JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antifungal Constituents from the Seeds of *Allium fistulosum* L.

SHENGMIN SANG,*,† AINA LAO,‡ YANSENG WANG,§ CHEE-KOK CHIN,§ ROBERT T. ROSEN,† AND CHI-TANG HO†

Department of Food Science and Center for Advanced Food Technology, and Department of Plant Science, Rutgers University, 65 Dudley Road, New Brunswick, New Jersey 08901-8520; and Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, People's Republic of China

A new unsaturated fatty acid monoglyceride (**1**), glycerol mono-(*E*)-8,11,12-trihydroxy-9-octadecenoate, was isolated from the seeds of *Allium fistulosum* L. along with five known compounds: tianshic acid (**2**), 4-(2-formyl-5-hydroxymethylpyrrol-1-yl) butyric acid (**3**), *p*-hydroxybenzoic acid (**4**), vanillic acid (**5**), and daucosterol (**6**). The structures of **1**−**3** were established by interpretation and full assignments of NMR spectroscopic data. Both **1** and **2** were found to inhibit the growth of *Phytophtohora capsici* on V8 media.

KEYWORDS: Monoglyceride; *Allium fistulosum*; Liliaceae; antifungal constituents

INTRODUCTION

Allium fistulosum L. (Liliaceae) is a perennial herb that is widely cultivated throughout the world, ranging from Siberia to tropical Asia. China, Japan, and Korea grow most of the world production. The common name "Welsh onion" derives from the German *welshche*, meaning foreign. Other local names include the following: in China, Cong; in English-speaking countries, Japanese bunching onion, Spanish onion, two-bladed onion, spring onion, green bunching onion, scallion, green trail, and Chinese small onion; in Japan, negi; and in Korea, pa. It is believed to have originated in northwestern China (*1*). Both the leaves and the bulbs are edible. It has also been used as an herbal medicine for many diseases. According to the dictionary of Chinese drugs (*2*), the bulbs and roots of this plant have been used for treatment of febrile disease, headache, abdominal pain, diarrhea, snakebite, ocular disorders, and habitual abortion, as well as having antifungal and antibacterial effects. The seeds are used as a tonic and an aphrodisiac. In earlier studies, various volatile compounds were reported from the edible parts of this plant (*3*−*5*). A novel antifungal compound, fistulosin, was isolated from the roots of *A. fistulosum* L. (*6*). Several steroidal saponins have been isolated and identified from the subterranean part of this plant (*7*, *8*). However, there are no reports on phytochemicals in the seeds of this plant. To find the bioactive constituents from this seed, we carried out systematic chemical analysis of this seed. In this report we describe the isolation and structure elucidation of a new unsaturated fatty acid monoglyceride (**1**), as well as five known compounds, tianshic acid (**2**), 4-(2-formyl-5-hydroxymethylpyrrol-1-yl) butyric acid (**3**), *p*-hydroxybenzoic acid (**4**), vanillic acid (**5**), and daucosterol (**6**), from the ethanol extract of the seeds of *Allium fistulosum* L.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were obtained on a JASCO p-1020 polarimeter at λ589 nm. FT-IR was performed on a Magna 550 spectrometer (Nicolet). ^{1}H (600 Hz), ^{13}C (150 Hz) and all 2-D NMR spectra were run on a Varian AM-600 NMR spectrometer, with TMS as internal standard. HRFAB-MS was run on a JEOL HX-110 double focusing mass spectrometer using glycerol or nitrobenzyl alcohol as matrix. The APCI MS was performed on a Fisons/VG Platform II mass spectrometer. Preparative thin-layer chromatography was performed on Sigma-Aldrich TLC plates (1000 μm thickness, 2−25 μm particle size). Thin-layer chromatography was performed on Sigma-Aldrich TLC plates (250 μm thickness, 2−25 μm particle size), with compounds visualized by spraying with 5% H_2SO_4 (v/v) in ethanol solution.

Plant Material. The seeds of *Allium fistulosum* were purchased from Shandong Traditional Chinese Medicine Inc. in June 1999, and were identified by Dr. Xiaoqiang Ma (Shanghai Institute of Materia Medica). A voucher specimen (HS18) was deposited at the Department of Food Science, Cook College, Rutgers University.

