JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

# Phytotoxic Allelochemicals from Roots and Root Exudates of *Trifolium pratense*

Quan Liu,[†] Rui Xu,[†] Zhiqiang Yan,[†] Hui Jin,[†] Haiyan Cui,[†] Liqin Lu,[†] Denghong Zhang,[†] and Bo Qin*[,†,‡]

[†]Key Laboratory of Chemistry of Northwestern Plant Resources and Key Laboratory for Natural Medicine of Gansu Province, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, People's Republic of China

[‡]State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, People's Republic of China

**ABSTRACT:** *Trifolium pratense*, a widespread legume forage plant, is reported to exhibit phytotoxic activity on other plants, but the active metabolites have not been clarified so far. A bioassay-guided fractionation of the root extracts led to the isolation of five isoflavonoids, which were elucidated by spectroscopic analysis. All of the purified compounds observably showed phytotoxic activities against *Arabidopsis thaliana*. Moreover, the inhibitory effects were concentration-dependent. The furan ring linked at C-4 and C-2′ positions by an oxygen atom and a 1,3-dioxolane at C-4′ and C-5′ positions are considered to be critical factors for the phytotoxic activity. The concentrations of (6a*R*,11a*R*)-maackiain and (6a*R*,11a*R*)-trifolirhizin, concluded to be allelochemicals from soil around plants of *T. pratense*, were determined by HPLC and LC-MS to be 4.12 and 2.37 μg/g, respectively. These allelochemicals, which showed remarkable activities against the weed *Poa annua* may play an important role in assisting the widespread occurrence of *T. pratense* in nature.

**KEYWORDS:** *Trifolium pratense, allelopathy, isoflavonoids, phytotoxicity*

## INTRODUCTION

Many legume plants biosynthesize a range of isoflavones and play an important role in traditional diets throughout the world.[1] Consumption of legumes has been linked to reduced risk of diabetes and obesity, coronary heart disease, colon cancer, prostate cancer, and gastrointestinal disorders.[2] The functions of isoflavones as well as their roles in human nutrition and pharmacology offer several potential applications in ecology and agriculture.[3,4]

*Trifolium pratense* L., also known as red clover, belonging to the family Leguminosae (Fabaceae), is an important forage plant that grows widely in many parts of the world. The plant is frequently used as an alternative medicine for hormone replacement therapy (HRT) to treat menopausal disorders.[5] It is also a health product for humans commonly used for the treatment of eczema and psoriasis[6,7] and relief of menopausal symptoms in clinic.[8] Many isoflavones that have been found in the plant have multiple pharmacological and biological activities.[9]

Previous research on red clover indicated that it significantly delayed the seed germination and inhibited the growth of other plants, which assists its widespread distribution in nature.[10−13] One important factor of its highly competitive nature and broad ecological adaptability was the allelopathic effect, which is due to the phytotoxic activities of secondary metabolites produced by the plant on neighboring plants. The existence of allelopathy as an ecological phenomenon has been well documented over the past few decades in both natural and agricultural ecosystems.[14,15] Plants could affect neighboring plants and other organisms by releasing chemicals into the environment. Once allelopathic plant species release allelochemicals from their roots to the soil, a series of interactions between allelochemicals with soil abiotic and biotic factors can take place.

In light of the wide occurrence of ecological benefits of *T. pratense*, we decided to investigate the chemical constituents with phytotoxic activities. In this study, using a bioactivity-guided approach, the phytochemical investigation led to the isolation of five active compounds that are responsible for the observed plant growth inhibitory activities on the model plant *Arabidopsis thaliana*. This study investigated the phytotoxic activities and structure−activity relationship of these compounds on seedling growth of *A. thaliana*. Furthermore, the allelochemicals were identified from the soil (soil rhizosphere plus surrounding soil) where the plant had grown by means of HPLC and UV spectroscopy, which were further confirmed by LC-MS. To our knowledge, this is the first report on the occurrence, structure, and content of phytotoxins and allelochemicals of this globally distributed plant.

## MATERIALS AND METHODS

**General Experimental Procedures.** $^{1}H$ and $^{13}C$ NMR spectra were performed on a Bruker AM-400BB instrument (Bruker, Karlsruhe, Germany) with TMS as internal standard, operating at 400 and 100 MHz, respectively. EI-MS was measured with a VG ZAB-HS instrument (VG, East Sussex, UK), and ESI-ION trap was measured with an Esquire 6000 instrument (Bruker Daltonics, Billerica, MA, USA). Column chromatography (CC) used Sephadex LH-20 (25−100 mm) (Pharmacia Fine Chemical Co., Ltd., Berlin, Germany), silica gel 60 RP-18 (230−400 mesh) (Merck, Rahway, NJ, USA), and silica gel (200−300 mesh) (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). TLC was performed on precoated silica gel 60 $F_{254}$ plates (Qingdao Haiyang Chemical Co., Ltd.), and spots

Received: January 18, 2013
Revised: May 23, 2013
Accepted: June 5, 2013
Published: June 5, 2013

