

## Phytotoxic Potential of *Onopordum acanthium* L. (Asteraceae)

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*Onopordum acanthium* L. (Asteraceae) is a plant native to southern Europe and southwestern Asia, but it is invasive in disturbed areas and agricultural fields around the world, causing many agronomic problems by interfering with crops or preventing animals from grazing on pastures. Allelopathy could be one of the reasons that this plant has spread over different continents. The aim of the present study was to bioprospect *O. acanthium* leaf extracts through the isolation and purification of allelopathic secondary metabolites with phytotoxicity to explain their invasive behavior. Phytotoxic activity was tested using etiolated wheat coleoptiles. The most active extract was selected to perform a bioassay-guided isolation of two flavonoids, pectolarigenin (**1**) and scutellarein 4'-methyl ether (**2**), and two sesquiterpene lactones, elemanolide 11(13)-dehydromelitensin  $\beta$ -hydroxyisobutyrate (**3**) and acanthiolide (**4**). All compounds were isolated for the first time from *O. acanthium*, and acanthiolide (**4**) is described for the first time. Compound **3** strongly inhibited the growth of wheat coleoptiles and **1** showed an intermediate effect. The results indicate that these compounds could contribute to the invasion of *O. acanthium* in ecological systems and agricultural fields.

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**Introduction.** – Invasive alien species are causing dramatic changes in worldwide ecological systems and agricultural fields, as they profoundly alter communities and ecosystems [1][2]. Invasive alien plants spread and persist in environments that are different from their natural habitat [3]. They are more competitive, they reproduce more rapidly and with higher quantities of seeds, survive under more adverse conditions [4], and have more leaf nitrogen than native species [5]. Furthermore, such plants can contain compounds or combinations of compounds that are new to the invaded herbivore and plant communities [6–8].

Allelopathy is a phenomenon that occurs between donor and target species, where plants, algae, bacteria, or fungus can liberate compounds (allelochemicals) to the environment that influence the growth and development of biological systems [9–11]. Allelopathy is recognized as an important ecological mechanism that influences the dominance and succession of plants, and the formation of communities, climax vegetation, agriculture management, and productivity [9][12–14].

Scotch thistle (*Onopordum acanthium* L.) is native to southern Europe and southwestern Asia, but it is an invasive alien species in disturbed areas and agricultural fields around the world, causing many agronomic problems by interfering with crops or

preventing animals from grazing on pastures [15][16]. It is a carduine thistle Asteraceae that is strongly competitive against other forage species in areas of USA [17], Canada [15], Australia [18], and Argentina [19]. The plant is commonly a monocarpic biennial, but, under certain conditions, it can be an annual or a short-lived perennial, reproducing almost entirely by cypselas (fruits) that it can produce up to 50,000 [20]. The cypselas are dormant, and they can persist in the soil before emerging after prolonged periods [21]. Leakage of compounds from the cypselas can inhibit the germination of the cypselas from which it was derived (autoinhibition) [22].

Flower extracts from this plant have been traditionally used as a medicine to treat cardiovascular diseases, urogenital diseases, as a diuretic, and to promote gastric secretion [23]. Stem and leaf extracts showed cytotoxic activities [24]. The sesquiterpene lactone onopordopicrin has been isolated from *O. acanthium* leaves [25], and it was very active on *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense* [26]. The phenylpropanoid glycoside 2-[3'-methoxy-4'-*O*-( $\beta$ -D-galactopyranos-1-yl)-benzyl]-3-(3'',4''-dimethoxybenzyl)-4-hydroxybutyric acid was isolated from the seeds of the plant [27]. Taraxasteryl acetate was isolated from the flowers, and taraxasterol from leaves and stems of the plant [28].

Our hypothesis is that allelochemicals could play an important role in the establishment of *O. acanthium* in invaded environments due to the complex bioactive chemical composition that has been found in previous studies. The aim of the present study was to bioprospect *O. acanthium* leaf extracts through the isolation and purification of secondary metabolites with phytotoxic activities to explain the invasive behavior of this plant. As part of this study, we selected the most phytotoxic extract from the leaves of *O. acanthium* by using etiolated wheat coleoptiles. A bioassay-guided fractionation of this extract was carried out in order to isolate and identify the chemical constituents. The structures of these compounds were characterized by  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectroscopy. The bioactivity profiles of the isolated compounds were also studied.

