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Synthesis, cannabinoid receptor activity, and enzymatic stability of reversed amide derivatives of arachidonoyl ethanolamide

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Abstract—Retroanandamide (**2f**) and its 10 analogues (**1a**–**e**, **2a**–**e**) were synthesized and evaluated for the cannabinoid receptor activation by a [^{35}S]GTPγS binding assay using rat cerebellar membranes, and Chinese hamster ovary cell membranes expressing human CB2 receptors. The primary goal of the study was to develop cannabinoid receptor agonists having improved enzymatic stability compared to endogenous *N*-arachidonoyl ethanolamide (AEA). Furthermore, by reversing the amide bond of AEA, the formation of arachidonic acid would be prevented. Finally, an effect of the carbonyl carbon position on the cannabinoid receptor activity was explored by synthesizing retroanandamide analogues having different chain lengths (**1a**–**e**, C_{19}; **2a**–**f**, C_{20}). All the synthesized compounds, except **2c**, behaved as partial agonists for the both cannabinoid receptors. In rat brain homogenate, the reversed amides possessed significantly higher stability against FAAH induced degradation than AEA. Therefore, the reversed amide analogues of AEA may serve as enzymatically stable structural basis for the drug design based on the endogenous cannabinoids.

1. Introduction

During the past 10 years the endogenous cannabinoid system (ECS) has shown its potential as a target for the drug discovery.[1] The quantity and the distribution of the cannabinoid receptors in the central nervous system (CNS) as well as in the peripheral tissues have suggested that the ECS has a significant role in certain physiological events. It is well known that the CB1 receptor participates, for example, in the appetite stimulation, nausea suppression, cognition and memory, pain perception, and the regulation of intra ocular pressure.[2–6] Few countries have already accepted cannabinoids (Marinol®, Cesamet®) for the treatment of chemotherapy related nausea and the appetite stimulation of AIDS patients, but several other therapeutic applications have also been suggested, such as pain, certain types of cancer, asthma, glaucoma, and especially various CNS disorders, like multiple sclerosis, Huntington's disease, Parkinson's disease, and ischemic stroke, are of great interest at the moment.[5–12]

So far, the most potent CB1 ligand in our experimental setting assessing CB1 receptor-dependent G protein activity has been the classical cannabinoid, HU-210 ($pEC_{50} \pm SEM = 8.3 \pm 0.1$, unpublished data). The most effective ligand is, however, the endocannabinoid 2-AG ($E_{max} = 620 \pm 5$, % Basal ± SEM),[13] which focused our interest in the molecular structure of endocannabinoids and their derivatives. Unfortunately, 2-AG's susceptibility for acid, base or heat promoted acyl migration makes it a difficult compound to synthesize and handle, and therefore, the other major endocannabinoid, arachidonoyl ethanolamide (AEA), and its derivatives have been synthesized and studied more extensively. Hillard reported that AEA acts as a partial agonist in a [^{35}S]GTPγS binding assay with the rat cerebellar membranes, nevertheless, being more potent than 2-AG.[14] The study of Savinainen et al.[13] confirmed the partial agonist behavior of AEA, but in their experimental setting 2-AG was clearly more potent than AEA (Table 1).[13,15]

Keywords: Cannabinoid; AEA; Reversed amide; Synthesis; CB1; CB2; [^{35}S]GTPγS binding assay; Enzymatic stability.
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Table 1. Comparison of efficacy (E_{max}) and potency (pEC_{50}) values of 2-AG, AEA, **1a–e** (C_{19} series), and **2a–f** (C_{20} series) at rat cerebellar membranes (means ± SEM, $n = 3$)

Compound	CB1 activation	
	Relative E_{max} (% 2-AG E_{max} ± SEM)	Potency (−log EC_{50} ± SEM)
2-AG	100	6.0 ± 0.0
AEA	67 ± 2	5.3 ± 0.1
1a	45 ± 4	5.4 ± 0.1
1b	36 ± 3	5.4 ± 0.2
1c	46 ± 6	4.4 ± 0.1
1d	38 ± 12	5.3 ± 0.3
1e	37 ± 4	6.1 ± 0.1
2a	49 ± 5	5.7 ± 0.1
2b	39 ± 2	5.6 ± 0.1
2c	NA	NA
2d	34 ± 1	5.8 ± 0.2
2e	40 ± 4	5.6 ± 0.3
2f	55 ± 7	4.9 ± 0.1

NA, no detectable CB1 activation at 10^{-4} M concentration.
The data represent means (±SEM) of [^{35}S]GTPγS binding as percentage of the maximal response evoked by 2-AG and are from at least three independent experiments performed in duplicate.