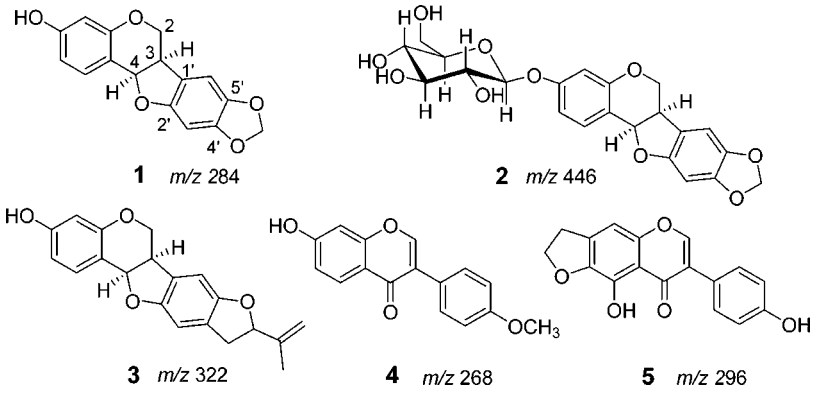


**Figure 1.** Structures of compounds (**1**−**5**) isolated from the roots of *T. pratense*.

were detected by ultraviolet (UV) illumination and sprayed with 5% $H_2SO_4$ in $C_2H_5OH$ (v/v).

**HPLC and LC-MS.** The HPLC analysis was carried out on a HP1200 system (Agilent, Santa Clara, CA, USA) with a model G1315D diode array, and the column used was a 250 mm × 4.6 mm, 5 μm, GraceSmart Rp C18 (Grace Corp., Deerfield, IL, USA). The solvent of methanol for HPLC analysis was of HPLC gradient grade (Anhui fulltime Co., Ltd., Hefei, China). Ultrapure water was prepared with a water purification system (Shanghai Laikie Instrument Co., Ltd., Shanghai, China). LC-MS spectra were acquired using an Agilent 1000 LC-MSD trap mass spectrometer equipped with an Agilent 1000 HPLC system and an electrospray ionization (ESI) source. Chromatographic separations were performed on stainless steel columns GraceSmart Rp C18 using a flow rate of 1.0 mL/min.

**Plant Material.** Roots and seeds of *T. pratense* were collected among the mountains in Min county, Gansu province, China, in 2008 and identified by Prof. Xun Pu from Lanzhou University. A voucher herbarium specimen has been deposited in the herbarium in Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences. The *A. thaliana* wild type seeds are of the Columbia ecotype and were kindly provided by Dr. Heng Liu from Lanzhou University. The soil and the seeds of *Poa annua* were purchased from a seed company in Lanzhou.

**Bioactivity-Guided Isolation of Phytochemicals.** Air-dried roots of *T. pratense* (4.5 kg) were chopped into small pieces, pulverized into fine powder, and then extracted with ethanol at room temperature five times. The roots stood in ethanol during the extraction for 1 day each time. The crude extracts were concentrated in vacuum to yield a residue (1.08 kg), which was then partitioned successively with petroleum ether, $CHCl_3$, and EtOAc, respectively. All of the fractionations and isolations were characterized with the *A. thaliana* seedling bioassays. Bioassay results indicated that the $CHCl_3$ and EtOAc fractions had significant inhibitory effects; in contrast, the petroleum ether fraction and water phase exhibited slight inhibitory activities. The $CHCl_3$ fraction (27.6 g) was separated on silica gel CC, eluting with $CHCl_3$/MeOH (1:0 to 0:1), to give fractions A−D on the basis of TLC analysis. Fraction B was subjected to silica gel CC, eluted with petroleum ether/$Me_2CO$ (15:1 to 0:1), and then purified with repeated silica gel CC to afford compounds **4** (29 mg) and **5** (76 mg) (Figure 1). Fraction C was passed through silica gel CC eluting with a $CHCl_3$/$CH_3OH$ gradient (20:1 to 0:1) and then was passed through a Sephadex LH-20 column ($CHCl_3$/$CH_3OH$ 1:1) and purified with repeated silica gel CC to yield compound **2** (1600 mg). The EtOAc fraction (47.7 g) was separated by silica gel column chromatography eluting with $CHCl_3$/MeOH in a gradient (20:1 to 0:1) to give fractions E−G. Fraction E was further fractionated via silica gel CC, eluting with petroleum ether/$Me_2CO$ gradient (15:1 to 0:1), to yield compound **1** (83 mg). Fraction F was subjected to silica gel CC eluting with a $CHCl_3$/$CH_3OH$ gradient (20:1 to 0:1) and further purified to produce compound **3** (16 mg).

**Bioassays.** *A. thaliana* seeds were surface-sterilized with 0.1% (w/v) mercuric chloride for 7 min, washed five times in distilled sterile water, and germinated on solid MS medium (a common medium used for the culture of plants in laboratory) at 22 °C in an incubator with a 16/8 h day/night photoperiod. Seven-day-old seedlings were transferred into 990 μL of liquid 1/2 MS medium supplemented with 1.5% sucrose in 24-well plates (diameter = 1.6 cm and height = 1.5 cm) (VWR Scientific Inc., USA). The plants were treated with different concentrations (50, 100, 200, and 400 μg/mL for crude extract and fractions prepared with different solvents; 25, 50, 100, 200, and 400 μg/mL for compounds **1**−**5**) of the test materials, with four replicates per treatment. Samples to be tested were dissolved in DMSO, and 10 μL was applied to each well containing the seedlings (10 μL of DMSO was added as control). Then the plates were sealed with parafilm and incubated in a rotary shaker at 95−105 rpm with a photoperiod of 16 h light/8 h dark at 22 °C for 7 days. After treatment, the fresh weights of each plant were measured, and the percentages of the fresh weights of the plants were determined by reference to the fresh weights of control. The phytotoxic bioassays of allelochemicals against *P. annua* were the same as for *A. thaliana*. Seven-day-old seedlings of *P. annua* were monitored for 7 days, and the fresh weights were measured at the end of the experiment. The results were analyzed by ANOVA ($p < 0.05$), and $IC_{50}$ values were calculated by probit analysis based on percent of either radicle growth or fresh weight inhibition.

