Evidence for Excitatory and Inhibitory Amino Acids Participation in the Neuropharmacological Activity of Alpha- and Beta-Amyrin Acetate

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Abstract: We evaluated the neuropharmacological profile of acetylated alpha- and beta-amyrin (AcAMY) obtained by the acetylation of the isomeric mixture of alpha- and beta-amyrin isolated from *Protium heptaphyllum*. Male Swiss mice were administered with AcAMY (2.5, 5, 10 and 25 mg/kg, i.p.), and anticonvulsant (pentylenetrazole- and pilocarpine-induced convulsions), sedative (barbiturate-induced sleep and open field tests) and anxiolytic (elevated plus maze test) activities were studied. Results showed that AcAMY administered intraperitoneally or orally, protected the animals against pentylenetetrazole- but not against pilocarpine-induced convulsions. The drug increased both the latency to the 1st convulsion and the latency to death. The barbiturate-induced sleeping time was also increased, as well as the ethyl ether-induced sleeping time, confirming the sedative nature of AcAMY. The acute administration of AcAMY also produced an anxiolytic effect. After the sub-chronic administration, both sedative and anxiolytic effects were manifested, at the two higher doses. Amino acids measurements in brain areas of mice treated with AcAMY (25 mg/kg, i.p., for 7 days) showed an 89% increase in tyrosine levels, in the hippocampus. In the striatum, tyrosine and taurine were increased by 97 and 79%, respectively, while decreases in the levels of aspartate, GABA and glutamate of 72, 55 and 60%, respectively were observed. In conclusion, our results showed that AcAMY presents sedative, anxiolytic and anticonvulsant properties. Although the drug mechanism of action is not completely clarified, it seems to involve a decrease in excitatory amino acids and an increase of inhibitory amino acids. Furthermore, the GABAergic system may also play a role.

Keywords: Burseraceae, *Protium heptaphyllum*, acetate of alpha- and beta-amyrin, sedative, anticonvulsant and anxiolytic effects.

INTRODUCTION

Protium heptaphyllum March. (fam. Burseraceae), a plant common to several regions of Brazil, is popularly used for inflammation. From the resin, an isomeric mixture of pentacyclic triterpenes, alpha- and beta-amyrin, is isolated [1]. Recently, we demonstrated the presence of sedative, anxiolytic and antidepressant activities in the mixture of alpha- and beta-amyrin, that possibly involve the GABAergic as well as the noradrenergic systems [2].

The triterpenes alpha-amyrin acetate, beta-amyrin acetate and beta-amyrin were tested for their effects on the synthesis of 5-lipoxygenase products in human neutrophils. All the triterpenes reduced 5-HETE synthesis without effect on LTB_4 synthesis. The relative effects suggest that 5-HETE inhibition can explain the antiarthritic activity possessed by these compounds [3].

Although the acetyl group may be easily removed metabolically, it may alter the biological activity of the original compound. Thus, it has been shown that acetylation can increase, decrease, or not change the compounds biological activity [4]. Acetylation and deacetylation studies on triterpenes from *Dysoxylum malabaricum* and *D. beddomei* indicated that all triterpenes examined showed an increased cytotoxicity with acetylation [5]. Others [6] demonstrated that acetyl-α-boswellic acid and acetyl-11-keto-β-boswellic acid, pentacyclic triterpenes, downregulate TNF-α expression. Acetyl-boswellic acids (acetyl BA) are cytotoxic for human glioma cell lines [7], and are effective anticancer agents [8].

In the present work, the neuropharmacological profile of the alpha- and beta-amyrin acetate (AcAMY) obtained by acetylation of the isomeric mixture of alpha- and beta-amyrin from *P. heptaphyllum*, was evaluated by the open field, elevated plus maze and barbiturate-induced sleeping tests, in order to assess locomotor, anxiolytic and sedative effects. We also used two experimental models of convulsions to evaluate anticonvulsant effects. Attempting to clarify the mechanism of action, through HPLC determination, we examined the concentrations of excitatory and inhibitory amino acids, in two cerebral regions, the hippocampus and striatum of mice treated with AcAMY for 7 days.

METHODS

Animals. Male Swiss mice (20-30 g) from the Animal House of the Federal University of Ceará were used throughout the experiments. Animals were housed under standard environmental conditions (23 ± 2°C, humidity 60 ± 5%, 12 h light:12 h dark cycle), with free access to a com-

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mercial diet and water. Each animal was used only once. Control animals were administered with the suspension of 0.5% Tween 80, in distilled water used as vehicle. Before its suspension, Tween 80 was weighted in order to give a 0.5% final concentration (0.5 g in 100 mL of distilled water). All experiments were performed according to the Guide for the Care and Use of Laboratory Animals, from the US Department of Health and Human Services, Washington, DC, 1985. The project was previously approved by the Animal's Ethics Committee, of the Faculty of Medicine of the Federal University of Ceará.

