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Phytotoxic Effect, Uptake, and Transformation of Biochanin A in Selected Weed Species

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ABSTRACT: Certain isoflavones are plant growth inhibitors, and biochanin A is a major isoflavone in clover species used for weed management. The effect of biochanin A on the monocot weed species *Echinochloa crus-galli* L. and *Lolium perenne* L. and dicot species *Silene noctiflora* L., *Geranium molle* L., and *Amaranthus caudatus* L. was evaluated in agar medium bioassays. *S. noctiflora* and *G. molle* root growth was progressively inhibited with increasing concentrations of biochanin A, whereas the monocot species were unaffected. With regard to the dicot species, *S. noctiflora* (EC_{50} = 35.80 μM and EC_{25} = 5.20 μM) was more susceptible than *G. molle* (EC_{50}, EC_{25} > 400 μM). *S. noctiflora*, *G. molle*, and *E. crus-galli* root and shoot samples, representing a susceptible, a less susceptible, and a nonsusceptible species, respectively, were analyzed by LC-MS to quantify biochanin A and its transformation products. Biochanin A and its known transformation products genistein, dihydrobiochanin A, pratensein, and *p*-coumaric acid were quantified. Sissotrin was identified and quantified while assigning unknown peaks. The treated root samples contained more biochanin A, genistein, pratensein, and dihydrobiochanin A than shoot samples.

KEYWORDS: *biochanin A, Silene noctiflora, Geranium molle, LC-MS, phytotoxic, uptake, transformation, sissotrin*

INTRODUCTION

Weeds compete with crops for nutrients, sunlight, and water and harbor insects and pests, thereby reducing yield and causing significant economic losses.[1−3] Weeds can be controlled mechanically,[4−6] but in modern agriculture weeds are controlled with synthetic herbicides, the widespread use of which has raised concerns about negative impacts on human health, wildlife, and the environment.[7−9] Integrated weed management is currently being promoted as the solution to reduce the reliance on herbicides and to minimize the adverse effects. For example, the European Union (EU) recently adopted Directive 2009/128/EC establishing a framework to promote the use of integrated pest management and alternative approaches to pest control to mitigate the negative consequences of pesticide use.[10]

Bioherbicides, which are organic compounds of biological origin, are an alternative to synthetic herbicides. In plants, these compounds are called allelochemicals, and they are believed to be the natural defense compounds of plants against weeds, diseases, and pests.[11,12] The isoflavonoids, including biochanin A, are an example of allelochemicals found in high concentrations in clover.[13,14]

The isoflavonoids are a large and distinctive subclass of the flavonoids. Some isoflavonoids have been found to be more potent antioxidants than the corresponding flavonoids.[15] Isoflavonoids are involved in interactions between plants and other organisms including bacteria, fungi, herbivorous insects, mollusks, and vertebrates.[16] Flavonoids are found throughout the plant kingdom, whereas isoflavonoids are largely restricted to members of the Fabaceae, such as soybean, mungbean, Japanese arrowroot, sprouts, kidney beans, and white clover.[13,17,18] They are, however, occasionally found in other families.[19]

Biochanin A (Figure 1) is an isoflavonoid present in legumes such as red[20] and white clover.[13,21] Biochanin A has been

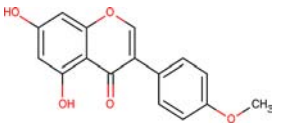

Figure 1. Structure of biochanin A.

reported to possess antifungal activity,[20,21] selective inhibitory effects on bacteria,[22] and a stimulating activity on soil microorganisms and the alga *Chlorella vulgaris*.[23,24] Furthermore, both biochanin A and its first degradation product, genistein,[25,26] can inhibit the growth of cucumber and certain plants.[27]

The objectives of this study were to investigate the phytotoxic effect of the isoflavonoid biochanin A on selected monocot and dicot weed species and a dicot crop, to quantify the uptake of biochanin A, and to identify and quantify the transformation products using LC-MS.

MATERIALS AND METHODS

Biological Materials. Seeds of two monocot weeds, *Echinochloa crus-galli* L. and *Lolium perenne* L., two dicot weeds, *Silene noctiflora* L. and *Geranium molle* L., and one dicot agricultural crop, *Amaranthus caudatus* L. (cultivated amaranth), were obtained from the seed bank of the Department of Agroecology at Aarhus University. The species were selected to represent commonly encountered weeds and for their

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high germination frequency. *A. caudatus* was included as a nontarget cultivated species.

Solvents and Chemicals. Standard HPLC grade solvents (methanol, acetone, acetonitrile, ethyl acetate, and ethanol) used for chromatography, extraction, and phytotoxicity tests were purchased from Rathburn (Walkerburn, Scotland). Glacial acetic acid and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Milli-Q water was used for chromatography. Biochanin A was obtained from Sigma-Aldrich (Schnelldorf, Germany). Phytochemical standards were purchased from several suppliers: Genistein was purchased from Lancaster Synthesis (Karlsruhe, Germany); *p*-coumaric acid, phloroglucinol, 3,4-dihydroxyphenylacetic acid, and *p*-hydroxybenzoic acid were purchased from Fluka (Schnelldorf, Germany); and pratensein and dihydrobiochanin A were purchased from Apin Chemicals (Abingdon, England).

