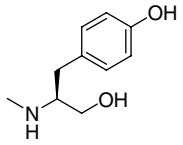
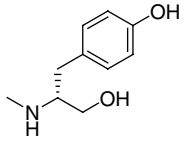
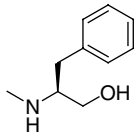
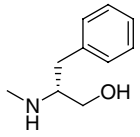
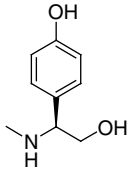
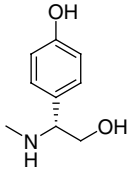
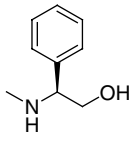
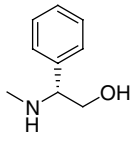
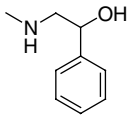
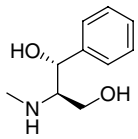
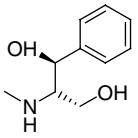
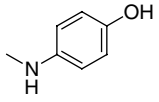
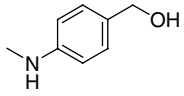
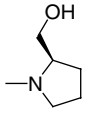
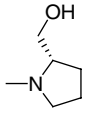
Compd	R	K_i (μM)
1a		2.4 ± 0.7[b]
1b		16.6 ± 0.9[b]
2a		3.0 ± 0.6[b]
2b		17.7 ± 1.5[b]
3a		>25
3b		>25
4a		11.1 ± 2.0
4b		>25
5a		>25
5b		17.5 ± 2.3
6a		>25
6b		21.2 ± 4.1
7a		12.9 ± 3.1
7b		19.4 ± 1.2
8a		16.6 ± 2.1
8b		>25

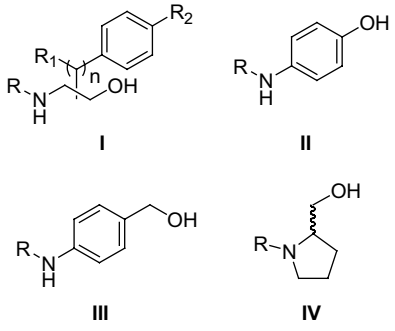
Table 1 (*continued*)

Compd	R	K_i (μM)
9a		>25
9b		>25
10a		>25
10b		15.7 ± 3.7
11a		6.4 ± 0.5
11b		>25
12a		>25
12b		8.1 ± 2.6[c]
13a		>25
13b		>25
14a		>25
14b		>25
15a		>25
15b		>25

[a] The inhibitory effect is expressed as the K_i calculated using the Cheng–Prusoff equation from the concentrations exerting 50% inhibition of [^{14}C]AEA uptake. Data are means ± SEM of $n = 3$ experiments.

[b] Data from Ref. 14.

[c] Value from Ref. 37.



R = arachidonoyl, oleoyl; R_1, R_2 = H, OH; n = 0, 1

Figure 1. General structures of the compounds synthesized.

Although it is clear, according to previous studies,[8] that the head group region in the AEA transporter can tolerate bulk additions, strict structural requisites appear to be necessary in order to observe a significant inhibition. Indeed, some of the compounds inhibited efficaciously the uptake process with K_i values in the low micromolar range (2.4–21.2 μM), while others were inactive at the highest concentration tested (25 μM). None of our compounds inhibited significantly FAAH from RBL-2H3 cells up to a concentration of 50 μM. Several conclusions on the transporter affinity can be drawn from data of Table 1. Removal of the hydroxy group in *para* on the phenyl ring of compounds **1**, **2** results in a decrease of the affinity for AMT (compounds **3** and **4**), which is particularly evident in the case of (*S*)-isomer of oleic acid series (**3a**). An analogous effect, concerned with the introduction of an hydroxy group in *para* of *N*-phenylarachidonylamide has been previously described by Piomelli et al.[42] and Hillard and co-workers[38] and was attributed to a stabilization of binding. An opposite trend is, however, observed here for the series of α-phenylethanolamine analogues (compounds **5**–**8**), where the presence of the *para*-OH group proves to be of little effect or even detrimental for the inhibitory ability. The decrease in the distance of the aromatic ring on the ethanolamine moiety by elimination of the methylene spacer produces variable effects on the inhibitory potency, depending on the presence of hydroxy groups, the stereochemistry and the position of the ring (compounds **1**, **2** and **3**, **4** compared with **5**, **6** and **7**, **8**, **9**, respectively). The introduction of an hydroxy group on the benzylic carbon atom affords appreciably active compounds only in the case of (*S*,*S*)-diastereomer of the oleic acid series (compound **11a**) and of (*R*,*R*)-diastereomer of arachidonic acid series (compound **10b**), a result difficult to interpret. The inactivity of the benzyl analogues **13** of AM404 seems to indicate that the stabilization of the binding to the carrier by a hydrogen bonding acceptor/donor group near to the head region binding site is very sensitive to the position of this group. Compound **13b** has been described by Makriyannis and co-workers as a poor FAAH substrate as well.[43] To study the effects of head group conformational preferences, a set of AEA analogues in which the hydroxyalkyl group was partially restricted by its incorporation into five- or six-membered rings has been previously synthesized.[42] The resulting 3-hydroxypyrrolidinyl- and 3- and 4-hydroxypiperidinylamides exhibited affinity for the transporter similar to that of AEA. In contrast, the conformationally restricted analogues of AEA and OEA **14** and **15** are, rather surprisingly, completely inactive, suggesting that the hydrogen of the hydroxyl group may be involved in an intramolecular hydrogen bond. The arachidonoyl 2-pyrrolidinemethanol derivatives **14b**, **15b** have been previously investigated for their effects on the intraocular pressure (IOP) in normotensive rats, causing a statistically significant reduction of IOP.[44] Regarding the stereochemical factor, the stereoselectivity of the transporter, already evidenced in previous studies, is further confirmed by the differences in the inhibitory potencies of various couples of stereoisomers such as **3a**–**4a**, **7b**–**8b**, **10a**–**11a** and **10b**–**11b**. Regarding the nature of the hydrophobic tail, compounds of the oleic acid series are able, generally, to compete with [^{14}C]AEA more efficaciously than arachidonoyl analogues or, at least, in a comparable fashion. The presence of only one *cis* double bond located in

