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Megalanthine, a Bioactive Sesquiterpenoid from *Heliotropium megalanthum*, its Degradation Products and their Bioactivities

Francisco A. Macías · Ana M. Simonet · Brigida D'Abrosca · Claudia C. Maya · Matías Reina · Azucena González-Coloma · Raimundo Cabrera · Cristina Giménez · Luis Villarroel

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Abstract The new bioactive sesquiterpenoid (3*R*,6*E*)-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyloxy)-dodeca-6,11-diene-2,10-diol, named megalanthine, was isolated from the resinous exudates of *Heliotropium megalanthum*. The degradation products of this compound were identified. Several plant-defensive properties (insecticidal, antifungal, and phytotoxic) were evaluated after obtaining positive results in a preliminary etiolated wheat coleoptile bioassay. This bioassay showed the need to have both the phenolic and sesquiterpene moieties of the natural product present to achieve a biological effect. This result was confirmed in phytotoxicity bioassays. Megalanthine was ruled out as a significant plant–plant defense agent because of its lack of stability. The positive results recorded in the antifungal and antifeedant tests suggest, however, that this chemical is relevant in several ecological interactions involving *H. megalanthum*.

Keywords *Heliotropium megalanthum* · Resin · Sesquiterpene phenylpropanoid · Megalanthine · Etiolated wheat coleoptile bioassay · Antifeedant · Antifungal · Allelopathic assay · STS

F. A. Macías (✉) · A. M. Simonet · C. C. Maya
Grupo de Alelopatía, Departamento de Química Orgánica,
Facultad de Ciencias, Universidad de Cádiz,
C/Avda. República Saharaui, s/n,
11510 Puerto Real, Cádiz, Spain
e-mail: famacias@uca.es

B. D'Abrosca
Dipartimento di Science della Vita, II Università di Napoli,
via Vivaldi 43,
81100 Caserta, Italy

M. Reina
Instituto de Productos Naturales y Agrobiología, CSIC,
38206 La Laguna, Tenerife, Spain

A. González-Coloma
Centro de Ciencias Medioambientales, CSIC,
Madrid, Spain

R. Cabrera · C. Giménez
Fitopatología, Departamento de Biología Vegetal,
Facultad de Biología, Universidad de La Laguna,
Avda. Astrofísico Francisco Sánchez s/n,
38206 La Laguna, Tenerife, Spain

L. Villarroel
Facultad de Química y Biología,
Universidad de Santiago de Chile,
Casilla 40, Correo-33,
Santiago, Chile

Introduction

Species of *Heliotropium* of the *Cochranea* section (Heliotropiaceae) are endemic to the coastal hills of northern and central Chile and southern Peru. Like many plants of that area, they characteristically produce resinous exudates that cover the leaves and stems (Johnston 1928). These exudates are associated with a complex defense mechanism that includes the prevention of excessive water evaporation and protection against UV radiation (80–320 nm), phytopathogens, and phytophagous organisms (Johnson 1983; Kelsey et al. 1984; Torres et al. 1994; Reina et al. 1997; Villarroel et al. 1997, 2001; Urzúa et al. 1998; Modak et al. 2004).

In this study, we describe the isolation and structural elucidation of megalanthine (**1**), a major component of the resinous exudates of *Heliotropium megalanthum* J. M.

Johnston (Boraginaceae), the alkaloidal fraction of which previously afforded the pyrrolizidine alkaloids (PAs) megalanthonine and lycopsamine (Reina et al. 1998).

Megalanthine spontaneously degrades. Of special interest is the comparison of the bioactivity levels of the degradation products with those of the original natural compound, since this may allow possible plant–plant ecological relationships to be established (Macías et al. 2005).

To explore the activity of the compounds, we used the etiolated wheat coleoptile bioassay, (Cutler 1984) antifeedant test, and toxic bioassays of selected compounds, against the herbivorous insect models *Spodoptera littoralis* (Boisduval), *Leptinotarsa decemlineata* (Say), and the aphid *Myzus persicae* (Sulzer), as well as antifungal studies on three *Fusarium* species. In addition, we tested the selective cytotoxicity on insect Sf9 cells derived from *S. frugiperda* pupal ovarian tissue and mammalian Chinese hamster ovary (CHO) cells. We also tested the phytotoxicity against the dicotyledonous (*Lactuca sativa*, *Lepidium sativum*, and *Lycopersicon esculentum*) and monocotyledonous (*Allium cepa* and *Triticum aestivum*) species, which are denoted as standard target species (STS) (Macías et al. 2000).

Methods and Materials

General Experimental Procedures Optical rotations were measured on a Perkin-Elmer 137 polarimeter. Infrared (IR) spectra were obtained on KBr disks on a Bruker IFS66V FTIR spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker AMX2 500 MHz, Varian UNITY-400, and INOVA-600 spectrometers with $CDCl_3$ as solvent. Chemical shifts are given in parts per million with respect to residual ^1H signals of $CDCl_3$ (δ 7.25), and ^{13}C shifts are with respect to the solvent signal (δ 77.0). High-resolution mass spectrometry was carried out on a VG AUTOESPEC mass spectrometer (70 eV). Column chromatography was performed on silica gel (63–200 mesh), and thin layer chromatography (TLC) analysis by using aluminum-packed precoated silica gel plates. For high-performance liquid chromatography (HPLC), LiChrosorb silica 60 was used in the normal-phase mode and LiChrospher RP-18 in the reverse-phase mode with a refractive index detector on a Hitachi L-6020A HPLC instrument. All solvents were spectroscopic grade or distilled from glass prior to use. Fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin were supplied by GIBCO-BRL (UK). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and *p*-nitrophenylphosphate were purchased from Sigma-Aldrich. All of these products were used as received.

Plant Material H. megalanthum J. M. Johnston was collected during the flowering season in October 1995 in the north of Chile (Atacama, III Region) and was identified by Dr. Sebastian Teiller from the Museo de Historia Natural de Santiago de Chile. A voucher specimen is deposited in the herbarium of this museum (number ST 2569).

Extraction and Isolation The resinous exudates of *H. megalanthum* were obtained by dipping 800 g of fresh plant in cold CH_2Cl_2 for 15 to 20 s. Extracts were concentrated to afford 8 g of residue. Resinous exudates were fractioned by flash column chromatography on silica gel, using CH_2Cl_2 with increasing amounts of MeOH as eluent. The major fraction was purified first by Sephadex LH-20 chromatography with *n*-hexane/$CHCl_3$/MeOH (2:1:1) as eluent and then by silica gel preparative TLC eluted with *n*-hexane/EtOAc (1:1) to give megalanthine (**1**) (300 mg, 0.0375% f.p.w.).

Megalanthine (**1**) was stored under dry conditions in the absence of light, at 4°C, but it underwent degradation under these conditions (about 20% in 1 month). Purification of the mixture with silica gel CC, eluted with a gradient of $CHCl_3$/Me_2CO, yielded a fraction with compounds of higher polarity. This fraction was found to be enriched with degradation products **2** and **3** after HPLC separation with MeOH/ACN/H_2O (2:1:2) on a RP-18 semipreparative column at a flow rate of 3 mL min^{-1} and with a refractive index detector.

