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Phytotoxic compounds from *Flourensia cernua*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Bioassay-directed fractionation of a CH_2Cl_2–MeOH (1:1) extract of the aerial parts of *Flourensia cernua* led to the isolation of three phytotoxic compounds, namely, dehydroflourensic acid (**1**), flourensadiol (**2**) and methyl orsellinate (**3**). Dehydroflourensic acid is a new natural product whose structure was established by spectral means. In addition, the known flavonoid ermanin and seven hitherto unknown γ-lactones were obtained, these being tetracosan-4-olide, pentacosan-4-olide, hexacosan-4-olide, heptacosan-4-olide, octacosan-4-olide, nonacosan-4-olide, and triacontan-4-olide. Compounds **1**–**3** caused significant inhibition of radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crus-galli*, interacted with bovine-brain calmodulin and inhibited the activation of the calmodulin-dependent enzyme cAMP phosphodiesterase.

Keywords: Asteraceae; *Flourensia cernua*; *Amaranthus hypochondriacus*; Dehydroflourensic acid; *Echinochloa crusgalli*; Sesquiterpenoids; Butanolides; Calmodulin; cAMP phosphodiesterase

1. Introduction

Continuing our research on bioactive agents from dry land plants of Mexico as part of the Latin American International Cooperative Biodiversity Group (Timmermann et al., 1999) we have investigated *Flourensia cernua* D.C. (Asteraceae). The genus *Flourensia*, tribe Heliantheae, consists of 32 species of resinous shrubs that grow from the southern United States south to Argentina and Chile. *Flourensia cernua* is a bitter-tasting shrub with a hop-like odor that grows in the deserts from northern Mexico and adjacent United States southward to the central Mexican states of Zacatecas and Hidalgo. This species has the largest geographic range of the nine Mexican taxa in the genus *Flourensia*. In the United States it is called ''tarbush'' while in Mexico it is referred to as ''hojasé'', ''hojasén'' or ''hoja ancha''. Throughout its Mexican range, an infusion of the leaves is drunk frequently to treat various gastrointestinal ailments; thus, a tea is imbibed to alleviate stomach ache, indigestion, diarrhea and dysentery. On the other hand, its uses as a purgative, expectorant and rheumatic remedy are restricted only to a few areas. Regarding the latter use, the leaves of the plant are mixed with those of *Datura stramonium* in hot water to bathe people suffering from rheumatism (Argueta et al., 1994; Bye, 2000; González, 1984; Martínez, 1989). In the United States, the leaves and flower heads are sold as a remedy for indigestion (Tellez et al., 2001). Tarbush is increasing in abundance within the Chihuahuan Desert and is currently the target of several studies involving interaction of herbivory and shrub chemistry at the Jornada Experimental Range (JER), Las Cruces, New Mexico (Tellez et al., 2001).

Previous phytochemical studies on *F. cernua* resulted in the isolation and characterization of several flavonoids (Rao et al., 1970; Dillon et al., 1976; Wollenweber and Dietz, 1981), sesquiterpenoids (Kingston et al., 1971; 1975; Pettersen et al., 1975; Estell et al., 1994; Tellez et al., 1997, 2001), monoterpenoids (Estell et al., 1994; Tellez et al., 1997, 2001), acetylenes, *p*-acetophenones,

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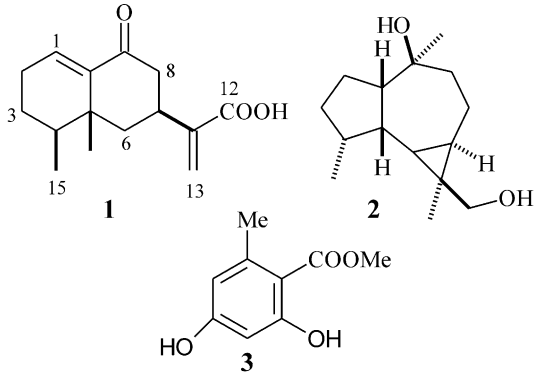
benzopyrans and benzofurans (Bohlmann and Grenz, 1977). Also, it has been also demonstrated that crude extracts and fractions from this species possess phytotoxic, antifungal, antialgal and antitermite activities, and reduce the consumption of alfalfa pellets by sheep (Dayan and Tellez, 1999; Estell et al., 1994; Tellez et al., 2001).