Extraction and Isolation. The powdered seeds of *A. fistulosum* (30 kg) were steeped successively with petroleum ether (×2) and 95% EtOH (×3). After evaporation of ethanol in vacuo, the residue was suspended in water and then extracted successively with petroleum ether, EtOAc, and *n*-BuOH. The EtOAc fraction was subjected to Sephadex LH-20 column chromatography (2.5 × 45.7 cm) eluted with 95% EtOH and collected with a fraction collector (10 mL per tube). After combination, 5 fractions were obtained. Subfraction 2 was isolated by preparative TLC eluted with $CHCl_3$/MeOH (20:1) to give compounds *p*-hydroxybenzoic acid (**4**) (10 mg, yield 0.33 mg/kg) and 3-methoxy-4-hydroxybenzoic acid (**5**) (10 mg, yield 0.33 mg/kg). The *n*-BuOH fraction was subjected to passage over Diaion HP-20 (5.0 × 45.7 cm)

* To whom correspondence should be addressed. Tel.: 732-932-9611, ext. 346. Fax: 732-932-6776. E-mail: ssang@rci.rutgers.edu.
† Department of Food Science and Center for Advanced Food Technology.
‡ Shanghai Institute of Materia Medica.
§ Department of Plant Science.

10.1021/jf025651o CCC: $22.00
Published on Web 09/14/2002

Table 1. ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR Spectral Data for **1** (C_5D_5N) and **2** (CD_3OD) (δ in ppm, J in Hz)

	1		2	
	δ_C	δ_H (J)	δ_C	δ_H (J)
1	173.7 s		177.9 s	
2	34.3 t	2.28 t (7.2)	35.1 t	2.29 t (7.2)
3	25.2 t	1.54 m	26.2 t	1.55 m
4	29.8* t	1.17 m	30.3* t	1.30 m
5	29.5* t	1.17 m	30.6* t	1.30 m
6	26.0 t	1.50 m	26.6 t	1.54 m
7	38.4 t	1.66 m	38.5 t	1.49 m
		1.77 m		1.55 m
8	71.8 d	4.45 m	73.2 d	4.05 dt 6.0
9	130.9 d	6.35 dd (15.6, 6.0)	131.2 d	5.68 dd (15.6, 6.0)
10	136.6 d	6.30 dd (15.6, 5.4)	136.7 d	5.73 dd (15.6, 5.4)
11	76.2 d	4.45 m	76.7 d	3.92 t 6.0
12	75.2 d	3.90 m	76.0 d	3.42 m
13	33.6 t	1.66 m	33.7 t	1.35 m
		1.83 m		1.52 m
14	26.2 t	1.77 m	26.8 t	1.30 m
15	29.7* t	1.17 m	30.7* t	1.30 m
16	32.3 t	1.20 m	33.3 t	1.30 m
17	22.9 t	1.22 m	23.9 t	1.30 m
18	14.2 q	0.74 t (7.2)	14.6 q	0.92 t (7.2)
1′	66.7 t	4.59 dd (11.2, 4.0)		
		4.67 dd (11.2, 4.8)		
2′	70.8 d	4.39 m		
3′	64.2 t	4.00 brd (5.2)		

*Assignments may be interchanged.

using an EtOH/H_2O gradient system (0−100%). The fraction eluted by 70% EtOH was subjected to silica gel column chromatography (3.8 × 45.7 cm) with a CH_2Cl_2/MeOH/H_2O solvent system (10:1:0.15−1:1:0.3). Finally, the fraction eluted by CH_2Cl_2/MeOH/H_2O (10:1:0.15) was subjected to RP-18 silica gel column chromatography (2.5 × 30.5 cm) with 60% MeOH to give compounds **1** (80 mg, yield 2.67 mg/kg), **2** (500 mg, yield 16.7 mg/kg), and daucosterol (**6**) (5 mg, yield 0.17 mg/kg); and the fraction eluted by CH_2Cl_2/MeOH/H_2O (5:1:0.2) was subjected to Sephadex LH-20 column chromatography (2.5 × 45.7 cm) with 95% EtOH to give 4-(2-formyl-5-hydroxymethylpyrrol-1-yl) butyric acid (**3**) (32 mg, yield 1.07%).

