Pergamon

0031-9422(95)00257-X

Phytochemistry, Vol. 40, No. 2, pp. 419–425, 1995

0031-9422/95 $9.50 + 0.00

BIOCHEMICALLY ACTIVE SESQUITERPENE LACTONES FROM *RATIBIDA MEXICANA**

MONICA R. CALERA,†‡ FERNANDO SOTO,† PERLA SANCHEZ,† ROBERT BYE,§ BLANCA HERNANDEZ-BAUTISTA,‡ ANA LUISA ANAYA,‡ BLAS LOTINA-HENNSEN† and RACHEL MATA†‖

†Facultad de Quimica, ‡Instituto de Fisiologia Celular, §Instituto de Biologia, Universidad Nacional Autónoma de México, México, D.F. 04510, México

(*Received in revised form* 20 *January* 1995)

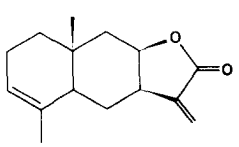
Key Word Index—*Ratibida mexicana*; Asteraceae; roots; *Amaranthus hypochondriacus*; Amaranthaceae; *Echinochloa crus-galli*; Poaceae; phytogrowth-inhibitory activity; cytotoxicity; phytopathogenic fungi; Hill's reaction inhibitor; sesquiterpene lactone; isoalloalantolactone; elema-1,3,11-trien-8,12-olide.

Abstract—Bioactivity-directed fractionation of the methanol extract of the roots of *Ratibida mexicana* resulted in the isolation of two bioactive sesquiterpene lactones, isoalloalantolactone and elema-1,3,11-trien-8,12-olide. Both compounds caused a significant inhibition of the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crus-galli*, exerted moderate cytotoxic activity against three different solid tumour cell lines and inhibited significantly the radial growth of three phytopathogenic fungi. Isoalloalantolactone also caused the inhibition of ATP synthesis, proton uptake and electron transport (basal, phosphorylating and uncoupled) from water to methylviologen, therefore acting as a Hill's reaction inhibitor. The lactone did not affect photosystem I but inhibited photosystem II. The site of inhibition of isoalloalantolactone is located in the span of P_{680} to Q_A redox enzymes because the uncoupled electron transport from water to silicomolybdate and, from DPC to DCIP are inhibited approximately to the same extent.

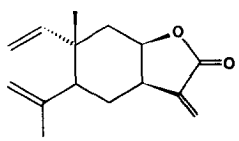
INTRODUCTION

Ratibida mexicana (Wats.) Sharp (Asteraceae) is a yellow-flowered perennial medicinal herb which grows scattered along the Sierra Madre Occidental in northwestern México. The Tarahumara Indians refer to the plant as Howinowa and crush the roots to use on legs for rheumatism. Other Indian tribes use the roots for a wash and a potion of the leaves in a beverage for headaches and colds [1].

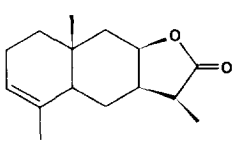
As a part of our search for biologically active compounds with medicinal and or agrochemical importance from plants of the *Ratibida* genus [2–4], we describe the isolation of isoalloalantolactone and elema-1,3,11-trien-8,12-olide, the major antifungal and phytotoxic principles of *R. mexicana*. In addition, the effect of isoalloalantolactone and three semi-synthetic derivatives (**1a–c**) on different photosynthetic activities was investigated.



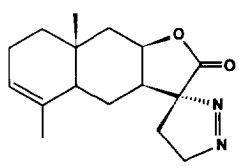
1



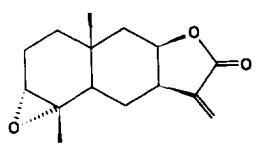
2



1a



1b



1c

*Taken in part from the PhD thesis of M. R. Calera and the BS theses of F. Soto and P. Sánchez.

‖Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Preliminary biological evaluations

The roots of *R. mexicana* were extracted with methanol. The methanol residue was then partitioned between hexane (F_0-1) and 10% aqueous MeOH (F_0-2). The original extract primary fractions F_0-1 and F_0-2 were biologically evaluated for their potential cytotoxic, phytogrowth-inhibitory and antifungal properties. The cytotoxic activity was determined against three human solid tumour cell lines and with the brine shrimp lethality test (BST) [5] (Table 1). The initial phytogrowth-inhibitory activity [6] was evaluated on seeds of *A. hypochondriacus* and *E. crus-galli* by using the Petri dish bioassay (PDPIB) (Table 2). Finally, the antifungal bioassays [7] were performed by measuring the inhibition of the radial growth of the phytopathogenic fungi *Fusarium oxysporum*, *Phythium* sp. and *Helminthosporium* sp. The minimum inhibitory concentrations (MIC) were > 1000, 200 and $\leqslant 50\ \mu g\ ml^{-1}$, respectively.

