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# Increasing Soy Isoflavonoid Content and Diversity by Simultaneous Malting and Challenging by a Fungus to Modulate Estrogenicity

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Supporting Information

**ABSTRACT:** Soybeans were germinated on a kilogram-scale, by the application of malting technology used in the brewing industry, and concomitantly challenged with *Rhizopus microsporus* var. *oryzae*. In a time-course experiment, samples were taken every 24 h for 10 days, and the isoflavonoid profile was analyzed by RP-UHPLC-MS. Upon induction with *R. microsporus*, the isoflavonoid composition changed drastically with the formation of phytoalexins belonging to the subclasses of the pterocarpans and coumestans and by prenylation of the various isoflavonoids. The pterocarpan content stabilized at 2.24 mg of daidzein equivalents (DE) per g after ∼9 days. The levels of the less common glyceofuran, glyceollin IV, and V/VI ranged from 0.18 to 0.35 mg DE/g and were comparable to those of the more commonly reported glyceollins I, II, and III (0.22−0.32 mg DE/g) and glycinol (0.42 mg DE/g). The content of prenylated isoflavones after the induction process was 0.30 mg DE/g. The total isoflavonoid content increased by a factor of 10−12 on DW basis after 9 days, which was suggested to be ascribable to de novo synthesis. These changes were accompanied by a gradual increase in agonistic activity of the extracts toward both the estrogen receptor α (ERα) and ERβ during the 10-day induction, with a more pronounced activity toward ERβ. Thus, the induction process yielded a completely different spectrum of isoflavonoids, with a much higher bioactivity toward the estrogen receptors. This, together with the over 10-fold increase in potential bioactives, offers promising perspectives for producing more, novel, and higher potency nutraceuticals by malting under stressed conditions.

**KEYWORDS:** Prenylation, isoflavonoids, soy, estrogenicity, phytoalexins, germination

## INTRODUCTION

The *Leguminosae* (or *Fabaceae*) is an economically important family of flowering plants. It comprises many edible legumes such as soybeans (*Glycine max*), beans (*Phaseolus* ssp.), peas (*Pisum sativum*), chickpeas (*Cicer arietinum*), alfalfa (*Medicago sativa*), peanut (*Arachis hypogaea*), and licorice (*Glycyrrhiza glabra*). Many edible legumes belong to the subfamily of the Papilionoideae. All species within this subfamily have in common the fact that they accumulate isoflavonoids, a subclass of flavonoids.[1] Soybean is considered to be one of the richest sources of isoflavones, a subclass of isoflavonoids. The three main isoflavones in soy are daidzein, genistein, and glycitein. These isoflavones are present as aglycone, glucoside, acetyl-glucoside, or malonyl-glucoside.[2] Soy isoflavones are known for their weak estrogenic properties, and it is assumed that soy consumption is associated with health-beneficial effects toward hormone-related conditions such as menopausal complaints and osteoporosis.[3] Isoflavones exert their in vitro estrogenicity by binding to the estrogen receptor (ER) and are, therefore, often referred to as phytoestrogens.

Application of stress to soy during germination is known to induce changes in the isoflavonoid profile, i.e., the formation of new isoflavonoids, also known as phytoalexins. Phytoalexins are defined as low molecular weight, antimicrobial compounds that are synthesized and accumulated in plants in response to, e.g., pathogen attack or application of chemical stimuli.[4] Soy phytoalexins comprise pterocarpans, coumestans, and isoflavones.[5] The formation of coumestans and pterocarpans is thought to result from the conversion of daidzein into coumestrol and glycinol, respectively.[6] Prenylation is a common feature to these phytoalexins. Coumestrol can be converted into phaseol by enzyme-catalyzed substitution with a prenyl chain on the 4-position. Glycinol can be enzymatically substituted with a prenyl chain to form glyceollidins I and II, which can subsequently ring-close into a pyran ring to form glyceollins I−III by action of a cyclase.[4,6,7] Furthermore, the occurrence of prenylated isoflavones in *Rhizopus*-challenged soy seedlings has been reported recently.[5]

Although the estrogenic activities of several soy phytoalexins have been described, surprisingly neither the formation of phytoalexins nor the changes in estrogenic activity in response to the altered isoflavonoid composition have been quantitatively monitored in time. In this study, soybeans were germinated in the presence of the food-grade fungus *Rhizopus microsporus* var.

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*oryzae*. Subsequently, both their isoflavonoid profile and estrogenic activity toward ERα and ERβ were assessed during 10 days with 24 h intervals. The treatment was performed on a kilogram-scale with malting technology commonly employed in the brewing industry. The aim of this study is to quantify the changes in isoflavonoid composition and the concomitant changes in the estrogenicity by means of a yeast estrogen bioassay.[8]

## EXPERIMENTAL SECTION

**Materials.** Soybeans, *Glycine max* (L.) Merrill, were provided by Frutarom Belgium (Londerzeel, Belgium). The authentic standards of daidzein, genistein, coumestrol, and estradiol ($E_2$) were purchased from Sigma Aldrich (St. Louis, MO, USA), and both *R,R*-THC and RU 58668 were purchased from Tocris Bioscience (Bristol, United Kingdom). UHPLC/MS grade acetonitrile (ACN) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were purchased from Merck (Darmstadt, Germany).

The fungus, *Rhizopus microsporus* var. *oryzae* (LU 581), was purchased from the Fungal Biodiversity Centre CBS (Utrecht, The Netherlands).

**Rhizopus-Challenged Germination of Soybeans.** The soybeans were subjected to a controlled malting protocol. This treatment consists of 3 stages: soaking, germination, and challenging by *Rhizopus*, and is further referred to as induction. All stages were performed in the absence of light. Samples were collected every 24 h and directly stored at −20 °C.

Soybeans were selected by visual screening. Damaged beans and debris were discarded (2−3% w/w). Before starting the treatment, surface-sterilization was performed by soaking the soybeans in a 1% (w/v) hypochlorite solution (5 L/kg beans) for 1 h at 20 °C. After surface-sterilization the soybeans were rinsed at least 3 times with (tap) water (5 L/kg beans). The treatment was performed in a Joe White micromalting system unit 90−102 (Perth, Australia) comprising 16 separate compartments. Compartments 1 to 3 and 14 to 16 were not used for the treatment. The micromalting system was cleaned and surface-sterilized before use, according to a cleaning-in-place protocol with the application of the commercial cleaning product Chlorosept (Sopura S.A., Courcelles, Belgium). For each time course experiment, a total of 4.0 kg of nonsoaked soybeans was divided into 10 portions of 400 g each. The portions were distributed over compartments 4 to 13.

In the first stage, the beans were soaked for 24 h at 22 °C (air flow parameters, 30% air (100% = 3000 rpm of ventilator); 85% recirculation, 15% fresh air). After soaking, the soaking water was drained, and the beans entered the second stage in which they were germinated for 48 h at 30 °C under 100% RH (30% air; 85% recirculation). The germination stage was followed by a third stage in which the soy seedlings were inoculated with a spore suspension of *R. microsporus*. After inoculation, the germination was continued for another 7 days at 30 °C, with adjusted air flow parameters (40% air; 25% recirculation).

For the fungal inoculation, a sporangiospore suspension was prepared by scraping off the sporangia from pure slant cultures of *R. microsporus*, grown on malt extract agar (CM59; Oxoid, Basingstoke, UK) for 7 days at 30 °C. Subsequently, the sporangia were suspended in sterile Milli-Q water with 0.85% (w/v) NaCl ($10^8$ CFU/mL). The beans were inoculated with the sporangiospore suspension (0.2 mL/g) by gently distributing the suspension manually through the germinated seeds.

**Soy Seedling Characterization.** The percentage of germination of the soybeans and the average root length were determined on 20 randomly picked seedlings from each of the 24 h interval samples. Root length was determined on the main root, excluding possible side roots. Germinated beans were defined as beans of which the root had a minimal length of 2 mm. The germination was expressed as the percentage of the germinated beans of the total amount of beans sampled. The averages (± standard deviation) of both percentage of germination and root length in the time course experiment were calculated on the basis of 5 individual time course experiments.

**Soy Seedling Extraction.** The frozen samples were freeze-dried and subsequently milled in a Retch Ultra centrifugal mill ZM 200 (Haan, Germany) using a 0.5 mm sieve. Ten grams of soybean powder was defatted with 250 mL hexane under continuous reflux conditions for 4 h. Defatted powder (0.5 g) was subsequently extracted with 50 mL of EtOH by sonication (20 min) and shaking (20 min; 250 rpm) at 20 °C in order to extract the isoflavonoids. The suspension was centrifuged (2500*g*, 10 min at 20 °C), and the pellet was rinsed with 10 mL of EtOH. This procedure was repeated four times, and the isoflavonoid contents of the supernatants were monitored by UV absorption at 280 nm to ensure that all isoflavonoids were extracted. The first four extracts and rinsing fractions were pooled, as the fifth supernatant did not contain isoflavonoids, i.e., did not show any UV absorption at 280 nm. The ethanol was removed under reduced pressure by a Savant ISS-110 SpeedVac concentrator (Thermo Fisher Scientific, Waltham, USA). The dried extracts were redissolved in EtOH to a concentration of 10 mg/mL and were stored at −20 °C. Prior to analysis, these stock solutions were thawed at room temperature (RT), vortexed, and centrifuged (5 min; 14000 rpm, RT).