Glycerol Mono-(*E*)-8,11,12-trihydroxy-9-octadecenoate (1). Amorphous solid, $[\alpha]_D^{25}$ −0.72° ($CHCl_3$, c 1.2). IR (film) ν_{max} 3400, 2926, 2850, 1730, 1558, 1507, 1471, 1456, 1300, 1190 cm^{-1}. ^1H NMR (C_5D_5N) and ^{13}C NMR (C_5D_5N) of **1,** see **Table 1**. Positive APCI-MS *m*/*z* 427 $[M + Na]^+$; negative APCI-MS *m*/*z* 403 $[M - H]^-$; positive FAB-MS *m*/*z* 427 $[M + Na]^+$; positive HRFAB-MS *m*/*z* 427.2671 $[M + Na]^+$, (calcd for $C_{21}H_{40}O_7Na$, 427.2672).

Alkaline Hydrolysis of 1. A 2-mg aliquot of **1** was dissolved in 10% KOH/H_2O and kept at room temp. for 4 h. The reaction mixture was neutralized with 2 N HCl and extracted with *n*-butanol. The residue solution was concentrated and then compared with **2** and glycerol on HR-TLC silica gel plate developed with $CHCl_3$/MeOH (15:1) and EtOAc/MeOH/H_2O (20:1.2:0.8) solvent system, detected by spraying with 5% (v/v) H_2SO_4 in ethanol solution, and then heating to 110 °C.

Tianshic Acid (2). Amorphous solid, $[\alpha]_D^{25}$ −0.28° ($CHCl_3$, c1.0). IR (film) ν_{max} 3400, 2930, 2852, 1756, 1560, 1507, 1472, 1456, 1300, 1190 cm^{-1}. ^1H NMR (CD_3OD) and ^{13}C NMR (CD_3OD) of **1**, see **Table 1**. Positive APCI-MS *m*/*z* 353 $[M + Na]^+$, negative APCI-MS *m*/*z* 329 $[M - H]^-$, (identical with the literature (*10*)).

4-(2-Formyl-5-hydroxymethylpyrrol-1-yl) Butyric Acid (3). Amorphous solid. ^1H NMR (CD_3OD, 600 MHz) δ 9.43 (1H, s, H-6), 6.98 (1H, d, J = 4.2 Hz, H-3), 6.27 (1H, d, J = 4.2 Hz, H-4), 4.66 (2H, s, H-7), 4.39 (2H, t, J = 7.8 Hz, H-4′), 2.22 (2H, t, J = 7.2 Hz, H-2′), 2.00 (2H, dt, J = 7.8, 7.2 Hz, H-3′). ^{13}C NMR (CD_3OD, 150 MHz) δ 181.4 (s, C-1′), 180.0 (d, C-6), 144.9 (s, C-5), 133.6 (s, C-2), 126.1 (d, C-3), 111.6 (d, C-4), 56.5 (t, C-7), 46.4 (t, C-4′), 35.4 (t, C-2′), 29.2 (t, C-3′). Positive APCI-MS *m*/*z* 234 $[M + Na]^+$, negative APCI-MS *m*/*z* 210 $[M - H]^-$, (identical with the literature (*11*)).

***p*-Hydroxybenzoic Acid (4).** White powder. APCI-MS, *m*/*z* 137 $[M - H]^-$, (identical with the literature (*12*)).

Vanillic Acid (5). White powder. APCI-MS, *m*/*z* 168 $[M - H]^-$, (identical with the literature (*13*)]).

Daucosterol (6). White powder. APCI-MS, *m*/*z* 575 $[M - H]^-$, (identical with the literature (*14*, *15*)).

Antifungal Assay (*16*). An isolate of *Phytophthora capsici* was furnished by Stephen Johnston of the Department of Plant Pathology, Rutgers University. The isolate was propagated as a mycelial culture on 1% agar medium containing 10% V8 juice and maintained in the dark at 20 °C. Seven-day-old cultures were used as the source of inoculum. The mycelia grew in radial fashion outward from the inoculum disk. The diameter of the mycelial mass was measured daily after inoculation. Compounds **1** and **2** were first dissolved in DMSO to produce 50 mM stock solutions, which were then added to V8 medium to give the appropriate final concentrations. Media containing only DMSO was used as control. Culture was initiated by placing a 2-mm disk of inoculum in the center of the medium in a 150-mm Petri dish.