Bioactivity guided isolation of isoalloalantolactone and elema-1,3,11-trien-8,12-olide

According to the results summarized in Tables 1 and 2 the methanol extract of *R. mexicana* and primary fraction F_0-1 showed significant cytotoxic and phytogrowth-inhibitory activities. The most bioactive fraction F_0-1 was further fractionated by column and thin layer chromatographies over silica gel using the BST and bioautographic phytogrowth-inhibitory bioassay (BPIB) [8] as activity directed fractionation. These procedures yielded two active sesquiterpene lactones, isoalloalantolactone (**1**) and elema-1,3,11-trien-8,12-olide (**2**), and three inactive known selinane type of sesquiterpenes.

Isoalloalantolactone (**1**) was identified by IR, NMR and mass spectral analyses and by comparison with the spectroscopic data previously described [9, 10]. In addition, the properties of the dihydro derivative (**1a**) and the monopyrazoline (**1b**), obtained from the reaction of lactone **1** with $NaBH_4$, and diazomethane, respectively, were identical to those previously reported [9]. Treatment of **1** with *m*-chloroperbenzoic acid afforded the epoxide (**1c**), not previously described, which was characterized by spectral means. The NMR spectra (see Experimental) of **1c** were similar to those of **1**, except that the signals for the double bond at C-3/C-4 were missing. In their place, bands for the epoxide group were observed at δ2.96 (H-3) in the ^{1}H NMR spectrum and at δ60.9 (C-3) and 57.6 (C-4) in the ^{13}C NMR spectrum.

Compound **2** was also characterized by spectral analysis. The NMR and the mass spectral data were identical to those previously reported [11, 12]. This compound might be an artifact of isolation since elemanolide sesquiterpene lactones are frequently found to be decomposition products of germacranolides.

The inactive selinanes were identified as (−)-cryptomeridiol, (−)-8α-acetoxycriptomeridiol and (−)-selin-11-en-4αol by comparison with authentic samples previously isolated from *Teloxys graveolens* (Willd.) Weber [13].

Phytogrowth-inhibitory activity of the natural lactones and derivatives **1a–c**

Natural lactones **1** and **2** significantly inhibited radicle growth of *A. hypochondriacus* and *E. crus-galli* in a concentration dependent manner with higher activity at increasing concentrations. The 50% phytogrowth inhibitory concentrations (IC_{50}) (Table 2) were in the range of $9.73\ \mu g\ ml^{-1}$ (4.19×10^{-5} M) to $89.02\ \mu g\ ml^{-1}$ (3.83×10^{-4} M). The phytogrowth-inhibitory activity of derivatives **1a–c** was also evaluated. Compounds **1**, **1a**, and **c** at the concentration of $200\ \mu g\ ml^{-1}$ strongly inhibited the radicle growth of *A. hypochondriacus* (Fig. 1). At the $50\ \mu g\ ml^{-1}$ level lactones **1a–c** were less active than the parent isoalloalantolactone (**1**). On the other hand, the seedlings of *E. crus-galli* were less sensitive to derivatives **1a c** at both concentrations (Fig. 1). These results revealed that in addition to the α methylene group in the γ-lactone, the $\Delta^{3,4}$ double bond in isoalloalantolactone is an important structural feature for high phytogrowth-inhibitory activity.

Table 1. Cytotoxic activity of the methanol extract, primary fractions and sesquiterpene lactones isolated from *Ratibida mexicana*

		Cell line†		
	BST*	HT-20	MCF-7	A-549
Methanol extract	62.27	2.60	4.00	3.40
F_0-1	37.40	ND	ND	ND
F_0-2	1288.9	ND	ND	ND
Compound **1**	45.31	1.00	2.00	1.00
Compound **2**	209.28	4.00	3.10	4.50
Adriamycin	ND	3.47×10^{-2}	3.32×10^{-1}	7.94×10^{-3}

*BST (brine shrimp test) results are expressed as LC_{50} values ($\mu g\ ml^{-1}$).

†Results are expressed as ED_{50} values ($\mu g\ ml^{-1}$).

HT-20 (human colon adenocarcinoma); MCF-7 (human breast carcinoma); A-549 (human lung carcinoma); ND (not determined).

Table 2. Phytogrowth-inhibitory activity of the methanol extract, primary fractions and isolated lactones from *Ratibida mexicana* on radicle elongation of *Amaranthus hypochondriacus* and *Echinochloa crus-galli*

	IC_{50} ($\mu g\,ml^{-1}$)	
	A. hypochondriacus	*E. crus-galli*
Methanol extract	41.18	213.73
F_0-1	31.74	205.42
F_0-2	347.49	563.47
Compound **1**	9.73	55.75
Compound **2**	27.72	89.02
Tricolorin A*	37.00	12.60

*Positive standard.
IC_{50} = 50% inhibitory concentration.