A CH_2Cl_2–MeOH (1:1) extract of *F. cernua* was selected for bioassay-guided fractionation in the present investigation on the basis of its phytogrowth inhibitory activity against *Amaranthus hypochondriacus* (IC_{50} = 300 μg/ml). Herein, we describe the isolation, structure elucidation and biological activity of the major phytotoxins from *F. cernua*, including their effect on the regulatory protein calmodulin (CaM) as a possible target of phytotoxic action.

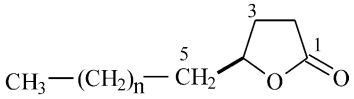
2. Results and discussion

2.1. Isolation and structure elucidation

Extensive chromatography of the crude active extract (IC_{50} = 300 μg/ml against *A. hypochondriacus*) furnished three phytotoxins (**1**–**3**), including dehydroflourensic acid (**1**), a new natural product. In addition, a known flavonoid and seven new lactones (**4**–**10**) were obtained. The structures of the phytotoxins **2** and **3** as well as that of the flavonoid were established as flourensadiol (Kingston et al., 1975), methyl orsellinate (Witiak et al., 1967) and ermanin (Dominguez et al., 1973), respectively, by comparison of their spectral data with values reported in the literature.



Dehydroflourensic acid (**1**) was isolated as a yellowish oil whose molecular formula was established as $C_{15}H_{20}O_3$ from HREIMS, ^{13}C NMR and DEPT analyses. The IR spectrum was consistent with the presence of conjugated carbonyl functionalities including a carboxylic acid (3500–3000, 1693 and 1623 cm^{-1}). The NMR spectra (see Experimental) indicated that **1** was an eremophilane type of sesquiterpene very similar to flourensic acid [9-oxoeremophila-11(13)-en-12-oic acid] (Kingston et al., 1971, 1975) and 9-oxoeremophila-10,11(13)-dien-12-al (Abell and Massy-Westropp, 1985). However, the NMR spectral data (see Experimental) indicated that **1** had one more double bond [δ_H/δ_c 6.60 (H-1, *t*, *J* = 3.9 Hz)/135.8 (C-1), 144.2 (C-10)] than flourensic acid; this extra double bond had to be conjugated with the ketone group at C-9 considering the chemical shift displayed by this carbonyl functionality in the ^{13}C NMR spectrum (δ_c 203.2). The fragment observed in the mass spectrum of **1** at *m*/*z* 260 [M^+–C_3H_6, base peak], resulting from a *retro*-Diels–Alder fragmentation, suggested the position of the ring double bond to be between C-1 and C-10, with the methyl groups at C-4 and C-5 (Abell and Massy-Westropp, 1985). The HMBC correlations C-10/ H-1 and H-15; C-9/H-1 and H-7; C-13/ H-7; C-5/ H-15, H-1 and H-4 provided further support to this proposal. The strong NOESY correlations between H-4 (δ_H 1.68) with H-7 (δ_H 2.88) and H-6_{eq} (δ_H 2.13) indicated that these three protons are in the same face of the molecule as in the case of flourensic acid. These observations as well as the coupling pattern of H-6_{eq} [δ_H 2.13, *ddd*, *J* = 13.8, 4.8, 2.4 Hz] and H-8 [H-8_{eq}: δ_H 2.51 (*ddd*, *J* = 17.4, 4.2, 2.4 Hz); H-8_{ax}: δ_H 2.42 (*dd*, *J* = 17.4, 12.3 Hz)] are consistent with the equatorial orientation of the side chain at C-7. The small coupling of 2.4 Hz is due to a *W* coupling between H-6_{eq} and H-8_{eq}. On the basis of the above data, the relative stereochemistry at the chiral centers of **1** was considered identical to that of flourensic acid. The absolute stereochemistry was tentatively assigned, as depicted, on biogenetic grounds.



Compound	n
4	18
5	19
6	20
7	21
8	22
9	23
10	24

Compounds **4**, **6** and **8** had the molecular formulas of $C_{24}H_{46}O_2$, $C_{26}H_{50}O_2$ and $C_{28}H_{54}O_2$, respectively. They exhibited nearly identical IR, UV, CD and NMR spectra, suggesting their close structural relationship. The IR spectra showed the presence of the γ-lactone ring (~1755 cm^{-1}). In all cases the EIMS showed a series of fragments indicative of a γ-lactone of 24-, 26- and 28-carbon hydroxy acids, respectively (Hussain and Waterman, 1982). The NMR spectra were in agreement with this proposal, and for the three compounds the spectra exhibited signals attributable to the γ-lactone moiety [δ_H 1.85 (*ddd*, *J* = 13.0, 9.5, 8.0 Hz, H-3), 2.32 (*dddd*, *J* = 13.0, 8.0, 6.6 Hz, H-3), 2.53 (*m*, H-2), and 4.48 (*dddd*, *J* = 8.0, 7.0, 6.0, 6.6 Hz, H-4); δ_C 28.0 (C-3),