**Sample Preparation Procedures of the Soil.** Seeds of *T. pratense* were sown into the soil and were grown for 1 year. The soil where *T. pratense* had grown was 1.08 kg (height = 8.5 cm; volume = $1.71 \times 10^{-3}$ $m^3$) and was extracted ultrasonically with MeOH three times (3 L of MeOH and 30 min each). The filtrate was concentrated in vacuum to near dryness on a rotary evaporator. Then the extract was dissolved in 100 mL of methanol to obtain the final solution. This solution was filtered through a 0.45 μm filter membrane prior to HPLC analysis.

**HPLC and LC-MS Analysis.** Analytical HPLC was performed using an Agilent apparatus equipped with a diode array detector (DAD) and a reverse-phase C18 column (5 μm particle size, 4.6 mm × 25 cm). Compounds were monitored at 254 and 275 nm, and UV spectra were recorded between 200 and 400 nm. The mobile phase, which was composed of water (A) and methanol (B), was programmed as follows: 0−5 min, B 40%; 5−10 min, B from 40 to 70%; 10−20 min, B 70%; 20−35 min, B from 70 to 85%; 35−40 min, B from 85 to 100%. The flow rate with an injection volume of 20 μL was 0.6 mL/min at the column temperature of 30 °C. The compound (6a*R*,11a*R*)-trifolirhizin was used to validate the analytical HPLC method described above. Mean recovery rates (accuracy) for (6a*R*,11a*R*)-trifolirhizin were >98% with a relative standard deviation of <5% and were determined by spiking a known amount of this authentic standard into the soil extract sample. Repeatability was determined by six injections of the extract sample in 1 day (intraday variability), and intermediate precision was determined on three different days (interday variability). The overall intraday and interday variations were found to be <5%.

The LC conditions for LC-MS analysis used solvents A (water) and B (methanol) as mobile phase. The gradient was identical to those used for HPLC analysis above. The injection volume of each sample

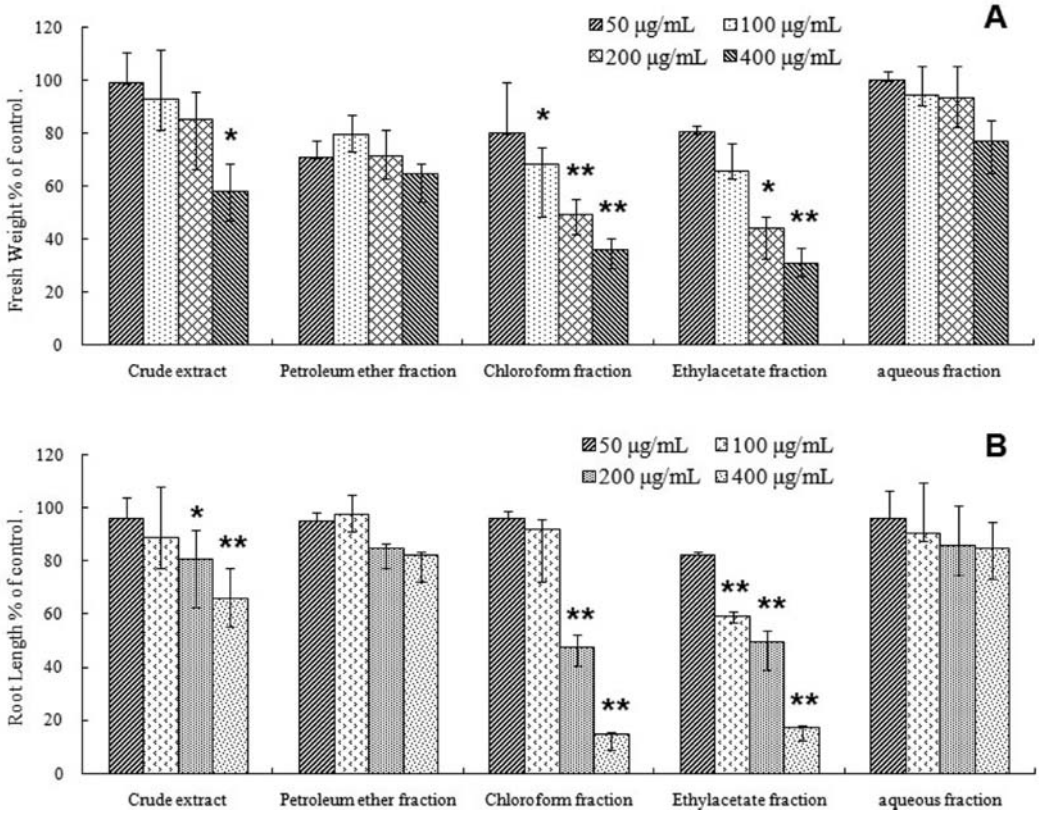


**Figure 2.** Effects of crude extract, petroleum ether, $CHCl_3$, EtOAc, and aqueous fractions from the roots of *T. pratense* on 7-day-old *A. thaliana* seedling fresh weight (A) and root length (B). Values are presented as percentage of the mean compared to the control. Means significantly lower than the DMSO controls are indicated with an asterisk (∗) (Dunnett's one-sided *t* test, $p < 0.05$) or two asterisks (∗∗) ($p < 0.01$). Error bars are one standard error of the mean. $N = 4$.

was 20 μL. For ESI-MS, the positive ion mode was used for further characterization of the phytochemicals. The capillary temperature and spray voltage were maintained at 325 °C and 5 kV, respectively. MS ionization was achieved using the Agilent ionization source with settings as follows: nebulizer gas ($N_2$) at 10 psi with drying gas at 8 L/min. The total ion current (TIC) chromatogram with a scan range from $m/z$ 150 to 1000 and fragmentation of precursor ions was recorded.

