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A Novel Antibiotic CJ-17,572 from a Fungus, *Pezicula* sp.

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A new antibiotic, CJ-17,572 (**I**) was isolated from the fermentation broth of a fungus *Pezicula* sp. CL11877. The structure of **I** was determined to be a new equisetin derivative by spectroscopic analyses. The compound inhibits the growth of multi-drug resistant *Staphylococcus aureus* and *Enterococcus faecalis* with IC_{50}s of 10 and 20 μg/ml, respectively.

Worldwide, many strains of *Staphylococcus aureus* have already been reported as resistants against various standard antibiotics except vancomycin. And the increasing incidence of hospital-acquired infections caused by multi-drug resistant (MDR) bacteria such as methicillin-resistant *S. aureus* has been a serious problem in the clinical area[1]. The recent emergence of vancomycin-resistant enterococci (VRE) and vancomycin-intermediate resistant *S. aureus* (VISA)[2~4] is raising serious public health concerns. Therefore, there is need for new, safe and effective antibiotics against MDR clinical strains.

In the course of screening for new antibiotics from microbial extracts, a fungal strain culture CL11877 was found to produce a new equisetin derivative, CJ-17,572 (**I**), which showed antibacterial activity against Gram-positive MDR bacteria. In this paper, we report the fermentation, isolation, structure elucidation and biological activity of **I**.

Results and Discussion

Isolation

The fermentation broth (900 ml) was filtered after the addition of the same volume of EtOH. The filtrate was concentrated to an aqueous solution (150 ml) and then adjusted to 50% aqueous MeOH solution. The solution was applied onto an ODS column (YMC-Pack ODS-AM 120-S50, 26×50 mm, YMC Co. Ltd.) and eluted with MeOH after washing with 50, 60 and 80% aqueous MeOH (100 ml each). The MeOH fraction was concentrated and resolved in the lower layer (300 ml) of *n*-hexane - MeOH - H_2O (10 : 9 : 1, v/v), and then partitioned with the upper layer (300 ml×3 times). The lower layer was evaporated to dryness (920 mg) and a part (200 mg) of them was applied to preparative HPLC on an ODS column (YMC-pack ODS AM-343 column, 20×250 mm+20×50 mm, YMC Co. Ltd.) with liner gradient system of MeOH - 0.05% TFA in H_2O (50 : 50 to 80 : 20 for 90 minutes) at a flow rate of 10 ml/minute. The eluted peak at 76 minutes was concentrated to afford **I** (41.7 mg) as white powder.

Structure Elucidation

The physico-chemical properties of **I** are summarized in Table 1. Compound **I** was obtained as white amorphous powder. The molecular formula of **I** was determined to be

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Abbreviation: cef: cefotaxime, cip: ciprofloxacin, chl: chloramphenicol, ery: erythromycin, *E. coli*: *Escherichia coli*, gent: gentamicin, kan: kanamycin, meth: methicillin, MLS_B: Macrolide, lincosamide, streptogramin B, pen: penicillin, str: streptomycin, tet: tetracycline, van: vancomycin.

Table 1. Physico-chemical properties of CJ-17,572 (**I**).

Appearance	White powder
Molecular weight	361
Molecular formula	$C_{21}H_{31}NO_4$
HRFAB-MS (*m/z*)	found 362.2341 $[M+H]^+$
	calcd. 362.2333
$[\alpha]_D^{25}$	+110.5 (*c* 0.42, MeOH)
UV λ_{max}^{MeOH} nm (ε)	255 (12,700), 292 (15,700)
UV $\lambda_{max}^{MeOH+HCl}$ nm (ε)	236 (11,500), 293 (17,000)
UV $\lambda_{max}^{MeOH+NaOH}$ nm (ε)	255 (14,000), 292 (15,700)
IR ν_{max}^{KBr} cm^{-1}	3445, 1649, 1569, 1470

Fig. 1. Structure of CJ-17,572 (**I**).

