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Phytotoxic polyacetylenes from roots of Russian knapweed (*Acroptilon repens* (L.) DC.)

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ABSTRACT

There are several factors thought to assist invasive weeds in colonization of ecosystems. One of these factors is allelopathy, the negative effect of chemicals produced by one plant on neighboring plants, frequently mediated through root exudates and other plant leachates. *Acroptilon repens* (Asteraceae) is one of the most invasive and ecologically threatening weed species in western North America. A bioassay-guided fractionation of the root extracts of this plant led to the isolation of five polyacetylenic compounds, of which one [5′-methoxy-1′-(5-prop-1-yn-1-yl-2-thienyl)-hexa-2′,4′-diyin-6′-yl acetate] was hitherto unknown. The structures of these compounds were elucidated on the basis of spectroscopic analysis (IR, ESIMS, ^{1}H, ^{13}C NMR and 2D NMR). All of the compounds obtained, except 1-chloro-4-(5-penta-1,3-diyn-1-yl-2-thienyl)but-3-yn-2-ol, showed phytotoxic activity against *Arabidopsis thaliana* seedlings. The presence of 4′-chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-3′-ol was detected in the root exudates of aeroponically grown *A. repens* plants. None of the polyacetylenes isolated in this study were found in Colorado soils collected between September 2006 and July 2007 in an *A. repens* colonized site. However, polyacetylene **5** in *A. repens* infested soil from Washington was found in June, 2007. Contrary to our previous report, the compound 7,8-benzoflavone (**6**) was not detected in root exudates, nor was it encountered in extracts of roots, aerial parts or infested soil. Since we could not repeat this work, the original report has been retracted [Stermitz, F.R., Bais, H.P., Foderaro, T.A., Vivanco, J.M., 2003. 7,8-Benzoflavone: a phytotoxin from root exudates of invasive Russian knapweed [A retraction]. Phytochemistry 64, 493–497.].

1. Introduction

Acroptilon repens (L.) DC (Russian knapweed; fomerly *Centaurea repens*) is a perennial herbaceous plant belonging to the family Asteraceae. Its highly competitive nature and broad ecological adaptability make it a persistent weed problem in North America (Goslee et al., 2001). This plant is native to Mongolia, western Turkestan, Iran, Turkish Armenia and Asia Minor (Maddox et al., 1985), and was introduced into North America in the early 1900's, primarily as a contaminant of Turkestan alfalfa (*Medicago sativa*) seeds (Watson, 1980). This exotic plant is now widely distributed in the United States, and has been reported in 22 western and mid-western states where it competes successfully with agricultural/range crops and native plants. Like most exotic species, *A. repens* primarily invades disturbed ecosystems, and it has invaded about 1.5 million acres in North America and each year its territory expands by circa 8% (Mullin et al., 2000).

A. repens has been shown to produce phytotoxic compounds, which may contribute to its competitive behavior. Studies of the extracts from root and callus tissue of *A. repens* have suggested the presence of plant growth inhibitors (Musiyaka et al., 1993). Fletcher and Renney (1963) found that soil where *A. repens* had grown showed inhibitory effects on the growth of tomato and barley plants. The chemical compounds responsible for this effect in soil were not identified in these studies. However, there has been some characterization of compounds produced by *A. repens*. Aerial tissues of *A. repens* possess aromatic amines and sterols (Mallabaev et al., 1982), and sesquiterpene lactones that have been reported as phytotoxic and neurotoxic compounds (Stevens and Merrill, 1985; Stevens et al., 1990a; Robles et al., 1998; Choi et al., 2000). Essential oils found in its aerial tissues have antimicrobial activity against gram-positive bacteria (Norouzi-Arasi et al., 2006). The triterpene euphorbol has been isolated from the whole plant (Ayneh-chi and Eshaghzadeh, 1974) and further studies have shown activity against the Epstein-Barr virus (Akihisa et al., 2002).

Roots of this species were reported to contain several polyacetylenic thiophenes (Stevens, 1986a,b) and some traces of

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guaianolides (Jakupovic et al., 1986). One of these thiophenic derivates showed some phytotoxic activity, inhibiting root growth of lettuce, alfalfa, barnyard grass, and red millet. This compound was also reported to be present at ecologically relevant concentrations in soil where *A. repens* was growing (Stevens, 1986a). Another compound, 7,8-benzoflavone (**6**), was reported from root exudates of *A. repens* grown in vitro (Stermitz et al., 2003) and to be phytotoxic, but roots themselves and soil were not investigated.