**Identification and Quantitation of Allelochemicals in the Soil.** Identification of isoflavones that were obtained from the soil was achieved by comparing their retention times and UV spectra with those of the standards of compounds **1**−**5**. The glycoside (6a*R*,11a*R*)-trifolirhizin, **2**, and its aglycone, (6a*R*,11a*R*)-maackiain, **1** (Figure 1), were detected in the soil, and their standards were used to obtain the standard curves. The standard curves were performed using the linear regression method, and peak areas at 275 nm were plotted versus concentrations. The regression equation of (6a*R*,11a*R*)-trifolirhizin, **2**, was $y = 6543x + 14.33$ ($r^2 = 0.9996$) in the concentration range of 0.001−0.2 mg/mL. The regression equation of its aglycone, **1**, was $y = 20705x - 23.924$ ($r^2 = 0.9999$) in the concentration range of 0.001−0.2 mg/mL. Limits of detection (LOD) were established at a signal-to-noise ratio (S/N) of 3. Limits of quantitation (LOQ) were established at signal-to-noise ratio (S/N) of 10. LOD and LOQ were experimentally verified separately by injections of compounds **1** and **2** at the LOD and LOQ concentrations. The LOD were calculated to be 0.03 μg/mL; the LOQ were calculated to be 0.1 μg/mL for compound **1**. The values of LOD and LOQ for compound **2** were the same as those for compound **1**. The internal standard method was used to verify the existence of allelochemicals in the soil. The method used compounds **1** and **2** as internal standards that were separately added into the prepared soil sample. Then the soil sample prepared for HPLC analysis was analyzed by LC-MS for further study.

## RESULTS AND DISCUSSION

**Isolation and Identification of Phytotoxic Chemicals from Roots of *T. pratense*.** The isolation of phytotoxic metabolites from roots of *T. pratense* was conducted by a bioassay-directed fractionation approach. The crude ethanol extract of *T. pratense* significantly inhibited the growth of *A. thaliana* seedlings (Figure 2). The crude extract was then extracted with different solvents. Bioassay showed that the $CHCl_3$ and EtOAc fractions had significant inhibitory effects; in contrast, the petroleum ether fraction and water phase exhibited slight inhibitory activities. Therefore, we selected the $CHCl_3$ and EtOAc fractions for further bioactivity-driven purification.

Five compounds, (6a*R*,11a*R*)-maackiain, **1**;[16] (6a*R*,11a*R*)-trifolirhizin, **2**;[17] barbacarpan, **3**;[18] formononetin, **4**;[19] and irilone, **5**,[20] were isolated from the $CHCl_3$ and EtOAc fractions and characterized by spectroscopic methods (Figure 1).

**Phytotoxic Activities of the Purified Metabolites on *A. thaliana* Seedlings.** Flavonoids are a major family of secondary metabolites that occur in a wide range of plant species and exhibit a number of functions in plant physiology, biochemistry, and ecology.[21] Two flavone *O*-glycosides isolated from rice seedlings had strong inhibitory activities on the growth of *Echinochloa crus-galli* and *Cyperus difformis*.[4]

In this study, isoflavonoids (compounds **1**−**5**) isolated in the bioassay-guided fractionations of *T. pratense* were assayed for growth-inhibiting effects on 7-day-old seedlings of *A. thaliana*. Results indicated that the fresh weights of *A. thaliana* seedlings were decreased by all of the compounds to different degrees.


