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Bioorganic & Medicinal Chemistry 16 (2008) 3377–3386
Bioorganic & Medicinal Chemistry

Synthetic derivatives of the α- and β-amyrin triterpenes and their antinociceptive properties

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Received 19 September 2007; revised 3 December 2007; accepted 4 December 2007
Available online 8 December 2007

Abstract—Fifteen different derivatives of an α- and β-amyrin mixture were synthesized by acylation with appropriate anhydride or acid chlorides and oxidation in the presence of *tert*-butyl chromate or PCC. The molecular structures of the obtained compounds were confirmed by means of IR and ^{1}H NMR spectra. The compounds were screened for antinociceptive activity using the acetic acid pain model. The 3-*O*-acyl derivatives α- and β-amyrin propionate **4**, α- and β-amyrin hexanoate **6**, and α- and β-amyrin octanoate **7** were found to be the most active compounds of the series. In addition, we also have found that α- and β-amyrin octanoate **7** was able to reduce acetic acid-induced abdominal constriction when administered by oral route. Furthermore, this compound reduced the nociceptive response induced by intraplantar injection of formalin.

1. Introduction

Pentacyclic triterpene compounds have been characterized by their wide range of pharmacological activities that include antioxidant, anti-allergic, anti-inflammatory, anticancerogenic, antiviral, antibacterial, antinociceptive, gastroprotective, hepatoprotective, cytotoxic, and cardioprotective properties.[1–13] In the past 10 years, there has been a boom in natural triterpenoid derivative synthesis, providing great advances in the pharmacological study of these compounds.

In this regard, α- and β-amyrin pentacyclic triterpenes, which occur naturally and are widespread in plants of the genus *Protium*, have been reported as possessing anti-inflammatory, antinociceptive, hepatoprotective, and anti-allergic properties.[4,6,7] These pharmacological activities seem to involve a reduction in mast cell degranulation and inflammatory cell migration.[4,9] In addition, it has been proposed that the intracellular messengers protein kinase A and C play a role in the antinociceptive effect of amyrin.[6] Reinforcing this idea, previous studies carried out with resinous exudates (resin) collected from *Protium heptaphyllum*, a folk remedy used in Brazil to treat inflammatory conditions and to hasten wound repair,[14] demonstrated that this resin possesses anti-inflammatory, gastroprotective, anti-allergic, and antinociceptive properties. These effects have since been attributed specifically to the presence of the pentacyclic triterpenoids α- and β-amyrin, maniladiol, and brein.[4,15]

The α-amyrin triterpene possesses a basic skeleton of the ursane type and the β-amyrin triterpene possesses a basic skeleton of the oleanane type, and the only difference between them is the methyl position in the E-ring. On examining the amyrin pentacyclic triterpenoid structure, a possible explanation for its valuable pharmacological activity is the presence of the perhydro-aromatic ring systems, which render the molecule very similar to steroid drugs.[4] On the other hand, small modifications to the molecule structure could inhibit its efficacy, since structural alteration of the molecule can alter the absorption, distribution, and excretion of the compound (pharmacokinetics), as well as the specificity of the

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Keywords: Structural modifications; Triterpene mixture; Amyrin derivatives; Antinociceptive activity.

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doi:10.1016/j.bmc.2007.12.008

molecule toward some targets (pharmacodynamics). In the latter case, we may find a more efficient substance with fewer side effects. The finding that α- and β-amyrins are natural antinociceptive compounds is of great importance due to their being widespread in the plant kingdom, easily available in huge amounts, and very cheap. Taking this into account, the aim of this study was to determine the effects of 15 semi-synthetic derivatives of an α- and β-amyrin triterpene mixture on acetic acid-induced visceral pain.

2. Results and discussion

2.1. Chemistry

The efforts to obtain new antinociceptive α- and β-amyrin derivatives were focused on structural modifications at positions C-3 and C-11. The synthetic routes are outlined in the scheme in Figure 1. Treatment of the α- and β-amyrin mixture with acetic anhydride/pyridine or RCOCl/pyridine afforded 3-*O*-acyl derivatives **2**–**13** in 45–95% yields. Oxidation of the α- and β-amyrin mixture **1** and its acetyl derivative **3** in the presence of *tert*-butyl chromate reagent or PCC in trichloromethane at reflux temperature gave 3-oxo and 11-oxo α- and β-amyrin derivatives **14**–**16** in 65–80% yields.

The infrared spectrum of compound **2** showed an absorption band at 1715 cm^{-1} and those for 3-*O*-acyl α- and β-amyrin **3**–**13** at 1729–1734 cm^{-1}, which are attributed to an ester carbonyl group. Characteristic absorption bands of the carbonyl group were observed for the synthesized compounds **14**–**16**.

In the ^{1}H NMR spectra of the synthesized compounds, signals with appropriate multiplicities for different types of hydrogens were observed as expected. The multiplets at δ 4.5 ppm are due to deshielding of the H-3 proton in the 3-*O*-acyl α- and β-amyrin derivatives. Compounds **15** and **16** showed the characteristic singlets of the deshielded olefinic-type hydrogen (H12) in the range 5.4–5.6 ppm due to the C-11 carboxy group.

2.2. Pharmacological investigations

In this study, we analyzed the antinociceptive response produced by administration of α- and β-amyrin derivatives. To this end, we chose the model of nociception induced by intraperitoneal injection of acetic acid, since the α- and β-amyrin mixture has been found to cause consistent antinociception in this model.[7] Moreover, the acetic acid-induced writhing reaction in mice, described as a typical model of inflammatory pain, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents.[16] In addition, it has been suggested that nociceptive activity of acetic acid may be due to the release of endogenous inflammatory mediators (e.g., kinins, substance P, prostanoids, and cytokines) and it is sensitive to non-steroidal anti-inflammatory drugs and to narcotics and other centrally acting drugs.[16–19] This method shows good sensitivity, as it allows for the effects of weak analgesics, but shows poor specificity because the abdominal writhing response may be suppressed by muscle relaxants and other drugs, leaving room for the misinterpretation of results. This can be avoided by complementing the test with other models of the nociception and locomotor activity test. For this reason, the most potent compound tested, α- and β-amyrin octanoate **7**, was examined for its action on formalin-induced pain and locomotor activity in the open-field test. We observed that α- and β-amyrin octanoate **7** did not significantly interfere with motor activity and produced marked suppression of the nociceptive response caused by acetic acid and formalin. In addition, the formalin-induced nociceptive behavior is characterized by stimulation of primary sensory afferent fibers inducing licking and biting of the footpad injected, following formalin injection. The initial nociceptive response normally peaked 5 min after formalin injection (early-phase) and 15–30 min after formalin injection (late-phase), representing the tonic and inflammatory pain responses, respectively.[20–22] Finally, to determine the accuracy of the bioavailability of the α- and β-amyrin derivatives, we administered the most potent compound, α- and β-amyrin octanoate **7**, by oral route and tested it against the acetic acid-induced visceral pain.

