Structure-Activity Relationships of Aromadendranes in Uterus-Relaxant Activity

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Aromadendranes belong to a class of sesquiterpenes present in higher plant essential oils and marine animals. Although the biological activities include antifungal, antibacterial, antiviral, plant growth regulatory, antifeedant, repellent and cytotoxic, there is only one precedent for spasmolytic effects. In a previous report we have shown that the aromadendrene molecule known as spathulenol, isolated from *Lepechinia caulescens,* efficiently relaxes rat uterus rings and therefore in the present work we describe structure-activity relationships of thirteen aromadendranes, most of them having the *trans*-fused perhydroazulene skeleton, with spasmolytic activity.

Key words: Aromadendranes, Structure-Activity Relationships, Uterus-Relaxant Activity

Introduction

Aromadendranes (Fig. 1) are dimethylcyclopropan[e]azulenes that take their name from the first isolated molecule, (+)-aromadendrene (**1**), found as a constituent of the essential oil of *Eucalyptus* trees (Smith, 1901; Birch and Lahey, 1953; Büchi *et al.*, 1966, 1969). The occurrence of these terpenes is high in nature; higher plant essential oils are the main source of aromandendranes, while liverworts and marine sponges are the source for *ent*-aromadendranes (Gijsen *et al.*, 1995). A common structural feature of aromadendranes is the oxidation at C-7 and/or C-10, while the oxidation at other carbon atoms except for C-5 and C-6 is less frequent (Gijsen *et al.*, 1995).

Some of the biological properties that have been reported for isolated aromadendranes include antifungal (Moreira *et al.*, 2003; Lago *et al.*, 2002; Gijsen *et al.*, 1992), antibacterial (Gaspar-Marques *et al.*, 2004; Yamakoshi *et al.*, 1992; Jacyno *et al.*, 1991; Murata *et al.*, 1990; Capon and Macleod, 1988), antiviral (Nishizawa *et al.*, 1992; De Tommasi *et al.*, 1990), plant growth regulatory (Matsuo *et al.*, 1981), antifeedant and repellent (Messer *et al.*, 1990; Thompson *et al.*, 1985; Hubert and Wiemer, 1985; Harada *et al.*, 1984; Asakawa *et al.*, 1980,) and cytotoxic activities (Su *et al.*, 2008; Phongmaykin *et al.*, 2008; Tada and Yasuda, 1985; Asakawa, 1984).

In a previous work we have shown that the aromadendrene molecule known as spathulenol (**5**), isolated from *Lepechinia caulescens* efficiently relaxes rat uterus rings (Perez-Hernandez *et al.*, 2008). This fact and the scarce information about spasmolytic activities of aromadendrane sesquiterpenes prompted us in the present work to look for structure-activity relationships of thirteen aromadendranes, most of them having a *trans*-fused perhydroazulene skeleton.

Material and Methods

General experimental procedures

Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Optical rotations were measured in $CHCl_3$ at 25 °C in a Perkin-Elmer 341 polarimeter. IR spectra were obtained using a Perkin-Elmer 16F PC FTIR spectrophotometer. NMR spectra were recorded in $CDCl_3$ on a Varian Mercury spectrometer working at 300 and 75.4 MHz for ^{1}H and ^{13}C NMR, respectively. Chemical shifts are reported in parts per million downfield compared to tetramethyl-

