Effects of Kaurane Diterpene Derivatives on Germination and Growth of *Lactuca sativa* Seedlings

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Kaurenoic and grandiflorenic acid, isolated from *Wedelia paludosa* (Asteraceae), some derivatives from these acids (alcohols, esters, amides, lactones, oximes) and other naturally occurring kaurane diterpenes were tested for their action on the growth of radical and shoot of *Lactuca sativa*. Gibberellic acid, GA₃, a commercially available phytohormone, belonging to the same class of diterpenes, was also tested. Some of the tested substances showed a remarkable activity either in the inhibition or in stimulation of *L. sativa* growth. The activity, in some cases, was even higher than that of GA₃.

Key words: Gibberellic Acid, Kaurenoic Acid, Allelopathic Activity

Introduction

A number of natural products with allelopathic activity has been reported and used in agriculture, as for example the gibberellins, a group of diterpene lactones. Kaurane diterpenes containing a rigid tetracyclic skeleton are intermediates in the biosynthesis of a number of plant and fungal metabolites, including gibberellins, and are widespread in the plant kingdom. Bioassay-guided fractionation in plant study made this class of diterpenes to be “rediscovered”, due to their many biological activities (Ghisalberti, 1997), including plant growth regulation (Torrenegra and Tellez, 1996; Villalobos et al., 1994; Hanson et al., 1980; Becker and Kempf, 1976; Hüneck and Scheiber, 1972; Cross et al., 1970; Katsumi et al., 1964).

In the phytochemical study of *Wedelia paludosa* (Asteraceae) we have isolated a high amount (ca. 10% of crude extract, in some cases) of ent-kaur-16-en-19-oic acid [kaurenoic acid, (2)], together with a minor proportion (ca. 2–3% of crude extract) of ent-kaur-Δ⁹(11),16-dien-19-oic acid [grandiflorenic acid (3)], among other kaurane diterpenes 11–13 (Fig. 1); ent-kauran-16/17-ol (5) was isolated from *Xylopia frutescens* (Anonaceae). Some bioassay systems were applied on these compounds (and derivatives obtained by chemical transformations of 2 and 3, Fig. 1), as for example, trypanocidal (Vieira et al., 2002) and allelopathic evaluations. We describe here the results of the lettuce (*Lactuca sativa*) hypocotyl assay.

Results and Discussion

Compounds 2–20 (Fig. 1) were obtained by isolation from plant extracts and/or chemical transformations (Vieira et al., 2001, 2002; Takahashi et al., 1995, 2001). Lactones 21 and 22 are described here for the first time to our best knowledge. The structure of lactone 23 was previously elucidated by X-ray (Doriguetto et al., 2002). Their syntheses are showed in Scheme 1. Intermediary ketones 24 and 25 were obtained from 2 and 3, respectively, initially by exocyclic double bond oxidation (Castellaro et al., 1990), followed by Baeyer-Villiger rearrangement (Anastasia et al., 1985). Lactone 22 was obtained from triol 8 by oxidation with KMnO₄/CuSO₄ (Jefford and Wang, 1988).

The choice of concentrations to be used in allelopathic experiments was based on the work of Macías et al. (1994, 2000). According to them, substances with inhibitory activity against species used in standard allelopathic bioassays show a strong inhibitory effect (alternative herbicides) only at concentrations between 10⁻² and 10⁻³ M; at lower concentrations (10⁻⁵–10⁻⁹ M) this effect disappears or becomes stimulatory. Plants were measured after 5 d standing in the dark, according procedures described by Hoad et al. (1981) and Macías et al.
(2000). The experimental results of germination, radical and shoot length in the lettuce hypocotyl assay for the kaurane diterpenoids 2–23 and gibberellic acid, GA₃ (1), are shown in Fig. 2–5. The numbers are expressed as percent units from the control: zero represents an observed value identical to the control, a positive value represents stimulation and a negative value represents inhibition.