Miscellaneous Supplies. Agar was purchased from Sigma-Aldrich (Schnelldorf, Germany). Polystyrene containers for plant cultivation were purchased from Greiner Bio-One (Kremsmünster, Austria). Ottawa sand was purchased from Fischer Scientific (Waltham, MA, USA).

Dose−Response Bioassays for Estimation of Inhibitory Effects. Eight doses of biochanin A (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μM) were applied to five plant species (*E. crus-galli*, *L. perenne*, *S. noctiflora*, *G. molle*, and cultivated amaranth). Four replicates were used for each treatment. Biochanin A solutions were prepared in methanol. One milliliter of methanolic biochanin A solution was poured into each Petri dish and allowed to evaporate. After evaporation, 20 mL of 0.5% agar solution was poured into the Petri dishes, agitated gently, and allowed to set. Seeds of each of the five weed species were pregerminated at room temperature for 48 h, and 10 pregerminated seeds (1−2 mm radicle length) were transplanted to each Petri dish. The plants were grown under controlled conditions at 24 ± 1 °C. Light was supplied by an Osram (Munich, Germany) 250 W HQI-T bulb of 46 mm diameter and 80 lm·W^{-1} luminous efficacy during a photoperiod of 16 h light and 8 h dark.

The plants were grown for 2 weeks, and on the basis of visual estimation of the inhibitory effect on root growth, *S. noctiflora* and *G. molle* were selected for root length measurement. The root growth of *S. noctiflora* and *G. molle* was measured, and dose−response curves were prepared (Figure 2). The root length of the other three species was not measured as no effect was visible. The experimental work was carried out between September 2010 and March 2011.

Statistical Analysis and Software Used for Evaluation of Bioassays. Nonlinear regression was applied to the data from the dose−response experiments using SAS (Cary, NC, USA) software. A four-parameter equation was used to describe the dose−response curves of biochanin A for each weed species. The relationship of root length (U) to dose was assumed to follow a log−logistic four-parameter model:[28]

$$U_i = C_i + \frac{D_i - C_i}{1 + \exp(b_i(\log(x) - \log(EC_{50i})))} \quad (1)$$

U_i denotes the root length of weed species i as a function of the dose (x). D_i and C_i denote the upper and lower asymptotes of root length at zero and infinite doses, respectively. EC_{50i} denotes the concentration of biochanin A required to reduce root length by 50% (halfway between D and C), and b_i is proportional to the slope of the curve at EC_{50i}.

By reparametrizing eq 1, the EC_{50} parameter can be replaced by any EC parameter, for example, EC_{25}. If the lower limit does not differ from zero, the equation can be reduced to a three-parameter model:

$$U_i = \frac{D_i}{1 + \exp(b_i(\log(x) - \log(EC_{50i})))} \quad (2)$$

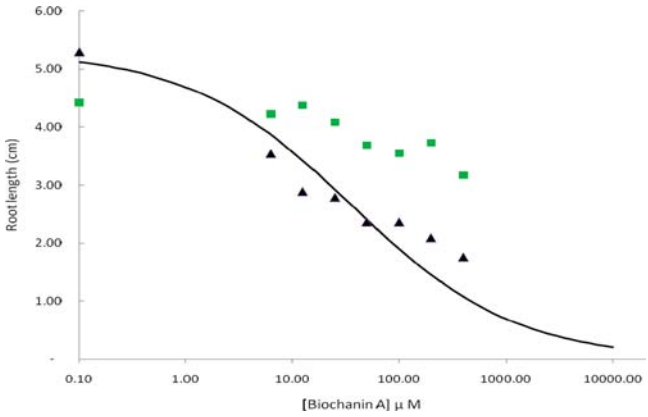

Figure 2. Log−logistic dose−response curve illustrating the growth inhibitory effect of biochanin A on the roots of *S. noctiflora*. Black triangles and line indicate the observed root length and the estimated dose−response curve of *S. noctiflora*, respectively. Green squares indicate the observed root length of *G. molle*.

Bioassays for Production of Plant Material for Chemical Analysis. Further experiments were performed in growth medium containing biochanin A in a setup where sufficient biomass could be produced for chemical analysis. The two dicot weed species, *S. noctiflora* and *G. molle*, and one monocot species, *E. crus-galli*, were selected for larger scale bioassays in polystyrene containers (diameter = 68 mm, height = 100 mm). Two replicates per treatment were used, each consisting of 14 polystyrene containers for *S. noctiflora* and 9 containers for *G. molle* and *E. crus-galli*. The seeds of *S. noctiflora*, *G. molle*, and *E. crus-galli* were pregerminated for 48 h at room temperature on filter paper that had been moistened with deionized water. One milliliter of one of three different concentrations of methanolic biochanin A (to produce a final concentration of 0, 20, or 400 μM) was dispensed into each of the containers and evaporated. Twenty milliliters of 0.5% agar medium was then added, and 14 pregerminated seeds were transplanted to the agar in each container. The plants were cultivated at 24 ± 1 °C using the light source and photoperiod previously described. Plants were harvested 14 days after transplanting to the containers. The roots and shoots of the plants were collected separately, dried with blotting paper, washed twice with Milli-Q water, and again blotted to make sure no agar was left on the plant material. The fresh and dry weights of the plant samples were recorded before and after freeze-drying, respectively. The samples were then stored at −18 °C until analysis.

