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A novel activity for a group of sesquiterpene lactones: inhibition of aromatase

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Abstract A group of eleven sesquiterpene lactones isolated from different Asteraceae species from north-western Argentina were investigated for their inhibitory action on the estrogen biosynthesis. Seven of them, of different skeleton types, were found to inhibit the aromatase enzyme activity in human placental microsomes, showing IC_{50} values ranging from 7 to 110 μM. The most active were the guaianolides 10-epi-8-deoxycumambrin B (compound 1), dehydroleucodin (compound 2) and ludartin (compound 3). These compounds were competitive inhibitors with an apparent $K_i = 4$ μM, $K_i = 21$ μM and $K_i = 23$ μM, respectively. Compounds 1 and 2 acted as type II ligands to the heme iron present in the active site of aromatase cytochrome P450 (P450arom). Besides, all of them failed to affect the cholesterol side-chain cleavage enzyme activity on human placental mitochondrias. This is the first report on the aromatase inhibitory activity of this group of natural compounds.

Key words: Aromatase inhibitor; Sesquiterpene lactone; Type II ligand; Cholesterol side-chain cleavage; Human placental microsome; Argentine; Asteraceae

1. Introduction

The biosynthesis of estrogens is catalysed by the enzyme complex aromatase. The complex is formed by a member of the superfamily of enzymes known collectively as cytochrome P450, namely, aromatase cytochrome P450 (P450arom, the product of CYP19 gene). Associated with the P450arom is a flavoprotein, the NADPH cytochrome P450 reductase. These two enzymes catalyse the aromatization of the A ring of androgens to form the phenolic ring characteristic of estrogens. This reaction involves three sequential oxidations to the C-19 methyl group of the substrate, followed by elimination of C-19 as formic acid and aromatization of the A ring. This reaction is the only one in vertebrates that introduces an aromatic ring into a molecule [1]. Regulation of this enzyme plays an important role in many physiological processes and in certain diseases, the most important of which is estrogen dependent breast cancer. The use of aromatase inhibitors for treatment of breast cancer is well demonstrated nowadays. The several different kinds of inhibitors investigated include steroidal and non-steroidal compounds which act in a competitive or in a mechanism-based manner [2].

The plant kingdom is a very important source of medicinal agents. However, few metabolites with significant aromatase inhibitory activity have been previously described [3]. The sesquiterpene lactones are a structurally diverse and extremely large group of metabolites with over 3500 structures thus far reported. Over 90% of them have been found in the Asteraceae, and also occur in the Acanthaceae, Anacardiaceae, Apiaceae, Euphorbiaceae, Lauraceae, Magnoliaceae, Menispermaceae, Rutaceae, Winteraceae and the Hepatidae. Biomedical research has established that sesquiterpene lactones have potential utility in the treatment of solid tumors, stomach ulcers, antimigraines, cardiotonics [4] and more recently as inhibitors of the expression of cyclooxygenase [5] and the transcription factor NF-κB [6]. No action on aromatase has been so far described for the sesquiterpene lactones. In this work we report and characterise the inhibitory effect of this group of compounds on human placental aromatase activity.

2. Materials and methods

2.1. Materials

The sesquiterpene lactones employed in the present study have been isolated and characterised as previously reported. Hence, they were 10-epi-8-deoxycumambrin B, ludartin [7], dehydroleucodin [8], peruvin, psilostachyin C, helenalin, psilostachyin, Sy II [9], achalensolide [10], Sy I [11] and eupahakonenin B [12].

[1,2,6,7-^{3}H]Testosterone (80 Ci/mmol) and [4-^{14}C]Cholesterol (51 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Their purities were checked by thin layer chromatography. All other non-radioactive steroids, NADPH, DL-dithiothreitol, ethylenediaminotetraacetic acid and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Biological preparations

Human placental microsomes (particles sedimenting at $105\,000 \times g$ for 60 min) were obtained as reported [13] and human placental mitochondrias were obtained using the procedure described by Tuckey [14]. The protein concentration was determined according to the Bradford procedure using BSA as standard [15].

2.3. Assay of aromatase activity

The enzyme activity was determined by measuring the conversion of [^{3}H]testosterone to [^{3}H]estradiol plus [^{3}H]estrone as described earlier [13]. To test the effect of different compounds on the aromatase activity present in the microsomal fractions, varying amounts of them were added to the microsomes from ethanolic stock solutions. Ethanol alone, which did not exceed a concentration of 1% was added to control incubations. Values of K_m and V_{max} were estimated graphically from plots of $1/V$ vs. $1/S$ using linear regression analysis. The replots from the slope of each reciprocal plot versus the corresponding inhibitor concentrations were generated and the K_i values for these compounds were determined.

