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Herbicidal Activity of Glucosinolate Degradation Products in Fermented Meadowfoam (*Limnanthes alba*) Seed Meal

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Meadowfoam (*Limnanthes alba*) is an oilseed crop grown in western Oregon. After extraction of the oil from the seeds, the remaining seed meal contains 2−4% of the glucosinolate glucolimnanthin. This study investigated the effect of fermentation of seed meal on its chemical composition and the effect of the altered composition on downy brome (*Bromus tectorum*) coleoptile emergence. Incubation of enzyme-inactive seed meal with enzyme-active seeds (1% by weight) resulted in complete degradation of glucolimnanthin and formation of 3-methoxybenzyl isothiocyanate in 28% yield. Fermentation in the presence of an aqueous solution of $FeSO_4$ (10 mM) resulted in the formation of 3-methoxyphenylacetonitrile and 2-(3-methoxyphenyl)ethanethioamide, a novel natural product. The formation of the isothiocyanate, the nitrile, and the thioamide, as a total, correlated with an increase of herbicidal potency of the seed meal ($r^2 = 0.96$). The results of this study open new possibilities for the refinement of glucosinolate-containing seed meals for use as bioherbicides.

KEYWORDS: Meadowfoam; seed meal; *Limnanthes alba*; glucosinolate; glucolimnanthin; herbicide; isothiocyanate; nitrile; thioamide

INTRODUCTION

The continuing growth of organic farming practices calls for increased use of pesticides derived from naturally occurring materials. Many plants produce toxic phytochemicals when attacked by herbivores (phytoalexins) or deposit protoxic glycosides in leaves that prevent seed germination of other, neighboring plants when the aglycones are released from the shed leaves (allelochemicals). Glucosinolates form a group of allelochemicals produced by many species of the Brassicales, including *Brassica* and *Sinapis* spp. (mustards), *Lepidium* and *Nasturtium* spp. (cresses), and *Limnanthes* spp. (meadowfoams) (*1*). The interest in *Brassica* glucosinolates is primarily due to their presence in oilseeds because they are retained in the marc after oil extraction (meal). Glucosinolate-containing seed meals are intensively investigated as biofumigants for weed control (*2, 3*).

White meadowfoam (*Limnanthes alba* Hartw. ex Benth., Limnanthaceae) is native to southern Oregon and northern California (*4, 5*). Several cultivars have emerged from a meadowfoam breeding program at Oregon State University (*6*). The species is cultivated in the Willamette valley of western Oregon for the seed oil, which is rich in unusual 20:1 and 22:1 fatty acids (*7*). The oil has commercial value as an ingredient of skin care products. The spent seed material (meal) can be used as a bioherbicide due to the presence of allelochemicals. The meal contains the glucosinolate glucolimnanthin **1** (*8*) and 3-methoxyphenylacetonitrile (also referred to as 3-methoxybenzyl cyanide, **2**), a known allelochemical (*9*) formed by heat-induced degradation of **1** (**Figure 1**) during the oil extraction process. Because water is added at an early stage of the industrial oil extraction process to facilitate seed crushing, conversion of **1** into 3-methoxybenzyl isothiocyanate **3**, catalyzed by myrosinase in the seed, would occur if the enzyme were not inactivated by application of heat. Early heat treatment is necessary to inactivate myrosinase and prevent contamination of the oil with nonpolar breakdown products of **1**, whereas late-stage heat treatment follows extraction of seed oil with an organic solvent with the purpose of removing residual solvent.

When seed meal is applied to soil for weed control, a disadvantage of the lack of myrosinase activity in seed meal is that breakdown of **1** depends on the soil microenvironment, which may lead to variable results regarding degradation kinetics, profile of degradation products, and weed suppression. In this study, the conversion of **1** into the allelochemicals **2**, **3** (*9*) and 2-(3-methoxyphenyl)ethanethioamide (**4**) in enzyme-inactive meal was investigated by making use of active enzymes present in meadowfoam seeds and a cofactor, $FeSO_4$. We

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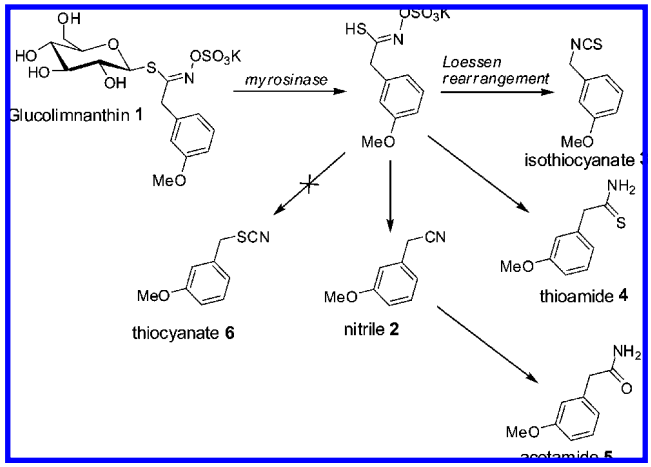

Figure 1. Enzymatic degradation of the glucosinolate glucolimnanthin **1**, forming the nitrile **2**, the isothiocyanate **3**, and the thioamide **4**. The nitrile **2** may hydrolyze to form the acetamide **4**.

demonstrate that enzyme-treated meal products contain greater amounts of **2**, **3**, or **4** and show greater inhibitory activity in a soil-based seed germination assay, compared to untreated meadowfoam seed meal. The results of this study open new possibilities for the refinement of glucosinolate-containing seed meals for use as bioherbicides in (organic) farming and horticulture.