Preparation of Standards for LC-MS Analysis. Standard stock solutions of biochanin A, genistein, pratensein, dihydrobiochanin A, *p*-coumaric acid, phloroglucinol, 3,4-dihydroxyphenylacetic acid, and *p*-hydroxybenzoic acid were prepared by dissolving the solid compounds in methanol. All of the reagents were weighed on a Sartorius (Göttingen, Germany) analytical balance and were dissolved in methanol in a volumetric flask to make a 10 mg·L^{-1} solution. The stock solution was diluted by a factor of 25 to produce a 400 μg·L^{-1} standard solution. Sequential dilutions were subsequently carried out to produce solutions of 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.39 μg·L^{-1}. Calibration curves were prepared by recording negative-mode LC-ESI-MS responses for the series of 11 solutions of known concentrations (see Tables 1 and 2 for source- and compound-dependent parameters).

Table 1. Source-Dependent Negative-Mode LC-ESI-MS Parameters

parameter	value
curtain gas (CUR)	11 psi
temperature (TEM)	600 °C
ion source gas 1 (GS1)	40 psi
ion source gas 2 (GS2)	65 psi
interface heater (ihe)	on
ion spray voltage (IS)	−4200 V
entrance potential (EP)	−4 V

Table 2. Compound-Dependent Negative-Mode LC-ESI-MS Parameters: Retention Time (t_R), Mass-to-Charge Ratio (m/z), and Declustering Potential (DP) for Selected Ion Monitoring of Isoflavonoids and Phenolic Acids

compound	$[M - H]^-$ (m/z)	t_R (min)	DP (V)
phloroglucinol	125.0	5.6	−22
p-hydroxybenzoic acid	137.2	16.5	−24
p-coumaric acid	163.2	17.9	−22
3,4-dihydroxyphenylacetic acid	167.0	14.2	−22
genistein	269.0	20.4	−55
biochanin A	283.4	23.4	−50
dihydrobiochanin A	285.4	22.6	−55
pratensein	299.3	20.6	−50

Extraction of Plant Samples Using Accelerated Solvent Extraction (ASE). Samples were lyophilized in a Heto Drywinner 6-85 freeze-dryer from Holm and Halby (Brøndby, Denmark) for 48 h and crushed to a powder. The crushed plant material was extracted using a Dionex (Sunnyvale, CA, USA) ASE 350 accelerated solvent extraction system. Thirty-three milliliter extraction cells were prepared as follows: A filter was placed at the bottom of the cell, and 5 g of chemically inert Ottawa sand was added on top of it. The entirety of each individual plant sample was then transferred to its respective cell. Finally, another 5 g of Ottawa sand followed by a second filter was added. The remaining volume of the extraction cells was filled with glass beads. The extraction solvent contained 70% methanol, 1% acetic acid, and 29% water by volume. The ASE extraction protocol was as follows: preheat for 5 min, heat for 5 min, 3 min static time, rinse volume 60%, purge for 60 s, 3 cycles, pressure 1600 psi, and temperature 40 °C.

The extracts were collected in 50 mL glass tubes, weighed, and stored at −18 °C. Each sample was concentrated using a Zymark Turbovap LV evaporator from Sotax (Allschwil, Switzerland) and stored at −18 °C until chemical analysis.

Analysis of Known and Unknown Transformation Products by LC-MS. The extracts were diluted with Milli-Q water in a 1:1 ratio and filtered through Frisenette (Knebel, Denmark) Q-Max 0.45 μm PTFE membrane syringe filters before analysis. Chromatography was carried out on an Agilent (Santa Clara, CA, USA) 1200 series HPLC using an AB Sciex (Framingham, MA, USA) 3200 QTrap system with electrospray ionization for mass spectrometric detection. The chromatographic data processing was carried out using Analyst 1.5.1 software.

The plant samples were analyzed for biochanin A, and its seven known transformation products. As standards for those eight compounds were available in the laboratory, they were termed "known compounds". Five of the known compounds were quantified. Chromatograms were recorded using negative-mode selected ion monitoring (SIM) for analysis of known compounds. See Tables 1 and 2 for the ESI-MS parameters used.

Unknown peaks were observed that shared masses with the known compounds, but differed in retention time. On the basis of the literature,[13,26] additional masses in the m/z range 120−540, corresponding to compounds of potential interest, including hypothesized derivatives of biochanin A, were monitored to determine whether biochanin A underwent any of several common transformation reactions (glycosylation, methylation, hydroxylation, oxidation, malonylation, or acetylation). For unknown compounds, both positive- and negative-mode SIM were used. The positive-mode parameters were identical in magnitude to the negative-mode parameters listed in Tables 1 and 2.