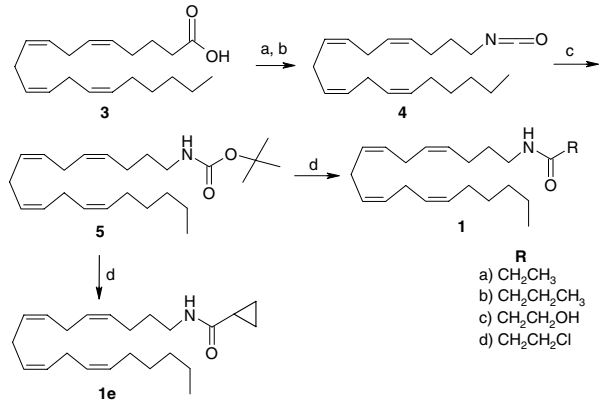
Although AEA and 2-AG are good ligands for the cannabinoid receptors, and they have been shown to act as therapeutic agents in several in vitro and in vivo models, they are enzymatically degraded too easily to serve as good probes for the cannabinoid research, not to mention as pharmaceuticals. There have been several attempts to stabilize the structure of AEA. Branching of an ethanolamine moiety stabilizes the amide bond; especially an addition of a methyl group adjacent to nitrogen ((*R*)-methanandamide) increases the enzymatic stability as well as activity in vivo.[16,17] Attachment of a methyl or dimethyl group on the α-position of AEA also increases the enzymatic stability and thereby the biological activity both in vitro and in vivo.[17,18] Nevertheless, the branching of the chain generates a chiral center to the molecule and it has been observed that enantiomers have differences in their CB1 receptor binding. Thus, to achieve reliable binding data, pure enantiomers must be provided, which is always an extra challenge for the synthesis. Makriyannis et al. observed indirectly that reversal of the carbonyl and NH in the amido group provides high metabolic stability.[19] However, this retroanandamide (**2f**, Scheme 3) showed weaker CB1 affinity than AEA; K_i values being 61 nM for AEA and 115 nM for retroanandamide when fatty acid amide hydrolase (FAAH) was inhibited by phenyl methylsulfonyl fluoride (PMSF). In contrast, when PMSF was not used, the K_i values were 5810 and 134 nM, respectively, indicating the increased enzymatic stability of retroanandamide.

In the present study, we have synthesized various reversed amides to study their cannabinoid receptor activity at rat cerebellar membranes and human CB2 (hCB2) receptors. In addition, we have determined their enzymatic stability using rat brain homogenate. Finally, we have also investigated the chain length effect (C_{19} vs C_{20}) on the cannabinoid receptor activity.

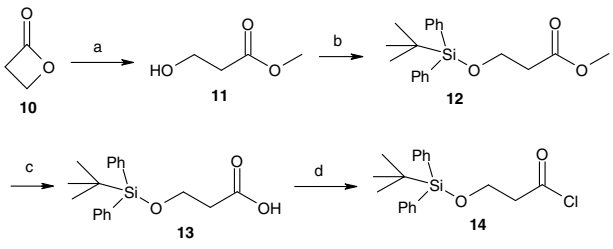
2. Chemistry

Norarachidonyl amides **1a–e** (C_{19} series) were prepared by the method published by Nazih and Heissler (Scheme 1).[20] This method allows one-pot conversion of *t*-butyl carbamates to amides. It is based on a cleavage of a *t*-butoxycarbonyl group with hydrogen iodide generated in situ by reaction of acyl halide with methanol and on an acylation of an intermediate amine with excess acyl halide in the presence of di-isopropylethylamine (DIPEA). The synthesis of the key intermediate norarachidonyl *N*-*t*-butylcarbamate **5** was started by converting arachidonic acid to acyl azide with diphenyl phosphorylazide (DPPA).[21] The acyl azide undergoes the Curtius rearrangement to norarachidonyl isocyanate **4** upon heating. Compound **5** is finally achieved by refluxing the isocyanate **4** with *t*-butanol for several days. Scheme 2 summarizes the synthesis method for 3-(*t*-butyldiphenylsilyloxy)-propionyl chloride **14** used in the synthesis of the final product **1c**.

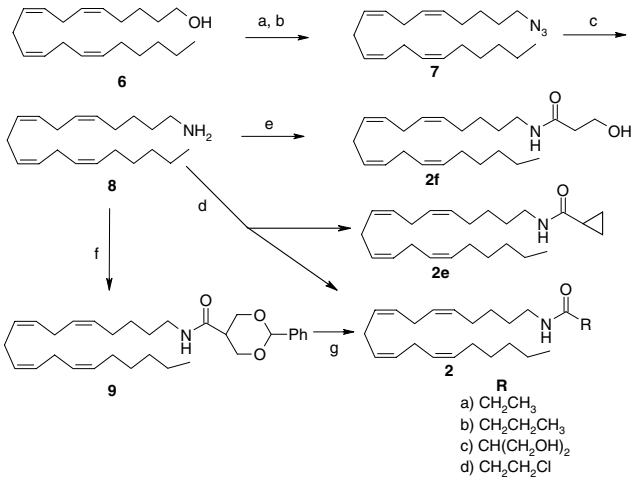
Synthesis methods for compounds **2a–f** (C_{20} series) are summarized in Schemes 3 and 4. Synthesis of a key intermediate, arachidonyl amine **8**, was performed by converting commercially available arachidonyl alcohol to arachidonyl azide **7** via more reactive mesylate and further reducing the azide to amine with $LiAlH_4$ (LAH).[19] Compounds **2a–b** and **2e–d** were synthesized from arachidonyl amine and appropriate acid halides. Retroanandamide **2f** was synthesized as previously described.[19] Compound **2c** was prepared by coupling *cis*/*trans*-2-phenyl-5-carboxy-1,3-dioxane **18** (Scheme 4)[22]



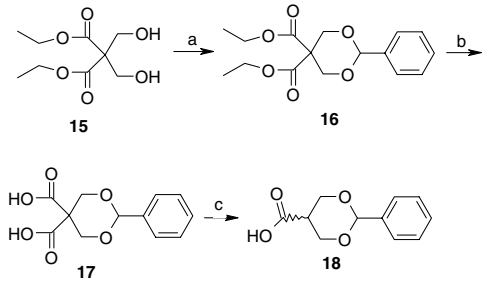
Scheme 1. Reagents: (a) DPPA, Et_3N, benzene; (b) toluene, Δ; (c) *t*-BuOH; (d) i—RCOCl, NaI, acetonitrile, MeOH; ii—DIPEA.



Scheme 2. Reagents: (a) NaOH, MeOH; (b) TBDPSCl, imidazole, THF; (c) 1M LiOH, MeOH, H_2O; (d) $(COCl)_2$, benzene.



