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Isolation and Characterization of Antimicrobial Substance Macrolactin A Produced from *Bacillus amyloliquefaciens* CHO104 Isolated from Soil

LEE, SEUNG-JE, JEONG-YONG CHO, JUNG-IL CHO[1], JAE-HAK MOON, KI DEOK PARK[2], YOUNG JU LEE[2], AND KEUN-HYUNG PARK*

Department of Food Science and Technology and Institute of Agricultural Science & Technology, Chonnam National University, Gwangju 500-757, Korea
[1]*Division of Food Science, Chosun College of Science and Technology, Gwangju 501-744, Korea*
[2]*Korea Basic Science Institute, Chonnam National University, Gwangju 500-757, Korea*

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Abstract A strain antagonistic to *Fusarium solani*, CHO104, was selected from approximately 100 microorganisms isolated from soil. Strain CHO104 was identified as *Bacillus amyloliquefaciens* and found to be Gram-positive based on the Biolog system and 16S rRNA methods. A culture broth of *B. amyloliquefaciens* CHO104 also exhibited antimicrobial activity against various microorganisms. As such, the EtOAc extract of the culture broth was isolated by various column chromatographic procedures and HPLC. The antimicrobial and antifungal substance was then characterized as macrolactin A ($C_{24}H_{34}O_5$) using high-resolution EI-MS and NMR analyses, and found to be very effective in inhibiting the growth of *Staphylococcus aureus*, *E. coli*, and *Botrytis cinerea*, even when using a concentration of one-twentieth of the benzoic acid as the control compound.

Key words: *Bacillus amyloliquefaciens*, macrolactin A, antimicrobial substance

Microorganisms such as fungi [22], bacteria [25], and viruses [8] cause various problems in the fields of agriculture, food, and medicine. In particular, the emerging antimicrobial resistance [10] of microorganisms has become a serious problem worldwide. In addition, within the food industry, there is an increasing demand for convenience foods with longer shelf-lives, yet consumers are also seeking the partial or complete removal of chemically synthesized preservatives from foods. As such, these consumer-led trends have promoted a renewed interest in the development of more 'natural' preservatives derived from plants [7], animal tissue [6, 17], and microorganisms [11, 15, 19, 20, 24] to maintain food safety. Although antimicrobial compounds have already been reported from various natural sources, their potential as natural food preservatives has not yet been fully investigated.

While screening for antimicrobial compounds useful for food preservation, we isolated a few potential antimicrobial substances from various plants [4, 5, 13, 14, 16, 26]. Therefore, the current study screened antagonistic microorganisms from soil and identified the selected strain CHO104. In addition, an antimicrobial, antifungal active substance was isolated and characterized from a culture broth of strain CHO104, and its antimicrobial and antifungal properties were determined.

MATERIALS AND METHODS

Screening and Isolation of Antagonistic Microorganisms

The screening and isolation of antagonistic microorganisms and culture conditions were carried out according to the method of Cho *et al.* [3]. The soil samples were mixed with 10 ml of Tris-HCl buffer solution (pH 7.5) and incubated in a nutrient medium at 25°C for 30 min with reciprocal shaking at 110 rpm. Next, the mixture was serially diluted with saline solution (0.85% NaCl) and spread on nutrient agar (NA) medium. The plates were then incubated at 25°C for 3 days, single colonies on the medium isolated, and each isolated strain inoculated with *Fusarium solani*. Previously, *F. solani* was inoculated into the center of a potato dextrose agar (PDA, Difco Laboratories, Detroit, MI, U.S.A.) plate and incubated at 25°C for 24 h. Then, the microorganisms isolated above were inoculated onto the plate-cultured *F. solani* and incubated at 25°C for 24 h. After being incubated for 7 days, the microorganisms inhibiting *F. solani* growth

*Corresponding author
Phone: 82-62-530-2143; Fax: 82-62-530-2149;
E-mail: khpark@chonnam.ac.kr

were selected [18]. The clear inhibition zones of the microorganisms exhibiting antagonistic activity against *F. solani* were compared, and the test for antagonistic activity was performed in triplicate.