Chromatographic separation was performed on a Phenomenex (Torrance, CA, USA) Synergi Fusion-RP 80A column (250 mm × 2.1 mm i.d., particle size = 4 μm) at a flow rate of 0.2 mL·min^{-1}, temperature of 30 °C, and injection volume of 20 μL. The mobile phase consisted of two eluents (eluent A, 7% acetonitrile, 93% water, and 20 mM acetic acid; eluent B, 78% acetonitrile, 22% water, and 20 mM acetic acid), and the following gradient was applied: 0−3 min, isocratic at 100% A; 3−16 min, ramp to 20% A; 16−27 min, ramp to 0% A; 27−28 min, isocratic at 0% A; and then 28−38 min, ramp back to 100% A. Source-dependent LC-ESI-MS parameters are given in Table 1, and the masses monitored as well as compound-dependent parameters are given in Table 2. Biochanin A (m/z 283, t_R = 23.4 min), pratensein (m/z 299, t_R = 20.6 min), genistein (m/z 269, t_R = 20.4 min), dihydrobiochanin A (m/z 285, t_R = 22.6 min), *p*-coumaric acid (m/z 163, t_R = 17.9 min), phloroglucinol (m/z 125, t_R = 5.6 min), *p*-hydroxybenzoic acid (m/z 137, t_R = 16.5), and 3,4-dihydroxyphenylacetic (m/z 167, $3t_R$ = 14.2) were identified in plant samples by comparison of masses and retention times with commercial standards (Table 2).

Recovery Assessment. Rye green leaves containing no biochanin A were used as the matrix for preparation of spiked samples for recovery assessment. Recovery assessments for biochanin A, genistein, pratensein, dihydrobiochanin A, and *p*-coumaric acid were performed at two concentration levels. Six replicates were performed at each concentration. A mixed standard solution was used to spike samples with biochanin A, genistein, pratensein, dihydrobiochanin A, and *p*-coumaric acid at concentrations of 5.0 and 50.0 ng·g^{-1} for the lower and higher doses, respectively. Two blank samples without standards were prepared as controls. All samples were extracted and analyzed as described previously.

Limits of Detection and Quantification. For each compound, the limit of detection (LOD) was determined as $3 \times s$ and the limit of quantification (LOQ) as $10 \times s$, s being the standard deviation of the concentration of the relevant compound over six spiked samples.[29]

Preparation of Standards in Matrix. To evaluate the matrix effect, a dilution series of standard compounds in rye top matrix was prepared from 800 to 0.19 ng·mL^{-1} of the stock solution containing biochanin A, dihydrobiochanin A, genistein, pratensein, and *p*-coumaric acid. The matrix mixture was made containing rye green leaf extract and Milli-Q water in the ratio of 1:1 for maintaining the same dilution level as the standard without matrix.

Standard Curves and Final Quantification. Standard curves were fitted in the range of 1.56−400 ng·mL^{-1}. Standard curves both with and without matrix were prepared for analyzing the different samples. Quadratic regressions with a $1/x$ weighting were used for each analyte. Five of eight known compounds were quantified.

Multivariate Data Analysis. Principal component analysis (PCA) was performed on the basis of peak areas of known and unknown compounds using LatentiX 2.00 software from Latent5 (Frederiksberg, Denmark). Two PCA models were constructed: One was a mean-centered model based on the areas of 73 assigned but unknown peaks in *S. noctiflora* root samples and was used to search for potential marker compounds of biochanin A exposure. The unknown peaks were assigned labels following manual alignment of the chromatograms by visual inspection and compensation for retention time drift. The other was a unit variance-scaled model restricted to the eight known compounds and used to illustrate the variation among species, tissues, and biochanin A doses applied.

The areas of visible and resolved peaks were collected and tabulated (after manual alignment) for all of the mass-to-charge ratios deemed from the literature to be of interest.[13,26] Assignment of peaks was verified both across and within sample chromatograms, because any given peak within a sample chromatogram was required to be similarly displaced from the mean retention time calculated for that peak across all sample chromatograms in which it occurred. If the observed shift in retention time was inconsistent with other assigned peaks, the assignment was discarded or revised. PCA of these data was used to suggest relevant peaks as candidates for identification efforts.

RESULTS AND DISCUSSION

Inhibitory Effect of Biochanin A on Weeds and Cultivated Amaranth. A test for lack of fit[28] did not reject the assumption that the three-parameter model could be used to describe the data from the dose−response experiments. The effect of biochanin A on *S. noctiflora* and *G. molle* root growth is presented in Figure 2. A reduction in root growth with

increasing concentrations of biochanin A was observed in *S. noctiflora* and *G. molle*, but even the highest dose did not produce a 100% effect. The effective concentration (EC) for inhibiting the root growth of *S. noctiflora* (see Table 3) was

Table 3. Effective Concentrations (EC, μM) for *S. noctiflora*

	estimated concn	standard error	95% confidence limit
EC_{25}	5.2	2.0	1.0−9.4
EC_{50}	35.8	9.9	15.5−56.1

determined using eq 2. The EC_{25} and EC_{50} values of *S. noctiflora* were 5.2 and 35.8 μM, respectively. Dose−response curves could not be fitted for *G. molle* because the highest applied dose (400 μM) produced <50% effect.

On the basis of visual estimation there was no difference in root inhibition between the control sample and higher doses of biochanin A in *E. crus-galli*, *L. perenne*, or cultivated amaranth. The differences in growth reduction between 100 and 400 μM concentrations were small. No references were found describing phytotoxic effects of biochanin A, although a study has been published showing that a biochanin A concentration of 100 μM inhibited the formation of adenosine triphosphate (ATP) in cucumber hypocotyl mitochondria,[27] and our study appears to be the first report of the effects of biochanin A on plant growth.

