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Structures and Phytotoxicity of Metabolites from *Valsa ceratosperma*

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Valsa ceratosperma, which is the pathogenic fungus of apple canker, was grown in a synthetic medium. The neutral extract from the culture filtrate was chromatographed on a silica gel column to give five isocoumarins. Their structures were determined by MS, UV, IR, ^{1}H and ^{13}C NMR, and CD spectra. Three of them were known compounds; (−)-5-methylmellein (**1**), (−)-5-carboxylmellein (**2**) and (−)-5-hydroxylmethylmellein (**3**). Since the absolute configurations at C-3 in **2** and **3** were not known until now, both were determined to be R by chemical correlations. The two were new compounds; (+)-(3*R*,4*S*)-*trans*-4-hydroxy-5-methylmellein (**4**) and (−)-(3*R*,4*R*)-*cis*-4-hydroxy-5-methylmellein (**5**). All the five compounds showed phytotoxicity in a bioassay using detached apple shoots and lettuce seedlings.

The fungus, *Valsa ceratosperma*, is the pathogen of apple canker, which is one of the most harmful diseases to apple growing in the northern parts of Japan. We took an interest in investigating secondary metabolites of the fungus and their influence on phytotoxicity in the host plant. Now, we wish to report the isolation and structural determination of new isocoumarins (**4** and **5**) and the known ones (**1**, **2** and **3**) produced by the title fungus, and their phytotoxicities against detached apple shoots and lettuce seedlings.[1)]

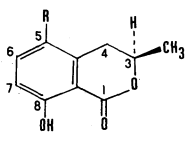
V. ceratosperma (VS-3) was grown in a synthetic medium by shaking culture for 10 days at 28°C. The filtrate was extracted with ethyl acetate to give a brown gummy substance. The crude extract was further examined to isolate its phytotoxic principle(s), becasue it caused a browning of the cambium and phloem tissues in detached apple shoots. The extract was chromatographed on a silica gel column, the acetone–chloroform eluates being combined into 10 fractions, four of which showed phytotoxicity against detached apple shoots. The crude crystalline substance in the first active fraction was purified by recrystallization to give the compound **1**, mp 124～125°C, $[\alpha]_D^{20}$ −105° (c=0.36). The molecular formula of **1** was determined to be $C_{11}H_{12}O_3$ by high resolution MS (m/z 192.0791, M^+). The UV, IR, ^{1}H NMR, ^{13}C NMR and optical rotation revealed **1** to be (−)-5-methylmellein (**1**), which is known as a metabolite of the almond pathogen *Fusicoccum amygdali*,[2a)] *Hypoxylon illitum*,[2d)] *Numularia* spp.,[2d)] *Phomopsis oblonga*[2e)] and *Semecarpus* spp. of plant.[2c)] The crystals contained in the third active fraction was recrystallized to give the pure substance **2**, mp 250～252°C, $[\alpha]_D^{20}$ −195° (c=0.14). High resolution MS (m/z 222.0529, M^+) revealed the molecular formula of **2** to be $C_{11}H_{10}O_5$. The UV and IR spectra suggested the presence of an isocoumarin skeleton. The ^{1}H NMR spectrum of **2** (see Table I) showed the presence of an acidic proton of a carboxyl group at δ 12.14, and the absence of the 5-methyl group. Additional proof was obtained from the ^{13}C NMR spec-