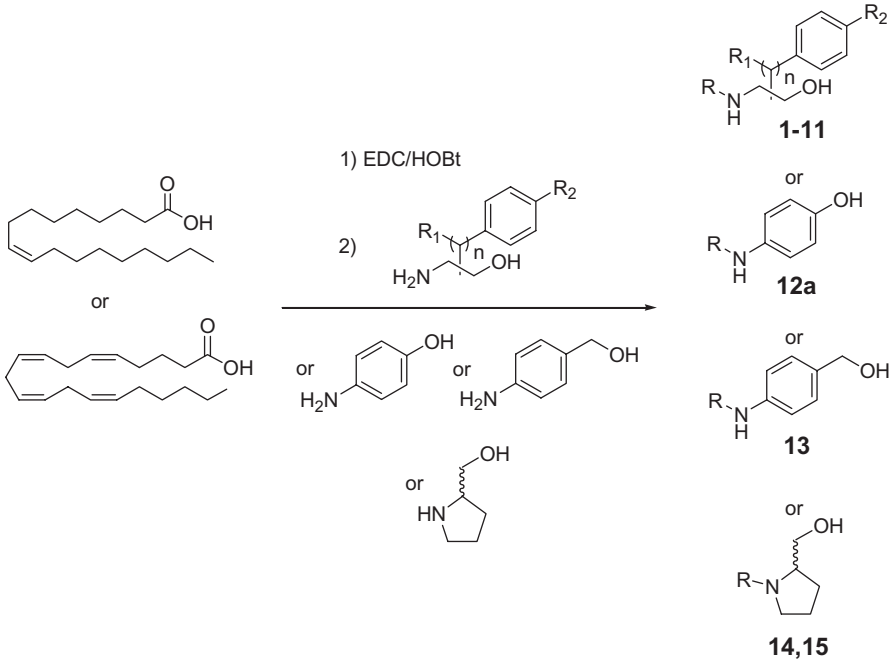


Figure 2. Synthesis of compounds **1**–**15**.

the middle of the fatty acid chain has been shown by Piomelli et al.[42] to represent a sufficient requisite for AMT recognition and, among the ethanolamides of fatty acids other than arachidonic acid, *N*-oleoylethanolamine proved to be the best inhibitor.[8] In this respect, the inactivity of the oleoyl analogue **12a** of AM404 (**12b**) is quite unexpected and deserves further investigation. In conclusion, it is evident that little structural variations in the aromatic moiety of the polar head region produce more or less profound differences in the affinity for AMT. Most importantly, the molecular requisites of the transport process differ significantly from those of the interaction with FAAH, despite the fact that the head group binding region in FAAH can also tolerate large head groups. The reactivity of the substrate[43,45] does not seem to be a major factor in determining the affinity for FAAH.

The three most potent new inhibitors (compounds **4a**, **7a** and **11a**) were also assessed for their activity on cannabinoid CB_1, CB_2 and TRPV1 receptors (Table 2). The three compounds exhibited weak affinity for CB_2 and almost no activity at TRPV1 receptors, while some notable affinity for CB_1 receptors was observed only for **4a**. Compounds **7a** and, particularly, **11a** can be viewed, therefore, as relatively selective inhibitors of the AMT.

Table 2. Effects of compounds **4a**, **7a**, **11a** on CB_1, CB_2 and TRPV1 receptors

Compd	hCB$_1$ (K_i μM)[a]	hCB$_2$ (K_i μM)[a]	hTRPV1 (efficacy)[b]
4a	0.5 ± 0.01	2.5 ± 0.02	9.3 ± 1.6
7a	2.7 ± 0.01	2.0 ± 0.1	10.5 ± 2.0
11a	>10	3.2 ± 0.2	10.5 ± 2.1

[a] The affinity for CB_1 and CB_2 receptors was measured in displacement assays carried out using transfected human COS cells and [^{3}H]CP55,940.

[b] Efficacy at human TRPV1 receptors transfected in HEK cells was measured by expressing the effect of a 10 μM concentration of each compound as percent of the effect of 4 μM ionomycin. Data are means ± SEM of $n = 3$ experiments.

3. Experimental

3.1. Chemistry

Melting points were determined with a Kofler hot stage apparatus and are uncorrected. Optical rotations were taken at 20 °C with a Schmidt–Haensch polartronic D polarimeter in a 1 dm cell. Infrared spectra were recorded with a Perkin–Elmer Spectrum 1000 FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured with a Varian Mercury 300 spectrometer using tetramethylsilane as internal standard. Column chromatographies were carried out using Merck silica gel 60 (230–400 mesh).