MATERIALS AND METHODS

General. NMR experiments were performed on a Bruker DPX400 instrument. High-resolution ESI-ToF MS measurements were performed on a Waters Micromass LCT Classic ToF instrument (Waters, Milford, MA) equipped with an electrospray ion source, interfaced to a Waters CapLC HPLC solvent delivery system.

Chemicals. HPLC water was produced from reversed-osmosis water by a Milli-Q water purification system. HPLC grade acetonitrile and methanol were purchased from EMD Chemicals, San Diego, CA. 3-Methoxyphenylacetonitrile (**2**) was purchased from Sigma Aldrich, St. Louis, MO. 3-Methoxybenzyl isothiocyanate (**3**) was obtained from Oakwood Products, West Columbia, SC. 2-(3-Methoxyphenyl)ethanethioamide (**4**) was purchased from Endeavour Specialty Chemicals, Daventry, Northamptonshire, U.K., and 2-(3-methoxyphenyl) acetamide (**5**) was from Maybridge, Trevillett, Tintagel, Cornwall, U.K.

HPLC. The HPLC equipment consisted of a Waters Delta 600 solvent delivery system, a Waters 717 plus autosampler, a Waters 2996 photodiode array detector, a Waters 600 controller, and a data acquisition/processing computer with Empower software (Waters). In HPLC system 1, separations were achieved on a reverse-phase Lichrosphere 5 μm C18 column (4 × 250 mm, Phenomenex, Torrance, CA). The HPLC solvents were 0.1% aqueous trifluoroacetic acid (solvent A) and MeCN (solvent B). A linear solvent gradient was employed starting from 5% solvent B in solvent A to 100% B over 30 min at a flow rate of 1.0 mL/min. After returning to the starting conditions in 1 min, the column was equilibrated for 10 min before the next injection. The injection volume was 10 μL. Online UV spectra were recorded in the range of 210−500 nm and the λ 274 nm trace was used for calculation of peak areas for all compounds, except for the thioamide **4**, which was recorded at λ 270 nm. Analyte concentrations were determined from calibration curves constructed for each analyte.

In HPLC system 2, used for monitoring fractions from a Sephadex LH-20 column, the HPLC column was an Agilent Zorbax 5 μm SB-C18 column (2.1 × 50 mm). The column was eluted with solvent A for 2 min, and then the proportion of solvent B was increased to 100% over 5 min, held at 100% for 0.5 min, and decreased to 0% solvent B (100% solvent A) in 0.5 min. The column was equilibrated at 100% solvent A for 2.4 min before the next injection. The flow rate was 0.3 mL/min, and the injection volume was 1.0 μL.

Mass Spectrometry. The LC-MS/MS instrument consisted of a 4000 QTrap hybrid linear ion trap−triple-quadrupole mass spectrometer equipped with a pneumatically assisted electrospray (Turbo V) source operated at 550 °C (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Liquid nitrogen was the source for heating and nebulizing gas, curtain gas, and collision gas. The spray needle was kept at +5.5 kV (positive ion mode). Product ion (MS/MS) spectra were recorded by scanning in the range of 50−200 amu at a cycle time of 0.6 s and at unit resolution for both Q1 and Q3. The collision energy was set at 27 eV, and the collision gas was set at "medium". Samples in MeCN/H_2O (1:1, v/v) were introduced into the mass spectrometer by loop injections (1 μL) in a flow of MeCN/H_2O/HCOOH (50:50:0.1 by volume) at 0.2 mL/min. An HPLC system consisting of two Shimadzu Prominence LC-20AD pumps and a Shimadzu SIL-HTC autoinjector (Shimadzu Scientific Instruments, Columbia, MD) provided the solvent flow and performed the injections.