Scheme 3. Reagents: (a) MsCl, pyridine; (b) NaN_3; (c) LAH; (d) RCOCl, Et_3N; (e) $Al(CH_3)_3$, β-propiolactone, CH_2Cl_2; (f) **18**, EDCl, DMAP, CH_2Cl_2; (g) HCl, MeOH.



Scheme 4. Reagents and condition: (a) benzaldehyde, toluene, H_2SO_4; (b) i—KOH, EtOH; ii—HCl; (c) Et_3N, Δ.

with arachidonyl amine using *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDCl) and 4-dimethylaminopyridine (DMAP) as coupling reagents. The desired product was obtained from compound **9** by removing the benzylidene acetal protective group with concd HCl/MeOH solution.

3. Results and discussion

3.1. [^{35}S]GTPγS membrane binding studies

3.1.1. CB1 receptor activation. The ability of the synthesized compounds to activate the CB1 receptor was determined by the [^{35}S]GTPγS membrane binding assay as previously described.[13] Comparison of the efficacy and the potency values are presented in Table 1. All the synthesized compounds, except for **2c**, showed dose-dependent CB1 activity, and their responses at 5×10^{-5} M were reversed by the CB1 receptor antagonist, AM251 at 10^{-6} M concentration (data not shown).

In the [^{35}S]GTPγS binding assay, the reversed amides, as well as AEA,[13,15] acted as partial agonists. As the previous affinity studies of retroanandamide suggested,[19] AEA has higher E_{max} value than **1c** and **2f** (retroanandamide) in the study conditions where function of FAAH is blocked with an enzyme inhibitor (MAFP). However, compounds appear to have comparable potency to AEA in the reversed amide series where hydroxyethyl group is replaced with alkyl chain (**1a**–**b**, **1d**–**e** and **2a**–**b**, **2d**–**e**); the results are in a good agreement with findings of Pinto et al. and Sheskin et al. who observed that by increasing the lipophilicity, the CB1 receptor affinity can be improved.[23,24]

It has been shown that naturally occurring anandamides, such as (22:4, *n*-6) and (20:4, *n*-6), that is, AEA, possess similar affinities for the CB1 receptor.[23] Reports about reversed amides with different chain lengths or different degree of double bonds have not been published. In our reversed amide series, a longer chain length (i.e., C_{20}) is preferred although the differences are not very significant.

3.1.2. CB2 receptor activation. The CB2 receptor activities for the compounds **1a**–**e** (C_{19} series) and **2a**–**f** (C_{20} series) were determined at the human CB2 (hCB2) receptor expressed in Chinese hamster ovary (CHO) cells as previously described.[25] As shown in Table 2, all the studied compounds behaved as partial agonists and had comparable efficacy and potency values to AEA. The most interesting finding in the both C_{19} and C_{20} series was that compounds with butanoyl tail (**1b**, **2b**) possess potencies that are equal to that of 2-AG (7.4 ± 0.1). In addition, compound **2b** is relatively efficacious. Although, Makriyannis et al. reported that retroanandamide has only weak affinity for the CB2 receptor,[19] our findings suggest that reversed amides are able to activate both subtypes of cannabinoid receptors.

3.2. Enzymatic stability of reversed amide derivatives

One of the main goals of this study was to stabilize the amide linkage of endogenous AEA against enzymatic

Table 2. Comparison of efficacy (relative E_{max}) and potency ($-\log EC_{50}$) values of AEA, **1a**–**e** (C_{19} series), and **2a**–**f** (C_{20} series) at CHO-hCB2 cells

Compound	CB2 activation	
	Relative E_{max} (% HU-210 E_{max} ± SEM)	Potency ($-\log EC_{50}$ ± SEM)
HU-210	100	9.8 ± 0.1[25]
AEA	48 ± 5	6.5 ± 0.2
1a	54 ± 3	6.7 ± 0.1
1b	53 ± 4	7.1 ± 0.2
1c	54 ± 15	5.8 ± 0.3
1d	59 ± 10	6.6 ± 0.5
1e	55 ± 4	6.7 ± 0.2
2a	64 ± 3	7.1 ± 0.2
2b	73 ± 3	7.0 ± 0.2
2c	ND	ND
2d	64 ± 4	6.8 ± 0.2
2e	67 ± 4	6.7 ± 0.2
2f	75 ± 7	6.6 ± 0.2

ND, not determined.
The data represent means (±SEM) of [^{35}S]GTPγS binding as percentage of the maximal response evoked by HU-210 and are from at least three independent experiments performed in duplicate.

hydrolysis. The stability of retroanandamide (**2f**) has not been studied, but it has been hypothesized to be more stable against FAAH mediated degradation than AEA, based on the results of affinity studies with and without a FAAH inhibitor.[19] The stability studies were performed by incubating the synthesized compounds **1a**, **1c**, **1e**, **2b**, **2e**, and **2f** in the rat brain homogenate for 90 min (37 °C, pH 7.4), taking the samples at time points 0 and 90 min, analyzing the samples by HPLC, and comparing the results to those using AEA. All the synthesized compounds were stable (no degradation during 90 min) in the rat brain homogenate, whereas AEA was almost fully degraded in 90 min. The degradation of AEA in the rat brain homogenate was inhibited also in the presence of a potent FAAH inhibitor URB597. URB597 was able to inhibit the AEA degradation with an IC_{50} value of 3.8 ± 0.6 nM ($n = 3$) which is comparable to the previously reported results, and it confirms that in the rat brain homogenate AEA is mainly metabolized by FAAH.[26]