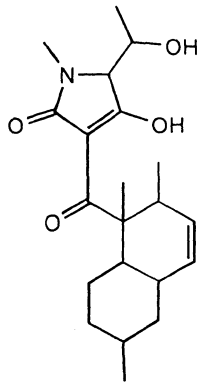
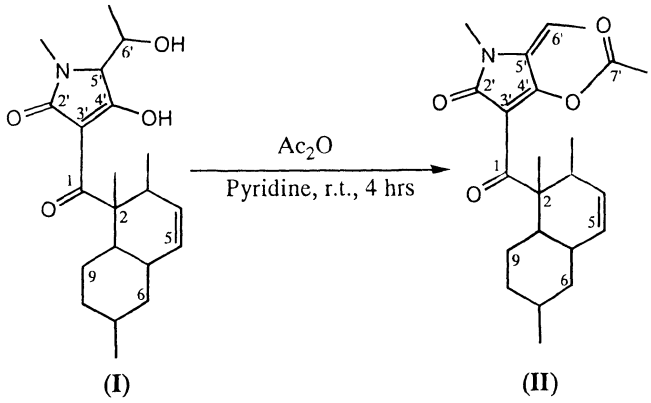


Fig. 2. Acetylation of CJ-17,572 (**I**) and its product.



$C_{21}H_{31}NO_4$ on the basis of HRFAB-MS [*m/z*, found 362.2341 $(M+H)^+$, calcd. 362.2333]. The organic soluble material exhibited intense electronic transitions in MeOH at λ_{max} 255 nm (ε 12,700) and λ_{max} 292 (ε 15,700). The ^{1}H-NMR spectrum, independent of solvent and temperature, gave abnormally broad signals, making it impossible to define individual resonances. The ^{13}C-NMR spectrum did not also show resolved 21 peaks appropriate for molecular formula, some of them were broadened and/or not observed. The presence of signals, δ_C 200.3 (s), δ_C 192.3 (s), δ_C 100.6 (s) in ^{13}C-NMR spectrum and the change of ultraviolet spectrum in an acidic condition; λ_{max} 236 nm (ε 11,500) and λ_{max} 292 (ε 17,000) suggested a substructure, 1,3-diketone, which may contribute to broaden some signals by its tautomeric features. In order to obtain the resolved NMR signal to help determine the structure, **I** was acetylated. The molecular weight of an obtained acetyl derivative (5.4 mg, **II**) was determined to be 385 (*m/z*, EI-MS) accounted for $C_{23}H_{31}NO_4$, which meant the product was dehydrated as well as acetylated (Fig. 2). The ^{13}C-NMR spectrum of **II** gave resolved 23 signals explained for the molecular formula (in order to improve the signals over noises, the NMR spectra for **II** were acquired with the $CDCl_3$-dissolved sample in a microcell produced by Shigemi).

The hydrocarbon domain of **II** was determined through the analysis of the COLOC observed from mainly methyl groups, selective INEPT spectra irradiated at methylenes and the ^{1}H-^{1}H COSY. The cross peaks on COLOC from 2-CH_3 (δ_H 1.26) to C-2 (δ_C 53.2), C-9a (δ_C 39.7) and C-3 (δ_C 36.8); from 3-CH_3 (δ_H 2.87) to C-2, C-3 and C-4 (δ_C 130.3); from 4-H (δ_H 5.53) to C-5a (δ_C 38.1); from 5-H (δ_H 5.32) to C-3 suggested a 2,3-dimetylated 6-membered ring (Fig. 3). ^{1}H-^{13}C long-range couplings observed through the irradiation at 4-H and 5-H in the selective INEPT experiments supported the presence of the partial structure. On the COLOC spectra, another doublet methyl signal at δ_H 0.89 was long-ranged coupled to C-6 (δ_C 42.2), C-7 (δ_C 33.2) and C-8 (δ_C 35.8), at which were irradiated in the selective INEPT experiments to give long-range couplings to C-8, C-5a and C-9a, respectively. Furthermore, the spin systems observed on ^{1}H-^{1}H COSY explained the connectivities (8-H–9-$H_{a,b}$–9a-H), which implied the presence of another 6-membered ring fused to the former 6-membered ring by two methines (5a and 9a). This proposed structure was proved by the observation of a long-range

Table 2. Chemical shifts of CJ-17,572 (**I**) and its acetyl derivative (**II**) in $CDCl_3$.