No information is available on the possible mode of action of biochanin A. We speculate that a likely mechanism may be that lipoxygenase enzymes used by *S. noctiflora* for fatty acid oxidation were inhibited by the biochanin A transformation product genistein, resulting in impaired root growth. A positive correlation between lipoxygenase enzymes and growth activity during the early stage of seedling growth was reported,[30] and a recent study showed that lipoxygenase activity was inhibited by the isoflavones genistein and daidzein.[31] Further study is needed to elucidate the mode of action of the inhibitory effect of biochanin A on plant growth.

Validation of Analytical Methods. Recovery experiments were carried out to validate the extraction protocol. Ground rye leaf samples were spiked at either 5 or 50 ng·g^{-1} to assess the recovery values of biochanin A, dihydrobiochanin A, genistein, pratensein, and *p*-coumaric acid. The results are summarized in Table 4. Recovery studies are an essential component of the validation and use of all analytical methods. In this study, the recovery values achieved were in the range of 65−100% for samples spiked to a concentration of 50 ng·g^{-1} and in the range of 76−104% for samples spiked to a concentration of 5 ng·g^{-1}. At the higher spiked concentration, relative standard deviations (RSD) were in the range of 7−18%, and at lower concentration they were 9−19%. According to the European Union Reference Laboratories for Residues of Pesticides (EURL RP),[32] the recovery values for a quantitative analytical method should be 70−120% and the RSD <20%. The recovery results from this study were within the range of 70−120% and agreed with the EURL RP, except for genistein (65%). Recovery values below 70% are acceptable, however, if the results are consistent, and for genistein the mean recovery value was 65% with an RSD of 12%, agreeing with the demands of the EURL RP.[32] In the case of *p*-coumaric acid spiked to 5 ng·g^{-1} the RSD was too large as the peaks were too small to be reliably integrated by the Analyst software. The recovery values were not used to correct the analytical results according to Fajgelj.[33] The LOD and LOQ of the analytical method were calculated according to Eurachem guidelines,[29] and their values are presented in Table 4. In all samples except controls, the concentrations of biochanin A, dihydrobiochanin A, genistein, and pratensein were found to be above the LOD. For *p*-coumaric acid, however, the concentrations were not always above the LOD. Values below the LOD are included because current IUPAC guidelines recommend that results below the LOD should be reported as found if the experimental data are reliable.[34]

Biochanin A Uptake in Weed Species. In *S. noctiflora*, there was no peak for biochanin A in the control plant but a visible peak in the sample treated with 400 μM biochanin A. In the case of *G. molle* and *E. crus-galli*, a very minor peak was visible in the control sample, whereas a distinctive peak was visible in plant samples that had been treated with biochanin A. *G. molle* showed a higher uptake than *S. noctiflora* and *E. crus-galli*.

Biochanin A and its transformation products (Figure 3) dihydrobiochanin A, genistein, pratensein, and *p*-coumaric acid were quantified in root and shoot samples of each species (Table 5). The uptake of biochanin A was higher in *G. molle* than in *S. noctiflora* and *E. crus-galli*.

The control samples of *G. molle* and *E. crus-galli* contained biochanin A, suggesting that the plants themselves contain some biochanin A. No literature was found, however, that

Table 4. Recovery Values, Limits of Detection (LOD), and Limits of Quantification (LOQ) of Biochanin A and Its Transformation Products from Rye Leaf Matrix, n = 6

compound	concn (ng·g^{-1})	% recovery	RSD (%)	LOD (μmol·g^{-1})	LOQ (μmol·g^{-1})
biochanin A	5	93	14	0.01	0.02
	50	93	16		
dihydrobiochanin A	5	104	18	0.01	0.03
	50	76	9		
genistein	5	81	18	0.01	0.03
	50	65	12		
pratensein	5	98	19	0.01	0.04
	50	101	17		
p-coumaric acid	5	79	81	0.06	0.19
	50	81	18		

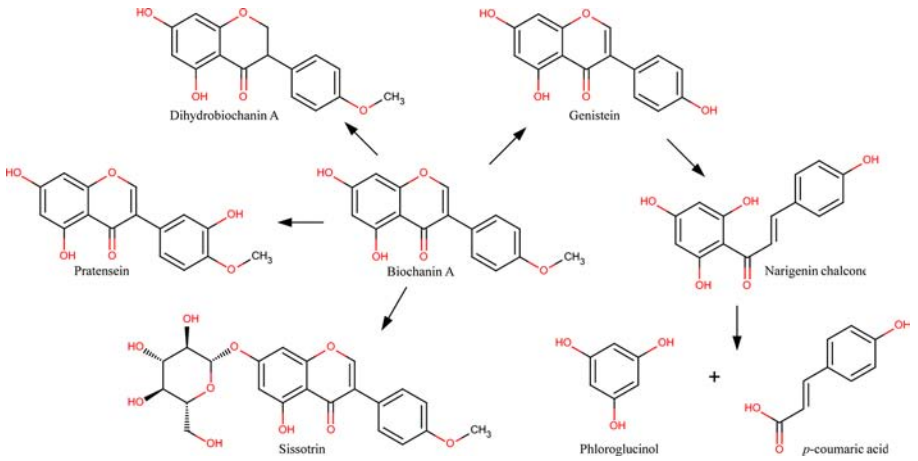

Figure 3. Partial transformation pathways of biochanin A (adapted from Rao and Cooper;[37] copyright 1995 APS Press).

