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CJ-15,696 and Its Analogs, New Furopyridine Antibiotics from the Fungus *Cladobotryum varium*: Fermentation, Isolation, Structural Elucidation, Biotransformation and Antibacterial Activities

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CJ-15,696 and 7 novel furopyridine antibiotics were isolated from the fungus *Cladobotryum varium* CL12284. Their structures were determined by X-ray crystallography and spectral analysis. Three biotransformed analogs were also prepared from CJ-15,696. CJ-15,696 showed moderate activity against various Gram-positive bacteria including some drug resistant strains such as methicillin resistant *Staphylococcus aureus* (MRSA).

There has been an alarming increase, recently, in the incidence of drug resistant infections that is limiting the utility of many standard agents[1]. For example, MRSA and vancomycin-resistant *Enterococcus* species are now co-resistant to essentially all other antibiotic classes[2~4]. *Streptococcus pneumoniae*, a frequent cause of upper and lower respiratory tract infections in both children and adults is rapidly becoming resistant to all presently available therapies[5]. Commonly prescribed anti-infectives such as β-lactams and the current macrolides are no longer reliably effective against such multidrug-resistant bacteria.

These multidrug-resistant bacteria are not limited to hospitals but also occur in a variety of human communities world-wide. In many cases, multidrug-resistant infections can lead to potentially fatal conditions and require hospitalization. A compound having good activity against various bacteria, including multidrug-resistant bacteria such as *Staphylococcus* sp., *Streptococcus* sp. and *Enterococcus* sp., would meet a serious medical need, and also provide health care cost savings by minimizing treatment failures, laboratory testing, and hospitalization[6].

In our search for new and effective antibiotics, CJ-15,696 and 7 novel analogs (**1**~**8**) were isolated from the fermentation broth of the fungus *Cladobotryum varium* CL12284, as new furopyridine type antibiotics[7]. This paper describes the fermentation, isolation and structure determination of the furopyridine antibiotics and their antibacterial activity. Biotransformation of CJ-15,696 to three novel compounds is also described.

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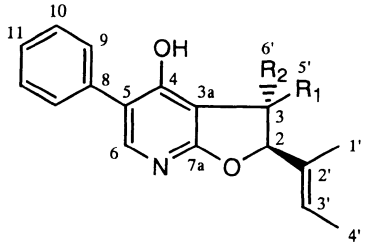
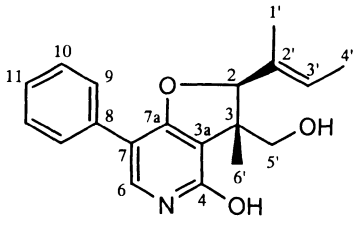
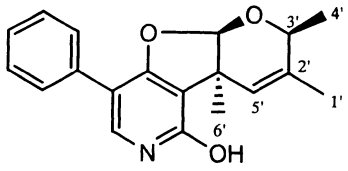
Fig. 1. Structures of furopyridines.



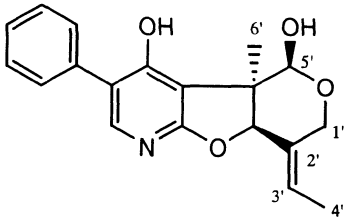
CJ-15,696 (**1**): R_1=CHO, R_2=Me
CJ-16,169 (**2**): R_1=CH_2OH, R_2=Me
CJ-16,174 (**6**): R_1, R_2=CH_2OH



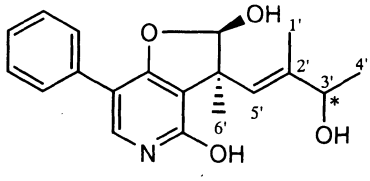
CJ-16,170 (**3**)



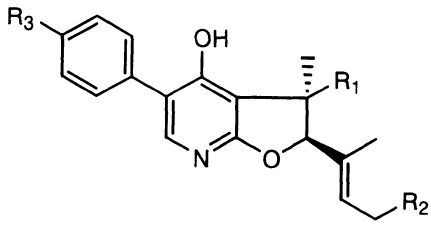
CJ-16,171 (**4**)



CJ-16,173 (**5**)



CJ-16,196 (**7**) and CJ-16,197 (**8**)
(epimers at C-3'* each other)



CP-471,326 (**9**): R_1=CHO, R_2=OH, R_3=H
CP-473,195 (**10**): R_1=CHO, R_2=H, R_3=OH
CP-473,198 (**11**): R_1=CH_2OH, R_2=OH, R_3=H

Producing Strain and Fermentation

The producing strain, designated as *Cladobotryum varium*[8] CL12284, was obtained from the New York Botanical Garden (New York, U.S.A.). It was deposited under the accession number FERM BP-5732 to National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Tsukuba, Japan).

The fermentation of *C. varium* was performed in both solid-supported media and liquid media.

For the solid supported fermentation, two 500-ml flasks containing 100 ml of Medium-1 (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%) were inoculated with vegetative cell suspension from a slant culture of FERM BP-5732. 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. Aliquots (5 ml, each) of the seed cultures were used to inoculate thirty 500-ml flasks containing 100 ml of Medium-2 (glucose 1%, glycerol 6.6%, NZ Amine Type A 0.5%, ammonium sulfate 0.2%, defatted soybean meal 0.5%, tomato paste 0.5%, sodium citrate 0.2%, adjusted to pH 7.0) and 30 g buckwheat. Incubation was carried out at 26°C for 14 to 21 days, standing still without shake.

For the liquid fermentation, a 500-ml flask containing Medium-1 (150 ml) was inoculated with 5 ml of a *C. varium* seed culture prepared as described above. The flask was shaken at 26°C for 3 days on a rotary shaker, to produce a second seed culture. The second seed culture was used to inoculate a 6-liter fermentation vessel containing 3 liters of sterile medium (Medium-3: glycerol 8.5%, soybean meal 0.5%, corn flour 1.0% and corn steep solid 0.25%, adjusted to pH 5.0). The fermentation was carried out at 26°C for 12 days with stirring at 1,700 rpm and aeration at 3 liters per minute.

