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Two Coumarins with Safener Activity from Rhizoma et Radix Notopterygii

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Rhizoma et Radix Notopterygii is often used as a traditional medicine in China. In our recent work, we found that the ethanol crude extract of Rhizoma et Radix Notopterygii could reduce phytotoxicity of acetochlor on rice. The crude extract of Rhizoma et Radix Notopterygii was isolated and purified by activity-guided fractionation. Two coumarins, isopimpinellin and 5-methoxypsoralen (5-MOP) were identified, and their bioactivity was tested in a growth chamber. The results showed that the two coumarins increased herbicide tolerance of rice shoots, and 5-MOP demonstrated better protection than isopimpinellin. The treatment of 5-MOP at 50 g ai ha^{-1} recovered the shoot height of rice from 42.6% (treated with acetochlor only) to 81.6% of the control treated without acetochlor, whereas the phytocidal activity of acetochlor on barnyardgrass was not impaired by 5-MOP. Further study suggested that 5-MOP increases herbicide tolerance of rice by enhancing the glutathione *S*-transferase level of activity in rice. Our findings suggest that isopimpinellin and 5-MOP have the potential to be applied as safeners for rice.

Nomenclature: Acetochlor, 2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide; isopimpinellin, 2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide; methoxypsoralen, 4-methoxyfuro[3,2-g]chromen-7-one; Rhizoma et Radix Notopterygii; barnyardgrass, *Echinochloa crus-galli* (L.) Beauv.; rice; *Oryza sativa* L.

Key words: Acetochlor, safener, Rhizoma et Radix Notopterygii, 5-methoxypsoralen.

Rhizoma et Radix Notopterygii es comúnmente usado como una medicina tradicional en China. En nuestro reciente trabajo encontramos que el extracto crudo con ethanol de Rhizoma et Radix Notopterygii pudo reducir la fitotoxicidad de acetochlor en arroz. El extracto crudo de Rhizoma et Radix Notopterygii fue aislado y purificado por medio del fraccionamiento guiado por actividad. Dos coumarins, isopimpinellin y 5-methoxypsoralen (5-MOP) fueron identificadas, y se evaluó su bioactividad en una cámara de crecimiento. Los resultados mostraron que estas dos coumarins aumentaron la tolerancia al herbicida de tejidos aéreos de arroz, y 5-MOP demostró una mejor protección que isopimpinellin. El tratamiento de 5-MOP a 50 g ai ha^{-1} recuperó la altura del arroz de 42.6% (tratado con sólo acetochlor) a 81.6% al compararse con el testigo tratado sin acetochlor, mientras que la actividad herbicida de acetochlor en *Echinochloa crus-galli* no fue afectada por 5-MOP. Estudios adicionales sugirieron que 5-MOP incrementa la tolerancia del arroz al herbicida al mejorar el nivel de actividad de glutathione-*S*-transferase en arroz. Nuestros descubrimientos sugieren que isopimpinellin y 5-MOP tienen el potencial de ser aplicados como antídotos en arroz.

Acetochlor, a chloroacetamide herbicide, is efficient in controlling annual graminaceous weeds and certain broadleaf weeds PRE (Nemeth-Konda et al. 2002). It is widely used in agriculture in China and other areas of the world, but its application in paddy fields is limited because of its phytotoxicity to rice (Huang and Xiong 2009). Safeners, also known as antidotes, are chemicals selectively protecting crop plants from herbicide toxicity without reducing the activity of herbicide to target weeds (Hatzios and Hoagland 1989; Ramsey et al. 2001). Some safeners for chloroacetanilide herbicides have been developed and used successively in the agricultural industry. For example, the safener fenclorim (4,6-dichloro-2-phenylpyrimidine) is mainly used in many rice-producing countries to protect rice from toxicity caused by pretilachlor (Christ 1985; Wu et al. 1996). Benoxacor, a safener developed for metolachlor in corn (*Zea mays* L.), can increase metabolism of metolachlor mainly in corn coleoptiles and reduce corn injury caused by metolachlor (Foy and Witt 1991; Kreuz et al. 1989). Many studies have suggested that the protection of crops by safeners is the result of a safener-mediated induction of glutathione *S*-transferase (GST) activ-

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ity that plays an important role in herbicide detoxification (Buono et al. 2006; Hatzios and Burgos 2004; Scarponi et al. 2005, 2006; Taylor et al. 2013). In our review of the current literature, there is no botanical safener reported, and the known safeners are all developed by chemical synthesis. In comparison, botanical safeners can play an important role in detoxifying herbicides in cereals with fewer environmental concerns than chemically synthesized safeners.