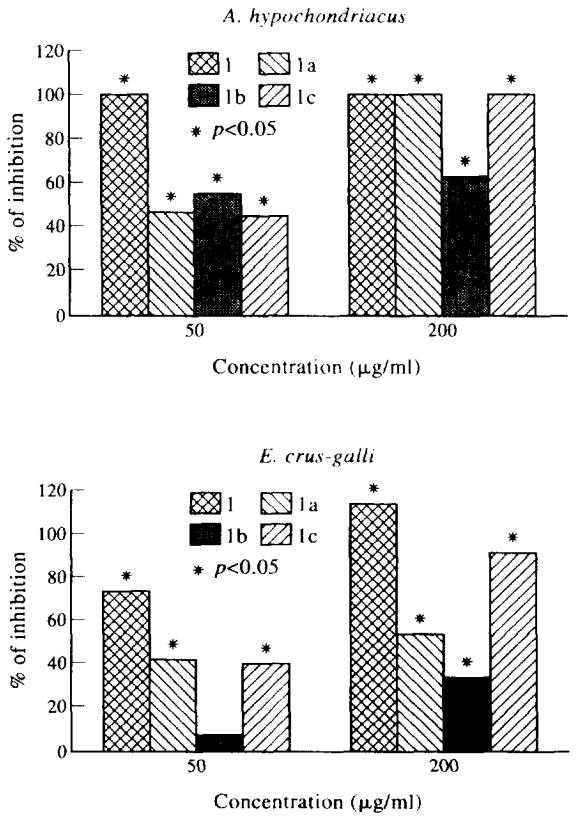


Fig. 1. Phytogrowth-inhibitory activity of isoalloalantolactone derivatives (**1a–c**) on radicle elongation of *A. hypochondriacus* and *E. crus-galli*.

Kaur and Kalsi [9] found that isoalloalantolactone (**1**) promoted root formation on stem cuttings of *Phaseolus vulgaris*. On the other hand, the corresponding enantiomer, diplophyllolide, showed inhibitory activity towards the germination and root elongation of rice husks [14].

It is important to point out that the concentration level of phytogrowth-inhibitory activity exhibited by **1** and **2** is comparable with those previously described for other sesquiterpene lactones [15]. Several other eudesmanolides have exhibited phytotoxic and plant growth regulatory properties. However, vernolepin has been the only elemanolide whose phytogrowth-inhibitory activity was previously described [15].

Cytotoxic activity of natural lactones **1** *and* **2**

The cytotoxic activity of lactones **1** and **2** is summarized in Table 1. Both compounds were active in the BST [5] and showed low cytotoxicity against A-549 (lung), MCF-7 (breast) and HT-29 (colon) in 7-day human solid tumour cell *in vitro* test, using adriamicyn as the positive control compound.

Antifungal activity

The radial growth of *Helminthosporium* was totally inhibited by lactones **1** and **2** at the tested concentrations. The minimum inhibitory concentrations were $\leqslant 50\ \mu g\,ml^{-1}$ in both cases. Concerning the three derivatives, only the epoxide (**1c**) showed comparable activity with a minimum inhibitory concentration of 75 $\mu g\,ml^{-1}$. *Pythium* was more susceptible to **1** and **1b** with minimum inhibitory concentrations of 125 and $\leqslant 50\ \mu g\,ml^{-1}$, respectively. Finally, *Fusarium* was considerably less sensitive and only isoalloalantolactone (**1**) inhibited at 200 $\mu g\,ml^{-1}$ 45% of the radial growth of this fungus. The 11,13-dihydro derivative (**1a**) was inactive against all three species at all concentrations thus indicating that the conjugated γ lactone is an important grouping for antifungal activity [16]. The potency of all the sesquiterpene lactones examined to inhibit the radial growth of the phytopathogenic fungi is less than that previously described for related compounds [16].

Effect of isoalloalantolactone on isolated spinach chloroplasts

Photosynthetic phosphorylation from water to methylviologen in spinach thylakoids was inhibited by isoalloalantolactone (**1**) and its derivatives (**1a–c**) (Fig. 2). Compound **1** inhibited ATP synthesis in a concentration-dependent manner, reducing it by 69% at 500 μM (IC_{50} was 200 μM). Also, the light-dependent proton uptake was decreased by 63% at 100 μM (Fig. 3). The dihydro derivative (**1a**) inhibited ATP synthesis by 71% at 500 μM. At this concentration the pyrazoline (**1b**) and the epoxide (**1c**) showed a weaker effect, inhibiting ATP synthesis only by 46 and 18%, respectively (Fig. 2). These data indicated that the $\Delta^{3,4}$ double bond of isoalloalantolactone (**1**) is an essential structural requirement for its inhibitory effect on ATP synthesis. However, the α-methyl-γ-lactone moiety was important but not essential for ATP synthesis inhibition. It is important to point out that the same trend was observed for the phytogrowth-inhibitory activity.