RESULTS AND DISCUSSION

The *n*-butanol fraction was chromatographed successively on Diaion HP-20, Si gel, Sephadex LH-20, and RP-18 Si gel columns to afford compounds **1**, **2**, 4-(2-formyl-5-hydroxymethylpyrrol-1-yl) butyric acid (**3**), and daucosterol (**6**). The ethyl acetate fraction was chromatographed successively on Sephadex LH-20 and then by preparative TLC to give *p*-hydroxybenzoic acid (**4**) and 3-methoxy-4-hydroxybenzoic acid (**5**).

Compound **1**, an amorphous solid, was assigned the molecular formula of $C_{21}H_{40}O_7$ with two degrees of unsaturation, determined by positive-ion HRFABMS and supported by ^{13}C NMR data. The IR spectrum indicated the presence of hydroxyl groups (3400 cm^{-1}), CH_2 moieties (2926 and 2850 cm^{-1}) and an ester group (1730 cm^{-1}). In the ^{13}C NMR spectrum (**Table 1**), one ester carbon (δ 173.7), one double bond (δ 130.9 and 136.6), four −CHOH− (δ 70.8, 71.8, 75.2, and 76.2), two $-CH_2OH-$ (δ 64.2 and 66.7), and 12 aliphatic carbons were identified. The ^1H NMR spectrum of **1** (**Table 1**) showed one triplet methyl group at δ 0.74; four oxygenated methine protons at δ 3.90, 4.39, and 4.45; two oxygenated methene groups at δ 4.00 (2H), 4.59, and 4.67 (each 1H), and two olefinic protons at δ 6.30 and 6.35. The $^1H-^1H$ COSY spectrum indicated the presence of one glycerol group [$CH_2(OH)-CH(OH)-CH_2-O-$] and the spin system [$-CH_2-CH(OH)-CH=CH-CH(OH)-CH(OH)-CH_2-$], [$-CO-CH_2-CH_2-CH_2-$], and [$-CH_2-CH_2-CH_3$]. All of this suggested that **1** is an unsaturated fatty acid glyceride. The NMR spectroscopic data of the fatty acid part of **1** was identical to that of **2** (**Table 1**), indicating that **1** was the ester of **2** and glycerol. This was supported by alkaline hydrolysis. After alkaline hydrolysis compound **1** gave **2** and glycerol. The *E*-configuration of the double bond was judged from its large $^3J_{H1,H2}$ coupling constants (J = 15.6 Hz) (*9*). The remaining outstanding issue was whether this linkage point in glycerol was at CH_2OH or CHOH. To resolve this question, we carefully analyzed the cross-peaks in the HMBC spectrum (**Figure 2**). The HMBC spectrum showed the cross-peaks between δ_C 153.6 and H-1′ (δ_H 4.67 and 4.59), indicating the fatty acid unit was attached at the C-1′ position of the glycerol by an ester group. Thus, compound **1** was determined as glycerol mono-(*E*)-8,-11,12-trihydroxy-9-octadecenoate (**Figure 1**). Full assignments of the ^1H and ^{13}C NMR signals were accomplished using HMBC, HMQC, $^1H-^1H$ COSY, and TOCSY experiments (**Table 1**).

In addition to the new unsaturated fatty acid monoglyceride, five known compounds, tianshic acid (**2**), 4-(2-formyl-5-

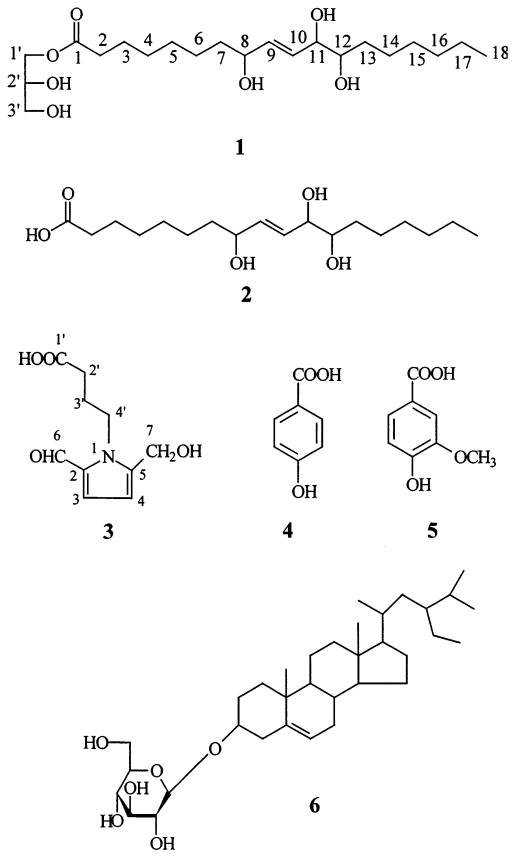

Figure 1. Structures of compounds **1**–**6**.