TABLE I. ^{1}H NMR RESONANCES[a] (δ, J IN HZ IN PARENTHESES[b]) FOR **1**, **2**, **3**, **4** AND **5**

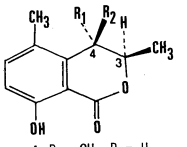
Compound	Solvent (M Hz)	Position								
		3	4	4	6	7	3-Me	5-Me	8-OH	Other
1	$CDCl_3$ (60 MHz)	4.68 m	3.00 dd (4.7, 16.2)	2.63 dd (10.6, 16.2)	7.28 d (8.5)	6.78 d (8.5)	1.54 d (6.1)	2.18 s	10.98 s	
2	DMSO-d_6 (60 MHz)	4.72 m	3.82 dd (3.0, 18.0)	2.96 dd (12.0, 18.0)	8.15 d (9.6)	6.94 d (9.6)	1.44 d (6.0)		11.61 s	12.94 s (COOH)
3	$CDCl_3$ (60 MHz)	4.69 m	3.20 dd (3.5, 16.5)	2.76 dd (10.5, 16.5)	7.43 d (8.5)	6.81 d (8.5)	1.52 d (6.0)		11.09 s	4.57 s, 2.40 s (CH_2OH)
4	$CDCl_3$ (400 MHz)	4.99 dq (1.4, 7.0)		4.72 dd (1.4, 6.6)	7.39 d (8.8)	6.96 d (8.8)	1.28 d (7.0)	2.35 s	11.10 s	2.01 d (OH) (6.6)
5	$CDCl_3$ (400 MHz)	4.63 dq[c] (1.5, 6.0)	4.64 br.d[c] (6.5)		7.38 d (8.8)	6.96 d (8.8)	1.63 d (6.0)	2.36 s	11.01 s	1.64 d (OH) (6.5)

[a] Signal assignments were confirmed by decoupling experiments.
[b] First-order approximations from line separation,
[c] These peaks were partially overlapped.

trum, which showed a signal at δ 160.5 assignable to a carboxyl carbon and no signal of an aromatic methyl carbon. These facts indicated the structure of **2** to be 5-carboxylmellein (**2**), which has been reported as a metabolite of *Hypoxylon illitum*[2d] and *Phomopsis oblongá*,[2e] and as an extract of fungus-infected woods.[2b]



1 R= CH_3
2 R= COOH
3 R= CH_2OH



4 R_1= OH, R_2= H
5 R_1= H, R_2= OH

The second active fraction was further separated by silica gel preparative TLC and the compounds **3**, **4** and **5** were isolated. The ^{1}H NMR spectrum (see Table I) of the compound **3**, mp 112~113°C, $[\alpha]_D^{20}$ −97.9° (c=0.5), closely resembled that of 5-methylmellein except for signals at δ 4.57 (2H, s) and 2.40 (1H, s, OH) attributed to a hydroxylmethyl group. Since signals arising from adjacent aromatic protons and a strongly hydrogen bonded hydroxyl group on the aromatic ring were present, but an aromatic methyl group signal was absent, the structure of **3** was determined to be 5-hydroxylmethylmellein (**3**) which has been reported as a metabolic procuct of *Hypoxylon illitum*.[2d] The absolute configurations of **2** and **3** have not been determined until now. The chemical conversion of (−)-5-hydroxylmethylmellein (**3**) into 5-methylmellein and 5-carboxylmellein by hydrogenation and Jones' oxidation, respectively, were then examined to confirm the structures involving the absolute configurations of **2** and **3**. The obtained 5-methylmellein showed an optical rotation, $[\alpha]_D^{20}$ −85° (c=0.25), which was the nearly the same value as that of natural (−)-5-methylmellein. The oxidation product of **3**, $[\alpha]_D^{20}$ −195° (c=0.2), was identified to (−)-5-carboxylmellein (**2**) by a comparison of the spectral data. These results revealed that both the compounds **2** and **3** have the 3R configuration as well as that of (−)-5-methylmellein (**1**).

The compounds **4**, mp 133~134°C $[\alpha]_D^{20}$ +85.0°(c=0.25), and **5**, mp 136~138°C, $[\alpha]_D^{20}$ −36.0°(c=0.23), gave the same molecular formula, $C_{11}H_{12}O_4$ (M^+, m/z 208.0721 and 208.0705, respectively), and their MS spectra showed almost the same fragmentation patterns. Both the UV spectra of **4** and **5** were very similar to that of 5-methylmellein (**1**) and therefore, the presence of the same chromophore was suggested. The ^{1}H NMR spectrum of **4** (see Table I) showed the presence of two