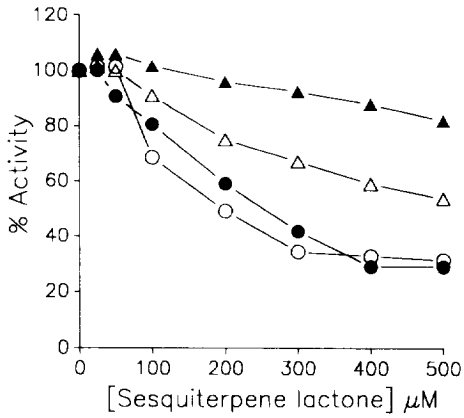


Fig. 2. Inhibitory effect of isoalloalantolactone (**1**) and its derivatives (**1a–c**) on photophosphorylation from water to methylviologen in chloroplast thylakoids isolated from spinach leaves. Photophosphorylation was measured in the presence of 1 mM ADP and 3 mM K_2HP_4. Each cuvette contained 20 μg chlorophyll per ml in the reaction medium. Other conditions as described in experimental section. Control value rate was 80.4 μmol ATP hr^{-1} mg Chl $^{-1}$. Isoalloalantolactone (○), dihydro (●), pyrazoline (△) and epoxide (▲).

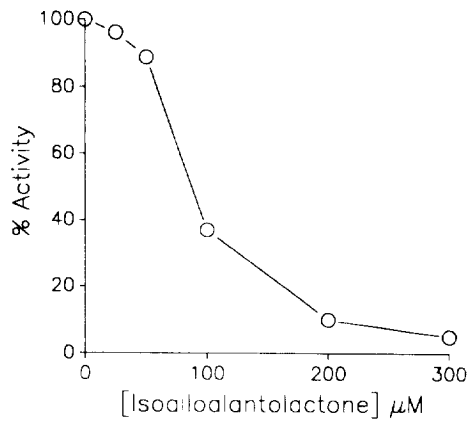


Fig. 3. Proton uptake as a function of sesquiterpene lactone concentration. In each case a cuvette contained 20 μg chlorophyll per ml in the reaction medium. Other conditions are as described in experimental section. Control value rate was 16.2 μeq H^+ hr^{-1} mg Chl $^{-1}$.

The light-dependent ATP synthesis by thylakoids may be inhibited by blocking electron transport, by uncoupling ATP synthesis from electron transport or by blocking the phosphorylation reaction itself. Isoalloalantolactone (**1**) could be acting at any of these levels.

To obtain further information, the effect of lactone **1** on the photosynthetic electron transport was investigated. Figure 4 shows that basal, phosphorylating and uncoupled electron flow from water to methylviologen were inhibited in a concentration-dependent manner. These results indicate that isoalloalantolactone behaves as a Hill's reaction inhibitor, since it has inhibited not only the electron transport but also the photophos-

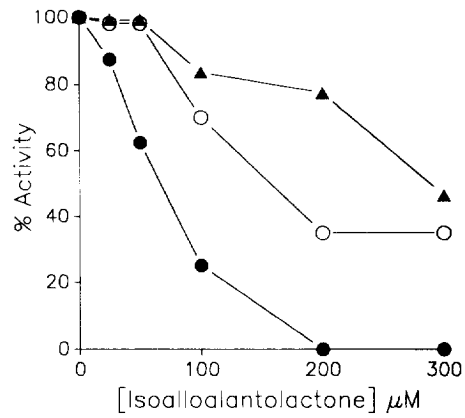


Fig. 4. Noncyclic electron transport (basal, phosphorylating and uncoupled) from water to methylviologen as a function of isoalloalantolactone concentration. Photophosphorylating electron transport was measured in presence of 1 mM ADP and 3 mM K_2HPO_4. A 6 NH_4Cl was added for measuring uncoupled electron transport. Each cuvette contained 20 μg chlorophyll per ml in the reaction medium. Other conditions were as described in the Experimental. Control values rates for basal, phosphorylating and uncoupled electron transport were 536, 954.8 and 2161, respectively in μeq e^- hr^{-1} mg Chl^{-1}. Basal (●), phosphorylating (○) and uncoupled (▲) electron transport.

phorylation and proton uptake process. The target of this lactone is exposed when the chloroplast is energized because in this condition the inhibition is stronger than when the chloroplast is unenergized (uncoupled state).