Intraperitoneal treatment of animals with all the synthesized compounds individually did not produce any behavioral alteration (result not shown). However, all structural derivatives from the α- and β-amyrin mixture tested exhibited a significant and dose-dependent inhibition of the acetic acid-induced abdominal constrictions. Morphine, used as a comparative substance, also inhibited the acetic acid-induced abdominal constrictions in a dose-dependent manner. The calculated mean ID_{50} (95% confidence limits) and inhibition values are shown in Table 1.

2.3. Pharmacological results

All the tested derivatives showed an interesting antinociceptive activity in the acetic acid pain model. Most of the compounds were less active than the starting material, the α- and β-amyrin mixture, and showed the same degree of potency. However, the 3-*O*-acyl derivatives α- and β-amyrin propionate **4**, α- and β-amyrin hexanoate **6**, and α- and β-amyrin octanoate **7** were found to be the most active compounds of the series. The antinociceptive activity of compounds **4**, **6**, and **7** was found to be approximately 2.1, 1.7, and 336 times more potent compared to that of the α- and β-amyrin mixture. On the other hand, the 2-carboxybenzoyl α- and β-amyrin derivative showed a more significant decrease in the antinociceptive activity, although it was 4.6 times more potent than aspirin. The order of potency for the compounds tested was octanoyl > hexanoyl > propionyl > 3-acetyl-11-oxo > decanoyl > palmitoyl > 3-oxo > acetyl > benzoyl > butyryl > *p*-bromobenzoyl > formyl > 3,11-dioxo > *p*-chlorobenzoyl > 3-carboxybenzoyl. When compared to standard commercially available aspirin, all drugs showed higher potency. The compounds

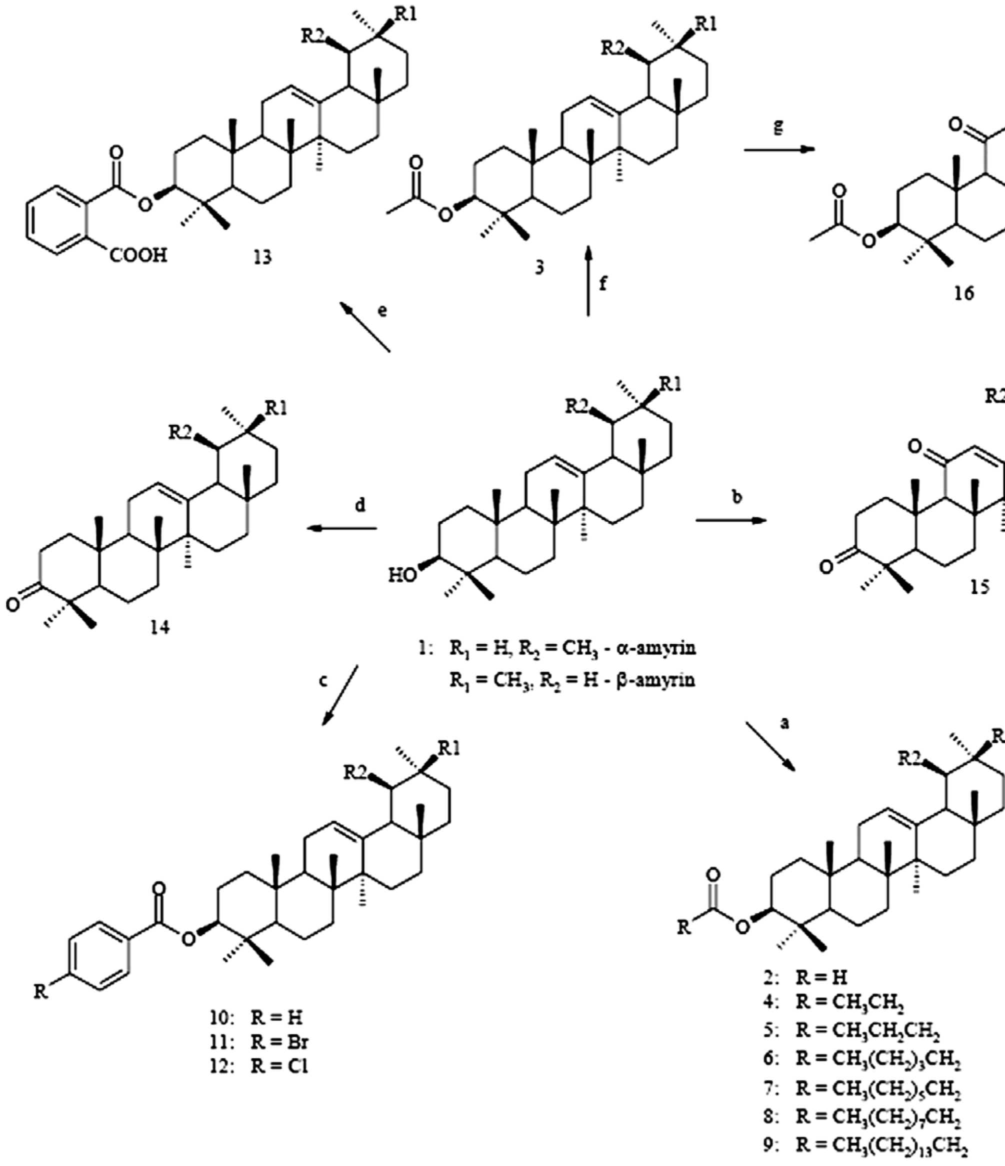

Figure 1. Synthetic scheme of α-amyrin ($R_1 = H$, $R_2 = CH_3$) and β-amyrin ($R_1 = CH_3$, $R_2 = H$) mixture derivatives. (a) Acyl chloride, Py, reflux; (b) *tert*-butyl chromate, $CHCl_3$, $(AcO)_2O$, AcOH, reflux during 6 h; (c) acyl chloride, Py, reflux; (d) PCC, CH_2Cl_2, rt; (e) phthalic anhydride, ethyl acetate, DMAP, reflux; (f) $(AcO)_2O$, ethyl acetate, DMAP, reflux; (g) *tert*-butyl chromate, $CHCl_3$, $(AcO)_2O$, AcOH, reflux during 6 h.

were around 4.6–24,000 times more potent than aspirin. On the other hand, when compared to morphine, the α- and β-amyrin propionate, α- and β-amyrin hexanoate, and α- and β-amyrin octanoate derivatives were found to be more effective in this pain model. These compounds were, respectively, 2.7, 2.2, and 427 times more potent than morphine.

