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Synthesis of all-*trans* anandamide: A substrate for fatty acid amide hydrolase with dual effects on rabbit platelet activation

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ABSTRACT

Anandamide (AEA) presents the four double bonds in the *cis* configuration, deriving from the arachidonic acid moiety. In the context of an antisense strategy based on the double bond configuration, all-*trans* AEA (*t*-AEA) was synthesized in high yield starting from all-*trans* methyl arachidonate and ethanolamine in the presence of KCN. *t*-AEA was assayed on rabbit platelet aggregation, obtaining effect only at high concentrations (>10^{-4} M) after an also concentration-dependent lag phase. At lower concentrations it inhibited PAF-induced rabbit platelet aggregation with an $IC_{50} = 4.6 \times 10^{-6}$ M. In contrast to anandamide, the activation of platelets was not due to the conversion of *t*-AEA to *trans* arachidonic acid, as ascertained by negative results with FAAH inhibitors. However, *t*-AEA was found to be a substrate for fatty acid amide hydrolase (FAAH), the enzyme that cleaves anandamide and regulates in vivo the magnitude and duration of the signaling induced by this lipid messenger.

1. Introduction

N-Arachidonoylethanolamine or anandamide (AEA), the most studied endocannabinoid, is a member of a large and diverse family of endogenous signaling lipids, the fatty acid amides (FAAs), which includes *N*-acyl ethanolamines, primary amides such as the sleep-inducing lipid, oleamide,[1] *N*-acyl aminoacids[2] and *N*-acyl taurines.[3] Since 1992 when AEA was recognized as the endogenous ligand for CB1 receptor,[4] a variety of functions of this endocannabinoid in the central nervous system have been reported. AEA stimulates signal transduction pathways by binding to its receptors,[5] mediates retrograde signaling at hippocampal synapses[6] and provides on demand defense against excitotoxicity.[7] Important physiological roles have been reported in many other systems including the cardiovascular system.[8,9] Human platelets are able to take up by facilitated diffusion and metabolize AEA that has been proposed as an agonist for these cells[10] by binding to a "platelet type" (CBPT) cannabinoid receptor.[11] On the other hand, rabbit platelets take up by simple diffusion and metabolize AEA,[12] which induces aggregation through its conversion to arachidonic acid (AA) by the action of fatty acid amide hydrolase (FAAH).[13] FAAH, a member of the amidase signature family, is the enzyme that cleaves the amide and regulates the magnitude and duration of the signaling induced by AEA and other FAAs.[14] Several studies suggest that FAAH may represent an attractive therapeutic target for a number of human disorders.[15]

In the structure of anandamide several changes have been produced in order to evaluate the structure–activity relationship. They involved the substitution of the amide chain or of the free hydroxyl group, introduction of an alkyl substituent in the carbon atom next to the carbonyl group, or changing the number of the double bond from four to three.[16] No data are available on the influence of the double bond configuration being *cis* the naturally occurring geometry determined by the desaturase enzyme activity.[17] The relevance of the double bond geometry for the biological activity of unsaturated lipids has been addressed by several studies on cell cultures and organisms.[18] The geometrical conversion of only one double bond to the *trans* configuration changed the effect of arachidonic acid in platelets.[19] More recently, the isomerization process has been related to a radical stress that occurs in vivo and affects membrane lipids.[20,21]

Based on this premise, we started to be interested in an antisense strategy based on the double bond isomerism of arachidonic acid and its derivatives, also in view of providing further insights on the interactions and cascades that lipid mediators are known to activate. In a previous report, all-*trans* arachidonic acid (*t*-AA) was synthesized and tested in the rabbit platelet aggregation, where it inhibited aggregation induced by the strong platelet agonist PAF with an IC_{50} at the micromolar range.[22]

Here, we present the synthesis of all-*trans* anandamide (**1** in Scheme 1, *t*-AEA) and its activity on platelet aggregation, as well as its interaction with FAAH, in comparison with *cis*-anandamide.

