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Article

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Antifungal, Phytotoxic, and Cytotoxic Activities of Metabolites from *Epichloë bromicola*, a Fungus Obtained from *Elymus tangutorum* Grass

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ABSTRACT: The development of high-quality herbage is an important aspect of animal husbandry. Inoculating beneficial fungi onto inferior grass is a feasible strategy for producing new varieties of high-quality herbage. *Epichloë bromicola* is a candidate fungus that is isolated from *Elymus tangutorum*. Seventeen metabolites, **1**−**17**, were obtained from *Epichloë bromicola,* and their biological activities were assayed. Metabolite **1** exhibited antifungal activities against *Alternaria alternata*, *Fusarium avenaceum*, *Bipolaris sorokiniana*, and *Curvularia lunata*. EC_{50} values ranged from 0.7 to 5.3 μM, which were better than the positive control, chlorothalonil. Metabolite **8** displayed obvious phytotoxic effects toward *Lolium perenne* and *Poa crymophila* seedlings, and it was as active as glyphosate. None of these isolated metabolites displayed cytotoxicity against Madin-Darby bovine kidney cells. The IC_{50} values were greater than 100 μM, and the metabolites increased the growth of the cells at a concentration of 12.5 μM. Bioassay indicated that *Epichloë bromicola* may be a beneficial fungus for producing new varieties of herbage with various resistances. Additionally, metabolite **7**, 3-(2′-(4′′-hydroxyphenyl)acetoxy)-2*S*-methylpropanoic acid, is a new natural product, and its stereochemistry was determined by means of optical rotation computation and chemical reactions.

KEYWORDS: *Epichloë bromicola*, *Elymus tangutorum*, *metabolites*, *antifungal activity*, *phytotoxicity*, *cytotoxicity*

INTRODUCTION

Animal husbandry comprises a considerable aspect of agriculture. The importance of animal husbandry has prompted a great effort to develop high-quality herbage with improved resistance to various insults. A majority of cool-season grasses of the subfamily Pooideae have been recognized as high-quality herbage containing fungi of the genus *Epichloë*. The genus includes previously reported species within the genus *Neotyphodium*.[1]

Asexual *Epichloë* endophytes often provide numerous benefits to their hosts. For instance, they can increase resistance to disease, undesired herbivory, parasitism, and drought. They can inhibit the growth of undesirable grasses, enhance reproductive growth, and provide other advantages.[2-7] It has been demonstrated that alkaloids from *Epichloë* endophytes played an important role in the provision of many of the resistances. For example, peramine and the loline alkaloids exhibit significant insecticidal activity.[8,9] However, asexual *Epichloë* endophytes also produce detrimental effects on their hosts, including toxicity to livestock, aversion by grazing animals, reduced livestock productivity, and impaired reproduction.[10,11] Therefore, it is highly desirable to find fungi that produce beneficial metabolites and lack harmful side effects.

As one of the cool-season grasses, *Elymus tangutorum* (*E. tangutorum*) adapts to drought and high salinity and is widely planted in temperate and subtropical regions, such as Qinghai, Xinjiang, Xizang, Sichuan, Gansu, Shanxi, Hebei and Mongolia provinces of China.[12] The frequency of infection of *E. tangutorum* by *Epichloë* spp.

ranges from 20-100% in China.[11] Therefore, *Epichloë* spp. may be new candidates for beneficial fungi carrying no side effects to their hosts. However, the resistances and cytotoxicity of *Epichloë* spp. are unknown. In this work, the isolation and identification of seventeen metabolites from an extract of *Epichloë bromicola* from *E. Tangutorum* were reported. The isolated metabolites were evaluated in vitro for antifungal activity, phytotoxicity, and cytotoxicity against Madin-Darby bovine kidney (MDBK) cells.

MATERIALS AND METHODS

General Experimental Procedures. Column chromatography employed silica gel (200−300 mesh) (Qingdao Marine Chemical Factory, China), Sephadex LH-20 (Amersham Pharmacia Biotech, Tokyo, Japan), and RP-18 (Merck, Darmstadt, Germany). TLC employed silica gel GF_{254} plates (10−40 μm) (Qingdao Marine Chemical Factory, China). Optical rotations were determined with a Perkin-Elmer 341 polarimeter (Perkin-Elmer Ltd, Waltham, MA). CD was determined with an Olis DSM 1000 spectrometer (Olis, Atlanta). IR spectra were obtained with a Nicolet FT-IR-360 spectrometer (Thermo Nicolet Inc., Waltham, MA). UV spectra were obtained with a Shimadzu UV-260 spectrophotometer (Shimadzu Instruments Co., Ltd, Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were determined with a Bruker AM-400BB (400 MHz) spectrometer (Bruker Ltd, Karlsruhe, Germany). Values of δ in ppm are relative to TMS. Values of J are given in Hz. HRESIMS was performed with a Bruker APEX-II mass spectrometer (Bruker Ltd, Karlsruhe, Germany), and results are presented in terms of *m/z*.