Plant Material. The resin of *Protium heptaphyllum* March was collected in the area of Crato, State of Ceará, Brazil, in November 2005, and authenticated by Dr. Afrânio G. Fernandes. A voucher specimen # 28509 is deposited at the Prisco Bezerra Herbarium, Department of Biology, Federal University of Ceará, Brazil. The resin was obtained by incision made on the plant trunk.

Extraction and Isolation. The resin (20 g) was crushed with silica gel (20 g), placed in a chromatography column and eluted with the following solvents: hexane (50 mL), chloroform (50 mL), ethyl acetate (50 mL) and methanol (30 mL). The chloroform fraction (5.2 g) was repeatedly chromatographed on silica gel (30 g), and eluted with a mixture of hexane-ethyl acetate (0-100; v/v). The fractions eluted with hexane-ethyl acetate (1:1; v/v) were purified by recrystallization with ethyl ether to afford 450 mg of a mixture identified by spectroscopic data as alpha- and beta-amyrin [9]. The percentages of each component present in the mixture were determined by ^{1}H NMR spectroscopy, based on the integration measure (three times) of absorption of $\delta_{H\text{-}12}$ (5.10 and 5.16 ppm) for alpha- and beta-amyrin, respectively. The mixture was quantified as having 67% of alpha- and 33% of beta-amyrin.

Acetylation of alpha- and Beta-Amyrin. The alpha- and beta-amyrin (0.23 mmol) mixture (Fig. **1**, **1/2**) and Ac_2O/pyridine (2 mL:1 mL) were stirred at room temperature, for a period of 24 h, and washed with a copper sulfate solution (20 mL). The reaction product was extracted with diethyl ether (20 mL), followed by solvent evaporation under reduced pressure to afford 98.6 mg, meaning that 100% of the mixture of alpha- and beta-amyrin were acetylated (**1a**/**2a**, Fig. **1**). The acetylated material was first analyzed by TLC, and presented a different Rf value as compared to the starting material (**1**/**2**). The acetylated material was identified by physical and spectroscopic data as 3-*O*-Acetyl-α/β-amyrin (**1a**/**2a**). The quantification of the mixture **1a**/**2a** (3-*O*-Acetyl-α/β-amyrin) was done based on ^{1}H NMR by the ratio measure (three times) of peak absorptions: H-12 at δ_H 5.15 ppm for **1a (**alpha isomer) and 5.20 ppm for **2a** (beta isomer), and defined as 64%:36% for the **1a**/**2a** mixture. This mixture was confirmed by IR analysis to be 100% acetylated. This method identified an absorption in 1735cm^{-1} of stretch C=O for ester and the absence of the absorption of stretch O-H (**1**/**2**). This was followed by analysis of ^{13}C NMR data, where the absorption of C-3 was observed at $\delta_{C\text{-}3}$ 79.0, characteristic of **1** /**2**, and a presence of absorption at $\delta_{C\text{-}3}$ 81 for **1a** /**2a** [1], confirmed by EI-MS.

The mixture of alpha- and beta-amyrin (**1**/**2**) is a white amorphous powder, m.p. 179-181°C; IR (KBr) $\nu_{max.}$ cm^{-1} 3300, 1480 and 1050; and ^{1}H and ^{13}C NMR ($CDCl_3$) agree with literature data [1,9]. 3-O-Acetyl-α/β-amirin (**1a/2a**) is a white amorphous powder, m.p. 192 - 193.5°C; IR (KBr) $\nu_{max.}$ cm^{-1} 2949, 1735, 1654, 1370, 1247, 1025); EI-MS: m/z 468 (M^+, 8.0%); 409 (9.7); 218 (100); 203 (33.9); 189 (25); 43 (32); and ^{1}H and ^{13}C NMR are also in agreement with the literature [1, 10].

Melting points (m.p.) were determined by a digital Mettler Toledo FP90 apparatus. IR spectra were obtained on a Perkin-Elmer FT-IR Spectrum 1000. EI-MS was obtained by direct insertion on a Shimadzu spectrometer at 70 eV. NMR spectra were done in $CDCl_3$ on a Bruker Avance DRX-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer. Silica gel 60 (70-230 mesh, VETEC, Brazil) was used for column chromatography, and TLC was carried out with pre-coated silica gel sheets on polyethylene (0.20 mm, MERCK, Germany). Fractions and compounds were monitored by TLC, and detected by spraying with a solution of vanillin/perchloric acid/EtOH, followed by heating for 5 min at 100°C.