These interesting preliminary studies led us to investigate the root and the above-ground tissues of this plant with advanced chromatographic and spectroscopic techniques in order to isolate other potentially phytotoxic compounds. It was also important to investigate the dichotomy between our previous results (Stermitz et al., 2003) and those of Stevens (1986a,b). This paper describes the isolation and structural identification of five polyacetylenic compounds (Fig. 1) and their phytotoxic activity against *Arabidopsis thaliana* seedlings. The presence of these compounds in root exudates and in collected soils from sites infested with *A. repens* was also investigated.

2. Results and discussion

2.1. Identification of chemicals

Bioassay of hexane, CH_2Cl_2 and *n*-BuOH extracts of dried *A. repens* roots showed that only the hexane and CH_2Cl_2 extracts significantly decreased the fresh weight of *A. thaliana* seedlings (Fig. 2). Chromatographic analysis of the CH_2Cl_2 extract of roots yielded 14 fractions. Fractions 1, 2 and 4 were found to be phytotoxic (Fig. 3). Fraction 1 (650 mg) was re-chromatographed by eluting with a hexane-EtOAc (flow rate 25 ml min^{-1}) gradient as a system solvent. Using this strategy the following compounds were separated and identified: 4′-chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-3′-ol (**1**, 120 mg) and 3′-chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-4′-ol (**2**, 15 mg). Fraction 2 (70 mg) was eluted on a gradient with hexane-EtOAc at 18 ml min^{-1}. This resulted in the isolation of 5′-methyl-1′-(5-prop-1-yn-1-yl-2-thienyl)-hexa-2′,4′-diyin-6′-yl acetate (**3**, 9 mg) and 3′-(5-penta-1,3-diynyl-thiophen-2-ylethynyl)-oxirane (**4**, 6 mg). Compound 1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yne-3′,4′-diol (**5**, 9 mg) was isolated from fraction 4 (100 mg) after using a gradient of hexane with increasing amounts of EtOAc at a flow rate 15 ml min^{-1}.

5′-Methoxy-1′-(5-prop-1-yn-1-yl-2-thienyl)-hexa-2′,4′-diyin-6′-yl acetate (**3**) had an UV spectrum typical of acetylenic thiophenes. The IR spectrum showed typical bands of acetylene (2359, 2342, 2232 cm^{-1}) and carboxyl (1642 cm^{-1}) groups. In its ^1H NMR spectrum, the two signals at δ 7.06 and 7.12 (each 1H, *d*, *J* = 4), corresponding to H-4 and H-3, respectively, indicated a thiophene ring substituted at C-2 and C-5 and the sharp singlet at 3.52 ppm suggested a methoxyl group. The ^{13}C NMR spectrum indicated three acetylenic units as singlets between 63 and 90 ppm [64.3 (C-1″), 66.6 (C-1″), 79.7(C-2′), 80.1 (C-3′), 83.8 (C-2″) and 89.9 (C-4′)]