**RP-UHPLC-MS Analysis.** Samples were analyzed on a Thermo Accela UHPLC system (San Jose, CA, USA) equipped with pump, autosampler, and PDA detector. Samples (1 μL) were injected on an Acquity UPLC BEH shield RP18 column (2.1 × 150 mm, 1.7 μm particle size) with an Acquity UPLC shield RP18 Vanguard precolumn (2.1 × 5 mm, 1.7 μm particle size; Waters, Milford, MA, USA). Water acidified with 0.1% (v/v) acetic acid, eluent A, and ACN acidified with 0.1% (v/v) acetic acid, eluent B, were used as eluents. The flow rate was 300 μL/min, the column temperature was controlled at 35 °C, and the PDA detector was set to measure at a range of 200−400 nm. The following elution profile was used: 0−2 min, linear gradient from 10%-25% (v/v) B; 2−9 min, linear gradient from 25%−50% (v/v) B; 9−12 min, isocratic on 50% B; 12−22 min, linear gradient from 50%−100% (v/v) B; 22−24 min, isocratic on 100% B; 24−25 min, linear gradient from 100%-10% (v/v) B; 25−27 min, isocratic on 10% (v/v) B. Mass spectrometric data were obtained by analyzing samples on a LTQ-XL (Thermo Scientific) equipped with an ESI-MS probe coupled to the RP-UHPLC. Mass spectrometric analysis in both positive (PI) and negative ion (NI) modes (data acquisition and reprocessing) was performed as described previously.[5] Because of the lack of commercially available references, daidzein was used as a generic standard to quantify the amounts of isoflavonoids expressed as mg daidzein equivalents per g dry weight (mg DE/g). The quantification was performed at 280 nm by means of Xcalibur (version 2.0.7 Thermo Scientific).

**Determination of Estrogenic Activity.** The protocol for the yeast-based estrogen bioassay measurements was adopted from Bovee et al.[8] with slight modifications. The yeast for this assay is genetically modified. It has a strong constitutive expression vector, stably integrated in the genome, to express either the human estrogen receptor α (ERα) or the human estrogen receptor β (ERβ). The yeast genome also contains a reporter construct, with an inducible yeast-enhanced fluorescent protein (yEGFP) regulated by the activation of a minimal promoter with estrogen responsive elements (EREs). Cultures of the yeast estrogen biosensor with either ERα or ERβ were grown overnight at 30 °C with shaking at 200 rpm. At the late log phase, the cultures of both estrogen receptors were diluted in selective minimal medium (MM) supplemented with either leucine (ERα) or histidine (ERβ) to an OD value (630 nm) between 0.04 and 0.06 (ERα) and 0.1−0.2 (ERβ). For exposure, 200-μL aliquots of this diluted yeast culture were combined with 2 μL of test compound or extract (in various concentrations) in a 96-well plate to test the agonistic properties of these

**Table 1. Growth Parameters of the Soybeans/Soy Seedlings during the Induction Process, Represented by the Average of 5 Experiments[a]**

| time (d) | stage in treatment | % of germination | | root length (mm)[c] | | absolute DW (g)[d] | | relative DW (g/100 g)[d] | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | nonsoaked[b] | 0 | | 0 | | 366.3 | (±0.2) | 100 | |
| 1 | soaking | 11 | (±14) | 5.3 | (±0.3) | 341.2 | (±20.1) | 93.2 | (±5.5) |
| 2 | germination | 53 | (±9) | 30.4 | (±2.3) | 343.9 | (±29.2) | 93.9 | (±8.0) |
| 3 | | 63 | (±11) | 41.9 | (±3.4) | 322.0 | (±4.6) | 87.9 | (±1.2) |
| 4 | challenging | 75 | (±8) | 54.1 | (±13.8) | 317.8 | (±43.1) | 86.8 | (±11.8) |
| 5 | | 83 | (±5) | 67.2 | (±6.9) | 314.8 | (±36.8) | 86.0 | (±10.1) |
| 6 | | 86 | (±4) | 82.0 | (±8.5) | 328.5 | (±3.1) | 89.7 | (±0.9) |
| 7 | | 88 | (±3) | 77.5 | (±9.0) | 333.3 | (±8.3) | 91.0 | (±2.3) |
| 8 | | 90 | (±0) | 78.2 | (±1.4) | 336.2 | (±7.4) | 91.8 | (±2.0) |
| 9 | | 90 | (±0) | 90.5 | (±1.8) | 340.6 | (±14.8) | 93.0 | (±4.0) |
| 10 | | 90 | (±0) | 87.7 | (±7.7) | 330.7 | (±4.7) | 90.3 | (±1.3) |

[a] Data are the mean ± SD values of measurements performed in 5-fold. [b] The weight of the experimental batch size of fresh beans was 400 g before drying. [c] The root length was defined as the length of the main root excluding possible side roots. [d] Absolute and relative DW refers to the whole seedling weight.

compounds. DMSO (blank) and control samples containing 17$\beta$-estradiol (E2) or genistein dissolved in DMSO were included in each experiment. Dilution series of each sample were prepared in DMSO, and the final concentration of DMSO in the assay did not exceed 1% (v/v). Each sample concentration was assayed in triplicate. Exposure was performed for 24 and 6 h for the ER$\alpha$ and ER$\beta$, respectively, in an orbital shaker (200 rpm; 30 °C).

Fluorescence and OD were measured at 0 and 24 h for the ER$\alpha$ and 0 and 6−8 h for the ER$\beta$ in a Tecan Infinite F500 (Männedorf, Switzerland) using an excitation filter of 485 nm (bandwidth 20 nm) and an emission filter of 535 nm (bandwidth 35 nm). The fluorescence signal of the samples was corrected with the signals obtained for the background signal of the culture medium with yeast. In order to verify the viability of the yeast in each well, the absorbance was measured at 630 nm. Each extract of the different time points in the treatment of a representative *Rhizopus*-challenged germination experiment was measured at 4 different dilutions, and every dilution was measured in triplicate: 1, 3, 10, and 30 $\mu$g/mL in both the ER$\alpha$ and ER$\beta$ bioassays. $EC_{50}$ calculations were performed in Sigma Plot (8.02, SPSS Inc.). The yeast-based assay was validated with a dilution series of reference compounds, estradiol and genistein. The $EC_{50}$ values in the ER$\alpha$ bioassay were 0.86 nM and 1.73 $\mu$M for estradiol and genistein, respectively, and 0.12 nM and 9.1 nM, respectively, in the ER$\beta$ bioassay. All $EC_{50}$ values were in line with those reported previously.[8]

For the determination of the potential antagonistic properties of the soy extracts toward ER$\alpha$ and $\beta$, the yeast cells were exposed to the $EC_{70}$ or $EC_{90}$ of estradiol, respectively, in combination with the 4 different extract concentrations (1, 3, 10, and 30 $\mu$g/mL; each measured in triplicates) of the same representative *Rhizopus*-challenged germination experiment. For each ER antagonist test, a reference compound was used as a positive control for antagonism, RU 58688 for ER$\alpha$[9] and *R,R*-THC for ER$\beta$.[10]

## RESULTS

**Growth Parameters of Soy Seedlings.** The different parameters of the soybean treatment that were monitored are shown in Table 1. The percentage of germination increased from 11% after 24 h to 90% after 6−7 days. The root development was characterized by a steady growth in the first 5 days after which a plateau was reached of approximately 90 mm. Furthermore, side-root formation was observed after 4 days of germination. These results are in line with similar soybean germination experiments

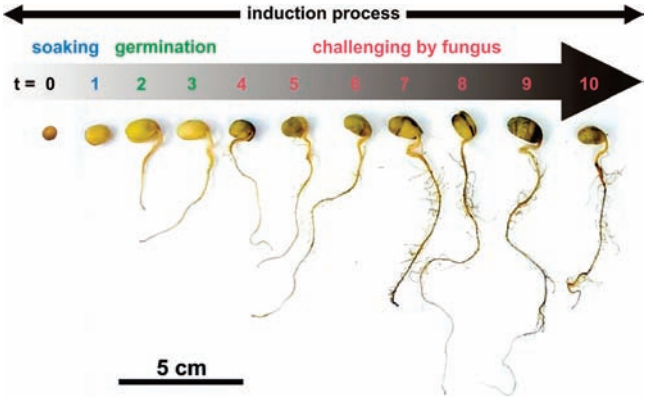


**Figure 1.** Soy seedling development vs time ($t$ in days) illustrated by representative samples. The induction process starts with nonsoaked beans ($t$ = 0) that were soaked ($t$ = 1), germinated ($t$ = 2,3), and challenged by *R. microsporus* ($t$ = 4−10).

in which an average germination percentage of 93% after 4 days was reported.[11] The seedling development in time is illustrated in Figure 1.

A weight loss of ~10% DW compared to the nonsoaked beans was observed after 10 days (Table 1). To account for this loss, the isoflavonoid content was recalculated to g DW of original soybean material at $t$ = 0. Without this correction, the total amount of isoflavonoid after germination would be overestimated.

**Identification of Compounds.** The UHPLC analysis of the extracts of nonsoaked beans, seedlings, and *Rhizopus*-challenged seedlings revealed that the UV-profiles changed drastically during induction (Figure 2). The identities of most peaks were established previously,[5] whereas peaks **1**, **8**, **10**, **13**−**15**, **24**, and **28** were now identified for the first time (Table 2). Figure 3 summarizes most of the structures found in this study. Furthermore, the metabolite flow from constitutive (isoflavones) to induced isoflavonoids (pterocarpans and coumestans) is visualized. Prenylation is indicated as the most downstream event in the biosynthetic pathway and relates to all three isoflavonoid subclasses mentioned.