To determine its antimicrobial activity, the culture broth of CHO104 was filtrated through a 0.22 µm nitrocellulose membrane filter (Micron Separation Inc., Westboro, MA, U.S.A.), then one-half was autoclaved at 121°C for 15 min. The culture filtrate and autoclaved filtrate were then both used to examine the inhibitory effects against fungal growth. The plates containing the culture filtrate were prepared as follows: PDA powder (39 g) dissolved in 250 ml of the culture filtrate was sterilized and cooled, then an additional 750 ml of the culture filtrate was added. Meanwhile, for the plates containing the autoclaved filtrate, the PDA powder (39 g) was dissolved in 1-l of the culture filtrate and autoclaved. The fungi were then inoculated onto separate plates and incubated for 7 days, after which the antimicrobial activities were determined by measuring the zone of inhibition.

Identification of Microorganisms

Preliminary identification of the antagonistic microorganism was carried out on the basis of morphological and biochemical analyses using Biolog™ Microlog3 4.01A (Biolog Inc., Hayward, CA, U.S.A.) [3] and the sequencing of the 16S rRNA gene after PCR (polymerase chain reaction). The PCR was performed to amplify a part of the 16S rRNA gene of the isolated CHO104. To determine the 16S rRNA sequence, the genomic DNA was isolated by the CTAB/NaCl method of Ausubel *et al.* [1]. The DNA manipulations for the cloning, transformation, plasmid isolation, ligation, and electrophoresis were carried out according to the method described by Sambrook *et al.* [23]. PCR was performed to amplify a partial 16S rRNA fragment of CHO104 using the universal primers (Y1:5'-TGGCTCA-GAACGCTGGCGGC-3', and Y2:5'-CCCACTGCCTCC-CGTAAGGAGT-3') [27]. The PCR was performed using a GeneAmp Thermal Cycler (model 2400, Perkin-Elmer, Norwalk, CT, U.S.A.). The amplified PCR product was then purified from the agarose gels using a gel elution kit (Bioneer Inc., Daejeon, Korea) and ligated into a pGEM-T vector (Promega Co., Madison, WI, U.S.A.) according to the manufacturer's instructions. The PCR products were sequenced using an Applied Biosystem (ABI373) DNA automated sequencer (PE Applied Biosystems, Foster City, CA, U.S.A.). The 16S rRNA sequence of strain CHO104 was then aligned with reference sequences obtained from the GenBank databases (NCBI, Bethesda, MO, U.S.A.).

Isolation and Purification of Active Compound

The isolated CHO104 strain was cultured for 48 h in LB (Luria Bertani, Detroit, MI, U.S.A.) liquid medium at 30°C, after which the cultured broth (9-l) was centrifuged for 15 min at 5,000 rpm. The supernatant (7-l) was adjusted to pH 3.0 with 1 N HCl and extracted with ethyl acetate (EtOAc, 7-l×3). The organic phase was also solvent fractionated using buffer solution of 2% $NaHCO_3$ at pH 8.0. The organic phase was obtained as an EtOAc-soluble neutral fraction, then the aqueous layer was adjusted to pH 3.0 with 1 N HCl and extracted with EtOAc to obtain an EtOAc-soluble acidic fraction [13].

The EtOAc-soluble neutral fraction was subjected to silica gel column chromatography (Kieselgel 60, 70-230 mesh, Merck, Darmstadt, Germany, 5 g, 1.5×11 cm), where the stepwise elution was carried out based on an increasing concentration of MeOH in EtOAc [26]. The active fraction was purified by Sephadex LH-20 column chromatography [2×97 cm, MeOH/$CHCl_3$ (4:1, v/v), 25-100 mesh, Pharmacia Fine Chemicals, Uppsala, Sweden] at a flow rate of 1.0 ml/min [4]. The active fraction was applied to a column of octadecylsilane (ODS, YMC-GEL, 70-230 mesh, YMC Co, Kyoto, Japan, 12 g, 1.5×13 cm, two times) and eluted stepwise based on an increasing concentration of MeOH in H_2O [14]. The active eluates were subjected to HPLC under the following conditions: ODS column, Senshu pak (8×250 mm, 5 µm, Kyoto, Japan); mobile phase, MeOH/H_2O (70:30, v/v); flow rate, 1.5 ml/min (model 510 solvent delivery system, Waters, Milford, MA, U.S.A.); detection, UV detector (254 nm, 486 tunable absorbance detector, Waters).