Recently, we found that the ethanol extract isolated from Rhizoma et Radix Notopterygii showed safening action against acetochlor on rice (Hu et al. 2012). Rhizoma et Radix Notopterygii, known as "qiang huo" in Chinese medicine, may contain the dried rhizome and root of *Notopterygium incisum* Ting ex H. T. Chang, *Notopterygium forbesii* Boiss, or a mixture of both plants. This herb has a relatively wide geographic distribution in China, including the Qinghai, Sichuan, and Gansu provinces and the autonomous region of Tibet. The extract of Rhizoma et Radix Notopterygii has been verified to have antifebrile and anodyne activities, as well as antilipid peroxidation, anti-inflammation, and anti–delayed-type hypersensitivity properties (National Pharmacopoeia Committee 2005; Qin et al. 2000; Sun and Xu 2003; Yang et al. 1991). A number of substances extracted from Rhizoma et Radix Notopterygii have been examined for medical purposes (Okuyama et al. 1993; Wu et al. 2010), but its detoxification activity in plant herbicides has not been reported until our recent study.

To explore Rhizoma et Radix Notopterygii as a potential protectant to rice, we isolated and purified the active constituents from the ethanol extract. The obtained compounds were identified with nuclear magnetic resonance (NMR) spectroscopy, and their protective effects on rice against acetochlor and their influence on the phytocidal activity of acetochlor were tested through indoor bioassay. The effect of a putative compound on induction of GST activity in rice shoots was also checked.

Materials and Methods

General Materials. NMR spectra were recorded in $CDCl_3$ on Varian INOVA-300 spectrometers (Varian Inc., now Agilent Technologies). The information provided by electron ionization mass spectrometry (MS) was obtained on Agilent 5973/6980 gas chromatography (GC)-MS. Silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China) was used for column chromatography. Silica gel GF254 plates (Qingdao Hai Yang Chemical Group Co., Qingdao, China) were used for thin-layer chromatography (TLC). Preparative high-speed countercurrent chromatography (HSCCC) was carried out with a model TBE-300A (Tauto Biotech Co. Ltd., Shanghai, China). An LC-10ATVP high-performance liquid chromatography (HPLC) pump (Shimadzu, Kyoto, Japan) equipped with a reversed-phase column Ultimate C_{18} (250 by 4.6 mm, 5 μm) was used in the analysis of this experiment. The methanol and water used for the HPLC mobile phase were of HPLC grade, and those used for other purposes were of analytical grade.

Extraction and Isolation. Dried Rhizoma et Radix Notopterygii rhizome and root (purchased from Hunan Medicine Company, Hunan, China) were crushed with a grinder and then extracted with ethanol in a Soxhlet extractor at 90 C. The extract solvent was evaporated under vacuum. Crude extract (100 g) was firstly run on a silica gel column (9.0 by 120 cm, 200–230 mesh, 1,600 g) and successively eluted with petroleum ether: ethyl acetate (90 : 0 to 0 : 100). The column fractions were analyzed by TLC, fractions with similar TLC patterns were pooled, and their safener activities against acetochlor on rice were evaluated. The fraction that showed an optimal protection effect was further separated with HSCCC, and *N*-hexane : ethyl acetate : methanol : water (5 : 5 : 4 : 5, v/v) was chosen as the solvent system. HSCCC was performed according to the method described previously by Lu et al. (2011). The peak fractions from HSCCC were analyzed by HPLC. The mobile phase was a linear gradient prepared from methanol and H_2O as follows: 0–15 min, ~ 4–50% methanol; 5–20 min, ~ 50–55% methanol; 20–25 min, ~ 55–55% methanol; 25–30 min, ~ 55–65% methanol. The flow rate was 1.0 ml min^{-1}, and the effluent was monitored at 330 nm by a UV detector. The compounds with high purity were identified by comparing the NMR data with literature values.

Evaluation of Safener Activity. Safener activity was evaluated by laboratory bioassay. Seeds of rice 'Jinyou 207' (Yuan Longping High-Tech Agricul-

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ture Co. Ltd, Changsha, China) were submersed in tap water for 24 h, sterilized in 3% sodium hypochlorite solution for 5 min, washed several times with sterile de-ionized water, and germinated in the dark for 48 h at 28 C. The uniformly germinated seedlings were selected before root emergence and transferred to a soil-based medium in a 13 by 8 by 5 cm plastic pot, each pot loaded with 200 g air-dried soil with an organic carbon content of 2.8%, pH 6.5, plus 100 ml of sterilized water stirred into a slurry. The pots were placed in the growth chamber under fluorescent lights delivering 540 μmol m^{-2} s^{-1} photosynthetic photon flux density with a 12/12 h light/dark photoperiod at 26 ± 2/22 ± 2 C and 80% relative humidity.