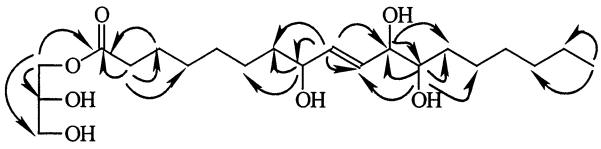

Figure 2. Significant HMBC (H→C) correlations of **1**.

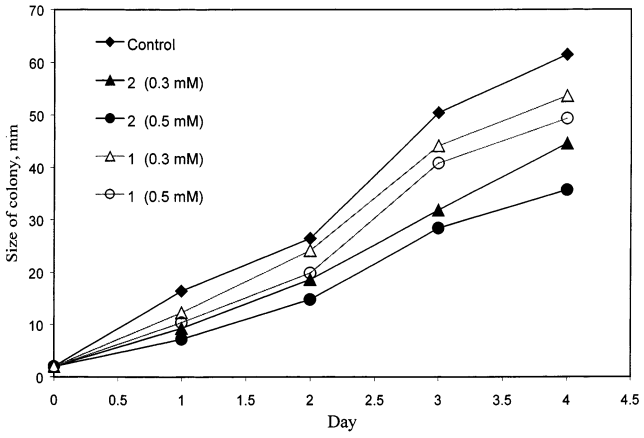

Figure 3. Effects of fatty acids on the growth of *Phytophthora capsici*.

hydroxymethylpyrrol-1-yl) butyric acid (**3**), *p*-hydroxybenzoic acid (**4**), vanillic acid (**5**), and daucosterol (**6**), were also isolated in this study. Their structures were identified by comparison of their NMR and MS data with those reported in the literature (*10*−*15*). Among them, the structures of **2** and **3** were confirmed by 2D NMR, including HMBC, HMQC, ^{1}H−^{1}H COSY, and TOCSY experiments. All of these compounds are reported from the seeds of this plant for the first time. It is notable that compound **3** is the Maillard reaction product of D-glucose and of 4-aminobutyric acid which is a major free amino acid found in coffee, cereals, and fruits. This compound has been reported to improve blood circulation (*17*) and promote hair growth (*18*). As a natural product, this compound was recently reported from "KAKO-BUSHI-MATSU" by Japanese researchers (*19*).

The effects of compounds **1** and **2** on *Phytophthora capsici*, the causal agent of the devastating blight disease of pepper, were studied. Both **1** and **2** were found to inhibit the growth of *P. capsici* on V8 media (**Figure 3**), and the free fatty acid **2** was clearly more inhibitory. This is consistent with the observations that most bioactive lipids are molecules released from glycerolipids (*20*). Because of the limited quantity of **1** and **2** isolated they were tested only on *P. capsici*. It will be of interest to examine the effects of these lipids on a broad range of fungi and bacteria. It will also be of interest to examine the contribution of **2** to the antifungal activity of the plant.

LITERATURE CITED

(1) Brewster, J. L.; Rabinowitch, H. D. *Onions and Allied crops; Japanese Bunching Onion (Allium fistulosum* L.*)*; Saito, S., Ed.; CRC Press: Boca Raton, FL, 1990; p 159.

(2) Jiangsu New Medical College. *Zhong-yao-da-ci-dian*; Shanghai Science and Technology Publisher: Shanghai, 1986; pp 2316−2318.

(3) Kameoka, H.; Iida, H.; Hashimoto, S.; Miyazawa, M. Sulphides and furanones from steam volatile oils of *Allium fistulosum* and *Allium chinense*. *Phytochemistry* **1984**, *23*, 155−158.