In order to localize the site of inhibition, the effect of lactone **1** on partial reactions (photosystem I and photosystem II) was measured using artificial electron donors and electron acceptors. Table 3 shows that the uncoupled photosystem I electron transport from dichlorophenolindophenol (DCIP) to methylviologen (MV) was not affected by this compound. However, at concentrations of 200 and 400 μM isoalloalantolactone (**1**) inhibited uncoupled electron transport in photosystem II from water to DCIP. More specifically, this reaction was inhibited by lactone **1** in the span of water to silicomolybdate, i.e. 71% at 400 μM.

To determine the site of electron transport inhibition between water to Q_A electron flows were measured from diphenylcarbazide (DPC) to DCIP in Tris-treated chloroplasts in the presence of **1**. The data showed that the target of lactone **1** was one of the redox enzymes in the span of P_{680} to Q_A electron transport chain (Table 3).

It is interesting to mention that other sesquiterpene lactones such as cacalol, zaluzanin C and ivalin [17–19], are also able to interfere with the photosynthetic process acting as Hill's reaction inhibitors. However, these terpenoids inhibit oxygen evolution. On the other hand, a number of well known photosynthetic inhibitors such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP), heavy metals ions as Cu(II), dinoseb (a phenolic herbicide) and cacalol derivatives have been found to inhibit redox enzymes at the same level as isoalloalantolactone does, i.e. in the span P_{680} to Q_A [17, 20–26].

Table 3. Effect of isoalloalantolactone on uncoupled photosystem I and II electron transport rate. Control values rates in μeq e$^-$ hr^{-1} mg Chl^{-1} for PSI (DCIP to MV), PSII (from H_2O to DCIP, H_2O to SiMo and DPC to DCIP) were 2000, 680, 140 and 105, respectively

Compound	Addition (μM)	DCIP MV (%)	H_2O (%)	H_2O SiMo (%)	DPC DCIP (%)
Control	none	100	100	100	100
Isoalloalan-tolactone	200	100	83	71	62
	400	100	50	29	28

The results of this study showed that natural lactones **1** and **2** posses remarkable antifungal and phytogrowth-inhibitory activities. Therefore, they might be developed into environmentally safe herbicide and/or fungicide agents. Also, it is probable that **1** and **2** are involved in the allelopathic interaction of *R. mexicana* [15]. In the case of isoalloalantolactone (**1**) the allelopathic mode of action could involve an alteration in the photosynthetic process by inhibiting the Hill's reaction and, therefore, the growth of photosynthetic organisms.

EXPERIMENTAL

Instruments. IR spectra were obtained in KBr on a Perkin Elmer 599 B spectrophotometer; ^{1}H and ^{13}C NMR spectra were registered at 300 MHz and 75 MHz, respectively, on a Varian VXR-300S apparatus. Mass spectra were taken on a Hewlett-Packard 599 B spectrometer. Mp: uncorr. Commercial silica gel 60 (70–230 mesh) Merck was used for CC. TLC was carried out using precoated silica gel 60 GF 254 plates (Merck).

Plant material. The plant material (roots) was collected in Municipio de Guachochic, Cusárare, State of Chihuahua, México in December 1991. Reference samples are deposited at the ethnobotanical collection of the National Herbarium (MEXU), Instituto de Biologia, UNAM, Voucher: Bye and Linares 18336.

Isolation. The air-dried plant material (3.2 kg) was ground into powder and extracted exhaustively by maceration at room temp. with MeOH. After filtration the extract was concd *in vacuo*. The MeOH residue (397 g) was partitioned between hexane (F_0-1) and 10% aqueous MeOH (F_0-2). After elimination of the solvent *in vacuo* F_0-1 yielded 56.5 g of a brown residue and F_0-2 340.5 g.

When fraction F_0-1 was tested by the phytogrowth-inhibitory bioautographic bioassay using the C_6H_6–EtOAc (4:1) system, the activity was found at a R_f of 0.5–0.6. F_0-1 (56 g) was subjected to CC over silica gel (523 g) eluted with a concn gradient of hexane–EtOAc starting with hexane only. A total of 110 frs (400 ml each) were collected and pooled based on TLC profiles to yield 7 major frs (F_1-1–F_1-7). Bioactivity in the BST bioassay showed two active pools: F_1-2 (BST, LC_{50} = 271.73 μg ml^{-1}] and F_1-3 [BST, LC_{50} = 90.77 μg ml^{-1}]. Similarly, using the bioautographic bioassay only fractions F_1-2 and F_1-3 also contained the phytotoxic activity, which was localized at R_f of 0.5 and 0.4, respectively. F_1-3 (15.03 g) was further chromatographed on silica gel (480 g), eluted with C_6H_6–EtOAc (4:1) to yield 1.70 g of isoalloalantolactone (**1**), mp 74–75° (Lit. mp 70°) [9] and 14 mg of (−)-selin-11-en-4α-ol, mp 82–84°.