The active compound: white plate (3.4 mg); HREIMS *m/z* 402.2401 (M^+; -0.6 mmu for $C_{24}H_{34}O_5$); $[\alpha]_D^{25}$=-10.36 (*c* 0.13, MeOH); ^{1}H- and ^{13}C-NMR data are summarized in Table 2.

Instrumental Analyses of Active Compound

Electron impact-mass spectrometry (EI-MS) was performed using a mass spectrometer (Jeol JMN AX 505 WA, Tokyo, Japan) under the following conditions: source temperature, 200°C; ionizing voltage, 30 eV; scanning mass range, *m/z* 25-500. The molecular formula was then analyzed through high resolution-mass spectrometry (HREIMS, Jeol).

The NMR (^{1}H and ^{13}C) spectrum was measured in CD_3OD using a ^{unit}INOVA 500 spectrometer (500 MHz, Varian, Walnut Creek, CA, U.S.A.), with tetramethylsilane (TMS, δ=0) as an internal standard.

Microorganisms, Bioassay, and Antimicrobial Activity

The antagonistic activity of CHO104 was tested against phytopathogenic fungi (*Botryosphaeria dothidea* var., *Glomerella ingulata*, *Alternaria mali*, *Valsa ceratosperma*, *Penicillium expansum*, *Fusarium solani*, *Botrytis cinerea*, *Rosellinia necatrx*, *Phomopsis* sp., *Fusarium oxysporium*, *Phythium ultium*, and *Helicobasidium mompa*). In addition, the antimicrobial activity of the filtrate and autoclaved filtrate of the CHO104 culture broth was also determined against the same phytopathogenic fungi. The fungi were

obtained from the Korean Culture Type Collection, and the medium for the pathogenic fungi was PDA. Also, foodborne bacteria (*Staphylococcus aureus* KCTC 1928, *Staphylococcus epidermidis* KCTC 1917, *Pseudomonas aeruginosa* KCTC 2513, *Escherichia coli* KCTC 2593, *Bacillus subtilis* KCTC 1021, *Micrococcus luteus* KCTC 3523, *Streptococcus pyogenes* KCTC 3096, and *Enterococcus faecalis* KCTC 3195) were used to determine the antimicrobial activity of each fraction obtained after solvent fractionation of the filtrate obtained from the CHO104 strain culture broth. The foodborne bacteria were incubated on nutrient or BHI medium. The antimicrobial activity was measured using the paper disc (6 mm, Whatman, Maidstone, England) agar diffusion method [28]. The paper discs treated with the fractions purified by the column chromatographies as well as the antimicrobial active substance isolated by HPLC were placed on NA or BHI solid medium. The diameter (mm) of the inhibition zone was measured after 24 h of incubation at 30°C or 37°C. The isolation of the antimicrobial active substance was guided by the activity against *S. aureus* and *E. coli*.

The antimicrobial activity of the compound isolated from the culture broth of CHO104 strain was determined against *S. aureus* and *E. coli* in a nutrient broth [14]. Briefly, an inoculum of the liquid culture of each test microorganism was prepared by a conventional method to a final microbial concentration of approximately 10^6 cfu/ml of bacteria. Each prepared culture broth (50 µl) was diluted with 150 µl of nutrient broth media, then inoculated into the wells at various concentrations (10, 50, 100, 150 µM). The growth inhibition was measured using an EL-800 microspectrophotometer (Bio-Tek instruments, Winooski, VT, U.S.A.) at 650 nm after incubation for 17 h with shaking (70 rpm) at 37°C. The concentration of the antimicrobial substance required for 50% growth inhibition (IC_{50}) was determined by means of a dose-response curve (percentage of growth inhibition *vs.* concentration) [5].

RESULTS AND DISCUSSION

Isolation and Identification of Antagonistic Microorganisms from Soil and Antimicrobial Activities of Culture Broth

About 100 microorganisms were isolated from a soil sample. These isolated microorganisms were then incubated with *F. solani* on PDA plates. Among the 20 microorganisms exhibiting antagonistic activity against *F. solani*, the 4 strains showing the highest levels of activity were selected. In particular, CHO104 strain showed the strongest inhibiting activity against *F. solani* (data not shown), and was therefore selected for further study. A culture broth of antagonist CHO104 also exhibited antifungal activity against various fungi such as *Botryosphaeria dothidea* var., *Glomerella ingulata*, *Alternaria mali*, *Valsa ceratosperma*, *Penicillium expansum*, *Botrytis cinerea*, *Rosellinia necatrx*, *Phomopsis* sp., *Fusarium oxysporium*, *Phythium ultium*, and *Helicobasidium mompa* (Table 1).