28.8 (C-2), 81.0 (C-4), 177.2 (C-1)] and an unbranched aliphatic side chain [δ_H 0.88 (*t*, *J* = 6.5 Hz, terminal-CH_3), 1.20–1.40 (*m*, –CH_2), 1.59 (*m*, H-5), 1.74 (*m*, H-5′); δ_C 14.1 (terminal-CH_3), 22.6, 25.2, 29.6, (–CH_2), 35.5 (C-5)] (Tumlinson et al., 1977; Hussain and Waterman, 1982; Solladié and Matloubi-Moghadam, 1982; Gräffe et al., 1982). Since the sign of the Cotton effect is known to reflect the chirality of the substituted lactone ring, the CD spectra of the three compounds were measured. The spectra of **4**, **6** and **8** displayed a positive Cotton effect at 214 nm (due to a π–π^* transition of the carbonyl group) compatible with an *R* absolute stereochemistry at C-4 (Beecham, 1968).

A mixture of lactones **4–10** was also isolated as a white powder and its NMR spectra were almost identical to those of the pure compounds **4**, **6** and **8**. The GC–MS analysis of the mixture (see Experimental and Fig. 1) indicated the presence of seven compounds, including **4**, **6** and **8**. The mass spectra of the individual components were very similar exhibiting identical fragmentation patterns. As expected in all cases the base peak arose from the loss of water from the molecular ion [M^+-18]. Accordingly, the components of the mixture were tentatively identified as tetracosan-4-olide (**4**), pentacosan-4-olide (**5**), hexacosan-4-olide (**6**), heptacosan-4-olide (**7**), octacosan-4-olide (**8**), nonacosan-4-olide (**9**), and triacontan-4-olide (**10**). In the case of compounds **4**, **6** and **8**, the major components of the mixture (25.23, 30.27 and 32.57%, respectively), the identification was confirmed by comparison of the GC mobilities and by co-injection with **4**, **6** and **8** isolated in pure form by HPLC during the course of this study. The stereochemistry of lactones, **5**, **7**, **9** and **10** was assumed on biogenetic grounds to be identical to that of **4**, **6** and **8**. Lactones **5**, **7**, **9** and **10** could not be isolated in enough amounts to pursue detailed NMR, IR and CD analyses.

2.2. Biological testing

Compounds **1–3** showed phytotoxic effects when tested against seedlings of *A. hypochondriacus* and *E. crus-galli* using a petri dish bioassay. Compounds **1** [$IC_{50} = 1.96\times10^{-4}$ M (*A. hypochondriacus*); $IC_{50} = 6.2\times10^{-4}$ M (*E. crus-galli*) and **3** [$IC_{50} = 9.2\times10^{-4}$ M (*A. hypochondriacus*); $IC_{50} = 3.1\times10^{-4}$ M (*E. crus-galli*)] inhibited radicle growth of both target species. However, compound **2** [$IC_{50} = 4.12\times10^{-4}$ M (*A. hypochondriacus*); $IC_{50}4.2\times10^{-3}$ M (*E. crus-galli*)], inhibited only seedling growth of *Amaranthus* with a similar potency to 2,2-dichlorophenoxyacetic acid [2,4-D; $IC_{50} = 1.8\times10^{-4}$ M (*A. hypochondriacus*); $IC_{50}2.3\times10^{-4}$ M (*E. crus-galli*), which was used as a positive control. The phytotoxic properties of compound **3** were previously demonstrated by Rojas et al. (2000) but the present work represents the first description of **1** and **2** as phytotoxins. The pure lactones **4**, **6** and **8** as well as the mixture **4–10** were not phytotoxic against the two seedlings.

Next the effect of the phytotoxins on calmodulin (CaM) was investigated. CaM is a major Ca^{2+}-binding protein, highly active, implicated in a variety of cell functions through the activation of CaM-dependent enzymes, such as phosphodiesterase, protein phosphatase, and nitric oxide (NO) synthase. CaM influences a number of important plant growth events. Accordingly, agents that inhibit the activity of CaM should have profound effects on the development of plants (Snedden and Fromm, 1998; Matthew, 2000). Indeed, certain fungal phytotoxins inhibit CaM (Au et al., 2000; Macías et al., 2000, 2001). According to an SDS–PAGE electrophoresis experiment (Fig. 2), it was found that compounds **1–3** interacted with bovine brain-CaM since CaM treated with the isolates had lower electrophoretic mobility than untreated CaM. The effect was comparable to that of chlorpromazine, a well-known CaM inhibitor (Hook and Means, 2001). In addition, the activation of the CaM-sensitive cAMP phosphodiesterase was inhibited in the presence of **1–3** and CaM (Fig. 3) with IC_{50} values of 23.2, 5.2 and 8.1 μM, respectively. Compounds **2** and **3** were more active than chlorpromazine (positive control, $IC_{50} = 10.2$ μM). Finally, lactones **4**, **6** and **8** were not CaM interactors.

