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Antifungal Activities of Dimeric Sesquiterpenes, Shizukaols C and F, Isolated from *Chloranthus japonicus* Sieb.

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Two dimeric sesquiterpenes were separated from *Chloranthus japonicus* Sieb. and identified as shizukaols C and F. They exhibited potent antifungal activities (MICs = 4–16 μg/ml) in vitro against various plant pathogenic fungi (*Pythium ultimum*, *Phytophthora infestans*, *Botrytis cinerea*, *Colletotrichum lagenarium*, *Alternaria kikuchiana*, and *Magnaporthe grisea*). Shizukaol C showed 88% and 91% protective activities in the greenhouse against *Puccinia recondita* (wheat leaf rust) and *Phytophthora infestans* (tomato late blight), respectively, at 100 μg/ml; shizukaol F exhibited 93% antifungal activity against *Puccinia recondita* at the same concentration. Therefore, these compounds might serve as interesting candidates for effective antifungal agents.

Keywords: *Chloranthus japonicus* Sieb., shizukaol C, shizukaol F, antifungal activity, *Phytophthora infestans*, *Puccinia recondita*

The extensive use of agrochemicals to protect crops against phytopathogenic fungi has led to soil pollution, the emergence of pesticide-resistant strains, and harmful effects on humans and the environment [1–3], despite their contribution to increase crop production [4]. Recently, the usage of agrochemicals in agricultural production has become restricted in many countries [5] as consumers increasingly demand pesticide-free crops or safe foods [6]. Thus, it is necessary to develop effective and safe agrochemicals against a variety of phytopathogenic fungi to maintain crop production [3].

Chloranthus japonicus Sieb. (Chloranthaceae) is mainly distributed in China, Japan, and Korea. It has long been acknowledged in South Korea as a traditional medicine for boils, dermatologic complaints, and enteric fever [7], and in China as a treatment for fractures, neurasthenia, pulmonary tuberculosis, rheumatic arthralgia, and traumatic injuries [8]. Various sesquiterpenoids, including eudesmane [9–12], lindenane [10, 13–15], germarcrane [9, 14, 16], acorane [9], lindenane-type sesquiterpenoid dimers [11, 17–20], and terpenoids [21], have been isolated from *C. japonicus* Sieb. Among them, the eudesmane-type sesquiterpenoid CJ-01 exhibited specific chitin synthase II inhibition [12]; and the dimeric sesquiterpenoids cycloshizukaol A, shizukaol B, and shizukaol F are shown to reduce the level of cell adhesion molecules [22]. Lindenane-type disesquiterpenoid chlorajaponilides A to E separated from *C. japonicus* Sieb. exhibited anti-HIV-1 activity [23]. Recently, the lindenane-type sesquiterpenoid chlojaponilactone B showed inflammatory suppression [24]. A number of sesquiterpenoids [25–30] have been identified from various *Chloranthus* species, and shizukaol D isolated from *C. serratus* has been shown to repress the growth of human hepatic cancer cells [31].

In the search for plant-derived antifungals with activity

against *Pythium ultimum*, the causative agent of rice damping-off, bioactive compounds were found in a methanol extract of *C. japonicus* Sieb. Here, we isolated and determined the structure of dimeric sesquiterpenes shizukaols C and F, and examined their antifungal activities against various plant pathogenic fungi.