Table 1. Antimicrobial activity of strain CHO104 selected from soil microorganisms on PDA medium, in filtrate and in autoclaved filtrate of culture broth.

Plant pathogenic fungi	Antimicrobial activity (%)[a]		
	A	B	C
Botryosphaeria dothidea var.	57	100	100
Glomerella ingulata	56	100	100
Alternaria mali	68	100	86
Valsa ceratosperma	52	100	100
Penicillium expansum	56	66	56
Fusarium solani	89	100	100
Botrytis cinerea	82	83	98
Rosellinia necatrx	72	100	100
Phomopsis sp.	68	100	98
Fusarium oxysporium	84	100	100
Phythium ultium	82	86	86
Helicobasidium mompa	79	100	100

[a]Antimicrobial activity (%)=[1-(T/NT)]×100.
NT: colony diameter of no treatment (mm).
T: colony diameter of treatment (mm).
A, antagonistic activity of strain CHO104 against plant pathogenic fungi on PDA medium; B, antimicrobial activity of filtrate of strain CHO104 culture broth against plant pathogenic fungi on PDA medium; C, antimicrobial activity of autoclaved filtrate of strain CHO104 culture broth against plant pathogenic fungi on PDA medium.

The antimicrobial activities of the filtrate and autoclaved filtrate of the antagonist CHO104 culture broth were also determined against various fungi in PDA plates. The resulting inhibiting rates were 80% or more, except for *P. expansum* (culture filtrate, 66%; autoclaved filtrate, 56%; Table 1). The inhibitory activities of the autoclaved filtrate of the antagonist CHO104 culture broth were very similar to those of the culture filtrate, regardless of the heat treatment (Table 1), suggesting that the antimicrobial activity was related to certain heat-stable substances. Therefore, an attempt was made to identify the antagonist CHO104, isolate and the inhibitory substance(s) produced by the strain.

First, to identify the antagonist CHO104, the morphological and biochemical characteristics were examined by Biolog™ MicroLog3. As such, strain CHO104 was found to utilize D-fructose, maltose, D-mannitol, D-mannose, D-psicose, L-sorbitol, and sucrose as carbon sources, but not L-arabinose, D-tagatose, lactamide, and D-maleic acid. The bacterium was also identified as *Bacillus amyloliquefaciens*, Gram-positive, and rod-shaped, with a 99% probability and similarity of 0.86. To confirm the identity of strain CHO104 more accurately, a part of the 16S rRNA gene of the antagonist CHO104 was amplified by PCR, then compared to the reference sequences of other bacterial 16S

rRNA genes using a Blast search at NCBI. The partial 16S rRNA sequence of strain CHO104 showed a 99% identity with that of *B. amyloliquefaciens*. Therefore, the antagonist CHO104 was identified as *B. amyloliquefaciens*, and named *B. amyloliquefaciens* CHO104.

Purification and Isolation of Antimicrobial Active Substance

A culture broth (9-l) of *B. amyloliquefaciens* CHO104 was centrifuged and the supernatant partitioned into the aqueous and organic phases with ethyl acetate and buffer solution. The resulting EtOAc-soluble acidic and EtOAc-soluble neutral fractions were then tested to determine their antimicrobial activity against various microorganisms by the paper disc method. Both fractions showed antimicrobial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, *M. luteus*, *S. pyogenes*, *E. faecalis*, and *B. cinerea* (data not shown). However, the EtOAc-soluble acidic fraction did not exhibit any antimicrobial activity against *S. epidermidis*, *B. subtilis*, as distinct from the EtOAc-soluble neutral fraction. In particular, the antimicrobial activity of the EtOAc-soluble neutral fraction against *S. aureus* and *E. coli* was stronger than that against any other bacteria. Therefore, the isolation of the antimicrobial compound was attempted from the EtOAc-soluble neutral fraction, as guided by the activity against *S. aureus* and *E. coli*.

