VOL. 54 NO. 11, NOV. 2001 THE JOURNAL OF ANTIBIOTICS pp. 911 — 916

A New Antibiotic CJ-17,665 from *Aspergillus ochraceus*

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(Received for publication June 27, 2001)

A new antibiotic, CJ-17,665 (**I**) was isolated from the fermentation broth of *Aspergillus ochraceus*, CL41582. It inhibits growth of multi-drug resistant *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Enterococcus faecalis*, with MICs of 12.5, 12.5 and 25 μg/ml, respectively. The structure contains a diketopiperazine and an indole *N*-oxide moiety that is unusual in natural products.

During the past decade, multi-drug resistant (MDR) bacteria such as methicillin-resistant strains of *Staphylococcus aureus* (MRSA) has caused a serious problem in clinics[1]. Vancomycin has been a drug of last resort for the treatment of MDR. But the recent emergence of vancomycin-resistant Enterococci (VRE) and vancomycin-intermediate resistant *Staphylococcus aureus* (VISA)[2~4] is raising serious public health concerns. Accordingly, there is a clinical need for novel antibacterial drugs with activities against MDR, VRE or VISA which differ from vancomycin in mode of action.

In our screening program to discover new antibacterial agents against MDR bacteria including VRE, a fungal strain CL41582 was found to produce a new antibiotic, CJ-17,665 (**I**). In this paper, we report the taxonomy of the producing organism and fermentation, isolation, structure elucidation and biological activity of **I**.

Results

Taxonomy

The strain CL41582 from a soil sample collected in Venezuela is characterized by the ochraceous conidia in mass, the globose vesicles, and the globose to subglobose conidia, which are delicately roughened. The colony reverse was buff to some shades of brown, with an orange-pink soluble pigment on some media. It showed good growth between 20 and 28°C but no growth between 37 and 50°C. Except for the longer primary phialides and the failure to produce lavender sclerotia, it fits into the description of *Aspergillus ochraceus* Wilhelm as defined by RAPER and FENNELL[5]. Thus, the culture CL41582 is a new strain of *Aspergillus ochraceus* Wilhelm.

Isolation

The static fermentation broth with buckwheat (900 ml) was filtered after the addition of EtOH (900 ml). The filtrate was concentrated to an aqueous solution, and then adjusted to 30% aqueous MeOH (200 ml). The 30% aqueous MeOH solution was loaded onto an ODS column (YMC-pack ODS-AM 120-S50, 26×50 mm, YMC Co. Ltd.) with 70% aqueous MeOH. The active eluate was evaporated to dryness and charged to a silica gel column (Mega Bond Elut, 5 g, Varian Ltd.) with EtOAc-*n*-hexane (1 : 1), and eluted with EtOAc. The eluate was applied onto a Sephadex LH-20 column (12×550 mm, Amersham Pharmacia

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Biotech) with MeOH. Fractions showing the antibacterial activity were combined and evaporated to dryness. The dried material (79.7 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 (9 : 11) at a flow rate of 10 ml/minute to yield semi-pure **I**. The semi-pure compound was further purified by a preparative silica gel TLC (Kieselgel $60F_{254}$, 1 mm thickness, Merck Co. Ltd.) developed with CH_2Cl_2 - MeOH (20 : 1) to give **I** (Rf value=0.18).

Structural Elucidation

The physico-chemical property of **I** is summarized in Table 1. Compound **I** was obtained as a white amorphous powder. The molecular formula of **I** was determined to be $C_{26}H_{27}N_3O_4$ on the basis of HRFAB-MS [*m/z*, found 446.2081 $(M+H)^+$, calcd. 446.2059]. The ^{13}C NMR spectrum (in $CDCl_3$) showed resolved 26 signals (Table 2), classified into 4 -CH_3, 4 -CH_2–, 6 -CH–, and 12 quaternary carbons by the aid of the DEPT spectra, the results of which were in harmony with the molecular formula.