	(I)		(II)	
	δ_C	δ_H	δ_C	δ_H
1	200.3		203.4	
2	48.6		53.2	
3	ND[a]	ND[a]	36.8	3.03 (1H, m)
4	129.3	5.50 (1H, ddd, *J*= 1.9, 4.9, 9.7 Hz)	130.3	5.53 (1H, m)
5	129.2	5.32 (1H, br.d, *J*= 9.7 Hz)	129.1	5.32 (1H, d, *J*= 10.0 Hz)
5a	38.6[b]	1.81[b] (1H, m)	38.1	1.74 (1H, m)
6	42.3	1.79 (1H, m), 1.85 (1H, m)	42.2	1.78 (1H, m), 0.85 (1H, m)
7	33.4	1.50 (1H, m)	33.2	1.46 (1H, m)
8	35.7	1.76 (1H, m), 1.12 (1H, m)	35.8	1.70 (1H, m), 1.04 (1H, m)
9	28.1	1.87, (1H, m), 1.00 (1H, m)	27.1	1.42 (1H, m), 1.00 (1H, m)
9a	38.9[b]	ND[a]	39.7	1.67 (1H, m)
2-CH_3	14.3	1.44 (3H, br s)	16.1	1.26 (3H, s)
3-CH_3	18.3	0.80 (3H, d, *J*= 7.0 Hz)	19.0	2.87 (3H, d, *J*= 7.0 Hz)
7-CH_3	22.5	0.90 (3H, d, *J*= 6.5 Hz)	22.5	0.89 (3H, d, *J*= 6.2 Hz)
2'	177.0		163.5	
3'	100.6		124.9	
4'	192.3		154.5	
5'	66.5	4.18 (1H, d, *J*= 4.3 Hz)	134.2	
6'	66.8	3.77 (1H, d, *J*= 4.3 Hz)	113.0	5.56 (1H, q, *J*= 7.2 Hz)
7'			166.9	
1'-*N*-CH_3	27.2	2.98 (3H, s)	25.1	3.07 (3H, s)
6'-CH_3	17.1	1.38 (3H, d, *J*= 7.0 Hz)	12.2	2.02 (3H, d, J= 7.8 Hz)
7'-CH_3			20.8	2.29 (3H, s)

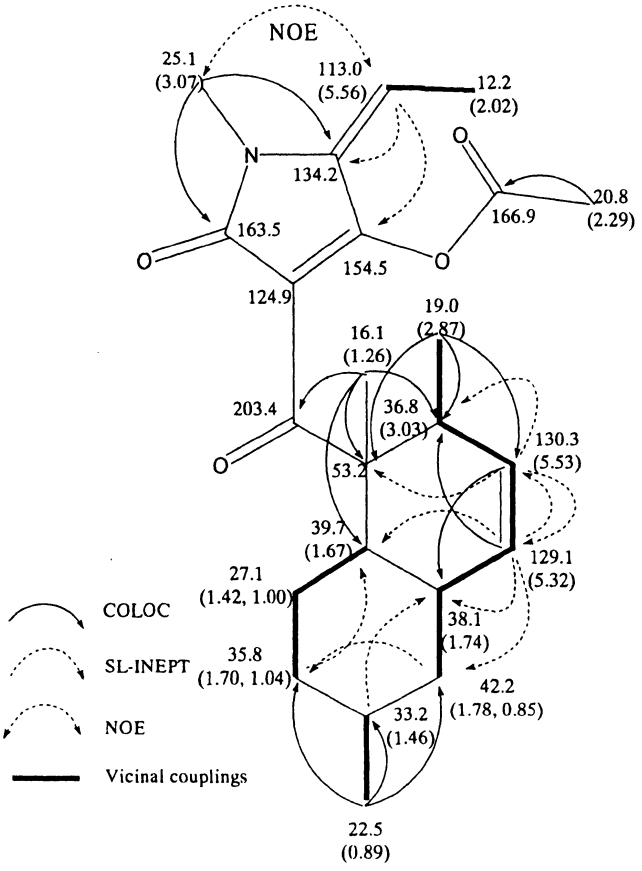
a: Not detected. b: Tentative assignment.

coupling from 5-H to C-6 in selective INEPT and the connectivities consisted of vicinal couplings in the ^1H-^1H COSY (3-CH_3–3-H–4-H–5-H–5a-H–6-$H_{a,b}$). The structure of hydrocarbon domain of **II** was thus determined as 2,3,7-trimethyl-2,3,5a,6,7,8,9,9a-octahydro-naphthalene.

