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Improved enzymatic procedure for the synthesis of anandamide and *N*-fatty acylalkanolamine analogues and combination strategy in antitumor activity

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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 yield and a highly chemoselective way. 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.). In order to get the best enzymatic way to synthesize the alkanolamides we evaluated the enzyme performance through three procedures: aminolysis of ethyl ester, direct condensation between the fatty acid and the alkanolamine and a one-pot two-steps conversion of the fatty acids into alkanolamides via *in situ* formation of the ethyl ester and subsequent aminolysis by the alkanolamine. The advantages showed by the enzymatic methodology, such as mild reaction conditions and low environmental impact, make the biocatalysis a convenient way to prepare the reported compounds. The cytotoxic activity of all compounds and mixtures of anandamide and its analogues was evaluated in rat glioma C6 cells. These studies demonstrated that some anandamide analogues are capable to enhance the antitumor effect 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 of neurotransmitters, AEA is an uncharged lipid capable of traversing the bilayer unaided.[6] Accordingly, several studies concluded 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 the immune response and reproduction.

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Moreover, they have been recognized as key mediators of several aspects of human pathophysiology and thus emerged to be among the most widespread and versatile signaling molecules ever discovered.[10, 11] One attractive strategy to elicit the desirable effects of cannabinoid activation, while 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 the endogenous concentrations of all of its substrates enhancing their biological effects. Thus, acting preferentially on active pathways, it might be expected to have a reduced risk of the psychotropic effects[12] In this concern, 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 on the polar head regarding FAAH activity.[13-15] A recent report, based on molecular modeling studies, shows that FAAH selectively accommodates anandamide into a multipocket binding site and confirms that the rate for substrate hydrolysis increase with the number of double bonds of the substrate lipid chain.[16]

Keeping in mind the above data we based our investigations on template of linolenoyl and arachidonoyl derivatives. 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**) through an enzymatic approach (Figure 1).

Biocatalysis proved to be a good alternative to the synthesis of organic compounds through a Green Chemistry approach. Pure enzymes and whole cells of microorganisms show interesting advantages, such as biodegradability and the possibility of working under mild reaction conditions.[17]

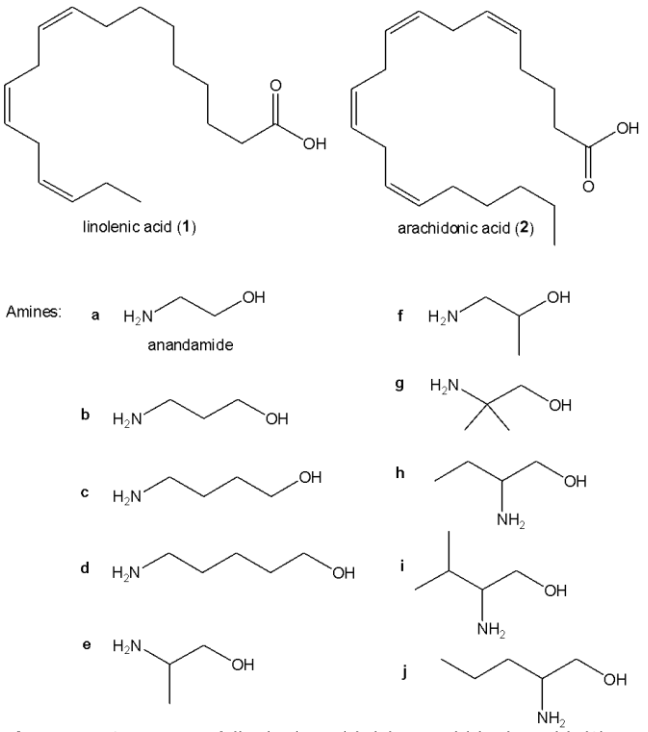


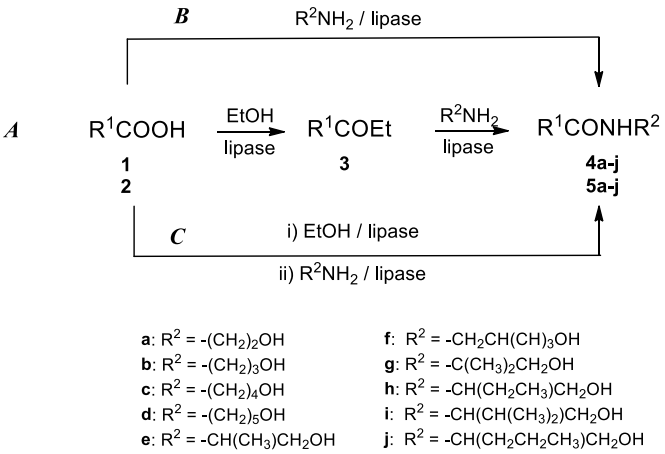
Figure 1. Structure of linolenic acid (**1**), arachidonic acid (**2**) and alkanolamines (**a**-**j**).

Moreover, they are capable of accepting a wide array of substrates and catalyze reactions in a chemo- and regioselelective way, carrying out different chemical transformations without the need for tedious protection and deprotection steps in compounds with several functional groups.[18] Over the last years, biocatalysis using lipases in non-aqueous media has been widely used in synthesis of pharmaceuticals catalyzing several synthetic reactions such as esterification, transesterification, aminolysis, polymerization, etc.[19-22] Enzymes are also well-known by their high enantioselective behavior and this property formed the basis for their widespread use for 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]
Finally, the effect of anandamide and the obtained analogues on glioma C6 cell viability is also reported.