Megalanthine (**1**): colorless oil; $[\alpha]^{25}_D$+28.6° (MeOH; c 0.77); IR (neat, KBr) ν_{max} cm^{-1}: 3,400 (OH), 2,973, 2,929, 1,710, 1,640, 1,614, 817; see Tables 1 and 2 for ^1H and ^{13}C NMR data; electron impact mass spectrometry (EIMS), *m/z* (rel. int.): 404 $[M]^+$ (0.5), 387 $[M–OH]^+$ (17), 386 $[M–H_2O]^+$ (14), 369 $[M–H_2O–OH]^+$ (35), 315 (2), 301 (12), 221 (20), 220 (22), 202 (32), 187 (10), 166 (20), 149 (30), 107 (100), 93 (15); high-resolution electron impact mass spectrometry (HREIMS) *m/z* calculated for $C_{24}H_{36}O_5$ 404.2563, found 404.2544.

(3R,6R,7S*,10S*)-7,10-Epoxy-2,6,10-trimethyl-3-(3-p-hydroxyphenyl propanoyloxy)-dodec-11-ene-2,6-diol* (**2**): colorless oil; $[\alpha]^{25}_D$+4.0° ($CHCl_3$; c0.26); IR (neat, KBr) ν_{max} cm^{-1}: 3,360 (OH), 2,971, 1,710, 1,616, 1,072, 920, 817; see Tables 1 and 2 for ^1H and ^{13}C NMR data; EIMS, *m/z* (rel. int.): 420 $[M]^+$ (2), 403 $[M–OH]^+$ (10), 166 (15), 149 (30), 111 (27), 107 (100), 93 (16); HREIMS *m/z* calculated for $C_{24}H_{36}O_6$, 420.2512, found 420.2500.

(3R,6S,7R*,10S*)-7,10-Epoxy-2,6,10-trimethyl-3-(3-p-hydroxyphenyl propanoyloxy)-dodec-11-ene-2,6-diol* (**3**): colorless oil; $[\alpha]^{25}_D$+11.33° ($CHCl_3$; c0.06); IR (neat, KBr) ν_{max} cm^{-1}: 3,362 (OH), 2,980, 1,714, 1,616, 1,074, 920, 817; see Tables 1 and 2 for ^1H and ^{13}C NMR data; EIMS, *m/z* (rel. int.): 420 $[M]^+$ (5), 403 $[M–OH]^+$ (15), 166 (17), 149 (26), 111 (30), 107 (100), 93 (16); HREIMS *m/z* calculated for $C_{24}H_{36}O_6$, 420.2512, found 420.2529.

Table 1 ^{1}H NMR chemical shift assignments for compounds **1**–**4** in $CDCl_3$

Position	1[a]	2[b]	3[c]	4[c]
1	1.12 (*s*)	1.14 (*s*)	1.14 (*s*)	1.14 (*s*)
3	4.74 (*dd*, *J*=10.5, 2.2)	4.78 (*dd*, *J*=9.0, 3.7)	4.78 (*dd*, *J*=10.2, 2.4)	3.33 (*dd*, *J*=10.4, 1.9)
4a	1.64 (*m*)	1.67[d]	1.78[d]	1.55 (*m*)
4b	1.56 (*m*)	1.46[d]	1.54[d]	1.39 (*dddd*, *J*=14.1, 10.4, 8.8, 5.7)
5a	1.79 (*dd*, *J*=7.8, 7.1)	1.44[d]	1.38 (*ddd*, *J*=12.4, 12.4, 4.4)	2.22 (*ddd*, *J*=13.9, 8.8, 5.6)
5b		1.24 (*m*)	1.14[d]	2.06[d]
7	5.02 (*t*, *J*=7.2)	3.77 (*dd*, *J*=7.4, 7.1)	3.77 (*dd*, *J*=7.1, 7.1)	5.20[d]
8a	2.00 (*m*)	1.79 (*ddd*, *J*=12.4, 8.7, 6.8)	1.78 (*m*)	2.06 (*m*)
8b		1.77 (*ddd*, *J*=12.4, 8.4, 5.6)		
9a	1.56 (*m*)	1.88 (*ddd*, *J*=11.8, 8.4, 6.8)	1.89 (*m*)	1.55 (*m*)
9b		1.69 (*ddd*, *J*=11.8, 8.7, 5.6)	1.54[d]	
11	5.90 (*dd*, *J*=17.3, 10.8)	5.85 (*dd*, *J*=17.3, 10.5)	5.94 (*dd*, *J*=17.3, 10.7)	5.90 (*dd*, *J*=17.3, 10.7)
12a	5.21 (*dd*, *J*=17.3, 1.2)	5.18 (*dd*, *J*=17.3, 1.6)	5.16 (*dd*, *J*=17.3, 1.2)	5.19 (*dd*, *J*=17.3, 1.3)
12b	5.06 (*dd*, *J*=17.3, 1.2)	4.98 (*dd*, *J*=10.5, 1.6)	5.00 (*dd*, *J*=10.7, 1.2)	5.05 (*dd*, *J*=10.7, 1.3)
13	1.12 (*s*)	1.13 (*s*)	1.13 (*s*)	1.18 (*s*)
14	1.52 (*s*)	1.16 (*s*)	1.15 (*s*)	1.60 (*s*)
15	1.29 (*s*)	1.28 (*s*)	1.30 (*s*)	1.27 (*s*)
2′6″	7.03 (*d*, *J*=8.5)	7.06 (*d*, *J*=8.4)	7.06 (*d*, *J*=8.6)	
3′5″	6.74 (*d*, *J*=8.5)	6.74 (*d*, *J*=8.4)	6.74 (*d*, *J*=8.6)	
7′	2.88 (*m*)	2.89 (*dd*, *J*=7.7, 7.5)	2.89 (*dd*, *J*=7.6, 7.3)	
8′	2.55 (*t*, *J*=7.7)	2.65 (*dd*, *J*=7.7, 7.5)	2.65 (*dd*, *J*=7.6, 7.3)	

Multiplicities are not repeated if identical with those in the preceding column
[a] 500 MHz
[b] 600 MHz
[c] 400 MHz
[d] Multiplicities could not be determined due to the overlap of signals

Saponification of Megalanthine Megalanthine (**1**) (20 mg) was dissolved in MeOH (0.5 mL) and 5% NaOH (3 mL) and the mixture was stirred continuously at room temperature for 12 h. After the usual workup, the product was purified by using HPLC with a silica gel analytical column eluted with $CHCl_3/Me_2CO$ (4:1) at a flow rate of 1 mL min^{-1} to yield 7 mg of dihydro-*p*-coumaric acid (**5**) and 8 mg of (3*R*,6*E*,10*S*)-2,6,10-trimethyl-3-hydroxydodeca-6,11-diene-2,10-diol (**4**).