The toxic fraction F_1-2 (14.45 g) was further resolved on another silica gel (280 g) column eluted with hexane–EtOAc (9:1) to yield impure elema-1,3,11,trien-8,12-olide (**2**). Further purification by prep. TLC on silica gel impregnated with $AgNO_3$ and using C_6H_6–EtOAc (9:1) as mobile phase, rendered 124 mg of pure **2**, mp 72–73° (Lit. mp, unreported).

When primary fraction F_0-2 was dissolved in MeOH, 63.3 g of a gummy solid pptd (F_4-1). F_4-1 (44.53 g) was subjected to CC over silica gel (600 g) eluted with a concn gradient of C_6H_6–EtOAc–MeOH, starting with C_6H_6 and ending with EtOAc–MeOH (1:1). A total of 112 frs were collected and combined into 9 groups (F_5-1–F_5-9) according to their TLC profiles. Fraction F_5–2, eluted with C_6H_6–EtOAc (1:1) yielded a mixture (37.1 mg) of two inactive compounds. The mixture was resolved by prep. TLC using hexane–EtOAc (1:1) as eluant to yield cryptomeridiol (26 mg) and (−)-8α-acetoxycryptomeridiol (8.60 mg), identical to standard samples [13].

Expoxidation of isoalloalantolactone. Compound **1** (50 mg) in $CHCl_3$ (10 ml) was treated with *m*-chloroperbenzoic acid (67.1 mg) for 90 min at room temp. The reaction mixture was washed with a 10% solution of $NaHCO_3$ and worked-up as usual to yield epoxide **1c** (53.2 mg), mp 133–135°; IR ν_{max}^{KBr} cm^{-1}: 2932, 2868, 1754, 1664, 1262; ^{1}H NMR (300 MHz, $CDCl_3$): δ6.16 (1H, *d*, J = 1.20 Hz, H-13), 5.64 (1H, *d*, J = 1.20, H-13'), 4.46 (1H, *ddd*, J = 4.95, 4.95, 1.5 Hz, H-8), 2.99 (1H, *m*, H-7), 2.96 (1H, *br s*, H-3), 2.07 (1H, *dd*, J = 15, 1 Hz, H-9b), 1.23 (3H, *s*, H-15), 0.91 (3H, *s*, H-14); ^{13}C NMR (75 MHz, $CDCl_3$): δ170.4 (C-12, *s*), 141.5 (C-11, *s*), 120.6 (C-13, *t*), 77.4 (C-8, *d*), 60.9 (C-3, *d*), 57.6 (C-4, *s*), 44.6 (C-5, *d*), 41.2 (C-7, *d*), 40.2 (C-9, *t*), 34.2 (C-1, *t*), 29.7 (C-10, *s*), 27.4 (C-6, *t*), 21.1 (C-15, *q*), 20.7 (C-2, *t*), 17.9 (C-14, *q*); EI-MS *m/z* (rel. int.) 248 ([M]$^+$, 1.0), 233 ([M − 15]$^+$, 16.0), 91 (17.0), 77 (17.0), 67 (18.0), 55 (33.0), 53 (34.0), 43 (100), 41 (47.0).

Reduction of isoalloalantolactone. To compound **1** (103.5 mg) in MeOH (10 ml) $NaBH_4$ (154.70 mg) was added and the solution was stirred for 36 hr at room temp. The viscous oil so obtained was purified by prep. TLC, using silica gel plates impregnated with $AgNO_3$ and C_6H_6–EtOAc (9:1) as eluant to yield 94.0 mg of **1a**.

Preparation of the pyrazoline **1b**. A solution of **1** (70 mg) in Et_2O was allowed to react with an Et_2O soln

of CH_2N_2 until the yellow color persisted for more than 20 min. After 24 hr the solvent was evapd and the residue recrystallized from Et_2O to yield 82.24 mg of **1b**.

Brine shrimp lethality test (BST) and cytotoxicity bioassays. The extract, fractions, isolated compounds and derivatives were evaluated for lethality to brine shrimp larvae as described. Cytotoxicities against human solid tumor cells were measured at the Cell Culture Laboratory, Purdue Cancer Center, for the A-549 lung carcinoma, MCF-7 breast, carcinoma and HT-29 colon adenocarcinoma, with adriamycin as a positive control, in 7-day assays [5].