dx.doi.org/10.1021/jf401241e | J. Agric. Food Chem. 2013, 61, 6321−6327

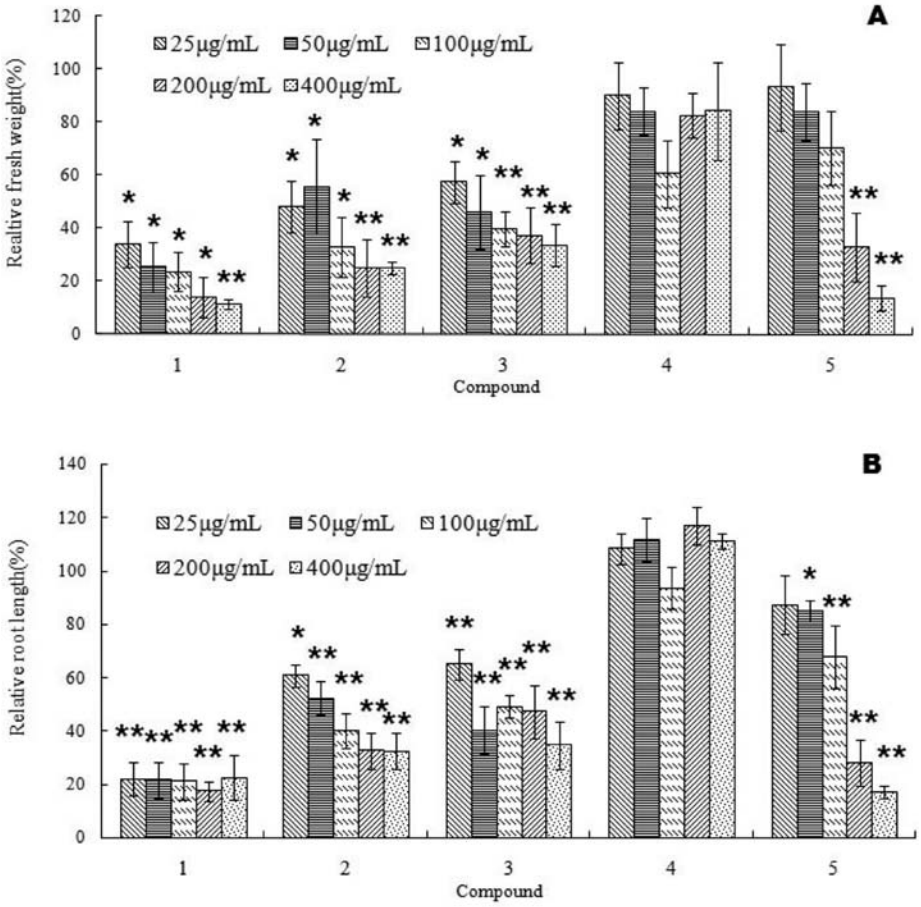


**Figure 3.** Phytotoxic effects of compounds **1**−**5** on *A. thaliana* seedling fresh weight (A) and root length (B) after 1 week of treatment. Values are presented as percentage of the mean compared to the control. Means significantly lower than the DMSO controls are indicated with an asterisk (∗) (Dunnett's one-sided *t* test, $p < 0.05$) or two asterisks (∗∗) ($p < 0.01$). Error bars are one standard error of the mean. $N = 4$.

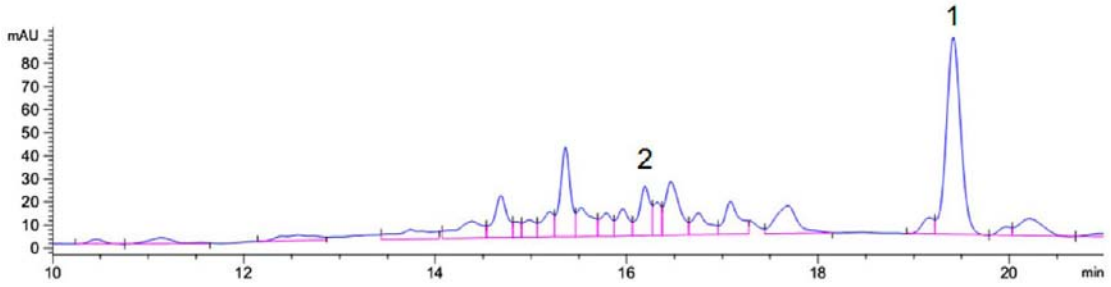


**Figure 4.** HPLC analysis of the soil where *T. pratense* had grown for 1 year. Compounds **1** and **2** are shown in the HPLC chromatogram at 275 nm and had LC retention times of 19.4 and 16.2 min, respectively.

Compounds **1**, **2**, **3**, and **5** had significant inhibitory effects (Figure 3), whereas compound **4** had only a slight inhibitory activity. After administration of compounds **1**−**5** for 7 days, treated plants showed wilting symptoms prior to senescence with morphological alterations of roots. The fresh weights of the *A. thaliana* seedlings treated with compounds **1**−**5** at 100 μg/mL were reduced by 76.4, 67.2, 60.4, 39.3, and 29.6% compared to the control, respectively. Compounds **1**, **2**, and **3** showed significant inhibitory activities on *A. thaliana* seedlings at low concentrations and almost arrested the growth of treated plants at higher concentrations, but did not kill them. The phytotoxic activities of all five compounds showed dose-dependent alterations. The $IC_{50}$ values of phytotoxic activity (50% of inhibition) treated by compounds **1**, **2**, **3**, and **5** on *A. thaliana* seedlings for 1 week were 21.2, 30.2, 42.7, and 176.8 μg/mL (0.075, 0.068, 0.13, and 0.60 μmol/mL), respectively.

Although compounds **1**−**3** possessed the same skeleton and compounds **4** and **5** had a different skeleton, the activities were distinctly different. The number and location of hydroxyl groups may influence the phytotoxic activity of the flavonoids. With comparison of plant growth inhibitory activities of compounds **1**−**5**, it is clear that the combination of a furan ring with an oxygen atom at C-4 and C-2′ positions in compounds **1**−**3** is an important factor responsible for appreciable phytotoxic activities. The activity of compound **2** with β-D-glucose at the C-7 position was slightly weaker than


