Malolactomycins C and D, New 40-Membered Macrolides Active against *Botrytis*

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A strain of *Streptomyces* was found to produce two new components of the 40-membered ring macrolides, malolactomycins C and D. They inhibited the growth of *Botrytis cinerea* in an agar medium and a detached leaf method.

In the course of our screening program for new antifungal antibiotics, strain KP-3144, isolated from a soil sample and identified as *Streptomyces* was found to produce two new components of 40-membered ring macrolide antibiotics of the malolactomycin group, and named malolactomycins C (**1**) and D (**2**). In this paper we describe the taxonomy of producing strain, and the fermentation, isolation, characterization, structure elucidation and biological activity of **1** and **2**.

Materials and Methods

Microorganisms

Strain KP-3144, a malolactomycin-producing culture, was isolated from a soil sample. A pure culture of strain KP-3144 was preserved under lyophilization. It was also maintained at 18°C for laboratory use as a slant on SEINO's agar.

Taxonomic Studies

The morphological properties were observed with a scanning electron microscope (model S-430, Hitachi, Tokyo). The isomer of diaminopimelic acid (DAP) was determined by the method of TAKAHASHI *et al.*[1] To investigate the cultural characteristics and physiological properties, the International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[2] and media recommended by WAKSMAN[3] were used. Cultures were observed after incubation at 27°C for two weeks. Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago)[4] was used for color names and hue numbers. The utilization of carbon sources was tested by growth at 27°C on PRIDHAM and GOTTLIEB's medium[5] containing 1% carbon sources.

Fermentation

Spores and mycelia on an agar slant of strain KP-3144 were scraped off to inoculate into a test tube (i.d. 2 × 20 cm) containing 10 ml of a seed medium composed of glucose 0.5%, soluble starch 2.4%, peptone 0.3%, meat extract 0.5%, yeast extract 0.5%, and $CaCO_3$ 0.4%, (pH 7.0). After incubation with reciprocal shaking (300 rpm) at 27°C for two days, 2 ml of this vegetative culture was transferred into flasks (500-ml volume) containing 100 ml of a seed medium of the same composition as above, incubated with rotary shaking (210 rpm) at 27°C for two days. The seed culture thus obtained was transferred into a jar fermenter (30-liter volume) containing 15 liters of a production medium. The production medium was composed of soluble starch 4%, defatted soybean meals 2%, KCl 0.03%, K_2HPO_4 0.05%, $FeSO_4 \cdot 7H_2O$ 0.05%, 0.1 N sodium thiosulfate 32 μg/liter and Adecanol (antifoam, Asahi Denka, Tokyo) 0.01%, pH 6.5 (before sterilization). The jar fermenter was run at 27°C for 4 days with aeration (7.5 liters/minute), and agitation (250 rpm).

Analytical Procedures

HPLC analyses were carried out using a Senshu HPLC system (model SSC-6530) equipped with a variable wave length UV detector (model SSC-6500), with either Senshu reverse phase columns (i.d. 2 × 30 cm) or a Asahi aminated silica gel NH2P-50 column (i.d. 2.15 × 25 cm). An open column chromatography was performed using reverse phase silica gel ODS-7515 (Senshu Scientific, Tokyo) with aqueous acetonitrile as eluant. UV spectrum was measured with a Shimadzu spectrophotometer model UV-240 (Shimadzu, Kyoto). FAB-MS was recorded by a JEOL spectrometer model JSM-A-505 HA. All NMR spectra were recorded on a Varian 400 MHz spectrometer.

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Biological Assay

Antibiotic activity in fermentation broths and purification steps was estimated by the conventional paper disc assay using *Botrytis cinerea* as indicator strain, which was incubated at 21~22°C for two days. The *B. cinerea* strain used in this study was resistant to benzimidazole fungicides (*e.g.* benomyl). MIC values were determined by the conventional serial two-fold dilution method against laboratory strains. Protective effect of malolactomycins against gray mold of kidney bean caused by *B. cinerea* was estimated by a detached leaf method. A primary leaf of kidney bean was cut into 3×3 cm pieces, which were dipped in solutions of varying concentrations of test compounds, followed by air drying. Agar pieces (about 5 mm in diameter) containing spores and mycelia of *B. cinerea* previously grown on potato glucose agar, were placed onto the above test compound-treated leaves, incubated at 20°C with 100% humidity. After 3 days, diameters of infectious lesions appearing on the leaves were measured, and compared with no drug control.