The fractions at each stage of the procedure were monitored by testing for safener activity against acetochlor (Monsanto Company, Beijing, China). To select the appropriate herbicide doses for the study, preliminary experiments were carried out to characterize the dose response of acetochlor applied to rice under indoor condition. Rice (3 d old) was treated with acetochlor doses ranging from 0 to 625 g ai ha^{-1}, and the shoot height and shoot fresh weight of rice seedlings were measured 7 d after treatment. As a result, the dose of 5 g ai ha^{-1}, which is close to the half maximal inhibitory concentration, was selected for the following experiment.

The isolated compounds were added to 3-d-old rice shoots at doses ranging from 0 to 100 g ai ha^{-1}, and the acetochlor dose was 5 g ai ha^{-1}. The rice seedlings were permitted to continue to grow in the growth chamber. Three replicates for each treatment were performed. The rice shoot heights and shoot fresh weights were measured after 7 d. Shoot heights were measured from 10 random plants per pot, and the fresh weight was the whole weight of the 10 plants. The protection effect was calculated as a percentage of shoot heights or weights of the control.

Barnyardgrass control was evaluated to assess the influence of putative safener compounds on the weed control effect of acetochlor. Seeds of barnyardgrass were sterilized in 2.5% sodium hypochlorite solution for 30 min and washed several times with sterilized de-ionized water. Acetochlor (5 g ai ha^{-1}) and putative safener compounds (0, 50, or 100 g ai ha^{-1}) were added to 0.3% agar media. Three replicates for each treatment were performed. Fifteen barnyardgrass seeds were planted per culture box and grown in the growth chamber under day/night photoperiod conditions of 12/12 h light/dark at 26 ± 2/22 ± 2 C. After 5 days, the bud lengths of each treatment were measured.

GST Activity Assays. 5-Methoxypsoralen (5-MOP) was added to 0.3% agar media in plastic pots at 100, 50, 25, 10, 5, 2, 1, and 0 g ai ha^{-1} (control). Acetochlor was added at 5 g ai ha^{-1} as another control. Three replicates for each treatment were performed. Fifty uniformly germinated rice seedlings before shoot emergence were planted in each culture box and grown in the growth chamber under dark conditions at 28 C for 5 d. The crude enzyme extraction from rice seedlings and the GST activity assay were performed according to the method represented by Deng and Hatzios (2002). Protein content was determined using a Bio-Rad assay (Bradford 1976).

Statistical Analysis. The values of shoot height, shoot weight, and bud length were expressed as means ± SD. Data were analyzed by one-way ANOVA, and Fisher's protected LSD test was used to separate means.

Results and Discussion

Purification and Identification of Active Compounds. Through the preliminary isolation by column chromatography, 21 different fractions were separated from the crude extract of Rhizoma et Radix Notopterygii and then tested for safener activity against acetochlor in rice. Fraction Q_5 was verified to possess the highest safener activity against acetochlor. Fraction Q_5 (100 g ai ha^{-1}) recovered shoot heights of acetochlor-treated rice seedlings from 36.7 to 82.7% of the shoot heights of rice seedlings without chemical treatment. Fraction Q_5 was further purified by HSCCC, and two different active compounds (compounds 1 and 2) were obtained. The relative content of these compounds, determined by peak area normalization, was 98.7 and 99.1%, respectively. The structure of isolated compounds 1 and 2 (Figure 1) were identified by NMR spectra and GC-MS.

Compound 1 was obtained as yellow needle crystals (GC-MS): m/z 246[M], 229, 188, 160, 132, 104; 1H NMR (CD_3Cl_3, 300 MHz, δ): 8.14 (1H, d, J = 9.6 Hz, H-4), 7.64 (1H, d, J = 2.4 Hz, H-12), 7.01 (1H, d, J = 2.1 Hz, H-11), 6.30 (1H,

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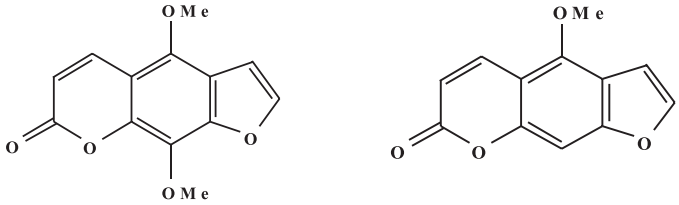

Figure 1. Structure of two components separated from the crude extract of Rhizoma et Radix Notopterygii: compound 1 (left) and compound 2 (right).

d, J = 9.9 Hz, H-3), 4.18 (6H, s, 2×OCH_3); 13C NMR (D_2O, 75 MHz, δ): 160.41(C-2), 148.17 (C-7), 142.65 (C-9), 141.95 (C-5), 145.14 (C-12), 139.44 (C-4), 128.13 (C-8), 116.53 (C-6), 112.88 (C-3), 107.16 (C-10), 105.10 (C-11), 61.17 (2×OCH_3). It was identified as isopimpinellin by comparing its spectral data with reported literature values (Liu et al. 2004).

