VOL. 54 NO. 11, NOV. 2001 THE JOURNAL OF ANTIBIOTICS pp. 917 — 925

New Pyrrolizidinone Antibiotics CJ-16,264 and CJ-16,367

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(Received for publication June 27, 2001)

Two new antibiotics, CJ-16,264 (**I**) and CJ-16,367 (**II**), were isolated from the fermentation broth of an unidentified fungus CL39457. These antibiotics have a pyrrolizidinone skeleton, first discovered in fungi. Compounds **I** and **II** inhibit the growth of Gram-positive multi-drug resistant bacteria and some Gram-negative strains such as *Moraxella catarrhalis* and *Escherichia coli* with altered permeability (*imp*). Comparison of an antibacterial profile between the two compounds suggested that the gamma-lactone portion of **I** is important for the activity.

The increasing incidence of nosocomial infections caused by multi-drug resistant (MDR) bacteria such as methicillin-resistant strains of *Staphylococcus aureus* has been a serious problem in the clinical area[1], although the mechanisms of antibiotic resistance have been elucidated[2,3]. Resistance to antibiotics may emerge within a few years after introduction of an antibiotic as a therapeutic agent. Recently, the situation became more critical by emerging enterococci resist to vancomycin, which has been used for the treatment of methicillin-resistant strains of *Staphylococcus aureus* (MRSA)[4]. Accordingly, there is need for new, safe and effective antibiotics against MDR (macrolides, quinolones, methicillin, tetracycline, gentamicin, *etc.*) clinical strains.

In the course of a screening for new antibiotics from microbial extracts, an unidentified fungus CL39457 was found to produce novel pyrrolizidinone compounds that show moderate antibacterial activity against Gram-positive MDR bacteria[5]. In this paper, we report the taxonomy of the producing organism and the fermentation, isolation, structure elucidation and biological activities of the pyrrolizidinone compounds.

Results

Taxonomy

The cultural characteristics of strain CL39457 are listed in Table 1. The strain is characterized by the slow growth; the buff, brown, gray to black colony and colony reverse; and the ochraceous buff to ochraceous orange soluble pigment on some media. It grew 20 and 37°C, but failed to grow between 45 and 50°C. Despite the attempt of an inducing sporulation by exposing the culture to black light for up to 9 weeks, it did not produce spores of either sexual or asexual types except that the sclerotia were produced on cornmeal agar and V-8 juice agar. The sclerotia closely resembled pycnidia typical of the Coelomycetes or Ascomata typical of the Ascomycetes. It is therefore considered as a strain of the sterile fungus class, Agonomycetes.

Isolation

The solid culture (2 liters) was filtered after the addition

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Table 1. Cultural characteristics of strain CL39457.

	Colony Diameter	Colony Texture	Colony Surface	Colony Reverse	Soluble Pigment
Malt Extract Agar	1.5 cm	Slightly raised, smooth, fluffy, lowly floccose to slightly funiculose	Avellaneous, vinaceous-fawn, army brown, natal brown, bone brown (XL) to fuscous black (XLVI)	Same	None
Cornmeal Agar	2.1 cm	Thin, smooth, submerged to fluffy	Pale ochraceous-salmon, pale ochraceous-buff to light ochraceous-buff (XV)	Pale ochraceous-buff to light ochraceous-buff (XV)	Cream color (XVI)
Czapek Dextrose Agar	2.5 cm	Thin, smooth, submerged to fluffy	Fawn color, army brown to natal brown (XL)	Same	None
Potato Dextrose Agar	2.5 cm	Slightly raised, smooth to slightly wrinkled, lowly floccose to slightly funiculose	Mikado brown, verona brown, warm sepia (XXIX), light olive-gray to olive-gray (LI)	Mikado brown, verona brown (XXIX), natal brown to bone brown (XL)	Pale ochraceous-buff to ochraceous-orange (XV)
V-8 Juice Agar	3.1 cm	Moderately raised, smooth to radiately wrinkled, floccose to funiculose	Pale mouse gray, light mouse gray, mouse gray to deep mouse gray (LI)	Army brown (XL), mouse gray to deep mouse gray (LI)	Ochraceous-buff to ochraceous orange
Phytone Yeast Extract Agar	4.8 cm	Raised, radiately wrinkled densely floccose	Pale ochraceous-buff (XL) but army brown to natal brown (XL) toward edge	Hair brown, grayish olive, deep grayish olive to dark grayish-olive (XLVI)	None