Phytogrowth-inhibitory bioassays. The phytogrowth-inhibitory activity of the MeOH extract, primary fractions and pure compounds was evaluated on seeds of *Amaranthus hypochondriacus* and *Echinochloa crus-galli* by using a Petri dish bioassay [6]. In addition, a direct bioautographic bioassay system [8] was employed to guide secondary fractionation and speed up the isolation of the active compounds.

The seeds of *E. crus-galli* were purchased from Valley Seed Service, Fresno, California, and those of *A. hypochondriacus* from Mercado de Tulyehualco, D.F., México. The data were analyzed by ANOVA ($P < 0.05$), and IC_{50} values were calculated by Probit analysis based on per cent inhibition obtained. The extract and primary fractions were evaluated at 50, 100 and 200 $\mu g\,ml^{-1}$. Compounds **1** and **2** were evaluated at 10, 30, 50, 70, 100, 150 and 200 $\mu g\,ml^{-1}$. Derivatives **1a–c** were evaluated only at 50 and 200 $\mu g\,ml^{-1}$. Tricolorin A was used as positive control [27].

The direct bioautographic assay, where the seeds of the target species grow directly on the thin layer chromatography plate, silica gel G60 F254 glass backed plates (20 × 5 cm, Merck), was used as previously described [8].

Bioassays with phytopathogenic fungi. The target species chosen were *Fusarium oxysporum*, *Helminthosporium* sp. and *Pythium* sp. The bioassays were carried out using the procedure previously described [7]. The measurements were made after 72 hr of incubation. The mean value of three replications were analyzed by ANOVA ($P < 0.05$). The extract, primary fractions and natural products **1** and **2** were evaluated at 50, 100, 125, 150 and 200 $\mu g\,ml^{-1}$. Derivatives **1a–c** were evaluated at 50, 75 and 100 $\mu g\,ml^{-1}$.

Chloroplasts isolation and chlorophyll determination. Chloroplast thylakoids were isolated from market spinach leaves (*Spinacea oleracea*. L.) as described earlier [17, 28] and suspended, unless indicated, in 400 mM sorbitol, 5 mM $MgCl_2$, 20 mM KCl and buffered with 0.03 M Na^+-tricine at pH 8.0. The chlorophyll concentration was measured spectrophotometrically as described [29].

Measurement of proton uptake, ATP synthesis and electron transport. Proton uptake was measured as a pH rise between 8.0 and 8.1 [30] using a combination microelectrode connected to a Corning potentiometer with expanded scale. The pH changes were recorded (Gilson recorder). The reaction medium was 100 mM sorbitol, 5 mM $MgCl_2$, 10 mM KCl, 1 mM tricine pH 8.0. ATP synthesis was measured as in proton uptake conditions in the presence of 1 mM ADP and 3 mM KH_2PO_4 [28]. Methylviologen (0.05 mM) was added as electron acceptor for Hill reaction.

Photosynthetic noncyclic electron transport activity from water to methylviologen was determined with an oxygraph 5300. The reaction medium was the same as in the proton uptake assay except that the tricine concentration was 15 mM and presence or absence of 6 mM NH_4Cl [17]. Photosystem I electron transport was determined in a similar form to noncyclic electron transport [17, 28]. The following reagents were added: 100 μM DCIP, 300 μM ascorbate, 10 μM DCMU and 6 mM NH_4Cl. Photosystem II was measured in presence of 50 μM DCIP, 1 μM DBMIB, 300 μM $[Fe(CN)_6]_3$ and 6 mM NH_4Cl [17, 28]. Uncoupled electron transport from water to silicomolybdate, was measured with a reaction mixture as in photosystem II except that 200 μM silicomolybdate and 10 μM DCMU were added [31]. Uncoupled electron transport from diphenylcarbazide (DPC) to dichlorophenol-indophenol (DCIP) was measured spectrophotometrically as reported [32], 200 μM DPC was added to the medium. All reaction mixtures were illuminated with actinic light of a projector lamp (GAF 2660) passed through a 5-cm filter of a 1% $CuSO_4$ soln.

Acknowledgements—This work was supported by grants IN-202493, IN204493 and IN203394, DGAPA-UNAM, and 0133-N9104 CONACYT. We thank M. en C. Atilano Gutiérrez, Instituto de Química, UNAM for the recording of the NMR spectra; QFB. Graciela Chávez, Q. Marisela Gutiérrez and Q. Georgina Duarte, Facultad de Química, UNAM, for IR, optical rotations and mass spectra measurements. M. R. Calera acknowledges the fellowship awarded by DGAPA-UNAM to carry out her PhD thesis. P. Sánchez and F. Soto acknowledge Facultad de Química, UNAM for the fellowship awarded, through Subprograma 127 to carry out their BS theses.