Compound 2 was obtained as white needle crystals (GC-MS): m/z 216 [M] (calc for $C_{12}H_8O_4$, 216.04), 201, 187, 173, 145, 89; ^{1}H NMR ($CDCl_3$, 300 MHz, δ): 8.17 (1H, d, J = 9.9 Hz, H-4), 7.60 (1H, d, J = 2.4 Hz, H-12), 7.14 (1H, s, H-8), 7.03 (1H, d, J = 2.4 Hz, H-11), 6.28 (1H, d, J = 9.6 Hz, H-3), 4.28 (3H, s, OCH_3); ^{13}C NMR (D_2O, 300 MHz, δ): 161.31 (C-2), 158.37 (C-7), 152.68 (C-9), 149.55 (C-5), 144.79 (C-12), 139.33 (C-4), 112.61 (C-3), 112.53 (C-6), 106.36 (C-10), 105.07 (C-11), 93.83 (C-8), 60.07 (OCH_3). The structure of compound 2 was established as 5-methoxypsoralen by spectral data similar to literature values (Chi et al. 2009; Liu et al. 2004).

To the best of our knowledge, this is the first report separating natural compounds from Rhizoma et Radix Notopterygii as safeners for rice. Isopimpinellin and 5-MOP are major coumarins present in many medicinal plant extracts and have demonstrated activity similar to skin photochemotherapy (Lee and Wu 2005), anticancer (Lee et al. 2003; Panno et al. 2010; Salvador et al. 2010), antidepressant (Darcourt et al. 1995), anticonvulsant (Tosun et al. 2008), and anti-inflammatory (Chen et al. 1995; Nicolis et al. 2009; Bose et al. 2011) compounds. None of the previous studies, however, have shown that these compounds protect crops from herbicidal injury.

Safener Activity of Separated Compounds. The safener activity of the two isolated compounds isopimpinellin and 5-MOP were evaluated using a growth chamber bioassay (Table 1). The shoot height and weight of rice seedlings treated with acetochlor were reduced compared with the control. Interestingly, both isopimpinellin and 5-MOP exhibited a protective effect on rice seedlings and reduced the toxicity of acetochlor. This is not surprising because isopimpinellin and 5-MOP have similar structures.

To analyze dosage response of the two coumarins, 5-MOP was selected for further evaluation (Table 2). The protective effect of 5-MOP increased after the increased dose of 5-MOP, until the dose was 50

Table 1. Safener activity of the two identified compounds on rice.[a]

Treatment			
Coumarin	Acetochlor	Shoot height	Shoot weight
g ha^{-1}		% of control[b]	
Isopimpinellin			
None	5	35.5 ± 3.5 a[c]	57.4 ± 2.4 a
50	5	68.4 ± 6.7 c	85.8 ± 2.4 c
25	5	54.3 ± 2.9 b	69.5 ± 4.2 b
5-MOP			
50	5	80.0 ± 3.2 d	88.1 ± 2.4 c
25	5	66.0 ± 3.3 c	69.0 ± 3.9 b
None	None	100.0 ± 2.8 e	100.0 ± 0 d

[a] 5-MOP, 5-methoxypsoralen.

[b] Control is the group without chemical treatment.

[c] Means within a column followed by the same letters are not significantly different at the 5% level according to Fisher's protected LSD test. Data represent the mean ± SD.

Table 2. Dose response of 5-methoxypsoralen (5-MOP) on rice seedlings treated with acetochlor.

Treatment			
5-MOP	Acetochlor	Shoot height	Shoot weight
g ai ha^{-1}		% of control[a]	
0	5	42.6 ± 4.3 a[b]	70.3 ± 6.6 a
12.5	5	55.6 ± 4.6 b	77.0 ± 4.9 a
25	5	70.5 ± 5.5 c	85.3 ± 3.2 b
50	5	81.6 ± 5.1 d	93.8 ± 7.3 b
75	5	78.3 ± 4.5 d	89.1 ± 5.6 b
100	5	64.7 ± 4.7 bc	76.7 ± 5.8 a
0	0	100.0 ± 0.0 e	100.0 ± 0.0 b

[a] Control is the group without chemical treatment.