of the equal volume of EtOH. The filtrate was concentrated to an aqueous solution (500 ml), and extracted 3 times with EtOAc (500 ml each). The combined organic layers were evaporated to dryness, and then reconstituted with 40% aqueous MeOH. The solution was loaded onto two ODS columns (YMC-AM 120-S50, 26×50 mm each, YMC Co. Ltd.), and eluted with MeOH - 0.05% TFA in H_2O (80 : 20). The eluates were combined, concentrated, and then applied to a Sephadex LH-20 column (160 ml, Amersham Pharmacia Biotech) with MeOH. Fractions showing antibacterial activity against *S. aureus* 01A1105 were combined and evaporated to dryness. The active residue (543 mg) was purified by preparative HPLC on an ODS column (Fluofix IEW 225, 20×250 mm, NEOS) with MeOH - 0.05% TFA in H_2O (60 : 40) at a flow rate of 10 ml/minute to yield two semi-pure solids of **I** and **II**. Each solid was further purified by preparative HPLC using an ODS column (J'sphere ODS-H80, 20×250 mm, YMC Co. Ltd.) with MeOH - 0.05% TFA in H_2O (75 : 25) at a flow rate of 10 ml/minute to give **I** (106.2 mg) and **II** (13.9 mg).

Physico-chemical Properties

The physico-chemical properties are summarized in Table 2. Compounds **I** and **II** were obtained as white amorphous powders. The molecular formulae of **I** and **II** were determined to be $C_{23}H_{31}NO_5$ and $C_{24}H_{33}NO_5$ by positive ion HRFAB-MS, respectively. The IR spectra suggested the presence of the carbonyl and the hydroxyl group.

Structural Elucidation of CJ-16,264 (**I**)

The ^{13}C NMR spectrum (in C_6D_6) showed 23 carbon signals (Table 3), classified into 4 -CH_3, 5 -CH_2–, 8 -CH– and 6 quaternary carbons through analysis of the DEPT spectra, which showed a good agreement with the molecular formula. Detailed NMR spectral analysis of **I** was aided by COLOC and selective INEPT spectra. The partial structure (1) of **I** was determined by analyzing the COLOC observed from methyl groups and the ^1H-^1H COSY (Fig. 2). The cross peaks on COLOC from 2-CH_3 (δ_H 0.96) to C-1 (δ_C 63.6), C-2 (δ_C 29.7) and C-3 (δ_C 133.1); from 3-CH_3 (δ_H 1.67) to C-2, C-3 and C-4 (δ_C 131.4); from 4a-CH_3 (δ_H 0.91) to C-4, C-4a (δ_C 37.0), C-8a (δ_C 39.0) and C-5 (δ_C 48.9); from 6-CH_3 (δ_H 0.87) to C-5, C-6 (δ_C 28.8) and C-7 (δ_C 34.3); and furthermore from a methine proton signal (δ_H 2.20, H-1) to C-4a and C-8 suggested the presence of tetramethyl-octahydro-naphthalene substructure. The spin systems observed on ^1H-^1H COSY explained the connectivities (2-CH_3–2-H–1-H–8a-H–8-$H_{a,b}$–7-$H_{a,b}$), which could not be proved by analysis of

Table 2. Physico-chemical properties of CJ-16,264 (**I**) and CJ-16,367 (**II**).

	CJ-16,264 (**I**)	CJ-16,367 (**II**)
Appearance	White powder	White powder
Molecular formula	$C_{23}H_{31}NO_5$	$C_{24}H_{33}NO_5$
Molecular weight	401	415
HRFAB-MS (*m/z*)		
found	402.2310 $[M+H]^+$	416.2429 $[M+H]^+$
calcd.	402.2282	416.2429
$[\alpha]_D^{24}$	+27.3 (*c* 0.11, MeOH)	+17.0 (*c* 0.11, MeOH)
$UV\lambda_{max}^{MeOH}$ nm (ε)	208 (12,000), 225 (6,200, sh)	207 (24,000), 226 (10,000,sh)
$UV\lambda_{max}^{MeOH+HCl}$ nm (ε)	205 (7,100), 231 (4,100), 260 (4,200)	205 (21,000), 256 (4,700)
$UV\lambda_{max}^{MeOH+NaOH}$ nm (ε)	205 (18,000)	205 (33,000), 288 (7,800)
$IR\nu_{max}^{KBr}$ cm^{-1}	3435, 1789, 1715, 1684, 1453	3455, 1714, 1454

Table 3. Chemical shifts of CJ-16,264 (**I**) and CJ-16,367 (**II**) in C_6D_6.