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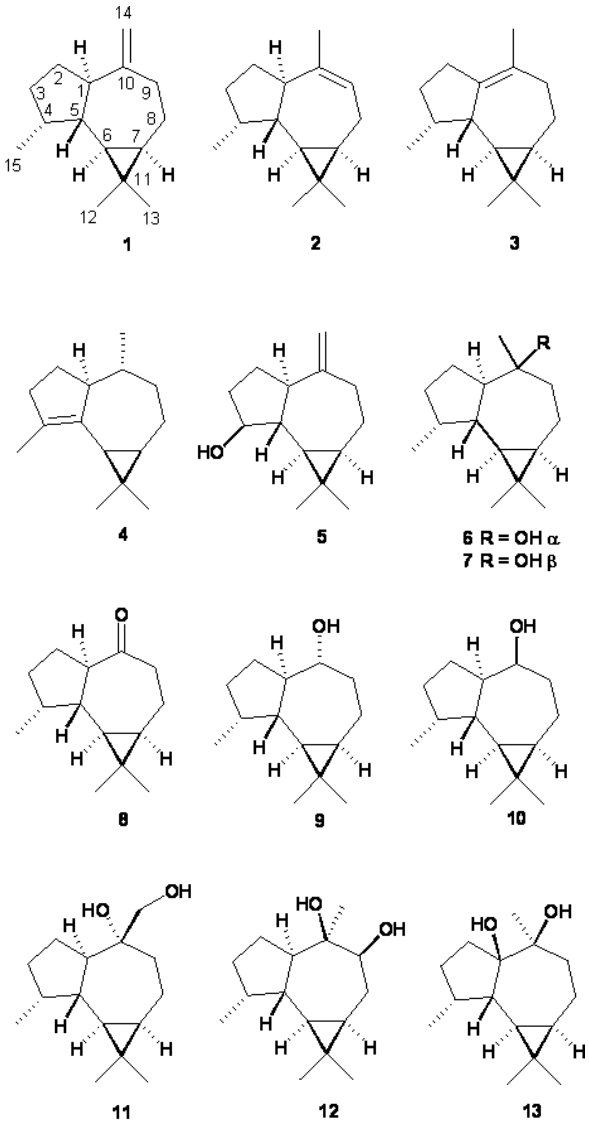

Fig. 1. Molecular structures of the thirteen aromadendranes studied.

silane. The spectral assignments were confirmed by standard procedures (HETCOR, COSY, gHMBC). Electron impact (EI) mass spectra were performed on a Varian Saturn 2000 ion trap (IT) spectrometer at 70 eV, using a column chromatography inlet. Flash chromatography was performed using Si gel Merck 70–230 or 230–400 mesh ASTM. Analytical thin-layer chromatography (TLC) was carried out on silica gel 60 F254 coated aluminum sheets (0.25 mm thickness) with a fluorescent indicator. Visualization was accomplished with UV light (254 nm) and ceric ammonium sulfate.

Compounds

The following compounds are commercially available (Fluka) and were used without further purification: (+)-aromadendrene (**1**), (–)-dehydroaromadendrane (**2**), (+)-ledene (**3**), (–)-*α*-gurjunene (**4**), (–)-globulol (**6**), and (–)-epiglobulol (**7**). (+)-Spathulenol (**5**) was isolated from leaves of *Lepechinia caulescens* following a described procedure (Perez-Hernandez *et al.*, 2008), while (+)-apoaromadendrone (**8**) (Henricus *et al.*, 1990; Graham *et al.*, 1960) and (–)-aromadendrane-1*β*,10*β*-diol (**13**) (Moreno-Dorado *et al.*, 2003; Ghisalberti *et al.*, 1994) were prepared according to literature procedures.

(–)-*nor-Aromadendr-10α- and -10β-ol* (**9** and **10**): To a stirred solution of 0.38 g (10 mmol) of $NaBH_4$ in 10 mL of 96% EtOH, at 0 ºC a solution of 0.2 g (96 mmol) of **8** in 5 mL of EtOH was added and maintained for 30 min. The reaction mixture was diluted with 10 mL of water and extracted three times with CH_2Cl_2 (15 mL). The organic phase was washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by column chromatograph (hexane/AcOEt 19:1) to give **9** (95 mg, 48%) followed by **10** (88 mg, 43%).

(–)-nor-Aromadendr-10α-ol (**9**): Colourless oil. – $[\alpha]_{589}$ –14º; $[\alpha]_{578}$ –15º; $[\alpha]_{546}$ –17º; $[\alpha]_{436}$ –28º; $[\alpha]_{365}$ –45º (*c* 2.0, $CHCl_3$). – IR ($CHCl_3$): ν = 2947, 2926, 2872, 1460, 1378 cm^{-1}. – EIMS: *m*/*z* = 208 $[M]^+$ (23), 191 (61), 175 (35), 147 (85), 134 (41), 119 (44), 105 (61), 95 (100). – ^1H NMR and ^{13}C NMR: see Tables II and III.