Results and Discussion

1. Enzymatic Synthesis

The most often described method for the synthesis of anandamide and its analogues is by reaction of a fatty acyl donor and the alkanolamine with catalysts such as sodium methoxide or 1-propylphosphonic acid cyclic anhydride or in the absence of the catalyst at temperatures about 180 °C.[25, 26] Fatty acyl donors include fatty acid chloride, free fatty acid and fatty acid methyl ester.[27] Another synthetic route is 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 including $Mg(OCH_3)_2$ and $CaCl_2$, sodium cyanide, metal catalysts. etc.[29]
All these methods are well established but they are faced with several limitations. Fastidious steps of alcohol group protection and deprotection for the control of chemo- and stereoselectivity are necessary to perform. The high temperatures often required for chemical synthesis also preclude the use of fragile molecules and may affect the color, odor and purity of final products. In addition, the coproduction of salts and the use of toxic reagents, that must be eliminated at the end of the reaction, increase the cost of the processes. Biocatalysis allows the use of esters or the direct use of carboxylic acid to obtain amides, which is advantageous from economic and environmental viewpoint.[30]
Regarding the biocatalytic approach, at this moment little has been reported about the enzymatic synthesis of endocannabinoids. Under various reaction conditions and using *Candida antarctica lipase* B immobilized on an acrylic resin, the preparation of some unsaturated fatty acylethanolamines was reported.[31]
In the present work, the synthesis of *N*-fatty acylalkanolamines was studied applying three different strategies in the enzymatic procedure (Scheme 1). The first approach (*A*) consists in the aminolysis of ethyl linolenate (**3**) (previously prepared enzymatically) by ethanolamine to afford *N*-linolenoylethanolamine **4a**. In the second approach *B* the product **4a** is obtained by direct condensation between linolenic acid and ethanolamine. Finally the third route *C* describes a one-pot two-steps procedure, previously reported in our laboratory,[32] which involves the fatty acid conversion to alkanolamides via *in situ* formation of the ethyl ester and subsequent aminolysis by the alkanolamines (**a**-**j**) affording two series of *N*-acylalkanolamines: linolenic acid derivatives **4a**-**j** and arachidonic acid derivatives **5a**-**j** (Scheme 1).



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

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

Route A
At the beginning of route *A*, ethyl linolenate **3** was prepared through esterification of linolenic acid with ethanol catalyzed by lipases. 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 used the lipase in the presence of activated molecular sieves in a reflux trap.[34]
In the present work, five lipases from several sources were applied: from the yeasts *Candida rugosa* (CRL) and *Candida antarctica* B (CAL B); Lipozyme from the fungus: *Rhizomucor miehei* (LIP) and the heterologous *Rhizopus oryzae* lipase (ROL) and from plants: the naturally immobilized *Carica papaya* lipase (CPL), which is the remaining solid fraction of papaya latex after wash off of proteases.
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**

Entry	Enzyme	Temp (°C)	Products coversion (%)[a] **3**[b] *A*	**4a**[c] *A*	**4a**[d] *B*	**4a**[e] *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.

E/S: 5; solvent: hexane; time: 48 h.
[a]Determined by GC or HPLC analysis.
[b]Ethanol/**1**: 1.2.
[c]Route *A*: ester aminolysis. Ethanolamine/ **3**: 1,2.
[d]Route *B*: direct condensation. Ethanolamine/ **1**: 2.
[e]Route *C*: one-pot two steps. Ethanol/**1**: 1.2; ethanolamine/ **3**: 1,2.

Among the five lipases tested in the esterification, CAL B gave the most satisfactory results at 55 °C. As it can be seen in Table 1, an increase in temperature improved the results in some lipases, showing CAL B the highest reactivity towards the esterification reaction. Thus CAL B was the enzyme of choice. Without enzymes, linolenic acid did not react at all.
Using CAL B as biocatalyst, the esterification was tested with ethanol as esterifying agent and solvent (solvent free system) and ethanol with hexane as solvent. The product was obtained at maximum conversion under both reaction conditions. In hexane this result was achieved in one hour and the solvent free system required a longer period of time of almost two days.
Regarding the influence of the enzyme: substrate ratio, we performed experiments varying the ratio from 0.1 to 5 (Table 2, column 3), and we observed that 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 E/S ratio of 1.

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

Entry	E / S	Products coversion (%)[a] **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

CAL B; 55 °C; solvent: hexane, time: 1h (**3**), 48 h (**4a**)
[a]Determined by GC and HPLC analysis.
[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. Working 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 days.
The product was isolated (yield 89%) and identified by spectroscopic methods as *N*-linolenoylethanolamine (**4a**). An important issue was chemoselectivity, since, under chemical conditions, alkanolamines are susceptible for acylation both at the amine and alcoholic group. Our results are in accordance with several studies which report that the lipase acts in a chemoselective manner, exclusively producing the amide.[32] In this case **4a** was obtained and the isomeric aminoester 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 equal to 1 and at 0.5 M ester concentration.
Considering that both substrate (ethyl linolenate) and nucleophile (ethanolamine) exhibit different polarity, we examined the aminolysis in various organic solvents (solvent free, hexane, diisopropyl ether and acetonitrile). Among them, the best results were obtained using hexane. In this solvent, ethyl linolenate was easily soluble and the system remained homogeneous even after the addition of ethanolamine. On the contrary, some precipitation was observed when ethanolamine was added to a solution of the fatty acid in a polar solvent such as acetonitrile. Finally, the reaction carried out in a solvent free system afforded a mixture of products accordingly with previous reports on related compounds.[31]

Route B
This approach consists of the direct condensation reaction between ethanolamine and linolenic acid.[31] We also studied the behavior of various lipases and some reaction parameters for this case. As it can be observed in Table 1 CAL B and hexane were the enzyme and solvent of choice. The reaction was performed at 55 °C, using an E/S = 2 (Table 2, entry 5) and 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 in the two routes. The yield in **4a** remarkably increased when the enzyme was washed several times with hexane.