	CJ-16,264 (**I**)		CJ-16,367 (**II**)	
Position	δ_C	δ_H	δ_C	δ_H
1	63.6	2.20 (1H, m)	59.2	3.47 (1H, m)
2	29.7	2.97 (1H, m)	29.7	3.40 (1H, m)
3	133.1		134.6	
4	131.4	5.08 (1H, s)	130.2	5.12 (1H, s)
4a	37.0		37.5	
5	48.9	1.42 (1H, m), 1.06 (1H, m)	50.5	1.42 (1H, m), 0.95 (1H, m)
6	28.8	1.42 (1H, m)	29.1	1.44 (1H, m)
7	34.3	1.60 (1H, m), 1.05 (1H, m)	35.5	1.65 (1H, m), 1.00 (1H, m)
8	31.9	1.42 (2H, m)	31.4	2.06 (1H, m), 1.65 (1H, m)
8a	39.0	2.52 (1H, m)	44.0	2.09 (1H, m)
2-CH_3	21.0	0.96 (3H, d, *J*=7.3 Hz)	21.3	1.15 (3H, d, *J*= 7.0 Hz)
3-CH_3	21.6	1.67 (3H, s)	22.1	1.71 (3H, s)
4a-CH_3	29.1	0.91 (3H, s)	30.4	1.05 (3H, s)
6-CH_3	22.3	0.87 (3H, d, *J*= 6.2 Hz)	22.6	0.86 (3H, d, *J*= 6.2 Hz)
1'			51.2	2.26 (1H, dd, *J*= 8.5, 10 Hz)
2'	174.2		29.2	2.68 (1H, m), 1.83 (1H, m)
2'a	47.6	2.75 (1H, d, *J*= 9 Hz)		
3'	29.7	2.10 (1H, m), 2.00 (1H, m)	41.2	3.35 (1H, m), 2.95 (1H, m)
4'	41.8	3.56 (1H, m), 2.76 (1H, m)		
5'			169.6	
6'	167.8		141.0	
7'	63.7	4.09 (1H, s)	151.8	7.76 (1H, s)
7'a	81.2	4.23 (1H, s)	98.3	
7'b	100.9			
8'	209.8		199.9	
1'-COOH			172.7	
7'a-O-CH3			50.8	2.86 (3H, s)

Fig. 1. Structures of CJ-16,264 (**I**) and CJ-16,367 (**II**).

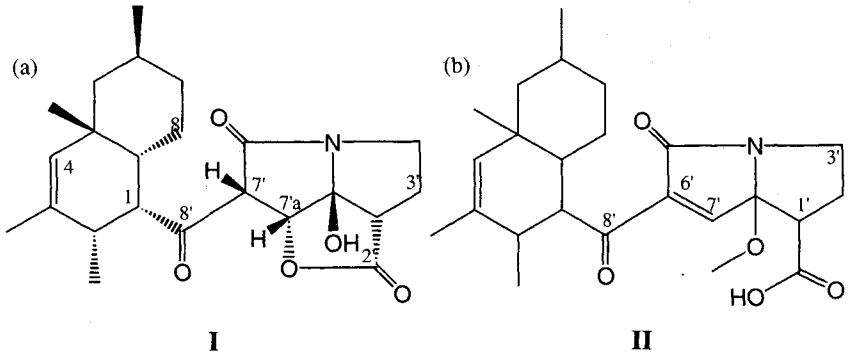
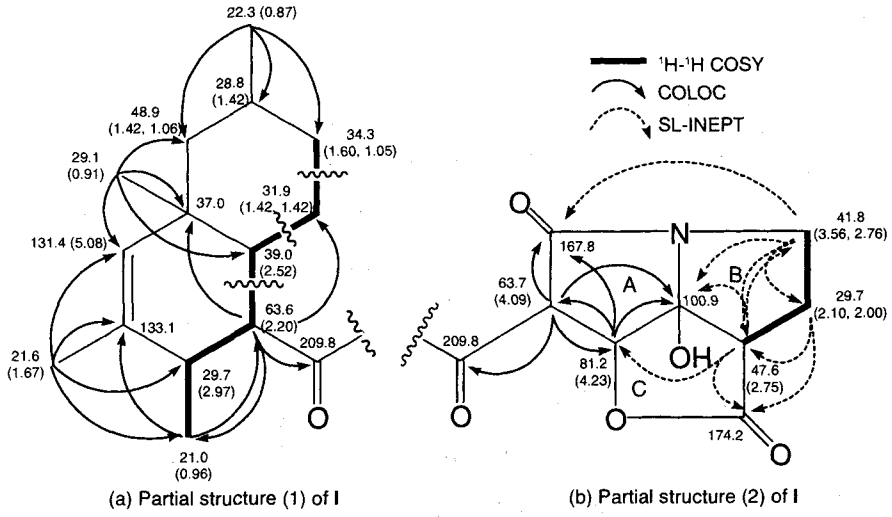


Fig. 2. Summary of COLOC, selective INEPT and ^{1}H-^{1}H COSY for **I**.



