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Bioactive apocarotenoids from *Tectona grandis*

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ABSTRACT

The bioactive fractions of *Tectona grandis* have yielded seven apocarotenoids, two of which have been isolated for the first time as natural products (tectoionols A and B). The chemical structures were determined through 1D and 2D nuclear magnetic resonance experiments. The absolute configuration of tectoionol A was determined using a modified Mosher methodology. Some NMR assignments for the compounds 9(*S*)-4-oxo-7,8-dihydro-β-ionol and 3β-hydroxy-7,8-dihydro-β-ionone have been corrected on the basis of g-HSQC and g-HMBC experiments. The general bioactivities of isolated compounds have been studied using etiolated wheat coleoptiles. Those compounds that presented higher levels of activity were assayed on standard target species (*Lactuca sativa*, *Lycopersicum esculentum*, *Lepidium sativum* and *Allium cepa*).

1. Introduction

Forest species produce large amounts of non-timber products in which bioactive substances are present in high percentages. These compounds can be transported to the ground by exudation from roots or leaching of the aerial parts. For this reason, agroforestry systems provide an excellent opportunity to explore the properties of these species in the control of weeds, insects and nematodes or for the improvement of grounds and as a source of bioactive products in Pharmacology.

Interest in allelopathic studies in agroforestry systems is growing, because knowledge of these interactions could provide powerful tools for the integrated management of plagues and a better exploitation of natural resources anywhere in the world.

Tectona grandis L.f. is a large natural tree from Southeast Asia and grows up to 50 m in height. It is commonly known as teak and it is the most important of the three species that belong to the genus *Tectona*. During the second half of the 19th century, the increasing demand for teak wood led to the implantation of the agroforestry system denominated taungya in Indonesia and other tropical countries in Asia. This consists of cultivating maize within young teak plantations (Wiersum, 1982). Since then, this species has been used successfully in culture rotation and is combined with agricultural species such as mountain rice, cotton, tapioca, chilli and ginger. In India it was found that teak plantations with peanut and soybean were very successful and negative effects on the growth of teak were not found (Mishra and Prasad, 1980).

On the other hand, Raets observed in Venezuela that maize seeded between young teak trees reduced the number of cleanings (Raets, 1965). Experiments with the taungya method in Costa Rica also showed good results. In Cuba, the use of this agroforestry system in maize or bean cultures has given excellent harvests. In addition, fields remained clean, without the competition of undesirable plants (Betancourt, 1983). Later studies proved the allelopathic effects of teak leaves on the germination of peanut and maize (Jayakumar et al., 1987).

The allelopathic effect of extracts from teak leaves has been tested on Solanaceae species such as the tomato (*Lycopersicum esculentum*), eggplant (*Solanum melongena*) and pepper (*Capsicum annum*) (Krishna et al., 2003). The extracts significantly inhibited germination and growth of these plant species. *T. grandis* has also shown high allelopathic activity on wheat (*Triticum aestivum*) (Krishna et al., 2003).

We report here the isolation of one monoterpene, seven apocarotenoids and the dehydrololiolide. Two of the apocarotenoids have been isolated for the first time as natural products – tectoionols A and B. The absolute configuration of the new compound tectoionol A was determined and the bioactivity profiles of the isolated compounds were studied.

2. Results and discussion

Dried leaves of *T. grandis* (5.5 kg) were extracted with water at room temperature for 24 h. This aqueous extract was extracted

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with methylene chloride (DCM/H_2O) and ethyl acetate (EtOAc/H_2O). The resulting aqueous layer was dried under reduced pressure (H_2O). The residual vegetal material was extracted with methylene chloride (DCM) and then with methanol (MeOH). The different extracts obtained were assayed on etiolated wheat coleoptiles (Macías et al., 2000) at 1000, 500, 250, 125 and 75 ppm (Fig. 1).

From these results the extracts can be put in decreasing order of activity as follows: DCM/H_2O > DCM > EtOAc/H_2O > MeOH > H_2O. Moreover, the differences in profile between DCM/H_2O and EtOAc/H_2O extracts suggest that the most active metabolites are those with lower polarity. The most active extract (DCM/H_2O) was chromatographed on silica gel using hexane/ethyl acetate mixtures of increasing polarity. The fractions E, F and I obtained using hexane/ethyl acetate (4:1, 1:1, and 1:4) mixtures yielded compounds **1**–**9** (Fig. 2). The spectroscopic data for the monoterpene **1** (Knapp et al., 1998), apocarotenoids **2**–**6** (Macías et al., 1999; Galbraith and Horn, 1972; Lin et al., 1999) and the catabolic product of carotenoids **9** (Ravi et al., 1982) were identical to those reported previously. This is the first time that a monoterpene and apocarotenoids have been described in the genus *Tectona* and that compounds **1**–**6** and **9** have been isolated from the family Verbenaceae. The assignment of the NMR spectra of compounds **4** and **6** is corrected and compounds **7** and **8** are described for the first time as natural products.