(4) Kuo, M.-C.; Ho, C.-T. Volatile constituents of the distilled oils of Welsh onions (*Allium fistulosum* L. variety maichuon) and scallions (*Allium fistulosum* L. variety caespitosum). *J. Agric. Food Chem.* **1992**, *40*, 111−117.

(5) Kuo, M.-C.; Ho, C.-T. Volatile constituents of the solvent extracts of Welsh onions (*Allium fistulosum* L. variety maichuon) and scallions (*Allium fistulosum* L. variety caespitosum). *J. Agric. Food Chem.* **1992**, *40*, 1906−1910.

(6) Phay, N.; Higashiyama, T.; Tsuji, M. Matsuura H.; Fukushi, Y.; Yokota, A.; Tomita, F. An antifungal compound from roots of Welsh onion. *Phytochemistry* **1999**, *52*, 271−274.

(7) Do, J. C.; Jung, K. Y.; Son, K. H. Steroidal saponins from the subterranean part of *Allium fistulosum*. *J. Nat. Prod.* **1992**, *55*, 168−173.

(8) Jung, K. Y.; Do, J. C.; Son, K. H. The structures of two diosgenin glycosides isolated from the subterranean parts of *Allium fistulosum*. *Han'guk, Yongyang Siklyong Hakhoechi* **1993**, *22*, 313−316.

(9) Masui, H.; Kondo, T.; Kojima, M. An antifungal compound, 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid, from *Colocasia antiquorum* inoculated with *Ceratocystis fimbriata*. *Phytochemistry* **1989**, *28*, 2613−2615.

(10) Chen, X. S.; Chen, D. H.; Si, J. Y.; Tu, G. Z.; Ma, L. B. Studies on chemical constituents from seeds of *Aesculus wilsonii* Rehd. *Yaoxue Xuebao* **2000**, 35, 198−200.

(11) Ninomiya, M.; Matsuzaki, T.; Shigematsu, H. Formation of reducing substances in the Maillard reaction between D-glucose and γ−aminobutyric acid. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 806−807.

(12) Wang, M.; Kikuzaki, H.; Zhu, N.; Sang, S.; Nakatani, N.; Ho, C.-T. Isolation and structural elucidation of two new glycosides from sage (*Salvia officinalis* L.) *J. Agric. Food Chem.* **2000**, *48*, 235−238.

(13) Sakushima, A.; Coskun, M.; Maoka, T. Hydroxybenzoic acids from *Boreava orientalis*. *Phytochemistry* **1995**, *40*, 257−261.

(14) Iribarren, A. M.; Pomilio, A. B. Sitosterol 3-*O*-β-xylopyranoside from *Bauhinia candicans*. *Phytochemistry* **1984**, *23*, 2087−2088.

(15) Sang, S. M.; Xia, Z. H.; Mao, S. L.; Lao, A. N.; Chen, Z. L. Studies on the chemical constitutents of the seeds of *Allium tuberosum* L. *Zhongguo Zhongyao Zazhi* **2000**, *25*, 286−288.

(16) Mackintosh, C. A.; Slater, L. A.; Walters, D. R.; Robins, D. J. Synthesis of six novel *N*,*N*-dialkyl derivatives of spermidine and effects on growth of the fungal plant pathogen *Pyrenophora avenae*. *FEMS Microbiol. Lett.* **2001**, *202*, 221−225.

(17) Mitsuo, M. Preparation and formulation of pyrrole derivatives for improving blood circulation. *Jpn. Kokai Tokkyo Koho* **1995**, 20 pp. Japan Patent JP 07138226 A2 19950530 Heisei.

(18) Mitsuo, M. Preparation of pyrrole derivatives as hair growth promoters. *Jpn. Kokai Tokkyo Koho* **1995**, 23 pp. Japan Patent JP 07138227 A2 19950530 Heisei.

(19) Miwa, M.; Yashie, S.; Hideo, B.; Mitsuo, M.; Takaomi, O.; Tsutomu, M. Studies on constituent of "KAKO-BUSHI-MATSU". *Nat. Med.* **1998**, *52*, 232−235.

(20) Wang, X. M. Plant phospholipases. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **2001**, *52*, 211−221.

Received for review May 5, 2002. Revised manuscript received August 8, 2002. Accepted August 8, 2002.

JF025651O