3.1.1. (*S*)-*N*-Boc-4-hydroxyphenylglycine methyl ester. To a stirred suspension of 4-hydroxyphenylglycine (1.672 g, 10 mmol) in 20 mL of dry MeOH, thionyl chloride (0.87 mL, 12 mmol) was added dropwise at 0 °C and the resulting mixture was stirred 15 min at 0 °C and 16 h at room temperature. Evaporation under reduced pressure of the solution afforded a residue, which was dissolved in dry CH_2Cl_2 (25 mL) and additioned of TEA (1.4 mL, 10 mmol), $NaHCO_3$ (1.26 g, 15 mmol) and $(Boc)_2O$ (2.40 g, 11 mmol). The reaction mixture was stirred at room temperature for 3 h, then concentrated under vacuum, acidified with 0.5 N HCl and extracted with AcOEt. The organic phase was washed with brine, 10% aqueous solution of $NaHCO_3$, brine, dried over Na_2SO_4 and evaporated to give crude (*S*)-*N*-Boc-4-hy-

droxyphenylglycine methyl ester (2.614 g), which was further purified by crystallization from CH_2Cl_2/hexane (2.30 g. 82%): mp 138–139 °C; $[\alpha]_D$ +110 (*c* 1.0, $CHCl_3$); IR (KBr) 3431, 3368, 2987, 1733, 1673, 1614, 1506, 1441, 1320, 1213, 1164, 1061 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.43 (9H, s), 3.70 (3H, s), 5.21 (1H, d, *J* = 7.2 Hz), 5.63 (1H, d, *J* = 7.2 Hz), 6.72 (2H, d, *J* = 8.7 Hz), 6.83 (1H, br s), 7.15 (2H, d, *J* = 8.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 28.27, 52.64, 57.00, 80.43, 115.64, 127.85, 128.19, 154.86, 156.14, 171.75.

3.1.2. (*S*)-*N*-Boc-4-hydroxyphenylglycinol. To a stirred solution of (*S*)-*N*-Boc-4-hydroxyphenylglycine methyl ester (2.117 g, 7.53 mmol) in dry THF (100 mL), $LiAlH_4$ (0.569 g, 15.0 mmol) was added portionwise in 30 min. The mixture was stirred at room temperature for 16 h and then Et_2O saturated with water was cautiously added. The suspension was poured into a cool 5% tartaric acid solution and extracted with AcOEt. The organic phase was washed twice with brine, filtered, dried over Na_2SO_4 and evaporated. The residue (2.291 g) was chromatographed on silica gel (70 g) using hexane/AcOEt = 1:1 as eluent to give pure (*S*)-*N*-Boc-4-hydroxyphenylglycinol (1.321 g, 69%): mp 176–178 °C (from acetone); $[\alpha]_D$ +69 (*c* 1.0, MeOH); IR (KBr) 3401, 3250, 1644, 1560, 1444, 1315, 1169, 1057 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3/CD_3OD$ = 9:1) 1.42 (9H, s), 3.62–3.76 (H, m), 4.59 (1H, m), 5.77 (1H, m), 6.79 (2H, d, *J* = 8.7 Hz), 7.12 (2H, d, *J* = 8.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ = 9:1) δ 28.31, 56.31, 65.93, 79.85, 115.30, 127.63, 156.01, 156.40.

3.1.3. (*S*)-4-Hydroxyphenylglycinol hydrochloride. To a solution of (*S*)-*N*-Boc-4-hydroxyphenylglycinol (0.253 g, 1.0 mmol) in dry MeOH (7.0 mL) cooled at 0 °C, thionyl chloride (0.073 mL, 1.0 mmol) was added and the mixture was stirred at 40 °C for 2 h. Evaporation under reduced pressure of the solution afforded (*S*)-4-hydroxyphenylglycinol hydrochloride (0.189 g), which was used in the next steps without further purification.

3.1.4. (*R*)-*N*-Boc-4-hydroxyphenylglycinol. Prepared following the same procedure used for the synthesis of (*S*) enantiomer. Yield 65.0%; mp 176–178 °C (acetone); $[\alpha]_D$ −69 (*c* 1.0, MeOH).

3.1.5. (*R*)-4-Hydroxyphenylglycinol hydrochloride. Prepared following the same procedure used for the synthesis of (*S*) enantiomer.

3.1.6. General procedure for the synthesis of compounds 1–15. To a solution of fatty acid in DMF at 0 °C, HOBt (1.05 equiv) and EDC (1.05 equiv) were added under stirring. After 15 min at 0 °C and 0.5 h at room temperature, the appropriate amine hydrochloride (1 equiv) and TEA (1 equiv) were added and the mixture was stirred at room temperature overnight. The mixture was diluted with brine and extracted with AcOEt. The organic phase was washed with 10% citric acid solution, saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using hexane/AcOEt or CH_2Cl_2/AcOEt mixtures as eluents.

3.1.7. (*Z*)-*N*-{(1*S*)-2-Hydroxy-1-[(4-hydroxyphenyl)methyl]ethyl}-9-octadecenamide (1a). Yield 82%; mp 88–89 °C; $[\alpha]_D$ −13 (*c* 1.0, $CHCl_3$); IR (KBr) 3432, 3325, 2928, 2856, 1651, 1515, 1234, 1201 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.87 (3H, t, *J* = 6.5 Hz), 1.26 (20H, m), 1.52–1.56 (2H, m), 1.96–2.00 (4H, m), 2.12 (2H, t, *J* = 7.6 Hz), 2.65–2.78 (2H, m), 3.12 (1H, br s), 3.47 (1H, dd, *J* = 11.1, 5.1 Hz), 3.56 (1H, dd, *J* = 11.1, 4.0 Hz), 4.07 (1H, m), 5.26–5.37 (2H, m), 6.38 (1H, d, *J* = 8.4 Hz), 6.70 (2H, d, *J* = 8.4 Hz), 6.97 (2H, d, *J* = 8.4 Hz), 8.05 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.09, 22.66, 25.72, 27.17, 27.21, 29.15, 29.30, 29.50, 29.74, 31.87, 36.15, 36.77, 52.73, 52.82, 63.63, 115.34, 128.46, 129.55, 129.81, 129.97, 155.05, 174.19. Anal. Calcd for $C_{27}H_{45}NO_3$ (431.7): C, 75.13; H, 10.51; N, 3.24. Found: C, 75.28; H, 10.44; N, 3.18.