**Results and Discussion.** – *Bioguided Isolation.* Dried leaves of *O. acanthium*, pre-defatted with hexane, were extracted with  $\text{CH}_2\text{Cl}_2$ , AcOEt, acetone, MeOH, and distilled  $\text{H}_2\text{O}$ . The extracts were subjected to an etiolated wheat coleoptile bioassay [29].

The wheat coleoptile bioassay is fast (24 h), sensitive to a wide range of bioactive substances [30–32], and can be considered as a first approach to identify phytotoxicity where undifferentiated tissue cells are used [33–35]. This bioassay has been proposed by Macías *et al.* as the first step in the search for potential new herbicides [29].

Three dilutions, 0.8, 0.4, and 0.2 mg ml $^{-1}$ , were used in this assay and these were prepared from dried extracts.

The results obtained in the bioassay are shown in *Fig. 1*. The extracts that showed the highest activity levels on the coleoptiles were those extracted with  $\text{CH}_2\text{Cl}_2$ , AcOEt, and acetone, which showed the highest inhibition values at 0.8 mg ml $^{-1}$  with values of –90, –89, and –82%, respectively. The activities of these extracts at 0.4 and 0.2 mg ml $^{-1}$  were:  $\text{CH}_2\text{Cl}_2$  (–78 and –50%, resp.), AcOEt (–55 and –38%, resp.), and acetone (–52 and –24%, resp.). The MeOH and  $\text{H}_2\text{O}$  extracts at the highest concentrations showed lower inhibitory activities at 0.8 mg ml $^{-1}$  (–56 and –41%, resp.).

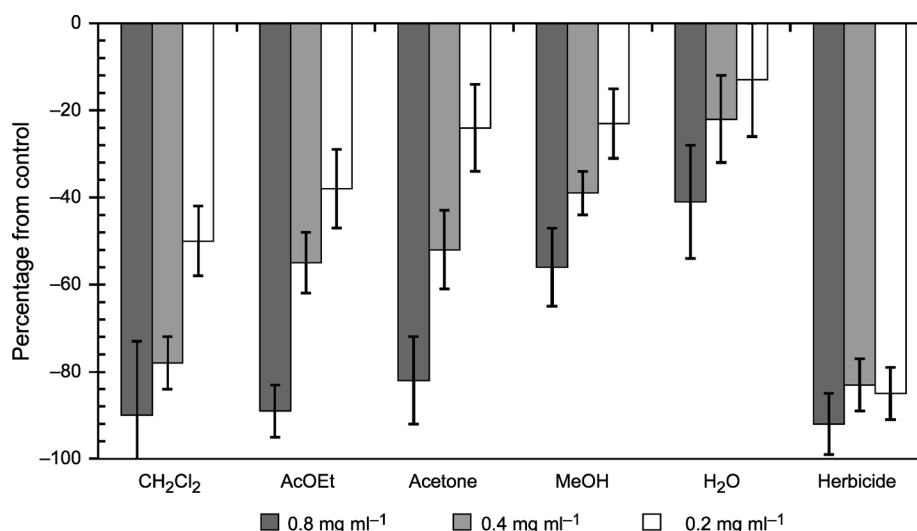


Fig. 1. Effects of  $\text{CH}_2\text{Cl}_2$ , AcOEt, acetone, MeOH, and  $\text{H}_2\text{O}$  leaf extracts of *Onopordum acanthium*, and the herbicide Logran<sup>®</sup> on the elongation of etiolated wheat coleoptiles. Values are expressed as percentage difference from control.