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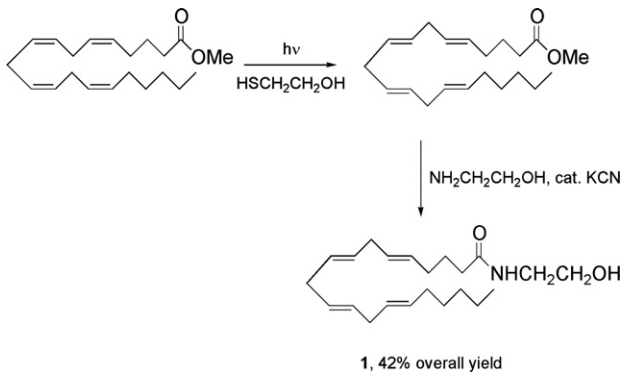


Scheme 1. Two-steps synthesis of all-*trans* anandamide (1, *t*-AEA).

We anticipate that *t*-AEA is able to inhibit PAF-induced platelet aggregation, and to interact with FAAH in a way not sensitive to FAAH inhibitors. This behavior highlights the antisense strategy based on the geometrical isomerism as a useful tool for understanding eicosanoids metabolic pathways.

2. Results

2.1. Chemistry

The radical-catalysed methodology is a very efficient procedure to convert the *cis* double bonds into the corresponding geometrical *trans* configuration and it was successfully applied to the preparation of arachidonic acid isomers.[23] In case of polyunsaturated fatty acids, it is known that the isomerization is a catalytic reaction, occurring by thiyl radical addition–elimination on the double bond, following a step-by-step mechanism, that produces mono-*trans* isomers first, and then di-*trans* isomers, and tri-*trans* isomers, until all-*trans* isomer is formed as the sole product of exhaustive isomerization.[24]

The synthesis of all-*trans* anandamide **1** was achieved using methyl arachidonate as starting material (Scheme 1). A mixture of methyl arachidonate in *i*-propanol was placed in a quartz photochemical reactor and bubbled with argon for 20 min. The thiol, 2-mercaptoethanol, in a 50% mol equivalent with respect to the methyl ester, was added and the mixture was exposed to low-pressure mercury lamp (5.5 W) at 25 °C for 150 min. The formation of all-*trans* methyl arachidonate was obtained together with a mixture of the tri-*trans* isomers. After work-up, purification was carried out by preparative Ag-TLC,[25] that provides separation of the desired product from the tri-*trans* isomer fraction. On the latter, the isomerization procedure was iterated and the exhaustive conversion of the double bonds from *cis* to *trans* was achieved with a 60% total yield (see Section 5). All-*trans* methyl arachidonate was then used for the next step in the presence of ethanolamine, following a reported procedure,[26] and afforded all-*trans* anandamide (**1**, *t*-AEA) in a 70% yield and an overall yield of 42%.

Anandamide was also synthesized starting from methyl arachidonate under the above described procedure in the presence of ethanolamine. In this case, the expected anandamide was accompanied by a side-product that, after isolation and characterization, was identified as 2-aminoethyl arachidonic acid ester, **2** (see Scheme 2, page 4). This compound can be formed by an intramolecular nucleophilic attack, a process that is known to occur in carboxylic acid derivatives.[27] By a careful examination of the reaction conditions and work up procedure, we concluded that the rearrangement could occur during the acidification step required to eliminate the amine excess. Indeed, this rearrangement also occurred by leaving the deuterated chloroform solution of *cis*-anandamide prepared for the NMR analysis at room temperature for 10 hours, reaching a 50% conversion. It is worth noting that such conversion was not found in case of *t*-AEA.

2.2. Induction of platelet aggregation by all-*trans* anandamide

As shown in Figure 1, all-*trans* anandamide caused a concentration dependent (irreversible) platelet aggregation with a lag phase dependent on the concentration: the lower the concentration, the longer the lag phase. The corresponding *cis* isomer, anandamide, under the same experimental conditions, caused concentration dependent platelet aggregation, at 10 times lower concentrations, with a much smaller lag phase and reversible at low concentrations (Fig. 2).

The integrity of platelets treated with high concentrations of *t*-AEA was tested by measuring LDH activity in the supernatant (after centrifugation of the platelets, when aggregation reaches a maximum) and as shown in Figure 3, platelets were not lysed: LDH activity was almost negligible, as compared to platelets treated with Triton X-100 (lysed, 100%), even after approx. 20 min in

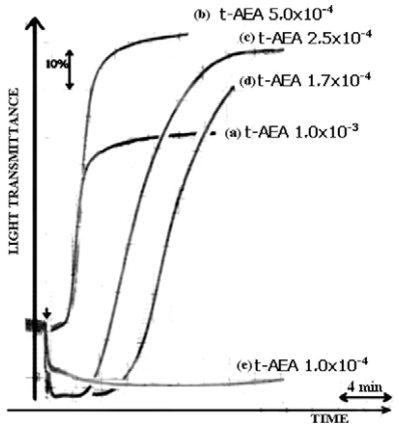


Figure 1. Effects of *t*-AEA on washed rabbit platelets. Typical aggregation traces in response to the addition of *t*-AEA (in ethanol) at concentrations indicated. *t*-AEA was added to cuvettes containing 0.5 mL of prewarmed (37 °C) platelet suspension (2.5×10^8 cells/mL).