Fungal Material. The fungus was isolated from the healthy stems of *E. tangutorum*, collected in Qinghai Province, China. The fungus was identified on the bases of morphology and molecular phylogenetic data. The organism was determined to be a common ancestor with an *E. bromicola* strain from *Hordeum brevisubulatum*. Therefore, the isolate was defined as *E. bromicola* (NI_201216) and was deposited at the College of Pastoral Agriculture Science and Technology, Lanzhou University.[13]

Preparative Scale Culture. *E. bromicola* was grown on a sterilized moistened rice medium in 1000-mL flasks (100 g/flask × 100) at 27 ℃ for 30 d to produce white moldy rice.

Extraction, Isolation, and Purification Process. The rice culture was extracted with EtOAc four times to yield 18 g of the crude extract. The crude extract was applied to a silica gel column (90 × 15 cm) and was eluted with $CHCl_3$/MeOH (4 L, 1:0-0:1 gradient system) to give four fractions labeled Fr-A through Fr-D.

Fr-A was chromatographed on Sephadex LH-20 column (120 × 2.5 cm) ($CHCl_3$/MeOH, 500 mL, 1:1) and silica gel column (30 × 4 cm) (petroleum ether/acetone, 800 mL, 1:0-0:1) to yield compounds **1** (478.0 mg), **2** (13.0 mg), **6** (6.6 mg), **8** (20.9 mg), and **12** (5.8 mg).

Fr-B was subjected to Sephadex LH-20 column (120 × 2.5 cm) (pure MeOH, 200 mL) and then silica gel column (20 × 4 cm) ($CHCl_3$/EtOAc, 700 mL, 1:0-0:1) to produce compounds **3** (98.0 mg), **13** (5.3 mg), **15** (7.5 mg), and **16** (6.9 mg).

Fr-C was purified by column chromatography on RP-18 column (30 × 2 cm) (500 mL, H_2O/MeOH, 1:0-0:1) and PTLC ($CHCl_3$/acetone, 80 mL, 1:0-0:1) to afford

compounds **4** (5.3 mg), **5** (12.6 mg), **7** (9.7 mg), **10** (5.1 mg), **11** (5.4 mg), and **14** (2.9 mg).

Fr-D was isolated by RP-18 column (30 ×2 cm) (H_2O/MeOH, 600 mL, 1:0-0:1) and then silica gel column (20 ×4 cm) ($CHCl_3$/MeOH, 550 mL, 1:0-0:1) to give compounds **9** (18.0 mg), and **17** (102.0 mg).

Cyclosporin T, **1**, was a white powder with $[\alpha]_D^{22}$ -197 (*c* 0.10 in MeOH). HRESIMS indicated *m/z* 1188.8337 $[M + H]^+$ (calculated for $[C_{61}H_{110}O_{12}N_{11}]$ 1188.8330). The spot color of **1** was red with 10% H_2SO_4 in EtOH followed by heating and under Dragendorff's reagent. ^{1}H NMR and ^{13}C NMR spectra of **1** in $CDCl_3$ were consistent with those reported in the literature.[14]

3-(2'-(4''-Hydroxyphenyl)acetoxy)-2*S*-methylpropanoic acid, **7**, was a yellow oil with $[\alpha]_D^{22}$ +50 (*c* 0.10 in MeOH). IR parameters (νKBr$_{max}$) were 3423, 1714, and 1632 cm^{-1}. ^{1}H NMR and ^{13}C NMR data are in Table 1.

Theoretical Calculations. To identify the absolute configuration of **7**, quantum mechanical calculations were performed. The B3LYP functional with the 6-31G(d, p) basis set was used.[15,16] The calculations were performed using the Gaussian 09 suite of programs.[17]

General Procedure for Synthesis of Compounds 7a and 7b. Thionyl chloride (0.65 mL, 8.9 mmol) and dimethylformamide (100.0 μL, 1.3 mmol) were added to a solution of 2-phenylacetic acid (0.40 g, 3.0 mmol) in dichloromethane (10.0 mL). After the mixture was stirred at r.t. for 1 h, the solvent was removed *in vacuo* to obtain 2-phenylacetyl chloride (0.45 g, 98%) as yellow oil, which was used directly in the next step.

To a solution of 2-phenylacetyl chloride (0.45 g, 2.9 mmol) in dichloromethane (2.0 mL), a solution of methyl 3-hydroxy-2*R/S* -methylpropanoate (0.21 g, 1.8 mmol) in dichloromethane (5.0 mL) was added. After the reaction mixture was stirred for 4 h, the reaction was quenched by addition of ice water and the organic phase was separated. The aqueous phase was then extracted with dichloromethane (2 × 10.0 mL). The organic phase was dried with $MgSO_4$, and the solvent was removed *in vacuo*. Purification by flash chromatography (ethyl acetate/petroleum ether, 1/10) gave methyl 2*R*-methyl-3-(2-phenylacetoxy)propanoate (**7a**) or methyl 2*S*-methyl-3-(2-phenylacetoxy)propanoate (**7b**) as an oil (0.31 g, 73%).