Reagents and Drugs. Pentobarbital sodium salt (98% purity), pilocarpine hydrochloride (98%), perchloric acid p.a., reagent grade ethanol, 2-mercaptoethanol (99%), o-phthadialdehyde (99%), pentylenetetrazole (99%) and all amino acids (98-99%) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Tween 80 and ethyl ether were from Vetec (Rio de Janeiro, Brazil). Diazepam 5 mg/mL ampoules from Cristália (São Paulo, Brazil) were diluted in distilled water for a 0.5 mg/mL final concentration, before use. All other drugs were of analytical grade.

Amino Acids Determination by HPLC. Mice were treated for 7 days with vehicle or AcAMY (10 and 25 mg/kg, i.p.). Thirty minutes after the last administration, animals were killed by cervical dislocation, and the hippocampus and striatum were immediately removed on ice. After dissection, each area was weighted and conserved at - 70ºC for use. A 10% homogenate was then prepared in 0.1 M perchloric acid, and derivatized with the same volume of the o-phthadialdehyde reagent solution. After a 60 s reaction period, a 20 μL aliquot was injected into the HPLC with a 4 mm C18 reverse phase column (Shimadzu, Japan). The mobile phase A (pH 6.95) was composed of sodium acetate (0.1 M), methanol (6%) and tetrahydrofuran (1.5%), and flowed at 1.0 mL/min in a 30 min gradient. The mobile phase B consisted of 100% methanol [11]. The fluorometric detector (model RF 535 from Shimadzu, Japan) had the excitation and emission wavelengths set at 370 and 450 nm, respectively. In the hippocampus and striatum, the concentrations of free amino acids were determined by comparison of their peak areas with those of standard amino acids injected into the HPLC column, at the day of the experiment. Calculations were, then, carried out on the basis of the standard amino acids known concentrations. The results were expressed as μg/g of wet tissue.

PTZ and Pilocarpine-Induced Convulsion Test. Animals were pretreated with AcAMY (2.5, 5, 10 or 25 mg/kg), 30 min (i.p.) or 1 h (p.o.), before the administration of PTZ (100 mg/kg, i.p.) or pilocarpine (400 mg/kg, i.p.). After that, animals were placed into individual cages and observed for 30 min. The latency time to the first convulsion and the death latency were the observed parameters [12]. Mice that did not show clonic or tonic convulsions, within 30 min of

PTZ or pilocarpine administration, were considered protected. The control group received 0.5% Tween 80.

Open Field Test. The open field arena was made of acrylic (transparent walls and black floor, 30 x 30 x 15 cm), divided into sixteen squares of equal areas. The open field was used to evaluate the exploratory activity of the animal [13]. The test was performed inside a room with controlled temperature (23°C), dim light and only one observer. Each animal was placed at the center of the arena, and allowed to explore it freely. The observed parameters were: ambulations (the number of squares crossed with all four paws), numbers for grooming and rearing, recorded for the last 5 min of the 6 min testing period. After each animal exposure, the apparatus was washed with a 5% alcohol/water solution (v/v), before using it again.

Elevated Plus Maze. This test has been widely validated for measuring anxiolytic and anxiogenic-like activities, in rodents [14]. The apparatus consisted of two opposite open arms (30 x 5 cm), crossed by two closed arms of the same dimensions, with 25 cm high walls. The arms were connected to a 5 x 5 cm central square. The apparatus was elevated 45 cm above the floor, in a dimly illuminated room. Mice were placed individually in the center of the maze, facing an enclosed arm, and the number of entries and time spent on the open arms were recorded for the next 5 min. Entry into an arm was defined as the animal placing all four paws onto the arm. After each test, the maze was carefully cleaned up with a wet paper (10% ethanol solution).

Sleeping Time Induced by Pentobarbital or Ethyl Ether. In this test, performed according to a previously described method [15], the sleep was induced by the intraperitoneal administration of 40 mg/kg pentobarbital, in mice pretreated 30 min before with AcAMY or vehicle. The sleep duration (min) of each animal was determined as the period for recovering the righting reflex, considered as the hypnosis endpoint. In the case of ethyl ether-induced sleeping time, the mice were treated with AcAMY or vehicle, and 30 min later each animal was placed under a glass funnel (500 mL capacity) with an ethyl ether saturated atmosphere. After sleeping induction, the animals were removed from the chamber, and the sleeping duration (s) was recorded, as described above.

Statistical Analysis. All data represent mean ± S.E.M. values. The data were analyzed by analysis of variance (ANOVA). Whenever ANOVA was significant, further multiple comparisons were undertaken, using the Dunnett or Tukey t-tests. All analyses were performed using the software Prism 3.0 for Windows. The level of statistical significance adopted was $p < 0.05$.