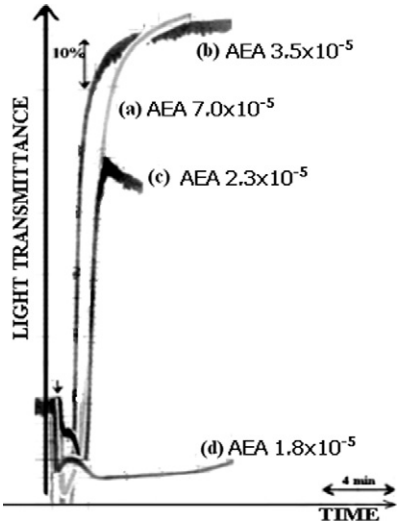


Figure 2. Effects of AEA on washed rabbit platelets. Typical aggregation traces in response to the addition of AEA (in ethanol) at concentrations indicated. AEA was added to cuvettes containing 0.5 mL of prewarmed (37 °C) platelet suspension (2.5×10^8 cells/mL).

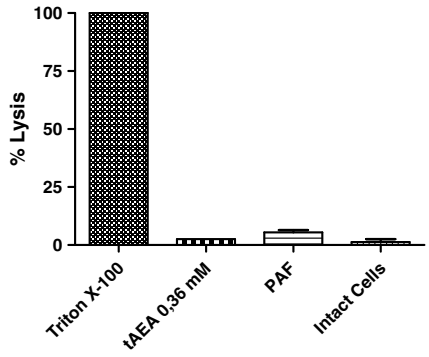


Figure 3. Effect of all-*trans* anandamide on platelet integrity. Platelets aggregated by *t*-AEA (at concentrations indicated) were centrifuged and LDH activity was measured in the supernatant. LDH activity from Triton X-100 treated cells corresponds to 100% lysis.

the presence of *t*-AEA under stirring. For comparison, LDH activity in the supernatant of platelets aggregated with PAF (4×10^{-10} M) was slightly higher (5.5%) (Fig. 3).These results clearly showed that all-*trans* anandamide is not toxic for platelets. Similar results were obtained for *cis* AEA (not shown).

In contrast to anandamide, which activated platelets through its conversion to arachidonic acid by the action of FAAH,[12,13] all-*trans* anandamide exerted its activity in a FAAH independent manner. PMSF (2 mM), a non-specific FAAH inhibitor, which blocks the aggregation by anandamide, caused only partial inhibition of aggregation (~20%), while the specific FAAH inhibitor URB597 (3 μM) did not cause any inhibition of all-*trans* anandamide induced platelet activation (not shown).

Indomethacin and aspirin were also unable to inhibit the aggregation induced by *t*-AEA suggesting that the cycloxygenase pathway and its metabolic products were not involved in the process; finally, aggregation was not mediated by ADP since was not affected by CP/CPK (not shown).

2.3. Inhibition of PAF induced platelet aggregation by all-*trans* anandamide

As shown in Figure 4, all-*trans* anandamide at μM concentrations, that, as shown previously, does not evoke platelet aggregation, was instead able to inhibit platelet aggregation induced by PAF in a dose dependent manner. This effect was rather specific since the platelet response to subsequently added thrombin (0.5 U/mL) remained unchanged (not shown). *t*-AEA displayed an IC_{50} ~4.7 μM when using 4×10^{-10} μM PAF.

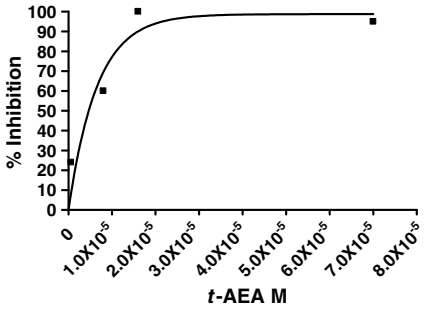


Figure 4. Inhibition of PAF induced platelet aggregation by all-*trans* anandamide. Dose-response curve of the inhibition of PAF (4×10^{-10} M) induced platelet aggregation, by *t*-AEA. Results are mean ± SEM (n = 3–5).