Methyl 2R-methyl-3-(2-phenylacetoxy)propanoate, ***7a***. The colorless oil exhibited $[\alpha]_D^{22}$ -70 (*c* 0.10 in MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ_H 7.27−7.34 (m, 5H, ArH), 4.19, 4.25 (dd, *J* = 7.2, 10.8 Hz; 2H, $-CH_2O-$), 3.64 (s, 3H, $-OCH_3$), 3.61 (s, 2H, $-CH_2-$), 2.78 (m, 1H, −CH−), 1.16 (d, *J* = 7.2 Hz, Me−H). ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 174.1 (−C=O), 171.2 (−C=O), 133.8, 129.2, 129.2, 128.5, 128.5, 127.1 (ArC × 6), 65.8 ($-CH_2O-$), 51.8 ($-OCH_3$), 41.2 (−CH−), 38.9 ($-CH_2-$), 13.7 (Me−C). HRESIMS *m/z* 237.1118 $[M + H]^+$ (calculated for $[C_{13}H_{17}O_4]$ 237.1121).

Methyl 2S-methyl-3-(2-phenylacetoxy)propanoate, ***7b***. The colorless oil exhibited $[\alpha]_D^{22}$ +70 (*c* 0.10 in MeOH). Compounds **7b** and **7a** shared the same NMR data. HRESIMS *m/z* 237.1120 $[M + H]^+$ (calculated for $[C_{13}H_{17}O_4]$ 237.1121).

Antifungal Assay. Antifungal activity against pathogenic fungi was tested using the previously reported method.[18] A sample was dissolved in DMSO to give a test solution at a defined concentration. This solution (0.1 mL) was thoroughly mixed with 20 mL of PDA molten agar medium. The mixture was then equally poured onto

three Petri dishes (6-cm diameter) to prepare treated culture. DMSO without sample was processed similarly to give control culture media. Chlorothalonil served as the positive control. Discs (0.4 cm) of hyphae were cut from the edge of a colony. These hyphae were placed in the middle surface of the culture medium with the hyphae downwards. The inhibition rate was obtained according to the following formula: inhibition rate (%) = (C − T)/(C− 0.4 cm). T is the average diameter of mycelia on PDA treated with sample, and C is the average number of mycelia on PDA treated with control compounds. The EC_{50} values of the tested compounds were thus determined. Each assay was performed in triplicate.

Phytotoxicity Bioassay. With glyphosate as the positive control, phytotoxic effects on *Lolium perenne* and *Poa crymophila* were examined using the method described in literature.[19] The seedlings were soaked in cold water for 100 min, then dipped in ethanol for 5 min and repeatedly rinsed until odorless. A 12-well microplate was placed on two sheets of aseptic filter paper. The samples to be tested were dissolved in MeOH and applied to the paper. Upon evaporation of the MeOH, aseptic water was added to the wells. *L. perenne* and *P. crymophila* seedlings of uniform size and shape were put on the papers. The seedlings were kept for 5 and 9 d, respectively, at 22 ℃ under dark conditions. The phytotoxicities were observed after 72 and 120 h, respectively. Methanol was used as a blank control. Each experimental result was expressed as the mean ± standard deviation (SD) of three trials.

Cytotoxicity Assay. MDBK cells were purchased from the Shanghai cell bank of the Chinese Academy of Sciences and maintained at 37 ℃ under an atmosphere of

95% air and 5% CO_2 in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and 1% antibiotic/antimicrobial solution. A cytotoxicity assay was performed as previously described.[20] MDBK cells were seeded in 96-well plates at a cell concentration of 5000 cells per well in 100 μL of DMEM medium. After incubation of the cells for 24 h at 37 ℃, various concentrations of compounds were added, and the incubation was continued for 24 h. Untreated cells were used as controls. After that, 10 μL of 5 mg/mL resazurin solution was added into each well, and incubation was continued for 2 h at 37 ℃. The fluorescence intensity was measured using FlexStation 3 (Molecular devices, Sunnyvale, California) at excitation/emission wavelengths of 555/585 nm. Each assay was performed in triplicate.

RESULTS AND DISCUSSION

Cultivation, Isolation, and Structure Elucidation. The fungal strain was cultured in rice medium at 27 ℃ for 30 d. The cultured material was extracted four times with EtOAc. The EtOAc extract was then fractionated by means of chromatography on silica gel, Sephadex LH-20, PTLC, and RP-18. A new compound (**7**) and sixteen known compounds (**1**−**6**, **8**−**17**), including a cyclic peptide (**1**) (Figure 1) were obtained from the EtOAc extract. It should be noted that these isolated compounds were not detected in the rice medium without the fungus (*Epichloë bromicola*). The structures of these obtained compounds were determined by combining the extensive spectroscopic analysis with computational optical rotation, chemical reaction, and comparison with the data in the literature.

