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iso-Kaurenoic acid from *Wedelia paludosa* D.C.

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ABSTRACT

A recent reinvestigation of aerial parts of *Wedelia paludosa* D.C. is described and reports, for the first time, the isolation of *iso*-kaurenoic acid from this species.

Key words: Asteraceae, *iso*-kaurenoic acid, silica gel impregnated with silver nitrate, *Wedelia paludosa* D.C.

INTRODUCTION

The genus *Wedelia* (Asteraceae, tribe Heliantheae, subtribe Ecliptinae) consists of about 60 species spread in tropical and warm temperate regions, including Brazil, India, Burma, Ceylon, China and Japan. Many plants of this genus, which are used as traditional herbal medicines throughout the world, have been reported to possess hepatoprotective, antipyretic-analgesic, bactericidal and molluscicidal activities (Li et al. 2007, García et al. 2007).

W. paludosa D.C. is a creeping plant frequently used as ornamental and is found in many regions of Brazil, especially in the states of Pernambuco, Bahia, Minas Gerais, São Paulo and Santa Catarina, where it is known as "pseudo-arnica", "pingo-de-ouro" or "margaridão" (Bresciani et al. 2000). The ethanol extract of its aerial parts was shown to exhibit *in vitro* trypanosomicidal activity against trypomastigote forms of *Trypanosoma cruzi*, which is the aetiological agent of Chagas Disease (Chiari et al. 1996). Bioassay-directed fractionation of this extract afforded the trypanosomicidal diterpenes *ent*-kaur-16-en-19-oic acid (**1**, kaurenoic acid) and *ent*-kaur-9(11),16-dien-19-oic acid (**2**, grandiflorenic acid) (Batista et al. 1999). Both of these diterpenes are major constituents of *W. paludosa* and occur along with other related diterpenes, triterpenes and eudesmanolide lactones (Roque et al. 1987, Ferreira et al. 1994, Block et al. 1998a, b, Batista et al. 1999, 2005, Carvalho et al. 2001).

The present paper describes a recent reinvestigation of aerial parts of *W. paludosa* D.C. and reports, for the first time, the occurrence in this species of *ent*-kaur-15-en-19-oic acid (*iso*-kaurenoic acid) (**3**). The isolation of the methyl ester of **3**, from a mixture of **1**+**2**+**3**, is also reported.

MATERIALS AND METHODS

General experimental procedures

Uncorrected melting point was measured with an APF-301 apparatus. IR spectrum was obtained using a Shimadzu IR-400 and Nicolet Impact 410 spectrophotometer. FAB High Resolution Mass Spectrometry (FAB-HRMS) spectrum was recorded on a VG TS250 mass spectrometer. Optical rotation was measured with a

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Perkin-Elmer 241 digital polarimeter. NMR spectra were recorded at 200 MHz for ^{1}H and 50 MHz for ^{13}C in deuterochloroform, added of TMS as internal reference, on a Bruker AC 200. Chemical shift values are expressed in ppm. Column chromatography (CC) and flash column chromatography (FCC) were performed on silica gel Merck 60 (0.063-0.200 and 0.040-0.063 mm, respectively). TLC was carried out on silica gel Merck 60 F254 (0.25 mm thick). Solvents and reagents were purified by standard procedures as necessary.

Plant Material

Aerial parts of *W. paludosa* D.C. were collected in October 2001 around the Federal University of Minas Gerais – UFMG, in Belo Horizonte, Minas Gerais, Brazil. This plant material has been previously identified by Dr. Telma S.M. Grandi (Chiari et al. 1996) and housed as a voucher specimen at the UFMG herbarium (BHCB 19033).

Extraction and Isolation of Kaurane Diterpenes

Air-dried aerial parts of *W. paludosa* D.C. (1.3 kg) were pulverized and extracted by percolation firstly with a 1:1 mixture of hexane-dichloromethane (10 L) and, then, with ethanol (20 L). The solvents of these extractions were removed under reduced pressure yielding a brown resinous oil (hexane-dichloromethane extract, **HDE**; 67.0 g) and a viscous greenish residue (ethanol extract, **EE**; 134.0 g) (Scheme 1).