The structure of heterocyclic domain of **II** was determined as follow: A methyl proton singlet at δ_H 3.07 (1'-*N*-CH_3) was coupled to an amide carbonyl group (δ_C 163.5) and an olefinic quaternary carbon (δ_C 134.2, C-5'). An olefinic methine signal at δ_H 5.56 (6'-H) vicinally connected with a methyl group (δ_H 2.02, 6'-CH_3) was coupled to C-5' and an oxygenated olefinic quaternary carbon (δ_C 154.5, C-4'). The formation of 5-membered ring at C-3' was established by the enol substructure consisted of –C1(=O)–C3'=C4'(O)–, derived from 1,3-diketone proposed in the structure of **I**. The C-1 was connected to C-2 for being long-ranged coupled with 2-CH_3. The remained acetyl group composed of C-7' (δ_C 166.9) and 7'-CH_3 (δ_C 20.8) was assigned to 4'-O. NOE spectrum obtained by irradiating at 1'-*N*-CH_3 group (δ_H 3.07) provided the stereochemistry of ethylidene group as *E* form. The structure of **II** was thus proposed as shown in Fig. 3.

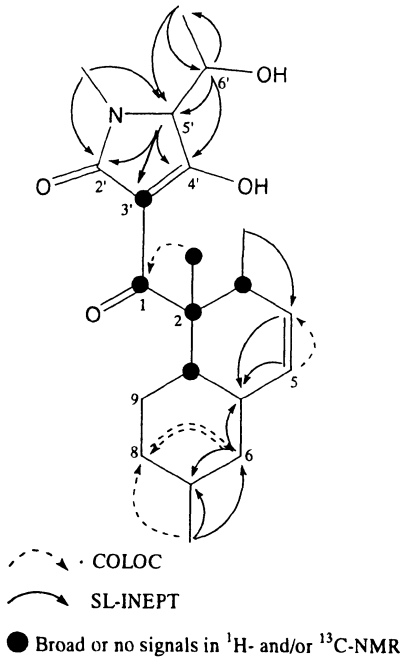
Owing to tautomerism between C-1 and C-4', 6 resonances (1, 2, 3, 9a, 1-CH_3 and 3') in ^1H- and ^{13}C-NMR spectra were broadened and/or not observed (● in Fig. 4). The partial structure of the hydrocarbon domain of **I** was found to be unaffected in the reaction of acetylation by the

Fig. 3. Correlations observed for **II** by COLOC, selective INEPT, ^{1}H-^{1}H COSY and NOE.



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

Fig. 4. Summary of COLOC and selective INEPT data for CJ-17,572 (**I**).



comparison of NMR spectra for **II** and **I**.

Acetylation of **I** gave rise to a dehydration as well as the acetylation to 4′-OH, which was explained by the drastic change of ^{13}C-NMR chemical shifts in the heterocyclic domain of **II**. The presence of 1,3-diketone of **I** [C1(=O)–C3′=C4′(–OH) ⇔ C1(=O)–C3′H=C4′(=O) ⇔ C1(–OH)=C3′–C4′(=O)] was proved by the formation of enol in **II**. The dehydration was found to be occurred at 1-hydroxyethyl group coupled to C-5′ in **I** to make ethylidene group. The presence of the 1-hydroxyethyl group at C-5′ of **I** was deduced from the selective INEPT irradiated at 6′-H (δ_H 3.77) and 6′-CH_3 (δ_H 1.38). A methine signal at (δ_H 4.18) was coupled to three quaternary carbons, C-2′ (δ_C 177.0, s), C-3′ (δ_C 100.6, s) and C-4′ (δ_C 192.3, s), which was in harmony with the 5-membered ring proposed in **II**. The heterocyclic domain of **I** was thus determined as a tetramic acid, 4-hydroxy-5-(1-hydroxyethyl)-1,5-dihydro-1-methylpyrrol-2-one. The ^{1}H-^{13}C long-range couplings observed in the COLOC and selective INEPT are summarized in Fig. 4. Among the possible 3 enol structures caused by 1,3-diketone, the structure of **I** in $CDCl_3$ was proposed as in Fig. 1, because of a long-range coupling observed from 2-CH_3 (δ_H 1.44) to C-1 (δ_C 200.3) in the COLOC and the presence of a quaternary carbon at C-3′ (δ_C 100.6).