Compound **7** was obtained as optically active yellow oil. The ^{13}C NMR and DEPT spectra, together with the molecular ion peak in the HRESIMS indicated a molecular formula of $C_{12}H_{14}O_5$. The IR spectrum of compound **7** showed absorption bands of hydroxyl (3423 cm^{-1}) and carbonyl groups (1632 and 1714 cm^{-1}). The ^1H NMR spectrum of compound **7** showed four aromatic protons at δ_H 6.72 (2H, d, J = 8.4 Hz) and 7.09 (2H, d, J = 8.4 Hz), implying that the aromatic ring had a 1,4-disubstitution pattern. Moreover, the ^1H NMR spectrum revealed the presence of a set of oxygenated methylene protons at δ_H 4.20, 4.28 (dd, J = 7.2, 10.8 Hz); a set of methylene proton at δ_H 3.55 (s); a methine proton at δ_H 2.82 (m), and a secondary methyl group at δ_H 1.21 (d, J = 7.2 Hz). The ^{13}C NMR and DEPT spectra of compound **7** displayed six aromatic carbons at δ_C 115.5, 115.5, 125.5, 130.4, 130.4, and 154.8; two carbonyl carbons at δ_C 172.1 and 179.4; an oxygenated methylene carbon at δ_C 65.6; a methylene carbon at δ_C 38.8; a methine carbon at δ_C 40.4, and a secondary methyl carbon at δ_C 13.5. The ^1H-^1H COSY spectrum of compound **7** revealed a spin-coupling system of H_2-3, H-2, and H_3-4, and the HMBC correlations of H-4 (δ_H 1.21) with C-3 and C-1; H-3 (δ_H 4.20, 4.28) with C-2′ and C-1; H-1′(δ_H 3.55) with C-2′ suggesting that compound **7** has a C_6 side chain. The C_6 side chain was positioned at C-1″ according to the HMBC correlations of the methylene group H-1′ (δ_H 3.55) with C-1″, C-2″ and C-6″ (Figure 2). Taking the above information into account, the hydroxyl was positioned at C-4″. Accordingly, the whole planar structure of compound **7** was completely elucidated.

A detailed analysis of the stereochemistry of compound **7** indicated only one

chiral center at C-2, thus making it possible to identify two candidate stereo structures as either 2*S* or 2*R*. The computational optical rotation approach was utilized to determine the configuration of C-2. All optical rotation calculations were performed using the IEFPCM solvation model, and, in agreement with the experimental condition, MeOH was specified as the solvent. The calculated optical rotation for 2*S* was 84.9 °, while the value of 2*R* was -84.9 °. The former is closer to the experimental value of 50.0°. Therefore, the absolute configuration of C-2 was 2*S*.

To further verify the absolute configuration of compound **7**, two model compounds (**7a** and **7b)** were synthesized through the esterification reactions using methyl 3-hydroxy-2*R*-methylpropanoate or methyl 3-hydroxy-2*S*-methylpropanoate as the substrates (Figure 3). The ECD spectra of **7a** and **7b** were tested in MeOH (Figure 4), and the ECD spectrum of **7b** is similar to that of **7**. Therefore, the absolute configuration of C-2 of **7** is 2*S*. Accordingly, the structure of the novel compound **7** was established and named as 3-(2'-(4''-hydroxyphenyl)acetoxy)-2*S*-methylpropanoic acid.

The spectral data (MS, ^{1}H and ^{13}C NMR spectra) of compounds **1**−**6** and **8**−**17** were identical to those reported in the previous literature. Accordingly, their structures were identified as those of cyclosporin T (**1**);[14] uracil (**2**);[21] 5-methyluracil (**3**);[21] 1*H*-indazole (**4**);[22] 1*H*-indazole-3-acetic acid (**5**);[23] cyclo-(L-leucyl-L-prolyl) (**6**);[24] 3,3'-dihydroxy-5,5'-dimethyldiphenyl ether (**8**);[25] (4-hydroxyphenyl)-acetic acid (**9**);[26] 2-(4-hydroxyphenyl)-ethanol (**10**);[27] *p*-hydroxybenzoic acid (**11**), vanillic acid (**12**);[28] (-)-sydonic acid (**13**);[29] furan-2-carboxylic acid (**14**);[30]

3-acetoxy-2*S*-methylpropanoic acid (**15**);[31] fumaric acid, methyl ester (**16**);[32] and dulcose (**17**).[33]

The obtained compounds were different from those alkaloids previously found in the fermentation of *Epichloë* spp., which may be due to the alteration of the cultivation parameters to one strain.[34] Analysis of the structures of the presently obtained compounds indicated that compounds **1** and **6** are peptides. In particular, compound **1** is a polypeptide. Compounds **2** and **3** are nucleobases. Compound **4** is an indazole. Compounds **7** and **9** are phenylacetic acid derivatives. Compounds **8** and **13** are a dimethyldiphenyl ether and a sesquiterpene, respectively. Compound **10** is 2-(4-hydroxyphenyl)-ethanol. Compounds **11** and **12** are substituted benzoic acids. The other compounds (**14**−**17)** were acids and sugar. These metabolites, except the alkaloid 1*H*-indole-3-acetic acid (**5**), were isolated from the genus *Epichloë* for the first time thorough literature search.