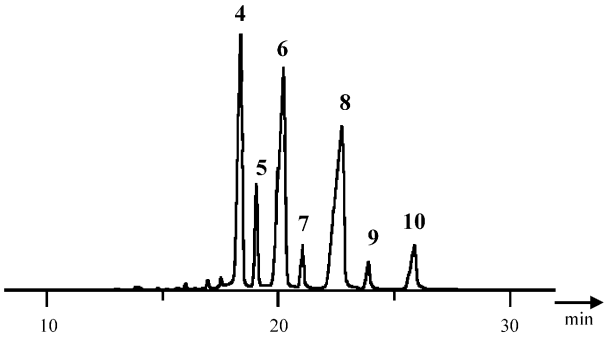


Fig. 1. Gas chromatogram (see experimental for conditions) of the γ-lactones **4–10** isolated from *Fluorensia cernua* and peak assignment.

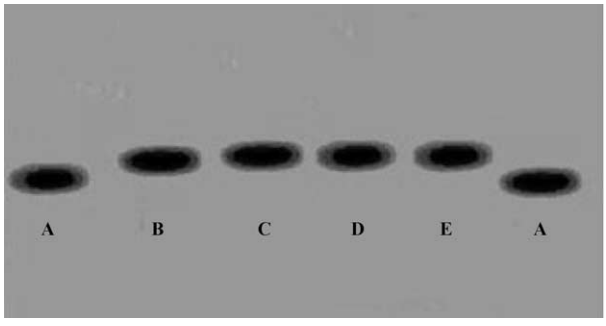


Fig. 2. SDS–PAGE of bovine-brain CaM after treatment with compounds **1–3**. Electrophoresis of 2 μg samples of bovine-brain CaM in the presence of 1 mM $CaCl_2$. Pretreatment of the CaM samples: 1.5 h at 30 °C in the presence of $CaCl_2$ (A); chlorpromazine in DMSO (B); **1** (C); **2** (D); **3** (E). In all cases, 0.33 μg of **1–3** in DMSO were applied.

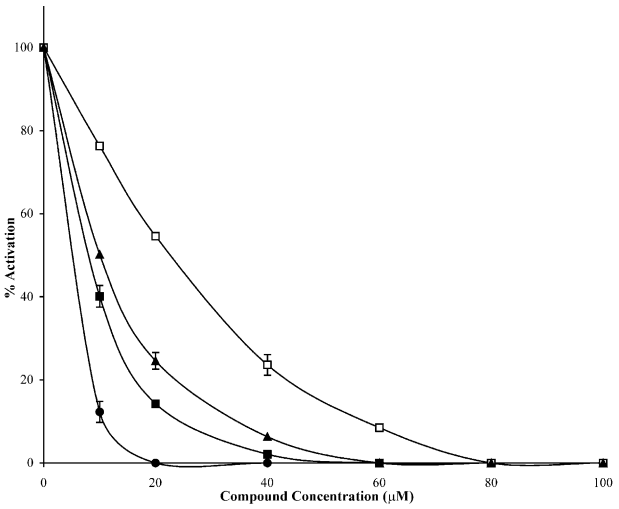


Fig. 3. Effect of compounds **1** (□), **2** (●), **3** (■), and chlorpromazine (▲) on CaM-dependent cAMP. Enzyme activity was measured as a function of compound concentration at saturating concentrations of bovine-brain CaM (0.2 μg). The values are expressed as a percentage of maximum activity obtained with each compound. Each data point represents the mean of analysis of three independent biological samples. Vertical bars represent maximum standard deviations.

3. Concluding remarks

Flourensia cernua accumulates phytotoxic compounds that interact with the regulatory protein CaM in vitro. Compounds **1–3** were isolated as the major phytotoxins of the crude extract of *F. cernua* analyzed in this study and could be involved in the allelopathic interactions of the plant in the Chihuahuan Desert. These and related compounds might also account for the phytotoxic and antialgal activities of some organic extracts of this species reported by other authors (Dayan and Tellez, 1999; Tellez et al., 2001). Since compounds **1–3** are CaM inhibitors they might also have physiological effects of medicinal interest.