2.4. Assay of cholesterol side-chain cleavage enzyme activity

The enzyme activity was determined by measuring the conversion of

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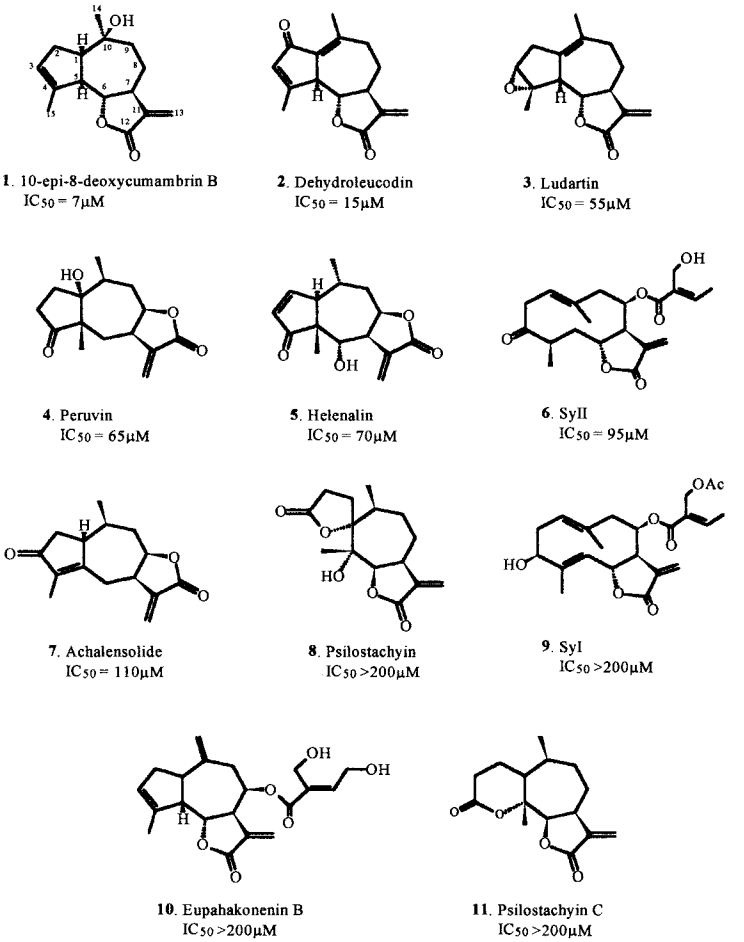

Fig. 1. Structures of sesquiterpene lactones and their IC_{50} values for aromatase activity in human placental microsomes. Values represent the average of duplicate determinations in two separate experiments. Substrate = 50 nM testosterone.

[^{14}C]cholesterol to [^{14}C]pregnenolone plus [^{14}C]progesterone as described by Robinson et al. [16] with some modifications. The compounds were added to the mitochondrias from ethanolic stock solutions. Ethanol alone, which did not exceed a concentration of 1%, was added to the control incubations. After a preincubation time of 5 min the reaction was started by adding 0.1 ml of a mixture of [^{14}C]cholesterol (80 000 cpm/ml) sonicated in the presence of Tween 80 [17]. The final concentration of substrate was 1.5 μM and the final concentration of detergent was 20 μg/ml. The reaction was linear for at least 4 h. Cholesterol side-chain cleavage enzyme activity was expressed as a percentage of radioactivity found in [^{14}C]pregnenolone plus [^{14}C]progesterone divided by the total radioactivity. The cholesterol side-chain cleavage enzyme inhibitory activity of the compounds was assessed in terms of 50% inhibitory concentration.