3.3. Conclusions

We have developed a synthetic method for arachidonoyl ethanolamide (AEA) derivatives having the chain length of C_{19}, synthesized reversed amide analogues of AEA, measured their activities via the rat brain CB1 and human recombinant CB2 receptors, and determined their metabolic stability in the rat brain homogenate. The reversal of the amide bond of AEA resulted in the weaker CB1 activity properties, but both efficacy and potency values were reverted or even enhanced by removing the hydroxyl group and therefore making the compound more lipophilic. In contrast, all the reversed amides studied had similar or improved activity properties for the CB2 receptor compared to AEA. Finally, the stability studies conducted with rat brain homogenate showed that the reversed amides remain intact in the studied conditions, whereas AEA is almost fully degraded. In conclusion, we have shown that by reversing the amide bond of AEA it is possible to develop cannabinoid receptor agonists with good CB1 and CB2 activity, and a structure which is stable against FAAH or other enzyme mediated metabolism.

4. Experimental

4.1. General

^{1}H NMR and ^{13}C NMR were recorded on a Bruker Avance 500 spectrometer operating on 500.1 and 125.8 MHz, respectively. $CDCl_3$ was used as a solvent if not otherwise noted, and tetramethylsilane (TMS) was used as an internal standard. The spectra were processed from the recorded FID files with MestRe-C software (version 2.3a, Departemento Quìmica Orgànica, Universidade de Santiago de Compostela, Spain). Chemical shifts (δ) are reported in parts per million (ppm) downfield from TMS. Following abbreviations are used: s = singlet, d = doublet, t = triplet, br s = broad singlet, qv = quartet, q = quintet, st = sextet, m = multiplet. Coupling constants are reported in Hz and letter J indicates ^5J if not otherwise noted. ESI-MS spectra were acquired using a LCQ ion trap mass spectrometer equipped with an electrospray ionization source (Finnigan MAT, San Jose, CA, USA). Elemental analyses for C, H, and N were performed on a Thermo-Quest CE Instruments EA1110-CHNS-O elemental analysator (ThermoQuest, Italy). TLC was performed using silica gel (60 F_{254}) coated aluminum sheets (Merck, Germany). Arachidonic acid and arachidonyl alcohol were purchased from Nu-Chek-Prep, Inc.

4.2. Norarachidonyl *N*-*t*-butylcarbamate (5)

Arachidonic acid (500 mg, 1.64 mmol) and dry Et_3N (0.34 ml, 2.46 mmol) were dissolved in dry benzene (6 ml) and stirred for 10 min. DPPA (0.53 ml, 2.46 mmol) was added, and stirring was continued at RT for 2 h. Benzene was evaporated, the residue was dissolved in petroleum ether/EtOAc 10:1 and filtered through the pad of silica gel. Solvents were evaporated and the oily residue was dissolved in dry toluene (3 ml). *t*-BuOH (0.31 ml, 3.28 mmol) in dry toluene (3 ml) was added, and the reaction mixture was stirred at 65 °C for 5 days. The reaction mixture was cooled to rt, water (20 ml) and EtOAc (20 ml) were added, and the solution was washed with brine. The organic layers were combined, dried over Na_2SO_4, and evaporated. The crude product was purified by flash chromatography eluting with 2.5% EtOAc in petroleum ether. Evaporation of solvents yielded 240 mg of yellowish oily product (39%). $R_f = 0.45$ (petroleum ether/EtOAc 6:1). ^{1}H NMR: δ 0.89 (t, $J = 7.0$, 3H), 1.26–1.40 (m, 6H), 1.44 (s, 9H), 1.56 (q, $J = 7.4$, 2H), 2.04–2.12 (m, 4H), 2.80–2.85 (m, 6H), 3.12–3.13 (m, 2H), 4.51 (br s, 1H), 5.31–5.43 (m, 8H).

4.3. General synthesis procedure for the compounds 1a–b and 1d–e

An appropriate acid halide (4 equiv) was added into the mixture of arachidonyl *N*-*t*-butylcarbamate (**5**) (1 equiv), MeOH (2 equiv), and NaI (2 equiv) in dry ACN (10 ml/mmol). The reaction mixture was stirred for 0.5 h. The yellow solution was cooled on an ice-bath and DIPEA (4 equiv) was added. Stirring was continued at rt for 1 h. 10% HCl was added and the solution was extracted with ether. The organic layer was washed with satd $NaHCO_3$ and evaporated. The crude product was purified by flash chromatography eluting with petroleum ether/EtOAc 5:1.

4.3.1. *N*-Propionyl norarachidonyl amide (1a). Colorless oil (65%). $R_f = 0.59$ (petroleum ether/EtOAc 1:1) ^{1}H NMR: δ 0.89 (t, $J = 7.0$, 3H), 1.15 (t, $J = 7.6$, 3H), 1.26–1.40 (m, 6H), 1.59 (q, $J = 7.2$, 2H), 2.03–2.13 (m, 4H), 2.2 (q, $J = 7.6$, 2H), 2.80–2.85 (m, 6H), 3.25–3.29 (m, 2H), 5.31–5.43 (m, 8H). ^{13}C NMR: δ 9.9, 14.1, 22.6, 24.7, 25.7 (2C), 27.3, 29.3, 29.6, 29.8, 31.6, 39.2, 127.6, 127.8, 128.1, 128.3, 128.7 (2C), 129.1, 130.6, 173.6. ESI-MS [M+H] 332.2. Elemental analysis. Calculated for $C_{22}H_{37}NO\cdot\frac{1}{3}H_2O$: C, 78.28%; H, 11.25%; N, 4.15%. Found: C, 78.30%; H, 11.48%; N, 4.07%.