3.1.8. (*all-Z*)-*N*-{(1*S*)-2-Hydroxy-1-[(4-hydroxyphenyl)-methyl]ethyl}-5,8,11,14-eicosatetraenamide (1b). Yield 74%; wax; $[\alpha]_D$ −17 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3433, 3331, 2930, 2870, 1656, 1615, 1515, 1456, 1261 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, *J* = 6.9 Hz), 1.25–1.37 (6H, m), 1.64 (2H, quintet, *J* = 7.5 Hz), 2.04 (4H, q, *J* = 6.9 Hz), 2.15 (2H, t, *J* = 7.6 Hz), 2.71–2.83 (8H, m), 3.49 (1H, m), 3.52 (1H, dd, *J* = 11.1, 4.9 Hz), 3.61 (1H, dd, *J* = 11.1, 3.3 Hz), 4.12 (1H, m), 5.26–5.41 (8H, m), 5.98 (1H, d, *J* = 7.8 Hz), 6.71 (2H, d, *J* = 8.4 Hz), 6.97 (2H, d, *J* = 8.4 Hz), 7.65 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.07, 22.54, 25.47, 25.60, 26.53, 27.18, 29.27, 31.46, 36.12, 36.21, 52.96, 63.76, 115.44, 127.30, 127.62, 127.91, 128.07, 128.42, 128.49, 128.67, 129.94, 130.32, 154.91, 173.90. Anal. Calcd for $C_{29}H_{43}NO_3$ (453.7): C, 76.78; H, 9.55; N, 3.09. Found: C, 77.02; H, 9.42; N, 3.00.

3.1.9. (*Z*)-*N*-{(1*R*)-2-Hydroxy-1-[(4-hydroxyphenyl)methyl]ethyl}-9-octadecenamide (2a). Yield 83%; mp 88–89 °C; $[\alpha]_D$ +13 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{27}H_{45}NO_3$ (431.7): C, 75.13; H, 10.51, N, 3.24. Found: C, 75.30; H, 10.42; N, 3.19.

3.1.10. (*all-Z*)-*N*-{(1*R*)-2-Hydroxy-1-[(4-hydroxyphenyl)methyl]ethyl}-5,8,11,14-eicosatetraenamide (2b). Yield 82%; $[\alpha]_D$ +18 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{29}H_{43}NO_3$ (453.7): C, 76.78; H, 9.55, N, 3.09. Found: C, 77.09; H, 9.40; N, 2.98.

3.1.11. (*Z*)-*N*-[(1*S*)-2-Hydroxy-1-(phenylmethyl)ethyl]-9-octadecenamide (3a). Yield 66%; wax; $[\alpha]_D$ −12 (*c* 2.0, $CHCl_3$); IR ($CHCl_3$) 3434, 2928, 2856, 1654, 1510, 1465, 1238, 1088 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, *J* = 6.6 Hz), 1.27 (20H, m), 1.51–1.58 (2H, m), 1.99–2.02 (4H, m), 2.11 (2H, t, *J* = 7.6 Hz), 2.79–2.93 (2H, m), 3.55 (1H, dd, *J* = 11.0, 5.1 Hz), 3.63 (1H, dd, *J* = 11.0, 3.9 Hz), 3.78 (1H, br s), 4.16 (1H, m), 5.28–5.40 (2H, m), 6.12 (1H, d, *J* = 7.5 Hz), 7.17–7.30 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.09, 22.65, 25.72, 27.16, 27.19, 29.13, 29.28, 29.49, 29.73, 31.86, 36.77, 36.95, 52.61, 63.72, 126.34, 128.31, 129.00, 129.47, 129.77, 137.58, 173.64. Anal. Calcd for $C_{27}H_{45}NO_2$ (415.7): C, 78.02; H, 10.91; N, 3.37. Found: C, 78.23; H, 10.79; N, 3.29.

3.1.12. (*all-Z*)-*N*-[(1*S*)-2-Hydroxy-1-(phenylmethyl)-ethyl]-5,8,11,14-eicosatetraenamide (3b). Yield 79%; wax; $[\alpha]_D$ –17 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3434, 2929, 2858, 1657, 1509, 1455, 1234, 1039 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, J = 6.9 Hz), 1.29–1.38 (6H, m), 1.64 (2H, quintet, J = 7.2 Hz), 2.01–2.08 (4H, m), 2.15 (2H, t, J = 7.5 Hz), 2.76–2.90 (8H, m), 3.17 (1H, br s), 3.57 (1H, dd, J = 11.1, 5.1 Hz), 3.66 (1H, dd, J = 11.1, 3.6 Hz), 4.17 (1H, m), 5.29–5.44 (8H, m), 5.83 (1H, d, J = 7.2 Hz), 7.20–7.33 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.06, 22.54, 25.45, 25.59, 26.52, 27.18, 29.27, 31.46, 36.08, 36.92, 52.68, 64.03, 126.44, 127.28, 127.61, 127.91, 128.02, 128.39, 128.56, 128.77, 128.95, 130.29, 137.39, 173.27. Anal. Calcd for $C_{29}H_{43}NO_2$ (437.7): C, 79.59; H, 9.90; N, 3.20. Found: C, 79.85; H, 10.02; N, 3.11.