The whole body of *C. japonicus* (1.58 kg) was ground and extracted three times using 20 L of methanol for 7 days, which was filtered and then evaporated to obtain crude extracts (253.86 g). The residue was added to water and partitioned with chloroform, hexane, ethyl acetate, and water. The active ethyl acetate fraction (32.30 g) was loaded into a silica gel column for chromatography (7.5 × 35 cm, 230–400 mesh, Kieselgel 60) and subjected to stepwise elution with hexane/ chloroform (1:1, 3:7, 1:9 (v/v), 1,000 ml each) and chloroform/ethyl acetate (9:1, 7:3, 5:5, 1,000 ml each). Each fraction was evaporated, solubilized in methanol, and used for bioassays against *Pythium ultimum* using an agar diffusion method [26]. The four bioactive fractions among six (3.66 g) were loaded into a C_{18} reversed-phase silica gel column for chromatography (3.5 × 25 cm, 40–63 μm, Lichroprep RP-18) and eluted using a gradient of acetonitrile/water (3:7, 5:5, 7:3, 10:0 (v/v), 0.5 L each). The two bioactive fractions among four (50% CH_3CN, 220 mg) were then loaded into a Sephadex LH-20 column for chromatography with methanol (0.4 ml/min, 8 ml each, 1.5 × 120 cm). The bioactive fractions against *P. ultimum* were collected and concentrated. For further purification, the active fractions (fraction 37–50, 158 mg) were loaded into a C_{18} reverse-phase column (YMC-Pack Triart, 5 μm, 4.6 × 250 mm) for HPLC and subjected to stepwise elution with the acetonitrile/water gradient (35:65, 60:40 (v/v), 50 min, 1 ml/min). The retention times of the two pure compounds were observed at 25.1 and 35.4 min using a UV spectrometric detector at 254 nm in HPLC analysis. The purity of the isolated compounds was confirmed by HPLC. Structural analyses with ESI-MS and various NMR techniques such as ^1H-^1H, DEPT, COSY, HMBC, and HMQC disclosed that the molecular formulas of the compounds were $C_{36}H_{42}O_{10}$ and $C_{40}H_{44}O_{13}$ with molecular weights of 634 and 732, respectively. These data identified the compounds as shizukaol C and shizukaol F (Fig. 1). Shizukaol C: Amorphorous powder; ESI-MS, *m/z* 633.7 $[M-H]^-$, ^1H NMR (400 MHz , CD_3OD) δ 0.32-0.42 (m, 1H), 0.64–0.78 (m, 1H), 0.83 (s, 3H), 0.82–1.01 (m, 1H), 1.04 (s, 3H), 1.21–1.34 (m, 1H), 1.42–1.53 (m, 1H), 1.61–1.74 (m, 1H), 1.78–1.98 (m, 6H), 1.82 (s, 3H), 1.89 (s, 3H), 2.37–2.60 (m, 3H), 2.75–2.88 (m, 2H), 3.38 (d, 1H), 3.69 (s, 3H), 3.79 (d, 1H), 3.88 (s, 1H),

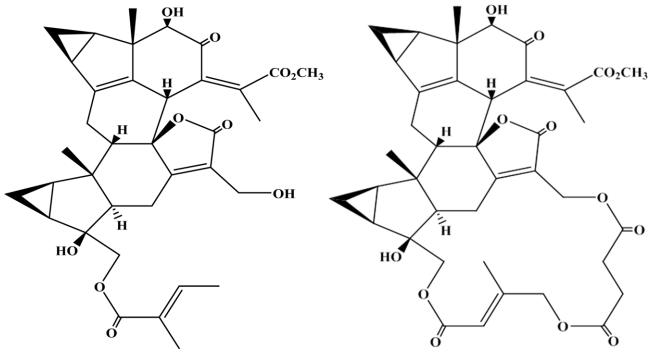

Fig. 1. Structures of shizukaol C (left) and shizukaol F (right) isolated from *Chloranthus japonicus* Sieb.

3.91 (br s, 1H), 4.16 (d, 1H), 4.23 (s, 1H), and 6.90 (q, 1H). Shizukaol F: Amorphorous powder; ESI-MS, *m/z* 731.7 $[M-H]^-$, ^1H NMR (CD_3OD, 400 MHz) δ 0.28–0.39 (m, 1H), 0.64–0.77 (m, 1H), 0.82 (s, 3H), 0.90–1.00 (m, 1H), 1.03 (s, 3H), 1.22–1.42 (m, 2H), 1.64–1.74 (m, 1H), 1.81 (s, 3H), 1.83–2.05 (m, 4H), 2.11 (s, 3H), 2.45–3.05 (m, 8H), 3.51 (d, 1H), 3.70 (s, 3H), 3.89 (s, 1H), 4.01 (br s, 1H), 4.49 (d, 1H), 4.62 (d, 1H), 4.73 (d, 1H), 4.81–5.01 (m, 2H), and 5.98 (br s, 1H). The spectral data for these compounds were identical with previously published data [22, 25].

The antifungal activities of both compounds against various phytopathogenic fungi were determined by the agar dilution method as described previously [32]. The MIC (minimum inhibitory concentration) is the lowest concentration that results in complete inhibition of organism growth as compared with a control plate without compounds [32].

The disease-control values of shizukaols C and F were investigated against various plant pathogens: *Blumeria graminis* f. sp. *hordei*, *Botrytis cinerea*, *Corticium sasaki*, *Magnaporthe grisea*, *Phytophthora infestans*, and *Puccinia recondita*. The in vivo assays of shizukaols C and F in the greenhouse were performed as described previously [26].