Compound **1** was tentatively assigned as phenylalanine on the basis of MS analysis. In addition, three other pterocarpans were

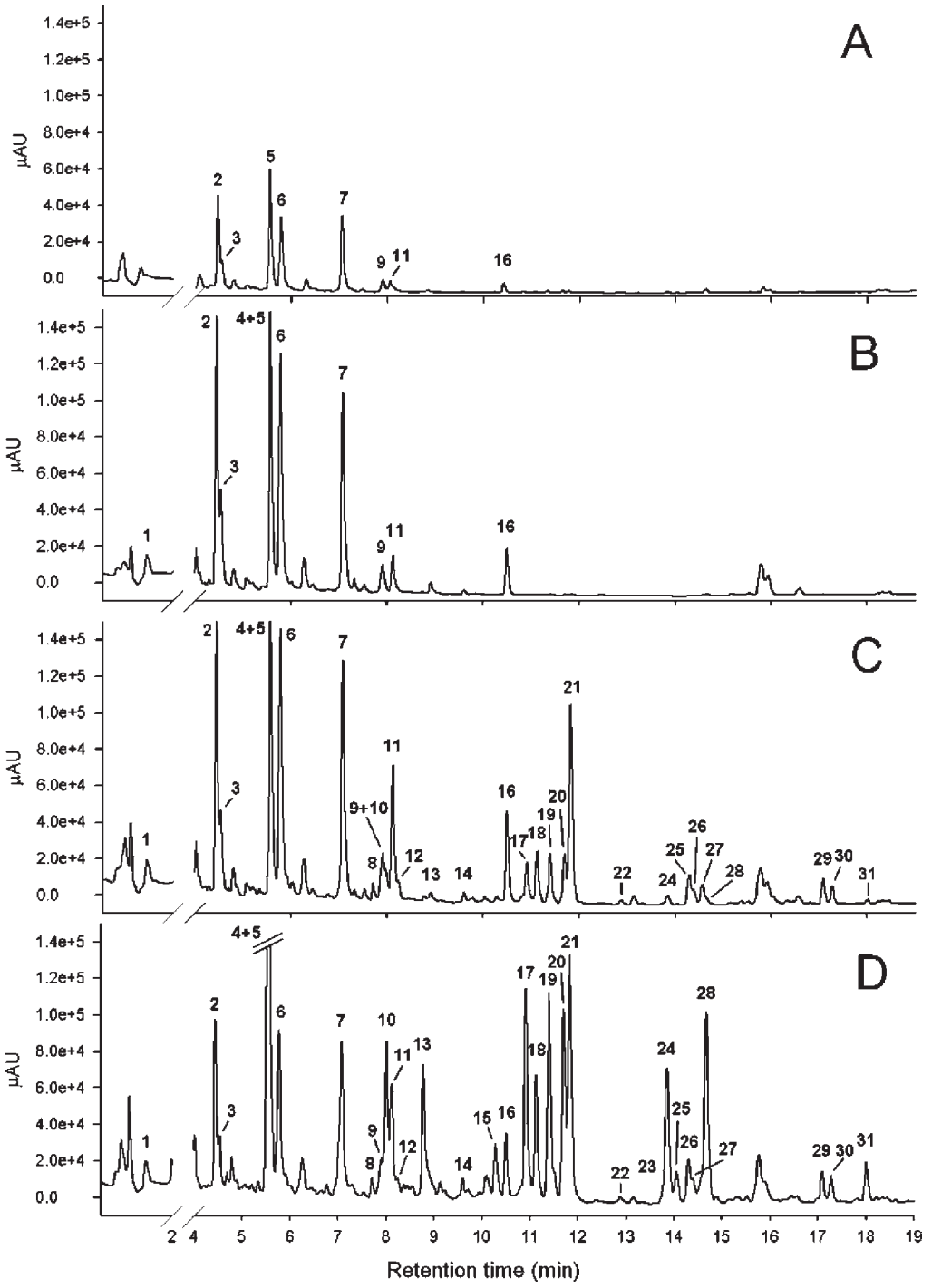


**Figure 2.** RP-UHPLC-UV-profile of EtOH extracts of nonsoaked soybeans (A), soybean seedlings at $t$ = 1 (B), and *Rhizopus*-challenged soybean seedlings at $t$ = 4 (C) and at $t$ = 10 (D). Peak numbers refer to the compounds in Table 2.

tentatively assigned at retention times (Rt) of 8.08 (**10**), 13.85 (**24**), and 14.67 (**28**) min with MW of 354, 336 and 354, respectively. The UV spectra of compounds **10**, **24**, and **28** were characterized by two $\lambda_{max}$ values of 227(±2) nm and 288(±3) nm. Glycinol, glyceollidins I/II, and glyceollins I, II, and III showed the same two $\lambda_{max}$ values. In addition, the NI $MS^2$ spectra of compounds **10**, **24**, and **28** displayed a characteristic fragment combination ($-H_2O^{x,y}$RDA fragments) that is also observed in the $MS^2$ spectra of glycinol, glyceollidins I/II, and glyceollins I−III, but not in any other $MS^2$ spectrum in the chromatogram.[5] Moreover, in PI mode, in-source fragmentation of compounds **10**, **24**, and **28** occurred, leading to the predominance of the $[M + H - H_2O]^+$ fragment ion in $MS^1$. These observations strongly suggest that **10**, **24**, and **28** belong to the subclass of the 6a-hydroxypterocarpans. Prenylation of **24** and **28** was confirmed by the characteristic neutral loss of 56 in PI mode $MS^2$,[12] whereas a loss of a methyl radical was observed in NI mode $MS^2$ of compound **28**. On the basis of all these findings,


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**Table 2. Compounds Tentatively Assigned by UHPLC-ESI-MS in the Extracts of *Glycine max* after Induction[a]**

| number | Rt. (min) | $UV_{max}$ (nm) | identification | class | molecular formula | $[M-H]^-$ | $MS^2$ NI product ions (*relative abundance*) | $[M+H]^+$ | $MS^2$ PI product ions (*relative abundance*) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.63 | 224,290 | phenylalanine | amino acid | $C_9H_{11}NO_2$ | 164 | 147(*100*) | 166 | 120(*100*) |
| 2 | 4.46 | 225,248 | daidzin[c] | isoflavone | $C_{21}H_{20}O_9$ | 415 | 253(*100*) | 417 | 255(*100*) |
| 3 | 4.56 | 225,256 | glycitin[c] | isoflavone | $C_{22}H_{22}O_{10}$ | 445 | 283(*100*) | 447 | 283(*100*) |
| 4 | 5.56 | 205,226,281 | glycinol[c] | pterocarpan | $C_{15}H_{12}O_5$ | 271 | 256(*34*),227(*28*),161(*100*) | 255[b] | 199(*100*),237(*52*) |
| 5 | 5.57 | 225,260 | genistin[c] | isoflavone | $C_{21}H_{20}O_{10}$ | 431 | 431(*100*),269(*11*) | 433 | 271(*100*) |
| 6 | 5.81 | 225,255 | malonyl-daidzin[c] | isoflavone | $C_{24}H_{22}O_{12}$ | 501 | 253(*100*) | 503 | 255(*100*) |
| 7 | 7.13 | 209,225,260 | malonyl-genistin[c] | isoflavone | $C_{24}H_{22}O_{13}$ | 517 | 269(*100*) | 519 | 271(*100*) |
| 8 | 7.74 | 224,257 | biochanin A | isoflavone | $C_{16}H_{12}O_5$ | 283 | 268(*100*) | 285 | 270(*100*) |
| 9 | 7.95 | 226,257 | glycitein[c] | isoflavone | $C_{16}H_{12}O_5$ | 283 | 268(*100*) | 285 | 270(*100*),229(*21*),225(*16*) |
| 10 | 8.03 | 225,257,291 | glyceofuran | pterocarpan | $C_{20}H_{18}O_6$ | 353 | 335(100),161(*6*),149(*25*) | 337[b] | 319(*87*),309(*100*),188(*25*) |
| 11 | 8.14 | 226,297 | daidzein[c] | isoflavone | $C_{15}H_{10}O_4$ | 253 | 253(*100*) | 255 | 227(*49*),199(*100*),137(*70*) |
| 12 | 8.26 | 212,226,257 | 2′-OH-genistein[c] | isoflavone | $C_{15}H_{10}O_6$ | 285 | 217(*100*) | 287 | 245(*49*),217(*100*),153(*57*) |
| 13 | 8.80 | 225,286,360 | C-methyl-coumestrol | coumestan | $C_{16}H_{10}O_5$ | 281 | 281(*11*),253(*100*) | 283 | 255(*100*) |
| 14 | 9.62 | 225,287 | naringenin | flavanone | $C_{15}H_{12}O_5$ | 271 | 177(*23*),151(*100*) | 273 | 243(56),215(73),253(100) |
| 15 | 10.30 | 225,351 | isotrifoliol | coumestan | $C_{16}H_{10}O_6$ | 297 | 297(*25*),282(*100*) | 299 | 284(19),271(*100*),267(15) |
| 16 | 10.52 | 224,260 | genistein[c] | isoflavone | $C_{15}H_{10}O_5$ | 269 | 269(*100*) | 271 | 153(*100*),215(*79*) |
| 17 | 10.93 | 209,227,285 | glyceollidin I/II[c] | pterocarpan | $C_{20}H_{20}O_5$ | 339 | 324(*45*),161(*100*) | 323[b] | 267(*100*) |
| 18 | 11.15 | 226,303,343 | coumestrol[c] | coumestan | $C_{15}H_8O_5$ | 267 | 267(*100*) | 269 | 241(*100*),225(*22*),197(*23*) |
| 19 | 11.41 | 211,227,284 | glyceollin III[c] | pterocarpan | $C_{20}H_{18}O_5$ | 337 | 319(*100*),293(*8*),149(*18*) | 321[b] | 306(*76*),297(*79*),251(*100*) |
| 20 | 11.72 | 211,228,283,307 | glyceollin II[c] | pterocarpan | $C_{20}H_{18}O_5$ | 337 | 319(*100*),293(*24*),149(*44*) | 321[b] | 306(*74*),297(*100*), 251(*94*) |
| 21 | 11.85 | 210,230,282 | glyceollin I[c] | pterocarpan | $C_{20}H_{18}O_5$ | 337 | 319(*100*),293(*34*),149(*95*) | 321[b] | 306(*71*),303(*100*), 293(*33*) |
| 22 | 12.92 | 225,292 | $A_{prenyl}$-2′OH-daidzein[c] | isoflavone | $C_{20}H_{18}O_5$ | 337 | 337(*37*),293(*100*),282(*84*) | 339 | 283(*100*) |
| 23 | 13.80 | 225,257 | $B_{prenyl}$-glycitein[c] | isoflavone | $C_{21}H_{20}O_5$ | 351 | 336(*100*) | 353 | 297(*100*),285(*11*) |
| 24 | 13.89 | 225,287,308 | glyceollin V/VI | pterocarpan | $C_{20}H_{16}O_5$ | 335 | 317(*100*),149(*39*) | 319[b] | 319(*28*),291(*63*),263(*100*) |
| 25 | 14.35 | 225,251,305 | $A_{prenyl}$-daidzein[c] | isoflavone | $C_{20}H_{18}O_4$ | 321 | 321(*16*),266(*100*) | 323 | 267(*100*) |
| 26 | 14.44 | 229,262 | $A_{prenyl}$-2′OH-genistein[c] | isoflavone | $C_{20}H_{18}O_6$ | 353 | 285(*95*),284(*100*),267(*37*) | 355 | 299(*100*) |
| 27 | 14.62 | 225,257,301 | $B_{prenyl}$-daidzein[c] | isoflavone | $C_{20}H_{18}O_4$ | 321 | 321(*100*),266(*16*),265(*68*) | 323 | 267(*100*),255(*8*) |
| 28 | 14.71 | 226,285 | glyceollin IV | pterocarpan | $C_{21}H_{22}O_5$ | 353 | 335(*100*),149(*20*),148(*5*) | 337[b] | 281(*100*),269(*49*) |
| 29 | 17.12 | 229,265 | $A_{prenyl}$-genistein[c] | isoflavone | $C_{20}H_{18}O_5$ | 337 | 337(*34*),283(*12*),282(*100*) | 339 | 283(*100*) |
| 30 | 17.30 | 229,260,334 | $B_{prenyl}$-genistein[c] | isoflavone | $C_{20}H_{18}O_5$ | 337 | 337(*100*),282(*16*),281(*51*) | 339 | 283(*100*),271(*11*) |
| 31 | 18.03 | 229,307,343 | 4-prenyl-coumestrol[c] | coumestan | $C_{20}H_{16}O_5$ | 335 | 335(*48*),281(*16*),280(*100*) | 337 | 281(*100*) |