Antifungal Activity of Metabolites. To determine the effects of these metabolites from *E. bromicola* on different phytopathogenic fungi, metabolites **1**, **3**, **9**, and **17** were tested for their inhibition of *A. alternata*, *B. sorokiniana*, *F. avenaceum*, and *C. lunata* in vitro (there were insufficient quantities of compounds **2**, **4**−**8**, and **10**−**16**). The phytopathogenic fungi were chosen because they are very common in the cool-season subfamily of Pooideae grasses and caused a substantial reduction in the yield of herbage.[35] As shown in Table 2, compound **1** was the most active among the assayed compounds. The other tested compounds were weaker than chlorothalonil as the positive control with EC_{50} values from 13.5 to 66.4 μM.[36] The EC_{50} values of

compound **1** against *A. alternata*, *B. sorokiniana*, *F. avenaceum*, and *C. lunata* were 5.3, 3.9, 0.7, and 2.9 μM, respectively, suggesting that it had broad-spectrum inhibitory activity against these four plant pathogenic fungi, and was stronger than chlorothalonil. Therefore, compound **1** may be considered as a lead compound for the development of new fungicides for suppression of common plant diseases, especially those caused by these tested phytopathogenic fungi. Furthermore, compound **1** was the most abundant of the seventeen isolated metabolites, indicating that **1** plays an important role in host disease resistance against these pathogens. Thus, *E. bromicola* is a promising lead organism for the development of a natural microorganism fungicide to allow *E. tangutorum* to resist plant pathogenic fungi.

Phytotoxic Activity of Metabolites. Metabolites **1**−**17** of *E. bromicola* were tested for their phytotoxic activities against *L. perenne* seedlings. As shown in Table 3, compound **8** was the most active against the roots and shoots with the inhibitory rates of 83.9% and 59.8%, respectively. Compound **5** (74.8%) showed the similar activity with glyphosate (72.0%) in inhibiting the root growth; it displayed weaker inhibition of the shoots (48.5%) than glyphosate as the positive control (82.3%). This result was consistent with reported literature.[38] Compound **9** exhibited moderate activity against the roots and shoots with inhibitory rates of 55.8% and 28.4%, respectively. Other metabolites showed weak activity toward the shoots and roots with the inhibition rates ranging from 0% to 35.6%.

To determine whether compound **8** possesses inhibitory activity against *P. crymophila*, its phytotoxic effects were assayed at different concentrations. As shown

in Figure 5, at high concentrations of 150−200 ppm, compound **8** exhibited inhibition rates in range of 74.9%−90.1% against roots, which was as active as glyphosate (73.1%−84.7%); it displayed weaker activity (23.5%−59.8%) than glyphosate (37.1%−63.3%) toward the shoots. At low concentrations of 25−100 ppm, compound **8** weakly inhibited the roots (27.8%−58.4%) relative to glyphosate (35.7%−68.3%); however, its inhibitory rates (3.6%−14.2%) were similar to that of glyphosate (2.4%−12.9%) against shoots. The results showed that compound **8** displayed marked phytotoxic effects toward the roots and shoots of various seedlings at concentrations of 50−200 ppm. Therefore, compound **8** may play a crucial role for *E. tangutorum* in inhibiting the growth of other grasses, and it has potential as a new type of natural microbial herbicide. The compound may be the reason for the wide distribution of *E. tangutorum* and its high frequency of infection of *Epichloë* spp. in China. Moreover, this is the first report of phytotoxic effects of dimethyldiphenyl ethers.

Cytotoxicity of Metabolites. Metabolites **1**−**17** were tested for their cytotoxicity against MDBK cells. As shown in Table 4, none of the obtained metabolites exerted cytotoxicity toward MDBK cells. The IC_{50} values were above 100 μM. Moreover, the inhibition rates of the metabolites were between -1.6% and -17.0% at a concentration of 12.5 μM, indicating that they may promote mammalian cell growth at low concentrations. Thus, the metabolites did not exhibit cytotoxicity to MDBK cells. The results were quite different from those observed with the alkaloids isolated from *Epichloë* spp.; those alkaloids produced detrimental effects on their hosts.[6] The present results indicate that it may be safe for animals to eat *E.*

tangutorum grass that is infected with *E. bromicola*. Accordingly, *E. bromicola* may be a beneficial fungus with desirable phytotoxic and antifungal properties, and a lack of cytotoxicity toward mammalian cells. The results indicate the possibility of raising new varieties of herbage with resistance against infection and competition.

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Figure captions

Figure 1. Structures of compounds **1**–**17**.

Figure 2. HMBC and COSY correlations of compound **7**.

Figure 3. Synthesis of compounds **7a** and **7b**.

Figure 4. ECD spectra of compounds **7**, **7a**, and **7b** (in MeOH).

Figure 5. Phytotoxic activities of compound **8** and positive control on *Poa crymophila*: A. root; and B. shoot (P<0.05).

426 **Table 1**. ^{1}H NMR and ^{13}C NMR data of metabolite **7**.[a]

No	**7**	
	δ_H multi (J in Hz)	δ_C
1″	-	125.5
2″	7.09 d (8.4)	130.4
3″	6.72 d (8.4)	115.5
4″	-	154.8
5″	7.09 d (8.4)	130.4
6″	6.72 d (8.4)	115.5
1′	3.55 brs	38.8
2′	-	172.1
1	-	179.4
2	4.83 m	40.4
3	4.20 dd (7.2, 10.8) 4.28 dd (7.2, 10.8)	65.6
4	1.21 d (7.2)	13.5

[a] ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz), TMS, Measured in $CDCl_3$.