As mentioned above, α- and β-amyrin octanoate **7** was the most potent amyrin derivative tested. When evaluated using the formalin-induced nociceptive response, the ip administration of α- and β-amyrin octanoate **7**, 30 min prior to i.pl. injection of formalin, decreased the licking behavior in mice in the inflammatory (late) phase of the test. The calculated mean of the inhibitory dose 50 (ID_{50}) was 5.36 (3.98–7.23) mg/kg and inhibition was 89 ± 6% at 30 mg/kg. On the other hand, the α- and β-amyrin octanoate **7** did not reduce the neurogenic (early) phase of formalin-induced nociceptive response (Fig. 2A and B). This result corroborates that obtained using the acetic acid-induced visceral pain model. Furthermore, in both models, in the inflammatory phase following formalin and acetic acid injection, the release of several inflammatory mediators was induced at peripheral levels with an increase in the release of excitatory neurotransmitters in the dorsal horn of the spinal medulla.[20,21,23,24]

In addition, we assayed the effect of α- and β-amyrin octanoate **7**, when administered by oral route, on acetic acid-induced visceral pain, because the oral route is the main site of medicament entry. Also, the way in which drugs enter the body is important in determining the final effect of compounds.[25,26] The oral administration of α- and β-amyrin octanoate **7** (1–30 mg/kg, po), 60 min prior to acetic acid injection, significantly reduced the

Table 1. Antinociceptive effect of structural derivatives from triterpenoid amyrin on the acetic acid-induced visceral pain in mice

Compound	ID_{50} (mg/kg)	ID_{50} (μmol/kg)	Inhibition (%)
1[a]	0.79 (0.63–1.01)	1.85	84 ± 3
2	2.93 (2.29–3.74)	6.45	94 ± 3
3	2.65 (2.24–3.13)	5.66	80 ± 5
4	0.42 (0.26–0.68)	0.87	95 ± 2
5	2.81 (1.82–4.35)	5.66	87 ± 6
6	0.54 (0.30–0.97)	1.04	95 ± 3
7	0.003 (0.001–0.005)	0.0055	85 ± 5
8	2.44 (1.82–3.26)	4.20	96 ± 3
9	2.60 (2.11–3.19)	3.91	80 ± 6
10	2.84 (2.29–3.52)	5.26	94 ± 3
11	2.86 (1.82–4.50)	4.69	93 ± 3
12	5.36 (4.25–6.76)	9.49	91 ± 6
13	16.37 (11.65–23.02)	28.52	70 ± 13
14	2.63 (1.72–4.03)	6.0	89 ± 4
15	3.17 (2.56–3.93)	7.23	86 ± 6
16	2.20 (1.92–2.52)	4.56	76 ± 9
Aspirin[b]	24.0 (13.1–43.8)	133	83 ± 1
Morphine	0.67 (0.56–0.81)	2.3	100 ± 0

Mice ($n = 8–10$ per group) were treated with compounds 30 min before acetic acid administration, by ip route, except morphine, which was administered by sc route. The ID_{50} values are shown with their respective confidence limits (95%). Data from [a]Otuki et al.[6,7]; [b]Vaz et al.[19]

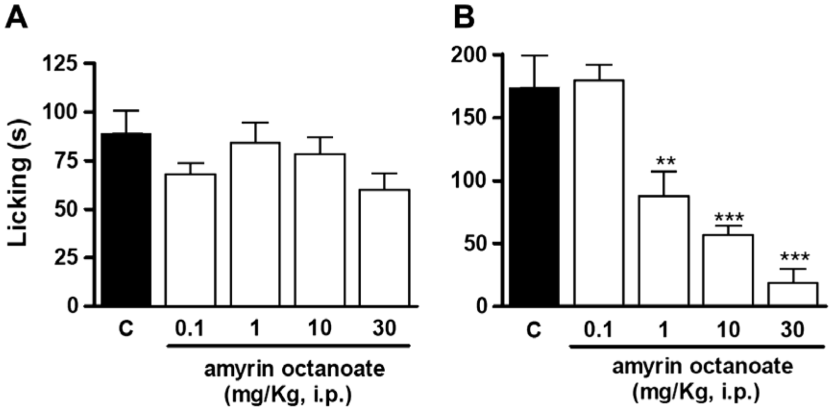

Figure 2. Effects of the α- and β-amyrin octanoate **7** on first phase (0–5 min) (**A**) or second phase (15–30 min) (**B**) of formalin-induced nociception in mice. Each bar indicates mean ± SEM of six to eight animals treated with vehicle (saline, C) or α- and β-amyrin octanoate **7** at different doses (white bars). The symbols denote the significance levels: $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control group (one-way ANOVA followed by Newman–Keuls test).

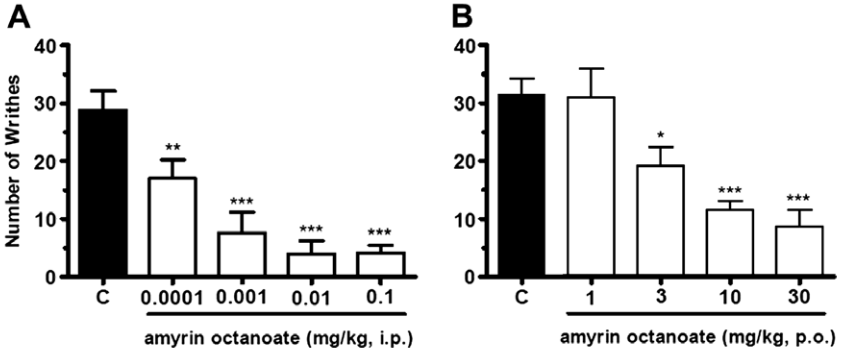

Figure 3. Effect of intraperitoneal (A) or oral (B) administration of α- and β-amyrin octanoate **7** on the abdominal constriction caused by intraperitoneal injection of acetic acid 0.6% in mice. Each bar indicates mean ± SEM of six to eight animals treated with vehicle (saline, C) or α- and β-amyrin octanoate **7** at different doses (white bars). The symbols denote the significance levels: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to control group (one-way ANOVA followed by Newman–Keuls test).

abdominal constriction induced by acetic acid (Fig. 3B). The calculated mean ID_{50} was 7.15 (5.94–8.60) mg/kg and the inhibition observed was 72 ± 9% at 30 mg/kg. Thus, this result demonstrated that α- and β-amyrin octanoate **7** was less potent in preventing the nociception caused by acetic acid when it was given orally in comparison with when it was given intraperitoneally (Fig. 3A and B). The calculated mean ID_{50} for intraperitoneal administration was 0.003 (0.001–0.005) mg/kg and the inhibition observed was 85 ± 5% at 0.1 mg/kg (Fig. 3A). Hence, α- and β-amyrin octanoate **7** was approximately 2300-fold more potent in preventing the nociception caused by acetic acid when it was given intraperitoneally than when given orally. Furthermore, α- and β-amyrin octanoate **7**, when administrated by either ip or po route, did not produce any change in the locomotor activity of the mice, since, there was no significant difference between control and treated groups (Table 2).