dx.doi.org/10.1021/jf401241e | J. Agric. Food Chem. 2013, 61, 6321−6327

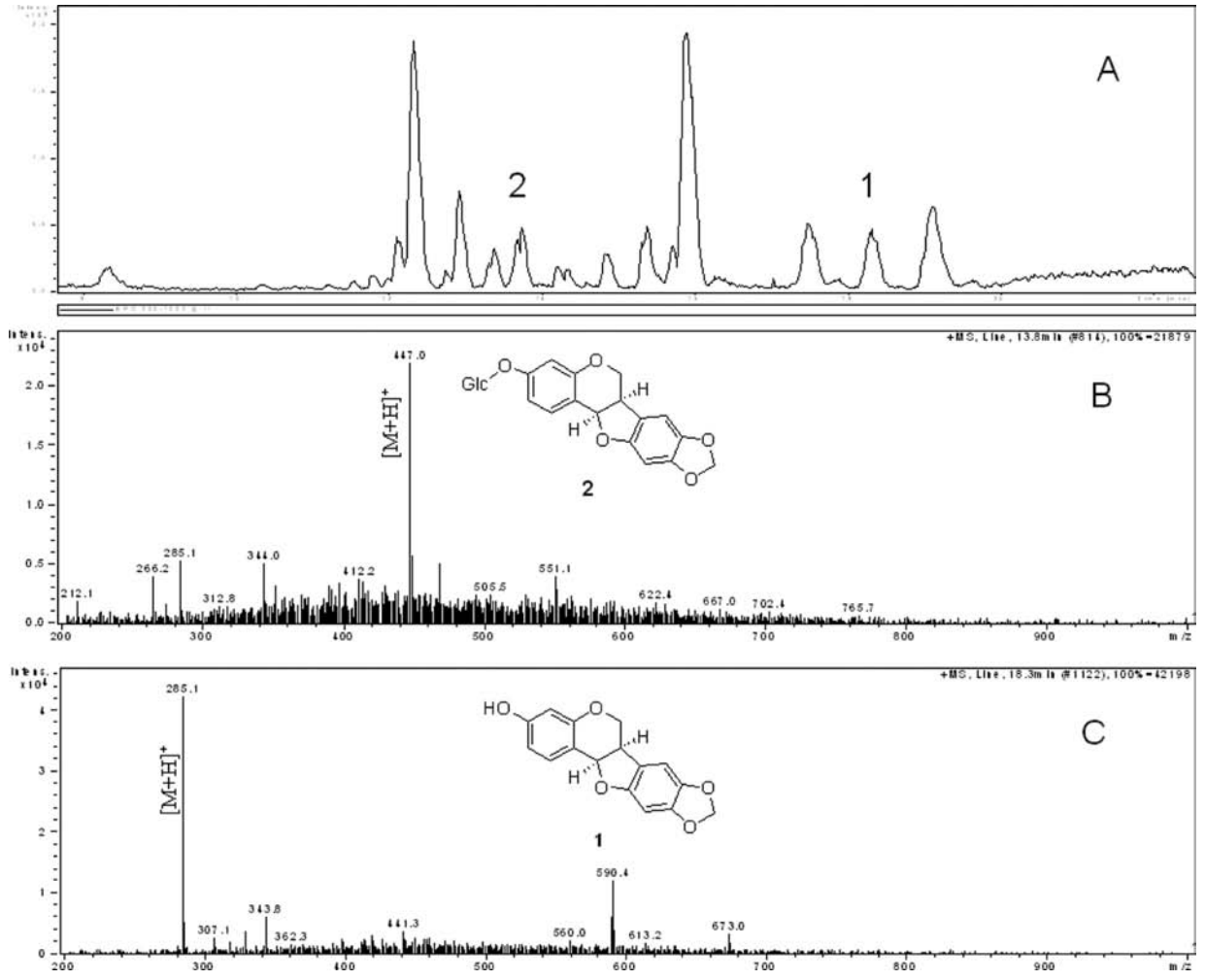


**Figure 5.** LC-MS analysis of the soil where *T. pratense* had grown for 1 year. Compounds **2** and **1** are shown in the total ion current (TIC) chromatogram with a scan range from $m/z$ 150−1000 (A) and have LC retention times of 13.8 and 18.3 min, respectively. Their mass spectra are listed above (B, C).

compound **1**, but the replacement with a hydrophilic group enlarged the applied range of compound **2**. Compound **1** exhibited stronger phytotoxic activity on *A. thaliana* seedlings than compound **3**, despite having the same skeleton, indicating that the existence of a 1,3-dioxalane at the C-4′ and C-5′ positions is also a critical factor for the phytotoxic activities in this kind of isoflavonoid. These compounds appear to be promising drugs of natural origin or biotic herbicides. However, further research will be required to determine their utility as pharmaceuticals or herbicides.

**Identification and Quantitation of Allelochemicals.** To elucidate the identities of allelochemicals that were produced by the plant during its periods of growth, HPLC analysis was carried out to detect the phytochemicals from the extracts of soil where the plants were grown. The HPLC chromatogram of the methanol extract from soil is shown in Figure 4. The detection at 275 nm was used in this study because most compounds had absorbance at this wavelength. Compounds **1** and **2** extracted from the soil were identified as allelochemicals, which was further confirmed by the internal standard method. Compared with the chromatogram of the soil, the peaks of compounds **1** and **2** used as internal standards were enhanced, and no new peak occurred. These compounds also appeared in the LC-MS chromatogram of the prepared soil sample (Figure 5). The standard curves were obtained using the linear regression method, and peak areas at 275 nm were plotted versus concentrations. Their concentrations in the soil were determined as 4.12 and 2.37 μg/g (14.5 and 5.31 nmol/g) of soil, respectively. Maackiain glycoside, **2**, was the most abundant in roots, whereas the concentration of its aglycone, **1**, was higher than that of the glycoside in soil. It has been reported that glucosidic conjugates serve as metabolic pools and can be mobilized by hydrolysis to release aglycones during elicitor treatment in red clover.[22] It can be suggested that conjugated forms of isoflavone may be hydrolyzed to the more active aglycone during periods of stress, and as suggested in the present study a higher concentration of compound **1** may also be transported into the soil.