[b] Means within a column followed by the same letters are not significantly different at the 5% level according to Fisher's protected LSD test. Data represent the mean ± SD.

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Table 3. Influence of 5-MOP on the weed control effect of acetochlor on barnyardgrass.

Treatment			
Acetochlor	5-MOP	Bud length	Inhibition rate
g ai ha^{-1}		mm	%
5	0	9.9 ± 1.6	81.7 a[a]
5	50	9.2 ±1.6	83.1 a
5	100	8.6 ± 1.7	84.2 a
0	0	54.4 ± 6.6	0.0 b

[a] Means within a column followed by the same letters are not significantly different at the 5% level according to Fisher's protected LSD test. Data represent the mean ± SD.

g ha^{-1}, and then decreased when the dose continued to increase. Thus, the optimal dose of 5-MOP is 50 g ha^{-1}, which allows for shoot height and weight to recover to 81.6 and 93.8% of the control, respectively.

Influence of 5-MOP on the Weed Control Effect of Acetochlor. To evaluate whether the safening activity of 5-MOP on rice also lessens weed control with acetochlor, barnyardgrass was chosen as a targeted weed. The bud lengths of barnyardgrass after 5 d of treatment with acetochlor and different concentrations of 5-MOP are shown in Table 3. No differences were observed between barnyardgrass treated with acetochlor alone or with acetochlor and 5-MOP, suggesting that 5-MOP has no influence on the weed control effect of acetochlor at the doses used in this paper.

Induction Effect of GST Activity by 5-MOP. To uncover whether the safening activity of 5-MOP results from the induction of GST activity and detoxification in rice, total GST activities in rice seedlings treated with 5-MOP or acetochlor were measured (Figure 2). Treatment of 5-MOP at 25, 50, and 100 g ha^{-1} significantly increased the GST activity in rice 20 to 40%, whereas neither treatment of 5-MOP at doses $<$ 10 g ha^{-1} nor treatment with acetochlor enhanced GST activity. Similar research has reported that the chemical safener fenclorim protects crops via induction of GST (Buono et al. 2006; Scarponi et al. 2005).

In summary, injury caused by acetochlor is a main hurdle for the application of this efficient and inexpensive herbicide in rice production. The safening effect of isolated isopimpinellin and 5-MOP from Rhizoma et Radix Notopterygii was demonstrated on rice seedlings treated with acetochlor. This study showed that isopimpinellin and 5-MOP could alleviate the injury of acetochlor to rice, while not reducing barnyardgrass control, and that 5-MOP can enhancing GST activity in rice seedlings.

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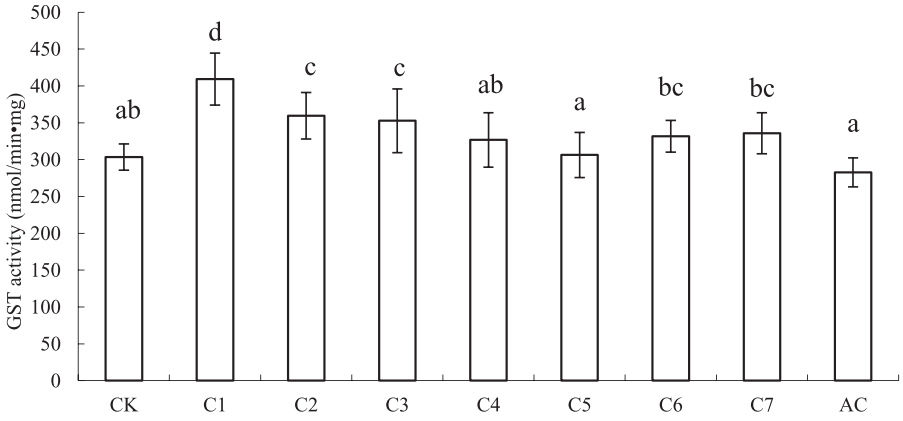

Figure 2. Influence of 5-MOP on glutathione *S*-transferase activity in rice shoots. CK, blank control; C1, C2, C3, C4, C5, C6, and C7 represent 100, 50, 25, 10, 5, 2, and 1 g ha^{-1} 5-MOP, respectively; AC, 5 g ha^{-1} acetochlor. The same letters at the top of columns indicate that values are not significantly different at the 5% level according to Fisher's protected LSD test.

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