(–)-nor-Aromadendr-10β-ol (**10**): Colourless solid, m.p. 57–58 ºC. – $[\alpha]_{589}$ –69º; $[\alpha]_{578}$ –73º; $[\alpha]_{546}$ –81º; $[\alpha]_{436}$ –130º; $[\alpha]_{365}$ –190º (*c* 2.0, $CHCl_3$). – IR ($CHCl_3$): ν = 2942, 2926, 2866, 2365, 1460, 1379 cm^{-1}. – EIMS: *m*/*z* = 238 $[M]^+$ (2), 220 (28), 203 (100), 177 (92). – ^1H NMR and ^{13}C NMR: see Tables II and III.

(–)-15-Hydroxy-epi-globulol (**11**): To a stirred solution of 0.5 g (2.4 mmol) of aromadendrene (**1**) in 20 mL of 96% EtOH, cooled at 0 ºC, a solution of 0.46 g (3.0 mmol) of $KMnO_4$ in 10 mL of water was added and maintained for 2 h. The

reaction mixture was diluted with 40 mL of water and extracted with three 20-mL portions of CH_2Cl_2. To remove the brown impurities, the organic layer was filtered through celite. The filtrate was washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was recrystallized twice from AcOEt/hexane to yield 0.53 g (52%) of pure **11**.

Colourless solid, m.p. 96–97 °C. – $[\alpha]_{589}$ –35°; $[\alpha]_{578}$ –37°; $[\alpha]_{546}$ –43°; $[\alpha]_{436}$ –70°; $[\alpha]_{365}$ –103° (*c* 3.4, $CHCl_3$). – IR ($CHCl_3$): ν = 3601, 2957, 2867, 1462, 1388, 1061, 1039 cm^{-1}. – EIMS: m/z = 238 $[M]^+$ (2), 221 (21), 189 (100). – ^1H NMR and ^{13}C NMR: see Tables II and III.

(–)-Aromadendrane-9β,10β-diol (**12**): To a stirred solution of 0.5 g (2.4 mmol) of (–)-dehydroaromadendrene (**2**) in 20 mL of 96% EtOH, at room temperature, a solution of 0.46 g (3.0 mmol) of $KMnO_4$ in 10 mL of water was added and maintained for 3 h. The reaction mixture was diluted with 40 mL of water and extracted with three 20-mL portions of CH_2Cl_2. To remove the brown impurities, the organic layer was filtered through celite. The filtrate was washed with brine, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was recrystallized twice from AcOEt/hexane to yield 0.69 g (76%) of pure **12**.

Colourless solid, m.p. 119–120 °C. – $[\alpha]_{589}$ –20°; $[\alpha]_{578}$ –22°; $[\alpha]_{546}$ –23°; $[\alpha]_{436}$ –38°; $[\alpha]_{365}$ –57° (*c* 2.0, $CHCl_3$). – IR ($CHCl_3$): ν = 3030, 2934, 1969, 1715, 1462, 1382, 1015 cm^{-1}. – EIMS: m/z = 208 $[M]^+$ (23), 191 (61), 175 (35), 147 (85), 134 (41), 119 (44), 105 (61), 95 (100). – ^1H NMR and ^{13}C NMR: see Tables II and III.

Tissue preparation

Animal experiments were performed following the recommendation of the "Guide for the care and use of laboratory animals" (US NIH # 85–23, revised in 1985).