RESULTS

In the PTZ-induced convulsions model, alpha- and beta-amyrin acetate (5, 10 and 25 mg/kg, i.p.) significantly increased the latency time to the first convulsion, in 42, 61 and 79%, as compared to controls. A similar increase was observed after oral administration with the doses of 10 (57%) and 25 mg/kg (77%). AcAMY also increased in 59, 97 and 127% the latency time to death, after intraperitoneal administration of 5, 10 and 25 mg/kg, respectively, while increases in this parameter of the order of 75 and 93% were observed after oral administration (10 and 25 mg/kg, respectively), as shown in Table **1**. In the pilocarpine-induced convulsions model, AcAMY at both doses was unable to alter the latency time to the first convulsion or the latency to death, unless after a higher dose (50 mg/kg, i.p.) was administered, as shown in Table **2**.

AcAMY significantly increased in 37 and 35% the barbiturate-induced sleeping time, after its administration at the doses of 10 and 25 mg/kg, i.p., respectively. After the oral administration and at the same doses, AcAMY also increased the sleeping time in 54 and 67%, respectively. AcAMY was also tested on the ethyl ether-induced sleeping time, in order to clarify the putative hypno-sedative effect of the drug. The prolongation of barbiturate-induced sleeping time may be due to pharmacokinetic interactions, what is not the case with ethyl ether. We showed that AcAMY also significantly increased in 97 and 162% the ethyl ether-induced hypnosis (Table **3**), confirming the AcAMY sedative action.

AcAMY, at the doses of 2.5, 5, 10 e 25 mg/kg/i.p., acutely administered 30 min before the experiment, did not alter the mice locomotor activity or the number of rearing, as compared to controls. However, significant decreases of the

Table 1. Anticonvulsant Effect of AcAMY on the PTZ-Convulsion Model in Mice

Group	Latency to the 1st Convulsion (s)	Latency to Death (s)
Control, i.p.	53.2 ± 1.9 (25)	185.3 ± 14.5 (20)
AcAMY 2.5 mg/kg, i.p.	61.2 ± 8.1 (8)	238.3 ± 54.9 (8)
AcAMY 5 mg/kg, i.p.	75.3 ± 16.9 (8)	294.8 ± 60.8 (8)
AcAMY 10 mg/kg, i.p.	85.9 ± 6.2* (16)	364.9 ± 43.5* (16)
AcAMY 25 mg/kg, i.p.	95.3 ± 10.4* (10)	420.9 ± 31.9** (10)
DZP 1 mg/kg, i.p.	97.1 ± 7.3* (8)	474.1 ± 38.3** (8)
Control, p.o.	57.4 ± 4.0 (7)	193.7 ± 37.3 (6)
AcAMY 10 mg/kg, p.o.	90.0 ± 9.2 (7)	338.6 ± 49.1* (8)
AcAMY 25 mg/kg, p.o.	101.7 ± 16.1* (7)	373.4 ± 42.8* (7)
DZP 1 mg/kg, p.o.	105.1 ± 10.1* (7)	381.4 ± 44.1* (7)

Data are means ± S.E.M of the number of animals in parentheses. * $p < 0.05$, ** $p < 0.01$; ANOVA followed by Dunnett as the *post-hoc* test, as compared to the control group (treated with 0.5% Tween 80).

Table 2. Anticonvulsant Effect of AcAMY on the Pilocarpine-Induced Convulsions in Mice

Group	Latency to the 1st Convulsion (s)	Latency to Death (s)
Control, i.p.	190.0 ± 19.2 (12)	321.8 ± 38.4 (10)
AcAMY 10 mg/kg, i.p.	192.6 ± 16.7 (8)	282.4 ± 29.0 (8)
AcAMY 25 mg/kg, i.p.	222.6 ± 28.8 (9)	395.0 ± 42.5 (8)
AcAMY 50 mg/kg, i.p.	326.0 ± 25.8**(11)	529.4 ± 39.5**(11)
DZP 5 mg/kg, i.p.	485.0 ± 14.8***(12)	735.4 ± 29.5***(10)

Data represent means ± S.E.M of the number of animals in parentheses. ** $p < 0.01$ and *** $p<0.001$, ANOVA followed by Dunnett as the *post hoc* test, as compared to the control group (treated with 0.5% Tween 80).

order of 38, 62 and 65% were observed in grooming behavior, after the administration of 5, 10 and 25 mg/kg, respectively. The sub-chronic administration of AcAMY for 7 days, at the doses of 10 and 25 mg/kg, i.p., produced significant reduction in the number of crossings (25 and 26 %, respectively). AcAMY also decreased the number of grooming (42 and 54%) as well as the number of rearing (45 and 28%). Diazepam (1 mg/kg, i.p.), used as a positive control, significantly decreased in 56, 58 and 27% the numbers of crossing, grooming and rearing, respectively (Table **4**).