To compare the activities of the extracts,  $IC_{50}$  values were calculated using a sigmoidal dose–response model. The results allowed the extracts to be arranged in decreasing order of activity as follows:  $\text{CH}_2\text{Cl}_2$  ( $IC_{50}=0.17 \text{ mg ml}^{-1}$ ,  $R^2=0.9868$ ) > AcOEt ( $IC_{50}=0.30 \text{ mg ml}^{-1}$ ,  $R^2=0.9697$ ) > acetone ( $IC_{50}=0.40 \text{ mg ml}^{-1}$ ,  $R^2=0.9635$ ) > MeOH ( $IC_{50}=0.72 \text{ mg ml}^{-1}$ ,  $R^2=0.9999$ ) >  $\text{H}_2\text{O}$  ( $IC_{50}=1.50 \text{ mg ml}^{-1}$ ,  $R^2=0.9918$ ). The  $\text{CH}_2\text{Cl}_2$ , AcOEt, and acetone extracts were the most active. The MeOH and  $\text{H}_2\text{O}$  extracts were not studied further due to their low activity levels. The differences in profiles between extracts suggest that the most active metabolites are those with lower polarity.

The most active extract ( $\text{CH}_2\text{Cl}_2$ ) was chromatographed on silica gel using hexane/AcOEt mixtures of increasing polarity. All fractions were bioassayed with etiolated wheat coleoptiles (Fig. 2). Three dilutions, 0.8, 0.4, and  $0.2 \text{ mg ml}^{-1}$ , were used in this assay, and these were prepared from dried fractions.

The results showed that fractions *Frs. 5–7* inhibit coleoptile elongation by more than  $-80\%$  at  $0.8 \text{ mg ml}^{-1}$ . In particular, *Fr. 5* presented values higher than  $-90\%$  at this concentration. As the  $IC_{50}$  values for *Frs. 5* ( $IC_{50}=0.06 \text{ mg ml}^{-1}$ ,  $R^2=0.9989$ ), *6* ( $IC_{50}=0.12 \text{ mg ml}^{-1}$ ,  $R^2=0.9924$ ), and *7* ( $IC_{50}=0.27 \text{ mg ml}^{-1}$ ,  $R^2=0.9822$ ) were high, they were selected for fractionation by chromatography.

The chromatographic separation of the bioactive fractions allowed the isolation of one flavonoid, **1**, from *Frs. 5* and *6*, one flavonoid, **2**, from *Fr. 6*, and two sesquiterpene lactones, **3** and **4**, from *Fr. 7* (Fig. 3). The spectroscopic data and physical constants for **1–3** were identical to those previously reported for pectolarigenin (**1**) [36], scutellarein 4'-methyl ether (**2**) [37], and 11(13)-dehydromelitensin  $\beta$ -hydroxyisobutyrate (**3**) [38]. Compound **4** has never been identified before, and it has been named acanthiolide. All compounds were isolated here for the first time from *O. acanthium*.

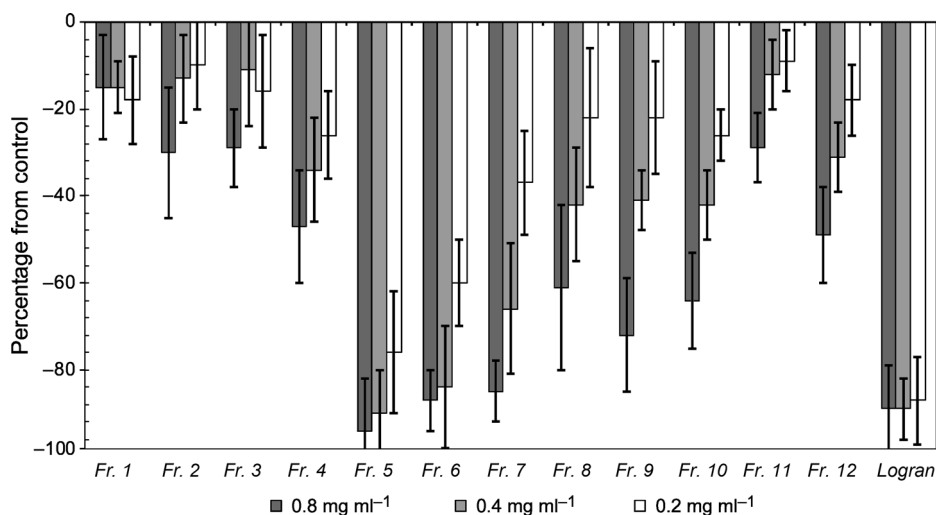


Fig. 2. Effects of fractions Frs. 5–7 of acetone leaf extracts of *Onopordum acanthium* on the elongation of etiolated wheat coleoptiles