Tissues were obtained from virgin female Wistar rats (240–300 g body weight) pretreated with estradiol-17-benzoate (40 μg/kg s.c.) 48 h before the experiments. The oestrus stage was confirmed by vaginal smear microscopic examination. Animals were killed by cervical dislocation, the reproductive tract was removed and placed in a Krebs-Ringer-Bicarbonate (KRB) solution, and for each uterus horn the connective tissue and fat were trimmed. Three 1-cm uterus sections were obtained from each uterus horn, giving a total of 6 rings. Uterus rings were placed vertically in a 3-mL organ bath containing KRB solution with the following composition (mM): NaCl 120, $NaHCO_3$ 20, KCl 4.4, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.5, and glucose 11, pH 7.4. The KRB solution was maintained at 37 °C and gassed continuously with a mixture of 95% O_2 – 5% CO_2. Each uterus ring was placed under an optimum resting force of 1 g and allowed to equilibrate for 1 h before the experiment. During this equilibration period, the uterus rings were washed with 3 mL of fresh KRB solution every 10 min. The detailed method used for tension studies has been described previously (Oropeza *et al.*, 2002).

Depolarizing solution (60 mM KCl) was prepared by equimolar substitution of NaCl by KCl. Tissues were stimulated with the depolarizing solution and those with two similar consecutive responses were included in the study. Each experiment was repeated 5–6 times using new uterus rings. Contractile responses were recorded isometrically with FT03 Grass tension transducers connected to a Grass Telefactor RPS312 RM polygraph.

Experimental procedure

Compounds **1**–**13** were tested for the contraction of uterus rings using the following protocol: The uterus rings were contracted with depolarizing solution, and the test substances were added upon tonic contraction 20 min after stimulus; this time corresponds approximately to a plateau phase of methods previously used (Parra *et al.*, 2000; Revuelta *et al.*, 2000; Shimizu *et al.*, 2000). The tested concentrations for all the compounds were 30, 10, and 3 μg/mL and were dissolved in an ethanol/DMSO (2:1) mixture in such a way that the final vehicle content in the organ bath was 0.15%, demonstrating the innocuity. For each uterus ring only one concentration was assayed. This was maintained during 20 min, the time in which the plateau phase of relaxation is reached.

After addition of compounds, KCl restimulation was made. The viability of tissues was verified by adding depolarizing solutions to control the uterus rings at the end of each experiment (approx. 150 min), which showed similar amplitude contractions as at the beginning of the assay (Gutiérrez *et al.*, 1994). The data were expressed

as percentage of the maximal tonic response induced by 60 mM KCl (100%). To establish that the relaxing effect was not originated by biological variability of tissues, the test was repeated for each concentration at least five times to obtain the standard deviation. Furthermore, one tissue control was maintained in each experiment, which showed no variation in response to KCl-induced contractions.

Data analysis

The contraction amplitude was measured. The maximal tissue contractile response was determined as the response elicited by 60 mM KCl. Relaxation induced by substances was assessed as percentage of relaxation of maximal contractile response. Results are expressed as mean ± S.E.M., $n = 4$. Statistical comparison was performed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test, whereby $p < 0.05$ was considered statistically significant.

Results and Discussion

In a previous work the ability of spathulenol (**5**) to induce spasmolytic action was evidenced (Perez-Hernandez *et al.*, 2008). On the base of this result, a structure-activity relationship study was made using aromadendrane analogues, most of them with the *trans*-fused hydroazulene skeleton. For initial evaluations the following compounds were selected: (+)-aromadendrene (**1**), (–)-dehydroaromadendrene (**2**), (+)-ledene (**3**), and (–)-α-gurjunene (**4**) representing the non-oxygen containing aromadendranes, as well as (+)-spathulenol (**5**) and (–)-globulol (**6**) representing the common oxidation at C-4 and C-10, respectively.

The biological evaluation of compounds **1**–**6**, expressed as the percentage relaxation of the initial contraction by 60 mM KCl, is presented in Table I. It follows that the presence of a hydroxy group in the aromadendranes is very important for the relaxing activity. Concerning the unsaturated compounds, (+)-ledene (**3**) has the most significant effect, 20.8% at 30 μg/mL, however, this percentage corresponds approximately to one fifth of the percentage shown by the oxidized sesquiterpenes **5** and **6**. At 10 and 3 μg/mL the remaining unsaturated compounds **1**, **2** and **4** have very weak or null activity.