3.1.13. (*Z*)-*N*-[(1*R*)-2-Hydroxy-1-(phenylmethyl)ethyl]-9-octadecenamide (4a). Yield 80%; wax; $[\alpha]_D$ +12 (*c* 2.0, $CHCl_3$). Anal. Calcd for $C_{27}H_{45}NO_2$ (415.7): C, 78.02; H, 10.91; N, 3.37. Found: C, 78.25; H, 10.79; N, 3.29.

3.1.14. (*all-Z*)-*N*-[(1*R*)-2-Hydroxy-1-(phenylmethyl)ethyl]-5,8,11,14-eicosatetraenamide (4b). Yield 76%; wax; $[\alpha]_D$ +18 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{29}H_{43}NO_2$ (437.7): C, 79.59; H, 9.90; N, 3.20. Found: C, 79.88; H, 10.03; N, 3.11.

3.1.15. (*Z*)-*N*-[(1*S*)-2-Hydroxy-1-(4-hydroxyphenyl)-ethyl]-9-octadecenamide (5a). Yield 73%; mp 134–135 °C; $[\alpha]_D$ +54 (*c* 1.0, EtOH); IR ($CHCl_3$) 3630, 3423, 3013, 2943, 2837, 1651, 1516, 1463, 1334, 1238, 1012 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3/CD_3OD$ = 9:1) δ 0.88 (3H, t, J = 6.6 Hz), 1.28 (20H, m), 1.62 (2H, m), 2.00 (4H, m), 2.22 (2H, t, J = 7.5 Hz), 3.76 (2H, m), 4.90 (1H, m), 5.34 (2H, m), 6.76 (2H, d, J = 8.9 Hz), 7.90 (2H, d, J = 8.9 Hz); ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD = 9:1) δ 14.10, 22.69, 25.77, 27.19, 27.22, 29.17, 29.27, 29.31, 29.52, 29.73, 31.90, 36.64, 55.12, 55.21, 65.91, 115.45, 127.73, 129.56, 129.83, 129.91, 156.09, 174.13. Anal. Calcd for $C_{26}H_{43}NO_3$ (417.6): C, 74.78; H, 10.38; N, 3.35. Found: C, 74.90; H, 10.45; N, 3.29.

3.1.16. (*all-Z*)-*N*-[(1*S*)-2-Hydroxy-1-(4-hydroxyphenyl)-ethyl]-5,8,11,14-eicosatetraenamide (5b). Yield 66%; wax; $[\alpha]_D$ +50 (*c* 1.0, EtOH); IR ($CHCl_3$) 3594, 3432, 3009, 2930, 2858, 1656, 1515, 1458, 1234, 1039 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.6 Hz), 1.26–1.37 (6H, m), 1.69 (2H, quintet, J = 7.5 Hz), 2.02–2.13 (4H, m), 2.23 (2H, t, J = 7.2 Hz), 2.77–2.84 (6H, m), 3.73 (2H, m), 4.87 (1H, m), 5.29–5.42 (8H, m), 6.68 (2H, d, J = 8.5 Hz), 7.16 (2H, d, J = 8.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.06, 22.55, 25.59, 26.64, 27.19, 29.28, 31.48, 35.94, 35.98, 55.14, 55.23, 65.81, 115.46, 127.31, 127.66, 127.91, 128.08, 128.42, 128.64, 128.75, 129.70, 130.32, 155.97, 173.92. Anal. Calcd for $C_{28}H_{41}NO_3$ (439.6): C, 76.50; H, 9.40; N, 3.19. Found: C, 76.78; H, 9.28; N, 3.10.

3.1.17. (*Z*)-*N*-[(1*R*)-2-Hydroxy-1-(4-hydroxyphenyl)-ethyl]-9-octadecenamide (6a). Yield 72%; mp 134–135 °C; $[\alpha]_D$ –54 (*c* 1.0, EtOH). Anal. Calcd for $C_{26}H_{43}NO_3$ (417.6): C, 74.78; H, 10.38; N, 3.35. Found: C, 74.89; H, 10.47; N, 3.27.

3.1.18. (*all-Z*)-*N*-[(1*R*)-2-Hydroxy-1-(4-hydroxyphenyl)-ethyl]-5,8,11,14-eicosatetraenamide (6b). Yield 74%; wax; $[\alpha]_D$ –50 (*c* 1.0, EtOH). Anal. Calcd for $C_{28}H_{41}NO_3$ (439.6): C, 76.50; H, 9.40; N, 3.19. Found: C, 76.77; H, 9.29; N, 3.09.

3.1.19. (*Z*)-*N*-[(1*S*)-2-Hydroxy-1-phenylethyl]-9-octadecenamide (7a). Yield 80%; mp 53–54 °C; $[\alpha]_D$ +31 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3436, 2928, 2856, 1662, 1505, 1465, 1234, 1069 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.3 Hz), 1.28 (20H, m), 1.62–1.67 (2H, m), 1.97–2.02 (4H, m), 2.24 (2H, t, J = 7.2 Hz), 2.83 (1H, br s), 3.88 (2H, m), 5.07 (1H, m), 5.31–5.36 (2H, m), 6.15 (1H, d, J = 6.3 Hz), 7.26–7.39 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.09, 22.66, 25.69, 27.16, 27.21, 29.11, 29.22, 29.50, 29.67, 29.75, 31.87, 36.74, 55.94, 66.77, 126.49, 127.74, 128.73, 129.51, 129.80, 138.78, 173.52. Anal. Calcd for $C_{26}H_{43}NO_3$ (401.6): C, 75.13; H, 10.51; N, 3.24. Found: C, 75.29; H, 10.43; N, 3.15.