(R)- and (S)-Methoxyphenylacetic Acid Derivatives of Compound **4** Compound **4** (4 mg) was treated with CH_2Cl_2 solutions of *N*′-dicyclohexylcarbodiimide (25 mg in 1 mL), *N*,*N*-dimethylaminopyridine (3.5 mg in 0.5 mL), and (*R*)- or (*S*)-methoxyphenylacetic acid (MPA) (10 mg in 0.5 mL); the mixture was stirred at room temperature for 22 h. Evaporation of the solvent under reduced pressure yielded a residue that was purified by HPLC with a Si 60 analytical column, eluting with $CHCl_3/Me_2CO$ (89:11) at a flow rate of 1 mL min^{-1} and a RI detector to yield 4 mg of (*R*)-MPA ester **4R** and 3.5 mg of (*S*)-MPA ester **4S**, respectively.

Preparation of **2** *and* **3** A solution of megalanthine (**1**) (60 mg) in CH_2Cl_2 (10 mL) was stirred with 1.1 equivalents of metachloroperbenzoic acid (MCPBA) at room temperature for 6 h. The crude product was purified by column chromatography $CHCl_3/Me_2CO$ (9:1). Compounds **2** (21 mg) and **3** (19 mg) were obtained after purification by HPLC by using a silica gel analytical column and eluting with $CHCl_3/Me_2CO$ (4:1) at a flow rate 1 mL min^{-1}.

Wheat Coleoptile Bioassay Wheat seeds (*T. aestivum* L. cv. Cortex) were sown on 15 cm diameter Petri dishes filled with Whatman #1 filter paper moistened with water and were grown in the dark at 24°C for 4 days. The etiolated seedlings were removed from the dishes and selected by size uniformity. Selected seedlings were placed in a Van der Wij guillotine, and the apical meristems (2 mm) were cut off and discarded. The next 4 mm of the coleoptiles were removed for bioassay and kept in aqueous nutritive buffer for 1 h to synchronize growth. Pure compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted to the appropriate concentration with a phosphate–citrate buffer containing 2% sucrose at pH 5.6 to give stock solutions with a final DMSO concentration of 0.5%. The buffer with 0.5% DMSO was used as a control. Assays were carried out in duplicate.

Test concentrations were obtained by dilution. The bioassay was performed in 10 mL test tubes: five

coleoptiles were added to each test tube containing 2 mL of the test solution. Three replicates were made for each test solution, and the experiments were run in duplicate. Test tubes were placed in a roller tube apparatus and rotated at 0.25 rpm for 24 h at 22°C in the dark. All manipulations were carried out under a green safelight. Coleoptiles were measured by digitalization of their photographic images.

Statistical Analysis Data were statistically analyzed by using Welch's test with significance fixed at 0.01 and 0.05. Results are expressed in bar charts in which the null value represents the control, negative values represent inhibition, and positive values represent stimulation of the studied parameter (Macías et al. 2000). Statistical significance is expressed by means of letters where "a" means significantly different from the control with 0.01 confidence and "b" means significantly different from the control with a confidence from 0.01 to 0.05. The absence of a letter indicates no significant difference from the control values.

Insect Bioassays *S. littoralis*, *L. decemlineata*, and *M. persicae* colonies were reared on an artificial diet (Poitout and Bues 1974), potato (*Solanum tuberosum* L), and bell pepper (*Capsicum annuum*) plants, respectively, and maintained at 22±1°C, >70% relative humidity with a photoperiod of 16:8 h (L/D) in a growth chamber.

Choice Feeding Assay These experiments were conducted with sixth instar *S. littoralis* larvae, *L. decemlineata* adults, and *M. persicae* apterous adults. Percentage feeding inhibition (%FI) indexes were calculated as described previously (Reina et al. 2001). For FI values >65%, compound **1** was tested in a dose–response experiment in order to calculate its effective antifeedant dose (EC_{50}, the effective dose for 50% feeding reduction), which was determined by linear regression analysis (%FI on log dose). The settling inhibition effect was calculated by the ratio $\%T/\%C$ where $\%T$ is the percentage of aphids settled on the treated disk and $\%C$ is the percentage of aphids settled on the control disk. Significant differences were tested by the Mann–Whitney test.

Oral Cannulation This experiment was performed with preweighed newly molted *S. littoralis* L6 larvae. A sixth instar was orally injected with 40 μg of the test compound in 4 μL of DMSO (treatment) or solvent alone (control) with a Rheodyne Hamilton syringe (50 μL) attached to a Hamilton microdispenser, as described by Reina et al. (2001). The syringe tip was inserted into the mouth of the larvae (maximum of 5 mm), and then larvae were forced to feed until no regurgitation was observed. In total, 20 larvae were "fed" this way per compound tested (Burgeno-Tapia et al. 2008). At the end of the experiments (72 h), larval consumption and growth were calculated on a dry weight basis. A covariance analysis (ANCOVA1) of food consumption (ΔI) and biomass gains (ΔB) with initial larval weight (BI) as covariate (covariate $P>0.05$) was performed to test for significant effects of the test compounds on these variables (Reina et al. 2001).

Cytotoxicity Sf9 cells derived from *S. frugiperda* pupal ovarian tissue (European Collection of Cell Cultures [ECCC]) and mammalian CHO cells (a gift from Dr. Pajares, I. C. Biomédicas, CSIC) were grown as described previously (González-Coloma et al. 2002). Briefly, Sf9 cells, derived from *S. frugiperda* pupal ovarian tissue (ECCC), were maintained in TC-100 insect cell medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin at 26°C. Mammalian CHO cells were grown in RPMI 1640 medium supplemented as above at 37°C under a humified atmosphere of 5% CO_2/95% air. Cells seeded in 96-well flat-bottom plastic microplates with 100 μL of medium per well (initial densities of 5×10^4 and 10^4 cells per well for the insect and mammalian cultures, respectively, were exposed for 48 h to serial dilutions of the test compounds). Cell viability was analyzed according to the MTT (Sigma) colorimetric assay method (Mosmann 1983). A 10-μL aliquot of stock MTT solution (5 mg/mL in PBS) was added to all wells, and the cultures were further incubated for 4 h. The medium was then removed by aspiration, 100 μL of dimethyl sulfoxide was added to dissolve the purple formazan precipitate, and the absorbance at 570 nm (reference wavelength of 630 nm) was measured on a microplate reader (SLT Lab Instruments, Groedig, Austria). For each treatment, cell viability was calculated as the percent absorbance of the control (untreated cells). The relative potency of **1** (EC_{50} values, the effective dose to give 50% cell viability) was determined by linear regression analysis (percent cell viability on log dose) for the sensitive cell lines.