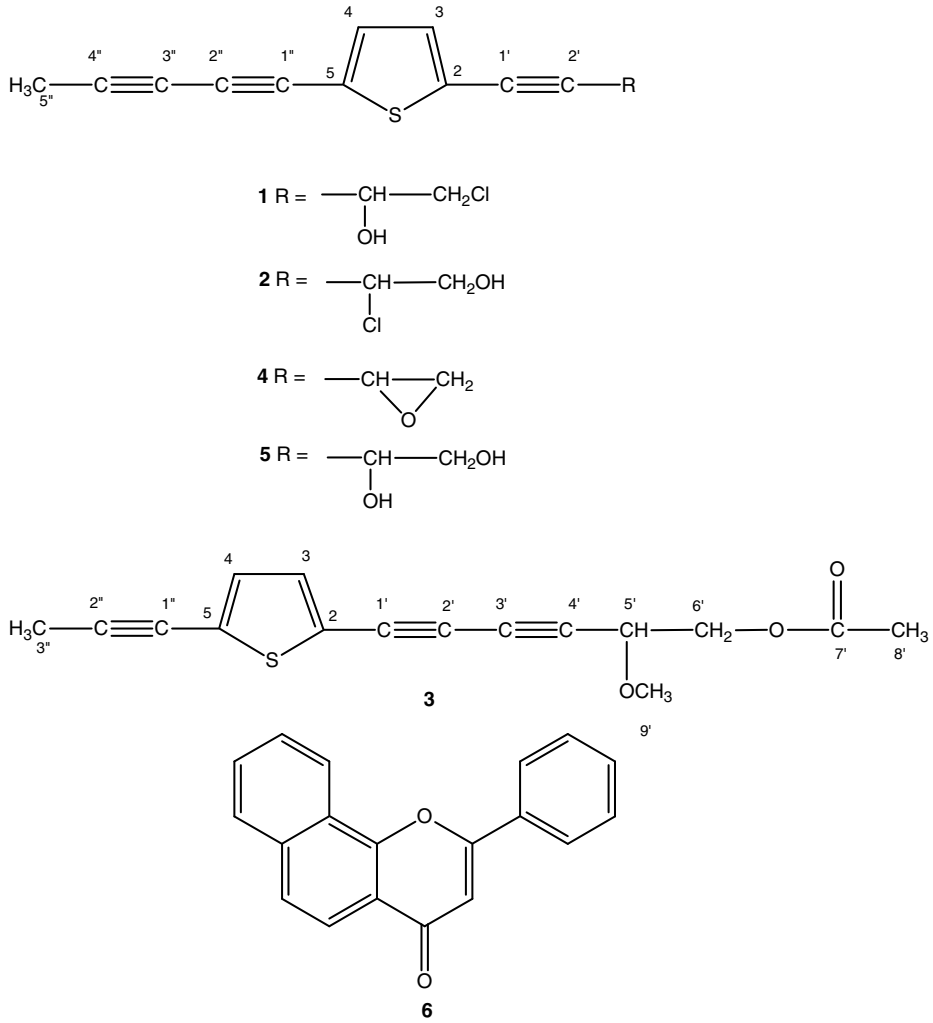

Fig. 1. Structures of compounds described in the text.

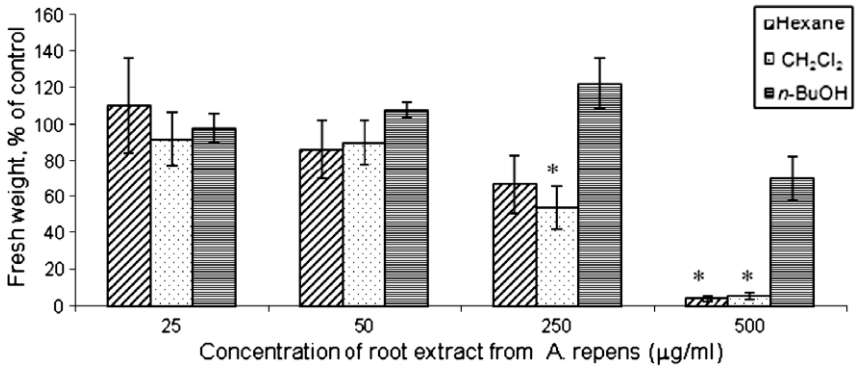

Fig. 2. Effect of hexane, CH_2Cl_2 and n-BuOH root extracts from *A. repens* on 7-day-old *A. thaliana* seedlings. Values are presented as percentage of the mean compared to the control. Errors bars are the s.e. of the mean, $n = 4$. $^{*}P = 0.0001$.

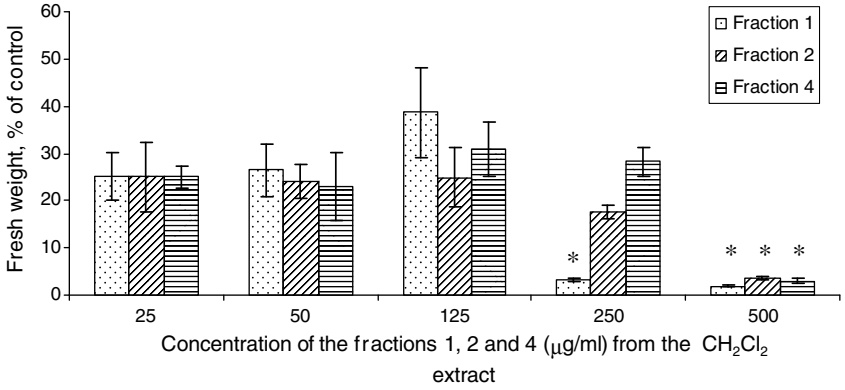

Fig. 3. Phytotoxic activity of fractions 1, 2 and 4 from the roots extracted with CH_2Cl_2 on 7-day-old *A. thaliana*. Errors bars are one the s.e. of the mean, $n = 4$. $^{*}P = 0.0001$.

and an acetate group with signals at 21.3 and 171.5 ppm. The HMBC spectrum showed a long-range correlation of H-5′ with C-9′, C-2′, C-3′ and C-4', suggesting the presence of an OMe-CH- moiety directly connected to the two acetylenic units. In addition, the correlation of H-6′ with C-9′ and one of the acetylenic carbons indicated that the CH_2OAc was attached to C-5′. Based on this analysis, compound **3** should show a molecular ion at *m/z* 286 in the mass spectrum. The EI-MS data showed a base peak at *m/z* 213, probably caused by the loss of the CH_2OAc from the molecular ion (Fig. 4). Fragments due to loss of methoxyl (*m/z* 255) and acetate (*m/z* 227) were also observed.