The EtOAc-soluble neutral fraction was subjected to silica gel adsorption column chromatography, and eluted stepwise with an increasing concentration of MeOH in the EtOAc. Activity was found in the 100% EtOAc (127.2 mg) and EtOAc/MeOH (90:10, v/v, 78.1 mg) eluates. Since the antimicrobial activity of the less polar fraction (100% EtOAc) was higher than that of the polar fraction (EtOAc/MeOH, 90:10, v/v), the less polar fraction was subjected to Sephadex LH-20 column chromatograpy and the activity was measured based on a Ve/Vt (elution volume/total volume) of 0.64-0.78 (90 mg). The active fraction was then further purified by ODS column chromatography using a MeOH-H_2O system in the mobile phase, yielding an active fraction in the 80% MeOH eluate (14.6 mg). Further purification was performed with reversed-HPLC (ODS, 70% MeOH, 254 nm), resulting in an active compound with a single peak (t_R 19.0 min, 3.4 mg).

Structural Elucidation and Antimicrobial Activity of Isolated Compound

The molecular formula $C_{24}H_{34}O_5$ of the active compound was determined from its HREIMS *m/z* 402.2401 (M^+; -0.6 mmu for $C_{24}H_{34}O_5$) together with other spectral data. The ^{13}C-NMR (CD_3OD, Table 2) and DEPT spectra revealed twenty-four carbon signals, including an ester carbonyl carbon (δ 166.83), methyl carbon (δ 20.27), six methylene carbons (δ 44.07-25.81), four oxygenated methine carbons (δ 69.37, 69.97, 72.37, 72.49), and twelve olefinic methine carbons (δ 145.12-118.15). The molecular formula, $C_{24}H_{34}O_5$, and ^{13}C-NMR data require eight degrees of unsaturation, indicating that the active compound was monocyclic. The ^{1}H-NMR spectrum showed signals corresponding to twelve sp^2-carbon protons (δ 7.22-5.52), a methyl carbon proton (δ 1.26, d, 3H), six methylene carbon protons (δ 2.48-1.53), and four methine carbon protons (δ 5.00-3.85). The ^{1}H-^{1}H COSY and TOCSY experiments, along with band-selective 1-dimensional NMR techniques, such as DPFSGE-NOE (Double Pulsed Field Gradient Spin Echo NOE) and 1-dimensional TOCSY, plus the coupling constants of the proton signals in the ^{1}H-NMR (Table 2) established a structure of twenty-three contiguous protonated carbons. For the heavily overlapped olefinic protons, DPFGSE-NOE and 1-dimensional TOCSY techniques, utilizing a waveform generator for the band-selective excitation along with a pulsed field gradient, were used for the peak assignment. For instance, when already assigned proton peaks from H-8 (chemical shift 5.75 ppm) and H-9 (chemical shift 6.55 ppm) were irradiated for the DPFGSE-NOE and 1-dimensional TOCSY experiments, respectively, a triplet centered at 6.14 ppm popped up from the heavily overlapped cluster of proton peaks from H-5, H-10, H-17, and H-18. This triplet was initially assigned to H-10 based on the bond distances in

Table 2. ^{1}H- and ^{13}C-NMR data of macrolactin A in CD_3OD.

Carbon No.	^{1}H (500 MHz)	^{13}C (125 MHz)
1		166.83
2	5.52 (1H, d, J=11.5 Hz)	118.15
3	6.63 (1H, dd, J=11.5, 11.5 Hz)	145.12
4	7.22 (1H, dd, J=11.5, 15.1 Hz)	130.42
5	6.17 (1H, dt, J=15.1, 11.9 Hz)	142.34
6	2.42 (2H, dd, J=6.4, 11.9 Hz)	43.02
7	4.26 (1H, td, J=6.4, 6.4 Hz)	72.37
8	5.75 (1H, dd, J=6.4, 15.3 Hz)	137.71
9	6.55 (1H, dd, J=15.3, 11.9 Hz)	126.10
10	6.14 (1H, dd, J=11.9, 11.9 Hz)	131.56
11	5.55 (1H, dt, J=11.9, 11.9 Hz)	128.54
12	2.48 (1H, m), 2.32 (1H, m)	33.67
13	3.85 (1H, tt, J=6.1, 6.1 Hz)	69.37
14	1.60 (2H, dt, J=6.1, 6.4 Hz)	44.07
15	4.30 (1H, td, J=6.4, 6.4 Hz)	69.97
16	5.67 (1H, dd, J=6.4, 15.0 Hz)	135.40
17	6.17 (1H, dd, J=15.0, 10.0 Hz)	131.36
18	6.05 (1H, dd, J=10.0, 15.0 Hz)	131.87
19	5.64 (1H, dt, J=15.0, 7.0 Hz)	135.29
20	2.19 (1H, m), 2.11 (1H, m)	25.81
21	1.53 (2H, m)	33.14
22	1.64 (1H, m), 1.58 (1H, m)	36.17
23	5.00 (1H, m)	72.49
24	1.26 (3H, d, J=6.4 Hz)	20.27

The assignments were aided by ^{1}H-^{1}H COSY, DEPT, HMQC, NOE, and HMBC.

