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Endocannabinoids

Improved Enzymatic Procedure for the Synthesis of Anandamide and *N*-Fatty Acylalkanolamine Analogues: A Combination Strategy to Antitumor Activity

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Abstract: Twenty *N*-fatty acylamines from linolenic and arachidonic acids, fifteen of them new compounds, were obtained through *Candida antarctica* B lipase-catalyzed esterification and aminolysis reactions in very good yields and with high chemoselectivity. The optimal reaction conditions were achieved by studying the reaction parameters (temperature, E/S ratio, alcohol and alkanolamine/fatty acid ratio, time, solvent, free-solvent system, etc.). To identify ideal enzymatic methods for generating the alkanolamides we evaluated enzyme performance in three procedures: i) aminolysis of ethyl ester, ii) direct condensation between the fatty acid and the alkanolamine, and iii) a one-pot/two-step conversion of fatty acids into alkanolamides via in situ formation of the ethyl ester and subsequent aminolysis by the alkanolamine. The advantages noted with the enzymatic methodology, such as mild reaction conditions and low environmental impact, underscore biocatalysis as a convenient way to prepare the reported compounds. The cytotoxic activities of all compounds and mixtures of anandamide and its analogues were evaluated in rat glioma C6 cells. These studies reveal that some anandamide analogues enhance the antitumor effects of anandamide, suggesting their possible application as therapeutic tools in cancer treatment.

Introduction

The endocannabinoid anandamide [*N*-(2-hydroxyethyl)arachidonoylamide or *N*-arachidonoyl-ethanolamine, AEA] is a neuromodulatory lipid that belongs to a family of signaling molecules collectively termed endocannabinoids.[1] The biological actions of AEA are tightly controlled through its enzymatic synthesis and degradation.[2] Like Δ^9-tetrahydrocannabinol, AEA activates the central and peripheral cannabinoid receptors CB_1 and CB_2 and is also a ligand for transient receptor potential vanilloid receptor 1 (TRPV1).[3–5] In contrast to most neurotransmitters, AEA is an uncharged lipid capable of traversing the bilayer unaided.[6] Accordingly, several studies have shown that AEA uptake occurs by passive diffusion,[7] although facilitated diffusion and/or endocytosis have also been proposed.[8] Under physiological conditions, AEA is rapidly hydrolyzed by fatty acid amide hydrolase (FAAH), the enzyme responsible for its degradation to arachidonic acid and ethanolamine.[9] Considerable research has shed light on the impact of endocannabinoids on human health and disease. Anandamide and congeners control basic biological processes in the brain, gastrointestinal tract, skeletal muscle, liver, bone and skin as well as in pathways associated with immune system function and reproductive systems.

Moreover, the endocannabinoids have been recognized as key mediators of several aspects of human pathophysiology and, thus, have been revealed as among the most widespread and versatile signaling molecules ever discovered.[10,11] One attractive strategy for eliciting the desirable effects of cannabinoid activation and avoiding the negative effects of global CB_1 stimulation is to modulate endogenous cannabinoid signaling by inhibiting the FAAH enzyme. This approach would be expected to increase endogenous concentrations of all FAAH substrates, thus, enhancing their biological effects. By acting preferentially on active pathways, such an approach might be expected to have a reduced risk of inducing psychotropic effects.[12] From this perspective, the employment of AEA analogues to compete for FAAH binding is an interesting tactic to generate inhibitors.

Thus, numerous endocannabinoid analogues were synthesized as tools to probe the influence of the lipid chain, the importance of the carbonyl function, and the tolerance to steric hindrance of the polar head with regards to FAAH activity.[13–15] A recent report, based on molecular modelling studies, have shown that FAAH selectively accommodates anandamide

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into a multipocket binding site and confirmed that the rate of substrate hydrolysis increases with the number of double bonds within the substrate lipid chain.[16]

Keeping in mind the above data, we focused on the use of linolenoyl and arachidonoyl derivatives as putative FAAH substrates. Thus, we report herein the synthesis of a series of *N*-linolenoyl- and *N*-arachidonoylalkanolamines by reaction of the corresponding ω-3 and ω-6 fatty acids (**1**, **2**) or their ethyl esters with various alkanolamines (**a**–**j**) using an enzymatic approach (Figure 1).

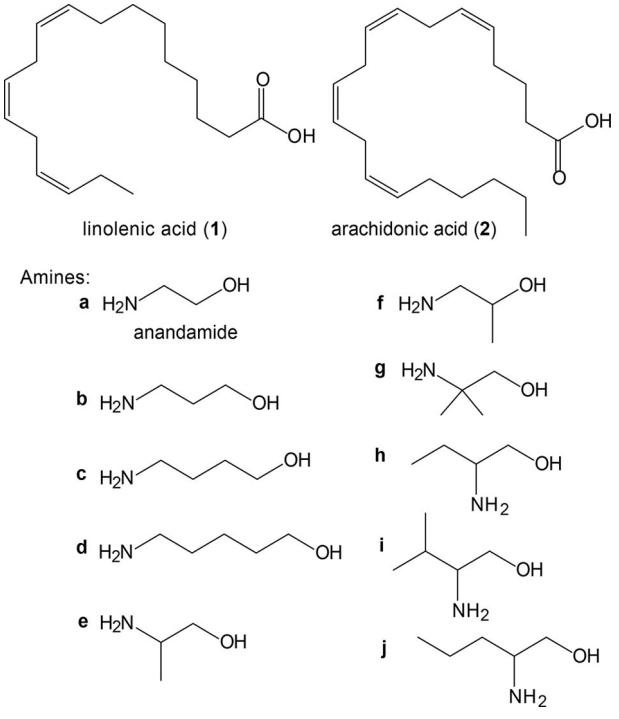


Figure 1. Structure of linolenic acid (**1**), arachidonic acid (**2**) and alkanolamines (**a**–**j**).

Biocatalysis has proven to be a valuable alternative to the synthesis of organic compounds using Green Chemistry approaches. The use of pure enzymes and whole microbes offers interesting advantages, such as biodegradability and the possibility of working under mild reaction conditions.[17] Moreover, these catalysts are able to accept a wide array of substrates catalyzing reactions in a chemo- and regioselelective way. Consequently, such systems circumvent tedious protection and deprotection steps often associated with compounds bearing diverse functional groups.[18]

Over the last several years, biocatalysis using lipases in non-aqueous media has been widely used in the synthesis of pharmaceuticals; examples include esterification, transesterification, aminolysis, polymerization, and numerous other chemistries.[19–22] Enzymes are also well-known for their high enantioselectivity and this property has provided the basis for their widespread use in the synthesis of enantiomerically pure compounds.[23] Studies carried out in our laboratory on the esterification and transesterification of multiple substrates have shown that lipases are useful in the synthesis of biologically active compounds from natural starting materials, particularly terpenes and steroids derivatives.[24]

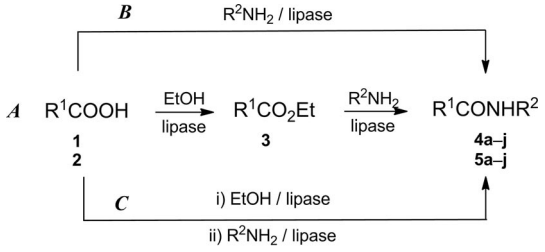
The most commonly described method for the synthesis of anandamide and its analogues entails the reaction of a fatty acyl donor and the alkanolamine with catalysts such as sodium methoxide or 1-propylphosphonic acid cyclic anhydride. Alternative schemes invoke temperatures of about 180 °C in lieu of catalysts.[25,26] Fatty acyl donors typically include fatty acid chlorides, free fatty acids and fatty acid methyl esters.[27] Alternative approaches call for the direct condensation between in situ pre-activated fatty acid, employing the coupling agents carbonyldiimidazole (CDI) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and the corresponding amine.[28] Moreover, several methods have been reported for the direct conversion of esters to amides involving $Mg(OCH_3)_2$ and $CaCl_2$, sodium cyanide, metal catalysts and other reagents.[29] All these methods are well established but are faced with several limitations. Fastidious steps of alcohol group protection and deprotection for the control of chemo- and stereoselectivity are often necessary and the high temperatures required for desired coupling chemistry often preclude the use of fragile molecules and may affect the color, odor and purity of final products. In addition, the formation of salts and the use of toxic reagents requiring removal at the end of the reaction often substantially increases the cost of production. Biocatalysis allows the use of esters or the direct use of carboxylic acids to obtain amides, which is advantageous from economic and environmental viewpoints.[30] To date, very little has been reported regarding enzymatic syntheses of endocannabinoids. Under various reaction conditions and using *Candida antarctica* B lipase immobilized on an acrylic resin, the preparation of some unsaturated fatty acylethanolamines has been reported.[31]

Finally, the effects of anandamide and the related analogues on glioma C6 cell viability is also reported.