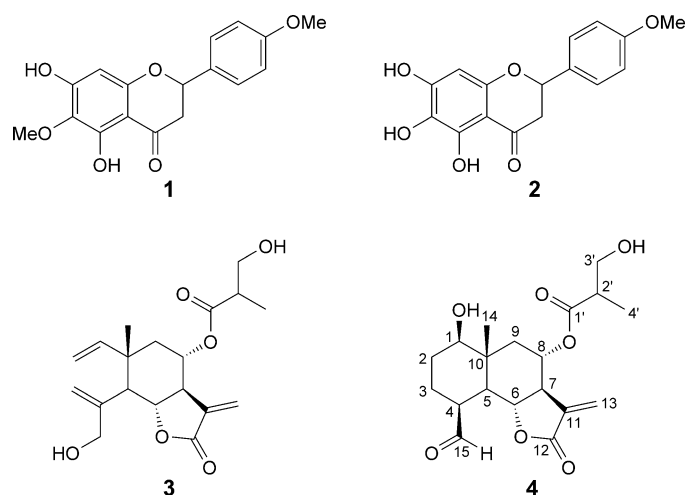


Fig. 3. Structures of compounds **1**–**4** isolated from *Onopordum acanthium*

**Structure Elucidation of 4.** The <sup>1</sup>H-NMR spectrum of **4** suggested a eudesmane backbone, showing a *singlet* at  $\delta(\text{H})$  0.90 (Me(14)) and signals close to those of a C(6)-lactonized eudesmanolide functionalized at C(1) and C(8) (3.38 (*dd*,  $J=4.6, 11.4$ , H–C(1)), 4.49 (*dd*,  $J=11.8, 11.8$ , H–C(6)), and 5.26 (*ddd*,  $J=4.6, 10.5, 10.5$ , H–C(8))) [39]. However, signals characteristic of aliphatic or olefinic H-atoms at C(15) were not observed. The signal due to H–C(15) appeared as a low-field *singlet* at  $\delta(\text{H})$  9.93, indicating the presence of an CHO group. The side chain was readily identified on the basis of characteristic <sup>1</sup>H-NMR signals [40] ( $\delta(\text{H})$  2.71 (*ddq*,  $J=5.0, 8.0, 7.1$ , H–C(2')),

3.77 (*dd*,  $J=8.0, 10.0$ ,  $H_a-C(3')$ ), 3.75 (*dd*,  $J=5.0, 10.0$ ,  $H_b-C(3')$ ), and 1.18 (*d*,  $J=7.2$ ,  $H-C(4')$ ) and EI-MS fragment-ion peak ( $[4\text{-hydroxyisobutyrate acylium}]^+$  at  $m/z$  87) as a 4-hydroxybutyrate. For this compound, the coupling constants for  $H-C(5)$  to  $H-C(8)$  and  $H-C(1)$  are consistent with a *trans* disposition of  $H-C(5)/H-C(6)$ ,  $H-C(6)/H-C(7)$ , and  $H-C(7)/H-C(8)$  and for the  $\alpha$ -orientation of  $H-C(1)$  and  $H-C(4)$ . The  $^1H$ - and  $^{13}C$ -NMR spectra were also in full agreement with those of the compound reported in [41], the structure of which differs only in the ester side chain. Since the absolute configuration of **4** was not established, that represented in Fig. 3 is the relative configuration. However, configuration denoted is that usually presented by the sesquiterpene lactones isolated from Asteraceae [42]. Thus, this new compound was established as  $8\alpha$ -[( $\beta$ -hydroxyisobutanoyl)oxy]-4-episonchucarpolide, named acanthiolide (**4**).