427

428 **Table 2**. The antifungal activities of metabolites **1**, **3**, **9**, and **17** against four pathogenic fungi (EC_{50},
429 μM).

Compound	*Alternaria alternat*	*Bipolaris sorokinian*	*Fusarium avenaceum*	*Curvularia lunata*
1	5.3	3.9	0.7	2.9
3	> 200	> 200	> 200	> 200
9	> 150	> 100	> 100	> 100
17	> 200	> 200	> 200	> 200
Chlorothalonil	56.8	66.4	13.5	38.8

430

431 **Table 3**. Inhibitory effects of metabolites **1**−**17** on the growth of *Lolium perenne* seedlings[a].

Compound	inhibition ratio (%)[b]	
	root	shoot
1	16.4 ± 1.1	9.1 ± 1.0
2	23.4 ± 2.1	35.6 ± 2.7
3	0	9.3 ± 3.4
4	19.3 ± 1.9	1.5 ± 1.4
5	74.8 ± 3.5	48.5 ± 3.2
6	5.6 ± 2.3	3.7 ± 2.1
7	10.1 ± 3.3	5.2 ± 2.1
8	83.9 ± 2.9	59.8 ± 2.1
9	55.8 ± 3.8	28.4 ± 1.7
10	13.8 ± 2.6	2.8 ± 5.7
11	2.6 ± 2.3	3.1 ± 2.2
12	8.7 ± 2.9	0
13	0	0
14	1.9 ± 1.3	2.5 ± 2.0
15	21.1 ± 3.8	11.9 ± 2.3
16	3.2 ± 1.9	2.6 ± 1.4
17	0	0
glyphosate	72.0 ± 3.5	82.3 ± 2.8

[a]All the metabolites were tested at the concentration of 200 ppm;

[b] Mean ± SD

432

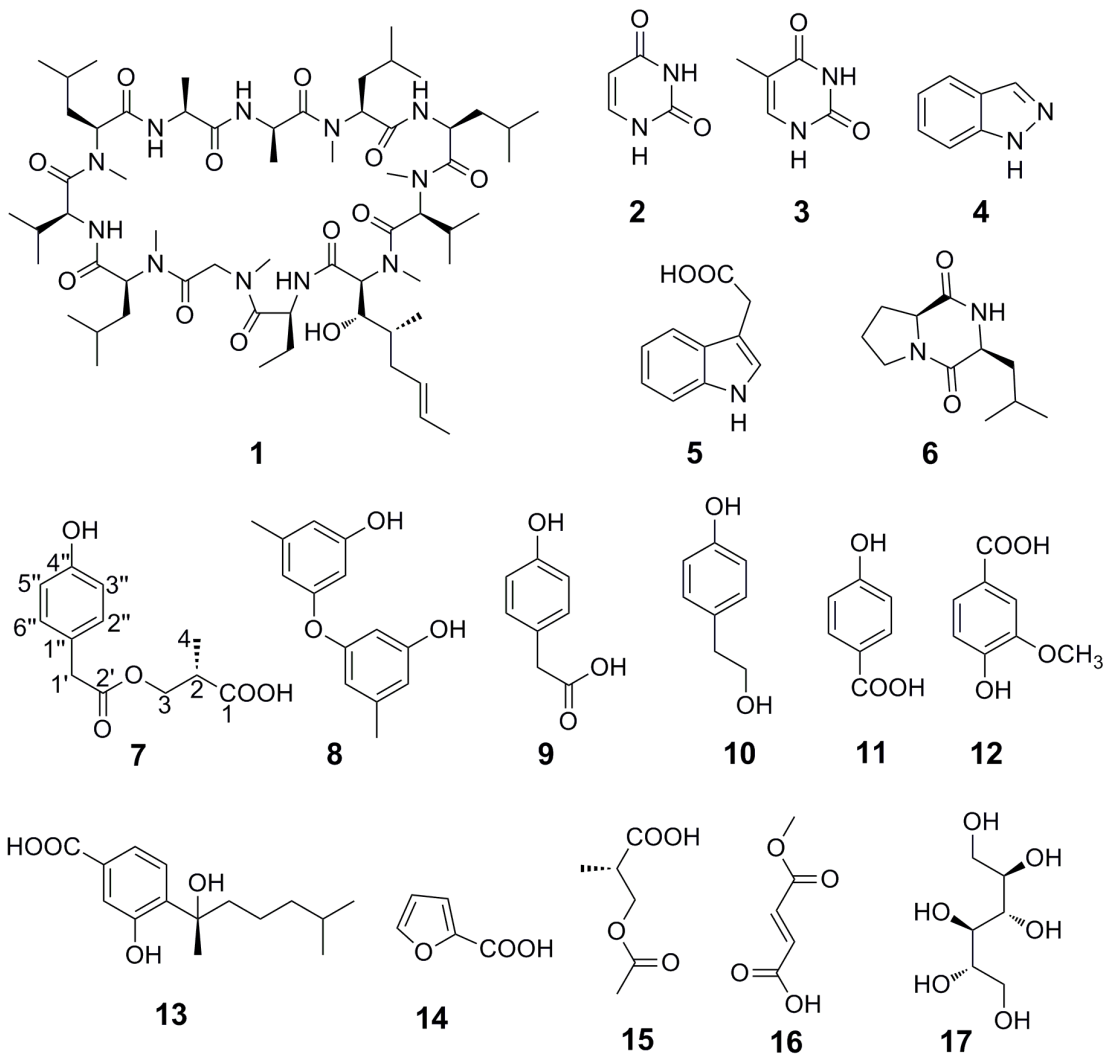
433 **Table 4**. Cytotoxic activities of metabolites **1**–**17** against Madin-Darby bovine kidney cells[a].