Thus, 3β-hydroxy-7,8-dihydro-β-ionone (**4**) showed ^1H and ^{13}C NMR spectra with signals identical to those described for this compound isolated from *Chamaecyparis formosensis* (Lin et al., 1999). Analysis of the correlations observed in g-HMBC experiment showed the following correlations: H-12 (δ 1.00) and H-13 (δ 1.02) with C-2 (δ 48.3); H-11 (δ 1.58) and H-2 (δ 1.37) with C-4 (δ 42.1); H-10 (δ 2.13) and H-7 (δ 2.25) with C-8 (δ 44.2); H-10 (δ 2.13) and H-8 (δ 2.45) with C-7 (δ 21.8); H-2 (δ 1.37) with C-3 (δ 65.0), C-4 (δ 42.1), C-1 (δ 37.8), C-12 (δ 28.3) and C-13 (δ 29.5); H-4 (δ 1.91) with C-3 (δ 65.0), C-2 (δ 48.3) and C-11 (δ 19.8); H-8 (δ 2.45) with C-7 (δ 21.8); and H-7 (δ 2.25) with C-8 (δ 44.2) and C-1 (δ 37.8). Thus, the assignments of protons H-2, H-4, H-7 and H-8 as well as carbons C-4, C-8, C-10 and C-12 must be corrected, as shown in Tables 1 and 2.

Similarly, 9(*S*)-4-oxo-7,8-dihydro-β-ionol (**6**) presents identical ^1H and ^{13}C NMR spectra to those described previously for this compound isolated from *C. formosensis* (Lin et al., 1999). Identical analysis of g-COSY, g-HSQC and g-HMBC experiments showed the following correlations g-COSY: H-8 (δ 1.56) with H-7 (δ 2.19-2.41); g-HSQC: H-2 (δ 2.45) with C-2 (δ 37.4); H-3 (δ 1.79) with C-3 (δ 34.3); and H-7 (δ 2.19-2.41) with C-7 (δ 26.8); g-HMBC: H-12 (δ 1.16) and H-13 (δ 1.16) with C-2 (δ 37.4). These data led us to correct the assignment of signals corresponding to H-2, H-3 and H-7, as well as their corresponding carbons C-2 and C-7, as shown in Tables 1 and 2.

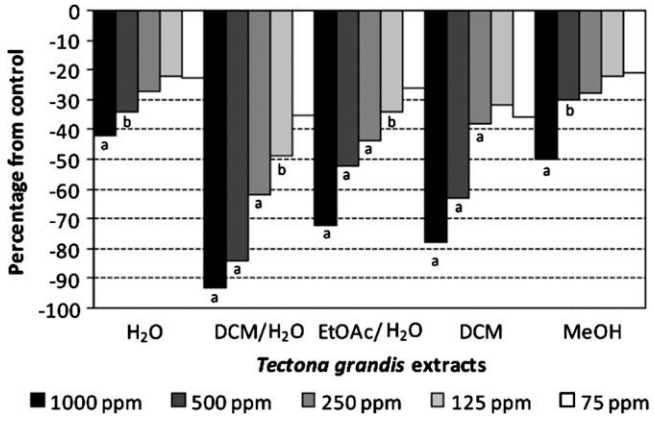


Fig. 1. Effect of *Tectona grandis* extracts on etiolated wheat coleoptile elongation. Values are expressed as percentage from the control and are not significantly different with $P > 0.05$ for tha Mann–Whitney's test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

2.1. Tectoionol A (**7**)

Compound **7** was isolated as a white amorphous solid from fraction I. The mass spectrum of **7** did not show a neat molecular ion. When it was recorded using chemical ionization, together with peak at *m/z* 211 $[M+1-H_2O]^+$ (100%) and 193 $[M+1-2H_2O]^+$ (45%) it is observed a quasi-molecular ion at *m/z* 227 $[M-1]^+$ (3%). This unusual peak has been explained in other cases with two possibles mechanisms (Ramalho et al., 1998). The first one implies a hydride ion transfer reaction to the methane ions. The second one proposed the loss of H_2 by an intramolecular reaction (Fig. 3). The quasi-molecular ion at *m/z* 227.1649 ($C_{13}H_{23}O_3$, calculated mass 227.1647) and fragmentations are consistent with the molecular formula $C_{13}H_{24}O_3$. The IR spectrum shows a broad band at 3400 cm^{-1} that suggests the present of hydroxyl groups.

The ^{13}C NMR spectrum shows the presence of 13 signals. Those that resonate at δ 76.9 (C-9), δ 64.3 (C-3), δ 89.9 (C-6) and δ 77.5 (C-5) indicate the existence of four carbon atoms bonded to oxygen. In addition, the IR spectrum and the molecular formula indicate the presence of two hydroxyl groups and an ether moiety.

The ^1H NMR spectrum shows three signals that integrate to three protons each at δ 1.08, δ 0.87 and δ 1.13. The chemical shifts of these protons and the molecular formula indicate that this compound should be a bisnorsesquiterpene with a ionane skeleton (López, 2003). These signals were assigned to H-13, H-12 and H-11, respectively. Additionally, a doublet at δ 1.15 (3 H, *d*, $J = 5.9$ Hz) coupled with a *ddq* at δ 4.01 were assigned to H-9, whose carbon signal appears at δ 76.9 and suggests that an oxygenated function is located at this position.

The ^1H NMR-2D-COSY spectrum of **7** showed the presence of the fragment CH_2–CH–(O)–CH_2–C–(O)–CH_3 by the following correlation series: signal at δ 1.69 (H-4β) with δ 1.61 (H-4α), δ 3.98 (H-3), and δ 1.43 (H-2β) with a long distance W coupling. The last two signals (H-3 and H-2β) correlated with that at δ 1.56 (H-2α). The chemical shifts of H-3 (δ 3.98) and C-3 (δ 64.3) determine another oxygenated function at this position. A third oxygenated function can be placed at C-5 and this does not present any signal in the ^1H NMR spectrum but has a shift of δ 77.5 in the ^{13}C NMR spectrum.