methyne groups bearing an oxygen atom and two hydroxyl groups, but the absence of a C-4 methylene group observed as the AB part of an ABX system in 5-methylmellein (**1**). The ^{13}C NMR spectrum also indicated the presence of the two methyne groups (δ 65.6 and 80.7). The ^{1}H and ^{13}C NMR spectra of **5** (see Table I and EXPERIMENTAL) indicated the presence of the same functional groups as those in **4**. These data suggested that both **4** and **5** were 4-hydroxy-5-methylmellein and stereoisomers at C-3 and/or at C-4 of each other. The configurations of C-3 and C-4 in **4** and **5** were determined by analyses of their ^{1}H NMR and CD spectra. In the ^{1}H NMR spectra of **4** and **5**, significant differences in the chemical shifts of the C-3 and C-3 methyl protons were observed (see Table I). The chemical shift of the C-3 methyl protons at δ 1.28 in **4** was in a significantly high field compared with those of them in **1**, **2**, **3** and **5**. This suggested that the C-3 methyl group of **4** was placed in a shielding zone of the benzene ring, while those of **1**, **2**, **3** and **5** were on a deshielding zone. Therefore, it was considered that the C-3 methyl group in **4** was axial or axial-like, and that those in **1**, **2**, **3** and **5** were equatorial or equatorial-like. These assignments were supported by the large $J_{3\sim4}$ values corresponding to a trans-diaxial vicinal coupling in **1**, **2** and **3**. The remarkable downfield shift of the C-3 proton at δ 4.99 in **4** also supported the above consideration. Assuming the configurations at C-3 of **4** and **5** were R, as are those of **1**, **2** and **3**, because oxidation at C-4 would occur after cyclization in biosynthesis, the small $J_{3\sim4}$ values (1.4 and 1.5 Hz in **4** and **5** respectively) in the ^{1}H NMR spectra suggested structures with $3\alpha H_{eq}$–$4\beta H_{eq}$ (*trans* isomer) in **4** and $3\alpha H_{ax}$–$4\alpha H_{eq}$ (*cis* isomer) in **5** (see Fig. 1). Consequently, the structures of **4** and **5** were determined to be (3*R*,4*S*)-*trans*-4-hydroxy-5-methylmellein and (3*R*,4*R*)-*cis*-4-hydroxy-5-methylmellein, respectively.

The CD spectra were measured for the compounds **1** ~**5**, although no CD spectrum of isocoumarins has so far been reported as we know. All the spectra showed Cotton effects at

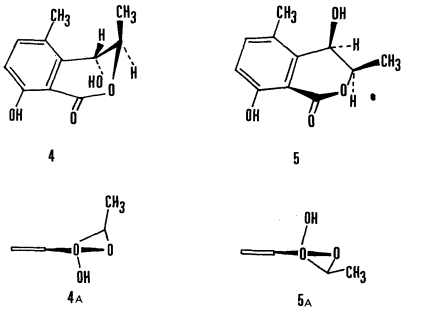


FIG. 1. Stereostructures and Octant Projections of **4** and **5**.

TABLE II. PHYTOTOXICITIES OF **1**, **2**, **3**, **4** AND **5**

(a) Browning activity on detached apple shoots.[a]

Concentrations	Samples				
	1	**2**	**3**	**4**	**5**
1000 ppm	++	++	++	++	++
100 ppm	+	++	+	+	+
10 ppm	−	+−	−	−	−

(b) Growth inhibition to lettuce seedlings.[b]

Concentrations	Samples				
	1	**2**	**3**	**4**	**5**
500 ppm	+−	+	+	+	+
100 ppm	−	+−	−	+	−
10 ppm	−	−	−	−	−

[a] The strength of browning activity is represented in the order of ++, +, +−, −.

[b] The inhibitory activity is represented in the order of +, +−, −.

ca. 220 nm and *ca.* 255 nm (see EXPERIMENTAL). In **1**, **2**, **3** and **5**, the sign of Cotton effects at *ca.* 255 nm was negative, and in **4** it was positive. Since in all the compounds **1**~**5**, the chromophore consisting of an aromatic ring and CO–O group was co-planer, a sector rule could be applied.[3a~c] The octant projections through the C-1 carbonyl group of **4** and **5** are shown in Fig. 1. These projections indicated an opposite cntribution of the C-3 methyl group to the sign of the Cotton effects in **4** and **5**, and the effect of the C-4 hydroxyl group was little. The predictions from the projections were in accordance with the em-

pirical observations if a reverse ketone octant rule was applied to them. A positive Cotton effect of **4** claimed the *R* configuration at C-3.