Isolation of Glucolimnanthin (1) from Meadowfoam Seed Meal. Factory grade meal (250 g; Natural Plant Products Inc., Salem, OR) was soaked in 500 mL of MeOH/H_2O (1:1, v/v) for 18 h and the slurry transferred to a 2 L percolator fitted at the bottom with a cotton plug and cotton cloth. MeOH/H_2O (1:1, v/v; 2.4 L) was passed through the column of seed meal at a flow rate of 0.2 L/h. Three fractions (0−0.8, 0.8−1.6, and 1.6−2.4 L) were collected and analyzed by HPLC using system 1. Fractions 1 and 2 were combined and taken to dryness by rotary evaporation (careful: foaming) and lyophilization. The residue was dissolved in 50 mL of water and diluted with 300 mL of MeOH. The resulting precipitate (carbohydrates and proteins) was removed by centrifugation, and the supernatant was concentrated in vacuo. The residue was taken up in 60 mL of MeOH, and four 15 mL portions were fractionated by column chromatography on Sephadex LH-20 using MeOH as the eluting solvent at a flow rate of 1.6 mL/min. Fractions (10 mL) were collected and monitored by HPLC (system 2) for the presence of glucolimnanthin (**1**), and the fractions containing **1** were combined and taken to dryness by rotary evaporation. Crude **1** obtained from two column runs (2.6 g) was redissolved in 6 mL of MeOH and purified on the same Sephadex LH-20 column using the same chromatographic conditions, yielding 1.8 g of >95% pure **1** by NMR analysis: ^{1}H NMR (400 MHz, MeOH-d_4) δ_H 7.26 (1H, t, J = 8 Hz, H-5′), 7.01 (1H, s, H-2′), 7.00 (1H, d, J = 8 Hz, H-6′), 6.83 (1H, d, J = 8 Hz, H-4′), 4.55 (1H, d, J = 9 Hz, H-1″), 4.25 (1H, d, J = 16 Hz, H-2), 4.05 (1H, d, J = 16 Hz, H-2), 3.87 (1H, d, J = 12 Hz, H-6″), 3.81 (3H, s, CH_3), 3.63 (1H, dd, J = 5, 12 Hz, H-6″), 3.37−3.25 (2H, m, H-3″ and H-4″), 3.21−3.12 (2H, m, H-2″ and H-5″); ^{13}C NMR (100 MHz, MeOH-d_4) δ_C 160.2 (C-3′), 159.5 (C-1), 137.6 (C-1′), 129.5 (C-5′), 120.2 (C-6′), 113.1 (C-2′), 112.6 (C-4′), 81.5 (C-1″), 80.9 (C-2″), 78.0 (C-3″), 72.8 (C-5″), 69.8 (C-4″), 61.4 (C-6″), 54.3 (CH_3), 38.3 (C-2). Assignment of these resonances was confirmed by ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and HMBC experiments. Compound **1** was obtained as the K^+ salt as determined by ESI-ToF MS: m/z 478.0269. Calcd for $C_{15}H_{21}NO_{10}S_2K$: 478.0244 (5.2 ppm).

Isolation of Compound 4. Two grams of meadowfoam seed meal was incubated overnight at room temperature with ground meadowfoam seeds (1% by wt) and 6 mL of an aqueous solution of $FeSO_4$ (10 mM). The incubation mixture was mixed with 6 mL of MeOH, sonicated for 1 min, and allowed to stand overnight. The mixture was centrifuged, and the resulting supernatant was chromatographed by HPLC on a 10 × 250 mm Phenomenex Ultrex 5 μm C18 column using a solvent gradient from 5 to 95% MeCN in H_2O containing 0.1% HCOOH over 30 min at a flow rate of 5 mL/min. The peak eluting at 17 min (UV_{max} 270 nm) was collected manually and analyzed by electrospray mass spectrometry.

Synthesis of 3-Methoxybenzyl Thiocyanate (6). The thiocyanate **6** was synthesized from 3-methoxybenzyl chloride and potassium thiocyanate using a general procedure for the conversion of alkyl halides into alkyl thiocyanates (*10*). Briefly, 3 g (17.2 mmol) of 1-*n*-butyl-3-methylimidazolium chloride [bmim]Cl (TCI America, Portland, OR) was dissolved in acetone (25 mL). After the addition of KSCN (34.4 mmol), the mixture was stirred for 48 h at room temperature. The resulting suspension was filtered, and the filtrate was concentrated in vacuo. The residue was dissolved in CH_2Cl_2 and filtered again. After evaporation of the solvent, [bmim]SCN was obtained as a reddish liquid.

A mixture of 395 mg of [bmim]SCN (2 mmol) and 157 mg (1 mmol) of 3-methoxybenzyl chloride (TCI America) was stirred for 3 h at room temperature and then kept overnight without stirring. The mixture was extracted with Et_2O (3 × 3 mL) by vigorous shaking. The combined Et_2O layers were dried with anhydrous Na_2SO_4 and concentrated in vacuo, yielding 3-methoxybenzyl thiocyanate (*6*) as a colorless oil (158 mg, 88% yield): ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.28−7.35 (1H, m, H-5), 6.91−6.99 (3H, m, H-2, H-4 and H-6), 4.16 (2H, s, $-CH_2-$), 3.85 (3H, s, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 160.0 (C-3), 135.8 (C-1), 130.2 (C-5), 121.2 (C-6), 114.54 (C-2), 114.46 (C-4), 112.0 ($-C\equiv N$), 55.3 ($-OCH_3$), 38.4 ($-CH_2-$); HR-EIMS (70 eV) $C_9H_9NOS^{\bullet+}$ observed *m*/*z* 179.0409 (44% rel int). Calcd 179.0405 (2.2 ppm), *m*/*z* 121 (100% rel int, $CH_3O-Ph-CH_2^+$).