4.3.2. *N*-Butanoyl norarachidonyl amide (1b). Colorless oil (52%). $R_f = 0.60$ (petroleum ether/EtOAc 1:1). ^{1}H NMR: δ 0.89 (t, $J = 7.0$, 3H), 0.95 (t, $J = 7.3$, 3H), 1.26–1.39 (m, 6H), 1.58 (q, $J = 7.3$, 2H), 1.66 (st, $J = 7.4$, 2H), 2.03–2.15 (m, 6H), 2.80–2.58 (m, 6H), 3.25–3.29 (m, 2H), 5.31–5.43 (m, 8H), 5.51 (br s, 1H). ^{13}C NMR: δ 13.8, 14.1, 19.2, 22.6, 24.7, 25.6, 25.7, 27.3, 29.3, 29.6, 31.5, 38.8, 39.2, 127.6, 127.8, 128.1, 128.3, 128.7 (2C), 129.1, 130.5, 172.9. ESI-MS [M+H] 346.2. Elemental analysis. Calculated for $C_{23}H_{39}NO\cdot\frac{1}{2}H_2O$: C, 77.91%; H, 11.37%; N, 3.95%. Found: C, 77.50%; H, 11.43%; N, 3.99%.

4.3.3. *N*-(3-Chloropropionyl)norarachidonyl amide (1d). Yellowish oil (43%). $R_f = 0.49$ (petroleum ether/EtOAc 1:1). ^{1}H NMR: δ 0.89 (t, $J = 6.9$, 3H), 1.26–1.39 (m, 6H), 1.61 (q, $J = 7.4$, 2H), 2.04–2.15 (m, 4H), 2.60 (t, $J = 6.4$, 2H), 2.80–2.85 (m, 6H), 3.30 (q, $J = 6.9$, 2H), 3.81 (t, $J = 6.5$, 2H), 5.32–5.44 (m, 8H), 5.60 (br s, 1H). ^{13}C NMR: δ 14.1, 22.6, 24.6, 25.6, 25.7, 27.2, 29.3, 29.4, 31.5, 39.4, 39.8, 40.3, 127.5, 127.8, 128.1, 128.4, 128.7, 128.8, 129.0, 130.5, 169.3. ESI-MS [M+H$^+$] 366.0.

4.3.4. *N*-(Cyclopropanecarbonyl) norarachidonyl amide (1e). Yellowish oil (99%). $R_f = 0.15$ (petroleum ether/EtOAc 6:1). ^{1}H NMR: δ 0.69–0.73 (m, 2H), 0.89 (t, $J = 6.9$, 3H), 0.94–0.97 (m, 2H), 1.26–1.39 (m, 7H), 1.59 (q, $J = 7.4$, 2H), 2.03–2.14 (m, 4H), 2.79–2.87 (m, 6H), 3.28 (q, $J = 7.0$, 2H), 5.31–5.43 (m, 8H), 5.68 (br s, 1H). ^{13}C NMR: δ 7.0, 14.1, 14.8, 22.6, 24.7, 25.6, 25.7, 27.2, 29.3, 29.6, 31.5, 39.4, 71.5, 127.5, 127.8, 128.2, 128.3, 128.6, 128.7, 129.1, 130.5, 173.4. ESI-MS [M+H$^+$] 344.1. Elemental analysis. Calculated for $C_{23}H_{37}NO\cdot\frac{1}{10}H_2O$: C, 79.99%; H, 10.86%; N, 4.0%. Found: C, 79.83%; H, 10.88%; N, 4.11%.

4.3.5. 3-Hydroxypropionylmethylester (11). β-Propiolactone (5 g, 69 mmol) was added dropwise into the precooled solution of NaOH (150 mg) in MeOH (50 ml). After the addition, stirring was continued on an ice-bath for 10 min. The solution was neutralized with 1 M HCl, and most of the solvent was evaporated. The remaining solution was extracted with EtOAc, the organic layers were combined, washed with brine, dried over Na_2SO_4, and evaporated. The crude product was purified by vacuum distillation (12 mbar, 67–72 °C). ^{1}H NMR: δ 2.57–2.59 (m, 3H), 3.72 (s, 3H), 3.87 (q, $J = 5.9$, 2H).

4.3.6. 3-(*t*-Butyldiphenylsilyloxy)-propionic acid (13). The mixture of 3-hydroxypropionylmethylester (**10**) (1 g, 9.6 mmol), *t*-butyldiphenylchlorosilane (3.17 g, 11.5 mmol), and imidazole (1.96 g, 28.8 mmol) in dry THF (18 ml) was stirred at rt for 3 h. The reaction mixture was washed with satd $NaHCO_3$, organic layers were combined, dried over Na_2SO_4, and evaporated. The crude product was purified by flash chromatography eluting with 2.5% EtOAc in petroleum ether. Evaporation of solvents yielded 2.78 g of desired product (85%). $R_f = 0.38$ (petroleum ether/EtOAc 9:1). 3-(*t*-butyldiphenylsilyloxy)-propionylmethylester (**11**) was dissolved in MeOH/H_2O (100 ml/20 ml), the solution was cooled on an ice-bath, and LiOH (1.02 g, 24 mmol) was added slowly. After the addition, stirring was continued at rt for 5 h. The reaction mixture was poured into ice-cold water and extracted with ether/hexane 1:1. The water layer was acidified with 0.5 M $KHSO_4$ and extracted several times with ether/hexane 1:1. The organic layers were combined, washed with water, dried over Na_2SO_4, and evaporated. The crude product was recrystallized from hexane. Yield 1.78 g (68%). $R_f = 0.14$ (petroleum ether/EtOAc 9:1). ^{1}H NMR: δ 1.04 (s, 9H), 3.05 (t, $J = 5.9$, 2H), 3.97 (t, $J = 5.9$, 2H), 7.36–7.46 (m, 6H), 7.64–7.67 (m, 4H).