On the basis of the above spectroscopic evidence, structure **3** was assigned to this new thiophene. Its alcohol has been isolated before from *Eclipta erecta* (Bohlmann and Zdero, 1970). In addition, we also isolated the compounds **1** and **2**, both of which have been previously isolated from *A. repens* (Stevens, 1986a); **4**, reported from *Ambrosia chamissonis* (Balza et al., 1989), and **5**, which has been isolated from *Pluchea dioscoridis* (Bohlmann et al., 1973); both of these plants belong to the Compositae family. As far as we know, the ^{13}C NMR spectroscopic data of compound **1** has been never reported before in the literature. Herein we report the ^{13}C NMR spectroscopic data of **1** to complete the characterization of this compound.

In previous work on *A. repens* roots (Stevens, 1986a,b), seven polyacetylenes were isolated, including **1** and **2**. Our work adds three polyacetylenes not found before in *A. repens*. In part, our method of following a bioassay-directed fractionation led us to the isolation of these new compounds. In spite of a detailed search in *A. repens* root tissues we were unable to confirm the previously reported root exudate compound, 7,8-benzoflavone (**6**) (Stermitz et al., 2003). In order to discard any possibility about the presence of this compound in *A. repens*, above-ground plant parts of this species were also analyzed by HPLC-MS, however no trace of this compound was detected.

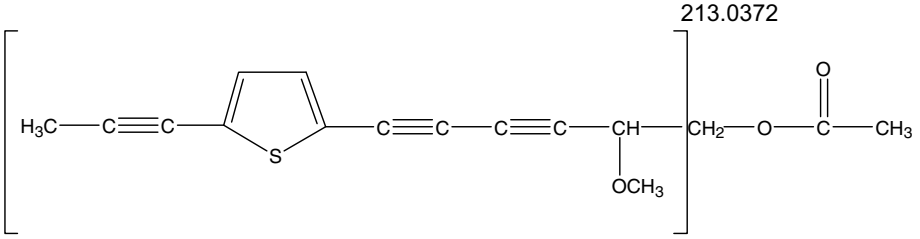

Fig. 4. Base peak in the EI-MS spectrum of **3**.

2.2. Phytotoxicity of the root polyacetylenes

Several polyacetylenes are already known to be phytotoxic, including *cis*-dehydromatricariaester (*cis*-DME) (Kawazu et. al., 1969), lachnophylum ester (LE), matricaria ester (ME) (Kobayashi et al., 1974), dehydromatricaria lactone (Ichihara et al., 1978), phenylheptatriyne (PHT) and α-terthienyl (Campbell et al., 1982).

A. repens has been reported to possess more thiophene analogs than other related noxious species (Stevens et al., 1990). The structure-activity relationship among several polyacetylenes was previously investigated by McLachlan et al. (1986), and it was found that polyacetylenes with more thiophene units were more active. Correspondingly, Stevens (1986a,b) found some acetylenic thiophenes isolated from roots that possessed phytotoxic activity. We repeated the phytotoxic bioassay of Stevens with some modifications (see Experimental), in order to examine the phytotoxic activity of the polyacetylenes isolated in the present study. Our data on the activity of the purified compounds against *A. thaliana* seedlings are shown in Fig. 5. All of the compounds isolated in the bioassay-guided fractionation of *A. repens* decreased the fresh weight of *A. thaliana* seedlings, except compound **2**. Stevens (1986a) also found that this compound was not phytotoxic. Compound **1** has already been reported to be phytotoxic, reducing the root length of lettuce 50% at a concentration of 12 ppm (Stevens, 1986a); our results are consistent with this report as we found that 12.5 μg ml^{-1} of this compound reduces *A. thaliana* fresh weight by 64%. Compound **5** was not as phytotoxic as **1**; however, **5** decreased the fresh weight of *A. thaliana* by 37% at 50 μg ml^{-1}.