Isolation of Furopyridine Antibiotics

Compounds **1**~**6** were isolated from the solid-supported fermentation of *C. varium*. The fermentation broth (3 liters) was paper-filtered after stirring with 3 liters of ethanol, overnight. The filtrate was concentrated to an aqueous solution (500 ml), applied onto a Diaion HP20 (Mitsubishikasei) column and eluted with 30%, 50% and 100% aqueous methanol followed by acetone. The methanol and acetone fractions were combined, evaporated

to dryness, reconstituted with 50% aqueous methanol. This material was then loaded onto an ODS open-column (YMC, ODS-AM 120-S50, 3×5 cm) and eluted with 70% aqueous methanol. The active fraction (3.1 g) was applied onto Sephadex LH-20 column (160 ml) and eluted with methanol. Fractions showing antibacterial activity were pooled and evaporated. The resulting material (1.7 g) was further purified by preparative HPLC: YMC-pack ODS AM SH-343-5AM column (20×250 mm), eluting with methanol - water (13 : 7) for 40 minutes at a flow rate of 10 ml/minute. The eluted compounds were collected by monitoring UV absorbance at 240 nm to yield CJ-15,696 (**1**, 700 mg), CJ-16,169 (**2**, 8.4 mg), CJ-16,170 (**3**, 18.3 mg), CJ-16,171 (**4**, 2.8 mg), CJ-16,173 (**5**, 16.2 mg), and CJ-16,174 (**6**, 3.3 mg).

CJ-16,196 (**7**, 5.6 mg) and CJ-16,197 (**8**, 7.3 mg) were also isolated from the liquid fermentation broth (3 liters) using same purification scheme described for compounds **1**~**6**, except that the final HPLC purification was performed with acetonitrile - water (1 : 4).

Physico-chemical Properties

Physico-chemical properties of furopyridines **1**~**8** are summarized in Table 1. The compounds are soluble in most organic solvents but insoluble in hexane and water. Furopyridines **1**, **2**, **5** and **6**, which showed very similar absorption maxima at 207.5~209.0 and 233.0~235.0 nm, were easily crystallized from MeOH, MeOH/H_2O or *n*-hexane/MeOH. On the other hand, furopyridines **3**, **4**, **7** and **8**, which showed very similar absorption maxima at 206.5~207.0 and 245.0~247.0 nm, were difficult to be crystallized, and only CJ-16,171 (**4**) was crystallized from MeOH after a number of trials. The crystals prepared for X-ray analysis were also used for melting point measurement. CJ-15,696 (**1**) was obtained as a MeOH adduct (as a hemi-acetal), as previously reported by another group[9], after crystallization from MeOH or *n*-hexane/MeOH. This MeOH is easily removable from the molecule by repeated evaporation with other organic solvents such as acetone as observed in the proton NMR by disappearance of a methoxy signal (δ 3.26 ppm) and appearance of an aldehyde signal (δ 9.57 ppm).

Structure Elucidation

The molecular formulas of all furopyridines were determined by HR-FAB-MS considering the number of protons and carbons from NMR data (Table 2).

Furopyridines **1**~**8** could be separated into two groups by the UV absorption maxima as described in the Physico-chemical Properties section: one is the group comprised of **1**, **2**, **5** and **6**; and the other is comprised of **3**, **4**, **7** and **8**. This suggested the furopyridines in each group have a common chromophore.

Structure Determination of **1**, **2**, **4**, **5** and **6**

NMR analysis, including 2D experiments, of compounds **1**, **2**, **4**, **5** and **6** revealed partial structures consisting of a mono-substituted benzene and an aliphatic portion for each structure. However, complete structure elucidation was not possible based on NMR alone, due to undetectable carbon signals on the pyridine ring. Fortunately, the compounds in the former group (**1**, **2**, **5** and **6**) were easily crystallized, and their structures were all determined by single crystal X-ray analysis (Fig. 2 and Table 3). These compounds possessed the 2,3-dihydro-4-hydroxy-5-phenylfuropyridine moiety, which is responsible for the UV absorption at 233.0~235.0 nm. From the latter group (**3**, **4**, **7**, and **8**), **4** was the only one succeeded on its crystallization, and the structure was determined by single crystal X-ray analysis (Fig. 2 and Table 3). Compound **4** was found to have a 2,3-dihydro-4-hydroxy-7-phenylfuropyridine moiety, which is responsible for the UV absorption at 245.0~247.0 nm.

The ^1H and ^{13}C NMR chemical shifts (Table 2) for **1**, **2**, **4**, **5** and **6** were assigned by analysis of ^1H-^1H COSY, ^{13}C-^1H COSY and COLOC spectra.

Structure Determination of **3**

The molecular formula of CJ-16,170 (**3**) was determined to be $C_{19}H_{21}NO_3$. Compared to the molecular formula of **4**, **3** has a gain of 2 hydrogens. It is also clear that **3** has the same 2,3-dihydro-4-hydroxy-7-phenylfuropyridine skeleton as that of **4**, based on the UV spectrum, ^1H and ^{13}C NMR spectra. Further, ^1H-^1H COSY and COLOC experiments (Fig. 3) allowed us to connect all attachments to the basic skeleton above. The ^1H-^1H COSY spectrum revealed the presence of a 2-butenyl group by vicinal coupling from methyl protons at H-4′ (δ 1.58) to an olefinic proton at H-3′ (δ 5.59), and *via* long range coupling between H-3′ and methyl protons at H-1′ (δ 1.67). This was also supported by the COLOC experiment that showed long range coupling from H-1′ to C-3′ (δ 124.29) and from H-4′ to C-2′ (δ 133.10). This 2-butenyl group was connected to C-2 position of the furopyridine skeleton based on the ^{13}C-^1H long range coupling from H-1′ and H-3′ to C-2 (δ 95.15), and from H-2 (δ 4.90) to C-1′ and C-3′. The residual methyl and hydroxymethyl groups were attached to the C-3 quarternary carbon (δ 52.04) based on the ^{13}C-^1H long range coupling from H-5′ (δ 3.66) and H-6′ (δ 1.17) to C-

Table 1. Physico-chemical properties of furanopyridines **1**~**8**.