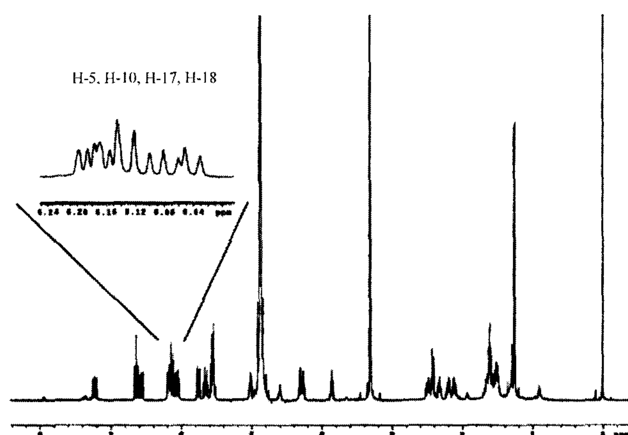
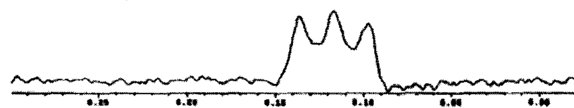


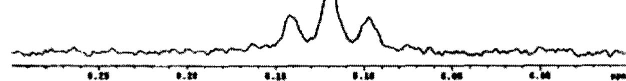
Fig. 1. ^{1}H-NMR spectrum (500 MHz) of macrolactin A in CD_3OD.
Insert is an expanded region of peaks from H-5, H-10, H-17, and H-18.

the assumed structure (Fig. 1 and 2), then later interpreted as a doublet of doublets arising from indirect couplings with the neighboring protons, H-9 and H-11. Similar procedures were repeated to assign the other overlapped proton peaks. In addition, the NOE data and coupling constants of the twelve sp^2-carbon protons (H-2, 3, 4, 5, 8, 9, 10, 11, 16, 17, 18, 19) provided partial structures corresponding to α, β, γ, and δ-unsaturated ester (C-1 to C-5) and two pairs of conjugated diens (C-8 to C-11 and C-16 to C-19). The geometries of the double bonds at C-2, 4, 8, 10, 16, and 18 based on the NOE data and ^{1}H coupling constants (Table 2) were assigned as *Z*, *E*, *E*, *Z*, *E*, and *E*, respectively. The complete planar structure, including the connection of C-23 and C-1 by ester bounding, was established by HMBC analyses (Table 3), which led to the identification of macrolactin A, previously isolated from a soil microorganism [12] and a deep-sea marine bacterium [9]. The stereochemistry of macrolactin A was recently established as 7*S*, 13*S*, 15*R*, and 23*R* by chemical synthetic

1D-TOCSY, Irradiated at H- 9



1D-NOE, Irradiated at H- 8



H-5, H-10, H-17, H-18

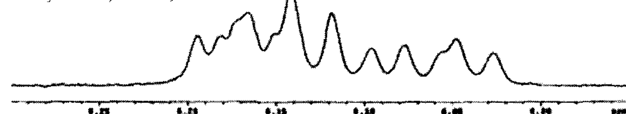


Fig. 2. Band selective 1D-TOCSY and DPFGSE-NOE experiment.

Table 3. HMBC correlation of macrolactin A.