A second correlation series is in good agreement with a side chain such as CH_2–CH_2–CH(O)–CH_3 in the ionane skeleton: the signal at δ 2.12 (H-7) with δ 1.89 (H-7′), δ 1.96 (H-8) and δ 1.44 (H-8′). H-8 and H-8′ with δ 4.01 (H-9) and, finally, H-9 with δ 1.15 (H-10). The absence of a signal corresponding to H-6, the multiplicity of protons attached at C-7, and the signal at δ 89.9 in the ^{13}C NMR spectrum indicate that the fourth oxygenated carbon should be C-6.

The chemical shifts of carbons 3 (δ 64.3), 5 (δ 77.5), 6 (δ 89.9) and 9 (δ 76.9) are consistent with hydroxyl groups attached at C-3 and C-5, as well as with an ether function between C-6 and C-9. This structure is consistent with the effect observed between signals corresponding with protons H-9 and H-12 in NOE diff experiments and the g-HMBC experiments (Fig. 4). The relative stereochemistry was established on the basis of the NOE effects represented in Fig. 5. These data match the structure proposed for this compound in Fig. 2, which has been isolated as a natural product for the first time and was named tectoionol A. In the literature we found a glycosylated derivative at C-3, isolated from the leaves of *Scorodocarpus borneensis* and denominated scoroposide (Abe and Yamauchi, 1993). Thus, the chemical shifts, multiplicities of signals

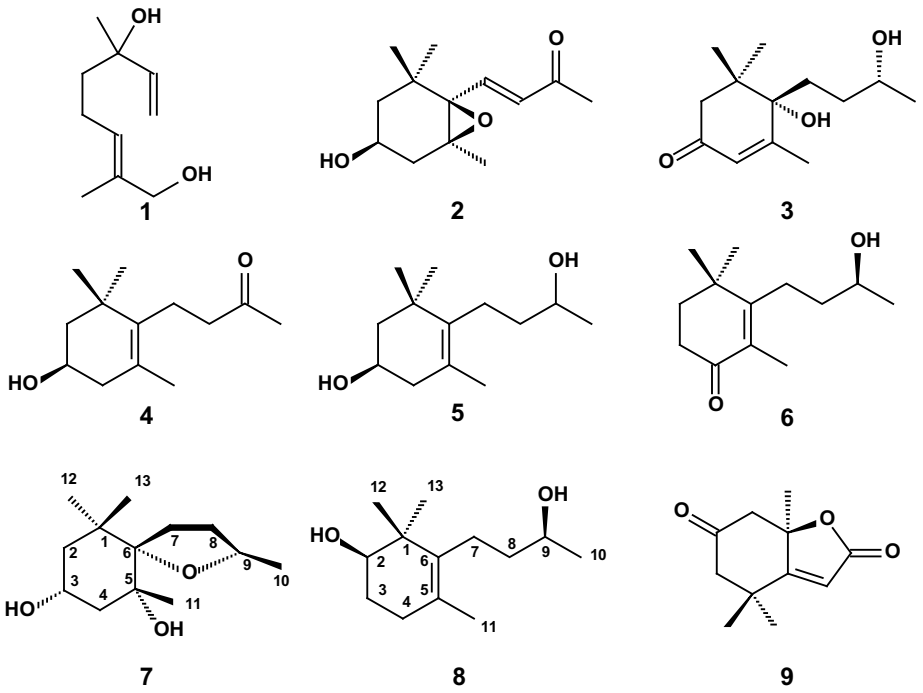


Fig. 2. Isolated compounds from *Tectona grandis*.

Table 1
^{1}H NMR data for compounds **4**, **6**, **7** and **8**

H	4	6	7	8
2	α 1.37 *dd* (*J* = 12.0, 12.0 Hz) β 1.67 *dd* (*J* = 12.0, 5.6, 2.4 Hz)	2.45 *dd* (*J* = 7.0, 7.0 Hz)	ax 1.56 (*J* = 12.2, 11.0 Hz) ec 1.43 (*J* = 12.2, 4.4, 2.2 Hz)	α 3.46 *dd* (*J* = 9.0, 3.0 Hz)
3	3.91 *dddd* (*J* = 12.0, 5.6, 9.6, 2.4 Hz)	1.79 *dd* (*J* = 7.0, 7.0 Hz)	β 3.98 *dddd* (*J* = 4.4, 11.0, 4.6, 11.1 Hz)	ax 1.64 m ec 1.76 *ddd* (6.0, 3.0, 2.0 Hz)
4	1.91 *ddd* (*J* = 9.6, 9.6, 0.8 Hz) 2.18 *d*	–	α 1.61 *dd* (*J* = 12.2, 11.1 Hz) β 1.69 *ddd* (*J* = 12.2, 4.6, 2.2 Hz)	2.03 *d*
5	–	–	–	–
6	–	–	–	–
7	2.25 *d*	2.19–2.41	1.89 *ddd* (*J* = 12.5, 11.2, 8.8 Hz) 2.12 *ddd* (*J* = 12.5, 8.6, 1.0 Hz)	2.16 *d*
8	2.45 *d*	1.56 *d*	1.44 *ddd* (*J* = 11.2, 11.2, 8.6 Hz) 1.96 *dddd* (*J* = 11.2, 8.8, 5.4, 1.0 Hz)	1.48 *ddd* (*J* = 8.8, 6.0, 2.0 Hz)
9	–	3.84 *dd* (*J* = 12.0, 6.0 Hz)	4.01 *ddd* (*J* = 5.4, 5.9, 5.6 Hz)	3.79 *ddq* (*J* = 6.0, 6.0, 6.0 Hz)
10	2.13 s	1.24 d (*J* = 6.0 Hz)	1.15 (*J* = 5.9 Hz)	1.20 (*J* = 6.0 Hz)
11	1.58 *s*	1.76 *s*	1.13 *s*	1.60 *s*
12	1.00 *s*	1.16 *s*	0.87 *s*	1.00 s
13	1.02 *s*	1.16 *s*	1.08 *s*	1.06 *s*

and the stereochemistry are identical to those of the aglycon obtained from scoroposide.