	CJ-15,696 (**1**)	CJ-16,169 (**2**)	CJ-16,170 (**3**)	CJ-16,171 (**4**)
Appearance	Colorless needle crystals	Colorless needle crystalss	Colorless glass	Colorless needle crystals
Molecular weight	309	311	311	309
Molecular formula	$C_{19}H_{19}NO_3$	$C_{19}H_{21}NO_3$	$C_{19}H_{21}NO_3$	$C_{19}H_{19}NO_3$
HRFAB-MS (*m/z*)	(positive)	(positive)	(positive)	(positive)
Found:	310.1429	312.1595	312.1598	310.1456
Calcd. for:	310.1444 ($C_{19}H_{20}NO_3$)	312.1601 ($C_{19}H_{22}NO_3$)	312.1601 ($C_{19}H_{22}NO_3$)	310.1444 ($C_{19}H_{20}NO_3$)
$[\alpha]_D$ (24°C, MeOH)	+21.2° (*c* 0.52)	+52.3° (*c* 0.09)	+15.4° (*c* 0.26)	-22.7° (*c* 0.33)
UV λ_{max} (MeOH) nm (ε)	209.0 (24,000) 234.0 (19,000)	208.5 (24,000) 233.0 (19,000)	206.5 (22,000) 246.0 (21,000)	207.0 (28,000) 247.0 (27,000)
IR ν_{max} (KBr) cm^{-1}	3025, 1724, 1645, 1593, 1440, 1413, 1374, 1289, 1201, 1063, 1049, 1013, 792, 696	3035, 1594, 1431, 1297, 1235, 1193, 1070, 1050, 945, 791, 698	3395, 2975, 1647, 1611, 1444, 1213, 1055, 782, 694	3435, 2940, 1659, 1617, 1428, 1215, 1157, 911, 838, 781, 693
Melting point	198~199°C	207~208°C	Not available	243~244°C
HPLC-RT (min.)[a]	6.4	7.9	7.6	8.2
TLC-RF[b]	0.50	0.51	0.14	0.26

	CJ-16,173 (**5**)	CJ-16,174 (**6**)	CJ-16,196 (**7**)	CJ-16,197 (**8**)
Appearance	Colorless needle crystals	Colorless needle crystals	Colorless glass	Colorless glass
Molecular weight	325	327	327	327
Molecular formula	$C_{19}H_{19}NO_4$	$C_{19}H_{21}NO_4$	$C_{19}H_{21}NO_4$	$C_{19}H_{21}NO_4$
HRFAB-MS (*m/z*)	(positive)	(positive)	(positive)	(positive)
Found:	326.1398	328.1534	328.1559	328.1570
Calcd. for:	326.1393 ($C_{19}H_{20}NO_4$)	328.1549 ($C_{19}H_{22}NO_4$)	328.1550 ($C_{19}H_{22}NO_4$)	328.1550 ($C_{19}H_{22}NO_4$)
$[\alpha]_D$ (24°C, MeOH)	+205.0° (*c* 0.12)	+40.0° (*c* 0.02)	-7.40° (*c* 0.11)	+2.17° (*c* 0.14)
UV λ_{max} (MeOH) nm (ε)	208.5 (28,000) 235.0 (25,000)	207.5 (15,000) 234.5 (13,000)	206.5 (23,000) 245.0 (23,000)	207.0 (22,000) 245.5 (22,000)
IR ν_{max} (KBr) cm^{-1}	3055, 1645, 1597, 1441, 1414, 1376, 1198, 1078, 947, 794, 697	3073, 1643, 1597, 1436, 1379, 1156, 1070, 944, 796, 697	3375, 1648, 1614, 1428, 1311, 1154,	3384, 1649, 1609, 1427, 1152, 1047, 874, 694
Melting point	234~235°C	214~216°C	Not available	Not available
HPLC-RT (min.)[a]	5.1	5.8	4.4	4.4
TLC-RF[b]	0.34	0.19	0.04	0.03

a) YMC-pack ODS-AM, AM-310-3 column (6.0 x 50 mm, YMC Co., Ltd.); eluted with acetonitrile-water gradient system: 10:90 (v/v) to 35:65 in first 2 min., 35:65 to 60:40 in subsequent 7.5 min., 60:40 to 100:0 in final 3 min.; at a flow rate of 0.9 mL/min.

b) Macherey-Nagel, Pre-coated TLC plates SIL G-25 UV_{254} (Art.# 809023, Chemco Co., Japan) developped with CH_2Cl_2:MeOH = 20:1 (v/v).

2, C-3 and C-3a (δ 117.05), respectively. The *cis*-geometry of the 2-butenyl group and the relative configuration of C-2 and C-3 were determined by the differential NOE experiments (Fig. 4). NOEs from H-6′ to H-1′ and H-3′ are consistent with *cis*-geometry. The same NOEs and, vice versa, a NOE between H-2 and H-5′ are consistent with the C-2, 3 configurations. From the above data, the structure of **3** was determined as shown in Fig. 1.

Structure Determination of **7**

The molecular formula of CJ-16,196 (**7**) was determined to be $C_{19}H_{21}NO_4$. Compared to the molecular formula of **4**,

Table 2-1. ^{1}H and ^{13}C NMR chemical shifts of furopyridines **1** to **4**.