Values and those in parentheses show ^{13}C- and ^{1}H-chemical shifts in ppm, respectively. Arrows mean long-range ^{13}C-^{1}H couplings.

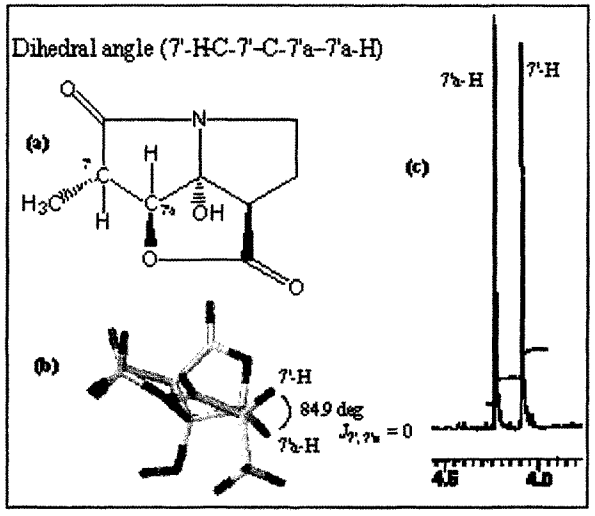
COLOC. Taken together, the partial structure (1) of **I** was thus determined as shown in Fig. 2 (a).

The observation of a weak NOE and a spin coupling in ^{1}H-^{1}H COSY between 7′-H (δ_H 4.09, s) and 7′a-H (δ_H 4.23, s) gave structural information that they are not separated, but vicinally coupled each other. The cross peaks observed on COLOC from 7′-H to amide carbonyl group C-6′ (δ_C 167.8), C-7′b (δ_C 100.9) and acylated oximethine signal C-7′a (δ_C 81.2); from 7′a-H to C-6′, C-7′b and C-7′ (δ_C 63.7) led to establish the 5-membered ring A of partial structure (2) of **I**. The analysis of ^{1}H-^{1}H COSY provided the connectivities of spin systems (2′a-H–3′-$H_{a,b}$–4′-$H_{a,b}$), which were irradiated to give selective INEPT spectra. The long-range couplings in the selective INEPT spectra from a 4′-H (δ_H 3.56) to C-2′a (δ_C 47.6), C-3′ (δ_C 29.7), C-7′b and C-6′; from a 3′-H (δ_H 2.00) to C-2′a and a carbonyl group C-2′ (δ_C 174.2); from 2′a-H (δ_H 2.75) to C-2′, C-7′a, C-7′b and C-4′ created the sub-structure ring B, fused with ring A in partial structure (2) in **I**. The formation of ring C at C-7′a in the partial structure (2) in **I** was indicated by the down-field shift of 7′a-H at 4.23 ppm. Thus, the partial structure (2) of **I** was determined to be 7′b-hydroxy-hexahydro-furo[2′,3′,4′-*gh*]pyrrolizine-2′,6′-dione, as shown in Fig. 2 (b). Molecular mechanic calculation (Gasteiger-

Hückel charge, Tropos force field in SYBYL) on the model compound shown in Fig. 3 (a) and (b) indicated that the dihedral angle (7′-H–C-7′–C-7′a–7′a-H) was 84.9°, which showed a good agreement with the observed coupling constants of 7′-H and 7′a-H (J≒0) as shown in Fig. 3 (c). The rare chemical shift of C-7′b (δ_C 100.9) was proved by comparison of those in known compounds such as pyrrolam C[6] (δ_C 98.2) and mitomycin C[7] (δ_C 111.0), as shown in Fig. 4.

The C-1 of the partial structure **I** was found to be connected to the C-7′ of the partial structure **II** by the remaining functional group, a ketone C-8′ (δ_C 209.8), since 1-H and 7′-H were long-range coupled to C-8′, and the structure of **I** was thus proposed as shown in Fig. 1 (a).