In order to establish the absolute configuration of this compound, we used the modified Mosher methodology (Sullivan et al., 1973; Latypov et al., 1996) by carrying out the synthesis of the (*R*)-(−)- and (*S*)-(+)-2-methoxy-2-phenylacetyl esters of tectoionol A (**7**). Tectoionol A has a secondary alcohol group and this is the main requirement for this methodology.

The method requires the assignments of as many proton signals as possible for the (*R*)- and (*S*)-MPA esters, which give the $\Delta\delta(R-S)$ values for the protons. Protons with positive $\Delta\delta$ values should subsequently be placed on the right-hand side, and those with negative values on the left-hand side of model A (Fig. 6). The application of model A shows that the absolute configuration of C-3 is *R* and thus the absolute configuration should be (3*R*, 5*S*, 6*S*, 9*R*).

Compound **7** is therefore (3*R*,5*R*,6*S*,9*R*)-6,9-epoxiionane-3,5-diol, which is described here for the first time and we have named it tectoionol A.

2.2. Tectoionol B (*8*)

This compound was isolated as colorless oil from fraction F of the DCM/H_2O extract. The mass spectrum of **8** shows the molecular ion at *m*/*z* 211.1688 $[M-1]^-$ in the HR-FAB-MS (negative mode) experiment, which corresponds to the molecular formula $C_{13}H_{24}O_2$.

Table 2
^{13}C NMR data for compounds **4**, **6**, **7** and **8**[a]

C	4	6	7	8
1	37.8 *s*	36.4	41.4	40.1
2	48.3 *t*	37.4	47.8	75.9 *d*
3	65.0 *s*	34.3 *t*	64.3 *d*	26.6 *t*
4	42.1 *t*	199.5 *s*	47.0 *t*	29.6
5	124.9 *s*	131.5	77.5	126.4
6	135.9 *s*	164.9	89.9	135.3
7	21.8 *t*	26.8	27.9	24.9
8	44.2 *t*	37.9	36.4	39.7
9	208.5 *s*	68.5 *d*	76.9	68.6
10	29.8 *q*	23.5	21.1	23.4
11	19.8 *q*	11.5	28.3	19.5
12	28.3 *q*	27.0	28.4	21.8
13	29.5 *q*	26.9	25.1	26.4

[a] Multiplicites are not repeated if identical with those in the preceding column.

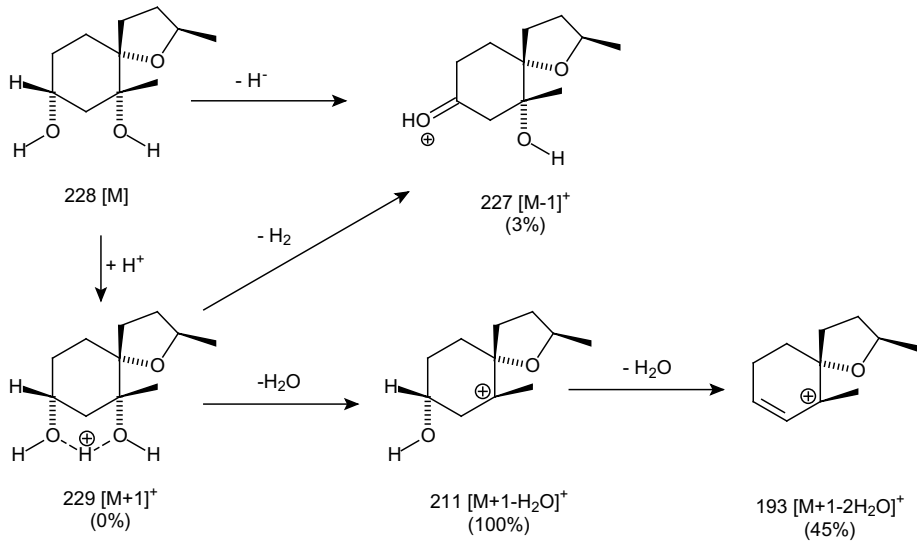


Fig. 3. Fragmentations in the mass pectrum of **7**.

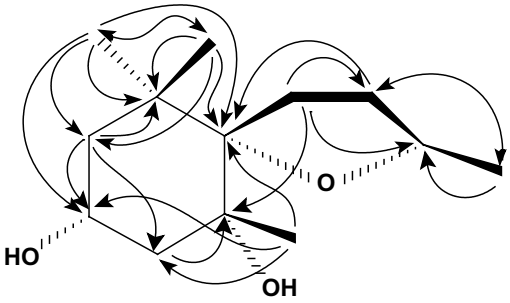


Fig. 4. g-HMBC correlations observed for compound **7**.