2.5. Spectral studies

Spectroscopic studies were carried out as described by Kellis and Vickery [18]. Spectra were recorded with an UV 1601 PC/UV Shimadzu spectrophotometer interfaced to an UV/PC personal spectroscopy software. The protein concentration was 6.0–10.0 mg/ml. Briefly, aromatase cytochrome P450 was extracted from human placental microsomes with 0.5% sodium cholate in the presence of 1 μM of testosterone. The detergent-solubilized enzyme was collected as a 55% saturated ammonium sulphate pellet and dialysed against 0.05 M sodium phosphate buffer (pH 7.2). A baseline spectrum was recorded with enzyme and substrate (0.25 μM testosterone) in the sample and reference cells and then, different concentrations of the compounds were added to the sample cell (solvent dimethyl sulfoxide alone was added to the reference). Repeated spectra were recorded to ensure sample equilibration and 3–5 spectra were routinely signal averaged. The reported apparents binding constants (K_{sapp}) were calculated using standard graphical analysis. Values represent the mean of three experiments.

2.6. Molecular modelling

The structures of the inhibitors and substrates were minimised using AM1 calculations with MOPAC.

3. Results

Eleven sesquiterpene lactones of different skeleton-type, shown in Fig. 1, were tested for aromatase inhibitory activity. None of the compounds were found to stimulate enzyme activity. Compounds **1–7** produced dose-dependent inhibition of aromatase with IC_{50} values ranging from 7 to 110 μM, while compounds **8–11** were inactive (Fig. 1).

The most active compounds, **1–3** (10-epi-8-deoxycumambrin B, dehydroleucodin and ludartin, respectively), belong to the same skeleton-type (guaianolide with trans-fusion of the γ-lactone ring to C-6). For this reason we performed a more detailed study of their inhibition mechanisms. Fig. 2A shows the time course of the aromatization of 50 pmol of testosterone in 1.0 ml (100 000 cpm/ml) by human placental microsomes in the presence and absence of compound **1**. Under these conditions, 10 μM of the compound inhibited the aromatization by approximately 70% (Fig. 2A, inset). The reaction rate in the presence of the inhibitor remains linear for 10 min. The lack of time-dependence of inhibition