Fig. 3. Coupling constants at 7′-H and 7′a-H in **I**.



(a) Model compound used in this experiment; 7b-Hydroxy-7-methyl-hexahydro-furo[2,3,4-*gh*]pyrrolizine-2,6-dione.

(b) Resulting structure by the computational calculation.

(c) Part of ^{1}H NMR spectrum for 7′-H and 7′a-H.

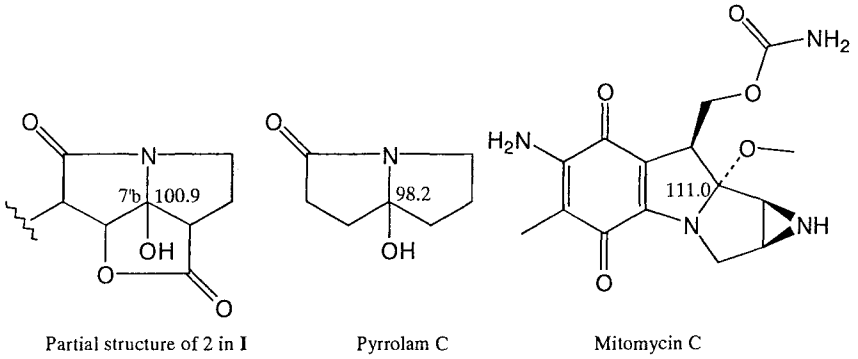
Structural Elucidation of CJ-16,367 (**II**)

The HRFAB-MS of **II** gave a parent ion peak at *m/z* 416.2429 [(M+H)$^+$; calcd. for 416.2429], indicating the gain of weight equivalent to a –CH_2– from **I**. ^{1}H and ^{13}C NMR spectra showed that signal's changes for the 7′ and 7′a positions of partial structure (2) in **I**, occurred together with addition of methoxy group (δ_C 50.8, δ_H 2.86). A singlet methine proton signal at 7.76 ppm (7′-H) was long-range coupled to C-6′ (δ_C 141.0), C-7′a (δ_C 98.3) and C-8′ (δ_C 199.9) in COLOC, which suggest the formation of double bond between 6′ and 7′, resulting in the opening of ring C in **I**. The position of the double bond is supported by the up-field chemical shift of a ketone at C-8′ (δ_C 209.8→ δ_C 199.9). The methoxy group (δ_C 50.8, δ_H 2.86) was assigned to be 7′a-O–CH_3 for being observed long-range coupling to C-7′a. The ring B was found to be unchanged, based on analysis of its 1D and 2D spectra, which led this partial structure to be 7′a-methoxy-5′-oxo-2′,3′,5′,7′a-tetrahydro-1′*H*-pyrrolizine-1-carboxylic acid. The partial structure of (1) in **II** was found to be identical with that of **I** through analysis of COLOC, selective INEPT and ^{1}H-^{1}H COSY, whose results are summarized in Fig. 5. The structure of **II** was thus determined as shown in Fig. 1 (b).

Stereochemistry

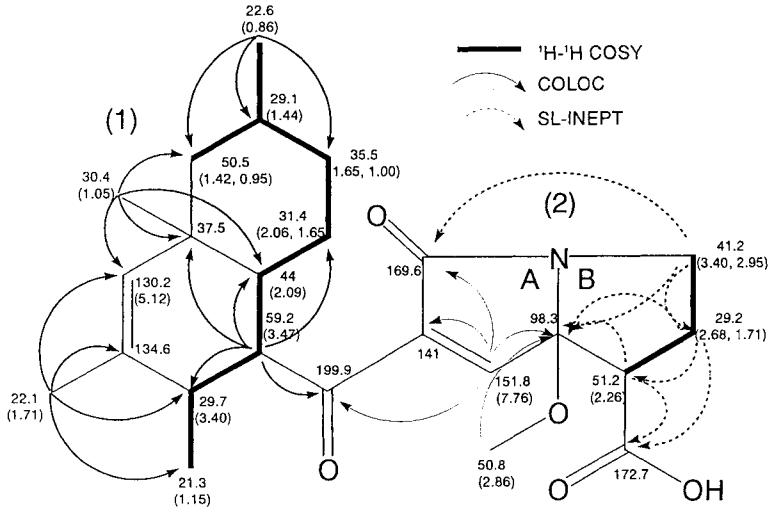
The relative configuration of **I** was determined by 1D-differential NOE spectra and a phase sensitive NOESY. As shown in Fig. 6a, NOEs were observed from 1-H to 2-H, methyl 2-H_3, 8-H_{eq} and 8a-H, from 2-H to methyl 4a-H_3,

Fig. 4. Comparison of chemical shift at C-7′b with known compounds.