Gibberellins are physiologically defined by their ability to induce shoot elongation in certain dwarf plants which was demonstrated in various plant extracts using dwarf maize plants and dwarf peas. With *Lactuca sativa*, in the dark, Frankland and Wareing (1960) demonstrated that hypocotyl growth was very little affected by gibberellic acid treatment at $10^{-4}$ M.
The acids 1–4 presented stronger action on radical than on shoot growth (Fig. 2). The best activity for 1 was at $10^{-7}$ M and for 3 at $10^{-3}$ M, although the latter was active in all three tested concentrations. Gibberellic acid (1) inhibited radical growth at higher concentrations, contrary to kaurenoic acid (2), that acted in an opposite way. Brian et al. (1967) did not find activity for 2 in the lettuce hypocotyl assay at 10 ppm ($3.3 \times 10^{-5}$ M) concentration.

Acids 1–3 were active on shoot growth (Fig. 2); compound 4 (ent-3β-hydroxy-kaur-16-en-19-oic acid) showed activity only at $10^{-7}$ M, the presence of the hydroxyl group at C-3 being the differentiating structural factor. The stereochemistry of hydroxyl groups in gibberellins is important: 3β-hydroxy gibberellins, in the absence of a 2β-hydroxy group, are more active than the correspondent α-hydroxy derivatives (Hoad et al., 1981). Therefore, the small activity of compound 4, compared to the
other acids, could be also associated with the \( \alpha \) stereochemistry of the hydroxyl group at C-3.

Oppositely to the acids, the alcohols 5–8 showed better stimulating activity on shoot growth (Fig. 2), mainly \( \text{ent-kauran}-16/\text{H}-17\)-ol (8) with the higher stimulatory effect at \( 10^{-3} \) M; in contrast, Brian et al. (1967) cited the slight activity presented by 5 in the lettuce hypocotyl assay at 10 ppm (3.5 \( \times 10^{-5} \) M) concentration. \( \text{Ent-kauran}-16\)-en-19-ol (6), the higher inhibitor among alcohols, and diol 7 showed analogous activity on radical elongation; triol 8 acted in the opposite way on promoting shoot growth and inhibiting radical growth. Only gibberellic acid (1) stimulated the germination of \( L. \) sativa. (Fig. 3A). Among the esters (9–13), \( \text{ent-3β-tigloyloxykauran-16-en-19-oic acid} \) (13) was the most active on radical growth, at \( 10^{-7} \) M, the lower dosis (Fig. 4). Methyl \( \text{ent-kauran-16-en-19-oate} \) (9) was the best inhibitor on shoot growth at \( 10^{-3} \) M (Fig. 4). According to Villalobos et al. (1994), the presence of an angeloyloxy group at C-18, associated with a methyl ester at C-19, improves the activity. Both \( E \) oximes 15 and 16 showed analogous results on radical and on shoot growth. Here, the \( E \) stereochemistry of the oxime group seems to be the determinant factor for growth activity. The kaurenoic ester 9 showed the best germination results, at \( 10^{-5} \) M, among all tested compounds, followed by tygloyloxy ester 13 and ester \( Z \) oxime 14 (Fig. 3B). Amide 18 (\( \text{ent-kauran-16-en-19-pyrrolidinamide} \) was active in both shoot and radical elongation and in all three concentrations (Fig. 5). In general, all three amides 17, 18 and 19 showed a total coherence in their results for radical and shoot growth. Tetrachirin (20) and lactone 23 showed similar activities in both radical and shoot growth (Fig. 5). Lactone 21, from kaurenoic acid, presented the best stimulatory \( (10^{-5} \) M) and the best inhibitory \( (10^{-3} \) M) effect on shoot elongation of \( L. \) sativa (Fig. 5).

Finally, only grandiflorenic acid (3) and \( \text{ent-kauran-16-en-19-pyrrolidinamide} \) (18) showed action on both shoot and radical length in all concentrations; kaurenoic acid (2) had analogous action in radical and shoot growth; gibberellic acid (1) acted
in an opposite way compared to lactone 23: its profile activity in radical corresponds to the shoot in 23 and vice-versa. Results found for the inhibitory effect for most of the tested substances corroborate with the proposition found in the literature (Macías et al., 2000) that inhibition occurs mainly at concentrations around at 10⁻³ M. These substances are consequently potential alternative herbicides.