EE (134.0 g) was coarsely fractionated on a silica gel column (8.0 × 25.0 cm) by elution with hexane (fractions from 1 to 3), hexane-dichloromethane 1:1 (fractions from 4 to 10) and dichloromethane (fractions from 11 to 15), collecting fractions of 500 mL that were concentrated in a rotavapor and combined according to their similarity on TLC. Fractions 5-12 were combined (22.9 g) and rechromatographed on a silica gel column (3.5 × 33 cm; 100 mL per fraction), eluting with mixtures of hexane/ethyl acetate of increasing polarities (100:0, 95:5, 90:10, 80:20) to give 34 final fractions, which were grouped together according to TLC analysis. A mixture of kaurenoic (**1**), grandiflorenic (**2**) and *iso*-kaurenoic (**3**) acids (14:17:8 respectively; 5.6 g) was obtained by crystallization of the grouped fractions 6-15. Attempts to isolate each constituent from this mixture of **1+2+3** (506 mg; 14:17:8) by FCC led to just 52 mg of grandiflorenic acid (2), which was eluted with hexane-diethyl ether 97:3.

Thus, this mixture of diterpenes **1+2+3** (1.14 g; 3.77 mmol, 14:17:8) was esterified by usual procedure with an ethereal solution (200 mL) of diazomethane, giving the mixture of methyl esters **4+5+6** (1.20 g; 3.77 mmol, 14:17:8) in quantitative yield, which was further submitted to column chromatography on silica gel (80 g) impregnated with 20% of silver nitrate, eluting with hexane-diethyl ether 97:3 (100 mL per fraction) to afford methyl *iso*-kaurenoate (**6**) (123 mg; 0.39 mmol; 50% yield) from combined fractions 21-46.

Methyl ent-kaur-15-en-19-oate (methyl *iso*-kaurenoate, 6). mp 73-74°C (*n*-hexane as solvent for crystallization); $[\alpha]^{25}D$ −48.9°, $CHCl_3$, *c* 0.90; IR (film/$CHCl_3$ solution, ν_{max}/cm^{-1}): 3020, 2928, 2848, 1728, 1646, 1443, 1231, 1158, 814. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 5.06 (s, 1H, H-15), 3.64 (s, 3H, H-1′), 2.30 (bs, 1H, H-13), 1.69 (s, 3H, H-17), 1.16 (s, 3H, H-18), 0.84 (s, 3H, H-20). ^{13}C NMR (50 MHz, $CDCl_3$) data, see Table I. HRMS (FAB-POSI, M+1) calcd 317.2481, found 317.2441.