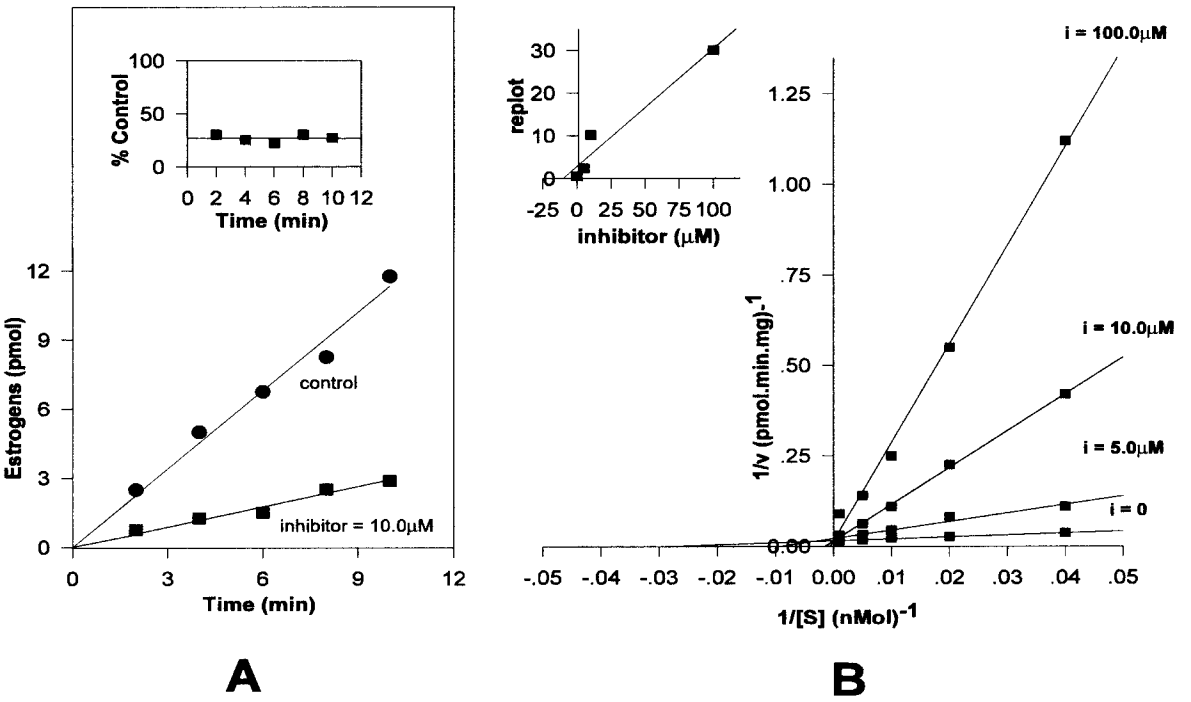

Fig. 2. (A) Time course of aromatization in microsomes in presence and absence of compound **1** (10 μM). Samples contained 100 μg of protein and 50 pmol of testosterone in 1.0 ml. The inset shows the activity of the inhibited sample as percentage of that of the control at each time. (B) Kinetic analysis of inhibition of testosterone aromatization in human placental microsomes by compound **1**. Inset, slopes of lines in double reciprocal plot vs. inhibitor concentration. Each point represents the mean of three experiments performed in duplicate.

indicates that no significant conversion of the inhibitor to more or less active forms, occurs during the incubation; similar results were obtained with compounds **2** and **3**. In addition, when human placental microsomes were pre-incubated in presence of 10 μM of compounds **1–3** and absence of substrate, variation of the pre-equilibration time failed to affect the fractional inhibition or time course (data not shown). Kinetic analyses were also performed to investigate the mechanism by which the sesquiterpene lactones inhibit aromatase enzyme activity. Fig. 2B shows a Lineweaver-Burk plot of the inhibition of human placental aromatase by compound **1**. Inhibition was competitive with respect to the substrate testosterone and a replot of the slopes of the lines (shown in the inset) yielded a K_i value of 4 μM for compound **1**. Similar results were obtained for compounds **2** ($K_i = 21$ μM) and **3** ($K_i = 23$ μM) (data not shown). In the absence of inhibitors, the K_m of testosterone averaged 38 nM.

The kinetic analyses suggested that compounds **1–3** may inhibit aromatization by competing with testosterone for the substrate binding site of the enzyme. This possibility was further investigated by monitoring the effects of these compounds on the absorption spectral properties of the enzyme. The difference spectra for compounds **1–3** together with that obtained for aminoglutethimide, a classical type II binder, are displayed in Fig. 3. When partially purified aromatase was first equilibrated with testosterone, addition of compound **1** produced a type II difference spectrum with a Soret peak at 419 nm. This type of spectrum reflected substrate displacement and conversion of the high spin aromatase cytochrome P450 to a low spin complex. Graphical analysis of the titration showed only one kind of binding site which exhibits a K_{sapp} of 29 μM. A similar difference spectrum was obtained for compound **2**, showing a spectral binding constant (K_{sapp}) of 40 μM. Compound **3** showed a plain curve difference spectrum. When compound **1** was added to aromatase in absence of the substrate (testosterone), no spectral changes were ob-

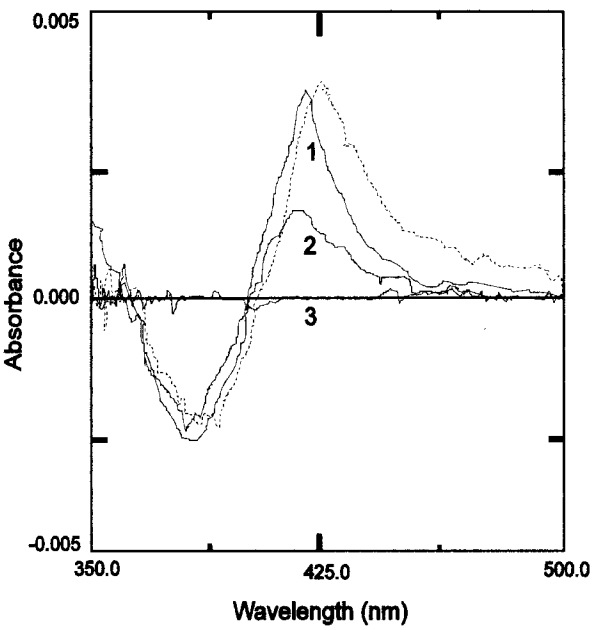

Fig. 3. Difference spectra obtained upon interaction of compound **1** [100 μM] (line 1), compound **2** [100 μM] (line 2), compound **3** [100 μM] (line 3) and aminoglutethimide [100 μM] (dotted line) with partially purified aromatase from human placenta. The samples contained 0.25 μM of testosterone and the protein concentration was 7.5 mg/ml.

served. This result is similar to that observed for 7,8-benzoflavone, another natural non-steroidal inhibitor [18].