The Phytotoxicity of the compounds **1**~**5** was examined by using young detached apple shoots and lettuce seedlings, and the results are summarized in Table II. All the five compounds caused browning of the cambium and pholeum tissues of apple shoots at a concentration of 100 ppm, and a growth inhibition of lettuce seedlings at a concentration of 500 ppm. The compound **4** showed the strongest inhibition to lettuce seedlings, but 5-carboxylmellein may be the most potent toxic substance among the five isocoumarins. These observations made us consider a role of the secondary metabolites of *V. ceratosperma*, in which the isocoumarins may play an important role in extending the infected area of apple canker on apple trees.

EXPERIMENTAL

Melting points were determined in sealed glass capillaries on a Mitamura-Riken micro-melting points apparatus and are uncorrected. IR and UV spectra were recorded with a Hitachi EPI-G and a Hitachi 124 spectrophotometer, respectively. Mass spectra were recorded on a JEOL JMS-D300 mass spectrometer. ^{1}H NMR spectra were recorded on a JEOL FX-60 Q or FX-400 spectrometer for solutions with TMS as an internal standard. ^{13}C NMR spectra were recorded on a JEOL FX-60 Q (15 MHz) spectrometer for solutions with TMS as an internal standard. Optical rotations were recorded on a JASCO DIP-4 spectrometer for solutions in ethanol unless otherwise stated. CD spectra were recorded on a JASCO J-20 spectrometer for solutions in methanol. Column chromatography was carried out on silica gel (Merck, Kieselgel 60, Art. 7729), and preparative TLC on glass plates coated with silica gel (Merck, Kieselgel 60F_{254}, Art. 5744). Analytical TLC was carried out on silica gel (Merck, Kieselgel 60F_{254}, Art. 5715) with benzene–ethyl acetate (80 : 20).

Fermentation. Valsa ceratosperma (VS-3) was grown at 120 rpm on a rotary shaking machine and 28°C for 10 days in a 500 ml flask containing 200 ml of a medium consisting of soluble starch (10.0 g), L-asparagine monohydrate (2.0 g), potassium dihydrogen phosphate (1.0 g), magnesium sulfate heptahydrate (0.5 g), zinc sulfate heptahydrate (0.88 g), ferric nitrate monohydrate (1.5 mg), manganese sulfate pentahydrate (0.44 mg), biotin (5 μg), and thyamine (100 μg) per litre of distilled water.

Isolation of metabolites. The culture broth (pH 7.0) was filtered through a filter paper (Toyo No. 2), then extracted with ethyl acetate to give a brown gummy extract (0.025 g/liter). The neutral extract (900 mg) was chromatographed on a silica gel column (30 g, 2 × 28 cm). Elution with acetone–chloroform (10 : 90) was separated into fifty fractions (each of 10 ml). The sixth and seventh fractions gave crystals of **1** (80 mg). Fractions from the eighteenth to twenty second gave an oil (70 mg) which was further purified by preparative TLC using acetone–chloroform (15 : 85) as the eluant. Three bands were scraped off and eluted with acetone to give crystals of **3** (30 mg), **4** (8 mg), and **5** (3 mg) in order of decreasing *Rf* values. Fractions from the twenty third to twenty eighth gave crude crystals of **2** (20 mg). The physico-chemical and spectral data of the above five compounds are subsequently given.

5-Methylmellein **1**, (−)-(*3R*)-*3,4-dihydro-8-hydroxy-3,5-dimethylisocoumarin*. mp 124~125°C (white needles from ethanol). $[\alpha]_D^{20}$ −105° (*c* = 0.36). *Rf* 0.86. MS *m/z*: 192.0784 (M^+, calcd for $C_{11}H_{12}O_3$: 192.0786), 177, 148 and 120. UV λ_{max}^{EtOH} nm (ε): 218 (19000), 247 (5500) and 322 (3500). IR ν_{max}^{Nujol} cm^{-1}: 3200~3000 (br.), 1660, 1605, 1240 and 820. CD λ nm ($\Delta\varepsilon$): 257 (−3.03), 242 (1.23) and 221 (−1.12). ^{1}H NMR (60 MHz, $CDCl_3$, δ): 1.54 (3H, d, *J* = 6.1 Hz), 2.18 (3H, s), 2.86 (2H, dq, *J* = 16.2, 10.6 and 4.7 Hz, AB part of ABX system), 4.68 (1H, m, X part of ABX system), 6.78, 7.28 (each 1H, dd, *J* = 8.5 Hz) and 10.98 (1H, s). ^{13}C NMR (15 MHz, $CDCl_3$, δ): 18.0 (q), 20.9 (q), 31.9 (t), 75.4 (d), 108.1 (s), 115.6 (d), 124.9 (s), 137.0 (s), 137.8 (d), 160.5 (s) and 170.2 (s).