2.4. Preliminary structure–activity relationship

The preliminary structure–activity relationship of these α- and β-amyrin triterpene derivatives revealed the following trends. The acylation of the free OH at C-3, introducing groups with linear chains forming compounds **2**, **3**, **5**, **8**, and **9** or with benzoyl chlorides forming compounds **10**, **11**, and **12**, reduced the potency of the standard compound (**1**) in this pain model. The benzoyl derivatives with electron releasing groups showed the same level of activity as the linear chain esters cited above. Also, when the α- and β-amyrin mixture (**1**) was acylated with phthalic anhydride forming the 2-carboxybenzoate derivative (**13**), we observed the greatest decrease in the effect of the series. Until now, we have too few results in relation to the benzoyl derivatives to draw conclusions regarding the electronic effects and antinociceptive activity. Oxidation at C-3 to form a keto group also led to an activity decrease in relation to the α- and β-amyrin mixture (**1**), being as active as the acylated compounds cited above. Additional oxidation at the allylic C-11 position of 3-oxo α- and β-amyrin (**14**) and α- and β-amyrin acetate (**3**) derivatives to give **15** and **16** did not produce any significant change in their activity, indicating that the conformation of the C ring is not very important for antinociceptive activity in this pain model. On the other hand, acylation at C-3 with propanoyl chloride, hexanoyl chloride, and octanoyl chloride led to a significant increase in the antinociceptive effect and the derivatives **4**, **6**, and **7** were more potent than aspirin and morphine in this pain model. Compound **7** was the most potent of the series showing a considerably greater effect in relation to the other derivatives and in relation to the standards aspirin and morphine. It should be noted that an important hydrophobic effect can be observed in relation to the hydrocarbon chain of the acyl derivatives. The maximum effect was observed with the octanoyl derivative suggesting a critical hydrophobic role in the antinociceptive activity of the α- and β-amyrin derivatives.

Table 2. Effect of α- and β-amyrin octanoate **7** on the locomotor activity (open-field test) in mice

Dose (mg/kg)	Route	Open-field (crossing number)
0	ip	120.7 ± 6.5
0.1	ip	127.6 ± 16.6
1	ip	122.3 ± 26.5
10	ip	96.8 ± 22.2
30	ip	87.2 ± 16.8
0	po	156.0 ± 13.8
10	po	146.7 ± 10.7
30	po	131.7 ± 16.9
100	po	174.0 ± 22.4
300	po	114.5 ± 25.6

Mice (*n* = 8–10 per group) were treated with vehicle (0) or α- and β-amyrin octanoate **7** at different doses, 30 or 60 min before open-field test by ip or po, respectively.

In the attempt to evaluate whether the lipophilicity is related to the antinociceptive activity, the results obtained in the induced model of pain for acid injection of acetic acid were used in the form of $\log 1/DI_{50}$ versus $\log P$ (Fig. 4). Figure 4A shows the relationship antinociceptive activity/lipophilicity of all derivatives. However, it is clearly shown that other effects can be influencing the results mainly in relation to the benzoyl and oxidized derivatives (**10–16**). So, on the graph 4B, only the linear chain ester derivatives (**1–9**) were plotted. The profile of these derivatives shows the important hydrophobic effect of α- and β-amyrin octanoate (**7**) described above.

The $\log P$ value, defined as the logarithm of the partition coefficient of *n*-octanol and water, is an important parameter for evaluating the likeness of a molecule to a drug. The lipophilicity is a fundamental physicochemical property that plays an important role in biological activity. This property can influence the pharmacological activity because it can encode the ability of the compound to be absorbed, transported, distributed, and excreted by the human organism. Often a parabolic relationship exists between the measured lipophilicity and biological activity. The increase in the activity of a substance with the number of methylene groups probably results in an increase in the lipophilicity, which leads to a better diffusion of the compounds through the membranes. Inversely, the decrease in activity as a function of the increase in methylene groups is attributed to the reduction in its solubility in the aqueous media intra- and extracellular. This trend was not evident in the present study, perhaps due to the range of the $\log P$ values for the increasing/decreasing activity being very narrow, with compound **7** being an exception. When a substrate is bound by a receptor it is possible that only part of the small molecule makes contact with the macromolecule. Those parts of the small molecules which do not make contact do not contribute to hydrophobic bonding. The linear chain of the octanoyl derivative may interact with a lipophilic pocket situated in the receptor. This could explain the enhanced antinociceptive activity for α- and β-amyrin octanoate **7** in relation to the other derivatives.

3. Conclusions

In conclusion, we have demonstrated that some synthesized α- and β-amyrin derivatives have very good antinociceptive activity when tested using the acetic acid model pain. The most promising compounds were found to be α- and β-amyrin propionate **4**, α- and β-amyrin hexanoate **6**, and α- and β-amyrin octanoate **7**. Further-

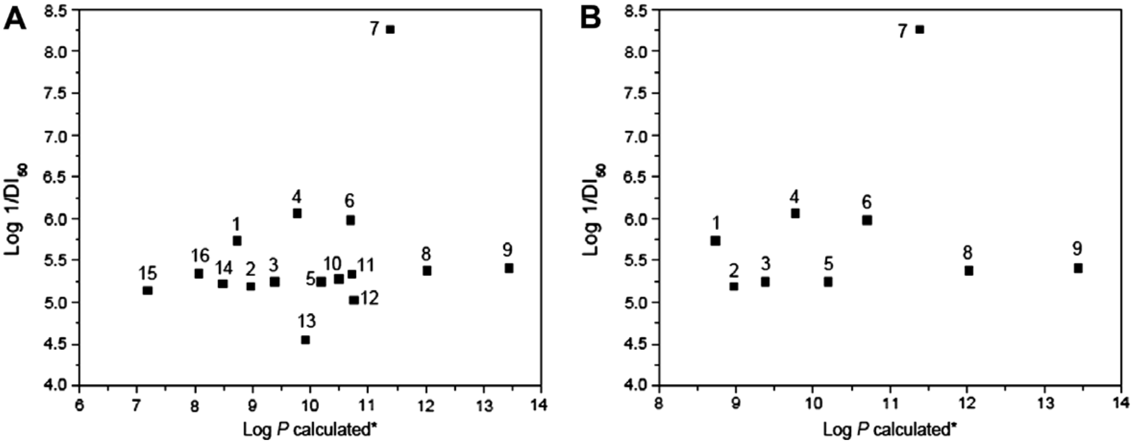

Figure 4. Relationship between antinociceptive activity (log 1/ID$_{50}$) and lipophilicity, expressed as log *P*. (A) Plot of all the derivatives of α- and β-amyrin mixture. (B) Plot of linear chain esters of α- and β-amyrin mixture. *Calculated by online software alogps in http://vcclab.org/lab/alogps.

more, the most potent compound, α- and β-amyrin octanoate **7**, was antinociceptive in the inflammatory phase of formalin, as observed through the effect of this compound on the inflammatory models of nociception. The doses required for the antinociceptive effect did not cause behavioral alteration, since no statistical difference was found in the open-field test. In addition, this compound was effective in preventing the nociception caused by acetic acid when it was given orally.