3.1.20. (*all-Z*)-*N*-[(1*S*)-2-Hydroxy-1-phenylethyl]-5,8,11,14-eicosatetraenamide (7b). Yield 70%; wax; $[\alpha]_D$ +26 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3434, 2930, 1662, 1505, 1455, 1234, 1070 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, J = 6.9 Hz), 1.29–1.31 (6H, m), 1.71 (2H, quintet, J = 7.5 Hz), 2.02–2.11 (4H, m), 2.23 (2H, t, J = 7.5 Hz), 2.77–2.84 (6H, m), 3.13 (1H, br s), 3.82 (2H, d, J = 4.8 Hz), 5.03 (1H, m), 5.29–5.44 (8H, m), 6.32 (1H, d, J = 7.2 Hz), 7.25–7.37 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.05, 22.54, 25.44, 25.60, 26.62, 27.18, 29.27, 31.47, 35.98, 55.79, 66.40, 126.47, 127.31, 127.62, 127.91, 128.04, 128.40, 128.49, 128.63, 128.78, 130.29, 138.84, 173.22. Anal. Calcd for $C_{28}H_{41}NO_2$ (423.6): C, 79.39; H, 9.75; N, 3.31. Found: C, 79.16; H, 9.88; N, 3.20.

3.1.21. (*Z*)-*N*-[(1*R*)-2-Hydroxy-1-phenylethyl]-9-octadecenamide (8a). Yield 76%; mp 55–56 °C; $[\alpha]_D$ –31 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{26}H_{43}NO_3$ (401.6): C, 75.13; H, 10.51; N, 3.24. Found: C, 75.31; H, 10.44; N, 3.19.

3.1.22. (*all-Z*)-*N*-[(1*R*)-2-Hydroxy-1-phenylethyl]-5,8,11,14-eicosatetraenamide (8b). Yield 71%; wax; $[\alpha]_D$ –26 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{28}H_{41}NO_2$ (423.6): C, 79.39; H, 9.75; N, 3.31. Found: C, 79.14; H, 9.87; N, 3.20.

3.1.23. (*Z*)-*N*-[(*R*,*S*)-2-Hydroxy-2-phenylethyl]-9-octadecenamide (9a). Yield 85%; wax; IR ($CHCl_3$) 3451, 2928, 2856, 1656, 1518, 1455, 1234, 1065 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.91 (3H, t, J = 6.6 Hz), 1.30 (20H, m), 1.57–1.61 (2H, m), 2.01–2.05 (4H, m), 2.15 (2H, t, J = 7.5 Hz), 3.27–3.36 (1H, m), 3.66 (1H, ddd, J = 13.8, 6.6, 3.3 Hz), 4.14 (1H, br s), 4.81 (1H, dd, J = 7.5, 3.3 Hz), 5.33–5.43 (2H, m), 6.21 (1H, m), 7.27–7.36 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.09, 22.65, 25.69, 27.16, 27.20, 29.13, 29.19, 29.23, 29.28, 29.49, 29.70, 29.73, 31.86, 36.51, 47.39, 73.44, 125.60,

127.51, 128.22, 129.50, 129.79, 141.66, 174.47. Anal. Calcd for $C_{26}H_{43}NO_3$ (401.6): C, 75.13; H, 10.51; N, 3.24. Found: C, 75.38; H, 10.42; N, 3.16.

3.1.24. (*all-Z*)-*N*-[(*R*,*S*)-2-Hydroxy-2-phenylethyl]-5,8,11,14-eicosatetraenamide (9b). Yield 76%; wax; IR ($CHCl_3$) 3450, 2929, 2858, 1659, 1518, 1455, 1234, 1206, 1065 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, J = 6.6 Hz), 1.26–1.38 (6H, m), 1.68 (2H, quintet, J = 7.5 Hz), 2.02–2.12 (4H, m), 2.17 (2H, t, J = 7.5 Hz), 2.79–2.85 (6H, m), 3.25–3.30 (1H, m), 3.67 (1H, ddd, J = 13.8, 6.9, 3.3 Hz), 3.77 (1H, br s), 4.80 (1H, d, J = 5.7 Hz), 5.28–5.43 (8H, m), 6.02 (1H, d, J = 7.2 Hz), 7.26–7.34 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.06, 22.54, 25.44, 25.61, 26.59, 27.19, 29.28, 31.47, 35.84, 47.40, 73.57, 125.60, 127.31, 127.63, 127.94, 128.05, 128.27, 128.41, 128.62, 128.79, 130.31, 141.60, 174.09. Anal. Calcd for $C_{28}H_{41}NO_2$ (423.6): C, 79.39; H, 9.75; N, 3.31. Found: C, 79.14; H, 9.88; N, 3.23.

3.1.25. (*Z*)-*N*-[(1*R*,2*R*)-Phenyl-1,3-propanodiol-2-yl]-9-octadecenamide (10a). Yield 82%; wax; $[\alpha]_D$ −28 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3433, 3025, 2928, 2856, 1655, 1509, 1465, 1199, 1062 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.6 Hz), 1.11–1.34 (20H, m), 1.39–1.48 (2H, m), 1.99–2.09 (6H, m), 3.66–3.77 (2H, m), 3.90 (1H, br s), 4.04–4.11 (1H, m); 4.35 (1H, br s), 5.18 (1H, d, J = 3.6 Hz), 5.29–5.40 (2H, m), 6.31 (1H, d, J = 7.8 Hz), 7.20–7.33 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.09, 22.65, 25.64, 27.17, 27.21, 29.02, 29.09, 29.22, 29.30, 29.49, 29.71, 29.74, 31.86, 36.64, 56.39, 63.12, 72.93, 125.58, 127.43, 128.14, 129.50, 129.79, 141.72, 174.31. Anal. Calcd for $C_{27}H_{45}NO_3$ (431.7): C, 75.13; H, 10.51; N, 3.24. Found: C, 75.34; H, 10.40; N, 3.16.