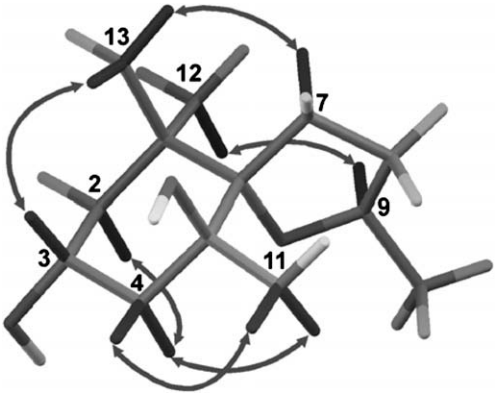


Fig. 5. NOE effects observed for tectoionol A (**7**) and represented on the most stable conformer calculated using PM3 parameters.

Thirteen signals appear in the ^{13}C NMR spectrum and this is consistent with a bisnorsesquiterpene structure.

The ^{1}H NMR spectrum of **8** is very similar to that of 3β-hydroxy-7,8-dihydro-β-ionol (**5**), which would indicate that it is a hydroxy derivative of 7,8-dihydro-β-ionol. The most significant difference between the two spectra is the chemical shift and the multiplicity of the proton geminal to the hydroxyl group at the ring (δ 3.46, *dd*, J = 9.0 Hz, J = 3.0 Hz, H-2). These data suggest that the two compounds differ in the position of this hydroxyl group, which must be located at C-2 or C-4. The correlation observed in the g-HMBC experiment between the methyl groups at δ 1.00 (3H, *s*, H-12) and δ 1.06 (3 H, *s*, H-13) and the signal at δ 75.9 confirms the position of the hydroxyl group at C-2. On the other hand, the values of the coupling constants of H-2 (1 H, *dd*, J = 9.0 Hz, J = 3.0 Hz) indicate an axial position for this proton.

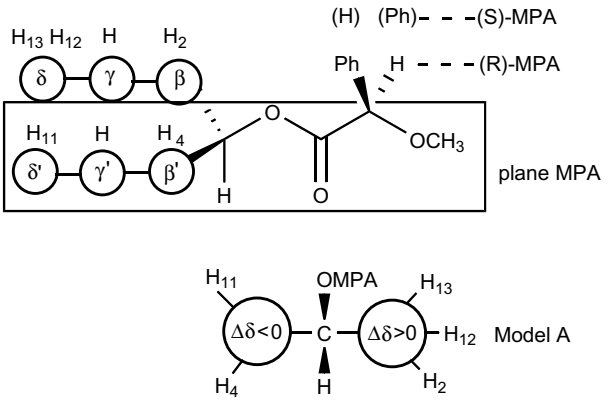
On the basis of these data the structure suggested for this compound is 2-hydroxy-7,8-dihydro-β-ionol, as shown in Fig. 2. A derivative glycosilated at C-9, named platanionoside J, has been described in the literature (Otsuka and Tamaki, 2002). The differences observed among the spectrocopic data of compound **8** and those of the product of hydrolysis of platanionoside J suggest that the relative stereochemistry of these compounds should be different [^{13}C NMR of platanionoside J, δ: 41.4 (C-1), 77.0 (C-2), 28.0 (C-3), 31.5 (C-4), 137.4 (C-6), 26.1 (C-7)]. This is the first time that this compound has been isolated and we have named it tectoionol B.

2.3. Bioassay results

In total, 1 mono terpene, 7 bisnorsesquiterpenes and 1 catabolic product of carotene, the dehydrololiolide, have been isolated from *T. grandis*. There are low number of precedents of activities studies of these compounds. Phytotoxicity of **2** has been tested previously (Macías et al., 1999). It showed low activity levels except over the monocotyledon species *Allium cepa* stimulating root growth. Compound **3** has also be assayed against different cancer cell lines and was found to be inactive (Youkwan et al., 2005; Phommart et al., 2005). Compound **3** has shown phytotoxicity against *Miscanthus floridulus* causing 20–25% inhibition of radical and shoot growth (Tseng et al., 2003).

Depending on the available quantities of these compounds, the bioactivities of 5 bisnorsesquiterpenes (**3**, **4**, **5**, **7** and **8**) and of the dehydrololiolide (**9**) have been evaluated. The highest concentration tested for **3**, **4**, **5** and **7** was 10^{-3} M, whereas **8** and **9** were tested from 5×10^{-4} M (Fig. 7). Compounds **4** and **5** show a level of activity higher than −50% at 10^{-3} M. On the other hand, **8** shows an activity of −59% at a concentration of 5×10^{-4} M. Compound **5** has greater persistence on dilution than compounds **4** and **8**, showing an inhibitory activity of −28% at 10^{-5} M. On the other hand, compounds **3**, **7** and **9** have low levels of activity, even at the highest concentration tested.

In order to compare activities of the compounds, IC_{50} values were calculated using a sigmoidal dose-response model. This technique



^{1}H	2	2'	12	13	4	4'	11
Δδ(**R-S**)	+0.091	+0.173	+0.043	+0.032	–0.117	–0.162	–0.04

Fig. 6. Configurational correlation model for the (*R*)- and (*S*)-MPA derivatives.