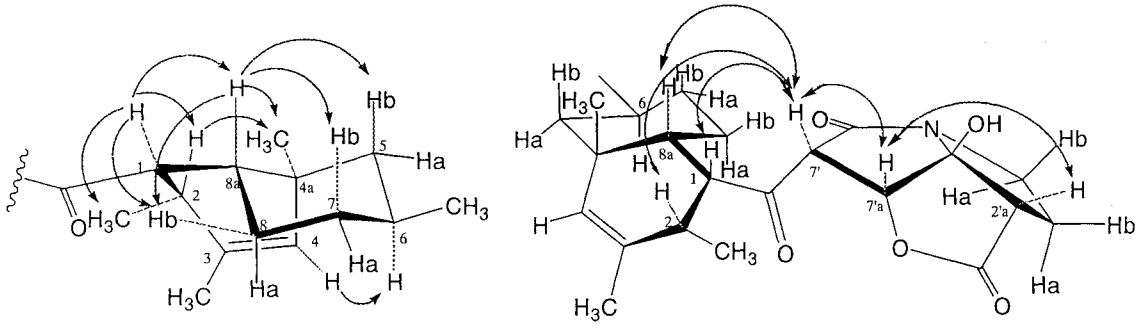
Partial structure of 2 in **I** Pyrrolam C Mitomycin C

Fig. 5. Summary of COLOC, selective INEPT and ^{1}H-^{1}H COSY for **II**.



Values and those in parentheses show ^{13}C- and ^{1}H-chemical shifts in ppm, respectively. Arrows mean long-range ^{13}C-^{1}H couplings.

Fig. 6. Net work of NOEs in **I**.



(a) NOEs in partial structure (1) of **I**.
Arrows mean NOEs observed in the differential NOE spectra.

(b) NOEs between partial structures (1) and (2) in **I**.
Arrows mean NOEs observed on NOESY.

from 4-H to 6-H, and also from 8a-H to methyl 4a-H_3, 5-H_{ax}, 7-H_{ax} and 8-H_{eq}, suggesting the configuration of partial structure (1) of **I**. Cross-peaks on the phase sensitive NOESY explained not only configuration of the partial structure (2) but also the structural correlation between partial structure (1) and (2) of **I** (Fig. 6b). Thus, the relative-stereochemistry of **I** was proposed as shown in Fig. 1.

Biological Activities

As shown in Table 4, **I** showed antibacterial activities (MIC from 0.39~12.5 μg/ml) against Gram-positive MDR strains with a broad spectrum including some Gram-negative ones, *Moraxella catarrhalis* 87A1055 and *E. coli* 51A1051 *imp* (increased membrane permeability) mutant. It also showed weak activity against *Haemophilus influenzae* strains. Compound **II** showed broad antibacterial activities against Gram-positive strains, *M. catarrhalis*

Table 4. Antibacterial activities of CJ-16,264 (**I**) and CJ-16,367 (**II**).

Microorganism	MIC (μg/ml)				
	CJ-16,264	CJ-16,367	Erythromycin	Azithromycin	Vancomycin
Staphylococcus aureus 01A1095	1.56	12.5	>100	>100	0.78
S. aureus 01A1105	1.56	12.5	>100	>100	1.56
S. aureus 01A1120	0.78	6.25	>100	>100	0.39
S. haemolyticus 01E1006	3.12	12.5	100	>100	0.78
Streptococcus agalactiae 02B1023	3.12	12.5	>100	>100	0.39
S. pyogenes 02C1068	3.12	25	>100	>100	0.39
S. pyogenes 02C1079	3.12	12.5	>100	>100	0.2
S. pneumoniae 02J1046	12.5	50	>100	>100	0.39
S. pneumoniae 02J1095	12.5	50	>100	>100	0.31
Enterococcus faecalis 03A1069	3.12	25	>100	>100	50
Haemophilus influenzae 54A0085	50.0	>100	3.12	$\leqq$0.2	Not tested
H. influenzae 54A0131	25.0	>100	3.12	$\leqq$0.2	>100
Moraxella catarrhalis 87A1055	0.39	1.56	0.78	$\leqq$0.2	50
Escherichia coli 51A0266	>100	>100	100	1.56	>100
E. coli 51A1051	6.25	50	$\leqq$0.2	$\leqq$0.2	>100
E. coli 51A1073	>100	>100	100	3.12	>100
E. coli 51A1074	>100	>100	100	1.56	>100
E. coli 51A1075	>100	>100	3.12	0.39	>100

87A1055 and *E. coli* 51A1051 *imp*, but the activities were rather weak. Compounds **I** and **II** showed cytotoxicity against HeLa cells with IC_{90} values of 8.0 and 6.8 μg/ml, respectively.