**Bioassays.** The bioactivities of the compounds isolated from *O. acanthium* were evaluated (Fig. 4). The quantities of the compounds obtained enabled us to test concentrations from  $10^{-3}$  to  $10^{-5}$  M for **1**, and from  $3 \cdot 10^{-4}$  to  $10^{-5}$  M for **2** and **3**. The small amount of **4** obtained did not allow its bioactivity to be tested. The results revealed that **1** and **3** had high bioactivities, with inhibitions of  $-44\%$  at  $10^{-3}$  M for **1** and  $-56\%$  at  $3 \cdot 10^{-3}$  M for **3**. Compound **3** was the most active despite its lower concentration. These results allow the metabolites to be arranged in decreasing order of activity: **3** ( $IC_{50}=1.794 \cdot 10^{-4}$  M,  $R^2=0.98$ ) > **1** ( $IC_{50}=1.263 \cdot 10^{-3}$  M,  $R^2=0.99$ ) > **2** ( $IC_{50}=1.709 \cdot 10^{-3}$  M,  $R^2=0.99$ ).

The elemanolide lactone, **3**, had higher bioactivity than the flavonoids **1** and **2**, but **1** was also active. The elemanolide lactones are a family of sesquiterpene lactones that

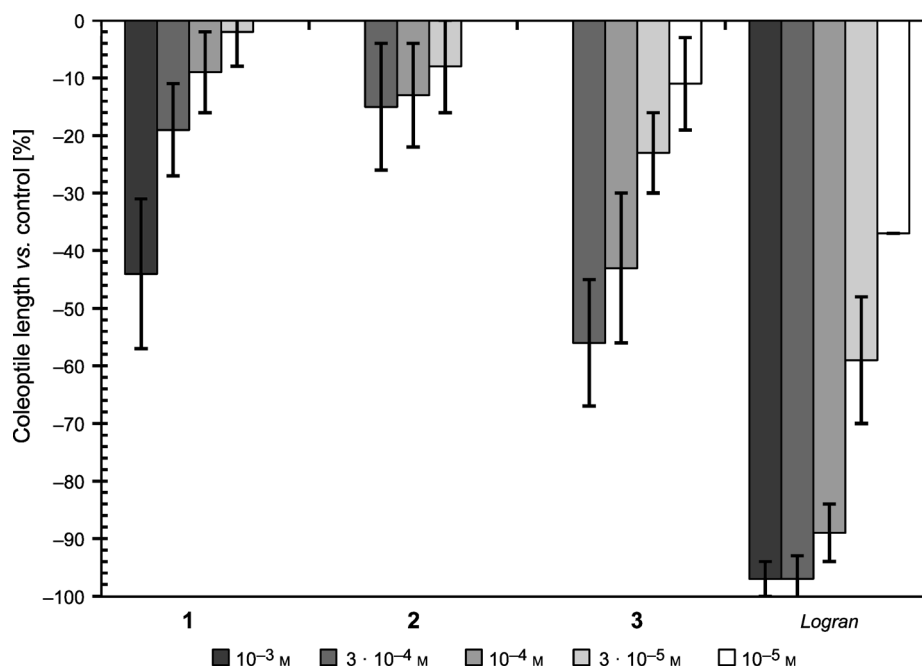


Fig. 4. Effects of **1–3** of *Onopordum acanthium* on the elongation of etiolated wheat coleoptiles

show pharmacological antibiotic and antifungal activities [43] and cytotoxic actions against cell lines [44]. Agricultural or ecological activities have not been reported previously for elemanolide lactones.

Sesquiterpene lactones are very abundant in Asteraceae, the family of which *O. acanthium* is a member [42]. These compounds have already been found in the weeds of *Centaurea tweediei* HOOK. and ARN., *Centaurea diffusa* LAM. [45], *Achillea millefolium* L. [46], and *Cyrtocymura cincta* (GRISEB.) H.ROB. [47]. They exhibit a range of biological properties, including insecticidal, fungicidal, bactericidal, and allelopathic activities [48]. Those that possess  $\alpha,\beta$ -unsaturated lactone moieties, as the isolated compounds, are well-known for their bioreactivity and particularly neurotoxicity [49]. Many sesquiterpene lactones have shown allelopathic activity, e.g., from sunflower (*Helianthus annuus* L.) on standard target species [48], from weed species on *Oryza sativa* L. [50], and from the invasive weed *Mikania micrantha* H. B.K. on target and native species [51].