Results and Discussion

Taxonomy of Producing Strain KP-3144

Morphological Properties

The vegetative mycelia grew abundantly on both synthetic and complex media, and did not show fragmentation into coccoid forms nor bacillary elements. The aerial mycelia grew abundantly on oatmeal agar and glycerol-asparagine agar. The spore chains were spiral in shape and each had more than 20 spores per chain. The spores were ovoid in shape, 1.2×0.6 μm in size, and had a spiny surface (Fig. 1). Neither whirls, sclerotic granules, sporangia nor flagellated spores were observed.

Chemical Composition

The DAP isomer in the cell walls of strain KP-3144 was determined to be LL-type.

Cultural Characteristics and Physiological Properties

The cultural characteristics and the physiological properties are shown in Tables 1 and 2. The vegetative mycelia showed yellow to brown color on various media. The aerial mass showed white to bluish gray color. Soluble pigment was not observed. The utilization of carbon sources is shown in Table 3.

Based on the taxonomic properties described above,

Fig. 1. Scanning electron micrograph of spore chain of strain KP-3144 grown on glycerol-asparagine agar for 14 days.

Bar represents 1 μm.

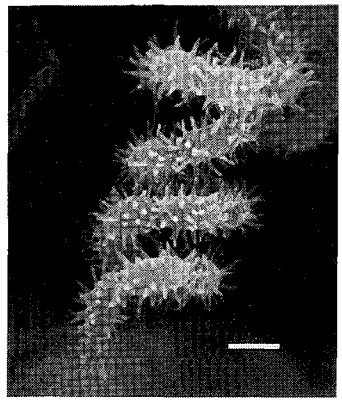


Table 1. Cultural characteristics of strain KP-3144.

Medium	Growth	Reverse color	Aerial mass color	Soluble pigment
Yeast extract-malt extract agar	Good, Lt. Mustard Tan (2ie)	Bamboo (2gc)	Abundant, Dusty Blue (16ge)	None
Oatmeal agar*	Moderate, Ivoly (2db)	Cream (11/2ca)	Moderate, Dusty Aqua Blue (17ge)	None
Inorganic salts-starch agar*	Good, Biscuit (2ec)	Biscuit (2ec)	Abundant, White~Dusty Aqua (a~18ge)	None
Glycerol-asparagine agar*	Good, Biscuit (2ec)	Golden Green (241/2ge)	Abundant, White~Dusty Blue (a~16ge)	None
Glucose-asparagine agar	Good, Biscuit (2ec)	Golden Green (241/2ge)	Abundant, White~Dusty Blue (a~16ge)	None
Peptone-yeast extract-iron agar**	Poor, Bamboo (2gc)	Bamboo (2gc)	None	None
Tyrosine agar*	Good, Bamboo (2gc)	Lt. Mustard Tan (3lg)	Abundant, White~Dusty Blue (a~16ge)	None
Sucrose-nitrate agar**	Poor, Ivory (2db)	Ivory (2db)	Poor, Blue Tint (15ba)	None
Glucose-nitrate agar**	Poor, Biscuit (2ec)	Biscuit (2ec)	None	None
Glycerol-calcium malate agar	Moderate, Biscuit (2ec)	Biscuit (2ec)	None	None
Glucose-peptone agar	Poor, Biscuit (2ec)	Biscuit (2ec)	None	None
Nutrient agar	Moderate, Biscuit (2ec)	Biscuit (2ec)	None	None

* Medium recommended by International Streptomyces Project.