Shizukaol F in vitro showed potent antifungal activities against diverse plant pathogenic fungi, including *P. ultimum*, *P. infestans*, *B. cinerea*, *C. lagenarium*, *A. kikuchiana*, and *M. grisea*, with MICs at concentrations of 4 to 8 μg/ml, whereas shizukaol C exhibited MIC values of 4–16 μg/ml. These compounds showed comparatively mild activities against *F. oxysporum* and *R. solani* (MICs 32–64 μg/ml) (Table 1). Moreover, shizukaol C showed 88% and 91% protective activities in the greenhouse against *P. recondita* and *P. infestans* at 100 μg/ml, respectively; shizukaol F exhibited 93% antifungal activity against *P. recondita* at the same concentration (Table 2). In addition, the disease-control values of shizukaols

Table 1. In vitro antifungal activities of shizukaols C and F against various phytopathogenic fungi.

Test microorganism	Minimum inhibitory concentration (μg/ml)	
	Shizukaol C	Shizukaol F
Alternaria kikuchiana	16	8
Botrytis cinerea	8	4
Colletotrichum lagenarium	8	4
Fusarium oxysporum	64	32
Magnaporthe grisea	16	8
Pythium ultimum	4	4
Rhizoctonia solani	64	64
Phytophthora infestans	4	8

C and F showed fluctuating control values for *M. grisea* or *P. infestans* at concentration of 33 μg/ml over 11 μg/ml. However, the disease-control values of these compounds did not show statistically significant difference. The fluctuating patterns of control values for the two compounds may be caused by weak phytotoxicities in rice and tomato seedlings. On the other hand, compounds having a strong phytotoxicity activity may have caused death on chemical-applied seedlings, whereas compounds with very weak phytotoxicity activity induced fluctuating patterns of control values for in vivo bioassays.

Although a number of compounds from *Chloranthus japonicus* Sieb. have been isolated to date [9–21], knowledge of their antifungal activities against phytopathogenic fungi is highly lacking. In particular, there is little information on the biological activities of the shizukaols C and F identified in this study against tomato late blight and wheat leaf rust. Interestingly, the disease-control value of CHE-23C isolated from *C. henryi*, which has an acetyl group instead of a hydroxyl group in the 13′ position of shizukaol C, indicated approximately 3-fold higher antifungal activity than that of shizukaol C against tomato late blight [30]. To our best knowledge, this is the first report regarding the potent protective activities of shizukaols C and F against *P. recondita* and *P. infestans*. Both may be utilized as lead compounds in the further development of antifungal compounds. Their detailed modes of action require further investigation.

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Table 2. In vivo disease-control activities of shizukaols C and F, isolated from *Chloranthus japonicus* Sieb., against various plant diseases[a].

Compounds	Concentration (μg/ml)	Control value[b] (%)					
		RCB	RSB	TGM	TLB	WLR	BPM
Shizukaol C	11	17 ± 23	25 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	43 ± 14	0.0 ± 0.0
	33	0.0 ± 0.0	44 ± 8.8	14 ± 0.0	14 ± 0.0	73 ± 9.4	8 ± 12
	100	80 ± 4.7	50 ± 18	0.0 ± 0.0	91 ± 2.0	88 ±9.4	0.0 ± 0.0
Shizukaol F	11	17 ± 23	38 ± 18	0.0 ± 0.0	43 ± 20	53 ± 0.0	0.0 ± 0.0
	33	58 ± 12	44 ± 8.8	7 ± 10	7 ± 10	88 ± 4.7	0.0 ± 0.0
	100	58 ± 12	69 ± 8.8	21 ± 10	36 ± 10	93 ± 0.0	0.0 ± 0.0
Chlorothalonil[c]	50				94 ± 2.0		
	100				100		
Dimethomorph[c]	2				64 ± 10		
	10				100		
Carboxin[c]	20					43 ± 14.1	
	50					100	

[a]RCB, rice blast (*Magnaporthe grisea*) ; RSB, rice sheath blight (*Corticium sasaki*); TGM, tomato gray mold (*Botrytis cinerea*); TLB, tomato late blight (*Phytophthora infestans*); WLR, wheat leaf rust (*Puccinia recondita*); BPM, barley powdery mildew (*Blumeria graminis* f. sp. *hordei*).

[b]Control value (%) = 100 × [(*A* − *B*)/*A*], where *A* = area of infection (%) on leaves or sheaths sprayed with Tween 20 solution alone, and *B* = area of infection (%) on treated leaves or sheaths. Values are expressed as the means ± SD of three independent experiments ($p = 0.05$).

[c]Commercially available chlorothalonil (Kyung Nong Co., Korea), dimethomorph (Dr. Ehrenstorfer GmbH Co., Germany), and carboxin (Dongbangagro Co., Korea) were used as positive controls against TLB and WLR, respectively.

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