Carbon	CJ-15,696 (**1**)		CJ-16,169 (**2**)	
No.	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
C-2	---	4.96 (1H, s)	95.31 d	4.80 (1H, s)
C-3	59.17 s		50.44 s	
C-3a	---		---	
C-4	---		---	
C-5	107.84 s		111.78 s	
C-6	---	7.74 (1H, s)	---	7.78 (1H, s)
C-7a	---		---	
C-8	---		137.31 s	
C-9	130.79 d	7.43 (2H, m)	130.57 d	7.49 (2H, m)
C-10	129.92 d	7.39 (2H, m)	129.29 d	7.33 (2H, m)
C-11	128.77 d	7.36 (1H, m)	127.95 d	7.23 (1H, m)
C-1'	13.53 q	1.60 (3H, s)	13.38 q	1.50 (3H, s)
C-2'	131.81 s		133.71 s	
C-3'	125.50 d	5.78 (1H, q, J = 6.6 Hz)	125.29 d	5.59 (1H, q, J = 6.8 Hz)
C-4'	13.52 q	1.61 (3H, d, J = 6.6 Hz)	13.53 q	1.58 (3H, d, J = 6.8 Hz)
C-5'	201.15 d	9.57 (1H, s)	67.22 t	3.75 (1H, d, J = 10.3 Hz)
				3.69 (1H, d, J = 10.3 Hz)
C-6'	20.68 q	1.53 (3H, s)	25.74 q	1.47 (3H, s)

Carbon	CJ-16,170 (**3**)		CJ-16,171 (**4**)	
No.	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
C-2	95.15 d	4.90 (1H, s)	112.79 d	5.90 (1H, s)
C-3	52.04 s		43.00 s	
C-3a	117.05 s		116.80 s	
C-4	166.80 s		164.61 s	
C-6	135.81 d	7.59 (1H, s)	135.87 d	7.48 (1H, s)
C-7	111.62 s		110.50 s	
C-7a	163.27 s		161.28 s	
C-8	134.75 s		135.61 s	
C-9	128.91 d	7.57 (2H, m)	128.84 d	7.55 (2H, m)
C-10	129.84 d	7.35 (2H, m)	129.79 d	7.35 (2H, m)
C-11	128.50 d	7.26 (1H, m)	128.31 d	7.25 (1H, m)
C-1'	14.39 q	1.67 (3H, s)	19.50 q	1.62 (3H, s)
C-2'	133.10 s		134.80 s	
C-3'	124.29 d	5.59 (1H, q, J = 7.0 Hz)	68.14 d	4.35 (1H, q, J = 7.6 Hz)
C-4'	13.61 q	1.58 (3H, d, J = 7.0 Hz)	19.89 q	1.25 (3H, d, J = 7.6 Hz)
C-5'	70.05 t	3.66 (2H, s)	124.67 d	5.71 (1H, s)
C-6'	17.54 q	1.17 (3H, s)	23.68 q	1.50 (3H, s)

Chemical shifts are referred to acetone-d_6 at 30.30 ppm for ^{13}C and at 2.00 ppm for ^{1}H.

7 has an increase of 2 hydrogens and 1 oxygen. The UV spectrum, ^{1}H and ^{13}C NMR spectra of **7** were very similar to those of **4**, suggesting that the 2,3-dihydro-4-hydroxy-7-phenylfuropyridine skeleton was unchanged. In the ^{1}H-^{1}H COSY experiment (Fig. 3), a large spin system between methyl protons H-4' (δ 1.12) and oxymethine proton H-3' (δ 4.08) was observed. Additionally, long range couplings were observed between H-3', methyl proton H-1' (δ 1.62) and olefinic proton H-5' (δ 5.77), respectively. In the COLOC experiment (Fig. 3), long range couplings from H-1' to C-2' (δ 143.27) and C-3' (δ 74.19), and from H-5' to C-1' (δ 12.98) and C-3' were observed. The above data suggested the presence of 3-hydroxy-2-methylbut-1-enyl group as a side chain moiety. The side chain moiety was connected to the C-3 (δ 49.78) position of the furopyridine skeleton by the observation of long range coupling from H-5' to C-3 and C-3a (δ 117.25). An additional methyl group was also connected to C-3 by the observation of long range

Table 2-2. ^{1}H and ^{13}C NMR chemical shifts of furopyridines **5** to **8**.

Carbon	CJ-16,173 (**5**)		CJ-16,174 (**6**)	
No.	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
C-2	92.29 d	4.81 (1H, s)	90.15 d	5.05 (1H, s)
C-3	53.21 s		56.30 s	
C-3a	---		---	
C-4	---		162.61 s	
C-5	---		---	
C-6	144.23 d	7.66 (1H, s)	148.90 d	7.73 (1H, s)
C-7a	---		---	
C-8	137.34 s		137.80 s	
C-9	130.47 d	7.51 (2H, m)	130.54 d	7.49 (2H, d, J = 7.0 Hz)
C-10	129.19 d	7.31 (2H, m)	129.20 d	7.31 (2H, t, J = 7.0 Hz)
C-11	127.94 d	7.24 (1H, m)	127.72 d	7.23 (1H, t, J = 7.0 Hz)
C-1'	64.11 t	4.44 (2H, s)	13.51 q	1.53 (3H, s)
C-2'	132.24 s		133.93 s	
C-3'	124.54 d	5.86 (1H, q, J = 7.0 Hz)	124.38 d	5.54 (1H, q, J = 7.0 Hz)
C-4'	13.53 q	1.61 (3H, d, J = 7.0 Hz)	13.61 q	1.58 (3H, d, J = 7.0 Hz)
C-5'	100.39 d	4.75 (1H, s)	63.94 t	3.98 (1H, d, J = 7.3 Hz)
				3.59 (1H, d, J = 7.3 Hz)
C-6'	22.25 q	1.41 (3H, s)	61.98 t	3.94 (1H, d, J = 7.3 Hz)
				3.51 (1H, d, J = 7.3 Hz)

Carbon	CJ-16,196 (**7**)		CJ-16,197 (**8**)	
No.	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)
C-2	111.23 d	5.98 (1H, s)	111.48 d	5.99 (1H, s)
C-3	49.78 s		49.70 s	
C-3a	117.25 s		117.40 s	
C-4	164.29 s		164.42 s	
C-6	135.61 d	7.51 (1H, s)	135.68 d	7.54 (1H, s)
C-7	110.80 s		110.92 s	
C-7a	161.69 s		161.83 s	
C-8	135.16 s		135.15 s	
C-9	128.90 d	7.55 (2H, m)	128.91 d	7.55 (2H, m)
C-10	129.72 d	7.33 (2H, m)	129.72 d	7.33 (2H, m)
C-11	128.27 d	7.24 (1H, m)	128.28 d	7.24 (1H, m)
C-1'	12.98 q	1.62 (3H, s)	12.94 q	1.62 (3H, s)
C-2'	143.27 s		143.31 s	
C-3'	74.19 d	4.08 (1H, q, J = 6.8 Hz)	74.26 d	4.08 (1H, q, J = 6.8 Hz)
C-4'	22.86 q	1.12 (3H, d, J = 6.8 Hz)	22.81 q	1.12 (3H, d, J = 6.8 Hz)
C-5'	128.90 d	5.77 (1H, s)	128.91 d	5.74 (1H, s)
C-6'	22.86 q	1.50 (3H, s)	22.81 q	1.49 (3H, s)