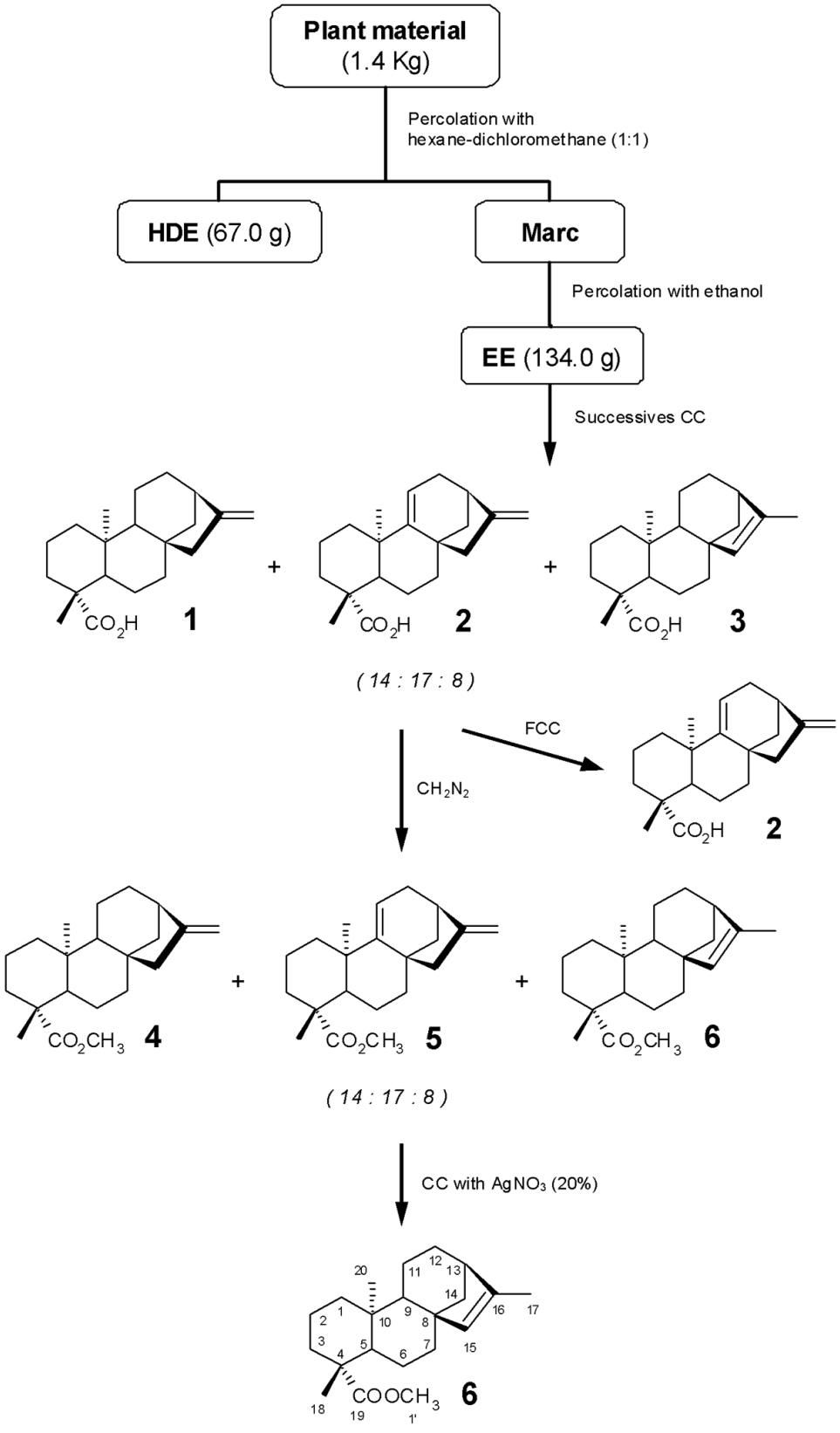
Preparation of Silica Gel Impregnated with Silver Nitrate (20%)

Column chromatography silica gel (64 g) was added to a solution of silver nitrate (16 g) in deionized water (100 mL). The aqueous mixture was concentrated in a rotary evaporator and dryed in an oven at 150°C for 12 hr. The resulting grey powder was stored in vacuo in the dark for further use.

RESULTS AND DISCUSSION

Isolation and Identification of *iso*-kaurenoic Acid

After extraction with a hexane-dichloromethane mixture (1:1), the aerial parts of *W. paludosa* were extracted with ethanol to give the ethanol extract (**EE**) that, after successive CC over silica gel, has yielded a mixture of diterpenes **1**, **2** and **3**, as described in the experimental section and depicted in Scheme 1. Attempts to separate these diterpenes by CC were unsuccessful, except when a careful FCC was carried on affording 52 mg of **2**. For this reason, part of the mixture was submitted



Scheme 1 – Isolation of diterpenes **2** and **6** from *W. paludosa* D.C.

TABLE I
^{13}C NMR data (δ/ppm; $CDCl_3$) of methyl kaurenoate (*4*) and methyl *iso*-kaurenoate (*6*).

Carbon	4*[a]	6	
		Literature**[b]	Present work[a]
1	40.8	40.9	40.8
2	19.1	19.2	18.9
3	38.1	38.2	38.1
4	43.8	43.9	43.8
5	57.1	56.9	56.8
6	21.9	21.0	20.8
7	41.3	43.9	43.8
8	44.2	49.2	49.3
9	55.1	48.1	48.0
10	39.4	39.6	39.5
11	18.4	19.0	19.1
12	33.1	24.9	24.8
13	43.8	44.8	44.7
14	39.7	39.6	39.4
15	48.9	135.1	135.1
16	155.9	142.5	142.3
17	102.9	15.2	15.3
18	28.7	28.7	28.7
19	178.1	178.0	178.0
20	15.4	15.2	15.1
1'	51.1	51.1	51.0

*Batista et al. 2007. [a]50 MHz. **Yamasaki et al. 1976. [b]25 MHz.