[a] n.a., not available; NI, negative ion mode; PI, positive ion mode. [b] The $[M+H-H_2O]^+$ dominated in the $MS^1$ compared to the $[M+H]^+$. [c] Previously assigned by Simons et al.[5]

compounds **24** and **28** were tentatively assigned as glyceollin V/VI and glyceollin IV, respectively. The absence of the characteristic neutral loss of 56 in PI mode $MS^2$ for compound **10** could be explained by the presence of a hydroxyl-group on the furan ring (hydroxyl-isopropenyl furan), as in glyceofuran. This hydroxyl-group altered its fragmentation: instead of a loss of 42 $[C_3H_6]$ or 56 $[C_4H_8]$, a loss of 28 [CO] was observed. Therefore, compound **10** was tentatively assigned as glyceofuran.

Five other compounds were observed. Compound **14** was tentatively assigned as the flavanone naringenin based on comparison of the NI mode $MS^2$ spectrum.[13] Compound **8** was assigned as biochanin A on the basis of comparative MS spectral analysis.[14−16] Two other unknown flavonoid peaks were observed at retention times 8.85 (**13**) and 10.33 (**15**). In the UV spectra of both **13** and **15**, two $\lambda_{max}$ values were observed around 224 and 355 nm, resembling the UV spectrum of coumestrol that has $\lambda_{max}$ values at 221 and 343−360 nm (data not shown). Moreover, coumestrol, **13** and **15**, have a predominant loss of 28 (CO) in PI mode $MS^2$ in common, and all three showed no retro-Diels−Alder (RDA) fragments in both PI and NI mode $MS^2$. Therefore, compound **13** was tentatively assigned as a C-methylated derivative of coumestrol, methyl-coumestrol. The predominant loss of a methyl radical observed in NI mode $MS^2$ of compound **15** led to its tentative assignment as a methyl ether of hydroxylated coumestrol. The absence of RDA fragments in $MS^2$ does not allow further speculation on the position of the C-methyl (**13**) or methoxyl (**15**) group. The only methoxylated coumestrol isolated from soy is isotrifoliol, 3,9-dihydroxy-1-methoxycoumestan.[17] Compound **15** was, therefore, tentatively assigned as isotrifoliol.

**Changes in Isoflavonoid Composition during the Induction Process.** *Isoflavones.* The quantification of all isoflavonoids

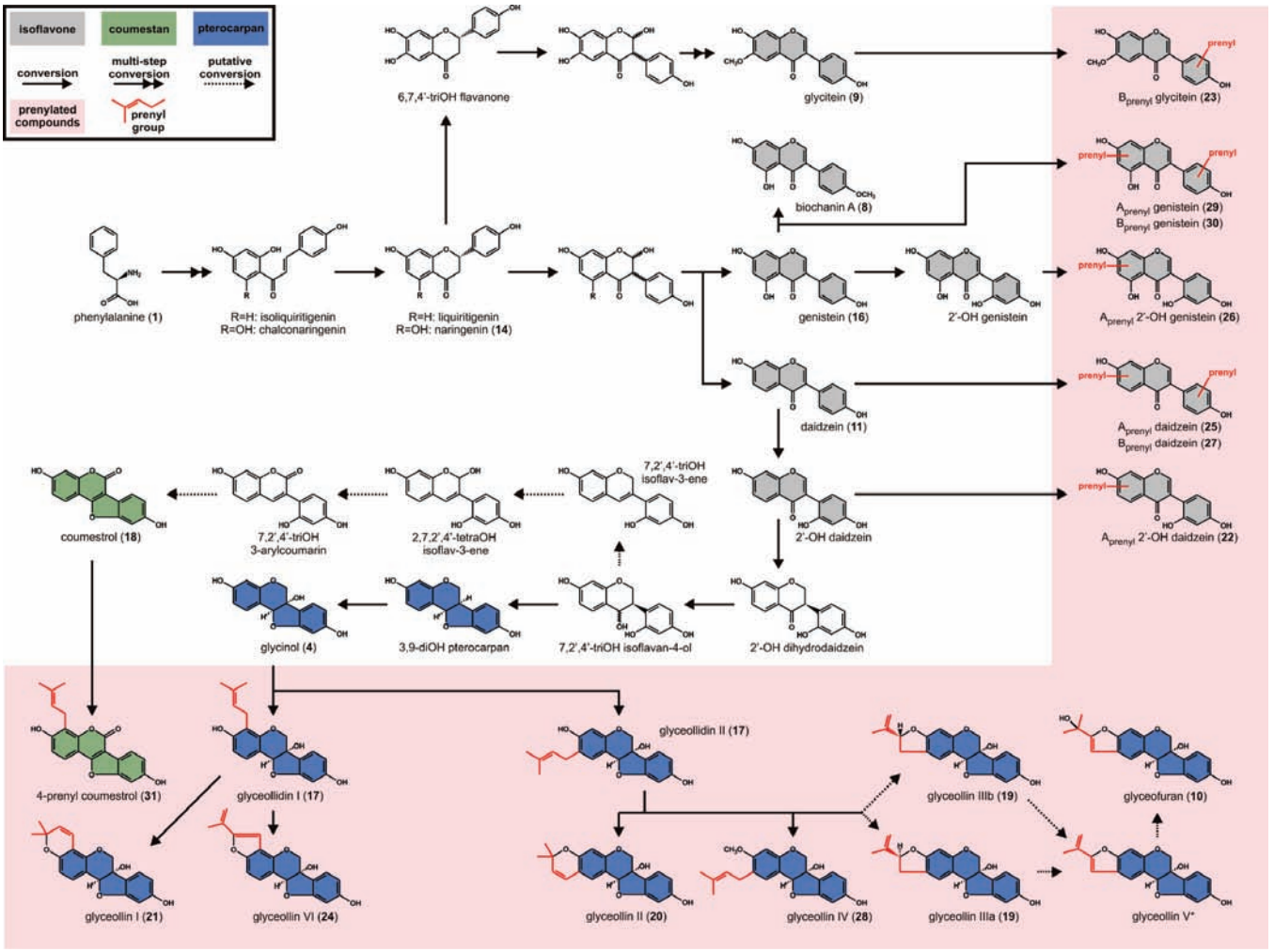


**Figure 3.** Biosynthetic pathway and structures of isoflavonoids in soybeans and *Rhizopus*-induced soy seedlings. In the literature, glyceollin V is often referred to as glyceollin IIIb. This scheme was compiled from refs 1, 5, 6, and 39.

annotated at each time point is given in Table 3. The changes in the isoflavone profile upon soaking and germination were mainly characterized by an increase of the aglycone, glucoside, and malonyl-glucoside forms of daidzein, genistein, and glycitein. This increase was most pronounced after soaking ($t$ = 1). The total isoflavone content increased from 0.37 to 1.54 mg DE/g during induction. The ratio of total (free and conjugated forms of) genistein, daidzein, and glycitein of approximately 0.45:0.45:0.10 remained constant throughout the whole process.

*Prenylated Isoflavones.* At $t$ = 3, the onset of the formation of most of the prenylated isoflavones was observed. The maximum content of all prenylated isoflavones was reached at $t$ = 9 after which a slight decrease was observed. The maximum content of prenylated isoflavones ranged from 0.01 mg DE/g for $B_{prenyl}$-glycitein (**23**) to 0.09 mg DE/g for $A_{prenyl}$-2′OH-daidzein (**22**).