** Medium recommended by S. A. WAKSMAN.

Table 2. Physiological properties of strain KP-3144.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin (21~23°C)	+
Peptonization of milk (27°C)	+
Coagulation of milk (27°C)	−
Cellulolytic activity	−
Hydrolysis of starch	+
Nitrate reduction	w
Temperature range for growth	15~35°C

+, Active; w, weakly active; −, not active.

Table 3. Utilization of carbon sources by strain KP-3144.

D-Glucose	+
D-Fructose	−
L-Rhamnose	+
D-Mannitol	+
L-Arabinose	+
i-Inositol	+
Raffinose	+
D-Xylose	+
Sucrose	+
Melibiose	+

+, Utilized; −, not utilized.

strain KP-3144 is considered to belong to the genus *Streptomyces*. The strain was deposited in the National Institute of Bioscience and Human Technology, Japan, under the name *Streptomyces* sp. KP-3144 and the accession No. is FERM P-15279.

Fermentation and Isolation

A typical time course of malolactomycins production by strain KP-3144 in a jar fermenter is shown in Fig. 2. The anti-*Botrytis* activity detected by a paper disc method appeared at day 2. The fermentation was stopped at day 4, and the mycelial cake obtained by centrifugation of the culture broth was used for isolation of active compounds.

The isolation procedures for malolactomycins is outlined in Fig. 3. The mycelial cake was extracted with acetone. The aqueous acetone layer was concentrated *in vacuo*. The residual aqueous solution was passed through a column of a porous adsorption resin, Diaion HP20 (Mitsubishi Chemicals, Tokyo) (1 liter), eluted stepwise with aqueous acetone. The active fractions eluted with 50~80% acetone were concentrated *in vacuo* to afford a brown material (9.2 g). The crude material, dissolved in a small amount of methanol was applied on a silica gel column (800 ml), which was developed with aqueous

Fig. 2. A typical time course of malolactomycin fermentation by *Streptomyces* sp. KP-3144 in a jar fermenter.

Diameter of inhibition zone (mm), ●; pH, △; and packed mycelial volume (ml/10 ml of culture broth), ○, are shown.

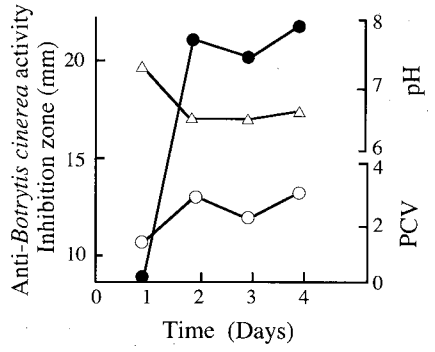
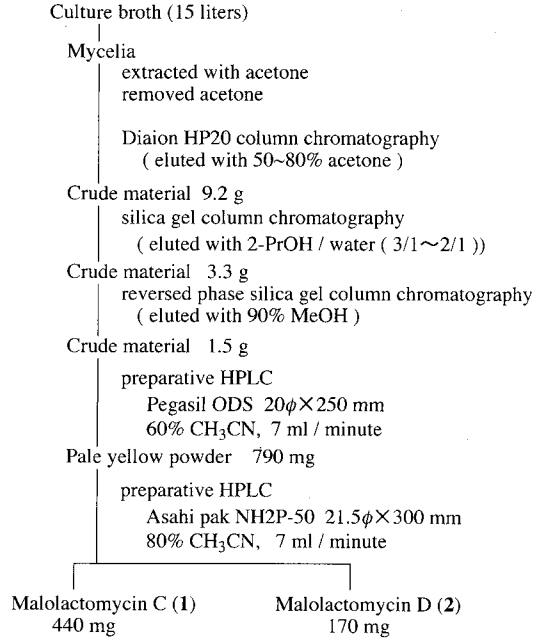


Fig. 3. Isolation procedures for malolactomycins C (**1**) and D (**2**).