to esterification with diazomethane to the corresponding mixture of methyl esters **4**, **5** and **6**, in the same proportion (14:17:8) of the starting material, according to the analysis of integral values observed on its ^{1}H NMR spectrum (Fig. 1). The composition of this mixture was evident by the presence of characteristic signals at δ 4.79 and 4.74 (s, H-17$_{\alpha,\beta}$) for compound **4**; δ 5.24 (bs, H-11), 4.91 and 4.79 (s, H-17$_{\alpha,\beta}$) for compound **5**; and, finally, δ 5.06 (s, H-15) for methyl *iso*-kaurenoate **6** (Wada et al. 1981, Batista et al. 2005, 2007). The unsuccessful attempts to isolate each constituent from the mixture **4+5+6** by usual CC on silica gel is in agreement with literature data, since a mixture of **4** and **6** was previously considered as an inseparable one (Wada et al. 1981). Thus, CC of this mixture on silica gel impregnated with silver nitrate (20%) was performed once this condition is recommended for separations that are otherwise carried on by often more difficult, less effective or tedious methods (Williams and Mander 2001). Then, methyl *iso*-kaurenoate **6**, the minor constituent of the mixture of acids **1+2+3**, was successfully isolated with a 50% yield from the mixture of the methyl esters **4+5+6**.

^{1}H and ^{13}C NMR data of compound **6** were found to be in agreement with those available for methyl *iso*-kaurenoate (Wada et al. 1981, Yamasaki et al. 1976). Besides the presence of the characteristic singlet at δ 5.06 (1H, H-15) on the ^{1}H NMR spectrum of methyl *iso*-kaurenoate (**6**), in opposite to two singlets at δ 4.74 and 4.79 (1H each, H-17$_{\alpha,\beta}$) observed for methyl kaurenoate (**4**), compound **6** can also be distinguished from **4** by comparison of their C-8, C-9, C-12, C-13, C-15, C-16 and C-17 chemical shifts (Table I), whose differences are due to the presence of the double bond at C-15/C-16 (**6**) or C-16/C-17 (**4**) positions. Recent data on *iso*-kaurenoic acid (**3**) and methyl *iso*-kaurenoate (**6**) have not been found in the literature. Miles et al. (1990) stated that physical and chemical properties of *iso*-kaurenoic acid (**3**) had been previously reported by Bohlmann et al. (1981) and Hayman et al. (1986), but such data were also not found in these references.

Biogenetic Aspects

Diterpenoids represent a vast class of isoprenoid natural products, which is biosynthesized from $2E,6E,10E$-geranylgeranyl pyrophosphate (GGPP) (Dewick 1999). They are classified in acyclic (phytanes), bicyclic (labdanes, clerodanes), tricyclic (pimaranes, abietanes, cassanes, rosanes, vouacapanes, podocarpanes), tetracyclic (trachylobanes, kauranes, aphidicolanes, stemodanes, stemaranes, beyeranes, atisanes, gibberellanes), macrocyclic diterpenes (taxanes, cembranes, daphnanes, tiglianes, ingenanes) and mixed compounds, in accordance with the number and the pattern of cyclizations shown by their skeleton (García et al. 2007).

It has been assumed until recently that all isoprenoids are exclusively formed from the C_5 compounds isopentenyl (IPP) and dimethylallyl (DMAPP) pyrophosphates, both of them derived from mevalonic acid (MVA) (Chappell 1995). In the cytosol, MVA is phosphorylated via two steps into MVA-5-diphosphate, which, after decarboxylation, yields IPP. However, new results indicate that IPP can also be formed via a non-mevalonate pathway in plastids (Lichtenthaler 1999, Rohmer 1999). In this pathway, D-glyceraldehyde 3-phosphate plus pyru-