The new polyacetylene **3** showed potent activity against *A. thaliana* seedlings by causing mortality one day after the application and by decreasing the plant fresh weight by 54.5% at 25 μg ml^{-1} compared to controls. Compound **4** was not previously reported as phytotoxic; however, treatment of *A. thaliana* seedlings at 500 μg ml^{-1} of compound **4** killed the plants and at 250 μg ml^{-1} reduced *A. thaliana* growth by 45%.

Different types of polyacetylenes have been reported to be phytotoxic (Kawazu et al., 1969; Kobayashi et al., 1974; Ichihara et al., 1978) but it is not clear which chemical groups present in those phytochemicals are needed for their phytotoxic activity. Among the compounds isolated in this study, **1** presented the strongest phytotoxic activity and unlike the other polyacetylenes tested, **1** has chlorine as the end group. The structural isomer of **1**, polyacetylene **2**, did not, however, show phytotoxic activity. Thus, the different position of the OH and Cl in both molecules may play an important role in the ability of these compounds to inhibit growth.

It has been reported that biological activity of polyacetylenes may be enhanced under sunlight or near-UV (Campbell et al., 1982). Whether the activity of the compounds isolated in this study is affected or not by these wavelengths of light is unknown at this point.

2.3. Root exudates of A. repens

If these phytotoxic substances found in root tissue are implicated in the invasiveness of the weeds it is necessary to demonstrate that the plant exudes them into the soil and that the allelochemicals are stable enough to persist under natural conditions. We analyzed the crude root exudates of *A. repens* plants grown under aeroponic conditions by LC-MS using the compounds previously isolated as standards. We observed the polyacetylene **1**, eluted at 42 min with a molecular ion of 249^{+}, in the root exudates. This compound was previously found in soil from plant communities infested with *A. repens* (Stevens, 1986a). The other polyacetylenes isolated from the roots were not detected in the root exudates under our experimental conditions. Previous work with *A. repens*

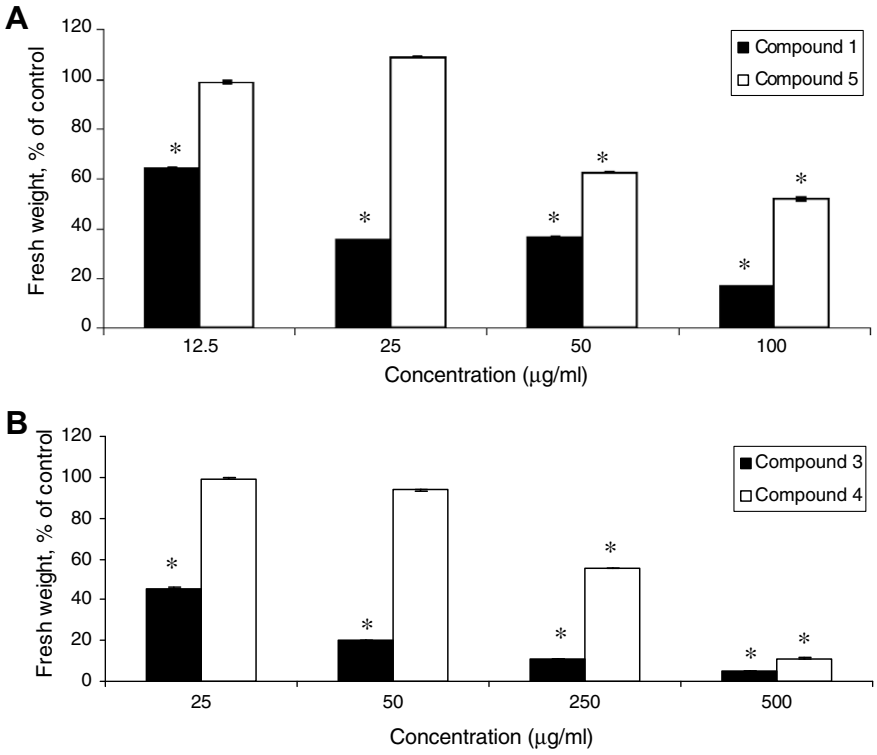

Fig. 5. Inhibition produced by pure compounds found in *A. repens* roots on *A. thaliana* growth at different concentrations: (A) Compounds 1 and 5; (B) Compounds 3 and 4. Compound 2 not shown. Compounds were dissolved in 50 μl MeOH as solvent. The control seedlings were only treated with MeOH. Values are presented as percentage of the mean compared to the control. Errors bars are the s.e. of the mean, $n = 4$. * $P = 0.0001$.