2-propanol (75~67%). Active fractions thus obtained (3.3 g) was applied on a column (300 ml) of reverse phased silica gel (ODS-7515, Senshu Scientific, Tokyo). Elution was conducted stepwise with aqueous methanol. The active fractions eluted with 90% aqueous methanol were concentrated to give a partially pure active material. This was then subjected to HPLC (column, Senshu Pegasil ODS, i.d. 2×25 cm; mobile phase, 60% acetonitrile; flow rate, 7 ml/minute; detection by UV at 210 nm). Concentration of active fractions gave a substance (790 mg), which was a mixture of two active components. Each component was separated by second HPLC

Table 4. Physico-chemical properties of malolactomycins C (1) and D (2).

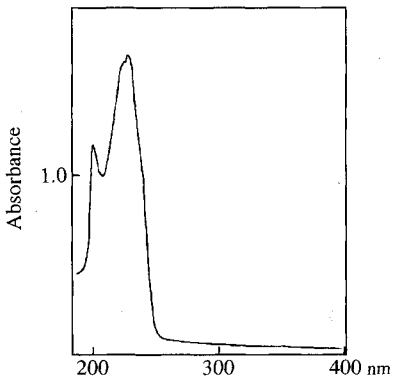
	Malolactomycin C (1)	Malolactomycin D (2)
Appearance	White powder	White powder
$[\alpha]_D^{25}$ (MeOH)	+20° (*c* 1.0)	+22° (*c* 1.0)
mp (°C)	138~141	140~143
Molecular formula	$C_{62}H_{109}N_3O_{20}$	$C_{61}H_{107}N_3O_{20}$
Molecular weight	1216.6	1202.5
HR FAB-MS (*m/z*): Calcd	1216.7682 (M+H)	1202.7526 (M+H)
Found	1216.7692	1202.7518
UV λ_{max}^{MeOH} nm	203, 225, 230, 238	203, 225, 230, 238
IR ν_{max} (KBr) cm^{-1}	3380, 2968, 2937, 1716, 1630, 1595, 1460, 1383, 1138, 1066	3380, 2968, 2935, 1714, 1633, 1600, 1456, 1385, 1066

(column, Asahipak NH2P-50, i.d. 2.15×30 cm; mobile phase, 80% acetonitrile; flow rate, 7 ml/minute; detection by UV at 210 nm). Two components **1** (440 mg) and **2** (170 mg) were obtained.

Characterization

Table 4 summarizes the physico-chemical properties of **1** and **2**. High resolution FAB-MS revealed the molecular mass (M+1) of 1216 for **1** and 1202 for **2**, and the molecular formulas $C_{62}H_{109}N_3O_{20}$ and $C_{61}H_{107}N_3O_{20}$ for **1** and **2**, respectively. The difference in molecular mass by 14 between **1** and **2** corresponded to the difference in molecular formula by CH_2. Compounds **1** and **2** show similar UV absorption spectra (Fig. 4).

Fig. 4. UV spectrum of malolactomycin C (**1**) (20 μg/ml in methanol).



Based on the physico-chemical data, **1** and **2** were compared with known microbial metabolites. They bear a certain resemblance to malolactomycins A[6,7] and B[6], but were discriminated from them in molecular mass and molecular formula. Therefore **1** and **2** were considered to be new antibiotics. This conclusion was confirmed by the structure elucidation described below.

Structure Elucidation

The structures of **1** and **2** were deduced on the basis of 1D and 2D NMR experiments in CD_3OD and CD_3OH. As the NMR spectra of **1** and **2** were very similar to that of malolactomycin A (**3**),[6,7] a 40-membered polyol macrolide, the assignments of the signals of **1** and **2** were accomplished by comparison with **3** and listed in Table 5.