*Pterocarpans.* The pterocarpan pool consisted of glyceollins I–IV (**19**–**21** and **28**) and VI/V* (**24**), glyceollidin I/II (**17**), glyceofuran (**10**), and glycinol (**4**). The pterocarpans represent the predominant group of phytoalexins that was induced in the soy seedlings and increased throughout the treatment, reaching a maximum level of 2.2 mg DE/g. The nonprenylated glycinol was the only pterocarpan that steadily accumulated throughout the whole time course experiment. The levels of all other pterocarpans appeared to stabilize at $t$ = 9.

*Coumestans.* The coumestans in the seedlings comprised coumestrol (**18**), *C*-methyl-coumestrol (**13**), isotrifoliol (**15**), and 4-prenyl-coumestrol (**31**). The levels of coumestrol showed the same trend as those of glyceollidin I/II, but they did not exceed the 0.16 mg DE/g. The total level of coumestans reached a maximum of 0.41 mg DE/g at $t$ = 9. Similar to most pterocarpans, the level of coumestans appeared to stabilize at $t$ = 9.

**Estrogenic Activity of Extracts from Induced Soybeans.** The estrogenic activity in response to the altered isoflavonoid profile in the extracts of soy during induction is shown in Figure 4. With 1 and 3 $\mu$g/mL extract, ERα showed too low responses, whereas a concentration of 30 $\mu$g/mL gave too high responses for most extracts (comparable with the maximum response obtained with a high dose of 17$\beta$-estradiol). With 10 $\mu$g/mL, a clear increase in estrogenic activity in the ERα bioassay in time was observed. For ER$\beta$, the three highest concentrations of extracts resulted in maximum responses in time, and no trend was observed. At 1 $\mu$g/mL of extract, saturation in signal (i.e., maximal response) was not reached yet, and an increase in estrogenicity for the ER$\beta$ in time was observed. The most representative experiments with respect

**Table 3. Quantitative Analysis of the Isoflavonoid Profile during Soybean Induction**[a]

| number | isoflavonoid | prenyl | $t = 0$ | $t = 1$ | $t = 2$ | $t = 3$ | $t = 4$ | $t = 5$ | $t = 6$ | $t = 7$ | $t = 8$ | $t = 9$ | $t = 10$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **5** | genistin | | 0.11(±0.00) | 0.30(±0.07) | 0.25(±0.04) | 0.18(±0.03) | 0.23(±0.04) | 0.23(±0.03) | 0.20(±0.01) | 0.23(±0.03) | 0.19(±0.04) | 0.26(±0.06) | 0.19(±0.02) |
| **7** | malonyl-genistin | | 0.06(±0.03) | 0.33(±0.07) | 0.29(±0.04) | 0.29(±0.07) | 0.30(±0.07) | 0.25(±0.06) | 0.25(±0.04) | 0.26(±0.02) | 0.22(±0.05) | 0.31(±0.13) | 0.20(±0.04) |
| **12** | genistein | | 0.01(±0.00) | 0.05(±0.01) | 0.05(±0.02) | 0.05(±0.02) | 0.05(±0.03) | 0.05(±0.04) | 0.06(±0.04) | 0.06(±0.03) | 0.09(±0.02) | 0.09(±0.04) | 0.06(±0.01) |
| **16** | 2′-hydroxygenistein | | | 0.01(±0.00) | 0.01(±0.00) | 0.02(±0.00) | 0.02(±0.01) | 0.02(±0.01) | 0.02(±0.01) | 0.02(±0.01) | 0.02(±0.00) | 0.08(±0.08) | 0.02(±0.00) |
| **26** | $A_{prenyl}$-2′OH-genistein | chain | | | | 0.01(±0.01) | 0.02(±0.01) | 0.03(±0.02) | 0.04(±0.02) | 0.04(±0.01) | 0.04(±0.02) | 0.04(±0.02) | 0.03(±0.01) |
| **29** | $A_{prenyl}$-genistein | chain | | | 0.01(±0.00) | 0.02(±0.01) | 0.03(±0.01) | 0.03(±0.01) | 0.04(±0.02) | 0.05(±0.02) | 0.05(±0.02) | 0.06(±0.02) | 0.03(±0.02) |
| **30** | $B_{prenyl}$-genistein | chain | | | | 0.01(±0.01) | 0.02(±0.01) | 0.03(±0.01) | 0.04(±0.02) | 0.04(±0.01) | 0.04(±0.02) | 0.05(±0.02) | 0.03(±0.01) |
| | **genistein pool** | | **0.18( ± 0.04)** | **0.70( ± 0.16)** | **0.60( ± 0.10)** | **0.58( ± 0.14)** | **0.66( ± 0.18)** | **0.64( ± 0.18)** | **0.65( ± 0.17)** | **0.70( ± 0.14)** | **0.65( ± 0.17)** | **0.89( ± 0.38)** | **0.56( ± 0.11)** |
| **3** | glycitin | | 0.02(±0.01) | 0.10(±0.02) | 0.09(±0.01) | 0.07(±0.02) | 0.07(±0.01) | 0.07(±0.01) | 0.06(±0.01) | 0.07(±0.01) | 0.05(±0.01) | 0.06(±0.01) | 0.05(±0.01) |
| **9** | glycitein | | 0.01(±0.01) | 0.06(±0.01) | 0.05(±0.01) | 0.06(±0.02) | 0.08(±0.01) | 0.06(±0.01) | 0.05(±0.01) | 0.06(±0.01) | 0.06(±0.01) | 0.07(±0.02) | 0.05(±0.02) |
| **23** | $B_{prenyl}$-glycitein | chain | | | | <0.01 | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.00) |
| | **glycitein pool** | | **0.03( ± 0.02)** | **0.16( ± 0.03)** | **0.14( ± 0.02)** | **0.13( ± 0.04)** | **0.15( ± 0.03)** | **0.13( ± 0.02)** | **0.12( ± 0.03)** | **0.13( ± 0.02)** | **0.12( ± 0.02)** | **0.15( ± 0.04)** | **0.10( ± 0.03)** |
| **2** | daidzin | | 0.07(±0.03) | 0.25(±0.04) | 0.22(±0.04) | 0.18(±0.03) | 0.19(±0.03) | 0.19(±0.01) | 0.16(±0.02) | 0.18(±0.03) | 0.14(±0.04) | 0.18(±0.03) | 0.12(±0.01) |
| **6** | malonyl-daidzin | | 0.07(±0.04) | 0.39(±0.07) | 0.34(±0.04) | 0.32(±0.08) | 0.32(±0.06) | 0.31(±0.05) | 0.27(±0.05) | 0.27(±0.05) | 0.22(±0.06) | 0.28(±0.10) | 0.21(±0.05) |
| **11** | daidzein | | 0.02(±0.01) | 0.05(±0.01) | 0.05(±0.02) | 0.07(±0.02) | 0.09(±0.04) | 0.09(±0.03) | 0.11(±0.05) | 0.13(±0.04) | 0.13(±0.02) | 0.16(±0.05) | 0.10(±0.01) |
| **22** | $A_{prenyl}$-2′OH-daidzein | chain | | | | 0.02(±0.01) | 0.04(±0.02) | 0.05(±0.02) | 0.07(±0.04) | 0.07(±0.02) | 0.08(±0.04) | 0.09(±0.04) | 0.06(±0.03) |
| **25** | $A_{prenyl}$-daidzein | chain | | | <0.01 | 0.02(±0.01) | 0.03(±0.01) | 0.03(±0.01) | 0.04(±0.02) | 0.03(±0.01) | 0.03(±0.02) | 0.04(±0.02) | 0.03(±0.01) |
| **27** | $B_{prenyl}$-daidzein | chain | | | <0.01 | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.01) | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.00) | 0.01(±0.00) |
| | **daidzein pool** | | **0.16( ± 0.07)** | **0.69( ± 0.13)** | **0.63( ± 0.10)** | **0.62( ± 0.15)** | **0.68( ± 0.17)** | **0.68( ± 0.14)** | **0.66( ± 0.18)** | **0.69( ± 0.16)** | **0.62( ± 0.18)** | **0.76( ± 0.24)** | **0.53( ± 0.12)** |
| **8** | Biochanin A | | | | | | 0.01(±0.01) | 0.01(±0.01) | 0.02(±0.01) | 0.02(±0.01) | 0.02(±0.01) | 0.02(±0.01) | 0.01(±0.01) |
| | **isoflavone pool** | | **0.37( ± 0.13)** | **1.54( ± 0.32)** | **1.38( ± 0.22)** | **1.32( ± 0.33)** | **1.50( ± 0.39)** | **1.46( ± 0.34)** | **1.46( ± 0.38)** | **1.54( ± 0.32)** | **1.40( ± 0.39)** | **1.83( ± 0.67)** | **1.21( ± 0.27)** |
| **4** | glycinol | | | <0.01 | <0.01 | 0.01(±0.00) | 0.04(±0.01) | 0.10(±0.03) | 0.17(±0.04) | 0.25(±0.03) | 0.27(±0.06) | 0.35(±0.03) | 0.42(±0.07) |
| **17** | glyceollidin I/II | chain | | <0.01 | <0.01 | 0.02(±0.01) | 0.06(±0.04) | 0.12(±0.06) | 0.16(±0.11) | 0.18(±0.07) | 0.20(±0.08) | 0.22(±0.05) | 0.23(±0.09) |
| **19** | glyceollin III | furan[1] | | <0.01 | 0.01(±0.00) | 0.02(±0.01) | 0.07(±0.04) | 0.12(±0.07) | 0.18(±0.11) | 0.21(±0.09) | 0.29(±0.09) | 0.32(±0.07) | 0.29(±0.06) |
| **20** | glyceollin II | pyran | | | 0.00(±0.00) | 0.01(±0.01) | 0.04(±0.02) | 0.08(±0.03) | 0.12(±0.07) | 0.17(±0.06) | 0.21(±0.04) | 0.24(±0.03) | 0.22(±0.04) |
| **21** | glyceollin I | pyran | | <0.01 | 0.02(±0.00) | 0.07(±0.04) | 0.17(±0.09) | 0.22(±0.10) | 0.24(±0.13) | 0.29(±0.11) | 0.31(±0.09) | 0.33(±0.05) | 0.32(±0.06) |
| **28** | glyceollin IV | chain | | | <0.01 | <0.01 | 0.02(±0.03) | 0.08(±0.08) | 0.16(±0.15) | 0.25(±0.13) | 0.27(±0.16) | 0.33(±0.20) | 0.35(±0.21) |
| **24** | glyceollin V/VI | furan[2] | | <0.01 | <0.01 | <0.01 | 0.03(±0.02) | 0.07(±0.04) | 0.13(±0.06) | 0.18(±0.04) | 0.22(±0.08) | 0.24(±0.05) | 0.22(±0.05) |
| **10** | glyceofuran | furan[3] | | 0.07(±0.00) | 0.06(±0.00) | 0.06(±0.00) | 0.04(±0.01) | 0.06(±0.02) | 0.08(±0.04) | 0.10(±0.04) | 0.15(±0.05) | 0.17(±0.03) | 0.18(±0.04) |
| | **pterocarpan pool** | | | **0.07( ± 0.00)** | **0.09( ± 0.01)** | **0.20( ± 0.09)** | **0.47( ± 0.26)** | **0.86( ± 0.43)** | **1.24( ± 0.70)** | **1.63( ± 0.57)** | **1.91( ± 0.64)** | **2.21( ± 0.51)** | **2.24( ± 0.62)** |
| **18** | coumestrol | | | | 0.01(±0.00) | 0.02(±0.01) | 0.06(±0.02) | 0.08(±0.03) | 0.10(±0.06) | 0.12(±0.04) | 0.15(±0.06) | 0.16(±0.05) | 0.13(±0.04) |
| **13** | C-methyl-coumestrol | | | | | | 0.01(±0.01) | 0.03(±0.01) | 0.06(±0.03) | 0.09(±0.04) | 0.15(±0.03) | 0.16(±0.04) | 0.15(±0.03) |
| **15** | isotrifoliol | | | | | 0.01(±0.00) | 0.01(±0.01) | 0.03(±0.02) | 0.05(±0.03) | 0.06(±0.03) | 0.09(±0.05) | 0.10(±0.04) | 0.08(±0.02) |
| **31** | 4-prenyl-coumestrol | chain | | | | 0.01(±0.01) | 0.01(±0.01) | 0.03(±0.02) | 0.05(±0.04) | 0.05(±0.03) | 0.07(±0.06) | 0.09(±0.06) | 0.06(±0.05) |
| | coumestan pool | | | | | **0.03( ± 0.02)** | **0.09( ± 0.05)** | **0.18( ± 0.09)** | **0.26( ± 0.17)** | **0.33( ± 0.13)** | **0.45( ± 0.20)** | **0.51( ± 0.20)** | **0.42( ± 0.14)** |
| | **total isoflavonoids** | | **0.37( ± 0.13)** | **1.61( ± 0.32)** | **1.48( ± 0.23)** | **1.54( ± 0.44)** | **2.06( ± 0.70)** | **2.49( ± 0.85)** | **2.95( ± 1.24)** | **3.50( ± 1.03)** | **3.77( ± 1.24)** | **4.54( ± 1.38)** | **3.87( ± 1.03)** |