Preparation of Fermented Meal Products (Treated Seed Meals, T1−T5). Fermented meal was prepared by grinding 9.9 g of meal together with 0.1 g of untreated meadowfoam seed (*L. alba* ssp. *alba* Benth. cv. Ross) in a coffee grinder (model E160B, Proctor Silex, Washington, NC) for 1 min. Ground batches were pooled and mixed with deionized water (3 mL/g of meal), sonicated for 5 min, allowed to incubate for 18 h at room temperature, freeze-dried, and reground for 30 s (**T1**). The freeze-drying step was considered necessary in order to be able to perform the germination assay. Iron-augmented meal was produced according to the same procedure except 10 mM $FeSO_4$ was substituted for deionized water (**T2**). Control incubations, all without seeds, included unaltered meal (ground but not incubated with water and not freeze-dried, **T5**), sham-augmented meal (meal plus water alone, **T4**), and iron only (meal plus 10 mM $FeSO_4$, **T3**).

For analysis by HPLC-UV, 1.0 g aliquots of the fermented and freeze-dried meal products were mixed with 6 mL of 50% methanol in screw-capped glass centrifuge tubes, vortexed for 30 s, and sonicated for 60 s. The glass tube contents were allowed to stand overnight in the dark at room temperature, vortexed and sonicated again, and centrifuged for 5 min on a clinical centrifuge. Supernatants were further centrifuged for 10 min at 13000 rpm using a microcentrifuge, diluted 1:9 with 50% methanol, and then analyzed directly using HPLC system 1. Samples were prepared in triplicate.

Assay for Herbicidal Activity. About 45 g of Walla Walla silt loam soil (coarse, silty, mixed, mesic Typic Haploxeroll, pH 6.1) was weighed into 10 cm diameter Petri dishes. The soil was taken from a grassland site to which no agrochemicals had been applied for 15 years, cleaned of straw and roots, and sieved through a 2.362 mm screen (no. 8). For germination testing of individual compounds (**1**−**5**), 15.0 mL of test solution was added to each dish. Test solutions of glucolimanthin **1** were made up in water and added to the dishes to give doses of 0 (control, $n = 12$), 0.09 ($n = 6$), 0.18 ($n = 12$), 0.28 ($n = 6$), 0.36 ($n = 12$), 0.47 ($n = 6$), 0.53 ($n = 6$), 0.71 ($n = 6$), and 0.89 ($n = 6$) mg/g of soil. Test solutions of compounds **2**, **3**, and **5** were made up in ethanol and added to the dishes to give doses of 0 (control), 0.06, 0.11, 0.22, 0.44, and 0.89 mg/g of soil. Test solutions of compound **4** were made up in ethanol and added to the dishes to give doses of 0 (control), 0.18, 0.36, 0.53, 0.71, and 0.89 mg/g of soil. The ethanol added to soil was allowed to evaporate overnight in a hood, and then 15 mL of water was added to the dishes. Freeze-dried meal products were mixed with soil, followed by the addition of 15 mL of water. Fifteen seeds of *Bromus tectorum* were placed in concentric circles within each dish. For each dose, the number of replicates was 6 or 12 for compound **1**, 4 for compounds **2**, **3**, and **5**, and 6 for compound **4**, and $n = 4$ for **T1**−**T5**. Petri dishes with lids were placed in an incubator at 20 °C during the day time (8 h) and at 15 °C at night (16 h) for 7 days. Coleoptile emergence was recorded at the end of the incubation period. Seeds with emerged coleoptiles were considered to be germinated.

Statistical Analysis. For compounds **1**−**5** and treated seed meals **T1**−**T5**, dose−response relationships were estimated using logistic regression (linear in dose) allowing (via quasilikelihood) for extrabinomial variation among the replicates at each dose (SAS 9.2 Genmod procedure, dscale option). For the compounds, the zero dose is the vehicle control (water for compound **1** and ethanol for compounds **2**−**5**). Therefore, compounds **2**−**5** have the same intercept in the model. For the treatments **T1**−**T5**, the zero dose is the same for all five groups. Therefore, in the model all five meal treatments have the same intercept. For compounds $i = 1,...,5$, the logistic regression equation is $\ln[p_{ij}/(1 - p_{ij})] = [\ln(OR)]_i x_{ij} + b_i$, where ln is the natural logarithm, OR is the odds ratio, x_{ij} is the dose, p_{ij} is the probability of germination for dose j of compound i, and $[\ln(OR)]_i$ is the slope and b_i is the intercept for compound i. The OR for compound i is the odds of germination at dose $x_{ij} = k + 1$ divided by the odds of germination at dose $x_{ij} = k$, where the odds of germination are $p_{ij}/(1 - p_{ij})$. Because compounds **2**−**5** have a common intercept, $b_2 = b_3 = b_4 = b_5$. The same logistic regression equation was used for the seed meals, except that all seed meals have the same intercept. From the equation, the ED_{50} is $-b_i/[\ln(OR)]_i$. Because the inverse of the ED_{50} is often used as an estimator of potency (*11*) and because the $ED_{50} = -b/\ln(OR)$ and b is the same for all seed meals, we expressed the herbicidal potency of the treated seed meals as the negative logarithm of the odds ratio, $-\ln(OR)$.