Some non-steroidal aromatase inhibitors exhibit dose-dependent effects on other cytochromes P450 mediated steroid hydroxylations. The cholesterol side-chain cleavage enzyme catalyses the three hydroxylations required for the conversion of cholesterol to pregnenolone. This enzyme is highly affected by aminoglutethimide [19]. We compared the capacity of aminoglutethimide and compounds **1** to **3** for inhibiting the activity of this enzyme present in human placental mitochondrias. Aminoglutethimide inhibited this enzyme activity at 100 μM by 50% but no significant inhibitory activity was obtained for compounds **1**–**3** in the range of concentrations tested (1–200 μM). Fig. 4 shows a dose-response curve for the activity of aminoglutethimide and compound **1**.

4. Discussion

Several natural products inhibit the aromatase enzyme, but the most relevant inhibitors belong to the groups of flavonoids [18,20], lignans [21] and tobacco alkaloids [22]. In this report we show that sesquiterpene lactones exhibit competitive inhibition of human placental aromatase activity in a range of concentrations similar to the other plant-derived inhibitors reported previously.

On the basis of the kinetic evidences, spectral data, molecular modelling of our compounds and previous work on three-dimensional model of aromatase [23], we propose a mechanism of action of compounds **1**–**3**. These compounds are structurally related and differ on the substitution pattern and oxidation state of carbons C-1, C-2, C-3, C-4, C-10, C-14 and C-15. At first glance, we observed a relationship between the activity and the position of the oxygen atom at C-10 (OH in compound **1**), at C-2 (carbonyl in compound **2**) and at C-3,

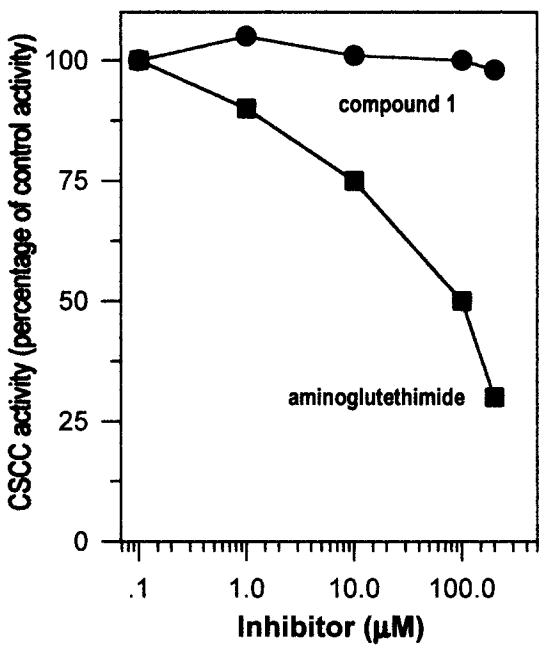

Fig. 4. Comparative effects of compound **1** and aminoglutethimide on cholesterol side-chain cleavage enzyme activity (CSCC) present in human placental mitochondrias. The cholesterol concentration was 1.5 μM. Each point represents the mean of two experiments performed in duplicate.

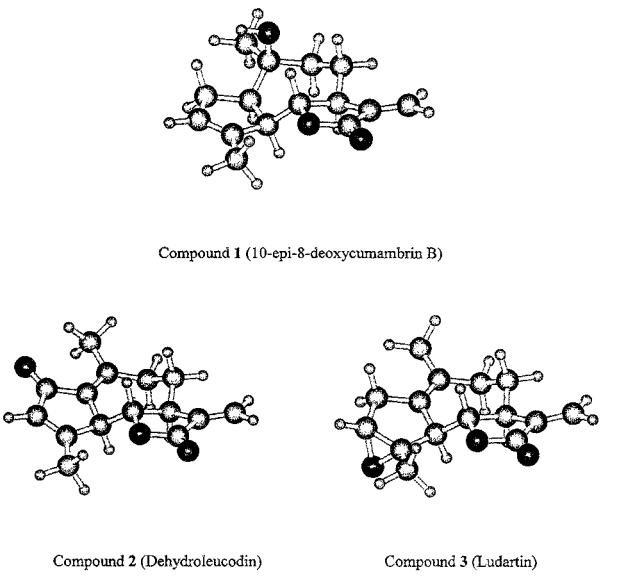

Fig. 5. Three-dimensional models of compound **1** (10 epi-8-deoxycumambrin B), compound **2** (dehydroleucodin) and compound **3** (ludartin).