^1H-^1H COSY of **I** showed two connectivities (–C^5H=C^6H–, –$C^{10}H$=$C^{11}H$–) which were irradiated in the selective INEPT experiment to give following long-range couplings: from 5-H (δ_H 7.34) to C-4 (δ_C 116.8), C-7 (δ_C 155.5) and C-13 (δ_C 133.9); from 6-H (δ_H 6.80) to C-4, C-7 and C-12 (δ_C 112.0); from 10-H (δ_H 5.79) to C-12, and an oxygenated quaternary carbon C-9 (δ_C 76.7); from 11-H (δ_H 7.79) to C-7, C-9, C-12 and C-13. Two singlet equivalent methyl groups were, in addition, coupled to C-9 and C-10. The couplings observed led to establish the partial structure (A) as 5,6-disubstituted 2,2-dimethyl-2*H*-chromene as shown in Fig. 2 (A), whereas the connection of C-7 and C-9 by an oxygen was determined by the oxygenated features of chemical shifts.

The partial structure (B) was determined by the aid of selective INEPT. Two singlet methyl protons at δ_H 1.62 and δ_H 1.21 (16-CH_3a and 16-CH_3b, respectively) were coupled to C-15 (δ_C 144.5), C-16 (δ_C 36.0) and C-17 (δ_C 52.8). An allylic singlet methine proton at δ_H 6.56 (2-H) was coupled to C-15 and C-17. Furthermore, a doublet methine signal at δ_H 2.45 (17-H) was coupled to C-15, C-16 and C-1 (δ_C 62.8). C-3 was tentatively assigned with the remaining allylic quaternary carbon at δ_C 139.9 as a counterpart of =C^2H–, though the coupling was not observed from 2-H. Figure 2 (B) exhibits the proposed partial structure (B) based on the above coupling data.

From each methylene signal of –$C^{20}H_2$–$C^{21}H_2$–$C^{22}H_2$–,

Table 1. Physico-chemical properties of CJ-17,665 (**I**).

Appearence	White powder
Molecular weight	445
Molecular formula	$C_{26}H_{27}N_3O_4$
HRFAB-MS (*m/z*)	found 446.2081 $(M+H)^+$ calcd. 446.2059
$[\alpha]_D^{25}$	+12.0 (*c* 0.6, MeOH)
UV λ_{max}^{MeOH} nm (ε)	240 (45,000), 308 (18,000), 354 (8,000, sh), 395 (5,000, sh)
IR ν_{max}^{KBr} cm^{-1}	3400, 1650, 1597, 1503

Table 2. Chemical shifts of CJ-17,665 (**I**).

No.	δ_C	δ_H
1	62.8	
2	119.9	6.56 (1H, s)
3	139.9	
4	116.8	
5	120.3	7.34 (1H, d, *J*= 8.1 Hz)
6	116.0	6.80 (1H, d, *J*= 8.1 Hz)
7	155.5	
9	76.7	
10	132.8	5.79 (1H, d, *J*= 10.0 Hz)
11	116.0	7.79 (1H, d, *J*= 10.0 Hz)
12	112.0	
13	133.9	
15	144.5	
16	36.0	
17	52.8	2.45 (1H, m)
18	30.9	2.17 (1H, m), 1.87 (1H, m)
19	67.0	
20	29.3	2.78 (1H, m), 1.87 (1H, m)
21	24.5	2.03 (2H, m)
22	44.3	3.50 (1H, m)
24	167.3	
26	172.8	
9-CH_3a	27.8	1.45 (3H, s)
9-CH_3b	27.8	1.45 (3H, s)
16-CH_3a	23.1	1.62 (3H, s)
16-CH_3b	15.6	1.21 (3H, s)

which was revealed their connectivities through the analysis of the ^{1}H-^{1}H COSY, couplings to C-19 (δ_C 67.0) were found, suggesting the 5-membered ring having nitrogen between C-19 and C-22 (δ_C 44.3). The non-equivalent methylene protons at δ_H 2.78 and 1.87 (H-20a and H-20b, respectively) were coupled to the C-18 (δ_C 30.9), one proton resonance of which at δ_H 2.17 was irradiated to provide long-range coupling to C-1. The methine proton at H-17, vicinally connected with H-18, was coupled to C-19 and the amide carbonyl group (C-24, δ_C 167.3), which was additionally coupled to H-22. This structural information made the preliminary partial structure C into hexahydro-indolizin-5-one. To the remaining amide carbonyl group at δ_C 172.8 (C-26), long range couplings were observed from 18-H, 20-Ha,b, which resulted in the formation of amide bridge between C-1 and C-19 to produce diketopiperazine substructure. The partial structure C was thus determined as shown in Fig. 2 (C).