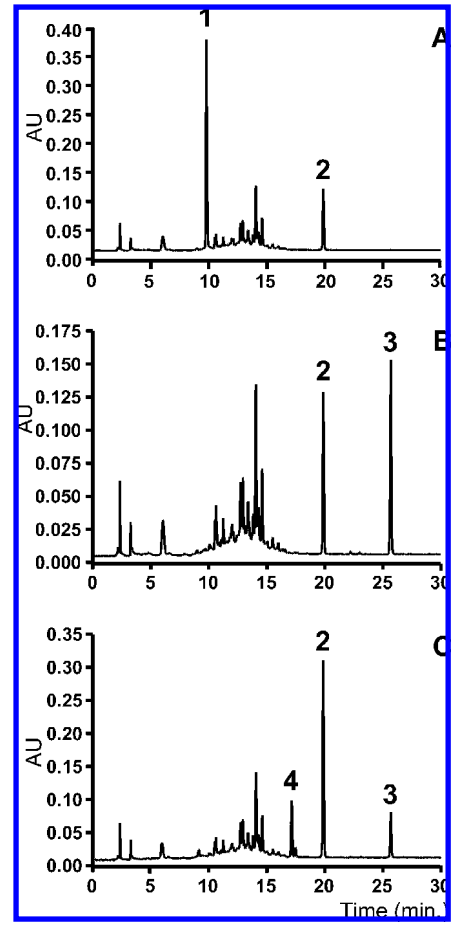

Figure 2. HPLC analysis of untreated meal (**A**), meal treated with 1% myrosinase-active meadowfoam seeds (**B**), and meal incubated with a 10 mM solution of $FeSO_4$ in the presence of 1% myrosinase-active meadowfoam seeds (**C**). The UV trace was recorded at 274 nm.

RESULTS AND DISCUSSION

Composition of Meadowfoam Seed Meal. Analysis of meadowfoam seed meal by HPLC-UV shows glucolimnanthin **1** (75 μmol/g) and its degradation product **2** (23 μmol/g) as the main constituents containing the 3-methoxybenzyl moiety (UV_{max} 274 nm, **Figure 2A** and **Table 1**). The presence of substantial amounts of nitrile **2** in the meal is attributed to heat-induced degradation of glucolimnanthin **1** during the industrial oil extraction process, because untreated meadowfoam seeds contain primarily **1** and only very small amounts of **2** (data not shown). This finding is consistent with continuous thermal formation of benzyl cyanide from benzyl glucosinolate in seeds of the garden cress (*Lepidium sativum*) after heat inactivation of myrosinase (*12*). The retention of **2** in meadowfoam seed meal after hexane extraction and **2** having sufficient solubility in

Table 1. Composition of Treated and Untreated Meadowfoam Seed Meal

	av μmol/g of meal ± SD (n = 3)				
	glucosinolate **1**	nitrile **2**	isothiocyanate **3**	thioamide **4**	total of **1**−**4**
meal + 1% seed (**T1**)	nd[a]	22.1 ± 0.3	20.8 ± 0.1	nd	42.9
meal + 1% seed + $FeSO_4$ (**T2**)	nd	49.4 ± 0.8	10.0 ± 0.2	0.82 ± 0.01	60.2
meal + $FeSO_4$ (**T3**)	55.8 ± 0.1	25.9 ± 0.1	0.76 ± 0.02	nd	82.5
meal + water (**T4**)	71.8 ± 0.3	18.3 ± 0.3	0.19 ± 0.01	nd	90.3
untreated meal (**T5**)	75.2 ± 0.8	23.2 ± 0.3	nd	nd	98.4

[a] nd, not detected.

hexane suggests that the thermal conversion of **1** took place after oil extraction when the meal undergoes steaming to remove residual extraction solvent. The acetamide **5** was detected at trace levels in the meal, indicating that hydrolysis of **2** during meal steaming is negligible.

Degradation of Glucolimnanthin in Seed Meal Mediated by Enzyme-Active Seeds. Fermentation of seed meal by inoculation with ground seeds resulted in complete degradation of glucosinolate **1** and formation of isothiocyanate **3** in 28% yield, whereas the content of nitrile **2** remained unchanged during fermentation (**Table 1** and **Figure 2B**). This is in agreement with earlier work by Vaughn and co-workers (*2*), who reported 25% recovery of isothiocyanate **3** from **1** in a mixture of meadowfoam seed meal and soil. The moderate yield of the isothiocyanate **3** can be explained by a combination of incomplete extraction and covalent binding of the isothiocyanate moiety to proteins and amino acids in the seed meal and ground seeds. It is also possible that isothiocyanate **3** releases SCN^-, which is known to be released from its analogue, 4-hydroxybenzyl isothiocyanate (*13*), and has phytotoxic properties (*14*). However, 3-methoxybenzyl isothiocyanate (*3*) is expected to be more stable than 4-hydroxybenzyl isothiocyanate, because **3** cannot dissociate into quinone methide and SCN^-, the presumed mechanism for spontaneous degradation of 4-hydroxybenzyl isothiocyanate (*13*).