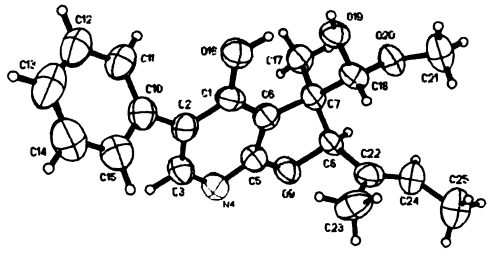
Chemical shifts are referred to acetone-d_6 at 30.30 ppm for ^{13}C and at 2.00 ppm for ^{1}H.

coupling from H-6' (δ 1.50) to C-2 (δ 111.23), C-3 and C-3a. Finally, the C-2 methine carbon of furopyridine must have a hydroxy group because of the chemical shift of C-2 and the molecular formula of **7**. The *trans*-geometry of the olefin was determined by NOEs observed between H-5' and H-3'/H-4' (Fig. 4). The relative stereochemistry between C-2 and C-3 was also determined as *cis*-configuration based on the NOE between methyl H-6' and methine H-2 (δ 5.98). From all the data above, the structure of **7** was determined as shown in Fig. 1.

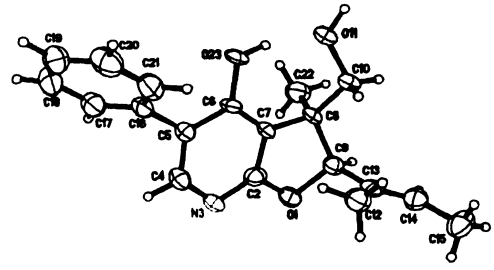
Structure Determination of **8**

The molecular formula of CJ-16,197 (**8**) was identical to that of **7**. The 1D and 2D NMR spectra and differential NOE experiments of **8** indicated the same carbon skeleton and relative configuration between C-2 and C-3 as those of

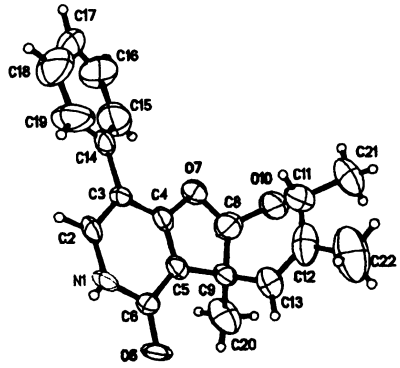
Fig. 2. Relative stereo-structures of furopyridines **1**, **2**, **4**, **5** and **6** determined by X-ray analysis: all atom numbers here are only for X-ray analysis.



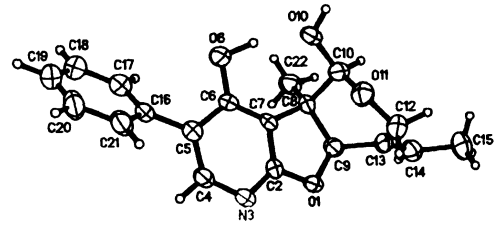
CJ-15,696 (1)



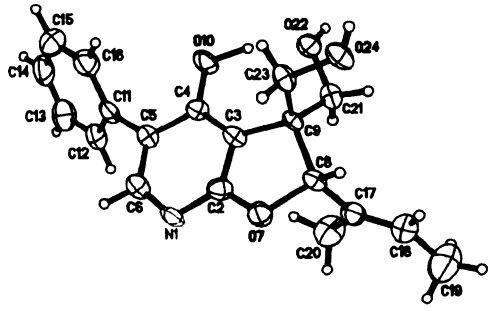
CJ-16,169 (2)



CJ-16,171 (4)



CJ-16,173 (5)



CJ-16,174 (6)

7. These observations together with the difference of optical rotations between **7** and **8** (−7.40° for **7** *vs.* +2.17° for **8**) indicated that **8** is the diastereomer of **7** at C-3′ (δ 74.26).

Biotransformation

Biotransformation of CJ-15,696 (**1**) was performed in an attempt to improve antibiotic activity. One day after inoculation, test tube cultures of 92 microorganisms were fed with CJ-15,696 at a final concentration of 0.1 mg/ml.

Table 3. Single crystal X-ray crystallographic analyses of furanopyridines **1**, **2**, **4**, **5** and **6**.

	CJ-15,696 (**1**)	CJ-16,169 (**2**)	CJ-16,171 (**4**)
A. Crystal Parameters			
formula	$C_{20}H_{23}NO_4$ (341.4)	$C_{19}H_{21}NO_3$ (311.4)	$C_{19}H_{19}NO_3$ (309.4)
crystallization medium	*n*-hexane/methanol	*n*-hexane/methanol	methanol
crystal size, mm	0.22 x 0.32 x 0.48	0.16 x 0.20 x 0.22	0.20 x 0.46 x 0.96
cell dimensions	a = 19.893 (1) Å	a = 9.859 (2) Å	a = 11.260 (1) Å
	b = 19.893 (1) Å	b = 8.014 (1) Å	b = 5.600 (1) Å
	c = 13.177 (1) Å	c = 11.010 (2) Å	c = 13.615 (2) Å
	α = 90.0°	α = 90.0°	α = 90.0°
	β = 90.0°	β = 107.34 (2)°	β = 107.90 (1)°
	γ = 120.0°	γ = 90.0°	γ = 90.0°
	V = 4515.0 (6) Å^3	V = 830.4 (2) Å^3	V = 817.0 (2) Å^3
space group	R3	$P2_1$	$P2_1$
molecules/unit cell	9.0	2	2
density calcd, g/cm^3	1.130	1.245	1.258
linear absorption factor, nm^{-1}	0.638	0.676	0.687
B. Refinement Parameters			
number of reflections	1060	932	955
nonzero reflections (I > 3.0σ)	1017	887	904
R-index[a]	7.16 %	4.09 %	8.56 %
GOF[b]	1.00	1.05	1.51
secondary extinction factor[c], χ	40 (6) x 10^{-4}	6 (1) x 10^{-3}	27 (2) x 10^{-3}