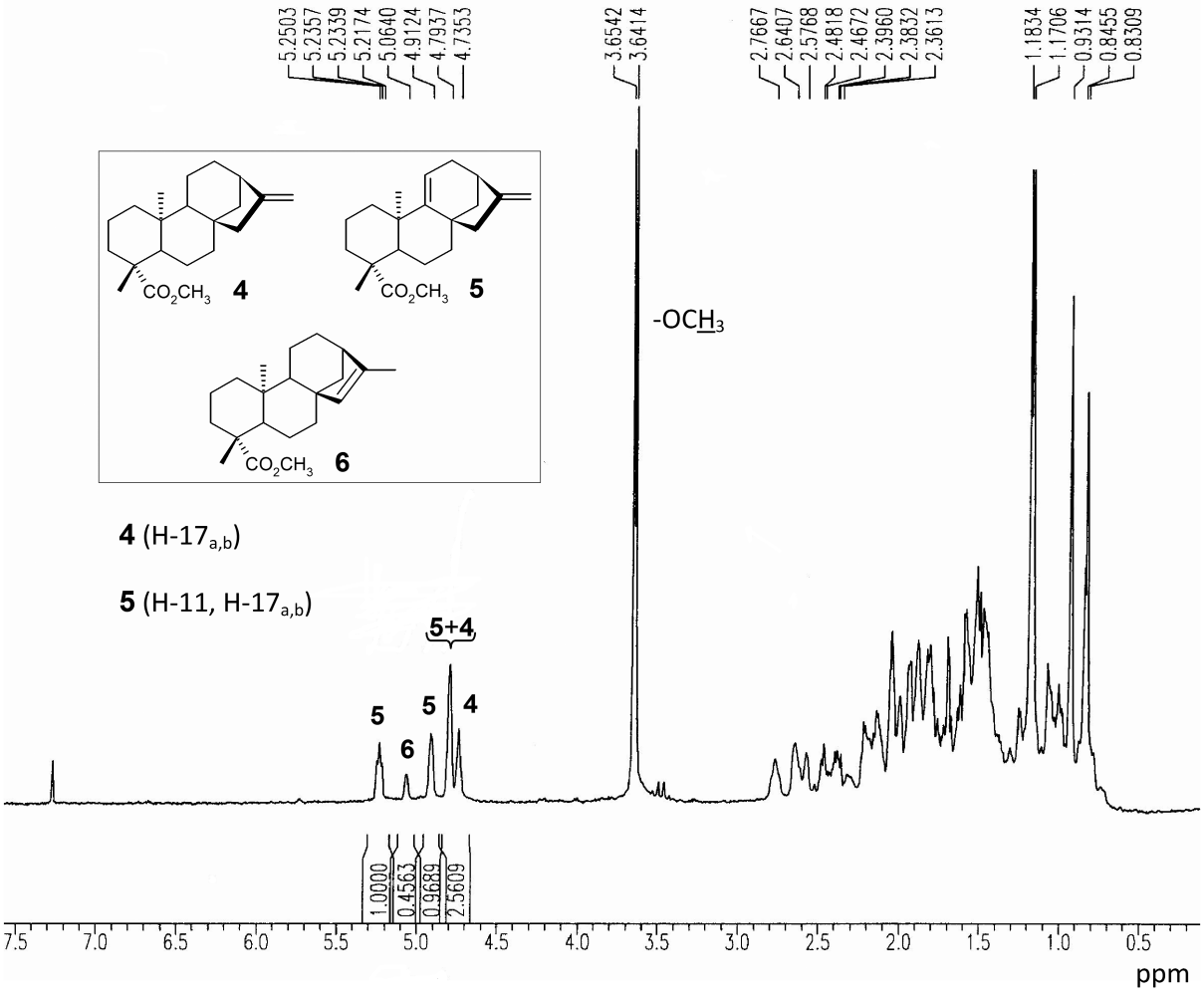