Antifungal Activity Assays *Fusarium moniliforme* (Sheldon), *F. oxysporum* fs. *lycopersici* (Scheldt), and *F. solani* (Mart) (Spanish Collection of Type Cultures CECT codes CECT 2152, CECT 2715, and CECT 2199, respectively) were grown in potato dextrose agar in Petri dishes and kept in the dark at 27°C. These cultures were frequently renovated in order to maintain the fungus in optimal conditions. The antifungal activity of **1** was tested at several doses (0.5, 0.1, 0.05, and 0.01 mg/mL) against the three species and estimated as mycelial growth inhibition. The relative potency of **1** (EC_{50} values, the effective dose to give 50% mycelial growth inhibition) was determined by linear regression analysis (percent inhibition on dose) for the sensitive species.

Standard Target Species Bioassays Seeds of lettuce (*L. sativa* L. cv. Roman), cress (*L. sativum* L.), tomato (*L. esculentum*), onion (*A. cepa* L. cv. Valenciana), and wheat

(*T. aestivum*) were obtained from FITÓ, S.L. (Barcelona, Spain). All undersized or damaged seeds were discarded, and the assay seeds were selected for uniformity. Bioassays were carried out in 9 cm diameter plastic Petri dishes with Whatman #1 filter paper as the support.

The general procedure for seedling bioassay was as follows: 25 seeds of each species per dish, except for *T. aestivum* (ten seeds per dish), were placed in 5 mL of the test solution and incubated in the dark at 25°C. Four replicates for each concentration were set up. Germination and growth time varied for each plant species: *L. sativum*, 3 days; *L. sativa*, *L. esculentum*, and *T. aestivum*, 5 days; and *A. cepa*, 7 days.

Test stock solutions (10^{-3} to 10^{-4} M) were prepared with DMSO and then diluted to 10^{-7} M with 10 mM 2-[*N*-morpholino]ethanesulphonic acid. The following solutions were obtained by dilution while maintaining the 1% DMSO percentage. Parallel blind (1% DMSO) and positive controls were performed. The positive controls used the commercial herbicide LOGRAN, a combination of *N*-(1,1-dimethylethyl)-*N*-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine (terbutryn, 0.6%), and 2-(2-chloroethoxy)-*N*-{[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl} benzenesulfonamide (triasulfuron, 59.4%), as an internal reference according to a previously reported study (Macías et al. 2000). LOGRAN stock solutions were prepared so that the major component (triasulfuron) was present at the same molarity as the materials to be tested.

Bioassay Data Acquisition The evaluated parameters (germination rate, root length, and shoot length) were recorded by using a Fitomed system (Castellano et al. 1999) that allowed automatic data acquisition and statistical analysis by its associated software.

Statistical Analysis Data were statistically analyzed with Welch's test with significance fixed at 0.01 and 0.05. Results are expressed in bar charts in which the null value represents the control, negative values represent inhibition, and positive values represent stimulation of the parameter under investigation (Macías et al. 2000). Statistical significance is expressed by letters with "a" meaning significantly different from the control with 0.01 confidence and "b" meaning significantly different from the control with a confidence from 0.01 to 0.05. The absence of a letter indicates no significant difference from the control values.

Results and Discussion

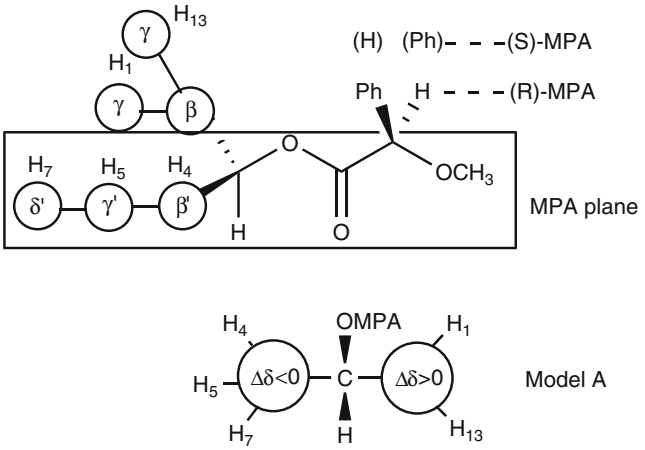
Megalanthine (**1**), the major compound of the resinous exudate (3.75%), showed a molecular ion at *m/z* 404.2544 in the HREIMS, and this is consistent with the molecular formula $C_{24}H_{36}O_5$. Additional peaks in the EIMS were observed at *m/z* 387 $[M–OH]^+$, *m/z* 369 $[M–OH–H_2O]^+$, and the base peak at *m/z* 107. A strong absorption was observed at 3,400 cm^{-1} in the IR spectrum, and this corresponds to the presence of two or more hydroxyl groups.

The ^{1}H NMR and COSY spectra (Table 1) showed signals for the protons of two subunits, a terpene and an aromatic moiety. The ^{13}C NMR spectrum (Table 2) confirmed the existence of a sesquiterpene unit with three oxygenated carbons [C-2 (δ 72.6), C-3 (δ 79.6), and C-10 (δ 73.9)] and two double bonds [C-6 (δ 134.3), C-7 (δ 125.1) and C-11 (δ 144.7), C-12 (δ111.9)]. The aromatic unit was identified as a dihydro-*p*-coumaric acid derivative. At this point, the number of unsaturations was justified and the sesquiterpene unit should be linear. Based on the structure of farnesyl sesquiterpene, the structure of **1** was proposed and this was subsequently confirmed by heteronuclear multiple bond correlations (HMBC). The position of the phenolic moiety was determined to be at C-3 because the ^{1}H NMR spectrum contained a signal at δ 4.74 *dd* for H-3, which is consistent with a vicinal acyloxy group, and a long-range correlation was observed between H-3 (δ 4.74) and C-9′ (δ 173.5) in the HMBC spectra. Compound **1** was saponified to obtain the dihydro-*p*-coumaric acid (**5**) and the sesquiterpene unit (**4**), which had the expected shielded signal for H-3 in the ^{1}H NMR spectrum (δ 3.33 *dd*). The

Table 2 ^{13}C NMR data for compounds **1**–**4** in $CDCl_3$ (100 MHz)

Position	1[a]	2	3	4
1	26.5	26.4	26.1	26.4
2	72.6	72.6[b]	72.7	73.0
3	79.6	80.2	80.6	78.2
4	27.8	23.4	23.4	29.6
5	35.9[b]	33.3	33.6	36.8
6	134.3	72.5[b]	72.5	135.3
7	125.1	85.0	85.1	125.0
8	22.7	25.9	26.0	22.7
9	41.7	37.4	37.8	41.9
10	73.9	82.9	82.6	73.5
11	144.7	143.6	144.3	144.9
12	111.9	111.4	111.7	111.8
13	24.6	25.0	24.8	23.2
14	15.8	23.9	24.0	15.9
15	27.5	26.8	26.7	27.9
1′	131.6	132.4	132.4	
2′6′	129.3	129.4	129.4	
3′5′	115.6	115.5	115.4	
4′	154.8	154.1	154.2	
7′	30.0	30.1	30.1	
8′	36.1[b]	36.1	36.0	
9′	173.5	173.1	173.0	