	CJ-16,173 (**5**)	CJ-16,174 (**6**)
A. Crystal Parameters		
formula	$C_{19}H_{19}NO_4$ (325.4)	$C_{19}H_{21}NO_4$ (327.4)
crystallization medium	*n*-hexane/methanol	methanol/water
crystal size, mm	0.14 x 0.26 x 0.40	0.40 x 0.58 x 0.60
cell dimensions	a = 6.858 (1) Å	a = 7.795 (1) Å
	b = 7.690 (1) Å	b = 10.448 (1) Å
	c = 7.976 (1) Å	c = 21.096 (5) Å
	α = 82.87 (1)°	α = 90.0°
	β = 85.66 (1)°	β = 90.0°
	γ = 78.42 (1)°	γ = 90.0°
	V = 408.33 (9) Å^3	V = 1718.1 (5) Å^3
space group	P1	$P2_12_12_1$
molecules/unit cell	1	4
density calcd, g/cm^3	1.323	1.266
linear absorption factor, nm^{-1}	0.762	0.724
B. Refinement Parameters		
number of reflections	907	1053
nonzero reflections (I > 3.0σ)	898	995
R-index[a]	3.51 %	4.51 %
GOF[b]	0.99	1.01
secondary extinction factor[c], χ	none	18 (7) x 10^{-4}

[a] R- index = $\Sigma \mid |Fo| - |Fc| \mid / \Sigma |Fo|$

[b] GOF = $[\Sigma w (Fo^2 - Fc^2)^2 / (m - s)]^{1/2}$
where $w = [\sigma^2 (F) + |g| F^2]^{-1}$ g = **1**: 0.0085, **2**: 0.0024, **6**: 0.0045, **5**: 0.0030, **4**: 0.0069

[c] $F^* = F [1 + 0.002\chi F^2 / \sin (2\Theta)]^{-1/4}$

Fig. 3. ^{1}H-^{1}H COSY and COLOC experiments of **3** and **7**.

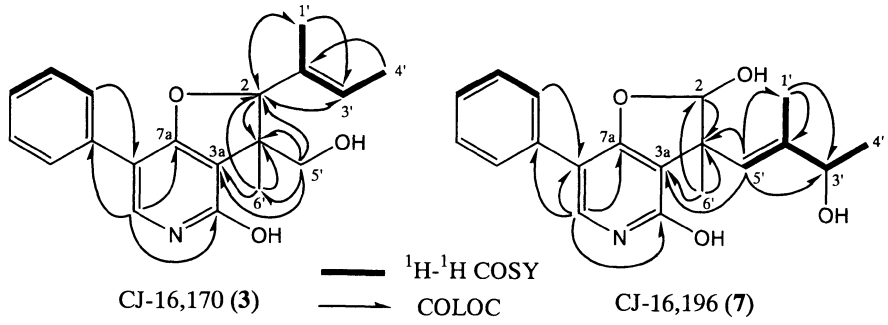
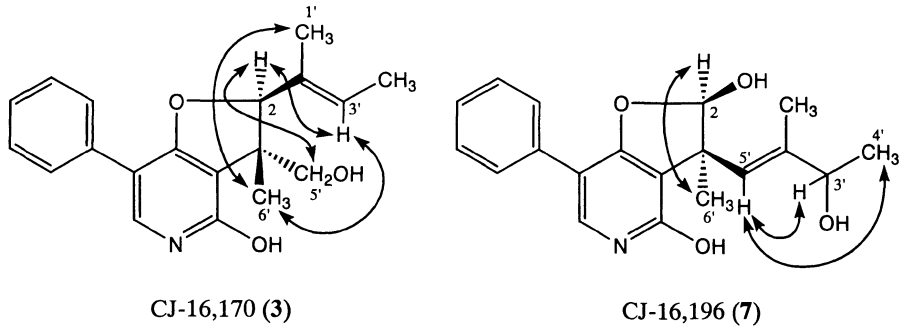


Fig. 4. NOE experiments of **3** and **7**.



The fermentation broths were analyzed by HPLC 1 to 6 days after substrate addition. As a result, *Calonectria decora* (FERM BP-6124), *Cunninghamella echinulata var. elegans* (FERM BP-6126), and *Actinomyces* sp. (FERM BP-6125) were found to produce new furopyridine derivatives that were later isolated from larger scale fermentations and assigned as CP-741,326 (**9**), CP-473,195 (**10**) and CP-473,198 (**11**). The structures of these biotransformation products were readily deduced as shown in Fig. 1 by the comparisons of molecular formula and ^{1}H NMR (Table 4) to those of **1** and **2** as described below.

CP-471,326 (**9**) has an additional oxygen compared to **1**, and the proton NMR spectrum differs from the spectrum of **1** in the absence of a signal for an allylic methyl (δ 1.61) and appearance of a signal for an oxymethylene (δ 4.14). Thus **9** was produced by hydroxylation at the 4′ position.

CP-473,195 (**10**) also has an additional oxygen compared to **1**, and the proton NMR spectrum clearly shows a *para*-substituted benzene moiety (δ 7.27, 2H and δ 6.83, 2H; coupled with 8.5 Hz) instead of mono-substituted benzene (δ 7.43, 2H; δ 7.39, 2H and δ 7.36, 1H). Thus **10** was derived from **1** by hydroxylation at the *para*-position of the benzene ring.

The molecular formula for CP-473,198 (**11**) indicated that **1** was modified by the addition of two hydrogens and an oxygen atom. The proton NMR spectrum of **11** showed the presence of a hydroxymethyl substituent on C-3 in place of the aldehyde moiety, thus accounting for the addition of the 2 hydrogens. Hydroxylation at C-4′ was also revealed by the proton NMR data, and accounted for the addition of oxygen.