2.4. All-*trans* anandamide as FAAH substrate

It is known that rabbit platelets contain FAAH activity,[12,13] and despite the fact that platelet aggregation by *t*-AEA was not found to be mediated by the enzyme, the next step was the investigation of the possibility that *t*-AEA could be a substrate for FAAH, such as its physiological counterpart (anandamide). Rabbit platelet homogenate was used as the source of the enzyme; [^{3}H]anandamide (labelled on the ethanolamine moiety, 20,000 cpm) "diluted" with non labeled anandamide or *t*-AEA to a final concentration 1.5 μM, was used as substrate. [^{3}H]anandamide was hydrolyzed by 10% in both cases (Fig. 5, columns 1 and 6). Furthermore when the enzymatic assay was carried out in the presence of 1.5 μM AEA, the addition of 1.5 μM and 7.5 μM *cis*- (Fig. 5 columns 2,3) or *trans*-anandamide (Fig. 5, columns 4 and 5, respectively), resulted to give identical inhibition of the hydrolysis of [^{3}H]anandamide. These results indicated that *t*-AEA is an equally good substrate for rabbit platelet FAAH compared to anandamide.

3. Discussion

In the present study, the geometrical isomer of anandamide, all-*trans* anandamide (**1** in Scheme 1, *t*-AEA), was synthesized for the first time in two steps, good overall yield (42%) and purity (>99%), starting from arachidonic acid methyl ester and using the thiyl radical mediated isomerization protocol to convert the four double bonds into the *trans* configuration.[22] *t*-AEA structure was confirmed by ^{1}H-NMR ^{13}C-NMR and GC/MS.

During the preparation of *cis* anandamide by the same procedure, 2-aminoethyl arachidonate was evidenced as side-product, due to an intramolecular nucleophilic attack, catalysed by acidic conditions (**2** in Scheme 2), that is, during the washing of the organic phase with an acidic aqueous solution without refrigeration, or by leaving the NMR sample in $CDCl_3$ for 10 hours at room temperature. *t*-AEA did not show such conversion suggesting that the conformation given by the *cis* double bonds could play a role in the rearrangement. This behaviour suggests to carefully control conditions and product when extraction of anandamide is performed from various sources. 2-Aminoethyl arachidonate could be useful for studying the in vivo stability of anandamide. Work is in progress for evaluating the occurrence of this transformation under physiological conditions.

There is a growing interest in the effects of *trans* fatty acid and/or their derivatives on biological systems, since their interference with the natural counterparts has been established for a

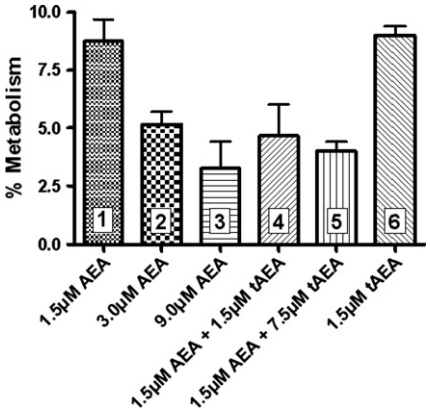
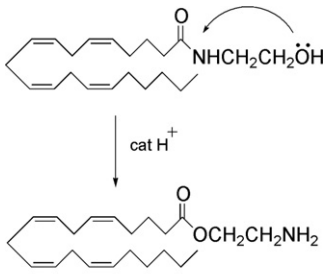


Figure 5. Rabbit platelet homogenate (50 μg protein/mL) was incubated with [^{3}H]anandamide (labelled on ethanolamine moiety, 20000 cpm) in the presence of non labelled AEA or *t*-AEA. Metabolic products were separated and radioactivity was measured as mentioned above.