The molecular formula of **1** was elucidated by HR-FAB-MS as $C_{62}H_{109}N_3O_{20}$, which was smaller than that of **3** by one CH_2 unit. The ^{13}C NMR spectrum of **1** showed 62 carbon signals in which one carbon at δ 45.8 was detected only in CD_3OH. Analysis of the DEPT spectra revealed that the ^{13}C NMR signals contained 10 methyl carbons, 17 methylene carbons, 28 methine carbons, one hemiacetal carbon (δ 99.8), two olefinic quaternary carbons, one guanidine carbon (δ 158.2), and three carbonyl carbons. The HMQC experiments indicated the connectivity of each proton and carbon. The structure of **1** was elucidated on the basis of ^1H-^1H COSY and HMBC experiments as shown in Fig. 5. Though proton and carbon signals of C-2′ methylene could not be detected in CD_3OD, they were detected clearly in CD_3OH (δ 3.24 for ^1H, δ 45.8 for ^{13}C). It may be due to the deuterium exchange of the methylene protons just as those of malonyl moiety of **3**[7]. Comparison of the ^1H NMR data of **1** with that of **3** revealed that a proton signal (δ 2.84, 6H) corresponding to two *N*-methyl groups in **3**[7] was observed as the signal (δ 2.84, 3H) corresponding to one *N*-methyl group in **1**.

The above results indicated that the structure of **1** was the same as that of **3**, except for an *N*-methyl group in the terminal guanidine group. Thus the structure of **1** was elucidated as *N*-demethyl **3** (Fig. 6).

The molecular formula of **2** was elucidated by HR-FAB-MS as $C_{61}H_{107}N_3O_{20}$, which was smaller than

Table 5. ^{1}H and ^{13}C NMR data of malolactomycins C (**1**) and D (**2**).