Compound	Inhibition ratio (%) at the concentration of 12.5 μM
1	-1.8
2	-2.9
3	-5.8
4	-5.3
5	-17.0
6	-12.1
7	-11.1
8	-11.1
9	-8.1
10	-8.8
11	-9.7
12	-5.1
13	-5.9
14	-1.5
15	-7.9
16	-1.6
17	-4.8
Ergonovine	29.3

[a]The IC_{50} values of metabolites **1**–**17** and ergonovine were above 100 μM.

434

435



436

437 **Figure 1**. Structures of compounds **1**–**17**.

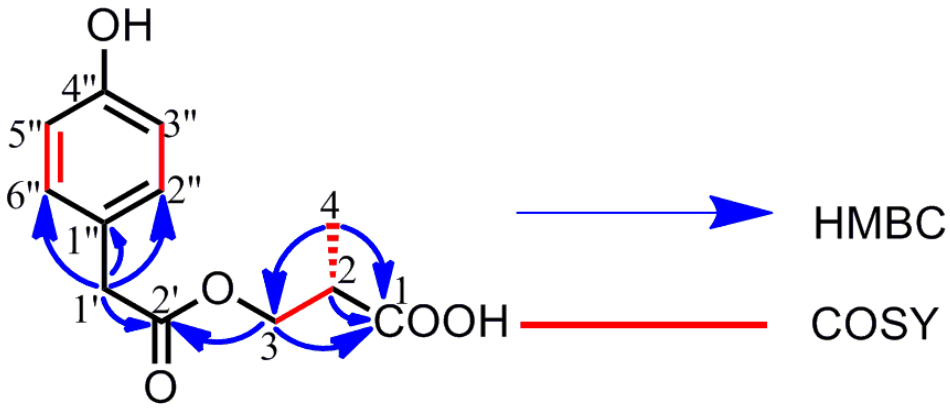


Figure 2. HMBC and COSY correlations of compound **7**.

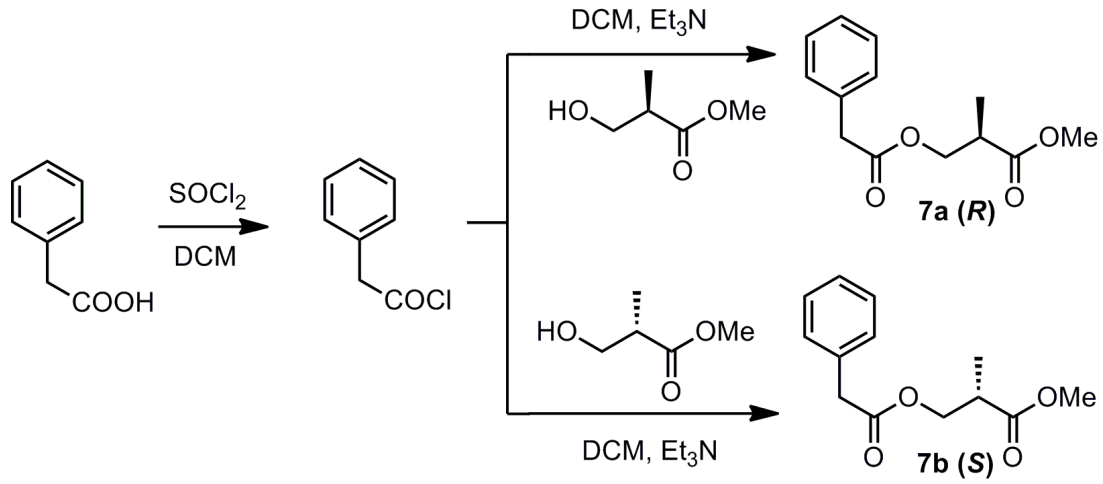
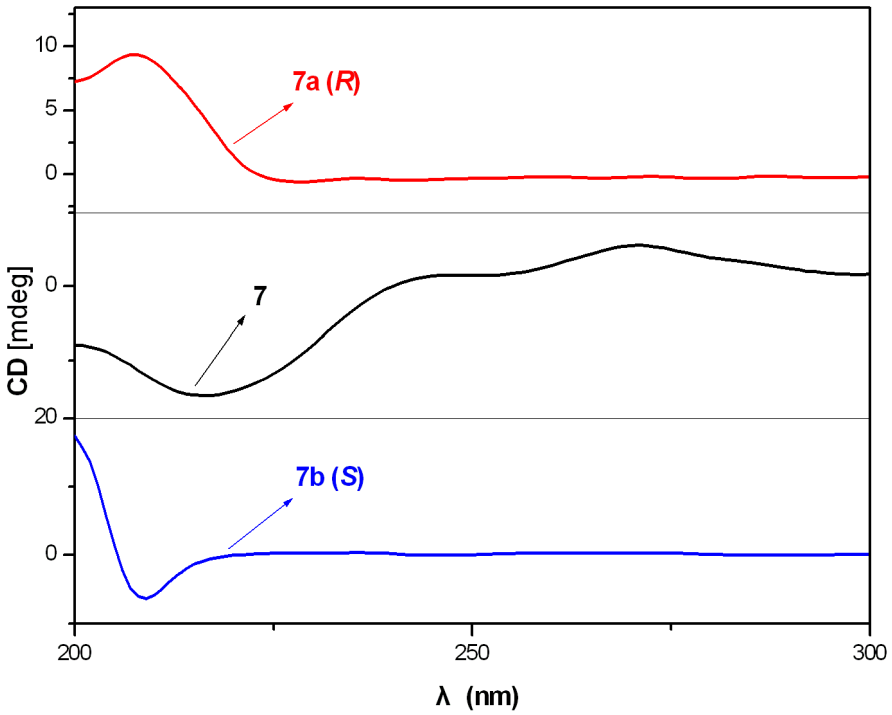