Flavonoids are related to flower pollination (due to the colors they provide) and to the protection of plants against UV light and diseases, and for signaling. However, there are some reports on phytotoxicity for these compounds, e.g., in kaempferol, quercetin, and naringenin [52][53]. Flavonoids have been found in other Asteraceae weeds such as *Blumea balsamifera* DC [54][55], *Bidens biternata* (LOUR.) MERR. & SHERFF [56], and *Chromolaena odorata* (L.) R. M. KING & H.ROB. [57]. Furthermore, the flavonoids from the weed *Jasonia montana* BOTSCH. proved to be allelopathic on Convolvulaceae weed species [58]. In the cases where flavonoids have shown allelopathic activity, it is normally connected to the regulation of auxin transport and degradation [59], inhibition of NADH oxidase, and the balance of reactive oxygen species [60] and their strong antioxidant potential [61].

The intermediate and strong activities of **1** and **3**, respectively, on wheat coleoptiles, along with the background of allelopathic activity of sesquiterpene lactones and flavonoids in the literature, indicate that these compounds could assist the invasion of *O. acanthium* in new environments. Although the limited amount of **4** precluded a study of its bioactivity, it should be considered along with **1** and **3** for the discovery of natural herbicides. In fact, sesquiterpene lactones have already proven active on the weed development of crenate broomrape (*Orobancha crenata* FORSSK.), field dodder (*Cuscuta campestris* YUNCK.) [62], and wild oat (*Avena fatua* L.) [63]. Furthermore, flavonoids from mango (*Mangifera indica* L.) were active on the weed *Parthenium hysterophorus* L. [64]. These compounds could also be important for the control of weeds that have shown resistance to herbicides.

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### Experimental Part

*General.* Prep. TLC and column chromatography (CC): silica gel (SiO<sub>2</sub>) from Merck (15111 and 5554). Semiprep. TLC: SiO<sub>2</sub> G 1500/LS 254 plates (200 × 200 × 0.25 mm, Schleicher & Schuell, Ref. 391132). HPLC: Merck-Hitachi instrument, with RI detection, Merck LiChrospher column: SI 60 (10 μm, 250 × 10 mm). Optical rotations: PerkinElmer model 241 polarimeter at the Na D-line; at r.t. IR Spectra: PerkinElmer FT-IR Spectrum 1000 or Mattson 5020 spectrophotometer; KBr pellets;  $\tilde{\nu}$  in cm<sup>-1</sup>. <sup>1</sup>H- and

$^{13}\text{C}$ -NMR spectra: *Varian INOVA-600* spectrometer;  $\delta$  in ppm rel. to residual  $^1\text{H}$  signals of  $\text{CDCl}_3$  and  $\text{CD}_3\text{OD}$  ( $\delta(\text{H})$  7.25 and 3.30, resp.);  $^{13}\text{C}$  signals are referenced to the solvent signal ( $\delta(\text{C})$  77.00 and 49.00, resp.),  $J$  in Hz. HR-MS: *VG AUTOESPEC* mass spectrometer (70 eV); in  $m/z$ .

*Plant Material, Extractions, and Isolation of Compounds.* The plant material was dried in an oven (for 72 h at  $40^\circ$ ) and powdered in an industrial mill. Dried material (215 g) was extracted with hexane at r.t. to yield 1.97 g of material in order to defat the material. The plant residue was re-extracted in two different steps.