Results and Discussion

1. Enzymatic Synthesis

In the present work, the synthesis of *N*-fatty acylalkanolamines was developed by applying three different strategies in an enzymatic procedure, using linolenic acid **1**, ethanol and ethanolamine (**a**) as model reactants (Scheme 1). The first approach (*Route A*) involves aminolysis of ethyl linolenate (**3**) (previously prepared enzymatically) with ethanolamine to afford *N*-linolenoylethanolamine **4a**. In the second approach (*Route B*), product **4a** is obtained by direct condensation between linolenic acid and ethanolamine. Finally, the third approach (*Route C*) describes a one-pot/two-step procedure, previously reported in our laboratory,[32] involving fatty acid conversion to alkanolamides via in situ formation of the ethyl ester and subsequent aminolysis with ethanolamine (Scheme 1).



a: $R^2 = -(CH_2)_2OH$
b: $R^2 = -(CH_2)_3OH$
c: $R^2 = -(CH_2)_4OH$
d: $R^2 = -(CH_2)_5OH$
e: $R^2 = -CH(CH_3)CH_2OH$
f: $R^2 = -CH_2CH(CH_3)OH$
g: $R^2 = -C(CH_3)_2CH_2OH$
h: $R^2 = -CH(CH_2CH_3)CH_2OH$
i: $R^2 = -CH(CH(CH_3)_2)CH_2OH$
j: $R^2 = -CH(CH_2CH_2CH_3)CH_2OH$

Scheme 1. Synthetic strategies in the enzymatic synthesis of *N*-linolenoyl-alkanolamines.

With the aim of achieving optimal reaction conditions, we studied the behavior of various lipases and select reaction parameters such as solvent, temperature, enzyme:substrate ratio (E/S) and nucleophile (alcohol or alkanolamine):substrate ratio (A/S).

Route A

To start off *Route A*, ethyl linolenate **3** was prepared by esterification of linolenic acid with ethanol in a lipase-catalyzed fashion. The enzymatic synthesis of linolenic acid ethyl ester has been reported by alcoholysis of soybean oil in hexane or compressed fluids, affording the ester in a complex mixture of fatty acid ethyl esters.[33] Another approach has employed lipase in the presence of activated molecular sieves in a reflux trap.[34]

In the present work, five lipases from several sources were applied. Lipases studied include i) from the yeasts *Candida rugosa* (CRL) and ii) *Candida antarctica* B (CAL B), iii) Lipozyme from the fungus *Rhizomucor miehei* (LIP), iv) heterologous *Rhizopus oryzae* lipase (ROL), and v) plant-derived *Carica papaya* lipase (CPL), which is the remaining solid fraction of papaya latex after protease removal by washing. The results of the lipase-catalyzed esterification of **1** are summarized in Table 1 (column 4).

Table 1. Lipase-catalyzed preparation of **3** and **4a**.

			Product conversion [%][a]			
Entry	Enzyme	Temp.	**3**[b]	**4a**[c]	**4a**[d]	**4a**[e]
		[°C]	*Route A*	*Route A*	*Route B*	*Route C*
1	CAL B	25	62	42	–	35
2	LIP	25	35	21	–	25
3	CRL	25	21	22	–	n.d.
4	ROL	25	14	10	–	n.d.
5	CPL	25	16	8	–	n.d.
6	CAL B	55	**100**	**92**	**90**	**96**
7	LIP	55	74	35	34	33
8	CRL	55	56	43	22	16
9	ROL	55	64	8	n.d.	n.d.
10	CPL	55	43	n.d.	n.d.	n.d.

[a] E/S: 5; solvent: hexane; time: 48 h. Conversions determined on basis of GC or HPLC analyses. [b] Ethanol/**1**: 1.2. [c] *Route A*: ester aminolysis. Ethanolamine/**3**: 1.2. [d] *Route B*: direct condensation. Ethanolamine/**1**: 2. Reaction was not tested at 25 °C. [e] *Route C*: one-pot/two-step approach. Ethanol/**1**: 1.2; ethanolamine/**3**: 1.2.

Among the five lipases evaluated, CAL B gave the most satisfactory esterification results at 55 °C. As shown in Table 1, temperature increases improved the results for some lipases, ultimately revealing CAL B as the most effective esterification agent. These data made clear that CAL B was the enzyme of choice. Notably, in the absence of enzyme, linolenic acid was found to be completely inert.

Using CAL B as biocatalyst, the esterification was evaluated using ethanol as the both esterifying agent and solvent (solvent free system). The same reaction, using hexane as solvent, was also carried out. Notably, desired product was obtained at maximum conversion under both reaction conditions. However, the use of hexane as a solvent enabled this result to be achieved in 1 h versus the solvent free system which required almost 2 d to achieve the same extent of product formation.

To probe the influence of enzyme:substrate ratio upon reaction efficiency, we performed experiments varying the enzyme:substrate ratios from 0.1 to 5 (Table 2, column 3). We found that an E/S = 1 gave the best results. Therefore, the following standard conditions were applied to the biocatalytic esterification: CAL B as biocatalyst, ethanol/**1** = 1.2, hexane as solvent, 55 °C and an E/S ratio of 1.

Table 2. Effect of enzyme:substrate ratio on lipase-catalyzed synthesis of **3** and **4a**.

		Products conversion [%][a]		
Entry	E/S	**3**	**4a**[b]	**4a**[c]
1	0.1	30	32	15
2	0.25	58	55	43
3	0.5	75	69	56
4	1	**97**	83	75
5	2	96	**93**	**89**
6	5	98	92	90

[a] CAL B; 55 °C; solvent: hexane, time: 1 h (**3**), 48 h (**4a**). Conversions determined on basis of GC and HPLC analyses. [b] *Route A*. [c] *Route B*.

Ethyl linolenate **3**, obtained in 95 % yield, was used as substrate in the screening of various lipases for the enzymatic aminolysis with ethanolamine (**a**) in the second step of *Route A*. The results are summarized in Table 1, column 5. Lipases showed variable activity; at 55 °C, CAL B gave the most satisfactory results using an E/S = 2 (Table 2). In the absence of enzyme, no product was detected within 2 d.

The amide product was isolated (yield 89 %) and identified by spectroscopic methods as *N*-linolenoylethanolamine (**4a**). An important issue was chemoselectivity, since, under certain conditions, alkanolamines are susceptible to acylation both at the amine and alcoholic group. Our results are in accordance with several studies reporting that the lipase acts in a chemoselective manner, exclusively producing the amide.[32] In this case, **4a** was obtained and the isomeric amino ester was not detected. The reaction was carried out at different ester and ethanolamine ratios and the best yield was obtained at an amine/ester ratio of 1 and at an ester concentration of 0.5 M.

Considering that both substrate (ethyl linolenate) and nucleophile (ethanolamine) exhibit different polarities, we examined the aminolysis in various organic solvents (solvent free, hexane, diisopropyl ether and acetonitrile). Among these conditions, the best results were obtained using hexane as solvent. Ethyl linolenate was easily soluble in hexane and the system remained homogeneous even after the addition of ethanol-

amine. Conversely, the use of polar solvents such as acetonitrile led to some level of precipitation upon addition of ethanolamine to the fatty acid solution. Finally, the reaction carried out in a solvent free system afforded a mixture of products in accordance with previous reports on related compounds.[31]

Route B

This approach consists of the direct condensation of ethanolamine with linolenic acid.[31] We also studied the behavior of various lipases and some reaction parameters for this case. As shown in Table 1 we identified CAL B and hexane as the enzyme and solvent of choice, respectively. Consequently, the reaction (*Route B*) was carried out at 55 °C, using an E/S = 2 (Table 2, Entry 5) and an A/S = 2. The isolated product **4a** was obtained in 85 % yield.

It is interesting to point out that a considerable amount of product **4a** remained adsorbed to the enzyme surface at the end of the reaction under both *Route A* and *Route B* conditions. Not surprisingly, the yield of **4a** remarkably increased when the enzyme was washed several times with hexane.

Route C

Taking into account our previous work,[32] we sought, in developing *Route C* approaches, a one-pot/two-step procedure for the preparation of **4a**. The two enzymatic steps yielding **3** and **4a**, respectively, were performed successively in the same pot. In this case, ethyl linolenate (**3**) was prepared from linolenic acid and ethanol using CAL B and hexane at 55 °C (as described in the first step of *Route A*). Upon complete conversion of acid into ethyl ester **3** (after 1 h of reaction), ethanolamine was added. The global yield for **4a**, resulting from application of both enzymatic steps, was 92 %. The notable simplicity of *Route C* makes this approach more convenient than *Route A*. The latter method calls for isolation of **3** and subsequent aminolysis thus requiring more work to achieve the desired product in lower yield (97 × 89 = 86 %).