Table I. Relaxation induced by compounds **1**–**6** in rat uterus rings. Results are expressed as means ± S.E.M. ($n = 4$).

Compound	Relaxation (%)		
	30 μg/mL	10 μg/mL	3 μg/mL
1	2.9 ± 0.2	1.0 ± 0.8	No effect
2	2.3 ± 0.7	1.0 ± 2.0	No effect
3	20.8 ± 4.8	6.6 ± 1.2	No effect
4	8.9 ± 2.1	2.9 ± 1.8	No effect
5	100 ± 0.2	75.7 ± 0.7	31.0 ± 3.9
6	100 ± 0.2	97.9 ± 2.1	73.0 ± 3.3

The hydroxylated compounds spathulenol (**5**) and globulol (**6**) have the strongest spasmolytic activity. At 30 μg/mL both sesquiterpenes are able to relax the contracted tissue by 100%, however, at low concentrations significant differences can be observed. At 10 μg/mL, **5** shows 75.7% of the relaxant activity against 97.9% for **6**, while at 3 μg/mL, the spasmodic effect for **5** is only 31.0% compared to 73.0% for **6**. These results suggest that the hydroxy group is more effective to induce the relaxation effect when placed at C-10 rather than at C-4.

Following these findings the uterus-relaxant activity of six additional aromadendrane analogues, **7**–**13** (Fig. 1) conserving the hydroxy group at C-10 or a carbonyl group, like **8,** was evaluated.

(+)-Apoaromadendrone (**8)** (Henricus *et al.*, 1990; Graham *et al.*, 1960) and the diol **13** (Moreno-Dorado *et al.*, 2003; Ghisalberti *et al.*, 1994) were prepared according to literature methods. Alcohols **9** and **10** are not described and were prepared by reduction of **8** with $NaBH_4$ at 0 °C. The purification was easily achieved by column chromatography in contrast to analogues with the *cis*-fused hydroazulene skeleton which are described as inseparable by chromatographic methods (Pires de Lima *et al.*, 1997). The complete NMR spectral assignment for both isomers is given in Tables II and III. Diols **11** and **12** were obtained by oxidation of **1** and **2**, respectively, with $KMnO_4$ and have no precedent in the literature. Their NMR data are also included in Tables II and III.

The relaxing effect of compounds **7**–**13** is presented in Fig. 2, from which the following assumptions can be done: The spasmolytic effect of (+)-apoaromadendrone (**8**, 50.5%) is smaller than that of (–)-globulol (**6**, 73%), suggesting that the oxygen functional group can modulate the bio-

Table II. ^{1}H NMR chemical shifts, multiplicity, and coupling constants of compounds **9**–**12**.

H	**9**	**10**	**11**	**12**
H-1	1.79–1.95 m	1.64–1.75 m	1.92–2.16 m	1.85–1.75 m
H-2	1.36–1.47 m	1.26–1.33 m	1.50 ddt (12.4, 8.5, 6.8)	1.77–1.60 m
	1.61–1.70 m	1.97–2.03 m	1.73–1.86 m	1.74–1.62 m
H-3	1.18–1.30 m	1.16–1.28 m	1.21–1.35 m	1.46–1.51 m
	1.71–1.78 m	1.73–1.77 m	1.63 dq (12.2, 6.0)	–
H-4	2.01–2.10 m	1.99–2.01 m	1.92–2.16 m	1.97–2.1 m
H-5	1.79–1.95 m	1.16–1.28 m	1.21–1.35 m	1.46–1.51 m
H-6	0.50 t (9.8)	0.51 t (10.0)	0.53 dd (10.5, 9.3)	0.50 t (9.8)
H-7	0.58 td (9.8, 5.2)	0.60 td (10.0, 5.8)	0.63 ddd (10.8, 9.3, 6.1)	0.58 td (9.8, 5.2)
H-8	1.36–1.47 m	0.96–1.04 m	0.86–1.0 m	1.15–1.22 m
	1.61–1.70 m	1.73–1.77 m	1.73–1.86 m	1.74–1.62 m
H-9	1.50–1.59 m	1.46–1.30 m	1.92–2.16 m	3.38 dd (10.5, 3.0)
	–	1.97–2.03 m	–	–
H-10	4.10 s broad	3.22 td (10.2, 4.0)	–	–
CH_3-12	1.04 s	0.98 s	0.97 s	1.04 s
CH_3-13	1.01 s	1.0 s	1.01 s	1.00 s
CH_3-14	0.91 d (7.2)	0.92 d (7.0)	3.62 s	1.30 s
CH_3-15	–	–	0.93 d (7.2)	0.92 d (7.0)