[a] The content of each flavonoid is expressed in mg daidzein equivalent (DE) per g DW, corrected for loss in dry weight during the induction process. Data are the mean ± SD values of five independent *Rhizopus*-challenged germination experiments. Chain, 3,3 dimethylallyl (prenyl chain); pyran, 2,2-dimethylchromeno; furan[1], 2″-isopropenyl-dihydrofuran; furan[2], 2″-isopropenyl-furan; furan[3], 2″-(2-hydroxy-)isopropenyl-dihydrofuran.



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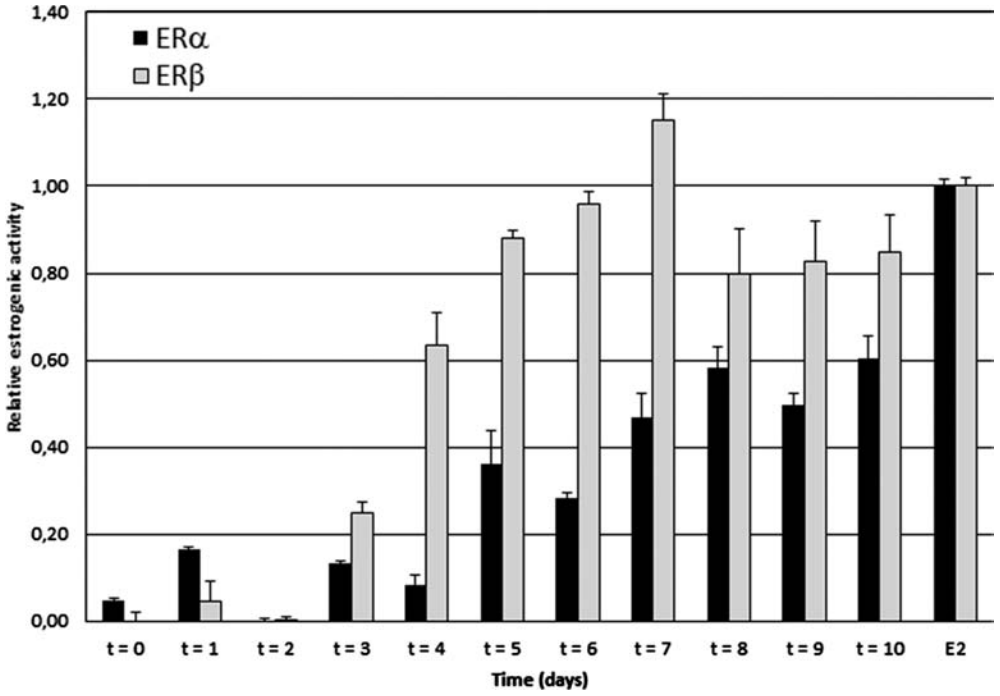


**Figure 4.** Estrogenic activity of the ethanol extracts of the soybean seedlings at different time points measured in the yeast estrogen bioassays. The extracts were measured at 10 $\mu$g/mL in the ERα bioassay and at 1 $\mu$g/mL in the ER$\beta$ bioassay. Data are the mean ± SD values of measurements performed in triplicate. $E_2$ is 1 $\mu$M 17$\beta$-estradiol.

to the increasing estrogenicity in time are shown in Figure 4. The estrogenicity toward the ER$\beta$ was relatively higher compared to that toward the ERα, even more so as the ER$\beta$ bar diagram was obtained with 10-fold lower extract concentrations than that of ERα. For 1 $\mu$g/mL, the estrogenicity toward the ER$\beta$ appeared to reach a plateau value at $t$ = 5.

The extracts were also tested for possible antagonistic activity toward both estrogen receptors. These experiments showed that the extracts were unable to suppress the agonistic response caused by the dose of 17$\beta$-estradiol that gave a 70 to 90% maximal response. On the contrary, the responses only increased by these coexposures, showing additive agonistic effects, e.g., the addition of the extracts of $t$ = 4 to $t$ = 10 enhanced the estrogenicity of estradiol by 20−25% (data not shown).

## DISCUSSION

By combining malting technology and fermentation with the food-grade *R. microsporus*, the induction of phytoalexins in soy seedlings was successfully performed in terms of increasing total isoflavonoid content, altering isoflavonoid composition, and enhancing the estrogenic potential of the soy material.

**De Novo Synthesis of Isoflavonoids during the Induction Process.** The isoflavonoid content of soy increased by a factor 10−12 suggesting the de novo synthesis of isoflavonoids. This is further corroborated by the appearance ($t$ = 1) of phenylalanine (**1**), the basic precursor of flavonoid biosynthesis, and naringenin (**14**), a precursor of genistein (**16**). The formation of the flavanone naringenin might thus explain the increase of the genistein pool.[18] These findings are supported by several reports in which de novo synthesis is suggested on the basis of the quantification of isoflavonoid content, up-regulation of enzymes involved in isoflavonoid biosynthesis and incorporation of $^{14}$C-labeled phenylalanine, daidzein, 2′OH-daidzein, and glycinol into glyceollins I−III.[6,19−22] Data on accurate quantification of the total isoflavonoid content during the induction process are very limited. It has been reported that levels ranging from 0.01 to 1 mg/g fresh weight (FW) of glyceollins I−III can accumulate in fungus-challenged soy seedlings.[23] With an approximate water content of 65−70% (w/w),[24] the glyceollin I−III levels in soy seedlings range from 0.03 to 3 mg/g DW, whereas a content of 0.9 mg DE/g DW was found at 9 days in our study (Table 3). The approximately 3-fold lower amount of glyceollins in our experiments compared to the highest accumulation reported in the literature might be explained as follows. In most studies, soybeans are wounded prior to inoculation. It has been shown that this additional treatment can increase the glyceollin I−III levels of soybeans inoculated with *Aspergillus sojae* by a factor 10.[25] This extra wounding step might explain the difference between our data and those in the literature. Moreover, a number of other pterocarpans (1.3 mg DE/g DW) were found in our study (Table 3), suggesting that the difference between our results and those in the literature might actually be smaller than they appear.

The stabilization of the glyceollins I−III contents at $t$ = 9 might be due to the inhibition of cyclase, the enzyme responsible for the last step in glyceollin biosynthesis, as this enzyme is inhibited by its reaction products.[26] In line with this, the accumulation of glycinol did not seem to level off.