REFERENCES

1. Richards, E. L. (1968) *Rhodora* **70**, 348.
2. Mata, R., Rojas, A., Soriano, M., Villena, R., Bye, R. and Linares, E. (1990) *Heterocycles* **31**, 1111.
3. Rojas, A., Villena, R., Jiménez, A. and Mata, R. (1991) *J. Nat. Prod.* **54**, 1279.
4. Jiménez, A., Pereda-Miranda, R., Bye, R., Linares, E. and Mata, R. (1993) *Phytochemistry* **34**, 1079.
5. Anderson, J. E., Goetz, C. M., McLaughlin, J. L. and Suffness, M. (1991) *Phytochem. Anal.* **2**, 107.
6. Anaya, A. L., Calera, M. R., Mata, R. and Pereda-Miranda, R. (1990) *J. Chem. Ecol.* **16**, 2145.
7. Castañeda, P., García, M. R., Hernández, B. E., Torres, B. A., Anaya, A. L. and Mata, R. (1992) *J. Chem. Ecol.* **18**, 1025.
8. Li, H. H., Nishimura, H., Hasegawa, K. and Mizutani, J. (1992) *J. Chem. Ecol.* **18**, 1785.

9. Kaur, B. and Kalsi, P. S. (1985) *Phytochemistry* **24**, 2007.
10. Marco, J. A. and Carda, M. (1987) *Magn. Reso. Chem.* **25**, 1087.
11. Bohlman, F. and Dutta, L. (1979) *Phytochemistry* **18**, 1228.
12. Herz, W., Kulanthaviel, P. and Goedken, V. L. (1985) *J. Org. Chem.* **50**, 610.
13. Mata, R., Navarrete, A., Alvarez, L., Pereda-Miranda, R., Delgado, G. and Romo de Vivar, A. (1987) *Phytochemistry* **26**, 191.
14. Asakawa, Y., Toyota, M., Takemoto, T. and Suire, C. (1979) *Phytochemistry* **18**, 1007.
15. Fischer, N. (1991) in *Ecological Chemistry and Biochemistry of Plant Terpenoids* (Harbone, J. B. and Tomas-Barberan, F. A., eds), pp. 377–398. Clarendon Press, Oxford.
16. Picman, A. K. and Schneider, I. E. F. (1993) *Biochem. Syst. Ecol.* **21**, 307.
17. Lotina-Hennsen, B., Roque-Reséndiz, J. L., Jiménez, M. and Aguilar, M. (1991) *Z. Naturforsch* **46c**, 777.
18. Lotina-Hennsen, B., Bernal-Morales, E., Romo de Vivar, A., Pérez-C, A. L., Castro-R, A. and Aguilar, M. (1992) *J. Chem. Ecol.* **18**, 1891.
19. Bernal-Morales, E., Romo de Vivar, A., Sánchez, B., Aguilar, M. and Lotina-Hennsen, B. (1994) *Can. J. Botany* **72**, 177.
20. Izawa, S. (1980) in *Encyclopedia of Plant Physiology* (Trebst, A. and Avron, M., eds), pp. 266–282. Springer, Berlin.
21. Hsu, B. D. and Lee, J. Y. (1988) *Plant Physiol.* **87**, 116.
22. Mohanty, N., Vass, I. and Demeter, S. (1989) *Plant Physiol.* **90**, 175.
23. Samson, G., Morissette, J. C. and Popovic, R. (1988) *Photochem. Photobiol.* **48**, 329.
24. Singh, D. and Singh, S. (1987) *Plant Physiol.* **83**, 12.
25. Yruela, I., Montoya, G., Alonso, P. J. and Picorel, R. (1991) *J. Biol. Chem.* **266**, 22847.
26. Rutherford, A. W., Zimmerman, J. L. and Mathis, P. (1984) *FEBS Letters* **165**, 156.
27. Pereda-Miranda, R., Mata, R., Anaya, A. L., Wickramaratne, M. D. B., Pezzuto, J. M. and Kinghorn, D. (1993) *J. Nat. Prod.* **56**, 571.
28. Peña-Valdivia, C. B., Rodríguez-Flores, L., Tuena de Gómez-Puyou, M. and Lotina-Hennsen, B. (1991) *Biophys. Chem.* **41**, 169.
29. Strain, H. H., Coppe, B. T. and Svec, W. A. (1971) *Methods Enzymol.* **23**, 452.
30. Dilley, R. A. (1972) *Methods Enzymol.* **24**, 68.
31. Giaquinta, R. T. and Dilley, R. A. (1975) *Biochim. Biophys. Acta* **387**, 288.
32. Vernon, L. P. and Shaw, E. R. (1969) *Plant Physiol.* **44**, 1645.