Biological Activities

Compound **I** showed moderate antibacterial activity against Gram-positive MDR strains (Table 3). MICs of **I** against staphylococci and enterococci is 10 and 20 μg/ml, respectively. It exhibits poor antibacterial activity against *Streptococcus pyogenes* with 20 μg/ml. The assay results of **I** were similar with those of equisetin analogs. There has been reported that equisetin derivative will decrease its antibacterial activity in the presence of 5% sheep blood owing to strong serum binding of the compound[5]. *S. pyogenes* requires lysed horse blood in assay medium. That suggests **I** lost its antibacterial activity in the assay medium of *S. pyogenes*. It showed cytotoxicity against HeLa cell with an IC_{90} of 7.1 μg/ml.

Table 3. Antibacterial activity of CJ-17,572 (**I**).

Microorganisms	MIC (μg/ml)			
	(I)	ERM	AZM	VAM
Staphylococcus aureus 01A1105	10	>100	>100	1.56
Streptococcus pyogenes 02C1068	>20	>100	>100	0.39
Enterococcus faecalis 03A1069	20	>100	>100	12.5
Escherichia coli 51A0266	>20	100	1.56	>100

ERM: erythromycin, AZM: azithromycin, VAM: vancomycin

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 a 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 $CDCl_3$ and peak positions are expressed in parts per million (ppm) based on the reference of $CDCl_3$ peak at 7.26 ppm for ^{1}H-NMR and 77.0 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 spectra were measured using glycerol-PEG400 mixture matrix.

Producing Microorganism

The producing strain, the fungus *Pezicula* sp. CL11877, was obtained from the New York Botanical Garden (strain number 78-203).

Fermentation

The culture CL11877 was maintained on a potato dextrose agar slant (Difco). A vegetative cell suspension from the slant culture was inoculated into a 500-ml flask containing 100 ml of a seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). The flask was shaken at 27°C for 7 days on a rotary shaker with 7-cm throw at 250 rpm to obtain seed culture.

The seed culture (5 ml) was used to inoculate into nine 500-ml flasks containing 100 ml of a production medium (glycerol 8.5%, corn flour 1%, oil-less soybean meal 0.5% and corn steep powder [SHONO DENPUN] 0.25%, pH 5.4). Static fermentation was carried out at 27°C on a rotary shaker at 250 rpm for 18 days.

HPLC Analysis

Analytical HPLC of **I** was performed using an ODS column (FL-ODS3 AM, 4.6×50 mm, YMC Co. Ltd.) and eluted with a linear gradient of MeCN - 0.05% TFA in H_2O (1 : 19 to 10 : 0 for 12.5 minutes) at a flow rate of 0.9 ml/minute. The retention time of **I** was 10.5 minutes.

Acetylation

One drop of $(CH_3CO)_2O$ was added to a solution of **I** (28 mg) in pyridine (1 ml). After being stirred at room temperature for 4 hours, the solution was evaporated under nitrogen gas. The residue was dissolved in small amount of hexane - CH_3OH (1 : 1) mixture and applied on the silica gel preparative TLC (Kieselgel $60F_{254}$, 0.5 mm, Merck Ltd.). The TLC was developed with CH_2Cl_2 to yield 5.4 mg of **II**.

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) is MDR clinical strain. *Streptococcus pyogenes* 02C1068 is MLS$_B^r$, kan^r and str^r. *Enterococcus faecalis* 03A1069 is an MDR clinical strain (cef^r, ery^r, gent^r, chl^r, kan^r, tet^s and van^r), confirmed to have an *ermB* gene. *E. coli* 51A0266 is a generally susceptible strain.

Antibacterial Assay

Preparation of the inoculum, antibacterial assay and microtiter-based MIC determinations were done according to the National Committee for Clinical Laboratory Standards[6]. Erythromycin, azithromycin and vancomycin were used as standard antibiotics.

Cytotoxicity

The HeLa cell line was cultured with Eagle's minimum essential medium containing 10% fetal bovine serum, 100 units/ml of pen and 100 μg/ml of streptomycin. An aliquot (180 μl) of cell suspension (5.5×10^4 cells/ml) were added into each well of a 96-well microtiter plate and incubated with 20 μl of test sample at 37°C with 5% CO_2. After 72-hour incubation, the medium was discarded, washed with PBS(−) once and then 50 μl of a 0.4% crystal violet solution was added. The plate was left at room temperature for 30 minutes. After dye removal, the plate was washed with tap water 10 times and air-dried. The pigment was eluted thoroughly with 50% methanol and quantitated by measuring absorbance at 490 nm. The percentage of inhibition of HeLa proliferation was calculated by the formula:

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

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

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