Degradation of Glucolimnanthin in Seed Meal Mediated by Enzyme-Active Seeds in the Presence of Fe^{2+}. Incubation of seed meal with ground seeds and 10 mM $FeSO_4$ resulted in complete degradation of glucosinolate **1**, formation of isothiocyanate **3** in 13% yield, and a doubling of the nitrile **2** content (**Table 1** and **Figure 2C**). Incubation of seed meal with 10 mM $FeSO_4$ showed a 26% decrease of glucosinolate **1**, which was not accounted for by an increase of nitrile **2** or isothiocyanate**3**. The origin of the apparent loss of **1** is unknown.

The enhanced formation of nitrile **2** in **T2** (active enzyme added) compared to **T3** (enzymes inactive) suggests a catalytic role for Fe^{2+} in conjunction with myrosinase or other enzymes to form nitrile **2**. Fe^{2+} has been identified by other researchers as a cofactor of nitrile-forming proteins that alter myrosinase-mediated hydrolysis of glucosinolates but lack hydrolytic activity by themselves (*15*, *16*). One form of nitrile-forming protein, epispecifier protein (ESP) from *Arabidopsis thaliana*, was shown by Burow et al. (*15*) to act in conjunction with myrosinase in the conversion of glucosinolates into the corresponding nitriles. They observed a 3-fold increase in ESP activity when Fe^{2+} was added at 0.01 or 0.5 mM. Similar results were obtained by Matusheski et al. (*17*), who furthermore observed that the myrosinase-mediated conversion of glucoraphanin into sulforaphane nitrile required higher concentrations of iron (0.1−1 mM Fe^{2+}) when ESP was absent. We observed very little conversion of glucosinolate **1** into nitrile **2** at $FeSO_4$ concentrations of 1 mM or less, which suggests that meadowfoam seeds have low nitrile-forming protein activity. On the other hand, meadowfoam seeds may contain normal levels of nitrile-forming

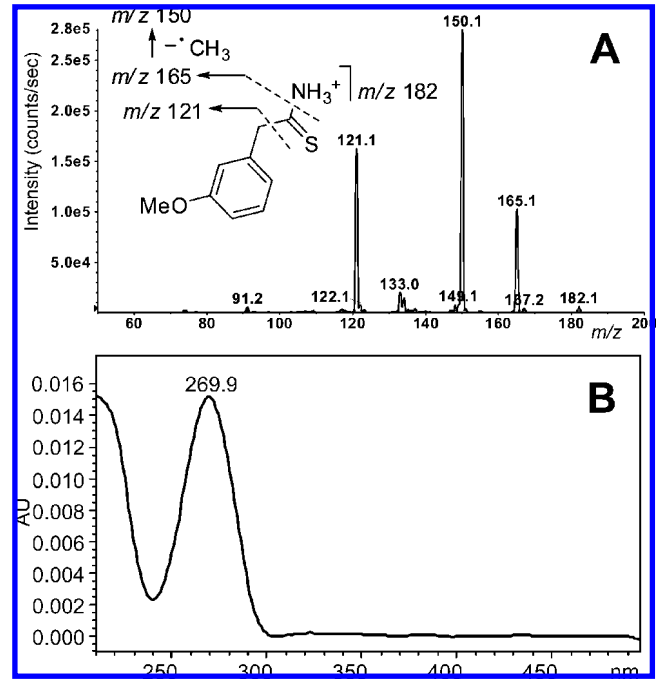

Figure 3. Identification of thioamide **4** in seed meal by electrospray MS/MS (**A**) and HPLC-UV (**B**).

protein compared to other species of Brassicales, but the added iron is not sufficiently available due to binding to proteins or other cellular macromolecules in the seeds or seed meal. In support of ESP-independent Fe^{2+} catalysis, Bellostas et al. (*18*) recently hypothesized that Fe^{2+} coordinates with the sulfur atom of the thioglucoside moiety, thereby preventing myrosinase-directed Loessen rearrangement and so directing glucosinolate degradation to the nitrile at the expense of isothiocyanate formation.