[a] 500 MHz
[b] These assignment may be interchanged in the same column



Δδ(R-S)	7	5	4a	4b	1	13
4	–0.33	–0.41	–0.09	–0.11	+0.21	+0.23

Fig. 1 Configurational correlation model for the (*R*)- and (*S*)-MPA derivatives. The MPA plane is shown; $H_{7,5,4a,4b}$ and $H_{1,13}$ are on the *right* and *left* sides of the plane, respectively. Model A, to determine the absolute configuration of C-3, is illustrated

spectroscopic data for the sesquiterpenic unit are in agreement with data previously described for 6*E*-2,6,10-trimethyl-3-hydroxydodeca-6,11-diene-2,10-diol (Miyase et al. 1987; Díaz et al. 1992; Miyazawa et al. 1996). Mosher's method was used to determine the absolute configuration of the chiral center (C-3) of compounds **1** and **4**. Compound **4**, which had a secondary alcohol at C-3, was esterified with the enantiomers of the chiral reagent, MPA (Latypov et al. 1996), and the resulting mixture was subjected to HPLC separation to afford compounds **4R** and **4S**.

The method requires the assignment of as many proton signals as possible of the (*R*)- and (*S*)-MPA esters, and this enables the $\Delta\delta$ (*R*–*S*) values to be determined for the protons. Protons with positive $\Delta\delta$ values should be placed on the right-hand side of model A while those with negative $\Delta\delta$ values are placed on the left (Ohtani et al. 1991) (Fig. 1). The application of the model confirmed that the absolute configuration of C-3 in compound **4** is *R*. The structure of the new sesquiterpene megalanthine (**1**) was thus determined to be (3*R*,6*E*)-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyloxy)-dodeca-6,11-diene-2,10-diol.

Compound **1** was stored under dry conditions in the absence of light at 4°C. About 20% was degraded to give two major compounds (**2** and **3**) within a month. Both compounds had very similar ^{1}H NMR spectra, and these corresponded to modifications at positions 6 and 7 of megalanthine (**1**). The H-7 signal at δ 3.77 (*dd*, *J*=7.1, 7.1 Hz) and the H-14 signal at δ 1.16/1.15 (*s*) for compounds **2** and **3** suggested oxygenation at C-6 and C-7. The ^{13}C NMR spectra of these compounds showed two signals at δ 72.5 and 85.1, which are assigned, respectively, to C-6 and C-7 by HMBC correlations. The

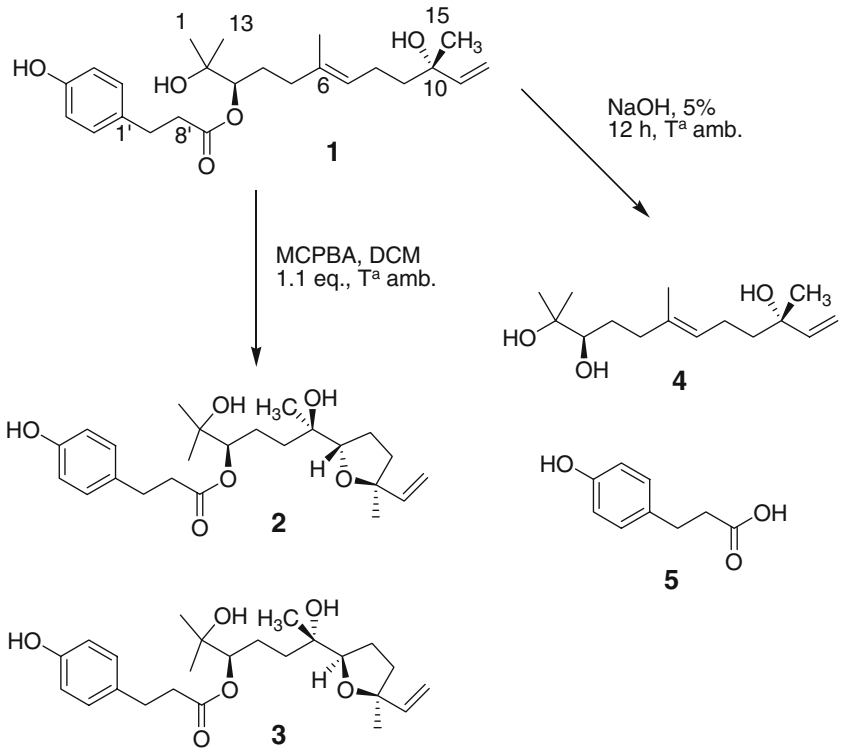
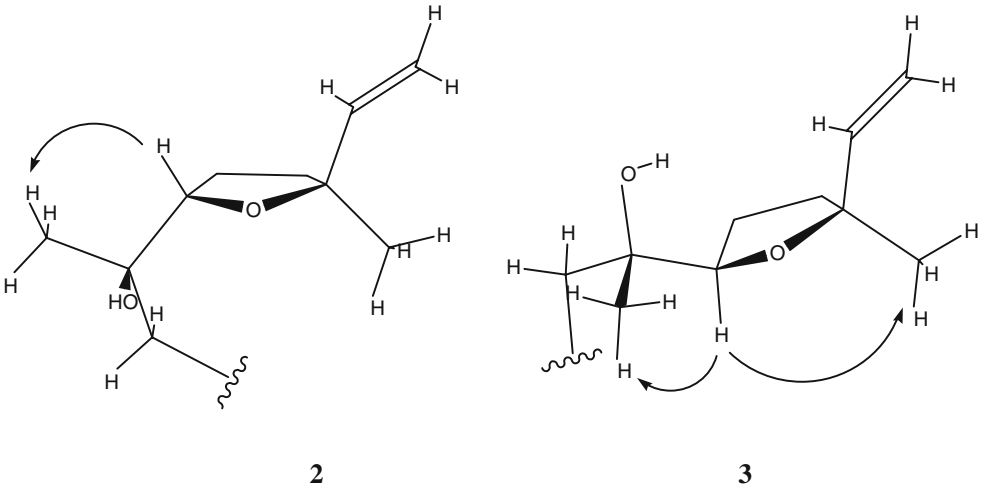


Fig. 2 Structures and chemical correlation of the new compound **1** (megalanthine), it degradation compounds **2** [(3*R*,6*R**,7*S**,10*S**)-7,10-epoxy-2,6,10-trimethyl-3-(3-*p*-hydroxyphenyl propanoyloxy)-dodec-11-ene-2,6-diol)] and **3** [(3*R*,6*S**,7*R**,10*S**)-7,10-epoxy-2,6,10-trimethyl-3-(3-p-hydroxyphenylpropanoyloxy)-dodec-11-ene-2,6-diol] and it saponification products **4** [(3*R*,6*E*,10*S**)-2,6,10-trimethyl-3-hydroxydodeca-6,11-diene-2,10-diol)] and **5** (dihydro-*p*-coumaric acid)



2 **3**

Fig. 3 NOE effects between H-7, H-14, and H-15 in the more stable conformers of compounds **2** and **3**, as identified by PM3 calculations

HREIMS data for the two compounds showed a molecular ion peak at *m*/*z* 420.2500 and 420.2529, respectively, which is consistent with a molecular formula $C_{24}H_{36}O_6$, and a base peak at *m*/*z* 107, corresponding to the *p*-hydroxyphenylmethyl radical, as in compound **1**. This molecular formula indicates the existence of an additional unsaturation in comparison to compound **1**, and this could be assigned to a cyclic ether. A study of the HMBC spectra (3 Hz) showed a correlation between C-7 and C-10, providing evidence for the presence of a tetrahydrofuranic cycle between these positions. It is proposed that compounds **2** and **3** are two isomers of (3*R*)-7,10-epoxy-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyloxy)-dodec-11-ene-2,6-diol.