Discussion

Many pyrrolizidines and pyrrolizidinones from plants and insects have been reported, whereas microbial origin has been reported only from a *Streptomyces olivaceus*[6]. Compounds **I** and **II** are the first pyrrolizidinones isolated from fungi. Moreover, the pyrrolizidinone with gamma-lactone has never been reported even in the synthetic compounds. Therefore, **I** is the first pyrrolizidinone compound containing gamma-lactone[5]. Similar compounds, USC1025A and USC1025B were reported to be novel antibiotics from *Acremonium* spp.[8].

While **I** showed moderate antibacterial activities against *Moraxella catarrhalis* 87A1055 and *E. coli imp* 51A1051, it had no activity against wild type strains of *E. coli*, an *acrAB* mutant (51A1074), and the wild type revertant of the *acrAB* strain (51A1075) (MIC>100 μg/ml). This suggests that **I** is not a substrate for the *acrAB* pump intrinsic to *E. coli*; however, the compound is likely to be excluded from its presumed intracellular target since **I** showed inhibitory activities against the *imp* strain as well as other Gram-negative bacteria having more permeable outer membranes. The presence of the gamma-lactone seemed to be critical to show stronger anti-bacterial activities in that **I** was more potent than **II**. It is known that dehydropyrrolizine alkaloids converted from pyrrolizidine alkaloids in mammals show acute toxicity, *e.g.*, hepatotoxic, mutagenic and carcinogenic activities[9]. In fact, **I** and **II** showed cytotoxicity against HeLa cells and acute toxicity in mice. Nevertheless, since **I** exhibits moderate antibacterial activity against MDR microorganisms, it could be worthwhile examining its mode of action.

Experimental

General

Spectral and physico-chemical data were obtained on the following instruments: IR, Shimadzu IR-470 spectrometer; UV, JASCO Ubest-30; Optical rotations, JASCO DIP-370 with a 5 cm cell; NMR, JEOL JNM-GX270 equipped with an LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; and FAB-MS, JEOL JMS-700. All NMR spectra were measured in benzene-d_6 and peak positions are expressed in parts per million (ppm) based on the reference of benzene peak at 7.2 ppm for ^{1}H NMR and 128.0 ppm for ^{13}C NMR. The peak shapes are denoted as follows: s (singlet), d (doublet), t (triplet), q (quartet), m

(multiplet) and br (broad). FAB-MS were measured using glycerol-PEG400 mixture matrix. Molecular modeling was performed by using SYBYL ver. 6.4 (Tropos Inc., St. Louis, MO).

Producing Microorganism

The strain CL39457 was isolated from a soil sample collected in Philippine. This strain was sub-cultured on plates of identification media after 3-week incubation on potato dextrose agar plates, and followed by the incubation at 25°C in the dark for a week. These plates were then cultured under black light and the dark for 12 hours each alternately for eight weeks. The plates were observed at every two weeks for studying its cultural characteristics and the temperature effects. The colors were determined by color chips from RIDGWAY[10]. Identification media used were Czapek-sucrose agar, cornmeal agar[11], malt extract agar[12], Phytone yeast extract agar (BBL), potato dextrose agar (peeled potato 100 g, dextrose 10 g, agar 20 g, tap water 1 liter), and V-8 juice agar (ATCC Medium #343). Malt extract agar was used for temperature study.

Fermentation

The unidentified strain CL39457 was maintained on a potato dextrose agar slant (Difco). A vegetative cell suspension from the slant culture was inoculated into two 500-ml flasks containing 100 ml of a seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). The flasks were shaken at 26°C for 4 days on a rotary shaker with 7-cm throw at 210 rpm to obtain seed cultures.

Every 5 ml aliquot of the seed cultures was inoculated into twenty 500-ml flasks containing 100 ml of a production medium (glucose 1%, glycerol 6.6%, NZ Amine Type A 0.5%, ammonium sulfate 0.2%, defatted soybean meal 0.5%, tomato paste 0.5% and sodium citrate 0.2%, and adjusted to pH 7.0) and 30 g buckwheat. Static fermentation was carried out at 26°C for 18 days.