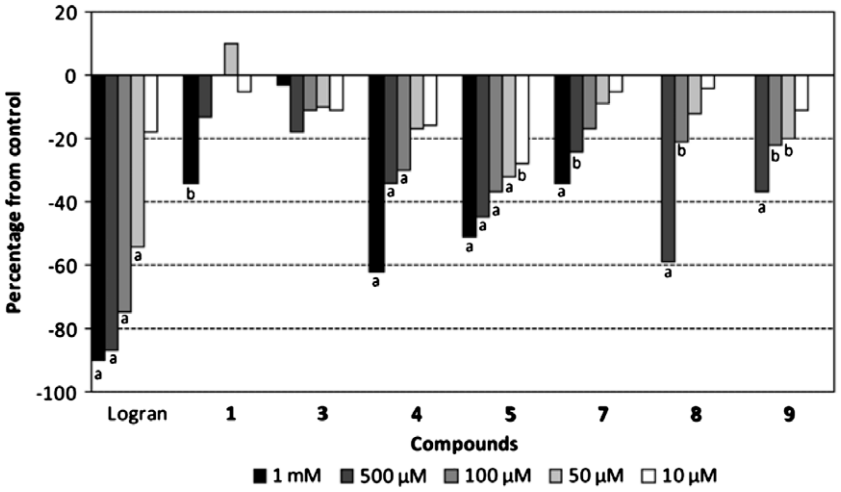


Fig. 7. Effects of compounds **1**, **3**–**5** and **7**–**9** on etiolated wheat coleoptile elongation. Values are expressed as percentage from the control and are not significantly different with $P > 0.05$ for tha Mann–Whitney's test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

allows comparison of the inhibitory activity of the active compounds even when, in some cases, different concentrations were used. The order of increasing activity of the compounds in this bioassay is: **8** (IC_{50} = 0.4074 mM, R^2 = 0.9972) > **4** (IC_{50} = 1.3660 mM, R^2 = 0.9714) > **5** (IC_{50} = 3.1700 mM, R^2 = 0.9922). The most active bisnorsesquiterpenes **4**, **5** and **8** are characterized by the double bond between C-5 and C-6, whereas the least active compounds **7** and **3** do not have this structural characteristic. Due to the small available amount of **8**, this compound could not be included in the phytotoxicity bioassay. Compounds **7**, **3** and **9** were also discarded because of the lower activity levels found.

The concentrations tested in the phytotoxicity assay were the same as in the previous bioassay. The standard target species (STS) were: *Lactuca sativa* (lettuce), *L. esculentum* (tomato), *Lepidium sativum* (cress) and *A. cepa* (onion) (Fig. 8). Neither compound showed a significant effect on germination and growth of *L. sativum*. Compound **5** showed inhibitory effects on the root and shoot lengths of *L. sativa*.

With regard to the third dicotyledonous species, *L. esculentum*, the tested compounds did not have significant effects on the germination of this seed. With regard to the activity shown on the root, the bisnorsesquiterpene **5** should be highlighted. Shoot length was also affected by this compound, which showed inhibitory activity at the highest concentrations.

The most sensitive parameter of the monocotyledon *A. cepa* was root growth and compound **5** showed inhibitory activity on this parameter.

In conclusion, we can state that *T. grandis* is an interesting source of active compounds. From the fractions studied, the terpenic compounds **4**, **5** and **8** presented the highest levels of bioactivity.

The results of a phytotoxicity assay of the tested compounds indicate that compound **5** shows a level of phytotoxicity on lettuce and tomato that is similar to that shown by the commercial herbicide Logran®. These results indicate that compound **5** could play a role in the allelopathic effects observed for this species.

3. Experimental

3.1. General

IR spectra (KBr) were recorded on a Perkin-Elmer FT-IR Spectrum 1000, Matton 5020 spectrophotometer. NMR spectra were run on Varian INOVA 400 and Varian INOVA 600 spectrometers.