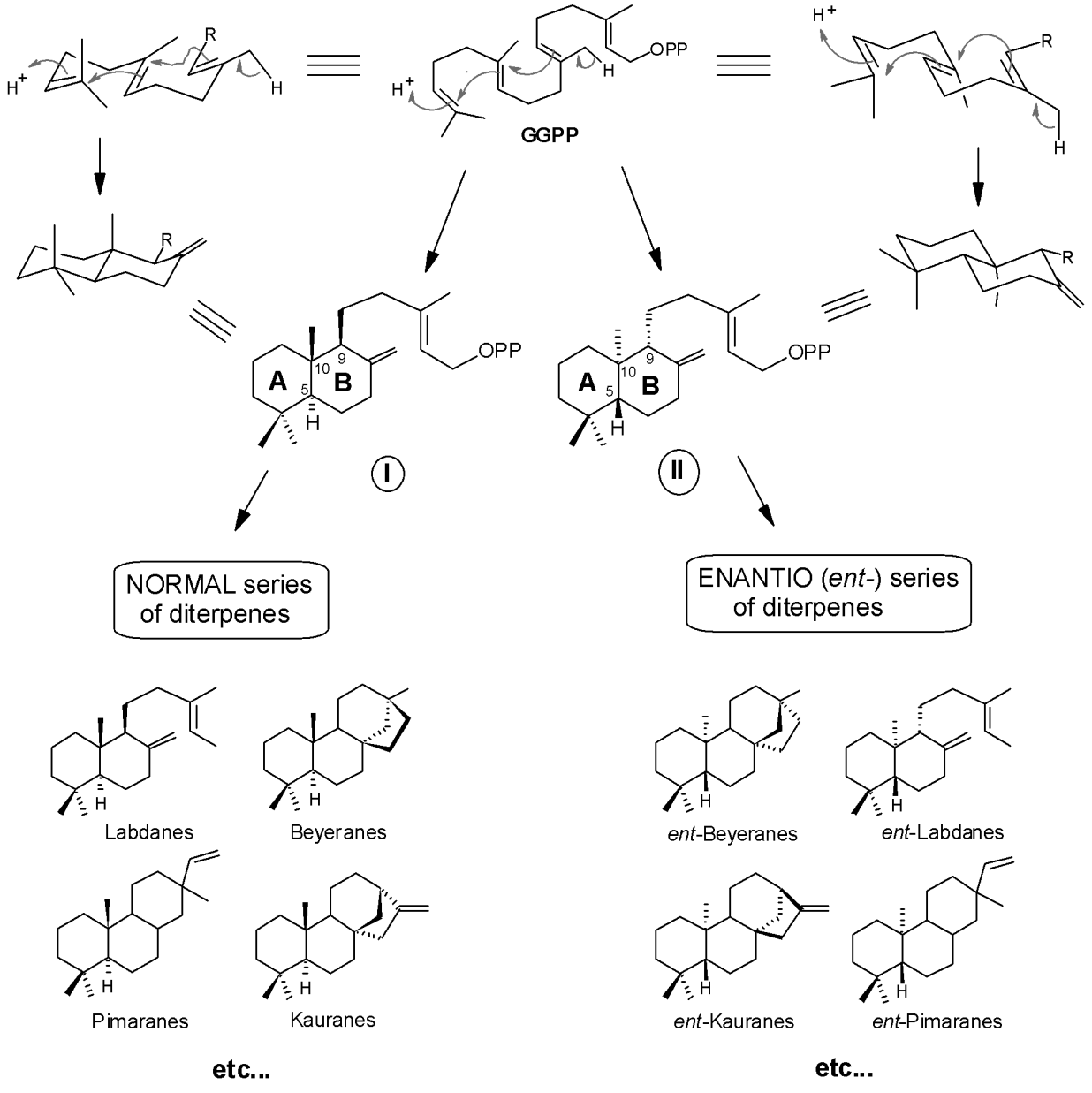