Figure 3. Synthesis of compounds **7a** and **7b**.



443

444 **Figure 4.** ECD spectra of compounds **7**, **7a**, and **7b** (in MeOH).

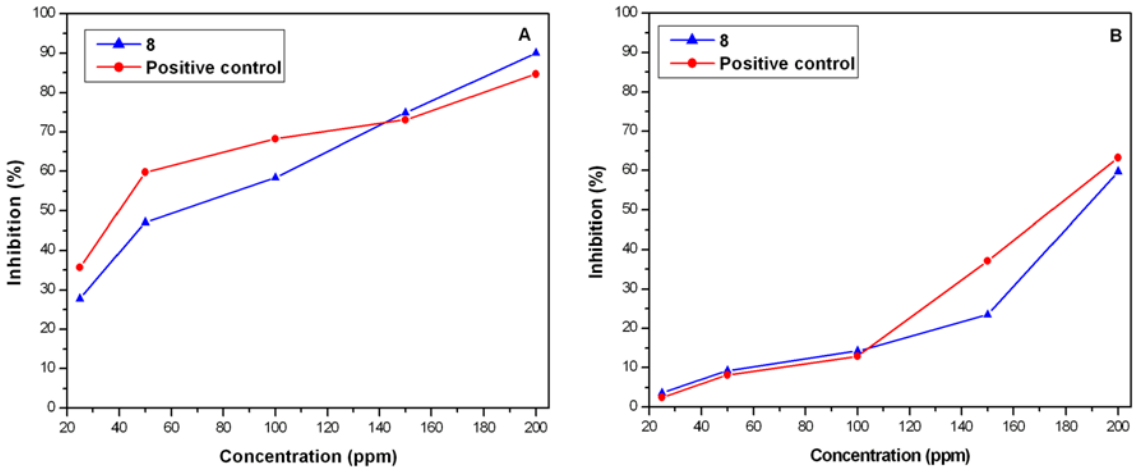
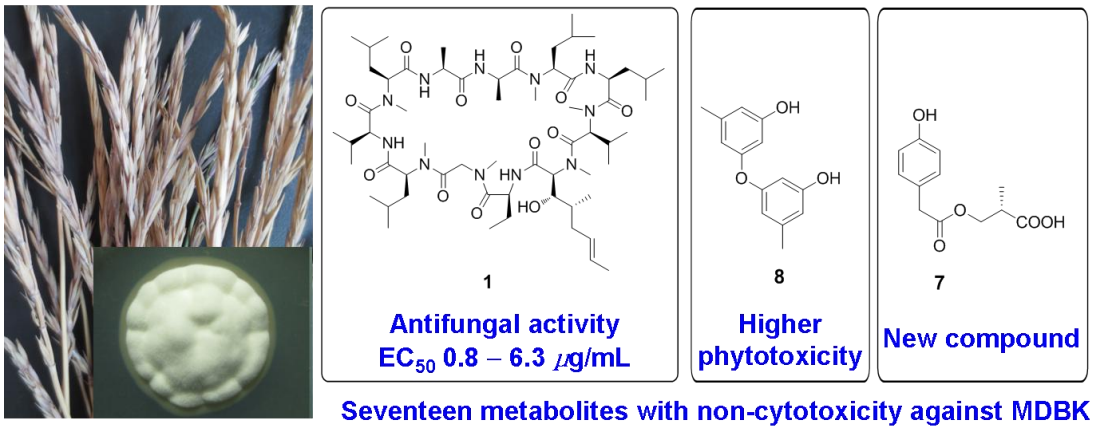


Figure 5. Phytotoxic activities of compound **8** and positive control on *Poa crymophila*: A. root; and B. shoot ($P<0.05$).

448

TOC Graphic:



449

Seventeen metabolites with non-cytotoxicity against MDBK