Route C
Taking into account our previous work,[32] in route *C* we tried a one-pot two-steps 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 as solvent at 55 °C (as described in the first step of route *A*). After one hour of reaction, when all acid was transformed into ester, ethanolamine was added. The global yield for **4a**, applying both enzymatic steps together, was 92%. It could be observed that route *C* is more convenient than route *A*. This latter involves the isolation of **3** and the subsequent aminolysis, requiring more work to achieve the product in lower yield (97 x 89 = 86%).
In summary, route *C* showed to be the best 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 the free fatty acid. The one-pot two-steps procedure described in route *C* allows the aminolysis without the isolation of ethyl linolenate, which is obtained in quantitave yield through enzymatic catalysis. Moreover, the use of linolenic acid as starting material is an additional advantage: it is more economic than ethyl linolenate (almost half the price). Regarding toxicity linolenic acid is non toxic, and known as an essential fatty acid acquired through diet. Although linolenic acid ethyl ester is not highly toxic, its manipulation may cause irritation of the skin.[35] The enzyme recycling is an additional advantage. As the immobilized lipase is insoluble in the reaction media, it is easily removed by filtration at the end of the process. It can be re-used and in this particular reaction, CAL B kept almost 80% of activity after three reaction cycles (Figure 2).

Application of the one-pot two-steps procedure to the synthesis of N-linolenoyl and N-arachidonoyl alkanolamines.

Once the experimental conditions were optimized, we decided to apply the route *C* to the synthesis of anandamide (**5a**) and analogues (**4b**-**j** and **5b**-**j**). The results, expressed as yield 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.

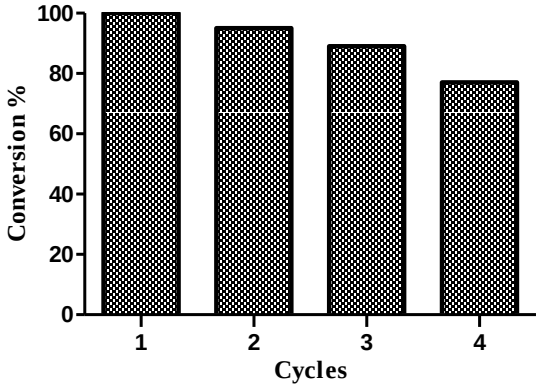


Figure 2. CAL B reuse in the synthesis of *N*-linolenoylethanolamine under standard conditions.

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

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

Standard conditions in route *C*.

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

It can be seen that the best yields were obtained with linear alkanolamines and linolenic acid (Table 3, entries 1-4), particularly ethanolamine (**a**) was found to be the best nucleophile for the reaction. It was observed a decrease in yield with the increase in insaturation in the fatty acid and the chain length in the alkanolamine. In every case linolenic acid derivatives were obtained in higher yield than those corresponding to arachidononic acid.

Regarding the branched alkanolamines derivatives (**4e**-**j** and **5e**-**j**), some differences in product yield were observed among the different examples studied, ranging from 60 to 87% (Table 3, entries 5-10). These nucleophiles have another polar group (hydroxyl) in β-position of the amino group which favors the *N*-acylation reaction.[36] The alkanolamines with two methyl groups (**4g** and **5g**) and isopropyl group (**4i** and **5i**) as substituent afforded the products in the lowest yield (Table 3, entries 7 y 9). These results were similar for both fatty acids and could be attributed to some steric hindrance in the alkanolamines. By 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 were obtained (Table 3, entries 4 and 5). These results could indicate that the position (1 or 2) of the alcohol and amine functions has no impact on the activity of the lipase.

At our best understanding, 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**, **5f** and **5g** have been already described although they have been synthesized through chemical methods involving oxalyl chloride.[37]

The possible stereoselective behavior of CAL B in the case of branched alkanolamines was studied through the determination of the optical rotation of the products. Unfortunately the results indicated that the lipase was not stereoselective in the acylation reaction.

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 a wide range of animal models.[39,40] Particularly, 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 could 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 could be caused by binding of AEA to serum proteins such as albumin. To circumvent this effect we conducted our experiments using 2% FBS.

First, we investigated the capability of enzymatically synthetized AEA and some analogues (**4a**, **4c**, **4g**, **4i**, **5b**, **5c**, **5d**, **5g** and **5 i**) to induce cell death.

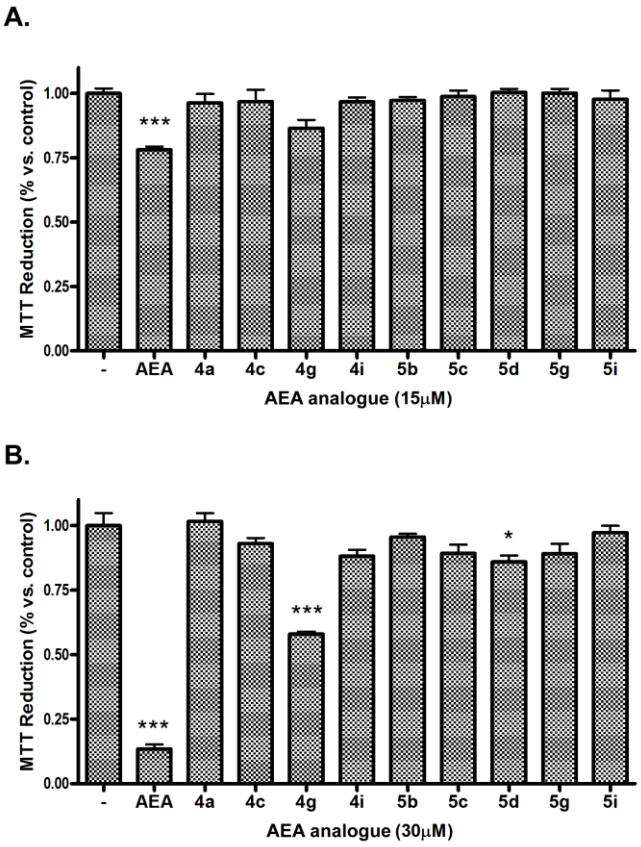


Figure 3. Cell viability in C6 glioma cells after exposure to AEA and its analogues. Cells were exposed to **(A)** 15μM and **(B)** 30μM of AEA and its analogues 4a, 4c, 4g, 4i, 5b, 5c, 5d, 5g, 5i for 24h in 2% FBS. Cell viability was assayed by MTT reduction. *p<0.05, ***p<0.001 vs. control.