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Scheme 2. Intramolecular attack with formation of 2-aminoethyl arachidonic acid ester **2**.

number of *trans* fatty acids. In addition, *trans* fatty acids especially those derived from PUFA are not an homogenous group and individual *trans* isomers might have distinct biological roles.[28] Various *trans* isomers have been shown to inhibit platelet aggregation,[29] modulate eicosanoid metabolism in endothelial cells and platelets, and most interestingly, be incorporated into cellular lipids.[30] The presence of *trans* isomers in human tissues has been also reported and many lines of evidence indicate that they do not derive only from exogenous sources,[31] but could be formed endogenously as well, by the action of free radicals.[20] Mono-*trans* arachidonic acid isomers have been detected in human plasma[32] and rat tissues,[33] but there is no evidence and it is highly improbable that all-*trans* arachidonic derivatives could be produced endogenously. Nevertheless, the investigation of biological activities given by an antisense geometrical configuration, where all the *cis* double bonds are changed to their *trans* isomers, can be interesting and may provide new insights in the mechanism of action and interference of lipid structures, both in vitro and in vivo, thus contributing to the ongoing debate of lipid relevance in health and disease.[34]

Platelets and their interaction with other cells, such as endothelial cells, play an important role in maintaining vascular homeostasis and many lines of evidence indicate the modulation of their function by *cis* unsaturated fatty acids (e.g., arachidonic acid, oleic acid) and/or their derivatives or metabolic products, while saturated and *trans* fatty acids have been accused for health risk including atherogenesis.[35] On the other hand, PAF is a strong platelet agonist and its levels are highly elevated during inflammation and other pathological situations, contributing to the atherogenesis as well.[36] We decided to investigate the effects of the synthesized *t*-AEA **1** on platelet function, as well as on the aggregation induced by PAF and thrombin.

We found that *t*-AEA had a dual effect on rabbit platelets: it caused aggregation in a concentration dependent manner, after an also concentration dependent lag phase (the lower the concentration, the longer the lag phase) (Fig. 1). At lower concentrations inhibited the aggregation induced by the strong platelet agonist PAF with an IC_{50} at the micromolar range (Fig. 4), and it did not have any effect on thrombin evoked aggregation. Such a dual effect on platelets is not uncommon and as it has been reported earlier by our group that other lipids, such as all-*trans* arachidonic acid (*t*-AA)[22] and oleic acid,[37] have a similar behavior. None of these isomers affected the integrity of aggregated platelets, as confirmed by measuring extracellular LDH activity (Fig. 3).

In an attempt to elucidate the mechanism of platelet activation by *t*-AEA, we compared it with the corresponding effects of the physiological mediator anandamide. As it has been reported earlier[13] and confirmed in this study (Fig. 2), anandamide induced washed rabbit platelet aggregation in a receptor independent manner through its conversion to AA, in contrast to the aggregation evoked in human platelets which is described as receptor mediated. Actually the presence of a putative platelet type cannabinoid (CBPT) receptor has been reported for human platelets.[11] In case of rabbit platelets, the presence of these receptors is questioned, being the effect of anandamide reported to be additive to the repetitive addition of cannabinoid receptor agonists and insensitive to the presence of cannabinoid receptor antagonists.[38,39] Therefore, we did not consider the system of rabbit platelets useful to gain insight in this interaction.

t-AEA-induced rabbit platelet aggregation was only partially (<20%) inhibited by PMSF, a widely used serine protease inhibitor and not responsive to URB 597, a specific FAAH inhibitor,[40–42] suggesting that the effect is not due to its enzymatic hydrolysis to *trans* arachidonic acid and/or to conversion to its metabolic products, by the action of FAAH and subsequently of cycloxygenase. In addition, aggregation was not due to metabolic products of *t*-AEA formed by a direct action of cycloxygenase, since indomethacin and aspirin (cycloxygenase inhibitors) did not have any effect on aggregation; as it has been reported, anandamide could be a substrate for cycloxygenase.[43,44]

We then investigated the possibility that rabbit platelet aggregation by *t*-AEA was receptor mediated, as it has been reported in the case of anandamide for human platelets.[11] Desensitisation experiments though, showed that the effect was additive since platelets treated with 1.0×10^{-4} M *t*-AEA in the absence of Ca^{2+}, were not desensitized to *t*-AEA and subsequent addition of the compound (under stirring) resulted to platelet aggregation (not shown); these results suggest that the aggregation by *t*-AEA is not mediated by specific receptors.