Among the new derivatives, the alkanoyl series showed the same level of activity as benzoyl derivatives suggesting that the electronic effect has little influence on antinociceptive activity. However, an important hydrophobic effect was observed for alkanoyl derivatives with a maximum effect for compound **7** which showed a critical hydrophobic role in the antinociceptive activity of the α- and β-amyrin derivatives. Further studies to acquire more information on the structure–activity relationships and action mechanism are in progress in our laboratory.

4. Experimental

4.1. Chemistry

IR spectra were taken on a Perkin-Elmer FT-IR 16 PC using KBr pellets. ^{1}H NMR spectra were recorded on Bruker AC (200 MHz) and Varian Mercury Plus (400 MHz) spectrometers using TMS as internal standard.

The triterpenes, α- and β-amyrin, were isolated as a mixture (1:2) from the crude resin of *Protium heptaphyllun* as previously described.[15] The purity and compositional proportions of the α- and β-amyrin mixture and obtained compounds were examined by gas chromatography using a Shimadzu gas chromatograph, model GC-14A, equipped with a flame ionization detector. Hydrogen was used as the carrier gas, at a flow rate of 1 mL min^{-1}. The injection temperature was 290 °C and detection temperature was 300 °C. The temperature program began at 200 °C and increased by 10 °C min^{-1} until 300 °C which was held for 70 min. The compounds were injected into a DB1 capillary column (30.0 m × 0.25 mm i.d. × 0.25 μm film thickness). A split injection with a ratio of 1:30 was used.

4.2. General procedure for the acyl chloride acylation

The starting material, α- and β-amyrin mixture (1:2), was dissolved in pyridine and treated with an excess of acyl chloride. The reaction mixture was heated under reflux until the substrate was consumed. The formation of products was monitored by TLC (SiO$_2$) using a hexane/ethyl acetate (95:5 v:v) mixture as the eluent. Then, an aqueous solution (100 mL) with hydrochloric acid at pH 2.0 ± 0.2 was added. The crude product was extracted with trichloromethane (3× 15 mL), washed with saturated NaHCO$_3$ solution (4× 25), dried over anhydrous Na$_2$SO$_4$, and purified using column chromatography (SiO$_2$). The solvent system used was a hexane/ethyl acetate (95:5 v:v) solution affording the α- and β-amyrin acyl derivatives as an inseparable mixture.

4.2.1. Urs- and olean-12-ene-3β-formiate [α- and β-amyrin formiate] (2). Compound **2** was prepared according to the general procedure described in Section 4.2, using a solution of α- and β-amyrin mixture (100.9 mg, 0.236 mmol) and formyl chloride (0.1 mL, 1.550 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (49.6 mg, 46% yield). R_f: 0.66 (SiO$_2$, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}) = 1715 (C=O); ^{1}H NMR spectrum (400 MHz, CDCl$_3$) δ (ppm) = 0.80–2.20 (characteristic profile of the substrate), 4.65 (m, H-3), 5.12 (m, H-12 of the β-amyrin derivative), 5.19 (m, H-12 of the α-amyrin derivative), 8.12 (s, H-aldehyde). GC (rt min): 16.8 (34%) α-amyrin formiate and 17.6 (66%) β-amyrin formiate.

4.2.2. Urs- and olean-12-ene-3β-propionate [α- and β-amyrin propionate] (4). Compound **4** was prepared according to the general procedure described in Section 4.2, using a solution of α- and β-amyrin mixture (302.7 mg, 0.710 mmol) and propionyl chloride (0.1 mL, 1.151 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid

(263.7 mg, 77% yield). R_f: 0.66 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}) = 1734 (C=O). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm); 0.80–2.20 (characteristic profile of the substrate), 2.32 (q, J = 7.6 Hz, H-2′), 4.51 (m, H-3), 5.13 (m, H-12 of the β-amyrin derivative), 5.18 (m, H-12 of the α-amyrin derivative). GC (rt min): 17.5 (34%) α-amyrin propionate and 18.3 (66%) β-amyrin propionate.

4.2.3. Urs- and olean-12-ene-3β-butyrate [α- and β-amyrin butyrate] (5). Compound **5** was prepared according to the general procedure described in Section 4.2, using a solution of an α- and β-amyrin mixture (152.4 mg, 0.358 mmol) and butyryl chloride (0.1 mL, 0.963 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (78.3 mg, 64% yield). R_f: 0.70 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}): 1728 (C=O). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.80–2.20 (characteristic profile of the substrate), 2.28 (t, J = 7.6 Hz, H-2′), 4.50 (m, H-3), 5.13 (m, H-12 of the β-amyrin derivative), 5.18 (m, H-12 of the α-amyrin derivative). GC (rt min): 21.8 (35%) α-amyrin butyrate and 23.0 (65%) β-amyrin butyrate.

4.2.4. Urs- and olean-12-ene-3β-hexanoate [α- and β-amyrin hexanoate] (6). Compound **6** was prepared according to the general procedure described in Section 4.2, using a solution of an α- and β-amyrin mixture (312.4 mg, 0.733 mmol) and hexanoyl chloride (0.1 mL, 0.715 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (253.5 mg, 67% yield). R_f: 0.74 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}): 1729 (C=O). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.80–2.20 (characteristic profile of the substrate), 2.28 (m, H-2′), 4.51 (m, H-3), 5.13 (m, H-12 of the β-amyrin derivative), 5.19 (m, H-12 of the α-amyrin derivative). GC (rt min): 25.5 (34%) α-amyrin hexanoate and 27.2 (66%) β-amyrin hexanoate.

4.2.5. Urs- and olean-12-ene-3β-octanoate [α- and β-amyrin octanoate] (7). Compound **7** was prepared according to the general procedure described in Section 4.2, using a solution of an α- and β-amyrin mixture (156.6 mg, 0.368 mmol) and octanoyl chloride (0.1 mL, 0.586 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (172.4 mg, 85% yield). R_f: 0.77 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}): 1729 (C=O). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.80–2.20 (characteristic profile of the substrate), 2.30 (t, J = 7.6 Hz, H-2′), 4.51 (m, H-3), 5.13 (m, H-12 of the β-amyrin derivative), 5.19 (m, H-12 of the α-amyrin derivative). GC (rt min): 40.1 (34%) α-amyrin octanoate and 43.1 (66%) β-amyrin octanoate.

4.2.6. Urs- and olean-12-ene-3β-decanoate [α- and β-amyrin decanoate] (8). Compound **8** was prepared according to the general procedure described in Section 4.2, using a solution of an α- and β-amyrin mixture (281.6 mg, 0.661 mmol) and decanoyl chloride (0.2 mL, 0.964 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (306.7 mg, 80% yield). R_f: 0.81 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}): 1734 (C=O). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.80–2.20 (characteristic profile of the substrate), 2.29 (m, H-2′), 4.51 (m, H-3), 5.12 (m, H-12 of the β-amyrin derivative), 5.18 (m, H-12 of the α-amyrin derivative). GC (rt min): 57.0 (36%) α-amyrin decanoate and 61.7 (64%) β-amyrin decanoate.