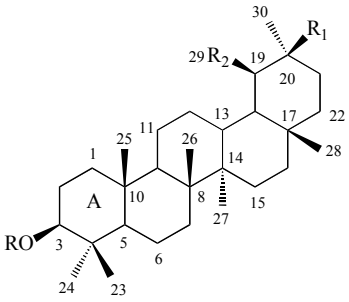

Fig. (1). (**1**) R = R_1 = H, R_2 = CH_3 (α-amyrin); (**2**) R = R_2 = H, R_1 = CH_3 (β-amyrin); (**1a**) R = $COCH_3$, R_1 = H, R_2 = CH_3 (Acetate of α-amyrin); (**2a**) R = $COCH_3$, R_1 = CH_3, R_2 = H (Acetate of β-amyrin).

AcAMY presented a significantly anxiolytic activity, at the doses of 5, 10 and 25 mg/kg, i.p., as assessed by the elevated plus maze test, where it increased the number of entrances in the open arms (NEOA) by 33, 39 and 82%, as well as the time spent in the open arms (TSOA) by 29, 88 and 97%, respectively, as compared to controls. AcAMY, at the doses of 10 and 25 mg/kg, i.p., also decreased by 42 and 46% the time spent in the closed arms (TSCA), The number of entrances in the closed arms (NECA) was altered only with the higher dose (a 38% decrease at the dose of 25 mg/kg), as compared to controls.

AcAMY, administered daily and intraperitoneally for 7 days, altered all parameters studied. Thus, NEOA was increased by 24 and 68%, at the doses of 10 and 25 mg/kg, while TSOA was increased by 50 and 105%, respectively. On the other hand, NECA was decreased by 30 and 49%, and the TSCA was also decreased by 36 and 61%, respectively, with the same doses. Diazepam (1 mg/kg, i.p.), used as a positive control, significantly increased NEOA and TSOA by 54 and 60%, and decreased NECA and TSCA by 45 e 60 %, respectively. Data from the elevated plus maze experiment are presented in Table **5**.

Only the 25 mg/kg AcAMY dose (i.p., for 7 days) was effective in modifying amino acids concentrations. Referring to both cerebral areas (striatum and hippocampus), there was an 89% increase of tyrosine levels in the hippocampus, whereas other amino acids were unaltered. In the striatum, alterations were more evident and complex. Thus, tyrosine and taurine were increased by 97 and 79%, respectively, while there was a decrease in aspartate, GABA and glutamate of the order of 72, 55 and 60%, respectively. Triptophan and glycine did not change in this brain area (Fig. **2**).

DISCUSSION

We recently showed [2] that the mixture of two pentacyclic triterpenes, alpha- and beta-amyrin, is highly active in the central nervous system, presenting anxiolytic and antidepressant activities, when acutely administered to mice, as assessed by the elevated plus maze and forced swimming tests. Single or subchronic administrations of AcAMY, the acetylated mixture, were used at doses ranging from 2.5 to 25 mg/kg, i.p.

We showed that AcAMY exhibits a potent anticonvulsant effect, in the model of PTZ-induced convulsions, but not in pilocarpine-induced convulsions, unless at a higher dose range. The pentylenetetrazole kindling model is characterized by an increased susceptibility to seizures, following injection of initially sub-convulsive doses of PTZ, culminating in generalized tonic-clonic seizures. PTZ is a selective blocker of the chloride ionophore complex to the $GABA_A$ receptor and, after repeated or single dose administrations, leads to a decrease in GABAergic function [16] and to the stimulation and modification of density or sensitivity of different glutamate receptor subtypes, in many brain regions [17]. PTZ may also trigger a variety of biochemical processes, including the activation of membrane phospholipases, proteases, and nucleases [18]. In the PTZ-induced convulsions, AcAMY administered by the oral or intraperitoneal routes showed a significant increase in the latency to the first convulsion, as well as an increase in the latency to death.

Recently, pilocarpine was reported to significantly increase brain malondialdehyde levels, a marker of lipid peroxidation [19]. An increase was also observed in catalase and superoxide dismutase activities, two important antioxidant enzymes. Furthermore, these authors also reported that pilocarpine induces oxidative damage, and increases antioxidant enzyme activities and expression in the brain cortex, leading to excitotoxicity and cell death [20]. In the model of