Position	1		2	
	^{13}C	^{1}H	^{13}C	^{1}H
1	177.1 s		177.2 s	
2	45.3 d	2.53 m	45.3 d	2.53 m
3	81.6 d	4.04 d (J=9.8 Hz)	81.6 d	4.03 d (J=9.5 Hz)
4	137.5 s		137.5 s	
5	127.6 d	5.42 br t (J=6.5 Hz)	127.6 d	5.42 br t (J=7.0 Hz)
6	34.3 t	2.26 m	34.3 t	2.24 m
7	71.8 d	4.02 m	71.9 d	4.03 m
8	43.2 d	1.54 m	43.2 d	1.55 m
9	75.9 d	3.82 m	75.9 d	3.82 m
10	39.2 t	1.52 m, 1.91 m	39.2 t	1.54 m, 1.91 m
11	76.0 d	3.82 m	75.9 d	3.82 m
12	44.5 d	1.57 m	44.5 d	1.56 m
13	72.2 d	3.88 m	72.2 d	3.87 m
14	33.9 t	1.38 m, 1.61 m	33.9 t	1.34 m, 1.61 m
15	30.7 t	1.32 m	30.7 t	1.32 m
16	40.9 d	1.63 m	40.9 d	1.63 m
17	72.2 d	3.92 m	72.3 d	3.92 m
18	41.4 t	1.82 m	41.4 t	1.82 m
19	99.8 s		99.8 s	
20	77.1 d	3.38 d (J=10.0 Hz)	77.2 d	3.36 d (J=8.0 Hz)
21	69.7 d	3.87 m	69.7 d	3.87 m
22	41.2 t	1.29 m, 1.91 m	41.1 t	1.29 m, 1.91 m
23	65.4 d	4.08 m	65.4 d	4.07 m
24	41.9 t	1.66 m, 1.76 m	41.9 t	1.66 m, 1.78 m
25	70.7 d	5.28 m	70.6 d	5.28 m
26	44.6 t	1.69 m	44.7 t	1.70 m
27	65.6 d	3.89 m	65.6 d	3.90 m
28	46.7 t	1.52 m	46.7 t	1.51 m
29	66.2 d	4.10 m	66.2 d	4.09 m
30	43.1 t	1.46 m, 1.64 m	43.1 t	1.46 m, 1.63 m
31	71.6 d	3.82 m	71.5 d	3.82 m
32	45.9 d	1.57 m	45.9 d	1.60 m
33	73.7 d	4.42 m	73.7 d	4.41 m
34	135.3 d	5.66 dd (J=14.8, 6.5 Hz)	135.3 d	5.65 dd (J=14.5, 6.5 Hz)
35	131.6 d	6.12 dd (J=14.8, 10.8 Hz)	131.6 d	6.10 dd (J=14.5, 10.5 Hz)
36	131.9 d	6.07 dd (J=14.8, 10.8 Hz)	131.8 d	6.07 dd (J=14.3, 10.5 Hz)
37	137.1 d	5.47 dd (J=14.8, 9.5 Hz)	137.1 d	5.46 dd (J=14.3, 9.5 Hz)
38	41.2 d	2.52 m	41.3 d	2.52 m
39	79.5 d	4.75 dd (J=8.5, 3.0 Hz)	79.3 d	4.75 dd (J=8.0, 3.0 Hz)
40	33.0 d	2.01 m	33.0 d	2.01 m
41	45.2 t	1.84 m, 2.08 m	45.2 t	1.84 m, 2.08 m
42	133.8 s		133.8 s	
43	128.2 d	5.19 br t (J=7.0 Hz)	128.3 d	5.19 br t (J=7.0 Hz)
44	28.7 t	2.03 m	28.7 t	2.05 m
45	30.4 t	1.41 m	30.4 t	1.42 m
46	27.3 t	1.41 m	27.2 t	1.42 m
47	29.9 t	1.60 m	29.9 t	1.61 m
48	42.6 t	3.17 t (J=7.0 Hz)	42.5 t	3.18 t (J=7.0 Hz)
2Me	15.4 q	0.87 d (J=7.0 Hz)	15.4 q	0.87 m (J=7.0 Hz)
4Me	10.7 q	1.62 s	10.7 q	1.62 s
8Me	10.0 q	0.94 d (J=7.0 Hz)	10.0 q	0.94 d (J=7.0 Hz)
12Me	10.3 q	0.91 d (J=7.0 Hz)	10.4 q	0.92 d (J=7.0 Hz)
16Me	14.7 q	0.92 d (J=7.0 Hz)	14.7 q	0.92 m
32Me	11.0 q	0.86 d (J=7.0 Hz)	11.0 q	0.86 m
38Me	17.9 q	1.02 d (J=6.5 Hz)	17.9 q	1.01 d (J=7.0 Hz)
40Me	13.9 q	0.90 d (J=7.0 Hz)	14.0 q	0.91 m
42Me	16.0 q	1.58 s	16.0 q	1.58 s
1′	171.5 s		171.6 s	
2′	45.8 t	3.24 m (in CD_3OH)	45.8 t	3.23 m (in CD_3OH)
3′	174.0 s		174.1 s	
$N=CN_2$	158.2 s		158.6 s	
NMe	28.3 q	2.84		

The CD_3OD signals (3.31 ppm of ^{1}H and 49.0 ppm of ^{13}C) were used as references.
The coupling constants (Hz) are in parentheses.

Fig. 5. NMR experiments for malolactomycin C (**1**).

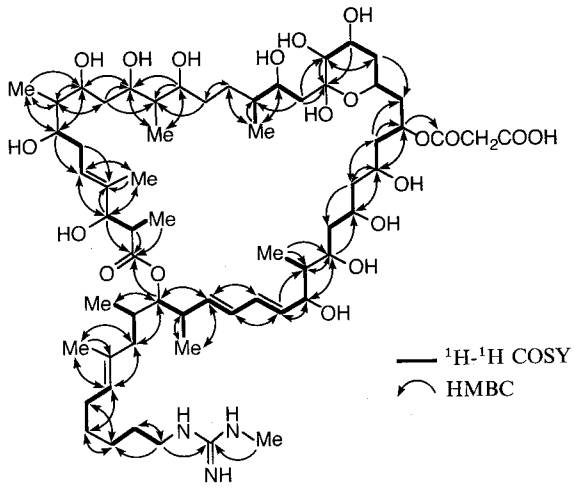
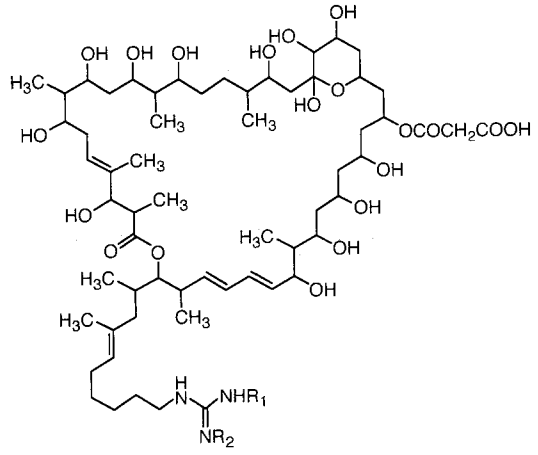