In the seed meal incubations with myrosinase-active seeds in the presence of 10 mM $FeSO_4$, we detected the formation of compound **4**, not present or present at very low levels in seed meal or in seed meal without the addition of iron and seeds (**Figure 2C**). When the experiments were repeated with the addition of pure glucolimnanthin **1** (60 mg/g of meal), a 4-fold increase in the formation of compound **4** was observed compared to incubations without addition of **1**, indicating that **4** is another degradation product of **1**. Compound **4** was collected upon elution from a semipreparative HPLC column. The peak fraction revealed a molecular ion with *m*/*z* 282 by ESI-MS and a fragment ion with *m*/*z* 121 (MeO − Ph − CH_2^+) (**Figure 3**). This suggested that compound **4** was identical with 2-(3-methoxyphenyl)ethanethioamide, which was confirmed by HPLC-UV and MS comparison of **4** with an authentic standard (**Figure 3**). Although Fe^{2+}-mediated hydrolysis and reduction of glucosinolates into the corresponding thioamides has previously been recognized by other researchers (*18*, *19*), thioamide **4** has not previously been reported as a natural product, nor

Table 2. Estimation of Herbicidal Potency for Test Compounds and Treated Meals[a]

	−ln(OR)[b] (SE)	−ln(OR) difference ($P < 0.05$)[c]
glucolimnanthin **1**	2.86 (0.602)	a, d
nitrile **2**	6.26 (0.592)	b
isothiocyanate **3**	2.15 (0.429)	a
thioamide **4**	10.47 (0.703)	c
acetamide **5**	3.77 (0.406)	d
meal + 1% seed (**T1**)	0.152 (0.0139)	f
meal + 1% seed + $FeSO_4$ (**T2**)	0.1913 (0.0156)	g
meal + $FeSO_4$ (**T3**)	0.0438 (0.0148)	h
meal + water (**T4**)	0.0223 (0.0162)	h
untreated meal (**T5**)	0.0412 (0.0149)	h

[a] Calculations were performed on mg/g of soil basis. [b] OR, odds ratio (see Statistical Analyses); −ln(OR) is the slope of the regression curve, a measure of herbicidal potency. [c] Within compound and seed meal groups, shared letters indicate lack of difference at the 95% confidence level.

have its herbicidal properties been examined. Our results indicate the involvement of an enzyme in the Fe^{2+}-mediated formation of thioamide **4** because **4** was not formed in the absence of enzyme-active seeds when iron was added (**Table 1**). Regarding the mechanism of thioamide formation, Bellostas et al. (*18*) proposed that Fe^{2+} coordinates with the imine nitrogen, a sulfate oxygen, and a hydroxyl group at C-2 in the side chain of the aglycone. The formation of thioamide **4** in our study suggests that there is no requirement for a C-2 hydroxyl to form a favorable glucosinolate−Fe^{2+} transition complex, because glucolimnanthin does not have such a hydroxyl group. An alternative explanation for the role of iron in the enzyme-mediated formation is that iron prevents Loessen rearrangement, possibly by coordination with the thioglucoside sulfur (*18*), resulting in an intermediate hydrolysis product, *N*-hydroxy-2-(3-methoxyphenyl)thioacetamide, that is subsequently reduced by 2 equiv of Fe^{2+} to form the thioamide **4**.

Thiocyanate (6) Formation. Thiocyanates are often reported as degradation products of glucosinolates (*1*, *2*, *20*), and therefore we investigated the presence of thiocyanate **6** in the fermented seed meals. Synthetic 3-methoxybenzyl thiocyanate (*6*) showed a retention time of 22.2 min in HPLC system 1 and a UV maximum at 280.5 nm, thus eluting between nitrile **2** and isothiocyanate **3**. Thiocyanate **6** was not detected in any of the (fermented) seed meals (**Figure 2**).

Herbicidal Activity of Glucolimnanthin (1) and Degradation Products (2−5). For compounds **1**−**5**, the estimated log odds ratios are given in **Table 2**. The predictions from the logistic regression model were back-transformed onto the percent germination scale to yield dose−response curves as shown in **Figure 4**. As can be seen in **Figure 4**, which shows the predicted dose−response curve together with the observed values, the regression model gives a good fit to the data. The absolute value of the log odds ratio, and therefore also the herbicidal potency of the compounds, decreases in the order thioamide **4** > nitrile **2** > acetamide **5** > glucolimnanthin **1** > isothiocyanate **3**. There was no significant difference between **1** and **3** or between **1** and **5** (both $P > 0.2$). All other pairs of compounds were significantly different (all $P < 0.001$). The fact that **3** and **5** are significantly different, but **1** and **5** are not, is largely due to the smaller standard error of the estimated difference between **3** and **5** as compared to **1** and **5**.

Herbicidal Activity of Fermented Meadowfoam Seed Meal Products. For seed meals **T1**−**T5**, the estimated log odds ratios are given in **Table 2** and the predicted dose−response curves are shown in **Figure 5**. As can be seen in **Figure 5** the regression

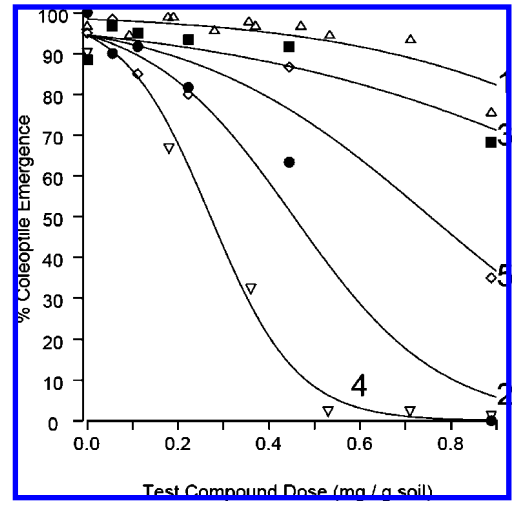

Figure 4. Herbicidal activity of compounds **1**−**5**. Symbols represent averages of measurements: (△) $n = 6$ or 12, compound **1**; (●) $n = 4$, compound **2**; (■) $n = 4$, compound **3**; (▽) $n = 6$, compound **4**; (◇) $n = 4$, compound **5**.