In the first step,  $\text{CH}_2\text{Cl}_2$ , AcOEt, acetone, MeOH, and  $\text{H}_2\text{O}$  were used to extract defatted material, and these extractions yielded, after removal of the solvent, 1.31 g ( $\text{CH}_2\text{Cl}_2$ ), 2.80 g (AcOEt), 0.26 g (acetone), 0.57 g (MeOH), and 1.26 g ( $\text{H}_2\text{O}$ ), resp. These extracts were bioassayed with etiolated wheat coleoptiles. The  $\text{CH}_2\text{Cl}_2$  extract was the most active.

In the second step, the rest of defatted material (179.57 g) was extracted with  $\text{CH}_2\text{Cl}_2$  to yield 4.15 g of material. This extract was chromatographed on silica gel using hexane/AcOEt mixtures of increasing polarity to afford twelve fractions: *Frs. 1–12*.

All fractions were bioassayed with etiolated wheat coleoptiles. *Frs. 5–7* showed bioactivity in the wheat coleoptile bioassay and were re-chromatographed. *Fr. 5* (hexane/AcOEt 60:40, 0.1723 g) was subjected to CC ( $\text{SiO}_2$ ; hexane/AcOEt of increasing polarity) to afford nine fractions, *Frs. 5.1–5.9*. *Fr. 5.4* (hexane/AcOEt 70:30, 0.0812 g) was subjected to CC ( $\text{SiO}_2$ ;  $\text{CH}_2\text{Cl}_2$ /acetone of increasing polarity) to give six fractions, *Frs. 5.4.1–5.4.6*. *Fr. 5.4.2* (53.2 mg,  $\text{CH}_2\text{Cl}_2$ /acetone 99:1) was purified by reversed-phase HPLC ( $\text{C}_{18}$ ;  $\text{H}_2\text{O}$ /MeOH) to yield **1** (9.5 mg, 20:80).

*Fr. 6* (hexane/AcOEt 50:50, 0.0563 g) was subjected to CC ( $\text{SiO}_2$ ; hexane/acetone of increasing polarity) to furnish six fractions (*Frs. 6.1–6.6*). *Fr. 6.3*, hexane/acetone 80:20, 0.0016 g was purified by reversed-phase (RP) HPLC ( $\text{C}_{18}$ ;  $\text{H}_2\text{O}$ /MeOH) to yield a further amount of **1** (2.7 mg;  $\text{H}_2\text{O}$ /MeOH 20:80). *Fr. 6.4* (hexane/acetone 70:30, 0.0055 g) was subjected to RP-HPLC ( $\text{C}_{18}$ ;  $\text{H}_2\text{O}$ /MeOH) to give a further amount of **2** (1.6 mg,  $\text{H}_2\text{O}$ /MeOH 30:70).

*Fr. 7* (hexane/AcOEt 40:60, 0.6367 g) was subjected to CC ( $\text{SiO}_2$ ;  $\text{CHCl}_3$ /MeOH of increasing polarity) to afford eight fractions, *Frs. 7.1–7.8*. *Fr. 7.4* ( $\text{CHCl}_3$ /MeOH 97:3; 0.0147 g) was submitted to CC ( $\text{SiO}_2$ ; hexane/acetone of increasing polarity) to furnish nine fractions, *Frs. 7.4.1–7.4.9*. *Fr. 7.4.4* (22.7 mg, 70:30 (v/v) was purified by HPLC ( $\text{SiO}_2$ ; hexane/acetone) to yield **3** (2.2 mg) and **4** (0.9 mg) at 55:45 v/v.