**Experimental**

**General procedure**

Melting points were determined with a Kofler hot plate apparatus and are uncorrected. The optical rotations were measured on a Perkin Elmer 341 polarimeter. IR spectra were recorded on a Shimadzu IR-408 spectrophotometer. IR absorption bands are expressed in cm⁻¹. ¹H and ¹³C NMR spectra were recorded in CDCl₃ at room temperature on a Bruker Advance DPX 200 operating at 200 and 50 MHz, respectively. The chemical shifts are reported in δ values (ppm) relative to the solvent CDCl₃ (δ = 7.26 for ¹H NMR and 77.01 ppm for ¹³C NMR). Mass spectra (GC-EI-MS) were obtained from a GC Finnigan-ION TRAP instrument and they were performed with an ionizing energy of 70 eV. Silica gel used for flash chromatography was obtained from Merck (WC4790-005, 230–400 mesh) and celite from Labsynth Ltda., São Paulo, SP, Brazil.

Gibberelic acid (1) was purchased from Sigma (USA). *Ent*-kaur-16-en-19-oic acid (kaurenoic acid, 2) was isolated from green fruits of both *Xylopia frutescens* (Takahashi et al., 1995) and *Xylopia sericea* (Takahashi et al., 2001) and aerial parts of *Wedelia paludosa* (9.6% of ethanolic crude extract). Grandifloric acid (3) was isolated from ethanolic extracts of aerial parts of *Wedelia paludosa* (2.4% of crude extract). *Ent*-3β-hydroxy-kaur-16-en-19-oic acid (4) was obtained by alkaline hydrolysis from *ent*-3β-cinamoyloxykaur-16-en-19-oic acid (12, yield: 84%). *Ent*-kaur-16β-H-17-ol (5) was isolated from the hexanic extract of green fruits of *Xylopia frutescens* (2.2% of crude extract). *Ent*-kaur-16-en-19-ol (6) was obtained by reduction of 9 with LiAlH₄ (yield: 86%). *Ent*-kaur-16β/H-17,19-diol (7) and *ent*-kaur-16β/H-11α,17,19-triol (8) were obtained from 2 and 3, respectively, by treatment with diborane/H₂O₂ (Vieira et al., 2002). Methyl *ent*-kaur-16-en-19-oate (9) and methyl *ent*-kaur-3β-hydroxy-16-en-19-oate (10) were obtained by treatment of 2 and 4, respectively, with diazomethane (quantitative yield). *Ent*-3β-angeloyloxykaur-16-en-19-oic acid (11, 2.4% of crude extract), *ent*-3β-cinamoyloxykaur-16-en-19-oic acid (12, 1.9% of crude extract) and *ent*-3β-tyglyloxykaur-16-en-19-oic acid (13, 0.065% of crude extract) were isolated from ethanolic extracts of aerial parts of *Wedelia paludosa* (Vieira et al., 2001). Methyl *ent*-16Z-oxime-17-norkauran-19-oate (14), methyl *ent*-16E-oxime-17-norkauran-19-oate (15), *ent*-16E-oxime-17-norkauran-19-ol (16), *ent*-kaur-16-en-19-piperidinamide (17) *ent*-kaur-16-en-19-pyrrolidinamide (18) and *ent*-kaur-16-en-19-N,N-diethylamide (19) were obtained according to the route described by Vieira et al. (2002). Tetrachirin (20, 0.068% of crude extract) was isolated from ethanolic extracts of aerial parts of *Wedelia paludosa* (Vieira et al., 2001).

*Ent*-kaur-16-oxo-17-nor-19 oic acid (24) and *ent*-kaur-16-oxo-17-nor-11(9)-en-19-oic acid (25) (Castellaro et al., 1990). To a suspension containing a mixture (500 mg) of kaurenoic acid (2) and grandifloric acid (3) and 1.6 g (8.0 mmol) of NaIO₄ in 50 ml of THF/H₂O 1:1 v/v a crystal of OsO₄ was added. After overnight stirring at room temperature, work-up (treatment by NaHSO₃) and flash chromatography, 173 mg of 24 and 198 mg of 25 were obtained.

*Ent*-13α-hydroxy-17-nor-13,16-seco-kaur-16,19-dioic acid 16→13-lactone (21) and *ent*-5α,15α-epoxy-9,10-friedo-10β,11β-dihydroxy-16,11ε,19,10β-diseco-17-norkauran-16,19-dioic acid 16→11:19→10-dilactone (23) (Anastasia et al., 1985). Trifluoroacetic acid was generated in situ by adding 148 mmol of trifluoroacetic anhydride to 31 mmol of 30% hydrogen peroxide at 0 °C in anhydrous CH₂Cl₂. Ketone (0.99 mmol), dissolved in anhydrous CH₂Cl₂, was added and the solution stirred for 1 h at room temperature. Work-up (2% K₂CO₃ solution) and flash chromatography (n-hexane/ethyl acetate) furnished pure lactones (yields: 21: 80% and 23: 19%).