4.2.7. Urs- and olean-12-ene-3β-palmitate [α- and β-amyrin palmitate] (9). Compound **9** was prepared according to the general procedure described in Section 4.2, using a solution of an α- and β-amyrin mixture (100.5 mg, 0.236 mmol) and palmitoyl chloride (0.2 mL, 0.659 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (121.4 mg, 79% yield). R_f: 0.84 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}): 1734 (C=O). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.80–2.20 (characteristic profile of the substrate), 2.29 (m, H-2′), 4.51 (m, H-3), 5.12 (m, H-12 of the β-amyrin derivative), 5.18 (m, H-12 of the α-amyrin derivative). GC was not carried out.

4.2.8. Urs- and olean-12-ene-3β-benzoate [α- and β-amyrin benzoate] (10). Compound **10** was prepared according to the general procedure described in Section 4.2, using a solution of an α- and β-amyrin mixture (100.7 mg, 0.236 mmol) and benzoyl chloride (0.15 mL, 1.293 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (99.7 mg, 80% yield). R_f: 0.74 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}): 1712 (C=O); ^1H NMR (200 MHz, $CDCl_3$) δ (ppm): 0.80–2.20 (characteristic profile of the substrate), 4.75 (m, H-3), 5.13 (m, H-12 of the β-amyrin derivative), 5.19 (m, H-12 of the α-amyrin derivative), 7.40–7.60 (m, H3′, H4′ and H5′), 8.05 (m, H2′ and H6′); GC (rt min): 44.0 (32%) α-amyrin benzoate and 47.3 (68%) β-amyrin benzoate.

4.2.9. Urs- and olean-12-ene-3β-*p*-bromobenzoate [α- and β-amyrin *p*-bromobenzoate] (11). Compound **11** was prepared according to the general procedure described in Section 4.2, using a solution of an α- and β-amyrin mixture (149.0 mg, 0.349 mmol) and 4-bromobenzoyl chloride (150.0 mg, 0.683 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (122.6 mg, 56% yield). R_f: 0.82 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}): 1712 (C=O); ^1H NMR (200 MHz, $CDCl_3$) δ (ppm): 0.80–2.20 (characteristic profile of the substrate), 4.73 (m, H-3), 5.12 (m, H-12 of the β-amyrin derivative), 5.18 (m, H-12 of the α-amyrin derivative), 7.57 (d, J = 8.4 Hz, H3′ and H5′), 7.89 (d, J = 8,4 Hz, H2′ and H6′). GC was not carried out.

4.2.10. Urs- and olean-12-ene-3β-*p*-chlorobenzoate [α- and β-amyrin *p*-chlorobenzoate] (12). Compound **12** was prepared according to the general procedure described in Section 4.2, using a solution of an α- and β-amyrin mixture (156.0 mg, 0.366 mmol) and 4-chlorobenzoyl chloride (0.1 mL, 0.414 mmol) in pyridine (5 mL). The purified product was obtained as a white amorphous solid (147.7 mg, 71% yield). R_f: 0.82 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}): 1719 (C=O); ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 0.80–2.20 (characteristic profile of the substrate), 4.73 (m, H-3), 5.13 (m, H-12 of the β-amyrin derivative), 5.19 (m, H-12 of the α-amyrin derivative), 7.40 (d, J = 8.4 Hz, H3′ and H5′), 7.97 (d, J = 8.4 Hz, H2′ and H6′). GC was not carried out.

4.3. General procedure for the carboxylic acid anhydride acylations

The starting material, the α- and β-amyrin mixture (1:2), was dissolved in ethyl acetate and to this solution an excess amount of carboxylic acid anhydride and catalytic amount of 4-dimethylaminopyridine (DMAP) were added. The mixture was heated under reflux until the substrate was consumed. The formation of product was monitored by TLC (SiO_2) using a hexane/ethyl acetate (95:5 v:v) mixture as the eluent. The evaporation of the reaction mixture gave a white solid that was purified by column chromatography (SiO_2). The solvent system used was a hexane/ethyl acetate (95:5 v:v) mixture affording the α- and β-amyrin acyl derivatives.

4.3.1. Urs- and olean-12-ene-3β-acetate [α- and β-amyrin acetate] (3). Compound **3** was prepared according to the general procedure described in Section 4.3, using a solution of α- and β-amyrin mixture (608.7 mg, 1.428 mmol) and acetic anhydride (8.0 mL, 84.7 mmol) in ethyl acetate (20 mL). The purified product was obtained as a white amorphous solid (638.0 mg, 100% yield). R_f: 0.60 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}) = 1734 (C=O); ^1H NMR spectrum (200 MHz, $CDCl_3$) δ (ppm) = 0.80–2.20 (characteristic profile of the substrate), 2.05 (s, CH_3 acetyl), 4.50 (m, H-3), 5.13 (m, H-12 of the β-amyrin derivative), 5.18 (m, H-12 of the α-amyrin derivative). GC (rt min): 17.2 (38%) α-amyrin acetate and 18.1 (62%) β-amyrin acetate.

4.3.2. Urs- and olean-12-ene-3β-*o*-carboxibenzoate [α- and β-amyrin *o*-carboxibenzoate] (13). Compound **13** was prepared according to the general procedure described in Section 4.3, using a solution of α- and β-amyrin mixture (105.5 mg, 0.247 mmol) and phthalic anhydride (45.0 mg, 0.304 mmol) in ethyl acetate (10 mL). The purified product was obtained as a white amorphous solid (50.0 mg, 35% yield). R_f: 0.15 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}) = 3434 (broad band, OH of acid), 1724 and 1704 (C=O); ^1H NMR spectrum (400 MHz, $CDCl_3$) δ (ppm) = 0.80–2.20 (characteristic profile of the substrate), 4.76 (m, H-3), 5.13 (m, H-12 of the β-amyrin derivative), 5.19 (m, H-12 of the α-amyrin derivative), 7.57 (m, H-3′ and H-4′), 7.73 (m, H-5′), 7.88 (m, H-2′). GC was not carried out.

4.4. Preparation of the 3-oxo-urs- and 3-oxo-olean-12-ene derivatives [3-oxo α- and β-amyrin] (14)[27]

The pyridinium chlorochromate was prepared according to the procedure described by Corey and Suggs.[27]

To a solution of 500.0 mg (1.17 mmol) of α- and β-amyrin mixture (1:2) in dichloromethane, 336.6 mg (1.56 mmol) of pyridinium chlorochromate was added. The mixture was stirred at room temperature and monitored by TLC (SiO_2), using a hexane/ethyl acetate mixture (95:5 v:v) as the eluent, until the substrates were consumed. The product was then extracted with diethyl ether, filtered over silica gel using hexane as the eluent, and dried to yield 452.7 mg (91% yield) of 3-oxo α- and β-amyrin R_f: 0.57 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}) = 1704 (C=O); ^1H NMR spectrum (200 MHz, $CDCl_3$) δ (ppm) = 0.80–2.20 (characteristic profile of the substrate), 2.46 (m, 2H-2), 5.12 (m, H-12 of the β-amyrin derivative), 5.18 (m, H-12 of the α-amyrin derivative); GC (rt min): 15.2 (38.2%) α-amyrin derivative and 15.8 (61.8%) β-amyrin derivative.