The two new chiral carbons, C-6 and C-7, can generate four diastereoisomers, and the synthesis of these compounds was achieved. The first approach involved epoxidation at the C-6–C-7 double bond and ether formation by nucleophilic ring opening of the epoxide with the hydroxyl group on C-10 (Fig. 2). The reaction was carried out by using MCPBA to yield compounds **2** and **3**. Epoxide ring opening occurred under the epoxidation conditions. This reaction is an *anti* addition that provided the isomers (6*R*,7*S*) and (6*S*,7*R*).

The most stable conformer of the isomer with the relative configuration 6*S**, 7*R**, 10*S** that was found by PM3 calculations (Fig. 3) suggested an alignment of H-7 and methyl groups at 14 and 15 with the presence of a hydrogen bond between the hydroxyl group on C-6 and the tetrahydrofuranic oxygen. The nuclear Overhauser effect (NOE) experiment on **3** showed a clear effect between H-7 and the methyl groups 14 and 15, and the structure of compound **3** is thus established as (3*R*,6*S**,7*R**,10*S**)-7,10-epoxy-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyl-oxy)-dodec-11-ene-2,6-diol.

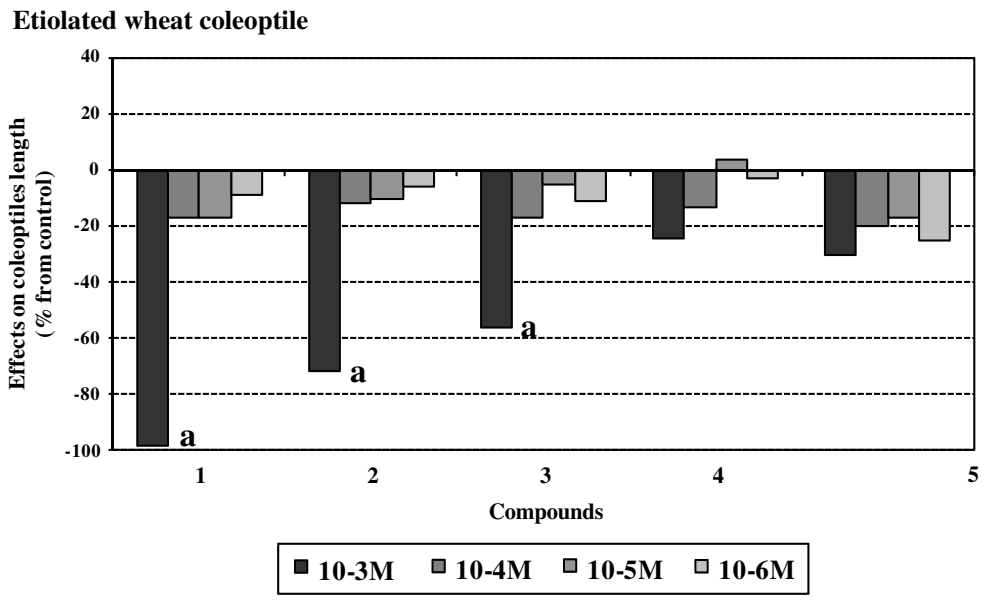


Fig. 4 Results of the etiolated wheat coleoptile assay on compounds **1**–**5**. Values are expressed as percentage differences from the control. If a letter is not indicated, then $P>0.05$ for Welch's test; *a* values significantly different at $P<0.01$

In contrast, the most stable conformer of the isomer with the relative configuration 6*R**, 7*S**, 10*S** (Fig. 3) had an alignment of H-7 and the methyl group 14 but not with methyl 15. The results of the nuclear Overhauser effect spectroscopy experiment on **2** confirmed this. The structure of compound **2** was established as (3*R*,6*R**,7*S**,10*S**)-7,10-epoxy-2,6,10-trimethyl-3-(3-*p*-hydroxyphenylpropanoyloxy)-dodec-11-ene-2,6-diol. These compounds have not been reported previously.

The first bioassay we carried out was the etiolated wheat coleoptile bioassay (Castellano 2002). This bioassay is intended to be a descriptive preliminary test. Our main objective was to perform a screening with low levels of compound, thus allowing a wider range of concentrations to be tested. This provides a general view of the possible bioactivities including plant growth regulators, herbicides (Cutler 1984; Macías et al. 2008), antimicrobials, mycotoxins, and pharmaceuticals (Jacyno and Cutler 1993).

Etiolated wheat coleoptile growth (Fig. 4) was inhibited ($P<0.01$, 98%) by a 10^{-3}-M solution of **1**. The degradation compounds **2** and **3** showed 72% and 56% of inhibition at 10^{-3} M, respectively. In contrast, the sesquiterpene and phenolic moieties (**4** and **5**) of compound **1** did not show significant activity.

The results of this screening showed that the natural product is the most active compound with the presence of sesquiterpene and phenolic moieties in the molecule as an apparent prerequisite for activity. The degradation of the compound lead to a diminution of the effect.