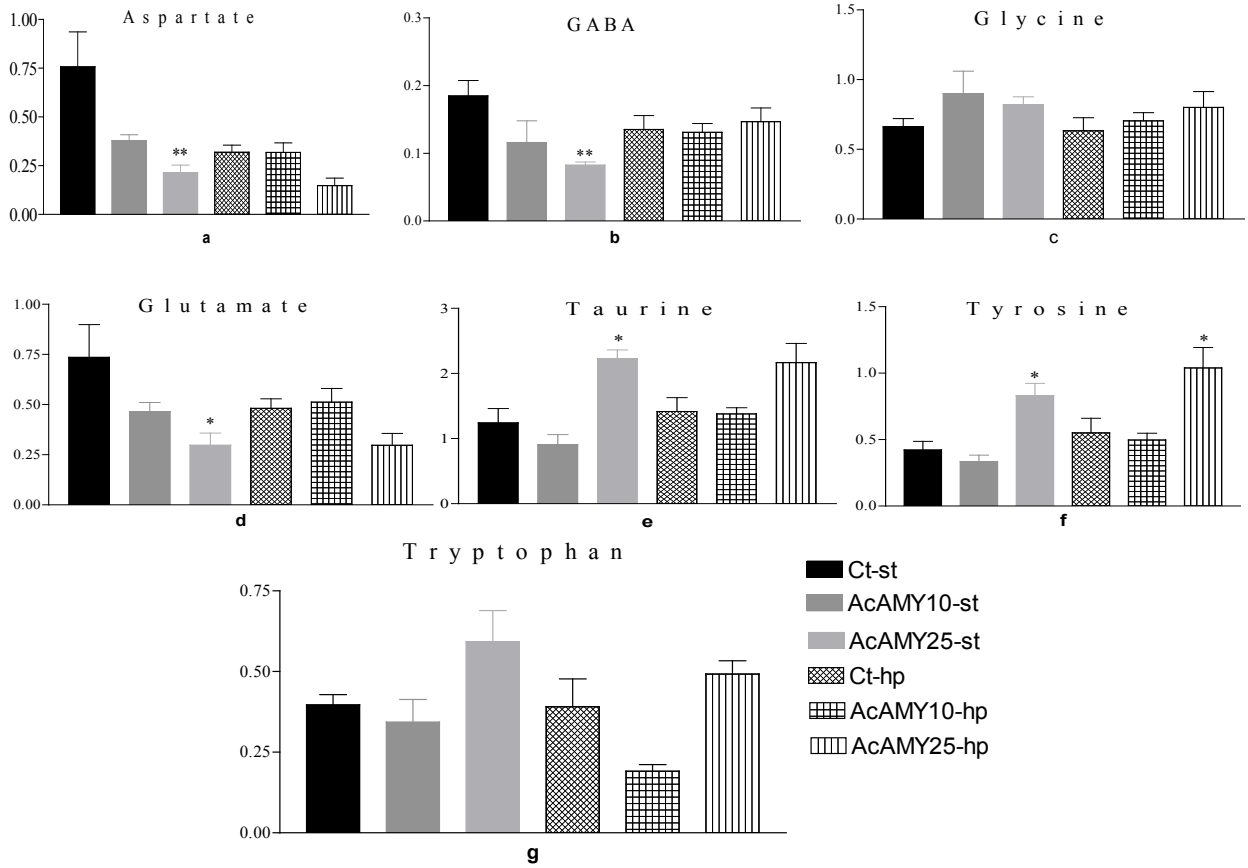

Fig. (2). Animals were treated for 7 days and killed by decapitation, 30 min after the last dose. The striatum and hippocampus were dissected and kept at - 70ºC for amino acid determination (µg/g de tecido) by HPLC. Each column represents the mean ± S.E.M of 5-14 animals per group. * $p < 0.05$ and ** $p < 0.01$; *** $p < 0.01$, ANOVA followed by Tukey as the *post hoc* test, as compared to the control group (treated with 0.5% of Tween 80). Legends: Ct = control, AcAMY = alpha- and beta-amyrin acetate, st = striatum and hp = hippocampus. 10 and 25 = doses of 10 and 25 mg/kg, i.p.

Table 3. AcAMY Increases (A) Pentobarbital- and (B) Ethyl Ether-Induced Sleeping-Times in Mice

Group	Sleep Latency
A. Pentobarbital-Induced Sleeping Time (min)	
Control, i.p	45.7 ± 6.0 (10)
AcAMY 10 mg/kg, i.p.	71.9 ± 5.0* (11)
AcAMY 25 mg/kg, i.p.	76.7 ± 5.3** (10)
DZP 0.5 mg/kg, i.p.	202.1 ± 7.8**(10)
Control, p.o.	48.5 ± 4.1 (13)
AcAMY 10 mg/kg, p.o.	74.6 ± 7.4** (14)
AcAMY 25 mg/kg, p.o.	80.9 ± 4.0** (14)
DZP 0.5 mg/kg, p.o.	180.4 ± 3.8** (10)
B. Ethyl ether-induced sleeping time (s)	
Control, i.p.	64.9 ± 5.4 (10)
AcAMY 10 mg/kg, i.p.	128.4 ± 7.6* (10)
AcAMY 25 mg/kg, i.p.	170.3 ± 15.8*** (10)
DZP 1mg/kg, i.p.	230.8 ± 26.2*** (10)

Data represent means ± S.E.M of the number of animals in parentheses. $p < 0.05$, ** $p < 0.01$ and *** $p<0.001$, ANOVA followed by Dunnett as the *post hoc* test, as compared to the control group (treated with 0.5% Tween 80).

pilocarpine-induced convulsions, AcAMY did not alter the latency time to the first convulsion, nor the latency time to death, unless at its highest dose (50 mg/kg, i.p.).