4. Experimental

4.1. General experimental procedures

Melting point determinations were carried out on a Fisher-Johns apparatus and are uncorrected. Optical rotations were recorded on a JASCO DIP 360 digital polarimeter. CD spectra were performed on a JASCO 720 spectropolarimeter at 25 °C in MeOH or $CHCl_3$ solution. IR spectra were obtained using KBr disks on a Perkin-Elmer 599B spectrophotometer. UV spectra were recorded on a Shimadzu 160 UV spectrometer in $CHCl_3$ solution. NMR spectra including COSY, NOESY, HMBC and HMQC experiments were recorded in $CDCl_3$ on a Varian Unity Plus 500 spectrometer or on a Bruker DMX500 spectrometer at 500 MHz (^{1}H) or 125 MHz (^{13}C) NMR, using tetramethylsilane (TMS) as an internal standard. MS were obtained on a Jeol JMS-AX505HA mass spectrometer. GC–MS [EI (ionization energy, 70 eV)] analyses were conducted on a JMX-AX-505H system. The GC column was HP 5% phenylmethylsilicone (Alltech) (30 m×0.32 mm i.d.). The linear temperature program was from 150 to 300 °C, at the rate of 10 °C/min, and the carrier gas was He (1 ml/min). Column chromatography: silica gel 60 (70–230 mesh, Merck). TLC (analytical and preparative) was performed on precoated silica gel 60 F_{254} plates (Merck). HPLC was carried out with a Waters HPLC instrument equipped with Waters 996 UV photodiode array detector (900) set at 209–214 nm, using a μPorasil column (19 mm i.d.×300 mm) at a flow rate of 8 ml/min. Control of the equipment, data acquisition, processing, and management of chromatographic information were performed by the Millennium 2000 software program (Waters).

4.2. Plant material

The aerial parts of *Flourensia cernua* D.C. were collected in San Luis Potosi, Mexico, on 28 October 1995 by Bye and Linares. A voucher specimen (Robert Bye and Edelmira Linares 20468) is deposited in the ethnobotanical collection of the Mexican National Herbarium (MEXU), Instituto de Biologia, UNAM.

4.3. Phytogrowth-inhibitory bioassays

The phytogrowth-inhibitory activity of the crude extract, fractions and pure compounds was evaluated on seeds of *Amaranthus hypochondriacus* and *Echinochloa crus-galli* using a Petri dish bioassay (Macías et al., 2000). In addition, a bioautographic phytogrowth inhibitory bioassay (Macías et al., 2000) was employed to guide secondary fractionation. The seeds of *E. crus-galli* were purchased from Valley Seed Service, Fresno, CA, and those of *A. hypochondriacus* from Mercado de Tulyehualco, D.F., Mexico. The results were analyzed by ANOVA ($P<0.05$) and IC_{50} values were calculated by probit analysis based on percent of radicle growth or germination inhibition. Samples were evaluated at 10, 100 and 1000 μg ml^{-1}. 2,4-D was used as the positive control. The bioassays were performed at 28 °C.

4.4. Evaluation of the interaction of compounds 1–3 with bovine brain calmodulin

The interaction of **1–3** with bovine-brain CaM (Sigma) was performed using a denaturing homogeneous electrophoresis (SDS–PAGE) procedure. SDS–PAGE was carried out according to a previously described procedure (Macías et al., 2002, 2001) using a 15% polyacrylamide gel. The interaction of the compounds with CaM was

evaluated by observing the difference in electrophoretic mobility in the presence of Ca^{2+}. Each electrophoretic run was performed in triplicate, and chlorpromazine was used as positive control. The experimental conditions are described briefly in the legend of Fig. 2.

4.5. Cyclic nucleotide phosphodiesterase assay

A cyclic nucleotide phosphodiesterase and bovine-brain calmodulin assay was performed by a modification of the method described by Sharma and Wang (1979). An aliquot (0.2 μg) of bovine brain CaM as enzyme activator was incubated with CaM-dependent cAMP (0.015 units) from bovine brain (Sigma) for 3 min in assay solution (800 μl) containing 0.3 units of 5′-nucleotidase, 45 mM Tris–HCl, 5.6 mM magnesium acetate, 45 mM imidazole, and 2.5 mM calcium chloride, at pH 7.0. Compounds were then added to the assay medium at 10, 20, 40, 60, 80, and 100 μM in DMSO, and the samples were incubated for 30 min. Then, 100 μl of 10.8 mM cAMP, pH 7.0, were added to start the assay. After 30 min, the assay was stopped by addition of a 55% trichloroacetic acid solution (100 μL). All of the above steps were carried out at 30 °C. The phosphodiesterase reaction was coupled to the 5′-nucleotidase (*Crotalus atrox* venom; Sigma) reaction, and the amount of inorganic phosphate released represented the activity of the phosphodiesterase. The phosphate produced in the assay was measured by the method of Sumner (1944). The wavelength used for the phosphate assay was 660 nm. Chlorpromazine was used as a positive control.