4.3.7. *N*-(3-Hydroxypropionyl)norarachidonyl amide (1c). 3-(*t*-Butyldiphenylsilyloxy)-propionic acid (**13**) was dissolved in benzene (12 ml) and oxalyl chloride (1.2 ml) and few drops of DMF were added. The mixture was stirred at rt for 1.5 h, after which unreacted oxalyl chloride and benzene were distilled off. Benzene (12 ml) was added and the distillation was repeated. 3-(*t*-Butyldiphenylsilyloxy)-propionylchloride (**14**) (291 mg, 0.84 mmol) was added into the mixture of arachidonyl *N*-*t*-butylcarbamate (**5**) (80 mg, 0.21 mmol), MeOH (0.018 ml, 0.42 mmol), and NaI (60 mg, 0.042 mmol) in dry ACN (2 ml). The reaction mixture was stirred for 0.5 h. The yellow solution was cooled on an ice-bath and DIPEA (0.14 ml, 0.84 mmol) was added. Stirring was continued at rt for 1 h. 10% HCl was added and the solution was extracted with ether. The organic layer was washed with satd $NaHCO_3$ and evaporated. The crude product was purified by flash chromatography eluting with petroleum ether/EtOAc 5:1. Evaporation of solvents yielded 107 mg of oily product (86%). $R_f = 0.22$ (petroleum ether/EtOAc 6:1). *N*-[(3-*t*-butyldiphenylsilyloxy) propionyl] norarachidonyl amide (107 mg, 0.18 mmol) and $Bu_4N^+F^-$ (86 mg, 0.274 mmol) in dry THF (3 ml) were stirred at rt for 2 h. The solvent was evaporated, and the crude product was purified by flash chromatography eluting 33–100% EtOAc in petroleum ether. Reaction proceeded quantitatively. $R_f = 0.10$ (petroleum ether/EtOAc 1:1). ^{1}H NMR: δ 0.89 (t, $J = 7.0$, 3H), 1.26–1.39 (m, 6H), 1.60 (q, $J = 7.3$, 2H), 2.06 (q, $J = 7.1$, 2H), 2.12 (q, $J = 7.1$, 2H), 2.41 (t, $J = 5.4$, 2H), 2.80–2.85 (m, 6H), 3.26–3.30 (m, 2H), 3.87 (t, $J = 5.4$, 2H), 5.31–5.43 (m, 8H), 5.93 (br s, 1H). ^{13}C NMR δ 14.01, 22.6, 24.7, 25.6, 25.7, 27.2, 29.3, 29.5, 31.5, 38.0, 39.1, 59.0, 127.5, 127.8, 128.1, 128.4, 128.7, 128.8, 128.9, 130.6, 172.4. ESI-MS [M+H] 348.1. Elemental analysis. Calculated for $C_{22}H_{37}NO_2\cdot\frac{1}{2}H_2O$: C, 74.11%; H, 10.74%; N, 3.93%. Found: C, 74.47%; H, 10.77%; N, 3.76%.

4.3.8. Arachidonylamine (8). Methanesulfonylchloride (0.16 ml, 2.07 mmol) was added slowly into the precooled solution of arachidonyl alcohol (400 mg, 1.38 mmol) in dry pyridine (8 ml). The reaction mixture was stirred on an ice-bath for 5 h, after which the solution was poured into ice-cold water and extracted with ether. The organic layers were combined, washed with 0.5 M H_2SO_4 and satd $NaHCO_3$, dried over Na_2SO_4, and evaporated. The residue was dissolved in dry DMF (10 ml) and NaN_3 (449 mg, 6.9 mmol) in dry DMF (30 ml) was added dropwise. After the addition, the reaction mixture was stirred at 90 °C overnight. The

solution was cooled to rt, poured into ice-cold water and extracted with ether. The organic layers were combined, dried over Na_2SO_4, and evaporated. The crude product was purified by flash chromatography eluting with 1% EtOAc in petroleum ether. The evaporation of solvents yielded 350 mg of colorless oil (80%). ^{1}H NMR δ 0.89 (t, J = 7.0, 3H), 1.26–1.39 (m, 6H), 1.42–1.49 (m, 2H), 1.59–1.35 (m, 2H), 2.04–2.13 (m, 4H), 2.80–2.85 (m, 6H), 3.27 (t, J = 6.9, 2H), 5.31–5.43 (m, 8H). Arachidonylazide (**7**) (105 mg, 0.33 mmol) in diethyl ether (3 ml) was added to the stirred solution of LAH (12.5 mg, 0.33 mmol) in dry THF (0.35 ml). The reaction mixture was refluxed for 4 h, cooled to rt and diethyl ether was added. The mixture was filtered and evaporated. The crude product was purified by flash chromatography eluting with 10–100% MeOH in DCM. Evaporation of solvents yielded 60 mg of oily product (64%). ^{1}H NMR: δ 0.89 (t, J = 6.9, 3H), 1.26–1.51 (m, 10H), 1.81 (br s, 2H), 2.07 (m, 4H), 2.69–2.72 (m, 2H), 2.80–2.84 (m, 6H), 5.31–5.43 (m, 8H).