5-Carboxylmellein **2**, (−)-(*3R*)-*5-carboxy-3,4-dihydro-8-hydroxy-3-methylisocoumarin*. mp 250~252°C (white needles from ethanol–benzene). $[\alpha]_D^{20}$ −195° (*c* = 0.14). *Rf* 0.68. MS *m/z*: 222.0529 (M^+, calcd for $C_{11}H_{10}O_5$: 222.0528), 207, 194, 189 and 150. UV λ_{max}^{EtOH} nm (ε): 228 (18900), 246 (7840) and 315 (2790). IR ν_{max}^{Nujol} cm^{-1}: 3190, 1685, 1645, 1580, 1235 and 820. CD λnm ($\Delta\varepsilon$): 255 (−5.12), 240 (−5.69) and 208 (2.48). ^{1}H NMR (60 MHz, DMSO-d_6, δ): 1.44 (3H, d, *J* = 6.0 Hz), 2.96 (1H, dd, *J* = 18.0 and 12.0 Hz), 3.82 (1H, dd, *J* = 18.0 and 3.0 Hz), 4.72 (1H, m), 6.94, 8.15 (each 1H, dd, *J* = 9.6 Hz), 11.61 (1H, s), and 12.94 (1H, s). ^{13}C NMR (15 MHz, DMSO-d_6, δ): 20.1 (q), 32.0 (t), 75.1 (d), 108.8 (s), 115.3 (d), 119.4 (s), 138.6 (d), 143.2 (s), 163.7 (s) and 169.2 (s).

5-Hydoxylmethylmellein **3**, (−)-(*3R*)-*3,4-dihydro-8-hydroxy-5-hydroxylmethyl-3-methylisocoumarin*. mp 111~112°C (white needles from benzene). $[\alpha]_D^{20}$ −97.9° (*c* = 0.56). *Rf* 0.14. MS *m/z*: 208.0743 (M^+, calcd for $C_{11}H_{12}O_4$: 208.0735), 193, 164, 149, 136 and 135. UV λ_{max}^{EtOH} nm (ε): 216 (15600), 246 (4440) and 319 (2920). IR ν_{max}^{Nujol} cm^{-1}: 3528, 1663, 1604, 1236 and 840. CD λnm ($\Delta\varepsilon$): 258 (−2.30), 243 (0.72) and 220 (−2.42). ^{1}H NMR (60 MHz,

$CDCl_3$ δ): 1.52 (3H, d, J=6.0 Hz), 2.40 (1H, s, OH), 2.76 (1H, dd, J=16.5 and 10.5 Hz), 3.20 (1H, dd, J=16.5 and 3.5 Hz), 4.57 (2H, s), 4.69 (1H, m), 6.81 and 7.43 (each 1H, dd, J=8.5 Hz). ^{13}C NMR (15 MHz, $CDCl_3$ δ): 20.7 (q), 31.1 (t), 62.3 (t), 75.6 (d), 108.3 (s), 115.7 (d), 127.9 (s), 136.6 (s), 138.4 (d), 161.6 (s) and 170.1 (s).

(3R,4S)-trans-4-Hydroxy-5-methylmellein **4**, *(+)-(3R,4S)-3,4-dihydro-4,8-dihydroxy-3,5-dimethylisocoumarin.* mp 133~134°C (white needles from benzene–hexane). $[\alpha]_D^{20}$ +85.0° (c=0.25). *Rf* 0.32. MS *m/z*: 208.0721 (M^+, calcd for $C_{11}H_{12}O_4$: 208.0735), 164, 136, 135 and 107. UV λ_{max}^{EtOH} nm (ε): 216 (17800), 245 (2600) and 324 (2350). IR ν_{max}^{Nujol} cm^{-1}: 3210, 1682, 1600, 1228 and 810. CD λnm ($\Delta\varepsilon$): 254 (4.84) 221 (3.03) and 205 (−3.53). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 1.28 (3H, d, J=7.0 Hz), 2.01 (1H, d, J=6.6 Hz, OH), 2.35 (3H, s), 4.72 (1H, dd, J=6.6 and 1.4 Hz), 4.99 (1H, dq, J=7.0 and 1.4 Hz), 6.96 (1H, d, J=8.8 Hz), 7.39 (1H, d, J=8.8 Hz) and 11.10 (1H, s). ^{13}C NMR (15 MHz, $CDCl_3$ δ): 17.1 (q), 18.1 (q), 65.6 (d), 80.7 (d), 106.7 (s). 118.1 (d), 127.5 (s), 135.1 (s), 139.0 (d), 160.1 (s) and 168.2 (s).