C-4 (epoxide in compound **3**). From the three-dimensional computer generated model of **1**–**3** shown in Fig. 5, it is possible to see that: (a) in compound **1** the OH group is above the plane of the C-10 member ring, (b) in compound **2** the oxygen appears as a carbonyl group attached to C-2 and aligned in the plane of the molecule and, (c) in compound **3** the oxygenation is below the plane of the molecule as an epoxide group at C-3, C-4. These displacements of the oxygenation in the guaianolide nucleus from top to bottom in the plane of the molecule is accompanied by a decreasing inhibitory activity.

The type II difference spectra obtained for compounds **1** and **2** and not for compound **3**, indicate that compounds **1** and **2** have the ability to coordinate to the heme iron at the active site. Besides, according to their spectra, compound **1** coordinates better than compound **2**.

During the aromatization reaction, the substrate (androstenedione or testosterone) has been suggested to position in the active site in such a manner that the methyl group C-19 is directed toward the heme iron, which directly participates in the three sequential oxidation of this methyl group. Besides, according to the aromatase model proposed by Graham-Lorence et al. [23], the substrate is fixed in the active site via interaction of the C-3 carbonyl group with K473 (lysine) and the 17-keto group of the androstenedione or the 17-OH of the testosterone to the H128 (histidine), stabilising the substrate and holding the C-19 methyl near the heme iron. In our case, we superimposed the models of compound **1** and testosterone by overlapping by hand the carbonyl group of compound **1** and the carbonyl group at C-3 of testosterone. We thus found a combination of both structures in which the hydroxyl group at C-10 of compound **1** is spatially close to the C-19 methyl group of testosterone (Fig. 6). This proximity in space would indicate that in analogy with the model proposed by Graham-Lorence et al. [23], the carbonyl group of the lactone would interact with the K473 residue and the OH group at C-10 would coordinate to the heme iron during the inhibition mechanism. In the case of compound **2**, which

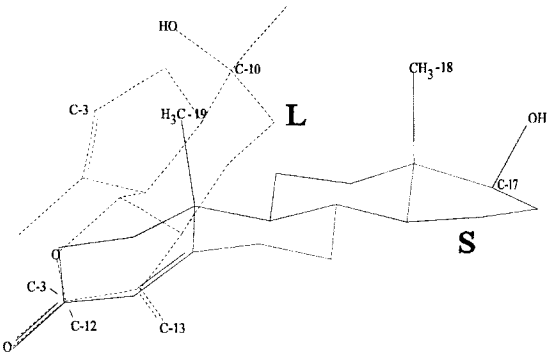

Fig. 6. Three-dimensional models of compound **1** (dotted line) and testosterone (full line). The models are superimposed by overlapping by hand the carbonyl group at C-12 of compound **1** and the carbonyl group at C-3 of testosterone. S, steroid; L, sesquiterpene lactone.

shows a weaker type II difference spectra, the carbonyl oxygen at C-2 seems to be responsible of coordinating to the heme iron. Considering that the coordination capacity of a ketone carbonyl is similar to an alcohol [24], the weaker type II spectra and the weaker activity shown by compound **2** may be due to the fact that carbonyl group at C-2 is farther from the heme iron than the OH of compound **1**. In the case of compound **3** the epoxide group is in the α face of the molecule and close to the lactone carbonyl group, which may explain why this compound is less active than **1** and **2**, and does not present a type II spectra. On the other hand, the small size of these inhibitors compared with the steroidal nucleus seems to be a limitation for the possible interactions with the relatively large active site of the enzyme.

5. Conclusions

As a result of our investigation we can conclude that we are in presence of a new group of natural non-steroidal inhibitors of the aromatase enzyme complex. Besides, the three most potent inhibitors here described (**1**, **2** and **3**) have shown to be: (a) competitive with respect to the substrate testosterone; (b) more specific than aminoglutethimide, since they do not affect the activity of the cholesterol side chain cleavage enzyme, the first step of the steroidogenesis; (c) compounds **1** and **2** act as type II ligands to the heme iron present in the active site of aromatase cytochrome P450.

To our knowledge there are few guaianolides with a C-10 hydroxyl group β-orientated and all were isolated from the genus *Stevia* from north-western of Argentina [7,25]. This uncommon stereochemistry opens interesting perspectives for the design of future modifications on the sesquiterpene nucleus in order to enhance its aromatase inhibitory activity.

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