Considering the facts that these compounds were simply derived from **1** and the good correspondence of ^{1}H NMR data, the relative stereochemistries of the dihydrofuran ring in **9**, **10**, and **11** were assumed to be the same as those of **1**.

Table 4. ^{1}H NMR chemical shifts of furopyridines **9** to **11**.

No.	CP-471, 326 (**9**)	CP-473,195 (**10**)	CP-473,198 (**11**)
C-2	4.88 (1H, s)	4.90 (1H, s)	4.86 (1H, s)
C-6	7.62 (1H, s)	7.65 (1H, s)	7.82 (1H, s)
C-9	7.50 (2H, d, J = 7.5 Hz)	7.27 (2H, d, J = 8.5 Hz)	7.53 (2H, d, J = 7.5 Hz)
C-10	7.32 (2H, t, J = 7.5 Hz)	6.83 (2H, d, J = 8.5 Hz)	7.38 (2H, t, J = 7.5 Hz)
C-11	7.22 (1H, t, J = 7.5 Hz)		7.28 (1H, t, J = 7.5 Hz)
C-1'	1.57 (3H, s)	1.55 (3H, s)	1.58 (3H, s)
C-3'	5.86 (1H, t, J = 6.0 Hz)	5.77 (1H, q, J = 6.6 Hz)	5.72 (1H, t, J = 6.0 Hz)
C-4'	4.14 (2H, d, J = 6.0 Hz)	1.62 (3H, d, J = 6.6 Hz)	4.14 (2H, d, J = 6.0 Hz)
C-5'	9.55 (1H, s)	9.57 (1H, s)	3.80 (2H, s)
C-6'	1.61 (3H, s)	1.59 (3H, s)	1.53 (3H, s)

Chemical shifts are referred to acetone-d_6 at 2.00 ppm for ^{1}H.

Table 5. Antibacterial activities of CJ-15,696 (**1**) and standard compounds.

Microorganism	MIC (μg/ml)			
	CJ-15,696	Erythromycin	Azithromycin	Vancomycin
Staphylococcus aureus 01A1095	25	>100	>100	0.78
S. aureus 01A1105	25	>100	>100	1.56
S. aureus 01A1120	25	>100	>100	0.39
S. haemolyticus 01E1006	50	100	>100	0.78
Streptococcus agalactiae 02B1023	25	>100	>100	0.39
S. pyogenes 02C1068	6.25	>100	>100	0.39
S. pyogenes 02C1079	12.5	>100	>100	0.2
S. pneumoniae 02J1046	25	>100	>100	0.39
S. pneumoniae 02J1095	6.25	>100	>100	0.31
Enterococcus faecalis 03A1069	12.5	>100	>100	50
Haemophilus influenzae 54A0085	50	3.12	< 0.2	Not tested
H. influenzae 54A0131	50	3.12	< 0.2	>100
Moraxella catarrhalis 87A1055	25	0.78	< 0.2	50
Escherichia coli 51A0266	>100	100	1.56	>100

Antibacterial Activity

The major component CJ-15,696 showed moderate antibacterial activity against some drug-resistant bacteria as shown in the Table 5. Since the other derivatives including the biotransformed products did not show any meaningful activity against the drug-resistant strains *Staphylococcus aureus* 01A1105, *Staphylococcus pyogenes* 02C1068, *Enterococcus faecalis* 03A1069 and *Escherichia coli* 51A0266, their antibacterial activity was not profiled further.

Discussion

When this work was started, the structure of CJ-15,696 (**1**)[7] had not been reported in the literature. Because of this novel structure and its modest activity against some antibiotic-resistant bacterial pathogens, it was of interest to search for analogs either coproduced with CJ-15,696 or made by microbial biotransformation of it. Unfortunately, none of the analogs discovered had any better antibacterial activity than CJ-15,696. In 1997 and 1998, another group described a compound isolated from *Cladobotryum* with the same structure as CJ-15,696 which they called

cladobotryal[9,10]. They reported it had antifungal activity but virtually no antibacterial activity. Besides these report, the partially related structures were found only in a synthetic study[11] and a patent on liqiud crystals[12].

Experimental

Structural Elucidation

Spectral and physico-chemical data for the furopyridine compounds **1**~**8** were obtained by the following instruments: mp (uncorrected), Yanako Micro Melting Point Apparatus; IR, Shimadzu IR-470; 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. Spectral data for the furopyridine compounds **9**~**11** were obtained by the following instruments: UV, Shimadzu UV160U spectrophotometer; NMR, Varian Unity Plus 400 MHz; and FAB-MS, VG Analytical ZAB 2SE high field mass spectrometer. All NMR spectra were measured in acetone-d_6 unless otherwise indicated and peak positions are expressed in parts per million (ppm) based on the reference of acetone peak at δ 2.0 ppm for ^{1}H NMR and δ 30.3 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). All FAB-MS spectra were measured using glycerol-matrix. All physico-chemical and NMR data on compounds **1**~**8** are summarized in Tables 1 and 2.

Single Crystal X-Ray Analysis

A representative crystal was surveyed and a 1 Å data set (maximum sin Θ/λ=0.5) was collected on a Siemens R3RA/v diffractometer. Atomic scattering factors were taken from the International Tables for X-Ray Crystalloagraphy (Vol. IV, pp. 55, 99, 149). All crystallographic calculations were facilitated by the SHELXTL system (Nicolet Instrument Co., 1981). All diffractometer data were collected at room temperature. A trial structure was obtained by direct methods and this trial structure refined by conventional means. Hydrogen positions were calculated wherever possible. The methyl hydrogens and the hydrogens on oxygen were located by difference Fourier techniques. The hydrogen parameters were added to the structure factor calculations but were not refined. The shifts calculated in the final cycles of least squares refinement were all less than 0.1 of the corresponding standard deviations. A final difference Fourier revealed no missing or misplaced electron density. Pertinent crystal, data collection, and refinement are summarized in Table 3, including the final R-index. The refined structure was plotted using the SHELXTL plotting package (Fig. 2). The absolute configuration was not determined in this analysis because no suitable "heavy atom" was present in the structure. The coordinates, anisotropic temperature factors, distances and angles of each structure were submitted as supplementary material.