(3R,4R)-cis-4-Hydroxy-5-methylmellein **5**, *(−)-(3R,4R)-3,4-dihydro-4,8-dihydroxy-3,5-dimethylisocoumarin.* mp 136~138°C (white needles from ethanol–benzene). $[\alpha]_D^{20}$ −36.0° (c=0.23). *Rf* 0.14. MS *m/z*: 208.0705 (M^+, calcd for $C_{11}H_{12}O_4$: 208.0735), 164, 136, 135 and 107. UV λ_{max}^{EtOH} nm (ε): 216 (12800), 245 (2560) and 322 (2240). IR ν_{max}^{Nujol} cm^{-1}: 3445, 3407, 1658, 1605, 1228 and 815. CD λnm ($\Delta\varepsilon$): 254 (−4.59) and 218 (2.53). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 1.63 (3H, d, J=6.0 Hz) 1.64 (1H, d, J=6.5 Hz), 2.36 (3H, s), 4.63 (1H, dq, J=1.5 and 6.0 Hz), 4.64 (1H, br d, J=6.5 Hz), 6.96 (1H, d, J=8.8 Hz), 7.38 (1H, d, J=8.8 Hz) and 11.01 (1H, s). ^{13}C NMR (15 MHz, $CDCl_3$, δ): 16.3 (q), 17.2 (q), 64.3 (d), 78.0 (d), 106.7 (s), 118.0 (d), 126.2 (s), 137.6 (s), 138.8 (d), 160.1 (s) and 169.7 (s).

Conversion of (−)-5-hydroxylmethylmellein **3** *into (−)-5-methylmellein* **1**. (−)-5-Hydroxylmethylmellein **3** (10 mg) dissolved in 5 ml ethanol was hydrogenated in the presence of 10% Pd–C as a catalyst. After completing the reaction by checking with TLC, the reaction mixture was filtered off. The filtrate was evaporated *in vacuo* to give crude crystals (7 mg). Recrystallization from ethanol gave pure crystals, mp 124~125°C, which were identified to be (−)-5-methylmellein by comparing with the spectral data and the value of optical rotation, $[\alpha]_D^{20}$ −105° (c=0.25).

Conversion of (−)-5-hydroxylmethylmellein **3** *into (−)-5-carboxylmellein* **2**. Jones' oxidation of (−)-5-hydroxylmethylmellein **3** (6 mg) gave (−)-5-carboxylmellein (4 mg), mp 182~184°C, $[\alpha]_D^{20}$ −195.0° (c=0.2), which was identical with the natural authentic sample in all respects.

Bioassays for phytotoxicity.

(a) *Detached apple shoot test.* Three detached young apple shoots were each immersed in 10 ml of aqueous solutions which contained the same amounts of each metabolite, and were allowed to stand for 72 hr at 25°C. The cut surfaces of the immersed edge and of the part at a distance of 5 mm from the edge were observed with respect to browning or damage at the phloem and cambium tissues with the unaided eye and microscope. Browning activity is indicated according to the following scale: ++, intensive browning; + clear browning; − no browning. No browning was observed in the shoots immersed in water only and in aqueous solutions of the other unidentified metabolites of *V. ceratosperma*.

(b) *Lettuce seedling test.* One ml of 1% Tween 20 solution containing a sample at a definite concentration (500, 100 and 10 ppm) was poured on to a filter paper (2.7 cm diameter) layered in a Petri dish (3 cm diameter). Seven lettuce seeds were placed on each filter paper and incubated at 20°C for 3 days. Growth inhibitory activity is indicated according to the following scale: +, clear inhibition; +−, doubtful inhibition; − no inhibition.

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