Table 5. Concentrations of Biochanin A and Its Known Transformation Products in the Roots and Shoots of Selected Weed Species after Treatment with Biochanin A

		av concn in plant samples (μmol·g^{-1} dry weight, n = 2)									
		biochanin A		dihydrobiochanin A		genistein		pratensein		*p*-coumaric acid	
species	dose (μM)	root	shoot	root	shoot	root	shoot	root	shoot	root	shoot
S. noctiflora	0	0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0	nd	nd
	20	4.65	0.76	0.01	0.00	0.08	0.24	nd	0.10	nd	nd
	400	4.54	1.11	0.07	0.02	0.13	0.15	0.11	0.06	nd	0.01
G. molle	0	0.14	0.00	0.16	0.06	0.90	0.18	0.00	0.00	0.16	nd
	20	9.93	1.00	0.38	0.01	0.05	0.09	0.66	0.02	0.06	nd
	400	11.46	1.13	0.31	0.02	0.08	0.12	0.32	0.05	0.05	0.03
E. crus-galli	0	0.20	0.00	0.00	0.00	0.00	0.06	0.41	0.13	0.21	0.15
	20	1.75	0.05	0.05	0.00	0.07	0.28	0.38	0.47	0.15	nd
	400	2.13	0.67	0.27	0.27	0.12	0.20	0.35	0.24	0.11	nd

reported biochanin A from the two species. One study reported that plants from the family Poaceae, to which *E. crus-galli* belongs, contained isoflavonoids.[19] This is consistent with our observation that *E. crus-galli* contained the isoflavonoid biochanin A. No literature was found describing the occurrence of isoflavonoids in the family Geraniaceae, to which *G. molle* belongs. Both replicates of the two species contained biochanin A, and the concentrations were above the LOD. Contamination of the containers used for cultivation with biochanin A is an unlikely explanation because each container was used only once for cultivating plants. There was thus no reason to immediately discard the result as an artifact, but further experiments should be carried out to confirm this unexpected finding.

Concentrations of Biochanin A and Its Known Transformation Products. Both control and treated samples of each species were analyzed for biochanin A and its transformation products. Quantification was performed separately for root and shoot samples, and the results are shown in Table 5.

Biochanin A concentrations were higher in root samples than in shoot samples. There was only a small difference in the uptake of biochanin A between the sample treated with 20 μM and the sample treated with 400 μM, and this may be due to the limited solubility of biochanin A in the agar medium. The applied method does not distinguish between surface adsorption and actual uptake of biochanin A. Concentrations are therefore reported in accordance with common practice in herbicide uptake studies, consisting both of the amount of a given metabolite taken up by the seedlings and that remaining adsorbed to the surface after the seedlings have been rinsed. The suitability of the method is evidenced by the general correlation between the amount of biochanin A applied and taken up, as well as that transformed to the 7-*O*-glucoside sissotrin (Table 6).

The quantification results illustrated the difference between the plant species. *S. noctiflora* was more susceptible to biochanin A, but the uptake of biochanin A in this species was less than in *G. molle*. A possible explanation is that due to the inhibitory effect of biochanin A on root growth, the roots did not penetrate the agar as well as the roots of *G. molle*. The uptake of biochanin A was less in *E. crus-galli* than in both of the two susceptible species, even though *E. crus-galli* had longer roots. The concentrations in the shoots of the two susceptible species were similar to each other, but the shoots of the nonsusceptible species contained less biochanin A.

Different concentrations of biochanin A transformation products (dihydrobiochanin A, genistein, pratensein, and coumaric acid) were observed in the analyzed samples. Higher concentrations of these transformation products were seen in the root samples than in shoot samples. In a previous study,[17] the total concentration of 23 flavonoids was determined in white clover (*Trifolium repens* L.) to be 8 μmol·g^{-1}, with the

Table 6. Concentration of Sissotrin in Roots and Shoots of Selected Weed Species

species	dose of biochanin A (μM)	av concn in sample (μmol·g^{-1} dry weight, n = 2) root	shoot
S. noctiflora	0	0.00	0.00
	20	1.72	2.36
	400	2.99	4.34
G. molle	0	0.00	0.00
	20	36.06	1.50
	400	123.61	2.41
E. crus-galli	0	0.00	0.00
	20	0.48	0.00
	400	1.00	0.00

concentration being larger in aboveground parts (6.3−16.1 μmol·g^{-1}) than in roots (3.5 μmol·g^{-1}). By comparison, the present study resulted in biochanin A concentrations of 10 μmol·g^{-1} in the roots of *G. molle* and 5 μmol·g^{-1} in the roots of *S. noctiflora* grown on 20 μM biochanin A and only a small increase in uptake when the dose was increased 20-fold. The lower aboveground biochanin A concentration observed in *S. noctiflora* and *G. molle* in the present study (approximately 1 μmol·g^{-1}) is consistent with an adverse reaction due to uptake of external biochanin A.