**Phytotoxic Activities of the Allelochemicals on Weed *P. annua*.** It is well-known that plants commonly produce and accumulate secondary metabolites to protect themselves.[23] Compounds **1** and **2** as the allelochemicals of red clover showed remarkable phytotoxic activities against *P. annua* (Figure 6), which is one of the world's most widely distributed weed species. The relative inhibition rate of compound **1** was 50.7% at 50 μg/mL, which was greater than the rate of compound **2** (36.6%) at the same concentration.


dx.doi.org/10.1021/jf401241e | J. Agric. Food Chem. 2013, 61, 6321−6327

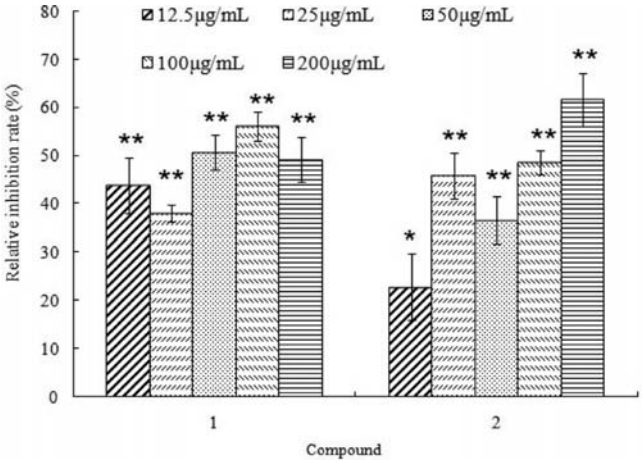


**Figure 6.** Phytotoxic activities toward *Poa annua* seedlings treated by compounds **1** and **2** for 1 week. Values are presented as percentage of the mean compared to the control. Means significantly lower than the DMSO controls are indicated with an asterisk (∗) (Dunnett's one-sided *t* test, $p < 0.05$) or two asterisks (∗∗) ($p < 0.01$). Error bars are one standard error of the mean. $N = 6$.

Compounds **1** and **2** possessed the same pterocarpan skeleton, a type of compound reported to exhibit varied activities, such as trypanocidal,[24] insect antifeedant,[25] and antibacterial[26] activities. Furthermore, compound **1** showed antifungal activity against *Candida* strains[27] and significant phytotoxic activities in this study. Compound **2** was also found to exhibit an anesthetic effect on juvenile stage 2 (J2) nematodes of *Ditylenchus destructor*.[28] These compounds in plants may contribute to the competition with neighboring plants and other organisms, and further research is needed to support this inference.

Compounds **1**−**5** were isolated as the major phytotoxins from the root extracts of *T. pratense* in the study, which had observably phytotoxic activities against *A. thaliana*. Compounds **1** and **2** were also identified as allelochemicals released into the soil and inhibited the growth of *P. annua*. Our findings suggest that the high levels of isoflavones found in red clover roots and soil may be utilized in many applications. Roots are able to exert an effect on other plants and soil microorganisms through the release of allelochemicals. The fitness of *T. pratense* appears to be increased through production of these chemicals, and then this plant becomes a successful competitor in natural ecosystems. The results suggest that *T. pratense* could be used as a component of a cropping system that exploits allelopathy as one stress factor in weed control, but for allelopathy to be fully exploited as a method of weed control, the mechanisms by which it operates must be better understood.

## AUTHOR INFORMATION

### Corresponding Author

*(B.Q.) Phone: +86-931-4968372. Fax: +86-931-8277088. E-mail: bqin@licp.cas.cn.

### Funding

This work was supported by the National Natural Science Foundation of China (No. 21102154), the Basic Research Program of Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences (080423SYR1), and the associate scholar program for talents cultivation plan of "Western Light" of the Chinese Academy of Sciences.

### Notes

The authors declare no competing financial interest.

## REFERENCES

(1) Wu, Z.; Song, L.; Feng, S.; Liu, Y.; He, G.; Yioe, Y.; Liu, S. Q.; Huang, D. Germination dramatically increases isoflavonoid content and diversity in chickpea (*Cicer arietinum* L.) seeds. *J. Agric. Food Chem.* **2012**, *60*, 8606−8615.

(2) Messina, M. J. Legumes and soybeans: overview of their nutritional profiles and health effects. *Am. J. Clin. Nutr.* **1999**, *70*, 439S−450S.

(3) Norbaek, R.; Aaboer, D. B. F.; Bleeg, I. S.; Christensen, B. T.; Kondo, T.; Brandt, K. Flavone *C*-glycoside, phenolic acid, and nitrogen contents in leaves of barley subject to organic fertilization treatments. *J. Agric. Food Chem.* **2003**, *51*, 809−813.

(4) Kong, C. H.; Zhao, H.; Xu, X. H.; Wang, P.; Gu, Y. Activity and allelopathy of soil of flavone *O*-glycosides from rice. *J. Agric. Food Chem.* **2007**, *55*, 6007−6012.

(5) Beck, V.; Unterrieder, E.; Krenn, L.; Kubelka, W.; Jungbauer, A. Comparison of hormonal activity (estrogen, androgen and progestin) of standardized plant extracts for large scale use in hormone replacement therapy. *J. Steroid Biochem. Molec. Biol.* **2003**, *84*, 259−268.

(6) Lin, L. Z.; He, X. G.; Lindenmaier, M.; Yang, J.; Cleary, M.; Qiu, S. X.; Cordell, G. A. LC-ESI-MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). *J. Agric. Food Chem.* **2000**, *48*, 354−365.

(7) Klejdus, B.; Vitamvásová, D.; Kubáň, V. Reversed-phase high-performance liquid chromatographic determination of isoflavones in plant materials after isolation by solid-phase extraction. *J. Chromatogr., A* **1999**, *839*, 261−263.