Table III. ^{13}C NMR chemical shifts of compounds **9**–**12**.

C	**9**	**10**	**11**	**12**
C-1	51.4	53.9	55.6	56.6
C-2	28.3	29.6	25.9	26.6
C-3	35.2	34.0	34.7	36.8
C-4	36.3	35.7	36.2	35.6
C-5	36.4	39.9	38.1	34.7
C-6	28.5	27.0	28.3	28.6
C-7	27.5	26.5	26.5	26.6
C-8	17.6	19.9	19.6	36.8
C-9	35.5	37.2	37.6	74.3
C-10	70.3	79.2	76.2	77.2
C-11	21.4	19.1	19.6	21.0
C-12	15.8	15.6	15.7	15.8
C-13	28.9	28.8	28.6	29.8
C-14	16.4	16.7	61.5	16.5
C-15	–	–	15.8	22.9

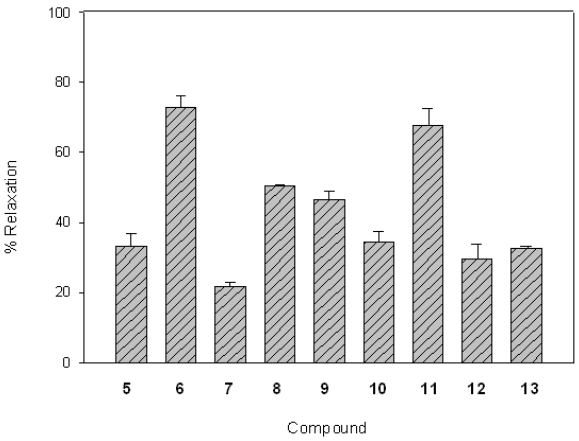

Fig. 2. Relaxation induced by compounds **5**–**13** at the minimum concentration tested (3 μg/mL) in rat uterus rings. Rings were contracted with potassium chloride (50 mM KCl) before being exposed to the test compounds. Results are expressed as means ± standard error of mean in terms of percentage relaxation of the respective contracting level (n = 4).

logical activity, *i.e.* the hydroxy group is more effective than the carbonyl group for inhibition of the uterus contraction. For alcohols **9** and **10**, in which the methyl group at position C-10 is absent, the relaxing effect is attenuated. The effect of **9** is 46.4% and of **10** it is 34.6%, which made these compounds week spasmodic agents compared to (–)-globulol (**6**). It is important to notice that the uterus-relaxant activity of the epimeric alcohols **9** and **10** is slight different, suggesting that the orientation of the hydroxy group has same importance to modify the biological activity. In order to corroborate this assumption (–)-epiglobulol (**7**) was tested for the spasmodic activity. The relaxing effect of **7** is only 22% against 73% of **6**, demonstrating that the α-oriented hydroxy group is adequate to obtain a better spasmolytic potency.

Finally, in order to determinate if an additional hydroxy group close to position C-10 improves the uterus-relaxamt effect, the diols **11**, **12** and **13**

were tested. It appears that the introduction of an additional hydroxy group in the vicinal position at C-1 and C-9, as in compounds **12** and **13**, respectively, does not increase the relaxant activity. Diol **11**, which contains a hydroxymethylene group at position C-10 exhibits almost the same effect like (–)-globulol (**6**) and these results support the hypothesis that the essential hydroxy group position to develop spasmolytic activity of the aromadendrane skeleton is C-10.

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