4.4. General synthesis procedure for the compounds 2a–b and 2d–e

Propionyl chloride (0.06 ml, 0.70 mmol) in dry DCM (2 ml) was added into the precooled solution of arachidonylamine (**8**) (100 mg, 0.35 mmol) and Et_3N (0.10 ml, 0.70 mmol) in dry CH_2Cl_2 (2 ml). After the addition, the cooling bath was removed and stirring was continued at rt for 2.5 h. CH_2Cl_2 was added and the solution was washed with water and brine. The organic layers were combined, dried over Na_2SO_4 and evaporated. The crude product was purified by flash chromatography eluting with petroleum ether/EtOAc 4:1.

4.4.1. *N*-Propionyl arachidonylamide (2a). Yellowish oil (27%). ^{1}H NMR: δ 0.89 (t, J = 6.9, 3H), 1.15 (t, J = 7.6, 3H), 1.26–1.42 (m, 8H), 1.49–1.55 (m, 2H), 2.03–2.17 (m, 4H), 2.20 (q, J = 7.5, 2H), 2.81–2.58 (m, 6H), 3.25 (t, J = 7.0, 2H), 5.31–5.43 (m, 8H), 5.50 (s, 1H). ^{13}C NMR: δ 10.0, 14.1, 22.6, 25.7, 26.9 (2C), 27.2, 29.3 (2C), 29.7, 29.8, 31.5, 39.4, 127.6, 127.9, 128.2, 128.3 (2C), 128.6, 129.7, 130.5, 173.8. ESI-MS [M+H$^+$] 346.2. Elemental analysis. Calculated for $C_{23}H_{39}NO\cdot\frac{1}{3}H_2O$: C, 78.58%; H, 11.37%; N, 3.98%. Found: C, 78.17%; H, 11.54%; N, 3.70%.

4.4.2. *N*-Butanoyl arachidonylamide (2b). Yellowish oil (83%). R_f = 0.12 (petroleum ether/EtOAc 4:1). ^{1}H NMR: δ 0.89 (t, J = 6.9, 3H), 0.95 (t, J = 7.4, 3H), 1.26–1.43 (m, 8H), 1.49–1.55 (m, 2H), 1.66 (q, J = 7.4, 2H), 2.04–2.11 (m, 4H), 2.13 (t, J = 7.5, 2H), 2.80–2.85 (m, 6H), 3.25 (q, J = 7.1, 2H), 5.31–5.43 (m, 8H). ^{13}C NMR: δ 13.8, 14.1, 19.2, 22.6, 25.7 (2C), 26.9 (2C), 27.2, 29.3, 29.4, 31.5, 38.8, 39.4, 127.6, 127.9, 128.2, 128.3 (2C), 128.6, 129.7, 130.5, 172.9. ESI-MS [M+H$^+$] 360.2. Elemental analysis. Calculated for $C_{24}H_{41}NO$: C, 80.16%; H, 11.49%; N, 3.90%. Found: C, 80.49%; H, 11.77%; N, 3.81%.

4.4.3. *N*-(3-Chloropropionyl)arachidonylamide (2d). Yellowish oil (69%). R_f = 0.18 (petroleum ether/EtOAc 4:1). ^{1}H NMR: δ 0.89 (t, J = 6.9, 3H), 1.26–1.44 (m, 8H), 1.54 (q, J = 7.2, 2H), 2.03–2.12 (m, 4H), 2.60 (t, J = 6.6, 2H), 2.80–2.85 (m, 6H), 3.30 (q, J = 7.1, 2H), 3.81 (t, J = 6.4, 2H), 5.31–5.43 (m, 8H), 5.59 (br s, 1H). ^{13}C NMR: δ 14.1, 22.6, 25.7, 26.8 (2C), 27.2, 29.2, 29.3, 31.5, 39.6, 39.8, 40.3, 127.6, 127.9, 128.2, 128.3 (2C), 128.6, 129.6, 130.5, 169.3. ESI-MS [M+H$^+$] 380.0.

4.4.4. *N*-Cyclopropanecarbonyl arachidonylamide (2e). Yellowish oil (82%). R_f = 0.14 (petroleum ether/EtOAc 4:1). ^{1}H NMR: δ 0.69–0.73 (m, 2H), 0.89 (t, J = 6.8, 3H), 0.94–0.97 (m, 2H), 1.26–1.44 (m, 9H), 1.49–1.56 (m, 2H), 2.04–2.12 (m, 4H), 2.80–2.87 (m, 6H), 3.27 (q, J = 6.9, 2H), 5.31–5.43 (m, 8H), 5.58 (br s, 1H). ^{13}C NMR: δ 7.0, 14.1, 14.8, 22.6, 25.7, 26.9 (2C), 27.2, 29.3, 29.4, 29.7, 31.5, 39.6, 127.6, 127.9, 128.2, 128.3 (2C), 128.6, 129.7, 130.5, 173.4. ESI-MS [M+H$^+$] 358.1.