4.6. Extraction and preliminary fractionation

The air-dried aerial parts (2.4 kg) were ground into a powder and extracted by maceration with CH_2Cl_2–MeOH (1:1) at room temperature during one week (three times). After filtration, the extract was evaporated under reduced pressure to yield 600 g of a dark-green residue, which was subjected to CC over silica gel (3 kg) and eluted with a gradient of hexane–EtOAc (10:0→0:10) and EtOAc–MeOH (10:0→5:5). Fractions of 1 l each were collected and pooled based on TLC profiles to yield ten major fractions (F_1–F_{10}). Fractions F_4, F_6 and F_7 concentrated the phytotoxic activity according to the PDPIB and BPIB bioassays.

4.7. Isolation of 3–10

From active fraction F_4 [IC_{50}: 143.82 μg/ml (*A. hypochondriacus*); IC_{50} = 162.25 μg/ml (*E. crus-galli*)], eluted with hexane–EtOAc 9:1, precipitated a white solid which was purified by CC on silica gel eluting with CH_2Cl_2 to yield 48.2 mg of the mixture of lactones **4**–**10**. The mixture (40 mg) was subjected to prep HPLC (hexane–CH_2Cl_2–MeOH, 95:3:2) to yield **4** (10 mg), R_t = 9.1 min, **6** (12 mg), R_t = 16 min and **8** (11 mg), R_t = 17 min. The mother liquors from fraction F_4 (18.9 g) were subjected to further silica gel column chromatography (215 g) eluting with hexane–EtOAc (10:0→0:10) to afford six secondary fractions ($F_{4\text{-I}}$–$F_{4\text{-VI}}$); of these only fraction $F_{4\text{-II}}$, eluted with hexane–EtOAc (8:2), showed phytotoxic activity in the BPIB assay. Prep TLC of this fraction (CH_2Cl_2–MeOH, 98:2) led to the isolation of **3** (14.4 mg), mp 136–138 °C.

4.8. Isolation of 1 and 2

Active primary fraction F_6 [16.6 g; IC_{50}: 316.22 μg/ml (*A. hypochondriacus*); IC_{50} = 152.98 μg/ml (*E. crus-galli*)], eluted with hexane–EtOAc (7:3), was subjected to CC on silica gel (270 g) eluting with a gradient of hexane–EtOAc (10:0→0:10). This process furnished eight secondary ($F_{6\text{-I}}$–$F_{6\text{-VIII}}$) fractions, of which $F_{6\text{-IV}}$–$F_{6\text{-VI}}$ showed phytotoxic activity. These fractions (4.3 g) were pooled on the basis of their TLC and activity profiles and further applied to silica gel column chromatography (60 g) to yield three tertiary fractions ($F_{6\text{-IV-VI-A-C}}$). Active fraction $F_{6\text{-IV-VI-B}}$ (2.66 g) was further resolved by prep TLC (CH_2Cl_2–MeOH, 97:3) to afford compound **1** (190 mg). From active fraction F_7 [IC_{50}: 135.39 μg/ml (*A. hypochondriacus*); IC_{50} = 358.31 μg/ml (*E. crus-galli*)], crystallized 533 mg of ermanin. The mother liquor from fraction F_7 (76.84 g) were also subjected to a silica gel column (533 g) using mixtures of hexane–EtOAc (10:0→0:10) as eluents. This process afforded eight secondary fractions ($F_{7\text{-I}}$–$F_{7\text{-VIII}}$). The phytotoxic activity was concentrated in fractions $F_{7\text{-III, IV}}$ and $F_{7\text{-VI}}$. Fractions $F_{7\text{-III}}$ and $F_{7\text{-IV}}$ were pooled on the basis of their TLC profiles and were further purified by TLC (CH_2Cl_2–MeOH 97:3) to yield **1** (64.1 mg). Secondary active fraction $F_{7\text{-VI}}$ (4.0 g) was reapplied on a silica gel column (60 g) eluting with a gradient of CH_2Cl_2–MeOH (10:0→9.8:0.2) to yield six tertiary fractions ($F_{7\text{-VI-A-F}}$) of which fraction $F_{7\text{-VI-D}}$ concentrated the phytotoxic activity. This fraction was performed by prep RP-TLC (MeOH-H_2O 9:1) to afford **2** (9.5 mg).