**Changes in Isoflavonoid Profile.** Although inoculation was performed at $t$ = 3, small amounts of pterocarpans and coumestans already occurred at $t$ = 2. This might be due to incomplete sterilization. Alternatively, the sterilization agent itself (hypochlorite) might have acted as a chemical elicitor.

The ratio of the commonly found glyceollins I, II, and III was 1.4:1:1.3 at $t$ = 9, respectively, and differed from the ratios of 6:1:2 previously reported.[27] More strikingly, the present study

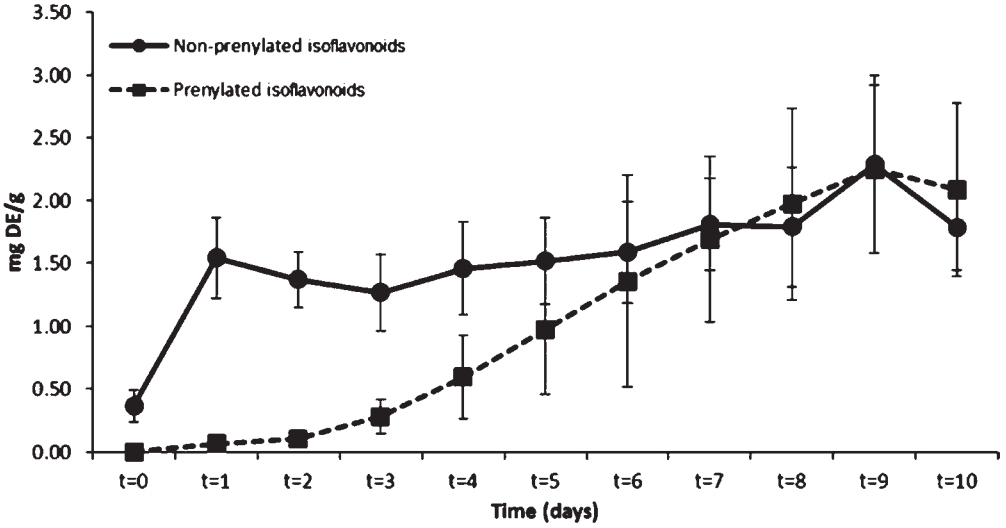


**Figure 5.** Quantitative analysis of the isoflavonoid profile in the time course experiments of the development of *Rhizopus*-elicited soy seedlings.

shows the additional formation of glyceofuran (**10**), glyceollin IV (**28**), glyceollin V/VI (**24**), coumestrol-derivatives (**13**, **15**, and **31**), and several prenylated isoflavones (**22**, **23**, **25**−**27**, **29**, and **30**). The simultaneous occurrence of these compounds has never been reported in *Glycine max*. However, glyceollin IV has been found in *G. tomentella* and glyceollin VI in *G. clandestine* and *G. tabacina*.[28,29] The isomer of glyceollin VI has recently been isolated and given the trivial name glyceollin V.[17] However, the name glyceollin V was already used for the enantiomeric form of glyceollin III.[28] To avoid confusion, the two enantiomers of glyceollins III are indicated as glyceollin IIIa and IIIb in Figure 3, whereas the isomer of glyceollin VI is indicated by glyceollin V.

The occurrence of phaseol (**31**) and isotrifoliol (**15**) has been reported before in *G. max*.[30,31] The formation of prenylated isoflavones has recently been reported for *G. max*, as well as in several other, often stress-induced, *Leguminosae* species.[5] After the 9 days of induction, these compounds have accumulated to an impressive 32% (w/w) of the total isoflavonoids in the soy material.

Taken together, the more extensive changes in isoflavonoid composition as observed in the present study compared to studies by others is most likely due to the combination of prolonged induction times and more favorable growth conditions in the micromalting system. Consequently, the altered ratio between the most common glyceollins and the formation of other compounds might represent the outcome of events occurring more downstream in the biosynthesis process. Possibly, the omission of the wounding pretreatment also plays a role.

**Accumulation of Phytoestrogens during the Induction Process.** Figure 4 shows a gradual increase in estrogenicity of the extracts during the induction process toward both the ERα and ER$\beta$. It is likely that the increased proportion of isoflavonoids and the change in isoflavonoid composition in the extract are responsible for this increase of estrogenic activity. Deglycosylation of isoflavones by $\beta$-glucosidase might be a driver behind this.[32] The proportion of glucosides (compounds **2**, **3**, and **5**−**7**) decreased from 90% ($t$ = 0) to approximately 20% ($t$ = 9). It is known that the glucosyl residues hamper binding to the ER.[8] More importantly, other isoflavonoids were formed of which several have been reported as potent agonists toward both ERs, particularly glycinol and coumestrol.[8,33] Differences in the outcome of various bioassays described in the literature, and lack of data for binding of individual isoflavonoids for both ERs, do not allow extrapolations concerning the contribution of each isoflavonoid to the estrogenicity of the current extract.

Besides agonistic activity, it is likely that the extracts contain compounds with antagonistic properties. For soybean, this has been demonstrated for glyceollin I,[34] which comprised 8% (w/w) of the total isoflavonoids at $t$ = 9. To determine to what extent phytoestrogens with a known $EC_{50}$ value can account for the estrogenic activity of a tested extract, the expected estrogenicity of the extract was calculated from the contribution of each phytoestrogen (expressed in $E_2$ equivalents, EEQ), using their relative estrogenic potency (REP) and their quantity in the extract. This was performed for extracts of $t$ = 10 and $t$ = 4 with ERα and ER$\beta$, respectively (Supporting Information). The calculated values were compared to those actually measured. The expected estrogenicity (expressed as the sum of the EEQ of genistein, coumestrol, and daidzein) was approximately 25% higher than the EEQ corresponding to the response measured in the assay. The same calculation was performed for the ER$\beta$ showing that the total expected estrogenicity was even a factor 7 higher than the EEQ of the response measured. Thus, the measured response in the assay was lower than the one calculated, which hints at the presence of antagonists in the extract. Even more so, as the calculated EEQ based on genistein, coumestrol and daidzein (phytoestrogens with a known $EC_{50}$) are most likely underestimated because glycinol, a potent phytoestrogen on both ER subtypes with an estrogenic activity similar to that of coumestrol, has not been taken into account. The data on glycinol available from the literature were obtained with a bioassay different from ours.[33] As mentioned earlier, glyceollin I has been reported to display antagonistic activity on the ERα. It has been suggested that prenyl substituents are important structural attributes for inducing antagonistic responses.[35−38] In Figure 5, the accumulation of prenylated isoflavonoids is compared with that of nonprenylated isoflavonoids. It is clearly seen that longer induction times promote a higher proportion of prenylated isoflavonoids, reaching similar amounts as nonprenylated isoflavonoids at around $t$ = 7. This suggests that the accumulation of prenylated isoflavonoids in time increases the antagonistic activity of the extracts.

In summary, we speculate that the increased agonistic ER activity of the extracts is mainly due to the formation of the nonprenylated pterocarpan, glycinol, and the nonprenylated coumestans, particularly coumestrol. Besides, the extracts might also be rich in ER antagonists, as the prenylation of isoflavonoids is known to correlate well with antagonistic properties (e.g., glyceollin I). Although antagonism was not established directly in our study, we found a lower estrogenic response with the extracts than might be expected from their amounts of agonists. Further investigation is necessary to substantiate this.

**Conclusions.** Malting of soybeans in the presence of a food-grade fungus yielded a completely different spectrum of isoflavonoids, with a much higher bioactivity toward both estrogen receptor subtypes than the starting material (nonsoaked beans). Together with the over 10-fold increase in potential bioactives, this offers promising perspectives for producing more, novel, and higher potency nutraceuticals by malting soybeans under stressed conditions.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Calculation of the estradiol equivalents (EEQ). This material is available free of charge via the Internet at http://pubs.acs.org.

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## ■ REFERENCES

(1) Veitch, N. C. Isoflavonoids of the Leguminosae. *Nat. Prod. Rep.* **2007**, *24* (2), 417–464.

(2) Wang, H. J.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42* (8), 1666–1673.

(3) Messina, M. J. Legumes and soybeans: Overview of their nutritional profiles and health effects. *Am. J. Clin. Nutr.* **1999**, *70* (Suppl. 3), 439S–450S.

(4) Dakora, F. D.; Phillips, D. A. Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. *Physiol. Mol. Plant Pathol.* **1996**, *49* (1), 1–20.

(5) Simons, R.; Vincken, J.-P.; Bohin, M. C.; Kuijpers, T. F. M.; Verbruggen, M. A.; Gruppen, H. Identification of prenylated pterocarpans and other isoflavonoids in *Rhizopus* spp. elicited soya bean seedlings by electrospray ionisation mass spectrometry. *Rapid Commun. Mass Spectrom.* **2011**, *25* (1), 55–65.

(6) Banks, S. W.; Dewick, P. M. Biosynthesis of glyceollins I, II and III in soybean. *Phytochemistry* **1983**, *22* (12), 2729–2733.

(7) Ingham, J. L.; Keen, N. T.; Mulheirn, L. J.; Lyne, R. L. Inducibly-formed isoflavonoids from leaves of soybean. *Phytochemistry* **1981**, *20* (4), 795–798.

(8) Bovee, T. F. H.; Helsdingen, R. J. R.; Rietjens, I. M. C. M.; Keijer, J.; Hoogenboom, R. L. A. P. Rapid yeast estrogen bioassays stably expressing human estrogen receptors α and β, and green fluorescent protein: A comparison of different compounds with both receptor types. *J. Steroid Biochem. Mol. Biol.* **2004**, *91* (3), 99–109.