In summary, *Route C* proved o in terms of yield and economy. Aminolysis of ester **3** in *Route A* afforded better results than the direct condensation in *Route B*, indicating that the ester is a better substrate for ethanolamine than is the free fatty acid. The one-pot/two-step procedure described in *Route C* enables the desired aminolysis without ethyl linolenate isolation, which is obtained in quantitative yield through enzymatic catalysis. Moreover, the use of linolenic acid as starting material is an additional advantage; this acid costs about half the price of its ethyl ester. With regards to toxicity, linolenic acid is nontoxic, and is actually well known as an essential fatty acid acquired through diet. Although linolenic acid ethyl ester is not highly toxic, its manipulation may cause skin irritation.[35] Additionally with regards to *Route C*, enzyme recycling represents an additional advantage. Since the immobilized lipase is insoluble in the reaction media, it is easily removed by filtration at the end of the process. The enzyme can be re-used and, in this particular reaction, CAL B has been found to retain ≈ 80 % of its activity after three reaction cycles (Figure 2).

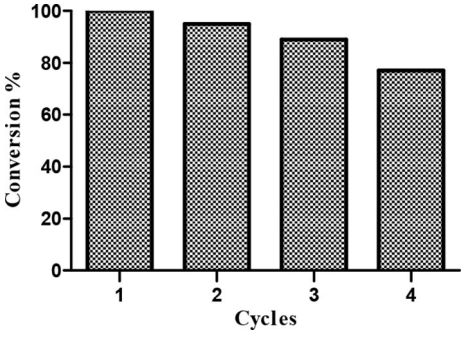
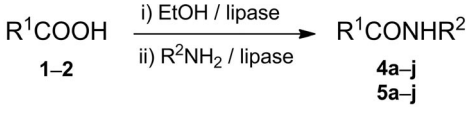


Figure 2. CAL B recycling in the synthesis of *N*-linolenoylethanolamine as described in the Experimental Section, *Route C*.

Application of the One-Pot/Two-Step Procedure to the Synthesis of N-Linolenoyl and N-Arachidonoyl Alkanolamines

Once the experimental conditions were optimized, we applied *Route C* to the synthesis of anandamide (**5a**) and related analogues (**4b**–**j** and **5a**–**j**), see Scheme 2.



a: $R^2 = -(CH_2)_2OH$ **f**: $R^2 = -CH_2CH(CH_3)OH$
b: $R^2 = -(CH_2)_3OH$ **g**: $R^2 = -C(CH_3)_2CH_2OH$
c: $R^2 = -(CH_2)_4OH$ **h**: $R^2 = -CH(CH_2CH_3)CH_2OH$
d: $R^2 = -(CH_2)_5OH$ **i**: $R^2 = -CH(CH(CH_3)_2)CH_2OH$
e: $R^2 = -CH(CH_3)CH_2OH$ **j**: $R^2 = -CH(CH_2CH_2CH_3)CH_2OH$

Scheme 2. One-pot/two-step procedure for synthesis of *N*-linolenoyl (**4a**–**j**) and arachidonoylalkanolamines (**5a**–**j**).

The results, expressed as yields of isolated products **4a**–**j** and **5a**–**j**, for linolenic acid (**1**) and arachidonic acid (**2**) respectively, with the series of alkanolamines **a**–**j** are summarized in Table 3.

Table 3. Enzymatic synthesis of *N*-linolenoyl and *N*-arachidonoylalkylamines.[a]

Entry	Alkanolamine		Product Yield [%]	
			4	**5**
1	$NH_2(CH_2)_2OH$	**a**	92	81
2	$NH_2(CH_2)_3OH$	**b**	83	73
3	$NH_2(CH_2)_4OH$	**c**	75	69
4	$NH_2(CH_2)_5OH$	**d**	68	66
5	$NH_2CH(CH_3)CH_2OH$	**e**	86	78
6	$NH_2CH_2CH(CH_3)OH$	**f**	87	80
7	$NH_2C(CH_3)_2CH_2OH$	**g**	63	60
8	$NH_2CH(CH_2CH_3)CH_2OH$	**h**	78	68
9	$NH_2CH[CH(CH_3)_2]CH_2OH$	**i**	62	63
10	$NH_2CH(CH_2CH_2CH_3)CH_2OH$	**j**	77	75

[a] Standard conditions in *Route C*.

Under these reaction conditions, CAL B dispayed high chemoselectivity, affording exclusively the *N*-acyl derivatives with both fatty acids and linear and branched chain alkanolamines.

Review of Table 3 reveals that optimum yields were obtained with linear alkanolamines and linolenic acid (Table 3, Entries 1–

4); ethanolamine (**a**), in particular, was found to be the best nucleophile for the reaction. Notably, a decrease in yield was observed to correlate with increases in fatty acid unsaturation and chain lengths of alkanolamines. In every case, linolenic acid derivatives were obtained in higher yields than those corresponding to arachidononic acid.

Regarding the branched alkanolamines derivatives **4e**–**j** and **5e**–**j**, some differences in product yields were observed among the different examples studied, ranging from 60–87 % (Table 3, Entries 5–10). These nucleophiles have another polar group (hydroxyl) in the β-position of the amino group favoring *N*-acylation reaction.[36] The alkanolamines with two methyl groups (**4g** and **5g**) and isopropyl groups (**4i** and **5i**) as substituents afforded products in the lowest yields (Table 3, Entries 7 and 9). These results were similar for both fatty acids and could be attributed to steric hindrance issues stemming from alkanolamine structures. In comparing the reactions with 2-amino-1-propanol (**e**) and 1-amino-2-propanol (**f**), for which the positions of the alcohol and amine functions are interchanged, similar results become evident (Table 3, Entries 4 and 5). These results imply that the position (1 or 2) of the alcohol and amine functionalities has no impact on lipase activity.

To the best of our knowledge, with the exception of **4a** and **5a**, this is the first enzymatic synthesis of the two series of *N*-fatty acid alkanolamines. In addition, **5e**–**g** have been previously reported following their chemical synthesis using oxalyl chloride-based chemistry.[37]

The possible stereoselective behavior of CAL B in the case of branched alkanolamines was studied through the determination of the optical rotation data for the respective products. Unfortunately, optical rotation data for these CAL B-derived products revealed that, under these reaction conditions, the lipase was not stereoselective.

2. Effect of AEA and Analogues on Glioma C6 Cell Viability

Numerous studies carried out during the past few years have demonstrated that cannabinoids exhibit antitumor effects in different cancer cell lines[38] and in a wide range of animal models.[39,40] In particular, the cytotoxic effect of AEA has been extensively investigated in the C6 cell line.[41–44] These cells are frequently employed as an in vitro model for glioma, the most common malignant brain tumor. C6 cells have a well characterized endocannabinoid system, and undergo AEA-induced apoptosis mediated by TRPV1 which can be counteracted by CB_1.[41] Previous findings[44] have demonstrated a significant dose-dependent cytotoxic effect of AEA on C6 cells when exposure was carried out in the absence of serum in the culture medium. In contrast, when AEA was added to the culture medium supplemented with 10 % fetal bovine serum (FBS), no cytotoxic effect was observed. This differential behavior may be caused by binding of AEA to serum proteins such as albumin. To circumvent this effect we conducted our experiments using 2 % FBS.

We first investigated the capability of enzymatically synthetized AEA and related analogues (**4a**, **4c**, **4g**, **4i**, **5b**–**d**, **5g** and **5 i**) to induce cell death.

For this purpose, cells were exposed to different concentrations of each compound for 24 h in 2 % FBS containing media. The cell viability was evaluated employing the standard MTT assay (Figure 3). The results showed that at 15 μM only AEA induced a decrease in cell viability (22 ± 3 % $p < 0.001$) (Figure 3, panel A). On the other hand, exposure of cells to 30 μM of AEA and analogues **4g** and **5d** induced cell death (88 ± 7 % $p < 0.001$, 42 ± 6 % $p < 0.001$ and 14 ± 6 % $p < 0.05$, respectively) (Figure 3, panel B).

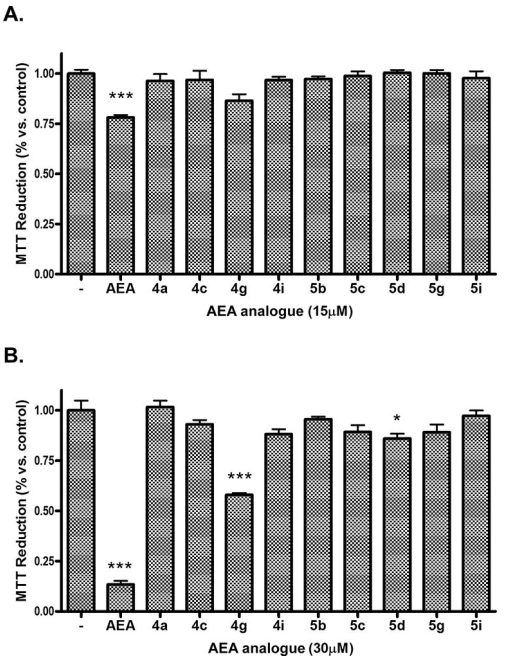


Figure 3. Cell viability in C6 glioma cells after exposure to AEA and its analogues. Cells were exposed to (A) 15 μM and (B) 30 μM of AEA and its analogues **4a**, **4c**, **4g**, **4i**, **5b**–**d**, **5g**, **5i** for 24 h in 2 % FBS. Cell viability was assayed by MTT reduction. * $p < 0.05$, *** $p < 0.001$ vs. control.