Fig. 6. Structure of malolactomycins.



Malolactomycin A (**3**) $R_1=CH_3$, $R_2=CH_3$
Malolactomycin C (**1**) $R_1=CH_3$, $R_2=H$
Malolactomycin D (**2**) $R_1=H$, $R_2=H$

that of **1** by one CH_2 unit. In the NMR spectra of **2**, the signals corresponding to an *N*-methyl group could not be observed. These results and the NMR assignments (Table 5) revealed that the structure of **2** is *N*,*N*-didemethyl **3** (Fig. 6). Compounds **1** and **2** were given names malolactomycins C and D, respectively.

Biological Activity

Compounds **1** and **2** showed moderate *in vitro* antifungal activity against *Cladosporium*, *Botrytis*, and *Pyricularia* (Table 6). No antimicrobial activity was observed against bacteria and yeasts tested. When tested in a detached leaf method (see Materials and Methods) the two compounds were effective in protecting kidney beans from infection by *Botrytis* at 500 ppm or above

Table 6. Antifungal activity of malolactomycin C (**1**) against plant pathogenic fungi.

Plant pathogenic fungi	MIC (μg/ml)
Fusarium oxysporum f. sp. *lycopersici*	>100
Phytophthora infestans	100
Trichoderma viridae	>100
Rhizoctonia solani	>100
Verticillium dahliae	>100
Alternaria kikuchiana	>100
Cladosporium fluvum	25
Botrytis cinerea	25
Pyricularia oryzae	25
Glomerella cingulata	>100
Cercospora beticola	100

Table 7. Protective effect of malolactomycins C (**1**) and D (**2**) against gray mold of kidney bean.

Compound	Control value (ppm)					
	1000	500	200	100	40	20
Malolactomycin C	100	100	100	60	0	0
Malolactomycin D	100	100	60	60	60	0

Table 8. Inhibitory effect of malolactomycin C (**1**) on spore germination of *Botrytis cinerea*.

Concentration (ppm)	Spore germination (%)	
	One day after treatment	One day after removal of malolactomycin C
100	0	40.6
25	0	56.0
6.25	0	85.7
1.56	92.7	ND
0	98.3	ND

ND: Not determined. Incubation at 20°C.

(Table 7). No phyto-toxicity was observed. Inhibitory activity of **3** on spore germination was studied by incubation at 20~21°C of spores of *B. cinerea* with varying concentrations of **3**, and the number of germinating spores was counted after one day of incubation. Table 8 shows that **3** inhibited spore germination at 6.25 ppm or higher. This inhibitory activity was not fungicidal but fungistatic, because removal of the antibiotic by centrifugation restored fungal growth after incubation for further one day (Table 8).

Malolactomycin A was reported to be active against bacteria and a wide range of fungi including yeast and filamentous fungi[7]. The LD_{50} value was 6.7 mg/kg (mice,

ip)[7]. Whereas, **1** and **2** appear to be less potent in antifungal activity and low in acute toxicity (no toxicity at 30 mg/kg, mice, ip).

These data suggest that the methylation of the guanidine moiety is important for antifungal activity, and probably for toxicity. Of the two new compounds reported here **2** has a free guanidine moiety, and thus provides a useful starting material for derivation of improved analogues by chemical and microbial means.

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