For this purpose, cells were exposed to different concentrations of these compounds for 24h in 2% FBS containing media. The cell viability was evaluated employing the MTT assay (Figure 3). The results showed that at 15µM only AEA induced a decrease in cell viability (22±3% p<0.001) (Figure 3A). On the other hand, exposure of cells to 30µM 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 3B).
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 (15µM) and AEA (15µM) during 24h was evaluated (Figure 4).

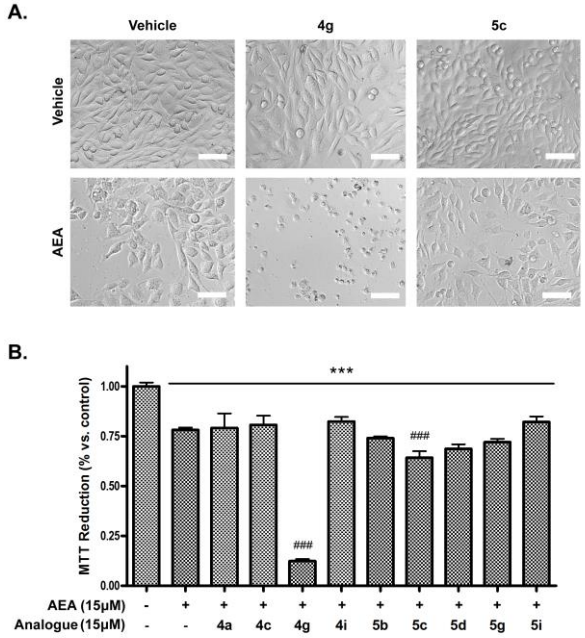


Figure 4. Products 4g and 5c increase AEA- induced cell death. C6 cells were exposed to a mixture of AEA (15µM) and its analogues **4a**, **4c**, **4g**, **4i**, **5b**, **5c**, **5d**, **5g**, **5i** (15µM) for 24h in 2% FBS. **A.** Representative phase contrast images of the combined effects of AEA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

Compounds **4g** and **5c** significantly augmented AEA-induced cell death observed by both phase contrast microscopy (Figure 4A) and MTT assay (66±2% and 15±4%, respectively; p<0.001) (Figure 4B). The fact that compound **4g** has exhibited the most efficient effect is in accordance with the rate for substrate hydrolysis increasing with the number of double bonds of the substrate lipid chain, as observed in previously published experimental data.[47] These findings support our premise that AEA analogues could increase AEA effect probably by increasing its availability through the competition for 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 increasing carbon number (**5b**: 3 methylene groups and **5d**: 5 methylene groups). The comparison of the data given by **4g** and **5g** shows the effect of length and unsaturation in the acyl group of the alkanolamide, because both have the same alkanolamine moiety, being **4g** a linolenic derivative and **5g** an arachidonic one. Regarding **5i**, which resulted ineffective, it could be possible that the combination of an arachidonoyl acyl chain and the isopropyl next to the amino group in the alkanolamide could generate steric hindrance for the interaction with FAAH.

3. Conclusions

This work describes the application of lipases to the synthesis of anandamide and two series of fatty acid derivatives with various alkanolamines and the biological studies carried out to evaluate their antitumoral activity.
The enzymatic approach used in the synthesis was performed applying three different strategies to a model reaction between linolenic acid and ethanolamine: *A*: aminolysis of ethyl linolenate (**3**) prepared enzymatically, by ethanolamine; *B*: direct condensation between linolenic acid and ethanolamine and *C*: one-pot two-steps procedure which involves the fatty acid conversion to alkanolamides via *in situ* formation of the ethyl ester and subsequent aminolysis by the alkanolamines (**a**-**j**) affording the two series of *N*-acylalkanolamines: linolenic acid derivatives **4a**-**j** and arachidonic acid derivatives **5a**-**j**. Route C was the most convenient and was selected as the way to obtain all the reported products.
Among the tested enzymes, *Candida antarctica* B lipase gave the best results in esterification and aminolysis reactions. Alkanolamides were obtained as the only products, showing a high regioselective behavior of the lipase under the reaction conditions and affording twenty products which were completely characterized by spectroscopic methods. Among them, fifteen were not described previously.
In summary, the one-pot two steps enzymatic reaction offers a good alternative to synthesize *N*-fatty acylalkanolamines. Although the synthesis of this class of compounds performed by chemical methods is well-known, it has the disadvantage of using polluting catalysts or reagents such as metals, carbodiimides, strong basic media, etc. The lipase-catalyzed procedure uses ethanol as reagent for esterification. As additional advantages, the enzymatic reaction is simple, it is performed at low temperature, and the products are isolated by simple filtration and solvent evaporation. The lipase is biodegradable and, consequently, more friendly to the environment than 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 steps route, CAL B retained 80 % activity after five reaction cycles.
On the other hand, the present report examines for the first time the combined effect of AEA and AEA analogues on C6 glioma cell viability. Our data demonstrate that AEA analogues **4g** and **5c** are capable to enhance the AEA cytotoxicity. This effect is probably achieved by inhibiting 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 effective drug target. Taking into account that the antitumor effect of AEA was extensively demonstrated,[48] our finding point out a possible role of AEA analogues as therapeutic tools in cancer treatment. In addition, considering the multiple vital functions in which AEA is involved, applications in other pathologies would not be discarded. Molecular modeling studies that will help to shed light about the interactions between FAAH and AEA analogues will be carried out in a near future. It is possible that they will allow elucidating the role of these molecules in the 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* lipase B (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 wash 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 vacuum drying oven before use (0.1 kPa, 30 °C), the other four lipases were used "straight from the bottle". Enzymatic reactions were carried out on 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 developing solvent and visualized by 254 nm UV or immersion in an aqueous solution of $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.04 M), $Ce(SO_4)_2$ (0.003 M) in concentrated H_2SO_4 (10%). % Conversion was determined using 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 led 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 a using SP-2330 column (30 m x 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. Column temperature was programmed from 60 to 160 °C at a rate of 10 °C /min followed by 2 °C/min to 230 °C and stable 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 $CHCl_3$. ^{1}H 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 ^{1}H and ^{13}C, respectively. The ^{1}H 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 linolenic acid ethyl ester (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. ^{1}H NMR ($CDCl_3$) δ 0.96 (t, *J* = 7.5 Hz, 3H, H-18); 1.24 (t, *J* = 7.2 Hz, 3H, -OCH_2CH_3); 1.26-1.30 (m, 8H, H-4, H-5, H-6, H-7); 1.61 (m, *J* = 7.0 Hz, 2H, H-3); 2.05 (m, 4H, H-8, H-17), 2.27 (t, *J* = 7.4 Hz, 2H, H-2); 2.80 (t, *J* = 5.2 Hz, 4H, H-11, H-14); 4.11 (q, *J* = 7.2 Hz, 2H, -OCH_2CH_3); 5.34 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16); ^{13}C NMR ($CDCl_3$) 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); 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. ^{1}H NMR ($CDCl_3$) δ 0.98 (t, *J* = 7.5 Hz, 3H, H-18); 1.26-1.39 (m, 8H, H-4, H-5, H-6, H-7); 1.63 (qn, *J* = 7.0 Hz, 2H, H-3); 2.06-2.10 (m, 4H, H-8, H-17), 2.21 (t, *J* = 7.5 Hz, 2H, H-2); 2.80 (t, *J* = 5.2 Hz, 4H, H-11, H-14); 3.41 (q, *J* = 5.0 Hz, 2H, H-1'); 3.71 (t, *J* = 5.0 Hz, 2H, H-2'); 5.28-5.41 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16), 6.18 (bs, 1H, N*H*) ^{13}C NMR ($CDCl_3$) 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); 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 during 48 h. Yield: 85%.