In line with the evidence demonstrating that the pharmacological activation of cannabinoid receptors reduces the tumour growth, the upregulation of endocannabinoid-degrading enzymes such as FAAH has been observed in both, aggressive human tumours and cancer cell lines.[45,46] Thus, we next investigated the possibility that AEA analogues behave as FAAH inhibitors increasing the cytotoxic action of AEA. The combined effect of non-toxic concentrations of AEA analogues (15 μM) and AEA (15 μM) during 24 h was evaluated (Figure 4).

Compounds **4g** and **5c** significantly augmented AEA-induced cell death observed by both phase contrast microscopy (Figure 4, panel A) and MTT assay (66 ± 2 % and 15 ± 4 %, respectively; $p < 0.001$) (Figure 4, panel B). The fact that compound **4g** exhibited the most pronounced effect is in accordance with previous reports in which the rate of substrate hydrolysis increased with the number of double bonds of the substrate lipid chain.[47] These findings support our premise that AEA analogues may increase AEA effectiveness by increasing its availability through competition for the FAAH active site.

Interestingly, compounds **5b**, **5d** and **5g** showed a slight trend to decrease cell viability. These analogues share with AEA the arachidonic chain carrying four unsaturated bonds in the carboxylic moiety of the alkanolamide with a variation in the alkanolamine moiety. Two of them have linear chains with in-

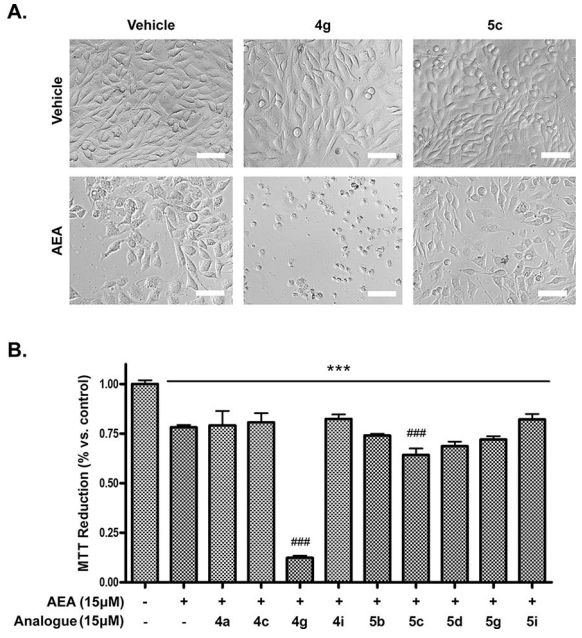


Figure 4. Products **4g** and **5c** increase AEA-induced cell death. C6 cells were exposed to a mixture of AEA (15 μM) and its analogues **4a**, **4c**, **4g**, **4i**, **5b**–**d**, **5g**, **5i** (15 μM) for 24 h in 2 %FBS. (A) Representative phase contrast images of the combined effects of AEA (15 μM) and its analogues **4g** and **5c** (15 μM). Scale bar: 50 μm. (B) Cell viability was assayed by MTT reduction. *** $p < 0.001$: significant differences between control whithout AEA and whithout analogues vs. all the treatments. ### $p < 0.001$: significant differences between cells treated with AEA plus analogues vs. cells treated with AEA.

creasing carbon number (**5b**: 3 methylene groups and **5d**: 5 methylene groups). Data comparisons for **4g** and **5g** reveal the effect of length and unsaturation in the acyl group of the alkanolamide, because both have the same alkanolamine moiety; **4g** being a linolenic derivative and **5g** being an arachidonic congener. Compound **5i**, which proved ineffective, may lack activity due to its converged arachidonoyl acyl chain and the isopropyl moiety next to the amino group of the alkanolamide; the combination of these spatially demanding groups in **5i** may pose too great a steric barrier for FAAH interactions.

3. Conclusions

This work describes the application of lipases to the synthesis of anandamide and two series of fatty acid derivatives with various alkanolamines along with biological studies aimed at evaluating their antitumor activity.

The enzymatic approach used in the synthesis was identified following development and study of three different strategies as applied to a model reaction between linolenic acid and ethanolamine. The approaches investigated include, i) *Route A*: aminolysis of ethyl linolenate (**3**) prepared enzymatically, by ethanolamine, ii) *Route B*: direct condensation between linolenic acid and ethanolamine, and *Route C*: a one-pot/two-step procedure involving fatty acid conversion to alkanolamides via in situ formation of the ethyl ester and subsequent aminolysis by alkanolamines (**a**–**j**) affording the two series of *N*-acylalkanolamines, linolenic acid derivatives **4a**–**j** and arachidonic acid derivatives **5a**–**j**. *Route C* was found to be the most convenient and was selected as the way to obtain all reported products.

Among the enzymes tested, *Candida antarctica* B lipase proved most effective in catalysing the required esterification and aminolysis reactions. Alkanolamides were obtained as the only products, showing the lipase to be highly regioselective under the reaction conditions. Under these conditions twenty products were generated and completely characterized by spectroscopic methods; among the products generated 15 have not been previously described.

In summary, the one-pot/two-step enzymatic reaction disclosed herein offers a good alternative to more classical syntheses of *N*-fatty acylalkanolamines. Although syntheses of this class of compounds are well-known, like many chemical syntheses, they have the disadvantage of using polluting catalysts or reagents such as metals, carbodiimides, strong basic media, etc. The lipase-catalyzed procedure uses ethanol as the reagent for esterification. As additional advantages, the enzymatic reaction is simple, does not require high temperatures, and the products are isolated by simple filtration and solvent evaporation methods. The lipase is biodegradable, and consequently, is more friendly to the environment than are chemical catalysts. In addition, because the enzyme is insoluble in the reaction medium, it is easily removed by filtration and can be reused. In the one-pot/two-step route, CAL B retained 80 % of its original activity after three reaction cycles.

In addition to synthetic efforts, the present report examines, for the first time, the combined effects of AEA and AEA analogues on C6 glioma cell viability. Our data demonstrate that AEA analogues **4g** and **5c** enhance AEA cytotoxicity. This is likely the result of inhibited FAAH activity. It is widely proposed that regulation of the endocannabinoid system is a promising strategy for treating pain, cancer, and other inflammatory-related diseases, pointing to FAAH as an effective drug target. Taking into account that antitumor effects of AEA have been extensively demonstrated,[48] our findings suggest that AEA analogues may serve as important therapeutic tools in cancer treatment. Additionally, given the multiple vital functions in which AEA is involved, applications of the reported AEA analogs to other pathologies clearly warrant consideration. Molecular modelling studies aimed at understanding the interactions between FAAH and AEA analogues will be carried out in a near future. From these studies we aim to elucidate the role of these molecules in modulating FAAH activity.

Experimental Section

General: Chemicals and solvents were purchased from Merck Argentina and Sigma–Aldrich de Argentina and used without further purification. Lipase from *Candida rugosa* (CRL) (905 U/mg solid) was purchased from Sigma Chemical Co.; *Candida antarctica* B lipase (CAL B): Novozym 435 (7400 PLU/g) and Lipozyme RM 1 M (LIP) (7800 U/g) were generous gifts of Novozymes Spain; *Carica papaya* lipase (CPL) is the remaining solid fraction of papaya latex, after washing off of proteases using distilled water. CPL is a naturally immobilized enzyme and was a generous gift of Dr. Georgina Sandoval, CIATEJ, México; heterologous *Rhizopus oryzae* lipase (ROL), immobilized on Octadecyl Sepabeads was a generous gift of Dr.

Francisco Valero, UAB, Spain. ROL was dried overnight in vacuo in a drying oven before use (0.1 kPa, 30 °C), the other four lipases were used "straight from the bottle". Enzymatic reactions were carried out in an Innova 4000 digital incubator shaker (New Brunswick Scientific Co.) at the corresponding temperature and 200 rpm. E/S is given as enzyme amount in mg/substrate amount in mg. To monitor the reaction progress aliquots were withdrawn and analyzed by TLC performed on commercial 0.2 mm aluminum-coated silica gel plates (F254), using EtOAc/hexane, 3:7 as the developing solvent and visualized by 254 nm UV or immersion in an aqueous solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.04 M), $Ce(SO_4)_2$ (0.003 M) in concentrated H_2SO_4 (10 %). The extent of conversion was determined using a Shimadzu HPLC LC-20A Prominence equipped with a vacuum degasser, a binary pump, manual injector and UV detector. The reactions were monitored employing a C-18 Kromasil column 5 μm, 250 × 4.6 mm. Mobile phases were A (methanol) and B (water), both containing 0.1 % of trifluoroacetic acid. LC gradient conditions were as follows: 0–5 min, 80 % A; 5–50 min, 0.44 %/min A to 100 % A; then, the column was brought back to the original ratio of 20 % B and 80 % A to enable equilibration of the column. The flow-rate was 0.27 mL/min and the column was operated at room temperature. Peaks were detected at 254 nm of UV detection. Ester derivatives were determined by gas chromatography on a Thermo Focus GC chromatograph equipped with a flame ionization detector and using an SP-2330 column (30 m × 0.25 mm ID, 0.25 thickness; Supelco, Sigma Aldrich, USA). Nitrogen was the carrier gas. Both injector and detector temperatures were set at 250 and 280 °C, respectively. The column temperature was programmed to ramp from 60 to 160 °C at a rate of 10 °C/min followed by 2 °C/min to 230 °C and then held constant at 230 °C for 5 min. Melting points were measured in a Fisher Johns apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer (Waltham, MA, USA) 343 polarimeter in $CHCl_3$. ^1H NMR and ^{13}C NMR spectra were recorded at room temperature in $CDCl_3$ as solvent using a Bruker AM-500 NMR instrument operating at 500.14 and 125.76 MHz for ^1H and ^{13}C, respectively. The ^1H NMR spectra are referenced with respect to the residual $CHCl_3$ proton of the solvent $CDCl_3$ at δ = 7.26 ppm. Coupling constants are reported in Hertz [Hz]. ^{13}C NMR spectra were fully decoupled and are referenced to the middle peak of the solvent $CDCl_3$ at δ = 77.0 ppm. Splitting patterns are designated as: s, singlet; d, doublet; t, triplet; q, quadruplet; qn, quintet; dd, double doublet, etc. High Resolution Mass Spectrometry was recorded with Thermo Scientific EM/DSQ II–DIP. The results were within ± 0.02 % of the theoretical values.