Principal Component Analysis for Known Compounds. PCA was carried out using the peak areas of four known isoflavonoids (biochanin A, dihydrobiochanin A, genistein, and pratensein) and four phenolic acids (*p*-coumaric acid, phloroglucinol, 3,4-dihydroxyphenylacetic acid, and *p*-hydroxybenzoic). Mean-centered data scaled to unit variance were used for PCA, and the first two components explained 63% of the total variation (see Figure 4). In the PCA score plot, control and shoot samples were observed to cluster, whereas root samples were separated according to the doses of biochanin A applied. This separation was particularly pronounced for *S. noctiflora* (in red). In the PCA loading plot, biochanin A, dihydrobiochanin A, and genistein appeared to be correlated. Pratensein, *p*-coumaric acid, and *p*-hydroxybenzoic acid were correlated, whereas phloroglucinol and 3,4-dihydroxyphenylacetic acid did not appear to be correlated.

Assignment of Unknown Peaks and Identification of Additional Transformation Products. For assignment of unknown peaks, all of the visible peak areas in the chromatograms were collected on the basis of retention time (t_R). Peak areas were collected from both the positive and negative mode data recorded for samples of *S. noctiflora* roots and used to try to identify unknown compounds.

PCA was performed by using areas of assigned but unidentified peaks in the root samples of *S. noctiflora* (Figure 5). The *S. noctiflora* root samples were selected because the samples were clearly separated along principal component 1 according to the dose of biochanin A (Figure 5) and because *S. noctiflora* was the most susceptible of the species tested. The resulting PCA model illustrated relevant peaks for identification on the basis that they appeared to be explanatory of the separation of samples according to dose of biochanin A. Six objects and 73 variables were included in the PCA, and mean-

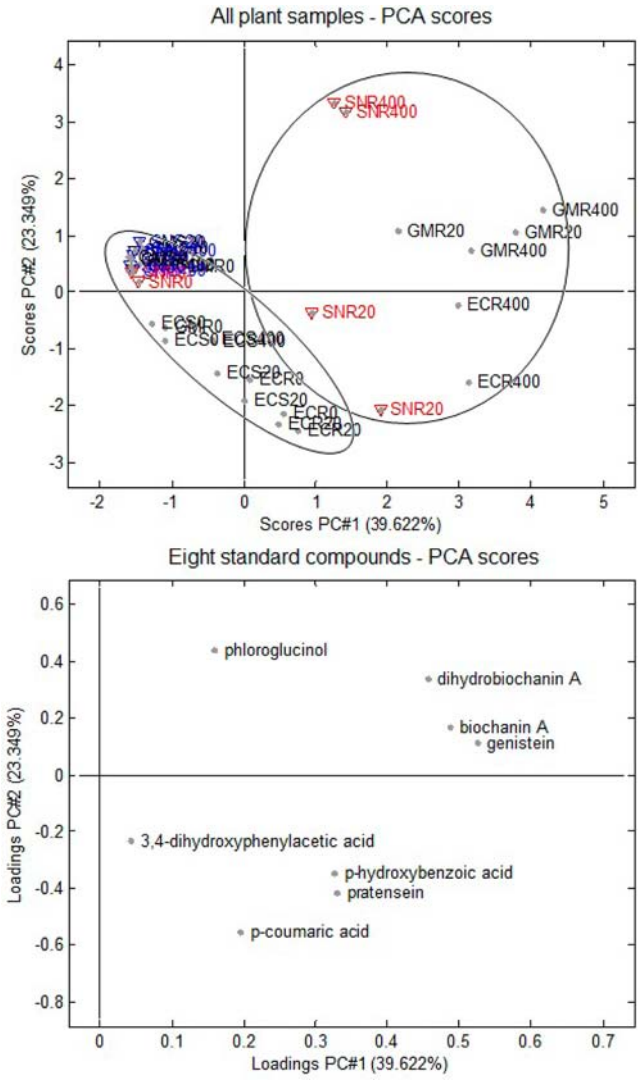

Figure 4. PCA scores and loadings for biochanin A and its transformation products in root and shoot samples. The small ellipse indicates the clustering of all shoot and control samples, and the large circle indicates the separation of different root samples. *S. noctiflora* root samples are indicated in red, and *S. noctiflora* shoot samples are indicated in blue. Data points are labeled according to plant sample (SN, *S. noctiflora*; GM, *G. molle*; EC, *E. crus-galli*), tissue (R, roots; S, shoots), and biochanin A dose (0, 20, or 400 μM).

centered but unscaled data were used. Both the positive- and negative-mode SIM data were used for PCA and were compared for identification of unknown compounds. Principal component 1 explained 96% of the variation because nearly all of the variation stemmed from the increasing doses of biochanin A.

Most of the labeled masses, for example, 285E (t_R 23.54 min), 285C (t_R 18.26 min), 271A (t_R 22.15 min), 285D (t_R 18.81 min), 301A (t_R 23.54 min), and 447A (t_R 18.81 min) were clustered near the center, but some of them were displaced from the origin (Figure 5). The compound labeled 447A had a mass of 447 in positive mode, corresponding to $[M + H]^+$. The actual mass was 446, and the retention time was 18.80 min, which suggested a biochanin A glycoside. The compound was shown to be biochanin A 7-*O*-glucoside, sissotrin, by comparison with a commercial standard. Recovery studies on sissotrin were performed following the same