Proton No.	δ_H	Carbon No. shown cross-peak with each ^{1}H signal
2	5.52	C-1, 4
3	6.63	C-1, 5
4	7.22	C-3, 6
5	6.17	C-3, 4, 6, 7
6	2.42	C-5, 8
7	4.26	C-5, 6, 8, 9
8	5.75	C-6, 10
9	6.55	C-7, 10, 11
10	6.14	C-8, 9, 12
11	5.55	C-9, 10, 13
12	2.48, 2.32	C-10, 11, 13, 14
13	3.85	C-11, 14
14	1.60	C-15
15	4.30	C-13, 14, 16, 17
16	5.67	C-14, 17, 18
17	6.17	C-15, 16, 18, 19
18	6.05	C-16, 17, 19, 20
19	5.64	C-17, 20, 21
20	2.19, 2.11	C-18, 19, 21, 22
21	1.53	C-19, 20, 22 ,23
22	1.64, 1.58	C-21, 23, 24
23	5.00	C-1, 21
24	1.26	C-22, 23

studies [2, 21], and as such the ^{1}H- and ^{13}C-NMR data of the isolated compound agreed well with those of macrolactin A. Furthermore, the optical rotation value $[\alpha]_D^{25}$=- 10.36 (*c* 0.13, MeOH) for the compound isolated in the present study was similar to those [- 9.6° (*c* 1.86, MeOH), - 20° (*c* 0.1, MeOH)] previously reported for macrolactin A [9, 12]. Consequently, it was determined that the absolute configuration of the active compound was identical to that of macrolactin A (Fig. 3).

The antimicrobial activities of macrolactin A against *S. aureus*, *E. coli*, and *B. cinerea* were tested using the paper disc agar diffusion method. As a result, the growth of the

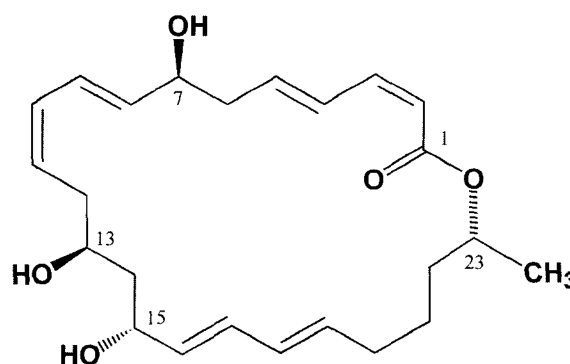


Fig. 3. Structure of macrolactin A.

Table 4. Antimicrobial activity of macrolactin A isolated from the culture broth of *Bacillus amyloliquefaciens* CHO104.

Microorganisms	Inhibition (Clear zone, mm)	
	Macrolactin A[a]	Benzoic acid[b]
Staphylococcus aureus KCTC 1928	12	10
Escherichia coli KCTC 2593	11	10
Botrytis cinerea	10	9

[a]10 μg of macrolactin A isolated from the culture broth of *Bacillus amyloliquefaciens* CHO104/6 mm paper disc.
[b]200 μg of benzoic acid/6 mm paper disc.

bacteria and fungi was inhibited even at a concentration of 10 μg (Table 4). Interestingly, the concentration of macrolactin A was only one-twentieth of that of the benzoic acid used as a control compound. To determine the inhibitory concentration of macrolactin A, dose-response curves were prepared for *S. aureus* and *E. coli*. The growth inhibition was expressed as the concentration of the antimicrobial substance required to inhibit 50% growth. *S. aureus* and *E. coli* were both found to be very sensitive to macrolactin A in the liquid culture at IC_{50} values lower than 50 μM (data not shown).

Kim *et al.* [12] previously isolated macrolactin A from *Actinomadura* sp. as a substance with neuronal cell protection activity, in which case the strain was cultured at 28°C for 6 days (yield, 1.6 mg/l). In addition, Gustafson *et al.* [9] isolated macrolactin A as an antimicrobial and antiviral compound from the culture broth of a deep-sea marine bacterium. According to their report, the deep-sea marine bacterium was cultured at 20°C for 5-15 days (yield, 0.4-0.5 mg/l) to produce macrolactin A. However, *B. amyloliquefaciens* CHO104 was incubated at 30°C for 2 days (yield, 0.4 mg/l) to obtain macrolactin A. Accordingly, the strain used for the present study would appear to be superior to the marine bacterium and *Actinomadura* sp. in terms of its production of macrolactin A, although the method of Kim *et al.* was more effective as regards the yield.

As such, the present work is apparently the first report on the isolation and characterization of macrolactin A as an antimicrobial substance from a culture broth of *B. amyloliquefaciens*. Therefore, further investigation of the biological effects and application of macrolactin A from *B. amyloliquefaciens* CHO104 may prove to be valuable.

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