4.4.5. *N*-(2-Hydroxymethyl-3-hydroxy-propanoyl)arachidonylamide (2c). Arachidonylamine (**8**) (120 mg, 0.41 mmol), EDCl (159 mg, 0.83 mmol), and DMAP (15 mg, 0.12 mmol) were dissolved in dry CH_2Cl_2 (8 ml) and stirred at rt for 20 h. The solvent was evaporated and the residue was dissolved in EtOAc. The solution was washed with water and brine, dried over Na_2SO_4, and evaporated. The crude product was purified by flash chromatography eluting with petroleum ether/EtOAc 4:1. The evaporation of solvents yielded 50 mg of white waxy product (25%). ^{1}H NMR: δ 0.89 (t, J = 7.0, 3H), 1.26–1.42 (m, 8H), 1.50–1.55 (m, 2H), 2.04–2.12 (m, 4H), 2.80–2.89 (m, 7 H), 3.25 (q, J = 7.3, 2H), 4.13–4.17 (m, 2H), 4.31–4.34 (m, 2H), 5.31–5.43 (m, 8H), 5.51 (s, 1H), 5.53 (br s, 1H), 7.33–7.39 (m, 3H), 7.46–7.48 (m, 2H). R_f = 0.33 (petroleum ether/EtOAc 6:1). Compound **9** was dissolved in concd HCl/MeOH (3 ml/7 ml) and the mixture was stirred at rt for 3 h. The solvents were evaporated, and the crude product was purified with flash chromatography eluting with petroleum ether/EtOAc 1:2. Evaporation of solvents yielded 40 mg of yellowish oil (98%). R_f = 0.2 (DCM/MeOH 9:1). ^{1}H NMR δ: 0.89 (t, J = 6.8, 3H), 1.26–1.43 (m, 8H), 1.54 (q, J = 7.4, 2H), 2.03–2.11 (m, 4H), 2.48–2.52 (m, 1H), 2.81–2.85 (m, 6H), 3.20 (s, 2H), 3.27 (q, J = 6.9, 2H), 3.84–3.93 (m, 4H), 5.31–5.43 (m, 8H), 6.64 (s, 1H). ^{13}H NMR: δ 14.1, 22.6, 25.7, 26.8, 26.9, 27.2, 29.1, 29.3, 29.7, 31.5, 39.3, 49.1, 62.0, 127.6, 127.9, 128.2, 128.3, 128.4, 128.6, 129.6, 130.5, 173.9. ESI-MS [M+H$^+$] 392.2. Elemental analysis. Calculated for $C_{24}H_{41}NO_3\cdot\frac{1}{10}H_2O$: C, 73.28%; H, 10.56%; N, 3.56%. Found: C, 73.24%; H, 11.00%; N, 3.24%.

4.4.6. *N*-(3-Hydroxypropionyl)arachidonylamide (2f). Arachidonylamine (**8**) (142 mg, 0.49 mmol) and 2M $Al(CH_3)_3$ (in heptane, 0.25 ml, 0.49 mmol) were dissolved in dry CH_2Cl_2 (6 ml) and stirred at rt for 20 min. β-propiolactone (0.03 ml, 0.49 mmol) was added and the reaction mixture was refluxed for 4 h. The solution was cooled to rt, 1M HCl was added, and the mixture was extracted with CH_2Cl_2. Organic layers were combined, dried over Na_2SO_4, and evaporated. The crude product was purified by flash chromatography

eluting with EtOAc. Evaporation of solvents yielded 70 mg of colorless oil (40%). ^{1}H NMR: δ 0.89 (t, J = 6.9, 3H), 1.26–1.43 (m, 8H), 1.43 (q, J = 7.5, 2H), 2.04–2.12 (m, 4H), 2.42 (t, J = 5.4, 2H), 2.80–2.85 (m, 6H), 3.27 (q, J = 6.7, 2H), 3.88 (t, J = 5.4, 2H), 5.31–5.43 (m, 8H), 5.80 (s, 1H). ^{13}C NMR: δ 14.5, 23.0, 26.0, 27.2, 27.3, 27.6, 29.6, 29.7, 32.0, 38.3, 40.0, 59.4, 127.9, 128.3, 128.6, 128.6, 128.7, 129.0, 130.0, 131.0, 172.8. ESI-MS 362.2. Elemental analysis. Calculated for $C_{23}H_{39}NO_2 \cdot \frac{1}{3}H_2O$: C, 75.16%; H, 10.88%; N, 3.81%. Found: C, 75.22%; H, 10.99%; N, 3.62%.

4.5. Stability study

Eight-week-old Wistar rats (200 g weight) were decapitated, whole brains minus cerebellum were dissected and homogenized in one times the volume (v/w) of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) with a Potter-Elvehjem homogenizer (Heidolph). The homogenate was centrifuged at 10,000g for 20 min at 4 °C and the resulting supernatant was used as a source of FAAH activity. The protein concentration of the supernatant (7.2 mg/ml) was determined by the method of Bradford with bovine serum albumin as a standard (Bradford 1976). Aliquots of the supernatant were stored at −70 °C until use. The experiments were initiated by a preincubation of a mixture (1380 μl, 37 °C, 10 min) containing 929 μg of a rat brain homogenate, 81 mM potassium phosphate buffer (pH 7.4), and 0.5% (w/v) BSA. The actual experiment was carried out by taking 1188 μl of the preincubated brain homogenate protein, adding 12 μl of studied compound (5 mM in ethanol, final volume 1200 μl), and incubating the mixture for 90 min at 37 °C. The final incubation mixture contained 800 μg rat brain homogenate protein, 80 mM potassium phosphate buffer (pH 7.4), 0.5% (w/v) BSA, and 50 μM of studied compound. At time points 0 and 90 min, 300 μl samples were taken and 600 μl of cold acetonitrile was added in order to stop the enzymatic reaction. The samples were centrifuged at 23,700g for 4 min at rt and the supernatant was analyzed for remaining compound by HPLC. The analytical HPLC system used in the stability studies consisted of a Merck Hitachi L-7100 pump, D-7000 interface module, L-7455 diode-array detector (190–800 nm, set at 211 nm), and L-7250 programmable autosampler. The separations were performed with Zorbax SB-C18 end capped reversed-phase column (4.6 mm × 150 mm, 5 μm). The injection volume was 50 μL. A mobile phase of 28% phosphate buffer (30 mM, pH 3.0) in acetonitrile was used at a flow rate of 2 ml/min. Retention times for AEA, **1a**, **1c**, **1e**, **2b**, **2e**, and **2f** were 4.5, 10.5, 4.9, 10.0, 17.2, 14.7, and 6.5 min, respectively.

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