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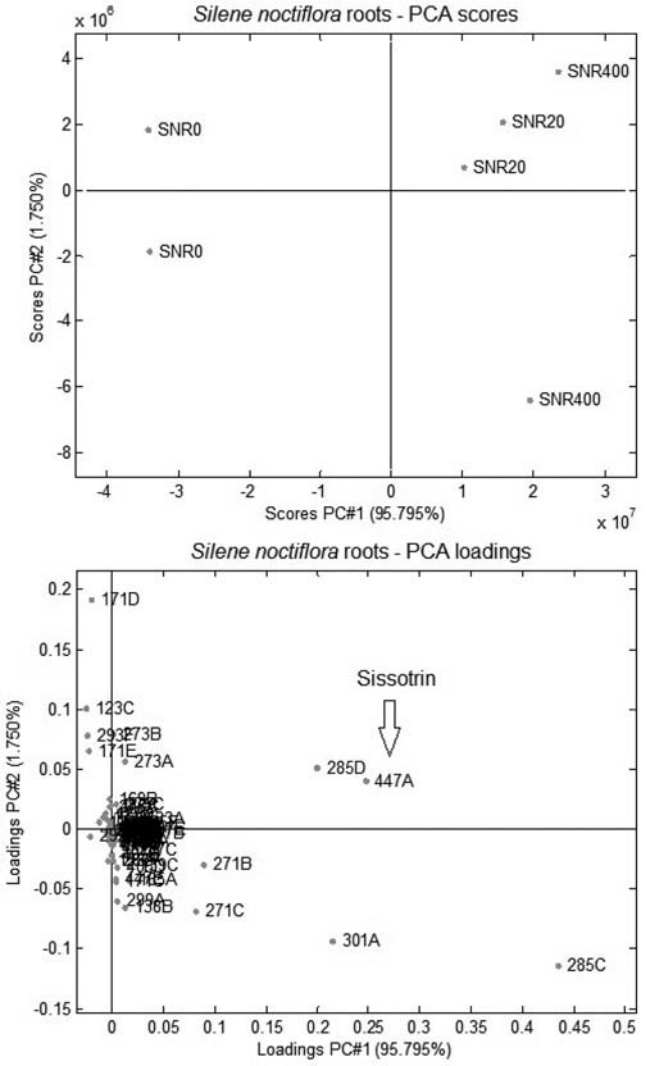

Figure 5. Principal component analysis of unknown peaks from *S. noctiflora* root samples. Principal components 1 and 2 explained 96 and 2% of the sample variation, respectively.

principles as described for the known compounds and used for correction in the quantification of sissotrin in the root and shoot samples of three weed species. The determined concentrations of sissotrin in different weed samples are shown in Table 6.

In the case of *S. noctiflora*, the shoot samples contained more sissotrin than the root samples. *G. molle* and *E. crus-galli* contained more sissotrin in the roots than in the shoots. In the present study *S. noctiflora* showed the most susceptibility to biochanin A and root growth was inhibited. Higher concentrations of biochanin A were found in root samples than in shoot samples. Shoot samples contained more sissotrin than root samples in the case of the susceptible weed species *S. noctiflora*. A possible explanation is that biochanin A was glycosylated to sissotrin as a detoxifying mechanism.

Enzymatic Transformation. Upon uptake of biochanin A, demethylation to genistein, hydroxylation to pratensein, reduction to dihydrobiochanin A, and glycosylation to sissotrin were seen. *S. noctiflora* itself did not contain biochanin A or any of its transformation products, but dihydrobiochanin A was nevertheless detected in the plants treated with biochanin A. Dihydrobiochanin A may be formed from biochanin A by the activity of the enzyme biochanin-A reductase.[35] *S. noctiflora* would not be expected to possess biochanin A reductase, given that it lacks the substrate, so it is possible that the reduction of biochanin A in *S. noctiflora* is therefore carried out by a general reductase enzyme. Pratensein is known to be formed by the activity of isoflavone 3′-hydroxylase on biochanin A,[35] and most samples in our study were observed to contain pratensein, with the exception of *S. noctiflora* control samples. The enzyme isoflavone 7-*O*-glucosyltransferase mediates the glycosylation of biochanin A to sissotrin (biochanin A-7-*O*-glucoside)[35] and was likely the enzyme responsible for the conversion to sissotrin in all investigated weed species. According to the KEGG biosynthetic pathway for isoflavonoids,[35] genistein is converted to biochanin A by the activity of methyltransferases. In our study, biochanin A instead appeared to be demethylated to genistein. To the best of our knowledge demethylation of biochanin A has until now only been shown in mammals, for example, in the human intestinal tract,[36] but never in plants. Identification of enzymes that effect transformations of biochanin A requires further study.

Conclusions. Biochanin A was shown to inhibit the growth of *S. noctiflora* and to a lesser extent *G. molle*, although solubility became a limiting factor. Biochanin A has the potential to be used for weed control and may play a role in weed suppression by clover. Although *S. noctiflora* was more susceptible to biochanin A, the concentrations of biochanin A and its transformation products were higher in the less susceptible *G. molle*. Glycosylation to sissotrin appeared to be a transformation used by *S. noctiflora* and *G. molle*. The latter was less susceptible and produced more sissotrin upon treatment with biochanin A than the former. By comparison, *E. crus-galli* took up almost no biochanin A and, thus, appeared to derive its resistance from lack of uptake rather than detoxification metabolism. Further experiments carried out in soil are needed to establish the ecological relevance of the findings presented, and the suggested enzymatic transformation pathways should be substantiated by genetic studies.

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Notes

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