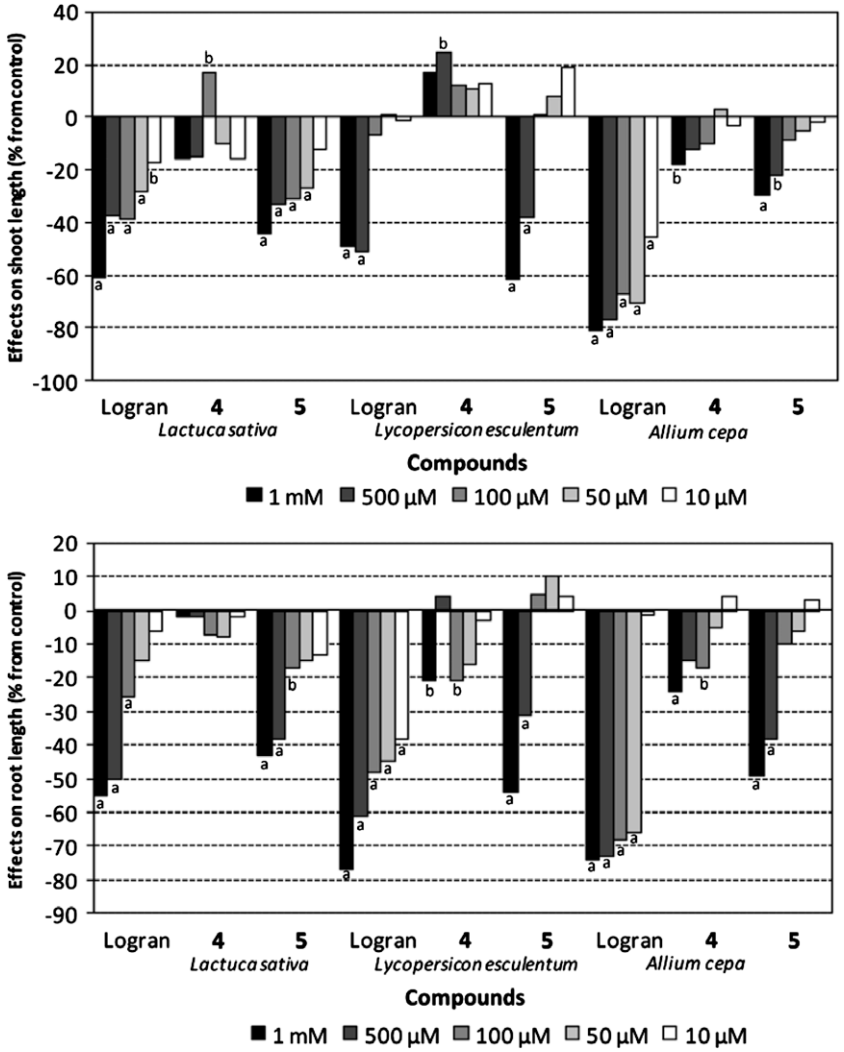


Fig. 8. Selected effects of compounds **4** and **5** on standard target species. Values are expressed as percentage from the control and are not significantly different with $P > 0.05$ for tha Mann–Whitney's test. [a]Values significantly different with $P < 0.01$. [b]Values significantly different with $0.01 < P < 0.05$.

Chemical shifts are given in ppm with respect to residual $CHCl_3$ or $CDCl_3$ signals (δ 7.25 and 77.00, respectively) and with respect to residual CH_3COCH_3 or CD_3COCD_3 signals (δ 2.04 and 29.80, respectively) Optical rotations were determined using a Perkin-Elmer model 241 polarimeter (on the sodium D line). HRMS were obtained on a VG AutoSpec-Q mass spectrometer.

3.2. Plant material

Leaves of *T. grandis* were collected between the months February and March (2003) in Ciudad de La Habana, and were identified by MsC. Lutgarda González. A voucher specimen (80613) was deposited at the Jardín Botánico de Cuba.

3.3. Extraction and isolation

Dried leaves of *T. grandis* (5 kg) were extracted in water (35 l) for 24 h at room temperature in the dark. The aqueous solution was extracted with CH_2Cl_2 and then with EtOAc at room temperature. The solvent from the organic layer was removed under reduced pressure to yield two extracts of 8.8 g (DCM/H_2O) and 9.8 g ($EtOAc/H_2O$), respectively. The remaining aqueous extract was dried under reduced pressure yielding 420 g (H_2O). The plant residue was dried at room temperature and re-extracted with CH_2Cl_2 and methanol yielding, after removal of the solvent, 120 g (DMC) and 53.3 g (MeOH), respectively. These extracts were bioassayed with etiolated wheat coleoptiles. The DCM/H_2O extract was the most active one. This was chromatographed on silica gel using hexane/EtOAc mixtures of increasing polarity as eluent. The fractions eluted with hexane/EtOAc (8:2) yielded compounds **1** (3 mg), **2** (2 mg), **5** (54 mg), **6** (2 mg), **8** (3 mg) and **9** (2 mg) and those eluted with hexane/EtOAc (3:7) yielded compounds **7** (30 mg) and **3** (18 mg).

3.3.1. Tectoionol A [(3R,5R,6S,9R)-6,9-epoxiionane-3,5-diol] (**7**)

Colorless oil; $[\alpha]_D^{25}$ +4.3 (*c* 0.01, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3320 (OH). ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; EIMS *m/z* (rel. int.): 228 $[M]^+$, 208 $[M-H_2O]^+$ (28), 107 (100); HREIMS *m/z* 227.1649 $[M-1]^+$ (calc. 227.1647).

3.3.2. Synthesis of (R)-MPA ester (***7a***)

Compound **7** (3 mg) was treated with CH_2Cl_2 solutions of dicyclohexylcarbodiimide (13 mg in 0.5 ml), *N*,*N*-dimethylaminopyridine (1.5 mg in 0.25 ml) and (*R*)-2-methoxy-2-phenylacetic acid (12 mg in 0.5 ml) and the mixture was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue was chromatographed by HPLC with an analytical silica gel column (5 mm) and hexane/$CHCl_3$ (2:3) as eluent, 1 ml/min

flow rate, and RI detector. This process afforded 2.5 mg of the (*R*)-MPA ester **7a**; ^{1}H NMR (400 MHz, $CDCl_3$): 0.86 (3H, *s*, H-12), 1.07 (3 H, *s*, H-13), 1.15 (3H, *s*, H-11), 1.18 (3 H, *d*, *J* = 5.9 Hz, H-10), 1.41 (1 H, *dd*, H-2), 1.66 (1 H, *dd*, *J* = 12.1 Hz, *J* = 12.1 Hz, H-2′), 1.77 (1 H, *ddd*, *J* = 5.9 Hz, *J* = 5.6 Hz, *J* = 5.4 Hz, H-4′), 1.87 (1 H, *dddd*, *J* = 11.1 Hz, *J* = 11.0 Hz, *J* = 4.6 Hz, *J* = 4.0 Hz, H-4).