*Ent*-13α-hydroxy-17-nor-13,16-seco-kaur-16,19-dioic acid 16→13-lactone (21): White powder, C₂₀H₃₆O₄ (334). m.p. 218–219 °C. [α]D²⁵ 110° (c = 0.002, CHCl₃). - IR (KBr) νmax = 3400, 1725, 1690, 1250–1111 cm⁻¹. - ¹H NMR (CDCl₃, 200 MHz): δ = 0.97 (3H, s, 20-CH), 1.26 (3H, s, 18-CH), 4.68 (1H, br s, 13-CH). - ¹³C NMR (CDCl₃, 50 MHz): δ = 40.9, 18.9, 37.3, 43.5, 56.6, 19.8, 42.9, 33.8, 53.0, 39.5, 16.3, 28.7, 75.8, 33.0,
To a solution containing 48.3 mmol of triol 8 in acetone, KMnO₄ (1.0 mmol) and CuSO₄ ·5 H₂O (0.06 mmol) were added and the mixture was stirred for 2 d at room temperature. After removing of acetone, the residue was chromatographed (celite followed by silica gel) and the lactone 22 was eluted with CH₃Cl₂ (47% yield) as a white powder, C₂₀H₃₀O₃ (318). δ (CDCl₃, 50 MHz): δ = 0.98 (3H, s, H-20), 3.46 (1H, d, J = 11.0 Hz, 19α-CH₂), 3.75 (1H, d, J = 11.0 Hz, 19β-CH₂), 0.99 (3H, s, 18-CH), 2.97 (1H, dd, J = 8.0 and 10.0 Hz, 16α-CH₂), 2.69 (1H, br s, 13-CH), 4.58 (1H, br s, 11-CH). δ ¹³C NMR (CDCl₃, 50 MHz): δ = 40.1, 18.1, 36.1, 38.6, 56.5, 20.4, 38.8, 44.2, 59.4, 37.4, 75.7, 35.2, 34.3, 41.9, 48.3, 42.5, 177.3, 27.2, 65.2, 17.9. - EI-MS: m/z (rel. int.) = 318 (18) [M⁺], 300 (10) [M⁺-H₂O], 287 (100) [M⁺-CH₂OH], 269 (40) [300-H₂O], 241 (35) [287-HCO₂H], 225 (20) [269-CO₂].

Bioassay

Seeds of Lactuca sativa (cv. Grand Rapids) were purchased from Isla Pak, RS, Brasil. All undersized and damaged seeds were discarded. In 100 mm Petri dishes containing a 90 mm sheet of Whatman no. 1 filter paper and 10 ml of a test (10⁻³, 10⁻⁵ and 10⁻⁷ m) or control solution 25 lettuce seeds were added. Test and control solutions were prepared with dionized water and their pH values [buffered with 10 mm 2-(N-morpholino)-ethanesulfonic acid, MES] were adjusted to 6.0–6.5 with NaOH solution. Lower concentrations than 10⁻³ m were obtained by dilution of the previous solution. There were 3 replicates for each concentration and for control. The dishes were sealed and incubated in the dark at 25 °C for 5 d. After this time the dishes were frozen during the measurement process to avoid subsequent growth (Macías et al., 2000). The osmotic pressure values were measured on a microosmometer (Precision Systems Inc., Natick, Mass. USA) and ranged between 30 and 38 mosmolar (Macías et al., 1994).

Statistic treatment

Statistic treatment was done according to Macías et al. (1994) and the results, presented in Fig. 2–5, consist of the differences (in cm) between mean values of seeds grown with tested compounds 1–23 and mean values for control (seeds grown without addition of tested compounds). Mean values of the controls (in mm) were: Fig. 2 = 2.1645 × 10⁻³; Fig. 4 = 2.6871 × 10⁻³; Fig. 5 = 2.3217 × 10⁻³. The germination, radical and shoot length values were tested by the Student’s t-test and the differences between the experiment and control were significant at a value of P = 0.05.

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