reported the presence of 7,8-benzoflavone (**6**) in the root exudates of plants grown in vitro (Stermitz et al., 2003); however, we found no trace of it in our reexamination of similarly grown exudates. Attempts to reevaluate the reported phytotoxicity of 7,8-benzoflavone (**6**) were frustrated by the extremely insoluble nature of that compound and no meaningful data were obtained. The sample purported to have been isolated from the exudates of *A. repens* (Stermitz et al., 2003) was not found in the very detailed reinvestigation described here, and we have retracted the original publication (Stermitz et al., 2003). Instead, the data reported here, along with the previous Stevens work, should be considered definitive.

2.4. Polyacetylenes in the surrounding soil of A. repens

Field soil collected from *A. repens*-invaded sites in Fort Collins, CO between September–October 2006 and April–July 2007 was analyzed. Polyacetylenes found in the roots of *A. repens* were not found in these soils at any of the time points.

In a separate study, we collected *A. repens* soil at Yakima, WA, during June 2007 and found polyacetylene **5** in the surrounding areas of *A. repens*. It is possible that the presence of this compound **5** in the soil is due to decomposition of leaves and stems by physical or biological processes. Also rain and fog could leach compounds from leaves into the soil.

In this soil we also detected two other polyacetylenes (M_r: 354 and 386), whose structures could not be further investigated due to a lack of material, but whose UV spectra were consistent with those of typical polyacetylenes. These compounds may represent conjugates. For example, the potassium sulfate conjugate of the diol 5 has the M_r 386.

Thus, we confirmed the Stevens report (Stevens, 1986a) of polyacetylenes in the Washington soil, although not from the Colorado site. We have not analyzed roots or root exudates from *A. repens* found growing at Yakima, WA soils, so there could be qualitative or quantitative differences in polyacetylenes content or processing between the Washington and Colorado *A. repens* populations. Root exudation may be influenced by several factors in natural ecosystems (Rovira, 1969), and different soil conditions (Blair et al., 2005) or microorganism content (Einhellig, 1999) could affect the stability of exuded polyacetylenes at different sites. Additionally, it is quite possible that different ecotypes of *A. repens* could produce and secrete slightly different quantities of phytotoxins.

An in-depth analysis of different soil and plant types or environmental conditions between the two sites of collection (Colorado and Washington) and independent study of root exudations of the Washington population are needed to clarify these results.

3. Concluding remarks

Our finding of phytotoxic thiophene polyacetylenes in roots, root exudates and, in one case in soil around plants, suggests that these compounds may be involved in the purported allelopathic behavior of Russian knapweed. A previous report on the presence of 7,8-dibenzoflavone (**6**) in *A. repens*, was not substantiated and this report has been withdrawn.

4. Experimental

4.1. General experimental procedures

Optical rotation was determined on an Autopol III automatic polarimeter (Rudolph Research Analytical). IR spectra were recorded using a Nicolet Avatar 320 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were performed on a Varian INOVA 400 instrument, operating at 400 MHz and 100 MHz, respectively. The spectra were run in $CDCl_3$; chemical shifts (δ are given in ppm and the coupling constant (J) in hertz (Hz), using TMS as an internal standard. ESIMS and FAB were measured with a Thermo Finnigan LCQ instrument and a Fision VG Autospec apparatus, respectively.

The HPLC system was equipped with a P680 pump, an ASI-100 autosampler, and a PDA-100 photodiode array detector (Dionex). Samples were run on an analytical Dionex Acclaim 120 C18 column (5 μm, 4.6 × 150 mm) using gradient elution. Mobile phase consisted of 0.1% (v/v) HOAc in water (A)-MeOH (B). The solvent system used was: three minutes at 10% B, a linear gradient to 90% B over 40 min, and held at 90% B for 8 min. The flow rate of the mobile phase was 0.7 ml min^{-1} and the injection volume was 20 μl. The HPLC was coupled to a Thermo Finnigan Surveyor MSQ mass spectrometer detector. Ionization for MS analysis was performed in both positive and negative ion mode using electrospray ionization with a nitrogen flow at 80 psi, a cone voltage of 70 V, a needle voltage of 3 kV, and a cone temperature of 600 °C. Mass data were collected over the range of the gradient program at a rate of one scan per two seconds. UV detection was recorded at 254, 280, and 310 nm. All solvents used were HPLC grade.