The phytotoxicity bioassay on STS (Macías et al. 2000) was applied to compounds **1**–**3** bearing in mind the possible degradation of compound **1** in the soil. The assay

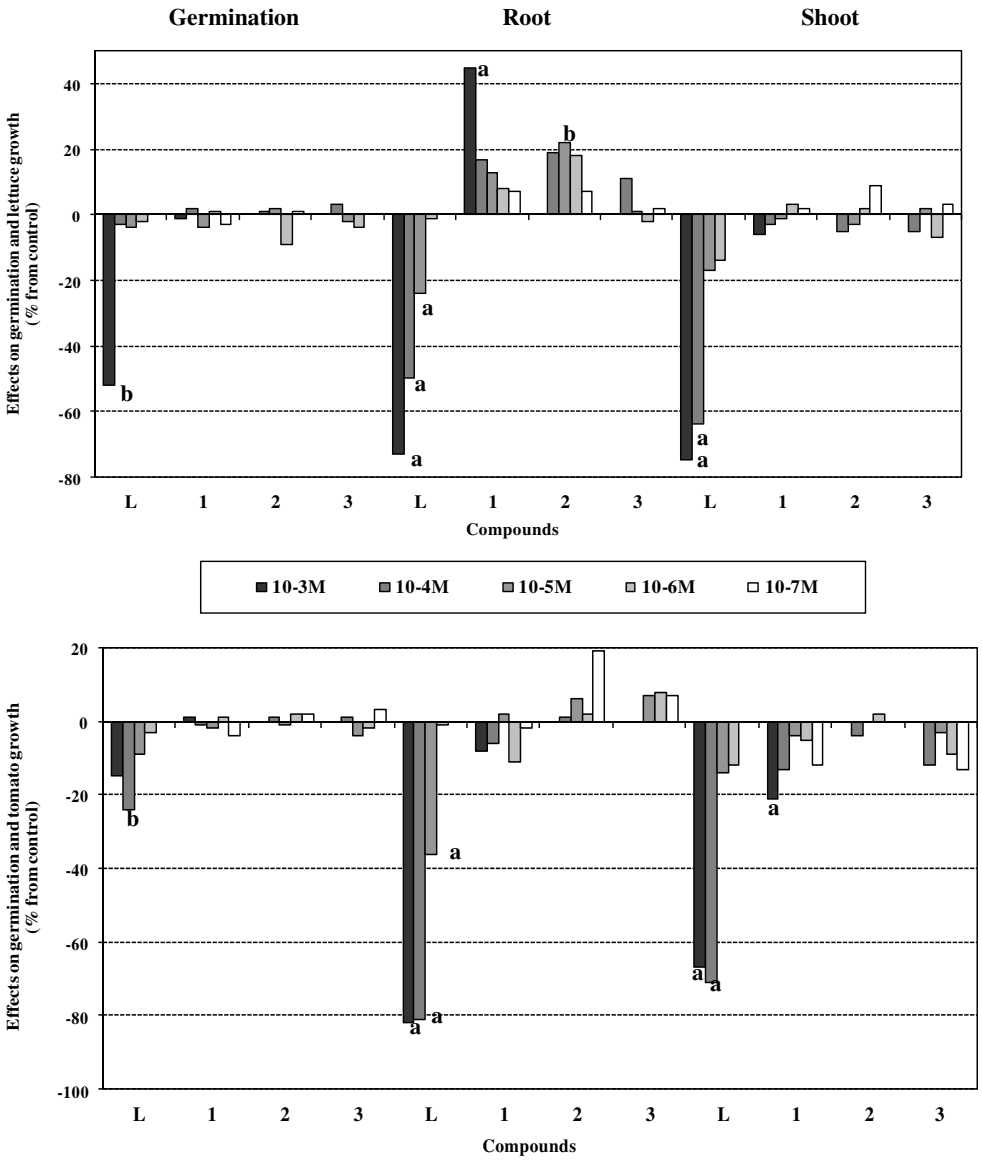


Fig. 5 Germination and growth effects of compounds **1**–**3** and LOGRAN on lettuce (*L. sativa*) and tomato (*L. esculentum*). Values are expressed as percentage differences from the control. If a letter is not indicated, $P>0.05$ for Welch's test; *a* values significantly different at $P<0.01$, *b* values significantly different at $0.01<P<0.05$

showed the effect of a series of aqueous solutions of compound **1** on the germination and growth of dicotyledonous (*L. sativa, L. sativum*, and *L. esculentum*) and monocotyledonous (*A. cepa* and *T. aestivum*) species. Data are presented as percentage differences from the control; positive values represent stimulation of the studied variable and negative values represent inhibition. The commercial herbicide LOGRAN® was used as a positive control.

Inhibition effects were not observed for lettuce (*L. sativa*), tomato (*L. esculentum*), onion (*A. cepa*), or wheat (*T. aestivum*) seeds (Figs. 5 and 6). However, there was a stimulation (average=40%) of the root growth of lettuce and wheat.

Cress seeds (*L. sativum*) were the most sensitive to the compounds tested (Fig. 7). Megalanthine (**1**) was phytotoxic to cress at 10^{-3} M, showing inhibition values of 34% for germination, 43% for root length, and 62% for shoot length. The degradation compounds **2** and **3** had reduced activity compared to compound **1**. Compounds **1**–**3** again showed a stimulation (average=40%) of the root length of cress at concentrations ranging from 10^{-4} to 10^{-7} M, as described previously for root growth of lettuce and wheat. These results suggest that megalanthine probably has no relevant role in the plant–plant defense.

In order to explore other possible roles for megalanthine (**1**), we tested megalanthine for antifeedant and antifungal effects on several target organisms. It had a moderate antifeedant effect (Table 3) on the lepidopteran *S. littoralis* without postingestive toxicity. It was a stronger antifeedant to *L. decemlineata*, but proved to be inactive to the aphid *M. persicae*. This compound was a selective cytotoxic