Synthesis of Alkanolamides. General one-pot two-steps 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. When the acid was converted into the ethyl ester, the corresponding amine (1.2 eq) was added. Once the reaction was finished the enzyme was filtered off, and the solvent evaporated under reduced pressure. The crude residue purified by column chromatography on silica gel employing mixtures of hexane-EtOAc as eluent (9:1 - 3:2). Yield : 92%.
Reuse experiments in the synthesis of **4a**: the filtered and washed enzyme was used in the next enzymatic one-pot reaction under the same reaction conditions.

1. *N*-linolenoylpropanolamine (4b). Yield 83% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, *J* = 7.5 Hz, 3H, H-18), 1.25-1.30 (m, 8H, H-4, H-5, H-6, H-7), 1.58-1.60 (m, 4H, H-3, H-2'), 2.05 (m, 4H, H-8, H-17), 2.16 (t, *J* = 7.6 Hz, 2H, H-2), 2.80 (t, *J* = 5.5 Hz, 4H, H-11, H-14), 3.29 (q, *J* = 5.6 Hz, 2 H, H-1´), 3.67 (t, *J* = 5.6 Hz, 2H, H-3'), 5.28-5.41 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16), 5.72 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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); $[M + Na]^+$ Calcd. $C_{21}H_{37}NNaO_2$ 358.2722. Found $C_{21}H_{37}NNaO_2$ 358.2729.

2. *N*-linolenoylbutanolamine (4c). Yield 75% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, *J* = 7.6 Hz, 3H, H-18), 1.25-1.38 (m, 8H, H-4, H-5, H-6, H-7), 1.59-1.65 (m, 6H, H-3, H-2', H-3'), 2.02-2.10 (m, 4H, H-8, H-17), 2.15 (t, *J* = 7.6 Hz, 2H, H-2), 2.80 (t, *J* = 6.2 Hz, 4H, H-11, H-14), 3.29 (q, *J* = 6.6 Hz, 2 H, H-1´), 3.67 (t, *J* = 6.0 Hz, 2H, H-2'), 5.28-5.41 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16), 5.72 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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); $[M + Na]^+$ Calcd. $C_{22}H_{39}NNaO_2$ 372.2879. Found $C_{22}H_{39}NNaO_2$ 372.2868.

3. *N*-linolenoylpentanolamine (4d). Yield 68% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, *J* = 7.5 Hz, 3H, H-18), 1.25-1.37 (m, 8H, H-4, H-5, H-6, H-7), 1.40 (m, 2H, H-3'), 1.50-1,63 (m, 6H, H-3, H-2', H-4'), 2.02-2.10 (m, 4H, H-8, H-17), 2.14 (t, *J* = 7.5 Hz, 2H, H-2), 2.80 (t, *J* = 5.5 Hz, 4H, H-11, H-14), 3.26 (q, *J* = 7.0 Hz, 2 H, H-1´), 3.64 (t, *J* = 6.4 Hz, 2H, H-2'), 5.28-5.42 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16),

5.48 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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); $[M + Na]^+$ Calcd. $C_{23}H_{41}NNaO_2$ 386.3035. Found $C_{23}H_{41}NNaO_2$ 386.3029.

4. *N*-linolenoyl(2-amino-1-propanol) (4e). Yield 86% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.91 (d, *J* = 6.8 Hz, 3H, H-3'), 0.97 (t, *J* = 7.5 Hz, 3H, H-18), 1.25-1.36 (m, 8H, H-4, H-5, H-6, H-7), 1.62 (qn, *J* = 6.8 Hz, 2H, H-3), 2.02-2.10 (m, 4H, H-8, H-17), 2.18 (t, *J* = 7.6 Hz, 2H, H-2), 2.80 (t, *J* = 5.2 Hz, 4H, H-11, H-14), 3.52 (dd, J_1 = 6.2 Hz, J_2 = 11.0 Hz, 1 H, H-2´), 3.65 (dd, J_1 = 5.0 Hz, J_2 = 6.2 Hz, 1 H, H-2´), 4.09 (m, 1 H, H-1´), 5.28-5.42 (m, 6H, H-9, H-10, H-12, H-13, H-15, H-16), 5.62 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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); $[M + Na]^+$ Calcd. $C_{21}H_{37}NNaO_2$ 358.2722. Found $C_{21}H_{37}NNaO_2$ 358.2718.