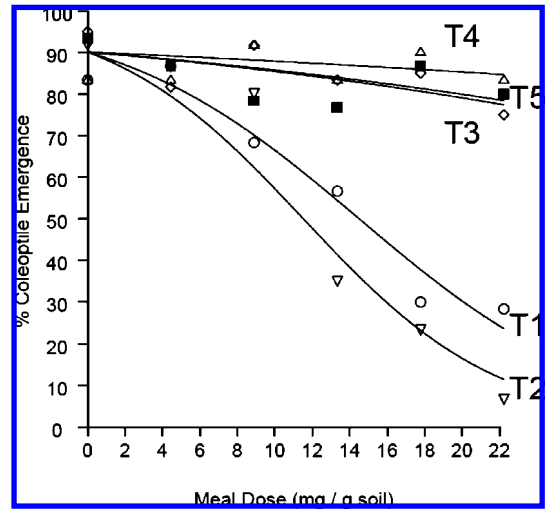

Figure 5. Herbicidal activity of seed meal treatments **T1**−**T5**. Symbols represent averages of measurements ($n = 4$): (○) **T1**; (▽) **T2**; (■) **T3**; (△) **T4**; (◇) **T5**.

model gives a good fit to the data. Seed meals **T3**, **T4**, and **T5** were not significantly different from each other ($P = 0.43$). Treatment of seed meal with enzyme active seeds significantly increased the herbicidal potency compared to the water treatment ($P < 0.001$) and compared to untreated meal ($P < 0.001$). Addition of 10 mM $FeSO_4$ to the incubation mixture of seed meal with enzyme active seeds further enhanced the herbicidal potency as compared to **T1** ($P = 0.012$). This treatment (**T2**) was also significantly different from **T3** ($P < 0.0001$).

To understand the increases in meal potency as a result of glucosinolate degradation, we examined the relationships between the content of the glucolimnanthin breakdown products and herbicidal potency. When the −ln(OR) was plotted separately against the content of the compounds **1**, **2**, **3**, and **4** in seed meals **T1**−**T5** (**Table 1**), only **1** showed a regression curve with a slope that was significantly different from 0 ($r^2 = 0.94$, $P = 0.0068$) (curve not shown). The negative sign of the slope indicated that the decrease of **1** in the meals correlated with an increase of meal potency. When the −ln(OR) was plotted against the total content of the compounds **2**, **3**, and **4** in the seed meals, a regression curve was obtained with a positive slope that was significantly different from 0 ($r^2 = 0.96$, $P = 0.0033$) (**Figure**

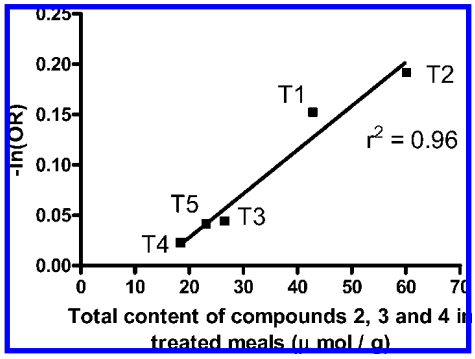

Figure 6. Correlation between herbicidal potency expressed as −ln(OR) and total content of compounds **2**−**4** in seed meals **T1**−**T5**.

6). These analyses demonstrate that the formation of **2**, **3**, and **4** from **1** strongly correlates with an increase in herbicidal potency of the treated seed meals. Diverting the degradation pathway away from **3** toward the formation of **2** and **4** results in further increases of meal potency, consistent with the potency order thioamide **4** > nitrile **2** > isothiocyanate **3** (**Figure 4** and **Table 2**).

We have explored a simple and inexpensive approach to convert the glucosinolate **1** into more potent herbicidal degradation products. The fermented seed meal products have greater herbicidal potency than factory-grade seed meal, the herbicidal activity of which largely depends on its constitutive content of nitrile **2** and soil factors for in situ degradation of **1**. *Brassica* manure is a well-documented source of myrosinase in the soil (*21*), but seed meals lack active myrosinase. *Aspergillus* fungi from soil have been reported to possess myrosinase activity (*22*, *23*). However, the soil microflora is variable and may not completely convert glucosinolate, resulting in underutilization of the seed meal's herbicidal potential. Therefore, fermented glucosinolate-containing seed meals may offer more effective bioherbicides with more predictable activity for use in (organic) farming.

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