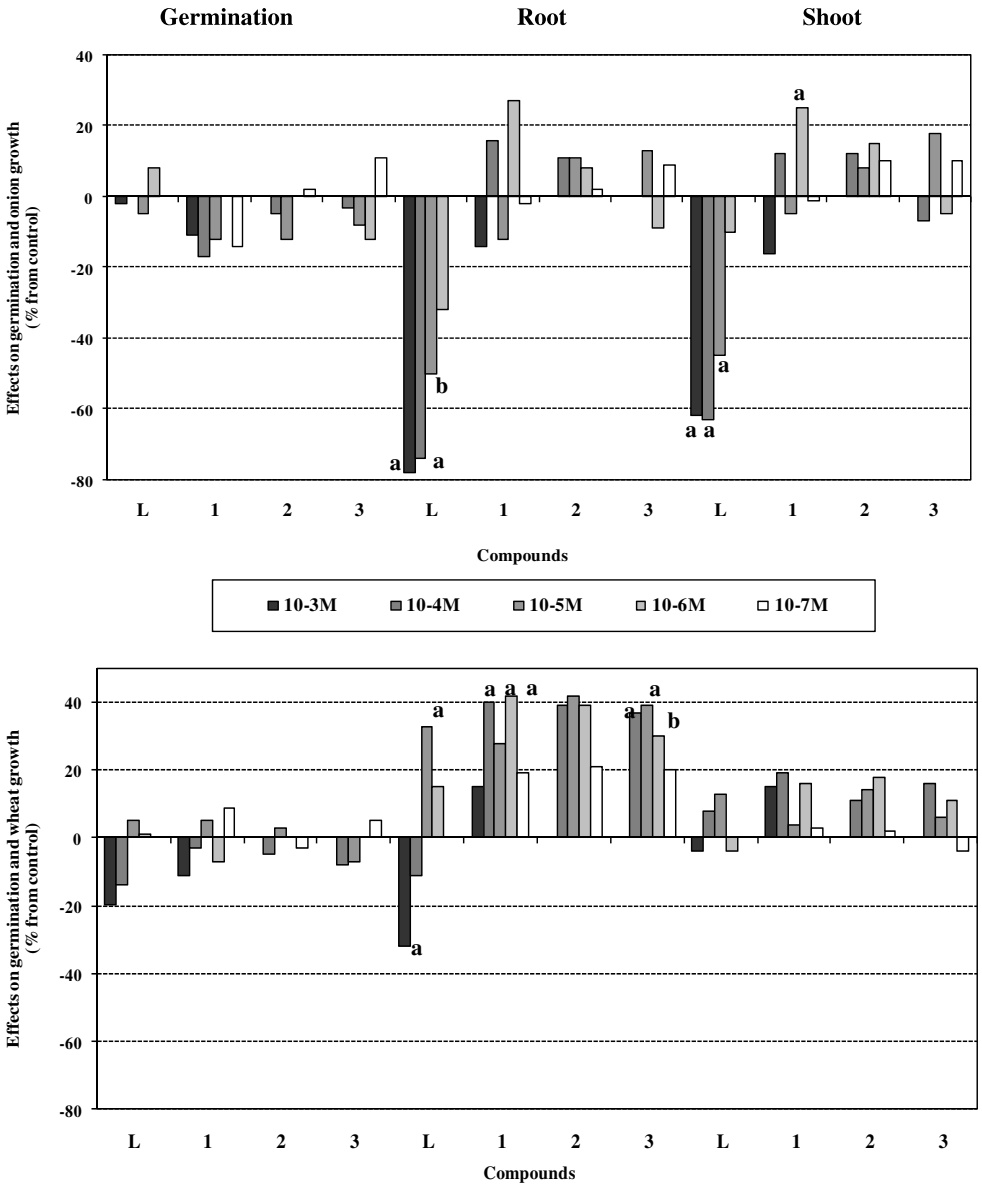


Fig. 6 Germination and growth effects of compounds **1**–**3** and LOGRAN on onion (*A. cepa*) and wheat (*T. aestivum*). Values are expressed as percentage differences from the control. If a letter is not indicated, $P>0.05$ for Welch's test; *a* values significantly different at $P<0.01$, *b* values significantly different at $0.01<P<0.05$

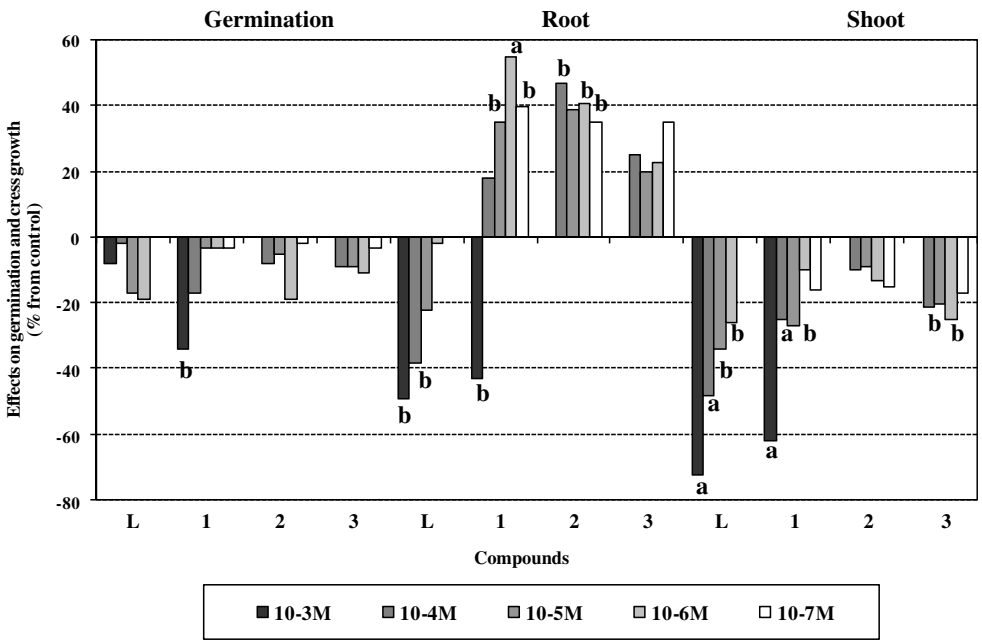


Fig. 7 Germination and growth effects of compounds **1**–**3** and LOGRAN on cress (*L. sativum* L.). Values are expressed as percentage differences from the control. If a letter is not indicated, $P>0.05$ for Welch's test; *a* values significantly different with $P<0.01$, *b* values significantly different with $0.01<P<0.05$

agent to insect-derived Sf9 cells, thus suggesting that the lack of *in vivo* toxicity on *S. littoralis* larvae could be the result of metabolic detoxification. Megalanthine (**1**) showed a significant and selective mycelial growth inhibition against the plant pathogen *Fusarium solani*.

The species-dependent antifungal and antifeedant effects with insect-selective cytotoxicity of megalanthine support a plant-defensive role against herbivorous insects and phytopathogens.

Our previous work on the alkaloidal fraction of this plant resulted in the isolation of an antifeedant with low to moderate selectivity to *S. littoralis*, the PA lycopsamine (Reina et al. 1998). However, the phenylpropanoid sesquiterpene (**1**) studied shows stronger antifeedant and antifungal action than lycopsamine. Phenylpropanoids have a multiplicity of functions in plants. The biosynthesis responds to environmental stresses such as wounding, pathogen infection, and UV radiation (Kliebenstein 2004; Dixon et al. 2002).

Table 3 Biological effects of megalanthine (**1**) on a series of biological targets related to plant defense: herbivorous insects (*S. littoralis*, *L. decemlineata*, and *M. persicae*), insect and mammalian cells (Sf9 and CHO), and phytopathogens (*Fusarium* spp.)

Target organism	Biological effects				
	Insecticidal			Cytotoxic	Antifungal
	Antifeedant	Postingestive			
		ΔB	ΔI		
S. littoralis	6.5×10^{-8} (1.8×10^{-8}, 23.2×10^{-8})[a]	109	99		
L. decemlineata	2.1×10^{-8} (1.0×10^{-8}, 4.2×10^{-8})[a]				
M. persicae	56:44[b]				
Sf9				1.09×10^{-7} (0.08×10^{-7}, 14.40×10^{-7})[a]	
CHO				≈100	
F. oxysporum					$>12.4\times10^{-4}$
F. moniliforme					$>12.4\times10^{-4}$
F. solani					9.9×10^{-4} (7.4×10^{-4}, 12.4×10^{-4})[a]

ΔB change in insect body weight (dry weight, in milligrams), ΔI food consumed (dry weight, in milligrams) expressed as a percentage of control
[a] Effective antifeedant, cytotoxic, or antifungal dose EC_{50} (in moles per square centimeter and molar)and 95% confidence limits (lower, upper)
[b] Percentage of aphids settled on treated (T) to control (C) leaf disk ratio (T/C)

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