4.9. γ-Lactone 4

White powder; mp 68 °C [hexane–EtOAc (9:1)]; IR (KBr) ν_{max} cm^{-1}: 2917, 2849, 1755, 1472, 1428, 1355, 1287, 1231; α_D −38° (hexane; *c*, 1); UV (MeOH) λ_{max} nm (log ε) 214 (3.11); CD (*c* 0.2, MeOH) (Δ_ε) 255 (−0.1), 241 (0), 214 (+6.6); ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.88 (3H, *t*, *J* = 6.5 Hz, H-24), 1.20–1.40 (38 H, *m*, H-6-H-23), 1.59 (1H, *m*, H-5), 1.74 (1H, *m*, H-5′), 1.85 (1H, *ddd*, *J* = 13.0, 9.5, 8.0 Hz, H-3), 2.32 (1H, *dddd*, *J* = 13.0, 8.0, 6.6 Hz, H-3′), 2.53 (2H, *m*, H-2), 4.48 (1H, *dddd*, *J* = 8.0, 7.0, 6.0, 6.6 Hz, H-4); ^{13}C NMR (125

MHz, $CDCl_3$) δ: 14.1 (C-24), 22.6 (C-23), 25.2 (C-6-C-22), 28.0 (C-3), 28.8 (C-2), 29.6 (C-6-C-22), 35.5 (C-5), 81.0 (C-4), 177.2 (C-1); EIMS *m/z*: 366 [M^+ (37)], 348 (100), 330 (57), 304 (77), 264 (28), 250 (17), 179 (15), 165 (23), 151 (31), 139 (46), 125 (68), 111 (77), 97 (80), 85 (97), 57 (89), 43 (98), 41(57); HREIMS 366.3546 (calc. for $C_{24}H_{46}O_2$, 366.3498).

4.10. γ-Lactone **6**

White powder; mp 69 °C [hexane–EtOAc (9:1)]; IR (KBr) ν_{max} cm^{-1}: 2917, 2849, 1756, 1472, 1425, 1357, 1287, 1230; α_D −38° (hexane; *c,* 1); UV (MeOH) λ_{max} nm (log ε) 214 (3.11); CD (*c* 0.2, MeOH) (Δ_ε) 255 (−0.1), 241 (0), 214 (+6.6); ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.87 (3H, *t*, *J* = 6.5 Hz, H-26), 1.20–1.40 (42 H, *m*, H-6-H-25), 1.60 (1H, *m*, H-5), 1.73 (1H, *m*, H-5′), 1.84 (1H, *ddd*, *J* = 13.0, 9.5, 8.0 Hz, H-3), 2.33 (1H, *dddd*, *J* = 13.0, 8.0, 6.6 Hz, H-3′), 2.55 (2H, *m*, H-2), 4.49 (1H, *dddd*, *J* = 8.0, 7.0, 6.0, 6.6 Hz, H-4); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.2 (C-26), 22.7 (C-25), 25.3 (C-6-C-24), 28.0 (C-3), 28.9 (C-2), 29.6 (C-6-C-24), 35.4 (C-5), 81.0 (C-4), 177.3 (C-1); EIMS *m/z*: 394 [M^+ (35)], 376 (100), 358 (51), 332 (80), 250 (14), 151 (25), 139 (38), 125 (65), 111 (72), 97 (78), 85 (98), 57 (89), 43 (95), 41 (46); HREIMS 394.3823 (calc. for $C_{26}H_{50}O_2$, 394.3811).