4.5. Preparation of the 3,11-dioxo-(urs- and olean-)12-ene derivatives [3,11-dioxo α- and β-amyrin] (15)[28]

The *tert*-butyl chromate reagent was prepared according to the procedure described by Pinto et al.[28]

To a solution of 504.2 mg (1.18 mmol) of an α- and β-amyrin mixture (1:2) in trichloromethane (50 mL), 9.01 mL (95.6 mmol) of acetic anhydride, 18.2 mL (315.9 mmol) of acetic acid, and 9.01 mL of *tert*-butyl chromate reagent were added. The reaction mixture was heated under reflux for 6 h. One hundred thirty milliliters of trichloromethane was then added and the solution was washed with a 5% aqueous solution of oxalic acid. The organic phase was washed with a 5% aqueous solution of $NaHCO_3$, dried over anhydrous $MgSO_4$, and evaporated. Column chromatography on silica gel using hexane as the eluent gave 414.0 mg (80% yield) of 3,11-dioxo α- and β-amyrin mixture: R_f: 0.24 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}) = 1708 (C=O), 1658 (C=C). ^1H NMR spectrum (200 MHz, $CDCl_3$) δ (ppm) = 0.80–2.20 (characteristic profile of the substrate), 5.58 (s, H-12 of the β-amyrin derivative), 5.63 (s, H-12 of the α-amyrin derivative). GC (rt min): 19.2 (22.8%) α-amyrin derivative and 19.8 (77.2%) β-amyrin derivative.

4.6. Preparation of the 11-oxo-(urs- and olean-)12-ene-3β-acetate derivatives [3-acetyl-11-oxo α- and β-amyrin] (16)[28]

To a solution of 205.7 mg (0.439 mmol) of compound **3** in trichloromethane, 9.01 mL (95.6 mmol) of acetic anhydride, 18.2 mL (315.9 mmol) of acetic acid, and 9.01 mL of *tert*-butyl chromate reagent were added. The reaction mixture was heated under reflux for a period of 6 h. Then, 130 mL of trichloromethane was added and the solution was washed with a 5% aqueous solution of oxalic acid. The organic phase was washed with a 5% aqueous solution of $NaHCO_3$, dried over anhydrous $MgSO_4$, and evaporated. Column chromatography on silica gel using hexane as the eluent gave 132.2 mg (62%) of 3-acetyl-11-oxo α- and β-amyrin: R_f: 0.38 (SiO_2, hexane/ethyl acetate 95:5 v:v). IR (KBr pellet): ν (cm^{-1}) = 1734 (C=O, ester) and 1655 (C=C). ^1H NMR spectrum (200 MHz, $CDCl_3$) δ (ppm) = 0.80–2.20 (characteristic profile of the substrate), 2.05

(s, CH_3 acetyl), 2.34 (s, H-9), 4.53 (m, H-3), 5.54 (s, H-12 of the β-amyrin derivative), 5.58 (s, H-12 of the α-amyrin derivative). GC (rt min): 22.2 (30%) α-amyrin derivative and 23.2 (70%) β-amyrin derivative.

4.7. Animals

Experiments were conducted using Swiss mice (25–35 g) of both sex, housed at 22 ± 2 °C under a 12-h light/12-h dark cycle (lights on at 6:00) and with access to food and water ad libitum. Animals (males or females) were homogeneously distributed among the groups and acclimatized to the laboratory for at least 1 h before testing and were used only once during the experiments. The experiments were performed after protocol approval by the Institutional Ethics Committee and were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations involving experimental pain in conscious animals as specified.[29] The number of animals and intensity of the noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

4.8. Abdominal constriction caused by intraperitoneal injection of acetic acid 0.6%

The abdominal constrictions were induced according to procedures described previously[17,18,30] and resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an intraperitoneal (ip) injection of acetic acid (0.6%; 0.45 mL/mouse) at the time of the test. Mice were treated, 30 min before irritant injection, with different doses (0.0001–30 mg/kg) of α- and β-amyrin derivatives **2–16** by ip route. Another group received morphine (0.1–5 mg/kg) by subcutaneous route (sc), positive control. To evaluate the bioavailability of the compound when administered by oral route, α- and β-amyrin octanoate **7** (1–30 mg/kg) was administered orally (po) 60 min before the irritant injection. Control animals received a similar volume of vehicle (10 mL/kg, ip or po). Morphine solution was prepared in saline sterile solution (NaCl 0.9%) and α- and β-amyrin derivatives were dissolved in Tween 80 plus saline solution. In the final solution, the Tween concentration did not exceed 5%. After injection, the mice were individually placed into glass cylinders of 20 cm diameter and the abdominal constrictions were counted cumulatively over a period of 20 min.

4.9. Formalin-induced nociception

The procedure used was essentially the same as that described previously.[30,31] Animals received 20 μL of a 2.5% formalin solution (0.92% formaldehyde) made up in saline, injected via intraplantar (i.pl.) administration in the ventral surface of the right hindpaw. Animals were observed from 0 to 5 min (neurogenic phase) and 15 to 30 min (inflammatory phase) and the time spent on licking the injected paw was recorded with a chronometer and considered as indication of nociception. Animals received an intraperitoneal injection of saline solution (10 mL/kg; control group) or α- and β-amyrin octanoate **7** (0.1–30 mg/kg) 30 min before formalin injection. Each animal was placed in the chamber for 5 min before irritant injection in order to allow acclimatization to the new environment.[20]

4.10. Measurement of locomotor activity—Open-field test

In order to evaluate the possible non-specific muscle relaxant or sedative effects of α- and β-amyrin octanoate **7**, mice were submitted to an open-field test as described previously.[32] The ambulatory behavior was assessed on apparatus that consisted of a wooden box measuring 40 × 60 × 50 cm. The floor of the arena was divided into 12 identical squares. The number of squares crossed with all paws (crossings) was counted in a session of 6 min. Mice were treated with α- and β-amyrin octanoate **7** (0.1–30 mg/kg, ip; or 10–300 mg/kg, po) and were tested in the open-field 30 and 60 min after treatment, respectively. Control animals received a similar volume of vehicle (10 mL/kg, ip or po).

4.11. Statistical analysis

The antinociceptive effects of the compounds were evaluated by means ± SEM of eight to 10 animals in each group. The significant difference between groups was analyzed following analysis of variance and Newman–Keuls multiple comparison test, $P < 0.05$ was considered as indicative of significance. The ID_{50} values (i.e., the dose of the compounds or morphine necessary to reduce the pain response by 50% in relation to the control value) were reported as geometric means accompanied by their respective 95% confidence limits. The ID_{50} values were determined by linear regression from individual experiments with linear regression GraphPad Software. The inhibition value was calculated using the most effective dose for each compound. A dose of 0.1 or 10 mg/kg was chosen for the inhibition evaluation of α- and β-amyrin derivatives **2–16**; and a dose of 5 mg/kg for morphine.