Fig. 1 – ^{1}H NMR spectrum (200 MHz, α/ppm, $CDCl_3$) for the mixture of methyl esters **4+5+6**.

vate yields 1-deoxy-D-xylulose 5-phosphate, which is converted into IPP. The mevalonate pathway gives rise to sterols, sesquiterpenes, and triterpenoids, whereas the pathway involving 1-deoxy-D-xylulose 5-phosphate yields carotenoids, phytol, plastoquinone-9, mono- and diterpenoids. Some interchanges among the pathways seem to exist (Rademacher 2000).

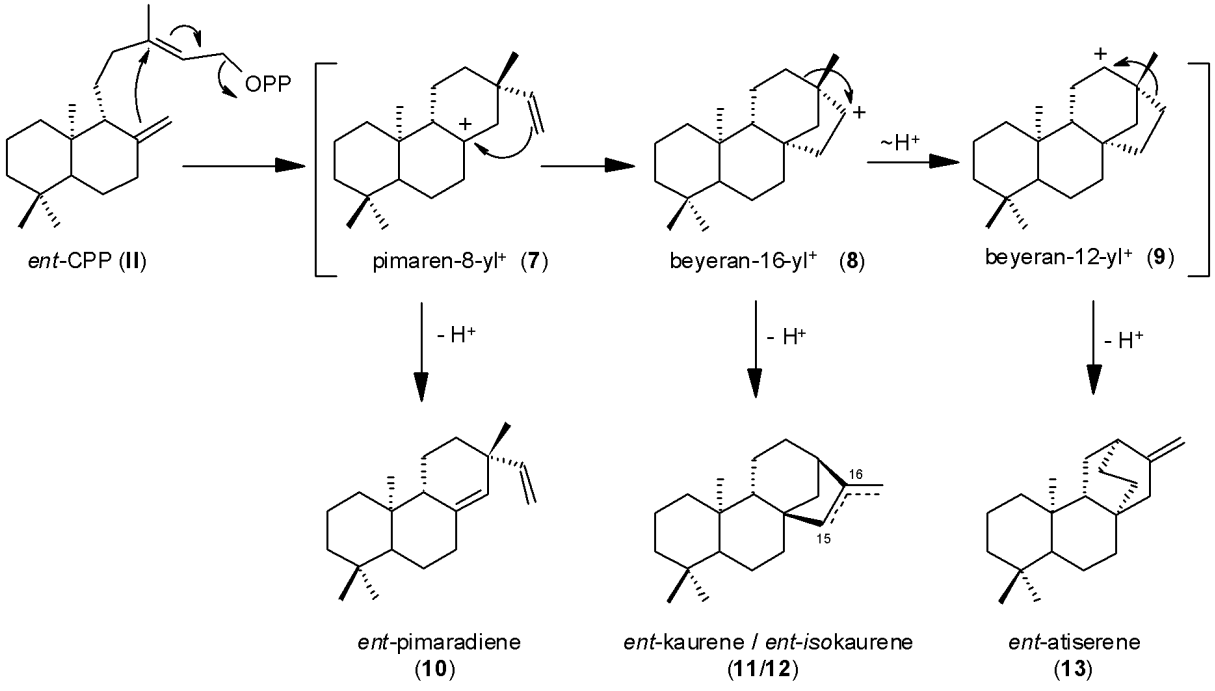
IPP is transformed via an isomerase-catalyzed reaction into dimethylallyl-PP. In head-to-tail condensations, three molecules of IPP are sequentially added to this compound to form geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}), and, finally, the C_{20} compound geranylgeranyl diphosphate (GGPP).

GGPP is cyclized via copalyl diphosphate (CPP) to *ent*-kaurene (Dewick 1999). This type of cyclization (Scheme 2) occurs in a very similar way to the cyclization that produces cyclic triterpenes. However, there is no previous epoxidation step as occurs in triterpene cyclization, but the double bond protonation on the initial isopropylidene unit of the GGPP chain leads to two perhydronaphtalene bicyclical intermediates [Scheme 2; copalyl diphosphate (CPP, **I**) and *ent*-copalyl diphosphate (*ent*-CPP, **II**)], resulting in the two enantiomeric series that differ from each other in their inverted configurations of the carbons C-5, C-9 and C-10. Namely, the "normal" series are the structures whose fusion between A and B rings occurs in the same way as in steroids, while the "enantiomeric" series (denoted as "*ent*-") are the corresponding structures of the specular images of the normal series (García et al. 2007).



Scheme 2 – Cyclization of geranylgeranyl pyrophosphate (GGPP) leading to the diterpenes of the "NORMAL" and "ENANTIO" (*ent*-) series (García et al. 2007).

Kaurene synthase (KS) catalyzes the cyclization of *ent*-copalyl diphosphate (*ent*-CPP, **II**) to *ent*-kaur-16-ene (*ent*-kaurene, **11**) through a multiple-step reaction mechanism that is depicted in Scheme 3. According to this scheme, diphosphate ionization-initiated cyclization of *ent*-CPP (**II**) to a pimaren-8-yl$^+$ (**7**) intermediate may be followed by secondary cyclization to a beyeran-16-yl$^+$ (**8**) intermediate that can either undergo ring rearrangement to the kauranyl ring structure, or, by a 1,3-hydride shift, to a beyeran-12-yl$^+$ (**9**) intermediate that undergoes ring rearrangement to the atiseranyl ring structure. In each case, the final carbocation intermediate is quenched by deprotonation [dotted bonds indicate alternative double-bond placement in *ent*-kaur-16-ene (*ent*-kaurene, **11**) *versus ent*-kaur-15-ene (*iso*-kaurene, **12**)] (Xu et al. 2007).