Microorganisms for Biotransformation

Cultures of *Calonectria decora* (FERM BP-6124), *Cunninghamella echinulata var. elegans* (FERM BP-6126), and *Actinomyces* sp. (FERM BP-6125) were maintained as spore suspensions (FERM BP-6124 and FERM BP-6126) or vegetative mycelium (FERM BP-6125) in 13.3% glycerol stored at −80°C.

Biotransformation Conditions

Biotransformation screening experiments were carried out in a soyflour medium described by R. V. SMITH and J. P. ROSAZZA[13] which contains glucose (20 g), NaCl (5 g), K_2HPO_4 (5 g), yeast extract (5 g), and soy flour (5 g) in 1 liter of deionized water. The mixture was adjusted to pH 7.0 and autoclaved at 121°C for 20 min. Screening was conducted in 16×125 mm test tubes containing 2.5 ml of medium which were inoculated with 0.05 ml of frozen glycerol stock. One day after inoculation, 0.05 ml of a 5 mg/ml solution of CJ-15,696 in dimethysulfoxide (DMSO) was added, giving a final broth concentration of 0.1 mg/ml. Tubes were incubated with shaking at 220 rpm at 28°C for 1 to 6 days.

For isolation of the bioconversion products, cultures FERM BP-6124 and FERM BP-6126 were scaled up into 125 ml Erlenmeyer or 2800 ml Fernbach flasks, containing 25 ml and 250 ml of the soyflour medium, respectively. Each flask was inoculated with a 10 percent preformed inoculum grown 2~3 days in the same medium. For culture FERM BP-6125, the inoculum and biotransformation media described by T. S. CHEN *et al.*[14] were used in the same proportions. CJ-15,696 was added to a final concentration of 0.1 mg/ml medium one day after inoculation and incubation continued 3 days.

Screening for Biotransformation Products

Biotransformation products were detected by HPLC on a Waters Millennium system comprised of a 600 controller, 717 autosampler and a 996 photodiode array detector. Broth samples (2.5 ml) were adjusted to pH 6 and extracted with an equal volume of ethyl acetate. The organic layer was removed and evaporated to dryness with nitrogen, after

which the solids were reconstituted in 1 ml of methanol. Samples were loaded on a 5 μ Inertsil C8 column (4.6×250 mm) and eluted with 20 mM KH_2PO_4, pH 6: acetonitrile (67 : 33) at a flow rate of 1 ml/minute. Eluate was monitored by UV absorbance at 233 nm.

Isolation of Biotransformation Products

Broths (total volumes: 4.2 liters for CP-471,326; 3.2 liters for CP-473,195 and 1.6 liters for CP-473,198) from the bioconversion fermentations were extracted three times with an equal volume of ethyl acetate. The extracts were concentrated to dryness under reduced pressureand then washed with 10 ml of hexane. The hexane fraction was discarded and the remaining extract was dissolved in 1.0 ml DMSO. Partial purification of the desired products was achieved by elution from a 10 g YMC-XQSFAQ100 solid phase extraction cartridge with a step-wise gradient of acetonitrile and water. Fractions containing the materials of interest were first stripped of solvent and then reextracted three times with ethyl acetate and subjected to further purification by HPLC using the system described above with the following modifications: 20×250 mm semi-preparative 5 μ Inertsil C8 column with a 50×20 mm 10 μ C8 Inertsil guard column and a modified mobile phase with a 78 : 22 ratio of 20 mM KH_2PO_4, pH 6 and acetonitrile. Fractions corresponding to the peaks of interest were pooled, stripped of solvent, extracted with ethyl acetate, and dried to yield the final products. Amounts recovered were: CP-471,326 (77.2 mg); CP-473,195 (8.1 mg); and CP-473,198 (35.4 mg).

CP-471,326 (**9**): White powder; molecular formula $C_{19}H_{19}NO_4$; LRFAB-MS *m/z* 326 $[M+H]^+$; HRFAB-MS *m/z* 326.1376 (calcd. for $C_{19}H_{20}NO_4$, 326.1392); $[\alpha]_D^{24}$ +28.4° (*c* 0.022, EtOH); UV λ_{max} (EtOH) nm (ε) 208.0 (33,400), 233.0 (24,200); ^{1}H NMR: see Table 4.

CP-473,195 (**10**): White powder; molecular formula $C_{19}H_{19}NO_4$; LRFAB-MS *m/z* 326 $[M+H]^+$; HRFAB-MS *m/z* 326.1380 (calcd. for $C_{19}H_{20}NO_4$, 326.1392); $[\alpha]_D^{24}$ +75.6° (*c* 0.045, EtOH); UV λ_{max} (EtOH) nm (ε) 211.0 (17,200), 245.0 (11,800); ^{1}H NMR: see Table 4.

CP-473,198 (**11**): White powder; molecular formula $C_{19}H_{21}NO_4$; LRFAB-MS *m/z* 328 $[M+H]^+$; HRFAB-MS *m/z* 328.1534 (calcd. for $C_{19}H_{22}NO_4$, 328.1549); $[\alpha]_D^{24}$ +37.2° (*c* 0.145, EtOH); UV λ_{max} (EtOH) nm (ε) 211.0 (52,000), 234.0 (45,300); ^{1}H NMR: see Table 4.

Test Bacterial Strains

S. aureus 01A1105 (cef^r, gent^r, meth^r, MLS_B^r, pen^r, tet^r, cip^r, van^s) and *S. aureus* 01A1095 (amp^r, cef^r, gent^r, imipenem^s, MLS_B^r, tet^r, van^s) are clinical multidrug-resistant 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 clinical multidrug-resistant strain [cef^r, ery^r, gent^r, chl^r, kan^r, tet^s, van^r], confirmed to have *ermB* gene. *Haemophilus influenzae* 54A0085 and 54A0131 are both type B and trm^r isolates; the former is pen^s whereas the latter is pen^r. *Moraxella catarrhalis* 87A1055 is pen^r and shows intermediate susceptibility to ery. *Escherichia coli* 51A0266 is a generally susceptible strain.

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].

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Superscripts ^{r} and ^{s} mean resistant and sensitive strain, respectively; 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 and van: vancomycin.

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