(9) Bovee, T. F. H.; Thevis, M.; Hamers, A. R. M.; Peijnenburg, A. A. C. M.; Nielen, M. W. F.; Schoonen, W. G. E. J. SERMs and SARMs: Detection of their activities with yeast based bioassays. *J. Steroid Biochem. Mol. Biol.* **2010**, *118* (1−2), 85–92.

(10) Meyers, M. J.; Jun, S.; Carlson, K. E.; Katzenellenbogen, B. S.; Katzenellenbogen, J. A. Estrogen receptor subtype-selective ligands: Asymmetric synthesis and biological evaluation of cis- and trans-5,11-dialkyl-5,6,11,12- tetrahydrochrysenes. *J. Med. Chem.* **1999**, *42* (13), 2456–2468.

(11) Choi, U. K.; Kim, M. H.; Lee, N. H.; Jeong, Y. S.; Kwon, O. J.; Kim, Y. C.; Hwang, Y. H. The characteristics of *cheonggukjang*, a fermented soybean product, by the degree of germination of raw soybeans. *Food Sci. Biotechnol.* **2007**, *16* (5), 734–739.

(12) Simons, R.; Vincken, J.-P.; Bakx, E. J.; Verbruggen, M. A.; Gruppen, H. A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography/electrospray ionisation mass spectrometry in licorice root extracts. *Rapid Commun. Mass Spectrom.* **2009**, *23* (19), 3083–93.

(13) Fabre, N.; Rustan, I.; de Hoffmann, E.; Quetin-Leclercq, J. Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2001**, *12* (6), 707–715.

(14) Prasain, J. K.; Wang, C. C.; Barnes, S. Mass spectrometric methods for the determination of flavonoids in biological samples. *Free Radical Biol. Med.* **2004**, *37* (9), 1324–1350.

(15) Kang, J.; Hick, L. A.; Price, W. E. A fragmentation study of isoflavones in negative electrospray ionization by MSn ion trap mass spectrometry and triple quadrupole mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21* (6), 857–868.

(16) Klejdus, B.; Vacek, J.; Benešová, L.; Kopecký, J.; Lapčík, O.; Kubáň, V. Rapid-resolution HPLC with spectrometric detection for the determination and identification of isoflavones in soy preparations and plant extracts. *Anal. Bioanal. Chem.* **2007**, *389* (7−8), 2277–2285.

(17) Yuk, H. J.; Lee, J. H.; Curtis-Long, M. J.; Lee, J. W.; Kim, Y. S.; Ryu, H. W.; Park, C. G.; Jeong, T. S.; Park, K. H. The most abundant polyphenol of soy leaves, coumestrol, displays potent α-glucosidase inhibitory activity. *Food Chem.* **2011**, *126* (3), 1057–1063.

(18) Yu, O.; McGonigle, B. Metabolic engineering of isoflavone biosynthesis. *Adv. Agronomy* **2005**, *86*, 147–190.

(19) Lozovaya, V. V.; Lygin, A. V.; Zernova, O. V.; Li, S.; Hartman, G. L.; Widholm, J. M. Isoflavonoid accumulation in soybean hairy roots upon treatment with *Fusarium solani*. *Plant Physiol. Biochem.* **2004**, *42* (7−8), 671–679.

(20) Graham, T. L.; Kim, J. E.; Graham, M. Y. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. *Mol. Plant-Microbe Interact.* **1990**, *3* (3), 157–166.

(21) Dhaubhadel, S.; McGarvey, B. D.; Williams, R.; Gijzen, M. Isoflavonoid biosynthesis and accumulation in developing soybean seeds. *Plant Mol. Biol.* **2003**, *53* (6), 733–743.

(22) Lozovaya, V. V.; Lygin, A. V.; Li, S.; Hartman, G. L.; Widholm, J. M. Biochemical response of soybean roots to *Fusarium solani* f. sp. *glycines* infection. *Crop Sci.* **2004**, *44* (3), 819–826.

(23) Bhattacharyya, M. K.; Ward, E. W. B. Resistence, susceptibility and accumulation of glyceollins I-III in soybean organs inoculated with *Phytophthora megasperma* f. sp. *glycinea*. *Physiol. Mol. Plant Pathol.* **1986**, *29* (2), 227–237.

(24) Shi, H.; Nam, P. K.; Ma, Y. Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (*Glycine max*) germination. *J. Agric. Food Chem.* **2010**, *58* (8), 4970–4976.

(25) Kim, H. J.; Suh, H. J.; Kim, J. H.; Kang, S. C.; Park, S.; Lee, C. H.; Kim, J. S. Estrogenic activity of glyceollins isolated from soybean elicited with *Aspergillus sojae*. *J. Med. Food* **2010**, *13* (2), 382–390.

(26) Welle, R.; Grisebach, H. Induction of phytoalexin synthesis in soybean: Enzymatic cyclization of prenylated pterocarpans to glyceollin isomers. *Arch. Biochem. Biophys.* **1988**, *263* (1), 191–198.

(27) Boué, S. M.; Carter, C. H.; Ehrlich, K. C.; Cleveland, T. E. Induction of the soybean phytoalexins coumestrol and glyceollin by *Aspergillus*. *J. Agric. Food Chem.* **2000**, *48* (6), 2167–2172.

(28) Keen, N. T.; Lyne, R. L.; Hymowitz, T. Phytoalexin production as a chemosystematic parameter within the Genus *Glycine*. *Biochem. Syst. Ecol.* **1986**, *14* (5), 481–486.

(29) Lyne, R. L.; Mulheirn, L. J. Minor pterocarpinoids of soybean. *Tetrahedron Lett.* **1978**, *19* (34), 3127–3128.

(30) Yuk, H. J.; Lee, J. H.; Curtis-Long, M. J.; Lee, J. W.; Kim, Y. S.; Ryu, H. W.; Park, C. G.; Jeong, T.-S.; Park, K. H. The most abundant polyphenol of soy leaves, coumestrol, displays potent [alpha]-glucosidase inhibitory activity. *Food Chem.* **2011**, *126* (3), 1057–1063.

(31) Caballero, P.; Smith, C. M.; Fronczek, F. R.; Fischer, N. H. Isoflavones from an insect-resistant variety of soybean and the molecular structure of afrormosin. *J. Nat. Prod.* **1986**, *49* (6), 1126–1129.

(32) Suzuki, H.; Takahashi, S.; Watanabe, R.; Fukushima, Y.; Fujita, N.; Noguchi, A.; Yokoyama, R.; Nishitani, K.; Nishino, T.; Nakayama, T. An isoflavone conjugate-hydrolyzing $\beta$-glucosidase from the roots of soybean (*Glycine max*) seedlings: Purification, gene cloning, phylogenetics, and cellular localization. *J. Biol. Chem.* **2006**, *281* (40), 30251–30259.

(33) Boué, S. M.; Tilghman, S. L.; Elliott, S.; Zimmerman, M. C.; Williams, K. Y.; Payton-Stewart, F.; Miraflor, A. P.; Howell, M. H.; Shih, B. Y.; Carter-Wientjes, C. H.; Segar, C.; Beckman, B. S.; Wiese, T. E.; Cleveland, T. E.; McLachlan, J. A.; Burow, M. E. Identification of the potent phytoestrogen glycinol in elicited soybean (*Glycine max*). *Endocrinology* **2009**, *150* (5), 2446–2453.

(34) Zimmermann, M. C.; Tilghman, S. L.; Boué, S. M.; Salvo, V. A.; Elliott, S.; Williams, K. Y.; Skripnikova, E. V.; Ashe, H.; Payton-Stewart, F.; Vanhoy-Rhodes, L.; Fonseca, J. P.; Corbitt, C.; Collins-Burow, B. M.; Howell, M. H.; Lacey, M.; Shih, B. Y.; Carter-Wientjes, C.; Cleveland, T. E.; McLachlan, J. A.; Wiese, T. E.; Beckman, B. S.; Burow, M. E. Glyceollin I, a novel antiestrogenic phytoalexin isolated from activated soy. *J. Pharmacol. Exp. Ther.* **2010**, *332* (1), 35–45.

(35) Jiang, Q.; Payton-Stewart, F.; Elliott, S.; Driver, J.; Rhodes, L. V.; Zhang, Q.; Zheng, S.; Bhatnagar, D.; Boue, S. M.; Collins-Burow, B. M.; Sridhar, J.; Stevens, C.; McLachlan, J. A.; Wiese, T. E.; Burow, M. E.; Wang, G. Effects of 7-O substitutions on estrogenic and antiestrogenic activities of daidzein analogues in MCF-7 breast cancer cells. *J. Med. Chem.* **2010**, *53* (16), 6153–6163.

(36) Ahn, E. M.; Nakamura, N.; Akao, T.; Nishihara, T.; Hattori, M. Estrogenic and antiestrogenic activities of the roots of *Moghania philippinensis* and their constituents. *Biol. Pharm. Bull.* **2004**, *27* (4), 548–553.

(37) Okamoto, Y.; Suzuki, A.; Ueda, K.; Ito, C.; Itoigawa, M.; Furukawa, H.; Nishihara, T.; Kojima, N. Anti-estrogenic activity of prenylated isoflavones from *Millettia pachycarpa*: Implications for pharmacophores and unique mechanisms. *J. Health Sci.* **2006**, *52* (2), 186–191.

(38) Boué, S. M.; Burow, M. E.; Wiese, T. E.; Shih, B. Y.; Elliott, S.; Carter-Wientjes, C. H.; McLachlan, J. A.; Bhatnagar, D. Estrogenic and antiestrogenic activities of phytoalexins from red kidney bean (*Phaseolus vulgaris* L.). *J. Agric. Food Chem.* **2011**, *59* (1), 112–120.

(39) Veitch, N. C. Isoflavonoids of the leguminosae. *Nat. Prod. Rep.* **2009**, *26* (6), 776–802.