Scheme 3 – Cyclization mechanism for pimaradienes, kaurenes and atiserenes (Xu et al. 2007).

KS is found in all higher plants because kaurene is an intermediate in the route to the diterpenoid gibberellin phytohormones required for normal growth and development. Hence, this enzyme participates in primary metabolism (Xu et al. 2007).

Occurrence and Biological Activities of Kaurenoic (1) and *iso*-kaurenoic (3) Acids

Kaurane diterpenes are widely found in different plant species belonging to several families such as Asteraceae, Annonnaceae, Euphorbiaceae, Celastraceae, Apiaceae, Velloziaceae, Lamiaceae (= Labiatae), Fabaceae, Rutaceae, Chrysobalanaceae, Jungermanniaceae, Erythroxylaceae and Rhizophoraceae, among others (García et al. 2007). A general survey and some taxonomic implications of the occurrence of kaurane and other classes of diterpenes in the Asteraceae family are discussed by Alvarenga et al. (2005).

Kaurenoic acid (**1**), an *ent*-kaurane diterpene, discloses a wide spectrum of bioactivities such as antiinflammatory, antibacterial, antifungal and moluscicide, among others (Ghisalberti 1997). It is relatively abundant in some species belonging to the genera *Wedelia*, *Mikania*, *Annona* and *Xylopia*, which has motivated its quantification in these plant species in order to enable all of them to be used as natural sources of this diterpene (García et al. 2007). It is one of the intermediate compounds in the biosynthesis of diverse kaurane diterpenes, including gibberellins, a group of growth phytohormones (Rademacher 2000). Therefore, it is not surprising that many naturally occurring kauranes act as growth regulators in plants (García et al. 2007).

On the other hand, *ent*-kaur-15-en-19-oic acid, an isomer of kaurenoic acid, also named "*iso*-kaurenoic acid" (**3**), is of very restricted occurrence in the plant kingdom. It has been found in a few number of Annonaceae and Asteraceae species, including *Annona glabra* (Hsieh et al. 2004), *Smallanthus maculatus* (Rios and Leon 2006), *Wedelia biflora* (Miles et al. 1990, Li et al. 2007) and some species belonging to the subtribe Espeletiinae (Asteraceae, tribe Heliantheae), such as *Espeletia semiglobulata*, *Coespeletia spicata* and *Libanothamus humbertii* (Viloria et al. 1997, Usubillaga et al. 2003). This is the first report on the occurrence of *iso*-kaurenoic acid (**3**) in *W. paludosa* D.C.

There are few reports on the biological activity of *iso*-kaurenoic acid (**3**), and it is worth to mention its effect as total feeding inhibition of boll weevils, at a con-

centration of 2.9 μg/ml, and potent antifungal activity against the soil-borne plant pathogenic fungi *Pythium ultimun* and *Rhizoctonia solani* (Miles et al. 1990). Antifeedant and antifungal activities have been extensively reported for kaurenoic acid (**1**) and related *ent*-kaurane diterpenes against several storage pest insects, such as *Homeosoma electellum*, *Trilobium confusum*, *Trogoderma granarium*, *Sitophilus granaries* and *Reticulitermes speratus*, as well as against some pathogenic fungi of agricultural importance, such as *Verticillium dahliae* and *Sclerotinium sclerotiorum* (Ghisalberti 1997).

CONCLUSION

W. paludosa is an abundant source of *ent*-kaurenoic acid (**1**), which is a bioactive diterpene showing a wide spectrum of biological effects and of interest as a starting compound for the production of bioactive derivatives. The presence in this species of *iso*-kaurenoic acid (**3**), which is a diterpene of very restricted occurrence, along with its effect as total feeding inhibitor of boll weevils and very potent antifungal compound against soil-borne fungi, brings naturally occurring kauranes to an important position as starting materials for the synthesis of new derivatives of promising agrochemical application as pesticides.

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RESUMO

Uma recente reinvestigação das partes aéreas de *Wedelia paludosa* D.C. é descrita e relata, pela primeira vez, o isolamento do ácido *iso*-caurenóico desta espécie.

Palavras-chave: Asteraceae, ácido *iso*-caurenóico, sílica gel impregnada com nitrato de prata, *Wedelia paludosa* D.C.

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