On the other hand, using rabbit platelet homogenate as a source of FAAH it was found that *t*-AEA was as good substrate as AEA for the enzyme (Fig. 5). Preliminary experiments using Tetrahymena cell homogenate as a source of the enzyme,[45] are in the direction of similar results, using various *cis* and *trans* ethanolamide isomers (not shown). It should be noticed that FAAH is an enzyme conserved during the evolution and its presence in the protist Tetrahymena has been described.[45,46] In mammalian cells the endogenous fatty acid amide substrates, hydrolyzed by FAAH, constitute multiple structural classes with distinct biological roles. These classes include *N*-acylethanolamines such as the endocannabinoid anandamide, the anti-inflammatory substance *N*-palmitoyl ethanolamine and the satiating factor *N*-oleoyl ethanolamine, primary amides such as the sleep inducing factor oleamide and *N*-acyl taurines.[14]

Subsequently, *t*-AA produced from *trans* anandamide by the action FAAH, (at concentrations not able to induce platelet aggregation) could be responsible for the inhibition of aggregation induced by PAF (Fig. 4) since, as reported earlier by our group, *t*-AA inhibited PAF induced platelet aggregation.[22] The direct effect of *t*-AEA though could not be excluded. It should be noted that the inhibitory effect on PAF-induced platelet aggregation was not due to a toxic effect of *t*-AEA, since sequential addition of thrombin resulted to the aggregation of platelets (not shown) suggesting also a rather specific effect on PAF.

As mentioned before, the possibility that all-*trans* anandamide could be formed endogenously is very unlikely, because all the four double bonds of the natural structure cannot be converted by an exhaustive isomerization in vivo.

4. Conclusions

In conclusion, the present study provides further knowledge on the antisense approach based on the geometrical change in unsaturated lipid molecules. The aim is to contribute to the overall understanding of the importance of the double bond configuration in biology, a subject that is emerging only recently in the evaluation of lipid structural and functional roles.

The antisense strategy carried out by all-*trans* arachidonic derivatives indicates so far that a dual effect can be obtained by such structural change: at concentrations much higher than *cis* analogues and with a different mechanism, *trans* isomers cause platelet aggregation while at lower concentrations they inhibit PAF-induced aggregation. Moreover, *t*-AEA can act as a competing substrate for FAAH, an enzyme that regulates the signaling induced by anandamide, as well as other signaling lipids and it represents a therapeutic target for several disorders. This study confirms that *trans* fatty acid derivatives could interact with the pathways followed by the natural *cis* compounds, involved in very important processes.

5. Experimental

5.1. Materials and methods

Anandamide, 5-*cis*,8-*cis*,11-*cis*,14-*cis*-eicosatetraenoic acid methyl ester (methyl arachidonate), arachidonic acid, platelet activating factor (PAF), thrombin, indomethacin, aspirin, bovine serum albumin (BSA), Triton X-100, phosphocreatine (CP) and creatine phosphokinase (CPK) 2-mercaptoethanol were commercially available (Sigma–Aldrich Co., Milan, Italy) and used without further purification. Phenyl methyl sulfonyl fluoride (PMSF) was from Serva (Heidelberg/New York, USA), and URB 597 from Cayman Chemical Co. (Ann Arbor, MI, USA). Chloroform, methanol, absolute ethanol, *i*-propanol, diethyl ether, ethyl acetate and *n*-hexane were purchased from Merck (HPLC grade) and used without further purification. [^{3}H]Anandamide (60 Ci/mmol, [ethanolamine 1-^{3}H]) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). Other chemicals were of the highest purity available.

Chromatography was performed on Florisil® (TLC grade, Aldrich Co.) by using the eluent specified in the protocols, under an argon stream to avoid lipid degradation. For the reaction monitoring and separation of *cis* and *trans* isomers, argentation of silica gel plates was done by a reported procedure.[25]

GC analysis for the determination of the isomeric ratio of the geometrical isomers of arachidonic methyl ester and anandamide was performed on an Agilent 5860 gas chromatograph equipped with a flame ionization detector and a Rtx-2330 column (90% biscyanopropyl-10% phenylcyanopropyl polysiloxane capillary column; 60 m, 0.25 mm i.d., 0.20 μm film thickness). Temperature started from 190 °C held for 15 min, followed by a decrease of 20 °C/min to 175 °C, held for 25 min, followed by an increase of 30 °C/min up to 250 °C. When necessary, GC/MS spectra were recorded on a Hewlett-Packard GC 5890 (series II) coupled to a Hewlett–Packard mass selective detector model 5971A.

NMR spectra were recorded on a Varian VXR 400 MHz instrument using $CDCl_3$ as the solvent and the reference peak (δ = 7.26 and 77.0 ppm).