4.2. Plant material

Acroptilon repens (L.) DC. roots and aerial part were collected at Fort Collins, Colorado, USA (40° 42' 35.25" N, 105° 06' 08.23" W) in the summer of 2006 and 2008, respectively. Seeds were obtained from the plants at the same location. A voucher specimen was deposited at the herbarium of Colorado State University.

Arabidopsis thaliana L. Columbia-0 seeds were purchased from Lehle Seeds (Roundrock, TX).

4.3. Isolation of phytochemicals

Freeze-dried roots (310 g) were ground and extracted twice over 48 h with EtOH—H_2O (95:5, v/v) at room temperature. The mixture was filtered and the solvent evaporated in vacuo. The crude extract (14 g) was dissolved in MeOH—H_2O and extracted with hexane, CH_2Cl_2 and *n*-BuOH (420 ml each) successively. These extracts were separately dried *in vacuo*.

The CH_2Cl_2 extract (1.7 g) was purified further by VLC (silica gel, 4 × 4 cm) using a hexane-EtOAc gradient to yield 14 fractions, which were tested for phytotoxic activity. Active fractions were further purified by CombiFlash™ RETRIEVE® system (ISCO, Lincoln, NE, USA), using different sizes of normal phase flash columns (RediSep™, ISCO).

Since polyacetylenes are labile compounds and *A. repens* was known to have polyacetylenes in its roots, light was excluded and the temperature of solvents were kept below 30 °C throughout the experiment to avoid degradation.

4.3.1. 4′-Chloro-1′-(5-penta-1,3-diyn-1-yl-2-thienyl)-but-2′-yn-3′-ol (*1*)

Polyacetylene **1** was previously isolated and its ^{1}H NMR spectrum described (Stevens, 1986a). However, this is the first time that the ^{13}C NMR data of this compound are presented.

^{13}C NMR δ: 5.1 (*q*, C-5″), 48.9 (*t*, C-4′), 63.3 (*d*, C-3′), 64.2 (*s*, C-3″)*, 66.6 (*s*, C-2″)*, 79.3 (*s*, C-1′), 80.1 (*s*,C-1″), 83.9 (*s*, C-4″), 91.3 (*s*, C-2′), 123.6 (*s*, C-2), 124.7 (*s*, C-5), 132.9 (*d*, C-3), 133.8 (*d*, C-4).

* = interchangeable carbon assignment data.

4.3.2. 5′-Methyl-1′-(5-prop-1-yn-1-yl-2-thienyl)-hexa-2′,4′-diyin-6′-yl acetate (*3*)

Oil. $[\alpha]_D^{25} = -17.5^{\circ}$ ($CHCl_3$; c 4 × 10^{-4}). UV λ_{max}^{EtOH} nm (log ε): 208 (5.07), 246 (5.15), 320 (5.26), 337 (5.27). IR ν_{max} (KBr) cm^{-1}: 2916, 2848, 2359 (—C≡C—), 2342 (—C≡C—), 2232 (—C≡C—),

1642 (C=O), 1462, 1260, 1106, 804; ^{1}HNMR δ: 2.05 (6H, *brs*, H-3″, H-8′), 3.52 (3H, *s*, H-9′), 3.80 (2H, *d*, H-6′, *J* = 5.6), 4.29 (1H, *t*, H-5′, *J* = 11.2 and 5.6), 7.06 (1H, *d*, *J* = 4, H-4), 7.12 (1H, *d*, *J* = 4, H-3). ^{13}C NMR δ: 5.0 (*q*, C-3″), 21.3 (*q*, C-8′), 57.3 (*q*, C-9′), 64.3 (*s*, C-1″), 65.2 (*t*, C-6′), 66.6 (*s*, C-1′), 72.9 (*d*, C-5′), 79.7 (*s*, C-2′), 80.1 (*s*, C-3′), 83.8 (*s*, C-2″), 89.8 (*s*, C-4′), 127.6 (*s*, C-2), 128.9 (*s*, C-5), 132.6 (*d*, C-4), 133.9 (*d*, C-3), 171.5 (*s*, C-7′). ESIMS positive mode: *m/z* 255 $[M-OMe]^+$ (50), 227 $[M-OAc]^+$ (18), 213 $[M-CH_2-OAc]^+$ (100), 173 $[213-CH_3-CC-H]^+$ (10). HRFABMS found: 213.0371 $[M-CH_2OAc]^+$ calc. for $C_{13}H_9OS$ 213.0374.