5. *N*-linolenoyl(1-amino-2-propanol) (4f). Yield 87% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, *J* = 7.6 Hz, 3H, H-18), 1.19 (d, *J* = 6.3 Hz, 3H, H-3'), 1.27-1.37 (m, 8H, H-4, H-5, H-6, H-7), 1.63 (qn, *J* = 7.3 Hz, 2H, H-3), 2.02-2.11 (m, 4H, H-8, H-17), 2.20 (t, *J* = 7.5 Hz, 2H, H-2), 2.80 (t, *J* = 5.4 Hz, 4H, H-11, H-14), 3.12 (m, 1 H, H-1´), 3.44 (ddt, J_1 = 2.3 Hz, J_2 = 6.5 Hz, J_3 = 12.1 Hz, 1 H, H-1´), 3.92 (m, 1 H, H-2´), 5.29-5.42 (m, 6H, H-9, H-10, H-12, H-13, H-15, H-16), 5.92 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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); $[M + Na]^+$ Calcd. $C_{21}H_{37}NNaO_2$ 358.2722. Found $C_{21}H_{37}NNaO_2$ 358.2729.

6. *N*-linolenoyl(2-methyl-2-amino-1-propanol) (4g). Yield 63% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, *J* = 7.5 Hz, 3H, H-18), 1.25-1.34 (m, 14H, H-4, H-5, H-6, H-7, H-3', H-4'), 1.60 (qn, *J* = 7.3 Hz, 2H, H-3), 2.03-2.11 (m, 4H, H-8, H-17), 2.16 (t, *J* = 7.6 Hz, 2H, H-2), 2.80 (t, *J* = 6.5 Hz, 4H, H-11, H-14), 3.58 (s, 2 H, H-2´), 5.28-5.42 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16), 5.48 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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); $[M + Na]^+$ 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 ($CDCl_3$) δ 0.92 (t, *J* = 5.0 Hz, 3H, H-4'), 0.97 (t, *J* = 7.4 Hz, 3H, H-18), 1.30 (m, 8H, H-4, H-5, H-6, H-7), 1.48 (m, 2H, H-3'), 1.62 (m, 2H, H-3), 2.04-2.09 (m, 4H, H-17, H-8), 2.19 (t, *J* = 7.5 Hz, 2H, H-2), 2.80 (m, 4H, H-11, H-14), 3.55 (dd, J_1 = 5.6 Hz, J_2 = 10.9 Hz, 1 H, H-2´), 3.67 (dd, J_1 = 3.3 Hz, J_2 = 10.8 Hz, 2 H, H-2´), 3.95 (m, 1 H, H-2´),5.32-5.38 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16), 5.57 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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); $[M + Na]^+$ Calcd. $C_{22}H_{39}NNaO_2$ 372.2879. Found $C_{22}H_{39}NNaO_2$ 372.2874.

8. *N*-linolenoyl(3-methyl-2-amino-1-butanol) (4i). Yield 62% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.7 Hz, 2H, H-18), 0.91 (d, 3H, H-4'), 0.96 (d, 3H, H-5'), 1.28 (m, 8H, H-4, H-5, H-6, H-7), 1.72 (qn, *J* = 7.2 Hz, 2H, H-3), 1.83 (m, 1H, H-3'), 2.03 (m, 4H, H-17, H-8), 2.21 (t, *J* = 7.4 Hz, 2H, H-2), 2.80 (m, 4H, H-11, H-14), 3.63 (dd, J_1 = 6.2 Hz, J_2 = 5.0 Hz, 1 H, H-1´), 3.69 (dd, J_1 = 6.0 Hz, J_2 = 9.5 Hz, 2 H, H-2´), 5.34-5.37 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16), 5.57 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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);$[M + Na]^+$ Calcd. $C_{23}H_{41}NNaO_2$ 386.3035. Found $C_{23}H_{41}NNaO_2$ 386.3040.

9. *N*-linolenoyl(2-amino-1-pentanol) (4j). Yield 77% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.97 (t, *J* = 7.5 Hz, 3H, H-18), 0.93 (t, *J* = 7.3 Hz, 3H, H-5'), 1.30 (m, 8H, H-4, H-5, H-6, H-7), 1.43 (m, 4H, H-3', H-4'), 1.62 (qn, *J* = 7.0 Hz, 2H, H-3), 2.04-2.09 (m, 4H, H-17, H-8), 2.19 (t, *J* = 7.6 Hz, 2H, H-2), 2.80 (m,4H, H-11, H-14), 3.55 (dd, J_1 = 6.2 Hz, J_2 = 11.0 Hz, 2 H, H-2´), 3.69 (dd, J_1 = 3.5 Hz, J_2 = 11.0 Hz, 1 H, H-2´), 3.95 (m, 1 H, H-1´), 5.29-5.42 (m, 6H, H-9, H-10; H-12, H-13, H-15, H-16), 5.57 (bs, 1H, N*H*). ^{13}C NMR ($CDCl_3$) 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); $[M + Na]^+$ Calcd. $C_{23}H_{41}NNaO_2$ 386.3035. Found $C_{23}H_{41}NNaO_2$ 386.3038.