Synthesis of Ethyl Linolenate (3) (*Route A*): CAL B (400 mg) was added to a solution of **1** (278 mg, 1 mmol) in ethanol (10 mL) or ethanol (0.5 mL, 1.2 mmol) and hexane (10 mL). The suspension was shaken at 200 rpm and 55 °C. Once the reaction was finished (1 h in hexane, 48 h in solvent free system), the enzyme was filtered off. After evaporation of the solvent under reduced pressure, 297 mg (97 %) of an oily residue were obtained. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.96 (t, J = 7.5 Hz, 3 H, 18-H); 1.24 (t, J = 7.2 Hz, 3 H, -OCH_2CH_3); 1.26–1.30 (m, 8 H, 4-H, 5-H, 6-H, 7-H); 1.61 (m, J = 7.0 Hz, 2 H, 3-H); 2.05 (m, 4 H, 8-H, 17-H), 2.27 (t, J = 7.4 Hz, 2 H, 2-H); 2.80 (t, J = 5.2 Hz, 4 H, 11-H, 14-H); 4.11 (q, J = 7.2 Hz, 2 H, -OCH_2CH_3); 5.34 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.2 (C-18), 20.5 (-OCH_2CH_3), 25.0 (C-3), 25.6 (C-11, C-14), 27.2 (C-8), 29.1 (C-4, C-5, C-6, C-7), 29.6 (C-17), 34.3 (C-2), 59.8 (-OCH_2CH_3), 127.1–131.9 (C-9, C-10, C-12, C-13, C-15, C-16), 176.9 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{20}H_{34}NaO_2$ 329.2457; found $C_{20}H_{34}NaO_2$ 329.2461.

Synthesis of *N*-Linolenoylethanolamine (4a) (*Route A*): To a solution of 1 mmol (300 mg) of **3** in hexane (10 mL), CAL B (600 mg) and ethanolamine (75 mg, 1.2 mmol) were added. The suspension was shaken at 200 rpm and 55 °C. Once the reaction was finished (48 h), the enzyme was filtered off and the solvent evaporated under reduced pressure. The residue was purified by column chromatography (silica gel) employing mixtures of hexane/EtOAc as eluent (9:1–3:2), yield 89 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.98 (t, J = 7.5 Hz, 3 H, 18-H); 1.26–1.39 (m, 8 H, 4-H, 5-H, 6-H, 7-H); 1.63 (qn, J = 7.0 Hz, 2 H, 3-H); 2.06–2.10 (m, 4 H, 8-H, 17-H), 2.21 (t, J = 7.5 Hz, 2 H, 2-H); 2.80 (t, J = 5.2 Hz, 4 H, 11-H, 14-H); 3.41 (q, J = 5.0 Hz, 2 H, 1′-H); 3.71 (t, J = 5.0 Hz, 2 H, 2′-H); 5.28–5.41 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H), 6.18 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.3 (C-18), 20.6 (C-17), 25.6 (C-11, C-14), 25.7 (C-3), 27.2 (C-8), 29.2 (C-4, C-5, C-6, C-7), 36.6 (C-2), 42.5 (C-1′) 62.3 (C-2′), 127.0–131.9 (C-9, C-10, C-12, C-13, C-15, C-16), 174.7 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{20}H_{35}NNaO_2$. 344.2566; found $C_{20}H_{35}NNaO_2$. 344.2572.

Synthesis of *N*-Linolenoylethanolamine (4a) (*Route B*): To a solution of linolenic acid (278 mg, 1 mmol) in hexane (10 mL), CAL B lipase (400 mg) and ethanolamine (150 mg, 2 mmol) were added. The mixture was shaken at 200 rpm and 55 °C over the course of 48 h, yield 85 %.

Synthesis of Alkanolamides. General One-Pot/Two-Step Procedure (*Route C*): CAL B (200 mg) was added to a solution of the linolenic acid (1 mmol) in 0.5 mL of ethanol and 5 mL of hexane. The suspension was shaken at 200 rpm at 55 °C and the progress of the reaction was monitored by GC. Once the acid was converted into the ethyl ester, the corresponding amine (1.2 equiv.) was added. Upon completion of the reaction the enzyme was filtered off and the remaining solvent evaporated under reduced pressure. The crude residue was purified by column chromatography over silica gel employing mixtures of hexane/EtOAc as eluent (9:1–3:2), yield 92 %.

For reuse experiments applicable to production of **4a**: the filtered and washed enzyme was used in the next enzymatic one-pot reaction under the same reaction conditions.

***N*-Linolenoylpropanolamine (4b):** Yield 83 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.97 (t, J = 7.5 Hz, 3 H, 18-H), 1.25–1.30 (m, 8 H, 4-H, 5-H, 6-H, 7-H), 1.58–1.60 (m, 4 H, 3-H, 2′-H), 2.05 (m, 4 H, 8-H, 17-H), 2.16 (t, J = 7.6 Hz, 2 H, 2-H), 2.80 (t, J = 5.5 Hz, 4 H, 11-H, 14-H), 3.29 (q, J = 5.6 Hz, 2 H, 1′-H), 3.67 (t, J = 5.6 Hz, 2 H, 3′-H), 5.28–5.41 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H), 5.72 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.3 (C-18), 20.6 (C-17), 25.7–25.8 (C-11, C-14), 25.9 (C-3), 27.3 (C-8), 29.3–29.4 (C-4, C-5, C-6, C-7), 32.6 (C-2′), 36.2 (C-2′), 39.2 (C-2), 62.4 (C-3′), 127.2–132.0 (C-9, C-10, C-12, C-13, C-15, C-16), 173.4 (C-1) ppm. $[M + Na]^+$ Calcd. $C_{21}H_{37}NNaO_2$ 358.2722; found $C_{21}H_{37}NNaO_2$ 358.2729.

***N*-Linolenoylbutanolamine (4c):** Yield 75 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.97 (t, J = 7.6 Hz, 3 H, 18-H), 1.25–1.38 (m, 8 H, 4-H, 5-H, 6-H, 7-H), 1.59–1.65 (m, 6 H, 3-H, 2′-H, 3′-H), 2.02–2.10 (m, 4 H, 8-H, 17-H), 2.15 (t, J = 7.6 Hz, 2 H, 2-H), 2.80 (t, J = 6.2 Hz, 4 H, 11-H, 14-H), 3.29 (q, J = 6.6 Hz, 2 H, 1′-H), 3.67 (t, J = 6.0 Hz, 2 H, 2′-H), 5.28–5.41 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H), 5.72 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.4 (C-18), 20.8 (C-17), 25.7–25.8 (C-11, C-14), 25.9 (C-3), 26.0 (C-2′), 27.3 (C-8), 29.3–29.4 (C-4, C-5, C-6, C-7, C-3′), 37.0 (C-2), 39.3 (C-1′), 62.5 (C-4′), 127.2–132.1 (C-9, C-10, C-12, C-13, C-15, C-16), 173.5 (C-1) ppm. $[M + Na]^+$. Calcd. $C_{22}H_{39}NNaO_2$ 372.2879; found $C_{22}H_{39}NNaO_2$ 372.2868.