3.3.3. Synthesis of the (S)-MPA ester (**7b**)

Treatment of compound **7** (3 mg) with CH_2Cl_2 solutions of dicyclohexylcarbodiimide (13 mg in 0.5 ml), *N*,*N*-dimethylaminopyridine (1.5 mg in 0.25 ml) and (*S*)-2-methoxy-2-phenylacetic acid (12 mg in 0.5 ml) as described above yielded 1.5 mg of the (*S*)-MPA ester 7 b. ^{1}H NMR (400 MHz, $CDCl_3$): 0.90 (3 H, *s*, H-12), 1.10 (3 H, *s*, H-13), 1.11 (3 H, *s*, H-11), 1.17 (3 H, *d*, *J* = 5.9 Hz, H-10), 1.58 (1 H, *m*, H-2′), 1.60 (1 H, *dd*, *J* = 2.8 Hz, *J* = 1.6 Hz, H-4′), 1.75 (1 H, *dd*, *J* = 8.0 Hz, *J* = 8.0 Hz, H-2), 1.76 (1 H, *dd*, *J* = 8.0 Hz, *J* = 8.0 Hz, H-4).

3.3.4. Tectoionol B [(2-hydroxy-7,8-dihydro-β-ionol] (**8**)

Colorless oil; $[\alpha]_D^{25}$ +3.9 (*c* 0.01, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}; 3400 (OH), 2932 (C–H). ^{1}H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HR-FAB-MS (negative-ion mode) *m*/*z* 211.1688 (calc. for $C_{13}H_{23}O_2$, 211.1698).

3.4. Coleoptiles bioassay

Wheat seeds (*T. aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moistened with water and grown in the dark at 22 ± 1 °C for 3 days (Hancock et al., 1964). The roots and caryopsis were removed from the shoots. The latter were placed in a Van der Weij guillotine and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for the bioassay. All manipulations were performed under a green safelight (Nitsch and Nitsch, 1956). Compounds were predissolved in DMSO and diluted to the final bioassay concentration with a maximum of 0.1% DMSO. Parallel controls were also run.

Crude extracts, fractions, or pure compounds to be assayed for biological activity were added to test tubes. The assays were carried out in duplicate. Phosphate-citrate buffer (2 ml) containing 2% sucrose (Nitsch and Nitsch, 1956) at pH 5.6 was added to each test tube. Five coleoptiles were placed in each test tube (three tubes per dilution) and the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptiles were measured by digitalization of their images. Data were statistically analyzed using Welch's test (Martín Andrés and Luna del Castillo, 1990). Data are presented as percentage differences from control. Thus, zero represents the control, positive values represent stimulation of the studied parameter, and negative values represent inhibition.

3.5. Phytotoxicity bioassay

Selection of target plants was based on an optimization process developed by us in our search for a standard phytotoxicity bioassay (Macías et al., 2000). Several Standard Target Species (STS) were proposed, including monocots *T. aestivum* L. (wheat) and *A. cepa* L. (onion) and dicots *L. esculentum* Will. (tomato), *L. sativum* L. (cress) and *L. sativa* L. (lettuce), which were assayed for this study.

Bioassays were conducted using Petri dishes (50 mm diameter), with one sheet of Whatman No.1 filter paper as support. Germination and growth were conducted in aqueous solutions at controlled pH using 10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1 M NaOH (pH 6.0). Compounds to be assayed were dissolved in DMSO (0.1, 0.02, 0.01 and 0.002 M) and these solutions were diluted with buffer (5 μl DMSO solution/ml buffer) so that test concentrations for each compound (10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5} and 10^{-5} M) were reached. This procedure facilitated the solubility of the assayed compounds. The number of seeds in each Petri dish depended on the seed size. 20 seeds were used for tomato, lettuce, cress and onion. Treatment, control or internal reference solution (1 ml) was added to each Petri dish. A similar procedure was used for wheat in 90 mm diameter Petri dishes with 10 seeds. Four replicates were used for tomato, cress, onion and lettuce (80 seeds); ten replicates (100 seeds) were used for wheat.

After adding seeds and aqueous solutions, Petri dishes were sealed with Parafilm to ensure closed-system models. Seeds were further incubated at 25 °C in a Memmert ICE 700 controlled environment growth chamber in the dark. Bioassays took 4 days for cress, 5 days for lettuce, tomato and wheat and 7 days for onion. After growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process.

The commercial herbicide Logran®, a combination of *N*-(1,1-dimethylethyl)-*N*′-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (Terbutryn, 59.4%) and 2-(2-chloroethoxy)-*N*-{[(4-methoxy-6-methyl-1,3,5-triazin-2yl)amino]carbonyl}benzene-sulfonamide (Triasulfuron, 0.6%), was used as an internal reference according to a comparison study reported previously (Macías et al., 2000). The herbicide was used at the same concentrations (10^{-3}, 5×10^{-4}, 10^{-4}, 5×10^{-5} and 10^{-5} M) and in the same conditions as those reported. Control samples (buffered aqueous solutions with DMSO and without any test compound) were used for all of the plant species assayed.

Evaluated parameters (germination rate, root length and shoot length) were recorded using a Fitomed© system (Castellano et al., 2001), which allowed automatic data acquisition and statistical analysis using its associated software. Data were analyzed statistically using Welch's test, with significance fixed at 0.01 and 0.05. Results are presented as percentage differences from the control. Zero represents control, positive values represent stimulation, and negative values represent inhibition.

IC_{50} values were obtained after adjusting phytotoxicity data to concentration (logarithmic scale), to a sigmoidal dose-response curve, defined by equation:

$$Y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + 10^{\log EC50 - X}} \quad (1)$$

where *X* indicates the logarithm of concentration, *Y* indicates the response (phytotoxicity) and Y_{max} and Y_{min} are the maximum and minimum values of the response, respectively. Goodness of fit is described by the determination coefficient (r^2). The adjustment and the r^2 were obtained using GraphPad Prism® software v. 4.00.

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