10. *N*-arachidonoylethanolamine (**anandamide, 5a).** Yield 81% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.83 (t, *J* = 6.7 Hz, 3H, H-20), 1.2-1.3 (m, 6H, H-17, H-18, H-19), 1.58 (bs, 1H, O*H*), 1.66 (qn, *J* = 7.5 Hz, 2H, H-3), 2.00 (q, *J* = 7.2 Hz, 2H, H-16), 2.06 (c, *J* = 7.0 Hz, 2H, H-4), 2.16 (t, *J* = 7.4 Hz, 2H, H-2), 2.75-2.78 (m, 6 H, H-7, H-10, H-13); 3.36 (q, *J* = 5.5 Hz, 2 H, H-1´); 3.67 (t, *J* = 5.0 Hz, 2 H, H-2´), 5.29-5.33 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.81 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: $[M + Na]^+$ Calcd. $C_{22}H_{37}NNaO_2$ 370.2722. Found: $C_{22}H_{37}NNaO_2$ 370.2726.

11. *N*-arachidonoylpropanolamine (5b). Yield 73% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.89 (t, *J* = 6.7 Hz, 2H, H-20), 1.25-1.35 (m, 6H, H-17, H-18, H-19), 1.60 (bs, 1H, O*H*), 1.67 (qn, *J* = 6.2 Hz, 2H, H-2'), 1.73 (qn, *J* = 7.5 Hz, 2H, H-3), 2.05 (q, *J* = 7.0 Hz, 2H, H-16), 2.11 (q, *J* = 7.0 Hz, 2H, H-4), 2.20 (t, *J* = 7.4 Hz, 2H, H-2), 2.80-2.84 (m, 6 H, H-7, H-10, H-13); 3.42 (q, *J* = 6.0 Hz, 2 H, H-1´); 3.62 (t, *J* = 5.8 Hz, 2 H, H-3´), 5.34-5.41 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.81 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: $[M + Na]^+$ Calcd. $C_{23}H_{39}NNaO_2$ 384.2879. Found: $C_{23}H_{39}NNaO_2$ 384.2883.

12. *N*-arachidonoylbutanolamine (5c). Yield 69% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.6 Hz, 2H, H-20), 1.25-1.35 (m, 6H, H-17, H-18, H-19), 1.59 (m, 4H, H-2', H-3'), 1.71 (qn, *J* = 7.5 Hz, 2H, H-3), 2.04 (q, *J* = 6.9 Hz, 2H, H-16), 2.10 (q, *J* = 6.9 Hz, 2H, H-4), 2.16 (t, *J* = 7.3 Hz, 2H, H-2), 2.80-2.84 (m, 6 H, H-7, H-10, H-13); 3.28 (q, *J* = 6.7 Hz, 2 H, H-1´); 3.67 (t, *J* = 6.0 Hz, 2 H, H-4´), 5.34-5.38 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.68 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: $[M + Na]^+$ Calcd. $C_{24}H_{41}NNaO_2$ 398.3035. Found: $C_{24}H_{41}NNaO_2$ 398.3038.

13. *N*-arachidonoylpentanolamine (5d). Yield 66% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.89 (t, *J* = 6.7 Hz, 2H, H-20), 1.28-1.40 (m, 8H, H-3', H-17, H-18, H-19), 1.59 (m, 4H, H-2', H-4'), 1.71 (qn, *J* = 7.5 Hz, 2H, H-3), 2.04 (q, *J* = 7.3 Hz, 2H, H-16), 2.10 (q, *J* = 7.0 Hz, 2H, H-4), 2.16 (t, *J* = 7.4 Hz, 2H, H-2), 2.80-2.84 (m, 6 H, H-7, H-10, H-13); 3.26 (q, *J* = 7.0 Hz, 2 H, H-1´); 3.65 (t, *J* = 6.4 Hz, 2 H, H-5´), 5.36-5.39 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.45 (bs, 1H,

N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: [M + Na]$^+$ Calcd. $C_{25}H_{43}NNaO_2$ 412.3191. Found: $C_{25}H_{43}NNaO_2$ 412.3196.

14. *N*-arachidonoyl(2-amino-1-propanol) (5e). Yield 78% of pure compound as a colorless oil. IR. ^{1}H NMR ($CDCl_3$) δ 0.89 (t, *J* = 6.7 Hz, 3H, H-20), 1.18 (d, *J* = 6.9 Hz, 3H, H-3'), 1.29-1.35 (m, 6H, H-17, H-18, H-19), 1.74 (qn, *J* = 7.5 Hz, 2H, H-3), 2.05 (q, *J* = 7.5 Hz, 2H, H-16), 2.11 (q, *J* = 7.0 Hz, 2H, H-4), 2.22 (t, *J* = 7.4 Hz, 2H, H-2), 2.80-2.83 (m, 6 H, H-7, H-10, H-13); 3.53 (dd, J_1 = 6.2 Hz, J_2 = 4.8 Hz, 1 H, H-2´); 3.67 (dd, J_1 = 9.0 Hz, J_2 = 6.2 Hz, 1 H, H-2´), 4.07 (m, 1 H, H-1´), 5.36-5.39 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.61 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: [M + Na]$^+$ Calcd. $C_{23}H_{39}NNaO_2$ 384.2879. Found: $C_{23}H_{39}NNaO_2$ 384.2881.

15. *N*-arachidonoyl(1-amino-2-propanol) (5f). Yield 80% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.7 Hz, 3H, H-20), 1.18 (d, *J* = 6.2 Hz, 3H, H-3'), 1.29-1.35 (m, 7H, H-17, H-18, H-19, O*H*), 1.73 (qn, *J* = 7.6 Hz, 2H, H-3), 2.05 (q, *J* = 7.1 Hz, 2H, H-16), 2.11 (q, *J* = 6.9 Hz, 2H, H-4), 2.21 (t, *J* = 7.5 Hz, 2H, H-2), 2.80-2.83 (m, 6 H, H-7, H-10, H-13); 3.11 (m, 1H, H-1'), 3.42 (ddd, J_1 = 2.9 Hz, J_2= 6.6 Hz, J_3 = 13.8 Hz, 1 H, H-1´); 3.89-3.93 (m, 1 H, H-2´), 5.36-5.39 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.91 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: [M + Na]$^+$ Calcd. $C_{23}H_{39}NNaO_2$ 384.2879. Found: $C_{23}H_{39}NNaO_2$ 384.2885.