***N*-Linolenoylpentanolamine (4d):** Yield 68 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.97 (t, J = 7.5 Hz,

3 H, 18-H), 1.25–1.37 (m, 8 H, 4-H, 5-H, 6-H, 7-H), 1.40 (m, 2 H, 3′-H), 1.50–1.63 (m, 6 H, 3-H, 2′-H, 4′-H), 2.02–2.10 (m, 4 H, 8-H, 17-H), 2.14 (t, J = 7.5 Hz, 2 H, 2-H), 2.80 (t, J = 5.5 Hz, 4 H, 11-H, 14-H), 3.26 (q, J = 7.0 Hz, 2 H, 1′-H), 3.64 (t, J = 6.4 Hz, 2 H, 2′-H), 5.28–5.42 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H), 5.48 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.4(C-18), 20.7 (C-17), 25.7–25.8 (C-11, C-14), 25.9 (C-3), 26.0 (C-2′), 27.3 (C-8), 27.4 (C-3′), 29.3–29.4 (C-4, C-5, C-6, C-7, C-4′), 37.1 (C-2), 39.4 (C-1′), 62.8 (C-5′), 127.2–132.1 (C-9, C-10, C-12, C-13, C-15, C-16), 173.3 (C-1) ppm. [M + Na]$^+$ Calcd. $C_{23}H_{41}NNaO_2$ 386.3035; found $C_{23}H_{41}NNaO_2$ 386.3029.

***N*-Linolenoyl(2-amino-1-propanol) (4e):** Yield 86 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.91 (d, J = 6.8 Hz, 3 H, 3′-H), 0.97 (t, J = 7.5 Hz, 3 H, 18-H), 1.25–1.36 (m, 8 H, 4-H, 5-H, 6-H, 7-H), 1.62 (qn, J = 6.8 Hz, 2 H, 3-H), 2.02–2.10 (m, 4 H, 8-H, 17-H), 2.18 (t, J = 7.6 Hz, 2 H, 2-H), 2.80 (t, J = 5.2 Hz, 4 H, 11-H, 14-H), 3.52 (dd, J_1 = 6.2, J_2 = 11.0 Hz, 1 H, 2′-H), 3.65 (dd, J_1 = 5.0, J_2 = 6.2 Hz, 1 H, 2′-H), 4.09 (m, 1 H, 1′-H), 5.28–5.42 (m, 6 H, 9-H, 10-H, 12-H, 13-H, 15-H, 16-H), 5.62 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.4(C-18), 17.2 (C-3′), 20.7 (C-17), 25.7–25.9 (C-11, C-14), 25.8 (C-3), 27.4 (C-8), 29.3–29.4 (C-4, C-5, C-6, C-7), 36.8 (C-2), 48.0 (C-1′), 67.6 (C-2′), 127.2–132.1 (C-9, C-10, C-12, C-13, C-15, C-16), 174.2 (C-1) ppm. [M + Na]$^+$ Calcd. $C_{21}H_{37}NNaO_2$ 358.2722; found $C_{21}H_{37}NNaO_2$ 358.2718.

***N*-Linolenoyl(1-amino-2-propanol) (4f):** Yield 87 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.97 (t, J = 7.6 Hz, 3 H, 18-H), 1.19 (d, J = 6.3 Hz, 3 H, 3′-H), 1.27–1.37 (m, 8 H, 4-H, 5-H, 6-H, 7-H), 1.63 (qn, J = 7.3 Hz, 2 H, 3-H), 2.02–2.11 (m, 4 H, 8-H, 17-H), 2.20 (t, J = 7.5 Hz, 2 H, 2-H), 2.80 (t, J = 5.4 Hz, 4 H, 11-H, 14-H), 3.12 (m, 1 H, 1′-H), 3.44 (ddt, J_1 = 2.3, J_2 = 6.5 Hz, J_3 = 12.1 Hz, 1 H, 1′-H), 3.92 (m, 1 H, 2′-H), 5.29–5.42 (m, 6 H, 9-H, 10-H, 12-H, 13-H, 15-H, 16-H), 5.92 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.4 (C-18), 21.1 (C-3′), 20.7 (C-17), 25.6–25.7 (C-3, C-11, C-14), 27.3 (C-8), 29.3–29.7 (C-4, C-5, C-6, C-7), 36.9 (C-2), 47.2 (C-1′), 67.5 (C-2′), 127.3–132.1 (C-9, C-10, C-12, C-13, C-15, C-16), 174.8 (C-1) ppm. [M + Na]$^+$ Calcd. $C_{21}H_{37}NNaO_2$ 358.2722; found $C_{21}H_{37}NNaO_2$ 358.2729.

***N*-Linolenoyl(2-methyl-2-amino-1-propanol) (4g):** Yield 63 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.97 (t, J = 7.5 Hz, 3 H, 18-H), 1.25–1.34 (m, 14 H, 4-H, 5-H, 6-H, 7-H, 3′-H, 4′-H), 1.60 (qn, J = 7.3 Hz, 2 H, 3-H), 2.03–2.11 (m, 4 H, 8-H, 17-H), 2.16 (t, J = 7.6 Hz, 2 H, 2-H), 2.80 (t, J = 6.5 Hz, 4 H, 11-H, 14-H), 3.58 (s, 2 H, 2′-H), 5.28–5.42 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H), 5.48 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.3 (C-18), 20.6, (C-17), 24.9 (C-3′, C-4′), 25.7 (C-11, C-14), 25.8 (C-3), 27.2 (C-8), 29.1–29.6 (C-4, C-5, C-6, C-7), 37.4 (C-2), 56.2 (C-1′), 71.0 (C-2′), 127.0–132.1 (C-9, C-10, C-12, C-13, C-15, C-16), 174.3 (C-1) ppm. [M + Na]$^+$ Calcd. $C_{22}H_{39}NNaO_2$ 372.2879; found $C_{22}H_{39}NNaO_2$ 372.2881.

7. *N*-Linolenoyl(2-amino-1-butanol) (4h): Yield 78 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.92 (t, J = 5.0 Hz, 3 H, 4′-H), 0.97 (t, J = 7.4 Hz, 3 H, 18-H), 1.30 (m, 8 H, 4-H, 5-H, 6-H, 7-H), 1.48 (m, 2 H, 3′-H), 1.62 (m, 2 H, 3-H), 2.04–2.09 (m, 4 H, 17-H, 8-H), 2.19 (t, J = 7.5 Hz, 2 H, 2-H), 2.80 (m, 4 H, 11-H, 14-H), 3.55 (dd, J_1 = 5.6, J_2 = 10.9 Hz, 1 H, 2′-H), 3.67 (dd, J_1 = 3.3, J_2 = 10.8 Hz, 2 H, 2′-H), 3.95 (m, 1 H, 2′-H),5.32–5.38 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H), 5.57 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-4′), 14.4 (C-18), 19.5 (C-3′), 20.7 (C-17), 25.5, 25.6 (C-11, C-14), 25.8 (C-3), 27.3 (C-8), 29.2–29.7 (C-4, C-5, C-6, C-7), 37.0 (C-2), 51.8 (C-2′), 66.3 (C-1′), 127.2–132.1 (C-9, C-10, C-12, C-13, C-15, C-16), 174.3 (C-1) ppm. [M + Na]$^+$ Calcd. $C_{22}H_{39}NNaO_2$ 372.2879; found $C_{22}H_{39}NNaO_2$ 372.2874.

***N*-Linolenoyl(3-methyl-2-amino-1-butanol) (4i):** Yield 62 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.87 (t, J = 6.7 Hz, 2 H, 18-H), 0.91 (d, 3 H, 4′-H), 0.96 (d, 3 H, 5′-H), 1.28 (m, 8 H, 4-H, 5-H, 6-H, 7-H), 1.72 (qn, J = 7.2 Hz, 2 H, 3-H), 1.83 (m, 1 H, 3′-H), 2.03 (m, 4 H, 17-H, 8-H), 2.21 (t, J = 7.4 Hz, 2 H, 2-H), 2.80 (m, 4 H, 11-H, 14-H), 3.63 (dd, J_1 = 6.2, J_2 = 5.0 Hz, 1 H, 1′-H), 3.69 (dd, J_1 = 6.0, J_2 = 9.5 Hz, 2 H, 2′-H), 5.34–5.37 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H), 5.57 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-18), 18.9 (C-5′), 19.5 (C-4′), 20.7 (C-17), 25.7–25.9 (C-3, C-11, C-14), 27.3 (C-8), 29.1 (C-3′), 29.3–29.7 (C-4, C-5, C-6, C-7), 36.3 (C-2), 57.2 (C-1′), 64.3 (C-2′), 127.5–130.6 (C-9, C-10, C-12, C-13, C-15, C-16), 174.0 (C-1) ppm. [M + Na]$^+$ Calcd. $C_{23}H_{41}NNaO_2$ 386.3035; found $C_{23}H_{41}NNaO_2$ 386.3040.