Acknowledgments

This research received financial support from CNPq, FAPESC, and CAPES.

References and notes

1. Susunaga, G. S. In *Estudo químico e biológico da resina produzida pela espécie Protium heptaphyllum* March. (Burseraceae). M.Sc Thesis, University of Amazon: Manaus, Brazil, 1996; 155 pp.
2. Ukiva, M.; Akihisa, T.; Tokuda, H.; Suzuki, H.; Mukainaka, T.; Ichiishi, E.; Yasukawa, K.; Kasahara, Y.; Nishino, H. *Cancer Lett.* **2002**, *177*, 7–12.
3. Katerere, D. R.; Grev, A. I.; Nash, R. J.; Waigh, R. D. *Phytochemistry* **2003**, *63*, 81–88.
4. Oliveira, F. A.; Lima-Júnior, R. C. P.; Cordeiro, W. M.; Vieira-Júnior, G. M.; Chaves, M. H.; Almeida, F. R. C.; Silva, R. M.; Santos, F. A.; Rao, V. S. N. *Pharmacol. Biochem. Behav.* **2004**, *78*, 719–725.

5. Oliveira, F. A.; Vieira-Junior, G. M.; Chaves, M. H.; Almeida, F. R. C.; Florêncio, M. G.; Lima-Junior, R. C. P.; Silva, R. M.; Santos, F. A.; Rao, V. S. N. *Pharmacol. Res.* **2004**, *49*, 105–111.
6. Otuki, M. F.; Ferreira, J.; Vieira-Lima, F.; Silva, C. M.; Malheiros, A.; Muller, L. A.; Cani, G. S.; Santos, A. R. S.; Yunes, R. A.; Calixto, J. B. *J. Pharmacol. Exp. Ther.* **2005**, *313*, 310–318.
7. Otuki, M. F.; Vieira-Lima, F.; Malheiros, A.; Yunes, R. A.; Calixto, J. B. *Eur. J. Pharmacol.* **2005**, *507*, 253–259.
8. Pawar, R. S.; Bhutani, K. K. *Phytomedicine* **2005**, *12*, 391–393.
9. Oliveira, F. A.; Chaves, M. H.; Almeida, F. R. C.; Lima, R. C. P., Jr.; Silva, R. M.; Maia, J. L.; Brito, G. A. A. C.; Santos, F. A.; Rao, V. S. *J. Ethnopharmacol.* **2005**, *98*, 103–108.
10. Sun, H. X.; Zheng, Q. F.; Tu, J. *Bioorg. Med. Chem.* **2006**, *14*, 1189–1198.
11. Baltina, L. A.; Flekhter, O. B.; Nigmatullina, L. R.; Boreko, E. I.; Pavlova, N. I.; Nikolaeva, S. N.; Savinova, O. V.; Tolstikov, G. A. *Bioorg. Med. Chem.* **2003**, *13*, 3549–3552.
12. Gauthier, C.; Legault, J.; Lebrun, M.; Dufour, P.; Pichette, A. *Bioorg. Med. Chem.* **2006**, *14*, 6713–6725.
13. Reyes, C. P.; Núñez, M. J.; Jiménez, I. A.; Busserolles, J.; Alcaraz, M. J.; Bazzocchi, I. L. *Bioorg. Med. Chem.* **2006**, *14*, 1573–1579.
14. Siani, A. C.; Ramos, M. F. S.; Lima, O. M., Jr.; Santos, R. R.; Ferreira, E. F.; Soares, R. O. A.; Rosas, E. C.; Susunaga, G. S.; Guimarães, A. C.; Zoghbi, M. G. B.; Henriques, M. G. M. O. *J. Ethnopharmacol.* **1999**, *66*, 57–69.
15. Susunaga, G. S.; Siani, A. C.; Pizzolatti, M. G.; Yunes, R. A.; Delle Monache, F. *Fitoterapia* **2001**, *72*, 709–711.
16. Tjolsen, A.; Hole, K. *The Pharmacol. Pain* **1997**, *130/I*, 1–20.
17. Collier, H. O. J.; Dinneen, J. C.; Johnson, C. A.; Schneider, C. *Br. J. Pharmacol. Chemother.* **1968**, *32*, 295–310.
18. Koster, R.; Anderson, M.; de Beer, E. J. *Fed. Proc.* **1957**, 18–412.
19. Vaz, Z. R.; Cechinel, V.; Yunes, R. A.; Calixto, J. B. *J. Phamacol. Exp. Ther.* **1996**, *278*, 304–312.
20. Hunskaar, S.; Hole, K. *Pain* **1987**, *30*, 103–114.
21. Shibata, M.; Ohkubo, T.; Takahashi, H.; Inuki, R. *Pain* **1989**, *38*, 347–352.
22. Moore, P. K.; Wallace, P.; Gaffen, Z.; Hart, S. L.; Babbedge, R. C. *Br. J. Pharmacol.* **1993**, *110*, 219–224.
23. Ribeiro, R. A.; Vale, M. V.; Thomazzi, S. M.; Paschoalato, A. B. P.; Poole, S.; Ferreira, S. H.; Cunha, F. Q. *Eur. J. Pharmacol.* **2000**, *387*, 111–118.
24. Feng, Y.; Cui, M.; Willis, W. *Anesthesiology* **2003**, *98*, 729–733.
25. Hodgson, E.; Levi, P. In *Introduction to Biochemical Toxicology*; Riviere, J. E., Ed., 2nd ed.; Appleton & Lange: Norwalk, Connecticut, USA, 1994; pp 11–48.
26. Nogueira, C. W.; Meotti, F. C.; Curte, E.; Pilissão, C.; Zeni, G.; Rocha, J. B. T. *Toxicology* **2003**, *183*, 29–37.
27. Corey, E. J.; Suggs, J. W. *Tetrahedron Lett.* **1975**, *31*, 2647–2650.
28. Pinto, A. C.; Pereira, A.; Kelecom, A.; Porreca, L.; Ribeiro, N. *Chem. Pharm. Bull.* **1988**, *36*, 4689–4692.
29. Zimmermann, M. *Pain* **1983**, *16*, 109–110.
30. *Drug Discovery and Evaluation. Pharmacological Assays*; Vogel, H. G.; Vogel, W. H., Eds.; Springer Verlag: Berlin, 1997.
31. Santos, A. R. S.; Calixto, J. B. *Neuropeptides* **1997**, *31*, 381–389.
32. Rodrigues, A. L.; da Silva, G. L.; Mateussi, A. S.; Fernandes, E. S.; Miguel, O. G.; Yunes, R. A.; Calixto, J. B.; Santos, A. R. S. *Life Sci.* **2002**, *70*, 1347–1358.