16. *N*-arachidonoyl(2-methyl-2-amino-1-propanol) (5g). Yield 60% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.89 (t, *J* = 6.7 Hz, 2H, H-20), 1.28 (s, 6H, H-3', H-4'), 1.28-1.30 (m, 6H, H-17, H-18, H-19), 1.61 (bs, 1H, O*H*), 1.70 (qn, *J* = 7.5 Hz, 2H, H-3), 2.05 (q, *J* = 6.9 Hz, 2H, H-16), 2.11 (q, *J* = 6.9 Hz, 2H, H-4), 2.16 (t, *J* = 7.1 Hz, 2H, H-2), 2.79-2.84 (m, 6 H, H-7, H-10, H-13); 3.58 (s, 2H, H-2´); 5.36-5.39 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.48 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: [M + Na]$^+$ Calcd. $C_{24}H_{41}NNaO_2$ 398.3035. Found: $C_{24}H_{41}NNaO_2$ 398.3040.

17. *N*-arachidonoyl(2-amino-1-butanol) (5h). Yield 68% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.89 (t, *J* = 6.7 Hz, 3H, H-4'), 0.95 (t, *J* = 6.9 Hz, 3H, H-20), 1.29-1.32 (m, 6H, H-17, H-18, H-19), 1.49 (m, 1H, H-3'), 1.60 (m, 1H, H-3'), 1.73 (qn, *J* = 7.5 Hz, 2H, H-3), 2.05 (q, *J* = 7.5 Hz, 2H, H-16), 2.12 (q, *J* = 7.0 Hz, 2H, H-4), 2.22 (t, *J* = 7.4 Hz, 2H, H-2), 2.80-2.84 (m, 6 H, H-7, H-10, H-13); 3.59 (dd, J_1 = 6.2 Hz, J_2 = 4.8 Hz, 1 H, H-2´); 3.69 (dd, J_1 = 9.0 Hz, J_2 = 6.2 Hz, 1 H, H-2´), 3.89 (m, 1 H, H-1´), 5.36-5.39 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.57 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: [M + Na]$^+$ Calcd. $C_{24}H_{41}NNaO_2$ 398.3035. Found: $C_{24}H_{41}NNaO_2$ 398.3039.

18. *N*-arachidonoyl (3-methyl-2-amino-1-butanol) (5i). Yield 63% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.87 (t, *J* = 6.7 Hz, 2H, H-20), 0.91 (d, 3H, H-4'), 0.96 (d, 3H, H-5'), 1.26-1.36 (m, 6H, H-17, H-18, H-19), 1.63 (bs, 1H, O*H*), 1.74 (qn, *J* = 7.5 Hz, 2H, H-3), 1.86 (m, 1H, H-3'), 2.05 (q, *J* = 7.5 Hz, 2H, H-16), 2.12 (q, *J* = 7.0 Hz, 2H, H-4), 2.23 (t, *J* = 7.4 Hz, 2H, H-2), 2.80-2.84 (m, 6 H, H-7, H-10, H-13); 3.65 (dd, J_1 = 6.2 Hz, J_2 = 4.8 Hz, 1 H, H-1´); 3.71 (dd, J_1 = 9.0 Hz, J_2 = 6.2 Hz, 2 H, H-2´), 5.36-5.39 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.60 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); HRMS: [M + Na]$^+$ Calcd. $C_{25}H_{43}NNaO_2$ 412.3191. Found: $C_{25}H_{43}NNaO_2$ 412.3195.

19. *N*-arachidonoyl(2-amino-1-pentanol) (5*J*). Yield 75% of pure compound as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, *J* = 6.8 Hz, 3H, H-5'), 0.93 (t, *J* = 7.0 Hz, 3H, H-20), 1.31-1.37 (m, 8H, H-3, H-17, H-18, H-19), 1.49 (m, 2H, H-4'), 1.74 (qn, *J* = 7.5 Hz, 2H, H-3'), 2.05 (q, *J* = 6.8 Hz, 2H, H-16), 2.11 (q, *J* = 6.5 Hz, 2H, H-4), 2.21 (t, *J* = 7.4 Hz, 2H, H-2), 2.81-2.84 (m, 6 H, H-7, H-10, H-13); 3.56 (dd, J_1 = 6.0 Hz, J_2 = 11.0 Hz, 2 H, H-2´); 3.69 (dd, J_1 = 3.5 Hz, J_2 = 11.0 Hz, 1 H, H-2´), 3.96 (m, 1 H, H-1´), 5.36-5.39 (m, 8 H, H-5, H-6, H-8, H-9, H-11, H-12, H-14, H-15), 5.54 (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$) 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); 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 ($1.5x10^4$ 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. Then, the product formazan was 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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Keywords: Biocatalysis / Aminolysis / Chemoselectivity/ Endocannabinoids / Antitumor agents

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Paula G. Quintana,[a] Guadalupe García Liñares,[a] Santiago N. Chanquia,[a] Roxana M. Gorojod,[b] Mónica L. Kotler,[b] and Alicia Baldessari[a]*

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Improved enzymatic procedure for the synthesis of anandamide and *N*-fatty acylalkanolamine analogues and combination strategy in antitumor activity

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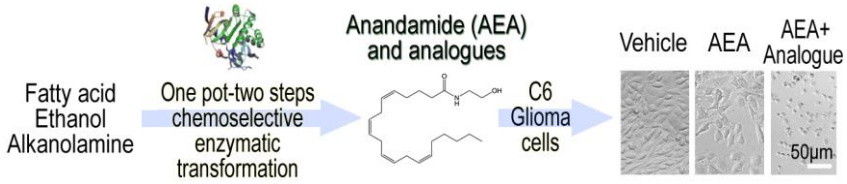

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