5.2. Synthesis of all-*trans* arachidonic acid methyl ester

A 15 mM solution of methyl arachidonate (33 mg; 0.10 mmol) in *i*-PrOH (7 mL) was placed in a quartz photochemical reactor and bubbled with argon for 20 min. 2-Mercaptoethanol (7.5 mM) was added and the mixture was exposed to a low-pressure mercury lamp (5.5 W) at 25 °C. The reaction was monitored by gas chromatography to ascertain the formation of all-*trans* methyl arachidonate, together with the corresponding four tri-*trans* isomers (reaction time: 150 min). The reaction was stopped and concentrated under vacuum. The residue was treated with absolute ethanol and evaporated under vacuum in order to be thiol-free. Separation was carried out by preparative Ag/TLC with hexane/diethyl ether in 8/2 ratio as the eluent and the fractions were detected by spraying a portion of the plate with cerium ammonium sulfate/ammonium molybdate reagent. The all-*trans* isomer fraction was scraped off the plate and the silica gel was washed using absolute ethanol. After filtration and ethanol evaporation, the residue corresponded to the Ag complex of all-*trans* methyl arachidonate, as a white powder insoluble in *n*-hexane. The complex was dissolved with 5% aq NH_4OH (10 mL) and extracted with chloroform/methanol (5:1 v/v). The organic phase was dried over anhydrous sodium sulfate and concentrated under vacuum to afford all-*trans* methyl arachidonic acid methyl ester. This compound was further purified by flash column chromatography on silica gel with diethyl ether as eluent, finally affording pure all-*trans* methyl arachidonate (oil; 5 mg; 0.016 mmol; 16% yield).

A second fraction was isolated from the preparative Ag/TLC containing the tri-*trans* geometrical isomers (12 mg; 0.037 mmol; 38% yield). This fraction was photo-irradiated using 1.9 mM 2-mercaptoethanol under the above described conditions and the reaction was monitored by gas chromatography. The all-*trans* isomer was formed in equal amount with the corresponding tri-*trans* isomers in 40 min. After solvent evaporation the residue was purified by preparative Ag/TLC, and all-*trans* arachidonate was isolated as previously described (2 mg; 0.006 mmol; 16% yield). Iterating the procedure of photoirradiation and product isolation, a total amount of all-*trans* methyl arachidonate was collected (18 mg; 0.06 mmol; yield 60%). Characterization by NMR and GC–MS analysis, gave results identical to previously reported data.[22]

5.3. Preparation of all-*trans N*-arachidonoylethanolamine (all-*trans* anandamide, *t*-AEA, 1)

A 0.2-M all-*trans* methyl arachidonate solution in methanol (18 mg; 0.057 mmol) was put in a vial equipped with open-top screw cap and a septum and added with a catalytic amount of KCN (0.02 M). A 10-fold excess of 2-ethanolamine (2 M) was added via syringe and the mixture was stirred overnight at 50 °C. The reaction was monitored by TLC with 1:4 hexane/ethyl acetate as the eluent and worked-up by addition of 1:4 hexane/ethyl acetate (2 mL) for four times, followed by evaporation under vacuum. The last addition was followed by rapid washing with cold 1 M HCl aqueous solution, then with sat. aq Na_2CO_3 and brine, and finally drying over $MgSO_4$. After solvent evaporation the crude was purified by flash column chromatography on Florisil, with a solvent mixture of ethyl acetate/hexane, varying the ratio from 1:4 to 4:1. All-*trans N*-arachidonoylethanolamine (all-*trans* anandamide, **1**) was isolated (14 mg; 0.040 mmol; 70% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.91 (s, 1H), 5.41 (m, 8H, vinylic), 3.72 (t, 2H, *J* = 5.0 Hz), 3.42 (q, 2H, *J* = 5.0 Hz), 2.70 (s, OH), 2.68 (m, 6H, bisallylic), 2.20 (t, 2H, *J* = 7.6 Hz), 2.05 (m, 4H), 1.71 (m, 2H), 1.30 (m, 6H), 0.88 (t, 3H, *J* = 6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.30, 131.31, 129.76, 129.69, 129.55, 129.32, 129.11, 128.96, 128.23, 62.68, 42.47, 35.84, 35.60, 35.57, 35.56, 32.53, 31.89, 31.41, 29.19, 25.26, 22.52, 14.07. GC/MS (*m*/*z*), 347 (M+), 332, 244, 218, 192, 178, 156, 145, 138, 125, 98, 85, 79, 67, 55.