In the barbiturate-induced sleeping time, AcAMY was able to increase the animal's sleeping time, after oral or intraperitoneal administration of both doses utilized. Drugs known to decrease the sleep latency or increase sleep time are considered as CNS depressors [21], and this effect was confirmed by the prolongation of the ethyl ether sleeping time by AcAMY. Unlikely ethyl ether, the prolongation of the pentobarbital-induced sleeping time may be due to pharmacokinetic interactions. Thus, some drugs can interact with the cytochrome P450 complex and promote a potentiation of the CNS depressant effect of barbiturates, without exerting any central action. The inhibitory neurotransmitter GABA is the molecular target of sedative/hypnotic drugs, and several of them bind to the $GABA_A$ receptor in the brain [22]. In the present case, similarly to what we showed before for AMY [2], mechanisms involving GABAergic neurotransmission will probably play a role in the potentiation of barbiturate-induced sleeping time, seen with AcAMY.

In order to evaluate the sedative activity of AcAMY, the open field test was used. We showed that AcAMY was ineffective when acutely administered by the oral or intraperito-

Table 4. Effect of the Acute and Sub-Chronic Intraperitoneal Administration of AcAMI (2.5 to 25 mg/kg) on the Open Field Test in Mice

Group	Number of Crossings	Grooming	Rearing
Control (30 min)	42.0 ± 1.8 (19)	6.2 ± 1.2 (20)	29.9 ± 2.1 (18)
AcAMY 2.5 (30 min)	36.7 ± 2.4 (7)	5.0 ± 0.8 (6)	31.8 ± 4.9 (6)
AcAMY 5.0 (30 min)	32.0 ± 1.5* (7)	3.8 ± 0.8 (6)	27.2 ± 3.1 (6)
AcAMY 10 (30 min)	45.6 ± 3,9 (7)	2.3 ± 0.4 (6)	32.8 ± 2.1 (6)
AcAMY 25 (30 min)	37.4 ± 1.8 (7)	2.2 ± 0.5 (6)	31.5 ± 3.0 (6)
DZP 1 (30 min)	31.4 ± 1.3* (7)	1.4 ± 0.5* (6)	20.5 ± 2.0* (6)
Control (7 d)	53.6 ± 2.5 (12)	8.2 ± 0.9 (11)	35.6 ± 1.3 (10)
AcAMY 10 (7 d)	40.2 ± 1.3** (8)	4.3 ± 0.6** (9)	19.6 ± 2.7* (9)
AcAMY 25 (7 d)	39.4 ± 3.9** (5)	3.8 ± 0.8** (6)	25.8 ± 2.2* (6)
DZP 1 (7 d)	23.8 ± 0.9** (10)	3.4 ± 0.8** (10)	26.0 ± 2.0* (6)

Data represent means ± S.E.M of the number of animals in parentheses. * $p < 0.05$, ** $p < 0.01$; ANOVA followed by Dunnett as the *post hoc* test, as compared to the control group (treated with 0.5% of Tween 80).

neal routes, at the two tested doses (10 and 25 mg/kg). Recent studies [23] reported that the mixture of alpha- and beta-amyrin (3 to 200 mg/kg, p.o.) neither significantly altered the pentobarbital sleeping time, nor impaired the ambulation or motor coordination, in the open field and rota rod tests, indicating the absence of sedative or motor abnormalities, in mice. In the present work, we observed that, after sub-chronic administration (once daily for 7 days), AcAMY presented a sedative activity, at both doses, altering all the parameters studied in the open field test, such as numbers of crossing, rearing and grooming, similarly to diazepam used as a positive control. Our results agree with earlier data [24] demonstrating a significant decrease of spontaneous locomotor activity of mice treated with beta-amyrin palmitate, at doses ranging from 2.5 to 10 mg/kg, and indicating a sedative property of this amyrin ester.

The evaluation of the anxiolytic property of AcAMY was carried out with the elevated plus maze test. According to Barrett, 1991 [25], an anxiolytic effect is suggested when the drug increases entries into the open arms, without altering the total number of arm entries. Mice acutely or sub-chronically treated with AcAMY altered all the parameters studied, and significantly increased the number of entrances as well as the time spent in the open arms, indicating a potent anxiolytic activity. We also showed recently [2] that the alpha- and beta-amyrin mixture exerts potent sedative, anxiolytic and antidepressant activities that probably involve GABAergic as well as noradrenergic mechanisms. We can not exclude that alterations in these neurotransmitter systems also play a role in the present work.