The C-17 and C-1 were common in the partial structures B and C. Taken together with long-range couplings from H-18a and H-2 to C-16 and C-24, respectively, the connection of the partial structures B and C were established (Fig. 3).

NOEs were observed between H-2 and H-5, which were irradiated in the selective INEPT experiments to give long-range couplings to C-4 and C-3, respectively (Fig. 3). These experiments led the connection between C-3 and C-4, and the confirmation of assignment of C-4 whose position was tentatively determined in the partial structure B. The remaining N in the molecular formula was assigned between C-13 and C-15 on the basis of chemical shifts.

The molecular formula of the established structure was $C_{26}H_{27}N_3O_3$, which was insufficient to satisfy the molecular formula of **I** by one mole of oxygen. This oxygen could be attached to one of the nitrogens 14-N, 23-N and 25-N, as an *N*-oxide form. GRIGOR'EV and his colleagues had previously reported on the effect of solvents on the ^{13}C NMR spectra of the cyclic nitrones of 3-imidazoline 3-oxide derivatives[6]. They found that in solvents capable of forming hydrogen bonds with the *N*-oxide group, the signal for the nitrone carbon atom is shifted down-field by 1.5~2.5 ppm for solution in $CDCl_3$ [δ_C ($CDCl_3$)] and 5~9

Fig. 1. Structure of CJ-17,665 (**I**).

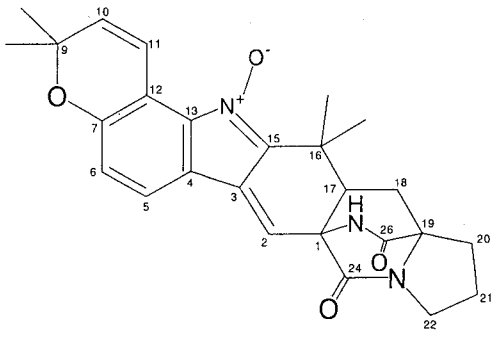


Fig. 3. Connectivities of partial structures A, B and C of CJ-17,665.

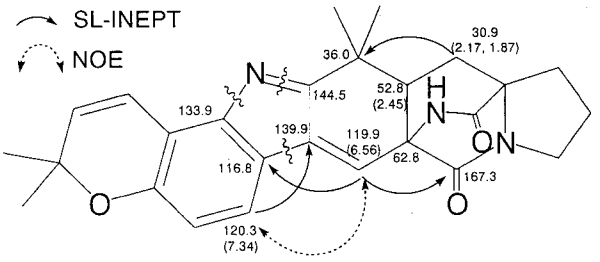
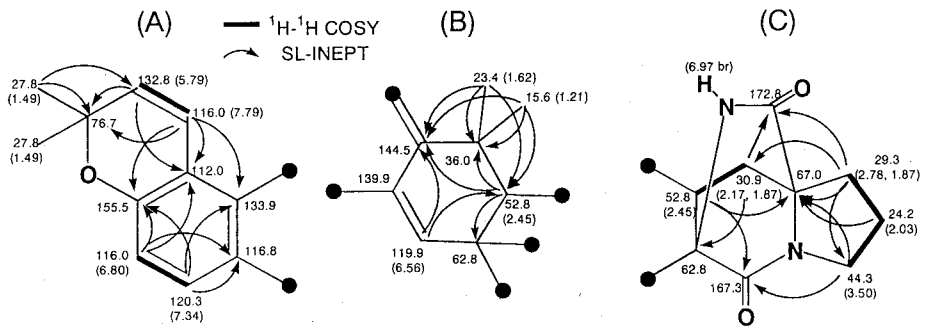


Fig. 2. Partial structures A, B and C.



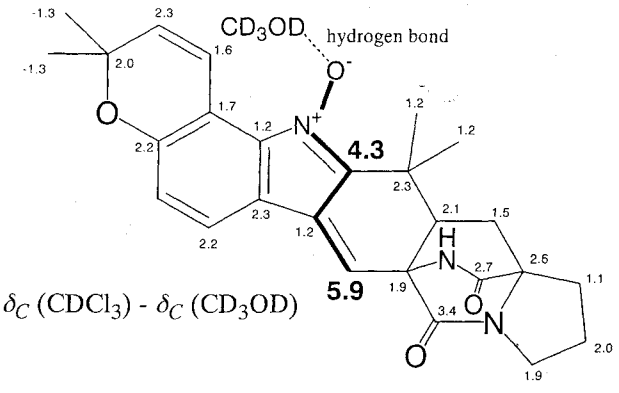
Values and those in parentheses show ^{13}C- and ^{1}H-chemical shifts in ppm, respectively. Arrows mean long-range ^{13}C-^{1}H couplings and bold lines exhibit vicinal couplings.