3.1.26. (*all-Z*)-*N*-[(1*R*,2*R*)-Phenyl-1,3-propanodiol-2-yl]-5,8,11,14-eicosatetraenamide (10b). Yield 86%; wax; $[\alpha]_D$ −31 (*c* 1.0, $CHCl_3$); IR ($CHCl_3$) 3432, 3009, 2958, 2929, 2858, 1656, 1508, 1453, 1210, 1063 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, J = 6.9 Hz), 1.26–1.38 (6H, m), 1.54 (2H, quintet, J = 7.5 Hz), 1.92–1.99 (2H, m), 2.02–2.12 (4H, m), 2.73–2.84 (6H, m), 3.73 (3H, br s), 4.06–4.10 (1H, m), 4.22 (1H, br s), 5.02 (1H, br s), 5.26–5.44 (8H, m), 6.26 (1H, d, J = 8.4 Hz), 7.24–7.32 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.06, 22.54, 25.45, 25.58, 26.47, 27.18, 29.27, 31.46, 35.98, 56.36, 63.19, 73.10, 125.56, 127.29, 127.49, 127.62, 127.92, 128.03, 128.16, 128.40, 128.54, 128.71, 130.29, 140.96, 173.93. Anal. Calcd for $C_{29}H_{43}NO_3$ (453.7): C, 76.78; H, 9.55; N, 3.09. Found: C, 77.11; H, 9.69; N, 2.97.

3.1.27. (*Z*)-*N*-[(1*S*,2*S*)-Phenyl-1,3-propanodiol-2-yl]-9-octadecenamide (11a). Yield 87%; wax; $[\alpha]_D$ +28 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{27}H_{45}NO_3$ (431.7): C, 75.13; H, 10.51; N, 3.24. Found: C, 75.28; H, 10.43; N, 3.15.

3.1.28. (*all-Z*)-*N*-[(1*S*,2*S*)-Phenyl-1,3-propanodiol-2-yl]-5,8,11,14-eicosatetraenamide (11b). Yield 87%; wax; $[\alpha]_D$ +31 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{29}H_{43}NO_3$ (453.7): C, 76.78; H, 9.55; N, 3.09. Found: C, 77.09; H, 9.67; N, 2.98.

3.1.29. (*Z*)-*N*-4-Hydroxyphenyl-9-octadecenamide (12a). Yield 64%; mp 104–105 °C; IR (KBr) 3278, 2917, 2849, 1647, 1615, 1559, 1537, 1518, 1466, 1256, 1097 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.6 Hz), 1.27–1.31 (20H, m), 1.64–1.71 (2H, m), 1.97–2.02 (4H, m), 2.30 (2H, t, J = 7.6 Hz), 5.32–5.36 (2H, m), 6.72 (2H, d, J = 8.7 Hz), 7.22 (2H, dd, J = 8.7, 1.8 Hz), 7.65 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.07, 22.65, 25.75, 27.16, 27.20, 29.13, 29.27, 29.49, 29.74, 31.86, 37.38, 115.47, 122.46, 122.58, 129.53, 129.79, 153.42, 172.51. Anal. Calcd for $C_{24}H_{39}NO_2$ (373.6): C, 77.16; H, 10.52; N, 3.75. Found: C, 77.41; H, 10.67; N, 3.67.

3.1.30. (*Z*)-*N*-[(4-Hydroxymethyl)phenyl]-9-octadecenamide (13a). Yield 75%; wax; IR ($CHCl_3$) 3285, 2917, 2849, 1654, 1605, 1542, 1466, 1412, 1260, 1015 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.6 Hz), 1.32 (20H, m), 1.70 (2H, m), 1.96–2.02 (4H, m), 2.32 (2H, t, J = 7.6 Hz), 2.65–2.78 (2H, m), 4.58 (2H, s), 5.32–5.36 (2H, m), 7.23 (2H, d, J = 8.4 Hz), 7.43 (2H, d, J = 8.4 Hz), 7.65 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.74, 15.30, 23.07, 26.01, 27.55, 27.60, 28.66, 29.53, 29.69, 29.90, 30.13, 30.91, 32.27, 38.01, 65.03, 120.34, 127.89, 129.89, 1130.20, 136.95, 137.45, 171.98. Anal. Calcd for $C_{25}H_{41}NO_2$ (387.6): C, 77.47; H, 10.66; N, 3.61. Found: C, 77.72; H, 10.77; N, 3.53.

3.1.31. (*all-Z*)-*N*-[(4-Hydroxymethyl)phenyl]-5,8,11,14-eicosatetraenamide (13b). Yield 77%; wax; IR ($CHCl_3$) 3605, 3433, 2929, 2858, 1686, 1596, 1518, 1411, 1306, 1224, 1004 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.9 Hz), 1.25–1.37 (7H, m), 1.80 (2H, quintet, J = 7.2 Hz), 2.05 (2H, q, J = 6.3 Hz), 2.16 (2H, q, J = 6.3 Hz), 2.34 (2H, t, J = 6.9 Hz), 2.78–2.83 (4H, m), 4.60 (2H, s), 5.34–5.41 (8H, m), 7.25 (2H, d, J = 8.1 Hz), 7.44 (2H, d, J = 8.1 Hz), 7.55 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.80, 23.66, 24.01, 24.52, 24.84, 25.79, 26.02, 26.42, 28.53, 30.71, 36.10, 63.92, 119.12, 126.53, 126.78, 126.86, 127.14, 127.30, 127.64, 127.98, 128.04, 129.58, 135.83, 136.26, 170.37. Anal. Calcd for $C_{27}H_{39}NO_2$ (409.6): C, 79.17; H, 9.60; N, 3.42. Found: C, 79.44; H, 9.49; N, 3.33.