4.11. γ-Lactone **8**

White powder; mp 70 °C [hexane–EtOAc (9:1)]; IR (KBr) ν_{max} cm^{-1}: 2918, 2850, 1757, 1473, 1429, 1351, 1282, 1235; α_D −38° (Hexane; *c,* 1); UV (MeOH) λ_{max} nm (log ε) 214 (3.11); CD (*c* 0.2, MeOH) (Δ_ε) 255 (−0.1), 241 (0), 214 (+6.6); ^1H NMR ($CDCl_3$, 500 MHz) δ: 0.86 (3H, *t*, *J* = 6.5 Hz, H-28), 1.20–1.40 (46 H, *m*, H-6-H-27), 1.57 (1H, *m*, H-5), 1.71 (1H, *m*, H-5′), 1.83 (1H, *ddd*, *J* = 13.0, 9.5, 8.0 Hz, H-3), 2.31 (1H, *dddd*, *J* = 13.0, 8.0, 6.6 Hz, H-3′), 2.51 (2H, *m*, H-2), 4.46 (1H, *dddd*, *J* = 8.0, 7.0, 6.0, 6.6 Hz, H-4); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.1 (C-28), 22.6 (C-27), 25.2 (C-6-C-26), 28.0 (C-3), 28.8 (C-2), 29.6 (C-6-C-26), 35.5 (C-5), 81.0 (C-4), 177.2 (C-1); EIMS *m/z*: 422 [M^+ (25)], 404 (100), 386 (34), 360 (51), 264 (8), 250 (8), 179 (8), 153 (15), 139 (25), 125 (46), 111 (66), 97 (72), 85 (95), 57 (83), 43 (67), 41 (31); HREIMS 422.4141 (calc. for $C_{28}H_{54}O_2$, 422.4124).

4.12. GC–MS analysis of mixture **4–10**

GC–MS of the lactone mixture **4–10** was performed using the conditions indicated in General Experimental Procedures. The retention times (min)/percentages (%, uncorrected)/EIMS *m/z* (rel. int.) of the lactones were: tetracosan-4-olide (**4**) 18.3/25.23/ 366 (M^+, 40), 348 (M-18, 100), 330 (57), 304 (85), 85 (97), 57 (88); pentacosan-4-olide (**5**) 18.96/6.88/ 380 (M^+, 16), 362 (M-18, 100), 344 (21), 318 (16), 85 (82), 57 (78); hexacosan-4-olide (**6**) 20.15/30.27/394 (M^+, 35), 376 (M-18, 100), 358 (48), 332 (80), 85 (98), 57 (86); heptacosan-4-olide (**7**) 20.94/0.46/ 408 (M^+, 10), 390 (M-18, 100), 372 (17), 346 (26), 85 (81), 57 (67); octacosan-4-olide (**8**) 22.7/32.57/ 422 (M^+, 28), 404 (M-18, 100), 386 (33), 360 (50), 85 (95), 57 (4), nonacosan-4-olide (**9**) 23.83/0.46/ 436 (M^+, 17), 418 (M-18, 90), 400 (19), 324 (27), 85 (100), 57 (70); triacontan-4-olide (**10**) 25.81/4.13/ 450 (M^+, 15), 432 (M-18, 91), 414 (16), 388 (32), 85 (100), 57 (81).

4.13. Dihydroflourensic acid (**1**)

Oil; IR (KBr) ν_{max} cm^{-1}: 3300–3000, 2960, 2929, 1693, 1623, 1421, 1088, 948; $[\alpha]_D$ −52° (CH_2Cl_2; *c,* 1); UV (MeOH) λ_{max} nm: 203, 237; ^1H NMR ($CDCl_3$, 300 MHz) δ: 6.60 (*t*, *J* = 3.9 Hz, H-1), 6.39 (*brs*, H-13′), 5.67 (*brs*, H-13), 2.88 (*m*, H-7), 2.51 (*ddd*, *J* = 17.4, 4.2, 2.4 Hz, H-8_{eq}), 2.42 (*dd*, *J* = 17.4, 12.3 Hz, H-8_{ax}), 2.23 (*m*, H-2), 2.13 (*ddd*, *J* = 13.8, 4.8, 2.4 Hz, H-6_{eq}), 1.50 (*m*, H-6_{ax}), 1.68 (*m*, H-4), 1.5 (*m*, H-3), 0.97, (*s*, H-15), 0.94 (*d*, *J* = 6.6 Hz, H-14); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 203.2 (C-9), 171.3 (C-12), 144.2 (C-10), 142.7 (C-11), 135.8 (C-1), 126.2 (C-13), 43.3 (C-8), 42.0 (C-6), 38.2 (C-4), 36.1 (C-5), 33.4 (C-7), 26.4 (C-3), 25.5 (C-2), 25.0 (C-15), 16.0 (C-14); EIMS *m/z* (rel. int.): 248 (M^+, 91), 233 (M^+-15, 48), 230 (M^+-H_2O, 15), 206 (M^+-42, 100), 191 (87), 160 (45), 145 (30), 135 (27), 107 (29), 91 (41), 79 (33), 65 (14), 53 (14), 41 (14); HREIMS 248.1429 (calc. for $C_{15}H_{20}O_3$, 248.1413).

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