The brain extracellular concentration of amino acids, such as glutamate, glycine, aspartate and GABA, has a particular importance in pathological processes, including epi-

Table 5. Effect of the Intraperitoneal Administration of AcAMY on the Elevated Plus Maze Test in Mice

Group	NEOA	NECA	TSOA	TSCA
30 min				
Control	4.4 ± 0.2 (20)	7.6 ± 0.4 (20)	88.5 ± 7.3 (20)	157.7 ± 6.8 (20)
AcAMY 2.5	4.5 ± 0.7 (8)	7.5 ± 1.2 (8)	81.8 ± 12.2 (8)	166.4 ± 6.8 (8)
AcAMY 5	5.9 ± 0.8 (8)	6.8 ± 0.8 (8)	114.3 ± 19.1 (8)	137.1 ± 16.3 (8)
AcAMY 10	6.1 ± 0.5(8)	5.9 ± 0.5 (9)	166.5 ± 9.7** (8)	91.6 ± 7.5** (9)
AcAMY 25	8.0 ± 0.5** (8)	4.6 ± 0.5** (9)	174.3 ± 12.5** (10)	85.8 ± 9.9** (9)
DZP	9.4 ± 0.4** (8)	4.5 ± 0.5** (10)	187.4 ± 10.9** (8)	79.2 ± 9.2** (8)
7 days				
Control	5.4 ± 0.4 (8)	8.8 ± 0.5 (10)	103.4 ± 11.3 (9)	148.2 ± 9.1 (10)
AcAMY 10	8.3 ± 0.6* (7)	4.9 ± 0.7* (8)	165.1 ± 20.5** (6)	99.9 ± 17.8* (8)
AcAMY 2	6.7 ± 0.9 (6)	6.2 ± 0.9* (6)	154.7 ± 13.6* (6)	95.4 ± 8.7* (5)
DZP	9.0 ± 0.8** (8)	4.5 ± 0.6** (8)	212.0 ± 8.4** (7)	57.5 ± 10.9**(8)

NEOA = number of entries in the open arms; NECA = number of entries in the closed armas; TSOA = time spent in the open arms; TSCA = time spent in the closed arms. Data represent means ± S.E.M of the number of animals in parentheses. * $p < 0.05$, ** $p < 0.01$; ANOVA followed by Dunnett as the *post hoc* test, as compared to the control group (treated with 0.5% of Tween 80).

lepsy, stroke, and other neurodegenerative disorders [26]. Steroid hormones and their metabolites, such as pregnenolone and allopregnenolone, are neuroactive, due to their ability to modulate activity at glutamate and $GABA_A$ receptors, respectively [27]. AcAMY is a triterpene chemically similar to steroids. Furthermore, the anticonvulsant, sedative and anxiolytic effects shown by AcAMY in the present study may reflect the observed alterations in amino acids contents. The increase of tyrosine in both brain areas may be related to the antidepressant activity, as that presented by AMY before esterification. In a recent work [2], we reported that this effect involves the noradrenergic system, but not the serotonergic one. This agrees with the fact that, in the present work, we did not find any alterations in tryptophan levels, the precursor of serotonin.

More than half of all CNS neurons utilize glutamate and aspartate, main excitatory neurotransmitters. Glutamate is responsible for 75% of the CNS depolarizing activity, and has been implicated in the initiation and spread of seizure activity [28]. Thus, AcAMY decreased not only glutamate and aspartate concentrations, but also GABA concentrations (GABA is synthesized from glutamate). An earlier study [29], performed with synaptosomes from brain tissue of patients with temporal lobe epilepsy, showed reductions in glutamine and GABA concentrations as well as the glutamine/glutamate ratio. These data suggest that alterations in the balance between excitatory and inhibitory amino acids may be involved in the expression of epilepsy. We also showed that, after AMY treatment, striatal taurine concentrations were increased, whereas no change was seen in the glycine content. Taurine, an amino acid that abounds in the brain, has been implicated in inhibitory neuromodulation and osmoregulation. Thus, increased taurine levels lead to a reduced frequency in epileptic crises, membrane stabilization and balance in glutamate levels, as assessed by animal models [30].

Furthermore, expressions of glutamate and GABA were shown to be inhibited in the hippocampus of rats submitted to chronic kindling by saikosaponins which are triterpenes, as alpha- and beta-amyrin. Also, ginsenoside Rh(2) inhibited the sodium channel function and sodium channel-activated release of neurotransmitters, including glutamate and GABA, in synaptic fractions from mouse brain [31, 32].

Recent studies showed that synthetic derivatives of alpha- and beta-amyrin, including their acetates, present mostly a qualitatively different pharmacological profile what agrees with our data [33, 34].

Altogether, we concluded that the mechanisms of action of the sedative, anticonvulsant and anxiolytic effects of AcAMY probably involve a decrease in excitatory amino acids contents (glutamate and aspartate) and an increase in the concentration of taurine, considered as an inhibitory amino acid. Furthermore, the GABAergic neurotransmission, but not the cholinergic one, may also play a role. However, other studies have to be carried out, in order to further clarify the mechanism of action of AcAMY.

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