3.1.32. (*Z*)-(*R*)-1-(1-Oxo-9-octadecenyl)-2-pyrrolidinemethanol (14a). Yield 78%; wax; $[\alpha]_D$ −32 (*c* 2.0, $CHCl_3$); IR ($CHCl_3$) 3346, 2928, 2855, 1612, 1445, 1340, 1234, 1015 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.85 (3H, t, J = 6.5 Hz), 1.23–1.28 (20H, m), 1.53–1.63 (3H, m), 1.78–2.04 (8H, m), 2.26 (2H, t, J = 7.6 Hz), 3.38–3.64 (4H, m), 4.14–4.22 (1H, m), 5.25–5.36 (2H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.09, 22.65, 24.38, 24.76, 27.14, 27.18, 28.26, 29.10, 29.30, 29.48, 29.73, 31.86, 35.06, 45.61, 48.04, 58.91, 61.00, 63.67, 67.37, 129.52, 129.74, 174.31. Anal. Calcd for $C_{23}H_{43}NO_2$ (365.6): C, 75.56; H, 11.85; N, 3.83. Found: C, 75.78; H, 11.98; N, 3.75.

3.1.33. (*all-Z*)-(*R*)-1-(1-Oxo-5,8,11,14-eicosatetraenyl)-2-pyrrolidinemethanol (14b). Yield 84%; wax; $[\alpha]_D$ −28 (*c*

1.0, $CHCl_3$); IR ($CHCl_3$) 3343, 2930, 1613, 1446, 1341, 1227, 1075 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.89 (3H, t, $J = 6.9$ Hz), 1.26–1.38 (6H, m), 1.57–1.65 (1H, m), 1.72–1.79 (2H, m), 1.84–2.09 (5H, m), 2.11–2.18 (2H, m), 2.31 (2H, t, $J = 7.5$ Hz), 2.77–2.83 (6H, m), 3.43–3.62 (4H, m), 4.18–4.25 (1H, m), 5.19 (1H, br s), 5.29–5.45 (8H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.05, 22.54, 24.37, 24.52, 25.62, 26.65, 27.19, 28.25, 29.29, 31.48, 34.26, 47.99, 61.02, 67.26, 127.32, 127.63, 127.99, 128.37, 128.56, 129.03, 130.26, 162.49, 173.93. Anal. Calcd for $C_{25}H_{41}NO_2$ (387.6): C, 77.47; H, 10.66; N, 3.61. Found: C, 77.79; H, 10.78; N, 3.52.

3.1.34. (*Z*)-(*S*)-1-(1-Oxo-9-octadecenyl)-2-pyrrolidinemethanol (15a). Yield 84%; wax; $[\alpha]_D$ +32 (*c* 2.0, $CHCl_3$). Anal. Calcd for $C_{23}H_{43}NO_2$ (365.6): C, 75.56; H, 11.85; N, 3.83. Found: C, 75.80; H, 11.96; N, 3.74.

3.1.35. (*all-Z*)-(*S*)-1-(1-Oxo-5,8,11,14-eicosatetraenyl)-2-pyrrolidinemethanol (15b). Yield 83%; wax; $[\alpha]_D$ +28 (*c* 1.0, $CHCl_3$). Anal. Calcd for $C_{25}H_{41}NO_2$ (387.6): C, 77.47; H, 10.66; N, 3.61. Found: C, 77.75; H, 10.78; N, 3.54.

3.2. Biology

3.2.1. Anandamide transporter assay. The effect of compounds **1**–**15** on the uptake of [^{14}C]AEA by intact RBL-2H3 cells was studied by using 5.0 μM (20,000 cpm) of [^{14}C]AEA as described previously.[35] Cells were incubated with [^{14}C]AEA for 5 min at 37 °C, in the presence or absence of varying concentrations of the inhibitors. Residual [^{14}C]AEA in the incubation media after extraction with $CHCl_3/CH_3OH$ = 2:1, determined by scintillation counting of the lyophilized organic phase, was used as a measure of the amount of AEA that was taken up by cells. Previous studies had shown that, after a 5 min incubation, the amount of [^{14}C]AEA disappeared from the medium of RBL-2H3 cells is found mostly (>90%) as unmetabolized [^{14}C]AEA in the cell extract.[38] Non-specific binding of [^{14}C]AEA to cells and plastic dishes was determined in the presence of 100 μM AEA and was never higher than 40%. Data are expressed as the K_i, calculated using the Cheng–Prusoff equation from the concentration exerting 50% inhibition of AEA uptake (IC_{50}).

3.2.2. Receptors binding assays. Substances were tested on recombinant human CB_1 or CB_2 receptors overexpressed in COS cells as described by the manufacturer (Perkin–Elmer). Briefly, increasing concentrations of the test compounds were incubated with 4–8 μg of membranes from transfected COS cells in the presence of 0.1–0.3 nM [^{3} H]CP55,940 for 90 min at 30 °C in binding buffer in the absence of PMSF. After the incubation bound and unbound [^{3}H]CP55,940 were separated by filtration. Non-specific binding was determined with 10 μM WIN 55–212 and was never higher than 10%. IC_{50} values in nM were obtained from the dose–response curves using GraphPad® and transformed into K_i by using the Cheng–Prusoff equation. The TRPV1 receptor functional assay was performed as described by Ortar et al.[14]

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