4.4. Bioassays

A. thaliana seeds were surface-sterilized with 100% commercial bleach for one min, washed five times in distilled sterile H_2O and germinated on solid Murashige and Skoog (Murashige and Skoog, 1962) medium in a 25 °C incubator with a 16 h/8 h day/night photoperiod. Seven-day-old plants were transferred into 1 ml of liquid MS medium supplemented with 3% sucrose in 24-well plates (VWR Scientific), then incubated on an orbital shaker at 90 rpm and under cool-white fluorescent lights (45 μmol m^{-2} s^{-1}) with a photoperiod of 16 h light/8 h dark at 25 °C.

After 24 h, the plants were treated with five concentrations of each test compound (0, 25, 50, 250, and 500 μg ml^{-1} or 0, 12.5, 25, 50 and 100 μg ml^{-1}), with four replicates per treatment. Samples to be tested were dissolved in MeOH, and applied to the 24-well plates containing the seedlings. MeOH (50 μl) was added to control wells. After treatment, plates were sealed with parafilm and incubated on rotary shakers for seven days. After seven days, the fresh weights of each plant were recorded and the percentage of the fresh weight of the plants was determined by reference to the fresh weight of control plants.

The effects of tested extracts, fractions and isolated compounds were visually assessed for phytotoxicity and statistical significance of plant growth inhibition relative to controls was determined using ANOVA and Dunnetts two-tailed comparisons (XLSTAT Pro 2006; Addinsoft, Paris, France).

In order to reevaluate the effect of 7,8-benzoflavone (**6**) on *A. thalina* seedlings this compound was dissolved as reported previously in MeOH (Stermitz et al., 2003) but it was insoluble in this solvent. Compound (**6**) was then dissolved in DMSO but this precipitated when added to the media containing *A. thaliana* seedlings.

4.5. Analysis of root exudates from A. repens

Seeds of *A. repens* were sown in a mixture of sand and peat and kept moist. When the seedling roots were at least 5 cm long, they were transferred to aeroponic culture chambers (EZ Clone 120, Green coast hydroponic). The aeroponic chambers were filled with half-strength Hoagland's solution using distilled H_2O. The aeroponic culture of *A. repens* provided 75 l of root exudates. In order to prepare the sample for LC-MS analysis, the exudates were concentrated through Oasis HLB 6 g extraction cartridges (Waters Corporation Milford, MA, USA). The column was equilibrated with MeOH (100 ml) followed by distilled H_2O (100 ml) and then the exudates were eluted through the column. Finally, the column was washed with distilled H_2O (500 ml) and the recovery of the metabolites was carried out eluting the column with MeOH. The MeOH extract was collected and evaporated to dryness under vacuum and re-dissolved with MeOH (1 ml) and subjected to LC-MS.

4.6. Soil analysis

A. repens infected soils were collected in two different locations: Fort Collins, Colorado and Yakima, Washington, USA. Soil from Fort Collins, CO (see 3.2), was collected from September to October, 2006 and from April to July, 2007. Ten samples from outside and ten from inside patches of *A. repens* were taken. Each sample was collected 10 cm deep from each of 10 selected locations within the site and then was passed through a 2-mm screen sieve to remove root contaminants. Soil from the Yakima Training Center in Yakima, Washington (46° 40' 27.36 N, 120° 26' 39.29″ W) was sampled in June 2007. The site was seeded in fall 2005 with restoration seed mixes but the dominant plant species was still *A. repens*. The soil samples from Washington were collected following a similar protocol to the one used in Colorado. Twenty soil samples were analyzed. The soil was stored at 4 °C until it was extracted and analyzed. The soil surrounding *A. repens* of each site was extracted with hexane and CH_2Cl_2 successively. Soil was extracted by incubating 2.5 g soil in 20 ml solvent on a rotary shaker (280 rpm) for 1.5 h at room temperature and then collecting the solvent in a vial. This procedure was then repeated twice per solvent. The solvent was evaporated under N_2 flow and then the residue was re-dissolved in MeOH and analyzed.

Soil sample analyses were carried out by LC-MS using the same conditions described above (Section 4.1). Polyacetylenes **1**, **3**, **4** and **5**, previously isolated from the roots, were used as standards and their retention time and molecular weight were compared with peaks obtained from the soil samples.

4.7. Analysis of above-ground plant parts

The air-dried and chipped aerial parts of *A. repens* were extracted and fractionated as described for root tissue in Section 4.3. The resulting extracts were concentrated on a rotavapor, redissolved in DMSO (1 ml) and subjected to HPLC-MS analyses using the same conditions as mentioned in Section 4.1.

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