HPLC Analysis

Analytical HPLC of **I** and **II** were performed by using an ODS column (J'sphere ODS-H80, 4.6×150 mm, YMC Co. Ltd.) and eluted with MeOH - 0.05% TFA in H_2O (40 : 1) at a flow rate of 0.9 ml/minute. The retention times of **I** and **II** were 6.1 and 7.6 minutes, respectively.

Test Strains

S. aureus 01A1105 (cef^r, $gent^r$, $meth^r$, $MLS_B{}^r$, pen^r, tet^r, cip^r and van^s, where r and s meant a resistant and sensitive strain, respectively) and *S. aureus* 01A1095 (amp^r, cef^r, $gent^r$, $imipenem^s$, $MLS_B{}^r$, tet^r and van^s) are MDR clinical strains. *S. aureus* 01A1120 exhibits a constitutive MLS_B-resistant phenotype due to the presence of a plasmid pE194 containing *ermC*. *Staphylococcus haemolyticus* 01E1006 is resistant to 14- and 15-membered macrolides, streptogramin B and trm. *Streptococcus pyogenes* 02C1068 is $MLS_B{}^r$, kan^r, and str^r and 02C1079 is $MLS_B{}^r$. *Streptococcus agalactiae* 02B1023 and *Streptococcus pneumoniae* (serotype 6) 02J1046 are $MLS_B{}^r$ and tet^r. 02J1095 (serotype 3) is $MLS_B{}^r$, pen^r, tet^r and trm^r. *Enterococcus faecalis* 03A1069 is also an MDR clinical strain [cef^r, ery^r, $gent^r$, chl^r, kan^r, tet^s and van^r], confirmed to have *ermB* gene. *Haemophilus influenzae* 54A0085 and 54A0131 are both type B and trm^r isolates; the former is pen-sensitive whereas the latter is pen-resistant. *Moraxella catarrhalis* 87A1055 is pen-resistant and shows intermediate susceptibility to erythromycin. *Escherichia coli* 51A0266 is a generally-susceptible strain. *E. coli* 51A1051 is a sensitive, *imp* (strain with increased outer membrane permeability) mutant (BAS901) from *E. coli* K12[13]. *E. coli* 51A1073 is an *E. coli* K-12 strain (MC4100). *E. coli* 51A1074 is an *acrAB* mutant strain of 51A1073. *E. coli* 51A1075 is an isogenic revertant of 51A1074, wild type for *acrAB* (WZM120)[14].

Preparation of Inoculum and MIC Determinations

Preparation of the inoculum, antibacterial assay and microtiter-based MIC determinations were made according to the National Committee for Clinical Laboratory Standards[15].

Cytotoxicity

The cytotoxicity test was performed according to the method of our previous paper[16]. Briefly, the HeLa cell line was cultured with Eagle's MEM containing 10% fetal bovine serum, 100 units/ml of pen and 100 μg/ml of streptomycin. The cell suspension (5.5×10^4 cells/ml, 180 μl) was added into each well of a 96-well microtiter plate, and incubated with 20 μl of test compound at 37°C with 5% CO_2. After 72-hour incubation, the medium was discarded, and the cells were washed with PBS, and then stained with 0.4% crystal violet solution (50 μl). The plate was left for 30 minutes at room temperature. After dye

Amp, ampicillin; cef, cefotaxime; cip, ciprofloxacin; chl, chloramphenicol; ery, erythromycin; gent, gentamicin; kan, kanamycin; meth, methicillin; MLS_B, Macrolide, lincosamide, streptogramin B; pen, penicillin; str, streptomycin; tet, tetracycline; trm, trimethoprim; van, vancomycin

removal, the plate was washed with tap water and air-dried. The pigment was eluted with 50% methanol and quantified by measuring absorbance at 490 nm. The cytotoxicity was expressed by the percentage of inhibition of HeLa proliferation,

$$\text{Inhibition (\%)} = 100 \times [A_{490}\text{ (no drug control)} - A_{490}\text{ (sample)} / A_{490}\text{ (no drug control)} - A_{490}\text{ (no growth control)}],$$

where A_{490} was the absorbance at 490 nm.

Acknowledgments

We would like to thank Dr. ARTHUR E. GIRARD and his colleagues for testing the compounds *in vivo*, *etc*. Thanks are also due to Dr. M. UCHIYAMA for his molecular mechanic calculation.

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