***N*-Linolenoyl(2-amino-1-pentanol) (4j):** Yield 77 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.97 (t, J = 7.5 Hz, 3 H, 18-H), 0.93 (t, J = 7.3 Hz, 3 H, 5′-H), 1.30 (m, 8 H, 4-H, 5-H, 6-H, 7-H), 1.43 (m, 4 H, 3′-H, 4′-H), 1.62 (qn, J = 7.0 Hz, 2 H, 3-H), 2.04–2.09 (m, 4 H, 17-H, 8-H), 2.19 (t, J = 7.6 Hz, 2 H, 2-H), 2.80 (m,4 H, 11-H, 14-H), 3.55 (dd, J_1 = 6.2, J_2 = 11.0 Hz, 2 H, 2′-H), 3.69 (dd, J_1 = 3.5, J_2 = 11.0 Hz, 1 H, 2′-H), 3.95 (m, 1 H, 1′-H), 5.29–5.42 (m, 6 H, 9-H, 10-H; 12-H, 13-H, 15-H, 16-H), 5.57 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-5′), 14.4 (C-18), 19.5 (C-4′), 20.7 (C-17), 25.7–25.9 (C-3, C-11, C-14), 27.3 (C-8), 29.3–29.7 (C-4, C-5, C-6, C-7), 33.5 (C-3′), 37.0 (C-2), 51.9 (C-1′), 66.3 (C-2′), 127.2–132.1 (C-9, C-10, C-12, C-13, C-15, C-16), 174.4 (C-1) ppm. [M + Na]$^+$ Calcd. $C_{23}H_{41}NNaO_2$ 386.3035; found $C_{23}H_{41}NNaO_2$ 386.3038.

***N*-Arachidonoylethanolamine (Anandamide, 5a):** Yield 81 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.83 (t, J = 6.7 Hz, 3 H, 20-H), 1.2–1.3 (m, 6 H, 17-H, 18-H, 19-H), 1.58 (br. s, 1 H, O*H*), 1.66 (qn, J = 7.5 Hz, 2 H, 3-H), 2.00 (q, J = 7.2 Hz, 2 H, 16-H), 2.06 (c, J = 7.0 Hz, 2 H, 4-H), 2.16 (t, J = 7.4 Hz, 2 H, 2-H), 2.75–2.78 (m, 6 H, 7-H, 10-H, 13-H); 3.36 (q, J = 5.5 Hz, 2 H, 1′-H); 3.67 (t, J = 5.0 Hz, 2 H, 2′-H), 5.29–5.33 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.81 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-20), 22.6 (C-19), 25.4 (C-10), 25.6 (C-3, C-7, C-13), 26.6 (C-4), 27.2 (C-16); 29.3 (C-17), 31.5 (C-18), 35.9 (C-2), 42.4 (C-1′), 62.5 (C-2′), 127.5–129.0 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.5 (C-5), 174.2 (C-1) ppm. HRMS: [M + Na]$^+$ Calcd. $C_{22}H_{37}NNaO_2$ 370.2722; found $C_{22}H_{37}NNaO_2$ 370.2726.

***N*-Arachidonoylpropanolamine (5b):** Yield 73 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.89 (t, J = 6.7 Hz, 2 H, 20-H), 1.25–1.35 (m, 6 H, 17-H, 18-H, 19-H), 1.60 (br. s, 1 H, O*H*), 1.67 (qn, J = 6.2 Hz, 2 H, 2′-H), 1.73 (qn, J = 7.5 Hz, 2 H, 3-H), 2.05 (q, J = 7.0 Hz, 2 H, 16-H), 2.11 (q, J = 7.0 Hz, 2 H, 4-H), 2.20 (t, J = 7.4 Hz, 2 H, 2-H), 2.80–2.84 (m, 6 H, 7-H, 10-H, 13-H); 3.42 (q, J = 6.0 Hz, 2 H, 1′-H); 3.62 (t, J = 5.8 Hz, 2 H, 3′-H), 5.34–5.41 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.81 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-20), 22.6 (C-19), 25.6 (C-10), 25.7 (C-3, C-7, C-13), 26.6 (C-4), 27.2 (C-16); 29.3 (C-17), 31.5 (C-18), 32.4 (C-2′), 36.0 (C-2), 36.2 (C-1′), 59.2 (C-3′), 127.5–129.0 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.5 (C-5), 174.2 (C-1) ppm. HRMS: [M + Na]$^+$ Calcd. $C_{23}H_{39}NNaO_2$ 384.2879; found $C_{23}H_{39}NNaO_2$ 384.2883.

***N*-Arachidonoylbutanolamine (5c):** Yield 69 % of pure compound as a colorless oil. ^{1}H NMR (500.14 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.6 Hz, 2 H, 20-H), 1.25–1.35 (m, 6 H, 17-H, 18-H, 19-H), 1.59 (m, 4 H, 2′-H, 3′-H), 1.71 (qn, J = 7.5 Hz, 2 H, 3-H), 2.04 (q, J = 6.9 Hz, 2 H, 16-H), 2.10 (q, J = 6.9 Hz, 2 H, 4-H), 2.16 (t, J = 7.3 Hz, 2 H, 2-H), 2.80–2.84 (m, 6 H, 7-H, 10-H, 13-H); 3.28 (q, J = 6.7 Hz, 2 H, 1′-H); 3.67 (t, J = 6.0 Hz, 2 H, 4′-H), 5.34–5.38 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.68 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-20), 22.6 (C-19), 25.6 (C-10), 25.7 (C-3, C-7, C-13), 26.3

(C-2′), 26.7 (C-4), 27.2 (C-16); 29.3 (C-17), 29.7 (C-3′), 31.5 (C-18), 36.2 (C-2), 39.2 (C-1′), 62.4 (C-4′), 127.5–129.1 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.5 (C-5), 173.0 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{24}H_{41}NNaO_2$ 398.3035; found $C_{24}H_{41}NNaO_2$ 398.3038.

***N*-Arachidonoylpentanolamine (5d):** Yield 66 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.89 (t, J = 6.7 Hz, 2 H, 20-H), 1.28–1.40 (m, 8 H, 3′-H, 17-H, 18-H, 19-H), 1.59 (m, 4 H, 2′-H, 4′-H), 1.71 (qn, J = 7.5 Hz, 2 H, 3-H), 2.04 (q, J = 7.3 Hz, 2 H, 16-H), 2.10 (q, J = 7.0 Hz, 2 H, 4-H), 2.16 (t, J = 7.4 Hz, 2 H, 2-H), 2.80–2.84 (m, 6 H, 7-H, 10-H, 13-H); 3.26 (q, J = 7.0 Hz, 2 H, 1′-H); 3.65 (t, J = 6.4 Hz, 2 H, 5′-H), 5.36–5.39 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.45 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-20), 22.6 (C-19), 23.0 (C-2′), 25.6 (C-10), 25.7 (C-3, C-7, C-13), 26.7 (C-4), 27.2 (C-16); 29.3 (C-17), 29.5 (C-3′), 31.5 (C-18), 32.2 (C-4′), 36.2 (C-2), 39.3 (C-1′), 62.6 (C-5′), 127.5–129.0 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.5 (C-5), 172.8 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{25}H_{43}NNaO_2$ 412.3191; found $C_{25}H_{43}NNaO_2$ 412.3196.

***N*-Arachidonoyl(2-amino-1-propanol) (5e):** Yield 78 % of pure compound as a colorless oil. IR. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.89 (t, J = 6.7 Hz, 3 H, 20-H), 1.18 (d, J = 6.9 Hz, 3 H, 3′-H), 1.29–1.35 (m, 6 H, 17-H, 18-H, 19-H), 1.74 (qn, J = 7.5 Hz, 2 H, 3-H), 2.05 (q, J = 7.5 Hz, 2 H, 16-H), 2.11 (q, J = 7.0 Hz, 2 H, 4-H), 2.22 (t, J = 7.4 Hz, 2 H, 2-H), 2.80–2.83 (m, 6 H, 7-H, 10-H, 13-H); 3.53 (dd, J_1 = 6.2, J_2 = 4.8 Hz, 1 H, 2′-H); 3.67 (dd, J_1 = 9.0, J_2 = 6.2 Hz, 1 H, 2′-H), 4.07 (m, 1 H, 1′-H), 5.36–5.39 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.61 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-20), 17.1 (C-3′), 22.5 (C-19), 25.6 (C-3, C-7, C-10, C-13), 26.6 (C-4), 27.2 (C-16); 29.3 (C-17), 31.5 (C-18), 36.1 (C-2), 47.8 (C-1′), 67.4 (C-2′), 127.5–129.0 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.6 (C-5), 174.1 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{23}H_{39}NNaO_2$ 384.2879; found $C_{23}H_{39}NNaO_2$ 384.2881.

***N*-Arachidonoyl(1-amino-2-propanol) (5f):** Yield 80 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.7 Hz, 3 H, 20-H), 1.18 (d, J = 6.2 Hz, 3 H, 3′-H), 1.29–1.35 (m, 7 H, 17-H, 18-H, 19-H, O*H*), 1.73 (qn, J = 7.6 Hz, 2 H, 3-H), 2.05 (q, J = 7.1 Hz, 2 H, 16-H), 2.11 (q, J = 6.9 Hz, 2 H, 4-H), 2.21 (t, J = 7.5 Hz, 2 H, 2-H), 2.80–2.83 (m, 6 H, 7-H, 10-H, 13-H); 3.11 (m, 1 H, 1′-H), 3.42 (ddd, J_1 = 2.9, J_2 = 6.6 Hz, J_3 = 13.8 Hz, 1 H, 1′-H); 3.89–3.93 (m, 1 H, 2′-H), 5.36–5.39 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.91 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-20), 21.0 (C-3′), 22.5 (C-19), 25.6 (C-3, C-7, C-10, C-13), 26.7 (C-4), 27.2 (C-16); 29.3 (C-17), 31.5 (C-18), 36.0 (C-2), 47.0 (C-1′), 67.6 (C-2′), 127.5–129.0 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.5 (C-5), 174.0 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{23}H_{39}NNaO_2$ 384.2879; found $C_{23}H_{39}NNaO_2$ 384.2885.