ppm for CD_3OD [δ_C (CD_3OD)], where the solvent used for comparison was DMSO-d_6. It could be deduced that the mathematically calculated range of the size (3.5~7.5 ppm) from δ_C (CD_3OD) - δ_C ($CDCl_3$) was sufficient to determine the assignment of an oxygen for **I**. Figure 4 shows the number on each carbon calculated using the proposed equation, δ_C (CD_3OD) - δ_C ($CDCl_3$), and the values for the nitrone carbon (C-15) and C-2 were 4.3 and 5.9, respectively, likely due to the formation of a stronger hydrogen bond between the methanol and *N*-oxide oxygen atom than that of chloroform, thereby affecting the electron density distribution of the molecule. The oxygen atom was thus assigned to be 14-N^+–O^-, and the structure of **I** was elucidated as shown in Fig. 1.

Biological Activities

Compound **I** showed antibacterial activities against MDR *S. aureus*, *S. pyogenes*, and *E. faecalis*, with MICs of 12.5, 12.5, and 25 μg/ml, respectively, whereas it showed no antibacterial activity against *E. coli* at 100 μg/ml (Table 3). It showed cytotoxicity against HeLa cell with an IC_{90} of 1.1 μg/ml.

Fig. 4. Effect of solvent on the chemical shift of cyclic nitrone carbon atom.



Values mean the differences of ^{13}C-chemical shifts in ppm between in CD_3OD and in $CDCl_3$.

Discussion

Compound **I** is a new diketopiperazine-related compound having an *N*-oxide oxygen atom. The presence of an *N*-oxide moiety is rare in natural products. It was determined based on the down-field shifts of the nitrone carbon atom, which was explained by the formation of a stronger hydrogen bond between the methanol and *N*-oxide group rather than that of chloroform. Tryptophan containing 2,5-dioxopiperazine type natural products, such as echinulin[7], sporidesmins[8], brevianamides[9], fumitremorgins[10], and tryprostatins[11] have been reported. These compounds are structurally similar to **I**, but show poor antibacterial activities against Gram-positive strains. While the biosynthetic pathway of this compound is considered to be entirely different from these compounds, suggesting that **I** arises by condensation of the 6-hydroxytryptophan, proline and two parts of isoprenoid. This kind of structure is unusual and has never been reported. Further studies will be required to examine the biosynthetic pathway and the mode of action of **I**.

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

Table 3. Antibacterial activities of CJ-17,665 (**I**).

Microorganism	MIC (μg/ml)			
	CJ-17,665	Erythromycin	Azithromycin	Vancomycin
Staphylococcus aureus 01A1105	12.5	>100	>100	1.56
Streptococcus pyogenes 02C1068	12.5	>100	>100	0.39
Enterococcus faecalis 03A1069	25	>100	>100	12.5
Escherichia coli 51A0266	>100	100	1.56	>100

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 ^1H 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.

HPLC Analysis

Analytical HPLC of **I** was performed using an ODS column (FL-ODS3 AM, 4.6×50 mm, YMC Co. Ltd.) and elution 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 6.7 minutes.

Fermentation

The culture CL41582 was maintained on a potato dextrose agar slant (Difco). A vegetative cell suspension from the slant culture was inoculated into 500-ml flask containing 100 ml of seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). The flask was shaken at 26°C for 4 days on a rotary shaker with 7-cm throw at 210 rpm to obtain seed culture.

This seed culture was used to inoculate 5 ml into nine 500-ml flasks containing 100 ml of 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.

Test Strains

S. aureus 01A1105 (cef^r, $gent^r$, $meth^r$, $MLS_B{}^r$, pen^r, tet^r, cip^r and van^s) is MDR clinical strain. *S. pyogenes* 02C1068 is $MLS_B{}^r$, kan^r and str^r. *E. 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 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 made according to the National Committee for Clinical Laboratory Standards [12].

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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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; van, vancomycin

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