5.4. Preparation of *N*-arachidonoylethanolamine (anandamide)

The above reported procedure was used starting from arachidonic acid methyl ester (20 mg; 0.063 mmol). The crude was purified by flash column chromatography on Florisil under the above reported conditions, affording 16 mg of *N*-arachidonoylethanolamine (anandamide) (16 mg; 0.046; 73% yield). The product was identical to the commercially available compound.

5.5. Conversion of anandamide to 2-aminoethyl arachidonate, 2

2-Aminoethyl arachidonate **2** was formed by an intramolecular transesterification of anandamide occurring under acidic condi-

tions (with HCl 1 M) used to eliminate from the organic phase the excess of 2-ethanolamine. The intramolecular transesterification was also occurring in the NMR tube containing 1 mL of $CDCl_3$ after 10 h. From the ^1H NMR spectrum, it was observed a 50% conversion to the corresponding ester, 2-aminoethyl arachidonate. Using the previously described condition of purification on Florisil, 2-aminoethyl arachidonate **2** was isolated. ^1H-NMR (400 MHz, $CDCl_3$): δ 5.72(s, 2H), 5.37 (m, 8H, vinylic), 4.16 (t, 2H, J = 5.6 Hz), 3.52 (q, 2H, J = 5.6 Hz), 2.81 (m, 6H, bisallylic), 2.20 (t, 2H, J = 7.2 Hz), 2.10 (m, 4H), 1.71 (m, 2H), 1.30 (m, 6H), 0.88 (t, 3H, J = 6.8 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 172.90, 130.52, 129.04, 128.79, 128.60, 128.24, 128.12, 127.82, 127.49, 63.39, 38.69, 35.98, 31.49, 29.30, 27.20, 26.62, 25.62, 25.39, 22.56, 14.06. GC/MS (*m/z*), 347 (M+), 332, 244, 218, 192, 178, 156, 145, 138, 125, 98, 85, 79, 67, 55.

6. Biology

6.1. Washed rabbit platelet preparation

Blood was withdrawn from the marginal ear vein of New Zealand white rabbits in tubes containing anticoagulant (sodium citrate), and platelet rich plasma (PRP) was obtained by centrifugation. The platelet suspension was obtained from EGTA-anticoagulated PRP according to a washing procedure described previously.[12,47] Platelets were counted and adjusted to 1.25×10^9 platelets/mL.

The anticoagulant solution, acid/citrate/dextrose (ACD), contained 1.36% citric acid, 2.5% trisodium citrate and 2.0% dextrose (w/v).

6.2. Washed rabbit platelet aggregation

Platelet suspension (2.5×10^8/mL with Tyrode's solution containing 1.3 mM calcium chloride) was incubated with solvent at 37 °C under stirring for appropriate time. Aggregation was monitored by light transmittance using a Chrono-log Corporation Aggregometer. The transmittance of the platelet suspension was taken as 0% aggregation and that of Tyrode's solution as 100% aggregation. The resuspension buffer for the washed rabbit platelets was Tyrodes/gelatin/Ca^{2+} pH 7.2 (Tg/Ca) which contained 0.8% NaCl, 0.02% KCl, 0.02% $MgCl_2$, 0.1% dextrose, 0.25% gelatin and 0.02% $CaCl_2$ (w/v).[22]

6.3. Measurement of extracellular LDH

The integrity of aggregated platelets in the cuvette, was evaluated by measuring the activity of the cytosolic enzyme lactate dehydrogenase (LDH) in the supernatant obtained after centrifugation of platelet suspension at 2000*g*, using an LDH kit (Sigma). Total LDH activity was determined in the platelet suspension after lysis of the platelets with 0.1% Triton X-100.[37]

6.4. FAAH assay

FAAH activity was determined using rabbit platelet homogenate that was incubated with [^3H]anandamide (20,000 cpm) "diluted" with non labeled anandamide or *t*-AEA to the desired concentration, for 15 min in 50 mM Tris–HCl pH 9 (1 mL final volume) at 37 °C. Tubes containing the reaction mixture were then transferred on ice and 0.1 mL 0.25% (w/v) BSA was added. Subsequently, 0.4 mL 40% (w/v) TCA was added and samples were centrifuged at 16000 g for 5 min at 4 °C. The supernatant containing the [^3H]ethanolamine and the pellet containing the [^3H]anandamide and arachidonic acid were separated and measured for tritium content by liquid scintillation counter.

Protein concentration was measured by the method of Lowry et al.[48]

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