***N*-Arachidonoyl(2-methyl-2-amino-1-propanol) (5g):** Yield 60 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.89 (t, J = 6.7 Hz, 2 H, 20-H), 1.28 (s, 6 H, 3′-H, 4′-H), 1.28–1.30 (m, 6 H, 17-H, 18-H, 19-H), 1.61 (br. s, 1 H, O*H*), 1.70 (qn, J = 7.5 Hz, 2 H, 3-H), 2.05 (q, J = 6.9 Hz, 2 H, 16-H), 2.11 (q, J = 6.9 Hz, 2 H, 4-H), 2.16 (t, J = 7.1 Hz, 2 H, 2-H), 2.79–2.84 (m, 6 H, 7-H, 10-H, 13-H); 3.58 (s, 2 H, 2′-H); 5.36–5.39 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.48 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-20), 22.6 (C-19), 24.9 (C-3′, C-4′), 25.5 (C-10), 25.6 (C-3, C-7, C-13), 26.5 (C-4), 27.2 (C-16); 29.2 (C-17), 31.5 (C-18), 36.6 (C-2), 56.3 (C-1′), 70.9 (C-2′), 127.5–128.9 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.6 (C-5), 173.9 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{24}H_{41}NNaO_2$ 398.3035; found $C_{24}H_{41}NNaO_2$ 398.3040.

***N*-Arachidonoyl(2-amino-1-butanol) (5h):** Yield 68 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.89 (t, J = 6.7 Hz, 3 H, 4′-H), 0.95 (t, J = 6.9 Hz, 3 H, 20-H), 1.29–1.32 (m, 6 H, 17-H, 18-H, 19-H), 1.49 (m, 1 H, 3′-H), 1.60 (m, 1 H, 3′-H), 1.73 (qn, J = 7.5 Hz, 2 H, 3-H), 2.05 (q, J = 7.5 Hz, 2 H, 16-H), 2.12 (q, J = 7.0 Hz, 2 H, 4-H), 2.22 (t, J = 7.4 Hz, 2 H, 2-H), 2.80–2.84 (m, 6 H, 7-H, 10-H, 13-H); 3.59 (dd, J_1 = 6.2, J_2 = 4.8 Hz, 1 H, 2′-H); 3.69 (dd, J_1 = 9.0, J_2 = 6.2 Hz, 1 H, 2′-H), 3.89 (m, 1 H, 1′-H), 5.36–5.39 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.57 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 10.6 (C-4′), 14.1 (C-20), 22.6 (C-19), 24.2 (C-3′), 25.6 (C-3, C-7, C-10, C-13), 26.6 (C-4), 27.2 (C-16); 29.3 (C-17), 31.5 (C-18), 36.2 (C-2), 53.4 (C-1′), 65.6 (C-2′), 127.5–129.0 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.5 (C-5), 173.9 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{24}H_{41}NNaO_2$ 398.3035; found $C_{24}H_{41}NNaO_2$ 398.3039.

***N*-Arachidonoyl (3-Methyl-2-amino-1-butanol) (5i):** Yield 63 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.87 (t, J = 6.7 Hz, 2 H, 20-H), 0.91 (d, 3 H, 4′-H), 0.96 (d, 3 H, 5′-H), 1.26–1.36 (m, 6 H, 17-H, 18-H, 19-H), 1.63 (br. s, 1 H, O*H*), 1.74 (qn, J = 7.5 Hz, 2 H, 3-H), 1.86 (m, 1 H, 3′-H), 2.05 (q, J = 7.5 Hz, 2 H, 16-H), 2.12 (q, J = 7.0 Hz, 2 H, 4-H), 2.23 (t, J = 7.4 Hz, 2 H, 2-H), 2.80–2.84 (m, 6 H, 7-H, 10-H, 13-H); 3.65 (dd, J_1 = 6.2, J_2 = 4.8 Hz, 1 H, 1′-H); 3.71 (dd, J_1 = 9.0, J_2 = 6.2 Hz, 2 H, 2′-H), 5.36–5.39 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.60 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 14.1 (C-20), 18.9 (C-5′), 19.5 (C-4′), 22.6 (C-19), 25.6 (C-3, C-7, C-10, C-13), 26.7 (C-4), 27.2 (C-16); 29.0 (C-3′), 29.3 (C-17), 31.5 (C-18), 36.3 (C-2), 57.2 (C-1′), 64.3 (C-2′), 127.5–129.0 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.5 (C-5), 174.0 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{25}H_{43}NNaO_2$ 412.3191; found $C_{25}H_{43}NNaO_2$ 412.3195.

***N*-Arachidonoyl(2-amino-1-pentanol) (5j):** Yield 75 % of pure compound as a colorless oil. ^1H NMR (500.14 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.8 Hz, 3 H, 5′-H), 0.93 (t, J = 7.0 Hz, 3 H, 20-H), 1.31–1.37 (m, 8 H, 3-H, 17-H, 18-H, 19-H), 1.49 (m, 2 H, 4′-H), 1.74 (qn, J = 7.5 Hz, 2 H, 3′-H), 2.05 (q, J = 6.8 Hz, 2 H, 16-H), 2.11 (q, J = 6.5 Hz, 2 H, 4-H), 2.21 (t, J = 7.4 Hz, 2 H, 2-H), 2.81–2.84 (m, 6 H, 7-H, 10-H, 13-H); 3.56 (dd, J_1 = 6.0, J_2 = 11.0 Hz, 2 H, 2′-H); 3.69 (dd, J_1 = 3.5, J_2 = 11.0 Hz, 1 H, 2′-H), 3.96 (m, 1 H, 1′-H), 5.36–5.39 (m, 8 H, 5-H, 6-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H), 5.54 (br. s, 1 H, N*H*) ppm. ^{13}C NMR (125.76 MHz, $CDCl_3$): δ = 13.9 (C-5′), 14.1 (C-20), 19.3 (C-4′), 22.6 (C-19), 25.6 (C-3, C-7, C-10, C-13), 26.6 (C-4), 27.2 (C-16); 29.3 (C-17), 31.5 (C-18), 33.3 (C-3′), 36.2 (C-2), 51.7 (C-1′), 66.1 (C-2′), 127.5–129.0 (C-6, C-8, C-9, C-11, C-12, C-14, C-15), 130.5 (C-5), 173.8 (C-1) ppm. HRMS: $[M + Na]^+$ Calcd. $C_{25}H_{43}NNaO_2$ 412.3191; found $C_{25}H_{43}NNaO_2$ 412.3187.

Drug Screening

Cell Culture and Treatments: Rat glioma C6 cell line (ATCC CCL-107), originally derived from an N-nitrosomethylurea-induced rat brain tumor,[49] was kindly provided by Dr. Zvi Vogel (Weizmann Institute of Science, Rehovot, Israel). C6 cells were maintained in DMEM (Sigma–Aldrich Co., St. Louis, MO, USA) supplemented with 10 % heat-inactivated FBS (Natocor, Córdoba, Argentina), 2.0 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin B (Richet, Buenos Aires, Argentina). Cells were cultured at 37 °C in a humidified atmosphere of 5 % CO_2-95 % air, and the medium was renewed three times a week. For experiments, C6 cells were removed with 0.25 % trypsin-EDTA (Sigma–Aldrich Co.), diluted with DMEM 10 % FBS and re-plated into 96-well plates (1.5×10^4 cells/well). After 24 h in culture, cells reaching ≈ 70–80 % confluence were exposed to AEA and its analogues for 24 h in 2 % FBS containing media. Images were obtained employing an Olympus IX71 inverted microscope.

MTT Reduction Assay: This assay was carried out to evaluate cell viability according to the protocol previously described[50] with

slight modifications.[51] After exposure, cells grown on 96-well plates were washed with Phosphate Buffered Saline (PBS) and incubated with MTT (Sigma–Aldrich Co.) (0.125 mg/mL) in culture media for 90 min at 37 °C. The formazan product was then solubilized in 200 µL of DMSO (Biopack, Buenos Aires, Argentina). Absorbance was measured at 570 nm with background subtraction at 655 nm in a BIO-RAD Model 680 Benchmark microplate reader (BIO-RAD laboratories, Hercules, CA, USA). Results are expressed as mean ± standard error of the mean. Experimental comparisons between treatments were made by one-way ANOVA, followed by Student-Newman-Keuls post hoc test with statistical significance set at $p < 0.05$.

Supporting Information (see footnote on the first page of this article): Spectral data for compounds **3**, **4a–j**, **5a–j**, associated with this article are supplied.

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