(8) Nissan, H. P.; Lu, J.; Booth, N. L.; Yamamura, H. I.; Farnsworth, N. R.; Wang, Z. J. A red clover (*Trifolium pratense*) phase II clinical extract possesses opiate activity. *J. Ethnopharmacol.* **2007**, *112*, 207−210.

(9) Kolodziejczyk-Czepas, J. *Trifolium* species-derived substances and extracts-biological activity and prospects for medicinal applications. *J. Ethnopharmacol.* **2012**, *143*, 14−23.

(10) Ohno, T.; Doolan, K.; Zibilske, L. M.; Liebman, M.; Gallandt, E. R.; Berube, C. Phytotoxic effects of red clover amended soils on wild mustard seeding growth. *Agric. Ecosyst. Environ.* **2000**, *78*, 187−192.

(11) Li, Z. H.; Shen, Y. X.; Ni, J. H.; Zhao, L. L. Apreliminary study on the allelopathy of legumes. *Pratacultural Sci.* **2002**, *19*, 28−31.

(12) Li, Z. H.; Shen, Y. X. Study on the allelopathy of the stems and leaves of red clover to the seeds and seedlings of several forage grasses. *Grassland of China* **2005**, *27*, 41−48.

(13) Wu, C. X.; Shen, Y. X.; Li, Z. H. Differences of allelopathic effect of different forage legumes on Italian ryegrass. *Grassland of China* **2005**, *27*, 39−43.

(14) Fischer, N. H.; Williamson, G. B.; Weidenhamer, D.; Richardson, D. R. In search of allelopathy in the Florida scrub: the role of terpenoids. *J. Chem. Ecol.* **1994**, *20*, 1355−1380.

(15) Queiroz, S. C. N.; Cantrell, C. L.; Duke, S. O.; Wedge, D. E.; Nandula, V. K.; Moraes, R. M.; Cerdeira, A. L. Bioassay-directed isolation and identification of phytotoxic and fungitoxic acetylenes from *Conyza canadensis*. *J. Agric. Food Chem.* **2012**, *60*, 5893−5898.

(16) Slade, D.; Ferreira, D.; Marais, J. P. J. Circular dichroism, a powerful tool for the assessment of absolute configuration of flavonoids. *Phytochemistry* **2005**, *66*, 2177−2215.

(17) Park, J. A.; Kim, H. J.; Jin, C.; Lee, K. T.; Lee, Y. S. A new pterocarpan, (−)-maackiain sulfate, from the roots of *Sophora subprostrata*. *Arch. Pharmacal Res.* **2003**, *26*, 1009−1013.

(18) Babu, U. V.; Bhandari, S. P. S.; Garg, H. S. Barbacarpan, a pterocarpan from *Crotalaria barbata*. *Phytochemistry* **1998**, *48*, 1457−1459.

(19) Donnelly, D. M. X.; Keenan, P. J.; Prendergast, J. P. Isoflavonoids of *Dalbergia ecastophyllum*. *Phytochemistry* **1973**, *12*, 1157−1161.

(20) Ali, A. A.; El-Emary, N. A.; El-Moghazi, M. A.; Darwish, F. M.; Frahm, A. W. Three isoflavonoids from *Iris germanica*. *Phytochemistry* **1983**, *22*, 2061−2063.
(21) Taylor, L. P.; Grotewold, E. Flavonoids as developmental regulators. *Curr. Opin. Plant Biol.* **2005**, *8*, 317−323.
(22) Tebayashi, S.; Ishihara, A.; Iwamura, H. Elicitor-induced changes in isoflavonoid metabolism in red clover roots. *J. Exp. Bot.* **2001**, *52*, 681−689.
(23) Hemaiswarya, S.; Kruthiventi, A. K.; Doble, M. Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine* **2008**, *15*, 639−652.
(24) Vieira, N. C.; Espíndola, L. S.; Santana, J. M.; Veras, M. L.; Pessoa, O. D. L.; Pinheiro, S. M.; Araújo, R. M.; Lima, M. A. S.; Silveira, E. R. Trypanocidal activity of a new pterocarpan and other secondary metabolites of plants from northeastern Brazil flora. *Bioorg. Med. Chem.* **2008**, *16*, 1676−1682.
(25) Morimoto, M.; Fukumoto, H.; Hiratani, M.; Chavasiri, W.; Komai, K. Insect antifeedants, pterocarpans and pterocarpol, in heartwood of *Pterocarpus macrocarpus* Kruz. *Biosci., Biotechnol., Biochem.* **2006**, *70*, 1864−1868.
(26) Rukachaisirikul, T.; Innok, P.; Aroonrerk, N.; Boonamnuaylap, W.; Limrangsun, S.; Boonyon, C.; Woonjina, U.; Suksamrarn, A. Antibacterial pterocarpans from *Erythrina subumbrans*. *J. Ethnopharmacol.* **2007**, *110*, 171−175.
(27) Meragelman, T. L.; Tucker, K. D.; McCloud, T. G.; Cardellina, J. H.; Shoemaker, R. H. Antifungal flavonoids from *Hildegardia barteri*. *J. Nat. Prod.* **2005**, *68*, 1790−1792.
(28) Xu, R.; Jin, H.; Liu, Q.; Yan, Z. Q.; Cui, H. Y.; Qin, B. Anesthetic activity of (6a*R*,11a*R*)-trifolirhizin from *Trifolium pratense* to *Ditylenchus destructor*. *Nat. Prod. Res. Dev.* **2011**, *23*, 820−823.