*Acanthiolide* (= (3*aR*,4*S*,5*aR*,6*R*,9*S*,9*bR*)-9-Formyl-6-hydroxy-5*a*-methyl-3-methylidene-2-oxododecahydronaphtho[1,2-*b*]furan-4-yl 3-Hydroxy-2-methylpropanoate; **4**). Oil.  $[\alpha]_D^{25} = +32$  ( $c=1$ ,  $\text{CHCl}_3$ ). IR: 3424 (OH), 1766 (C=O), 1730 (C=O), 1715 (C=O).  $^1\text{H}$ -NMR (600 MHz,  $\text{CDCl}_3$ ): 3.38 (*dd*,  $J=4.6$ , 11.4,  $\text{H}-\text{C}(1)$ ); 1.74 (*dddd*,  $J=3.5$ , 3.5, 3.5, 13.0,  $\text{H}_a-\text{C}(2)$ ); 1.61 (*dddd*,  $J=4.2$ , 11.4, 13.8, 12.0,  $\text{H}_b-\text{C}(2)$ ); 2.44–2.46 (*m*,  $\text{H}_a-\text{C}(3)$ ); 1.41–1.48 (*m*,  $\text{H}_b-\text{C}(3)$ ); 2.79 (*br. dd*,  $J=7.1$ , 4.2,  $\text{H}-\text{C}(4)$ ); 2.00 (*dd*,  $J=5.7$ , 12.0,  $\text{H}-\text{C}(5)$ ); 4.49 (*dd*,  $J=11.8$ , 11.8,  $\text{H}-\text{C}(6)$ ); 2.81 (*dddd*,  $J=2.9$ , 2.9, 10.5, 10.5,  $\text{H}-\text{C}(7)$ ); 5.26 (*ddd*,  $J=4.6$ , 10.5, 10.5,  $\text{H}-\text{C}(8)$ ); 1.25 (*dd*,  $J=11.0$ , 12.6,  $\text{H}_a-\text{C}(9)$ ); 2.43 (*dd*,  $J=4.2$ , 12.6,  $\text{H}_b-\text{C}(9)$ ); 6.18 (*d*,  $J=2.9$ ,  $\text{H}_a-\text{C}(13)$ ); 5.71 (*d*,  $J=2.9$ ,  $\text{H}_b-\text{C}(13)$ ); 0.90 (*s*, Me(14)); 9.93 (*s*,  $\text{H}-\text{C}(15)$ ); 2.71 (*ddq*,  $J=5.0$ , 8.0, 7.1,  $\text{H}-\text{C}(2')$ ); 3.77 (*dd*,  $J=8.0$ , 10.0,  $\text{H}_a-\text{C}(3')$ ); 3.75 (*dd*,  $J=5.0$ , 10.0,  $\text{H}_b-\text{C}(3')$ ); 1.18 (*d*,  $J=7.2$ , Me(4')).  $^{13}\text{C}$ -NMR (125 MHz,  $\text{CDCl}_3$ ): 78.1 (C(1)); 27.3 (C(2)); 22.4 (C(3)); 44.9 (C(4)); 48.8 (C(5)); 76.2 (C(6)); 53.7 (C(7)); 69.0 (C(8)); 44.0 (C(9)); 41.4 (C(10)); 136.3 (C(11)); 169.3 (C(12)); 120.7 (C(13)); 13.9 (C(14)); 201.7 (C(15)); 174.6 (C(1')); 64.6 (C(3')); 41.9 (C(2')); 13.4 (C(4')). HR-EI-MS: 366.1682 ( $M^+$ ,  $\text{C}_{19}\text{H}_{26}\text{O}_7$ ; calc. 366.1679).

*Coleoptile Bioassay.* Wheat seeds (*Triticum aestivum* L. cv. DURO) were sown in 15-cm diameter *Petri* dishes, moistened with  $\text{H}_2\text{O}$ , and grown in the dark at  $25 \pm 1^\circ$  for 4 d [34]. The roots and caryopses were removed from the shoots. The latter were placed in a *Van der Weij* guillotine, and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassays. All manipulations were performed under a green safelight [33]. Compounds were predissolved in DMSO and diluted to the final bioassay concentration with a maximum of 0.1% DMSO. Parallel controls were also run.

Crude extracts, fractions, or pure compounds to be assayed for biological activity were added to test tubes. Each assay was carried out in duplicate. Phosphate–citrate buffer (2 ml) containing 2% sucrose [33] at pH 5.6 was added to each test tube. Five coleoptiles were placed in each test tube (three tubes per dilution), and the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at  $25^\circ$  in the dark. The

coleoptiles were measured by digitalization of their images. Data were statistically analyzed using *Welch's* test [65]. Data are presented as percentage differences from control. Thus, zero represents the control; positive values represent stimulation of the studied parameter, and negative values represent inhibition.

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