NEW β-LACTAM ANTIBIOTICS, CARPETIMYCINS C AND D

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Two new carbapenem antibiotics, carpetimycins C and D have been isolated from the culture broth of *Streptomyces* sp. KC-6643, which produced carpetimycins A and B. The structures of carpetimycins C and D have been determined to be (5*R*,6*R*)-3-[2-acetamidoethyl-(*R*)-sulfinyl]-6-(1-hydroxy-1-methylethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid and (5*R*,6*R*)-3-[2-acetamidoethyl-(*R*)-sulfinyl]-6-(1-hydroxysulfonyloxy-1-methylethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, respectively. Studies on the fermentation, isolation and physico-chemical properties of these antibiotics are also described.

In a previous paper[1], we reported that *Streptomyces* sp. KC-6643 produced new carbapenem antibiotics, carpetimycins A and B. These antibiotics have a broad antimicrobial spectrum and show strong inhibitory activity against β-lactamases[2]. The structures and absolute configurations of carpetimycins A and B were determined[3].

Upon further investigation, it has been found that two new carbapenem antibiotics, carpetimycins C and D* are produced simultaneously as minor products, together with penicillin N, olivanic acid derivatives and asparenomycin group antibiotics. This paper describes the fermentation, isolation, physico-chemical properties and structures of these two minor components.

Fermentation

Spores of *Streptomyces* sp. KC-6643 were inoculated into 100 ml of a medium composed of 3.5% starch, 1.5% soybean meal, 0.02% L-cysteine and inorganic salts including 0.31% $Na_2HPO_4 \cdot 12H_2O$, 0.05% KH_2PO_4, 0.05% Na_2SO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 0.0005% $CoCl_2 \cdot 6H_2O$ in a 500-ml Erlenmeyer flask, and cultured at 28°C on a rotary shaker for 72 hours. Four hundred ml of the culture broth were then transferred into a 200-liter fermentor containing 130 liters of a medium consisting of 5.0% starch, 3.0% soybean meal, 2.0% cotton seed oil, 0.02% L-cysteine and inorganic salts as described above. The fermentation was conducted at 28°C under aeration of 130 liters/minute, agitation of 240 rpm and inner pressure of 0.5 kg/cm^2. The production of carpetimycin D was followed by HPLC using a Radial Pak C_{18} (0.8×10 cm, Waters Assoc.). After incubation for 168 hours, the antibiotic concentration reached the maximum (0.4 μg/ml). The concentration of carpetimycin C was so low that it was detectable only in partially purified preparations.

Isolation

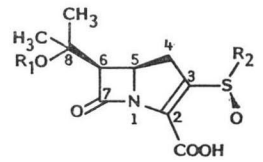
The 168-hour culture broth was filtered using Dicalite (Dicalite Orient Co.) as filter aid. The filtrate (280 liters) was passed through a column (21×83 cm) of Diaion PA-306. The adsorbed antibio-

* Carpetimycins C and D were initially designated as KA-6643 I and KA-6643 J, respectively.

tics were eluted with 20% NaCl. The active eluate was desalted on 30 liters of Diaion HP-20. The desalted eluate was charged on a column (7.5×80 cm) of Amberlite IRA-458. Carpetimycins C and D were eluted from the resin with 0.9% and 20% NaCl, respectively.

The eluate containing carpetimycin C was concentrated, and chromatographed on a column (6×75 cm) of Diaion HP-20 with water. The active eluate was applied on a column (3.5×40 cm) of QAE-Sephadex A-25 and eluted by gradient elution between 0.1% NaCl and 1.1% NaCl. The active eluate was desalted with Diaion HP-20 and purified by gel filtration on Sephadex G-10. The partially purified carpetimycin C (8 mg) was recovered from the eluate by lyophilization. The crude powder was further purified by preparative HPLC on a Bondapak C_{18}/porasil B column (0.8×120 cm, Waters Assoc.) with 2.5% MeOH in 0.1 M phosphate buffer pH 6.8. The active eluate was desalted with Diaion HP-20 and lyophilized to afford the sodium salt (0.5 mg) of pure carpetimycin C.

Fig. 1. Structures of carpetimycins A, B, C and D.



	R_1	R_2
Carpetimycin A	-H	-C(H)=CNHCOCH$_3$
Carpetimycin B	-SO_3H	-C(H)=CNHCOCH$_3$
Carpetimycin C (**1**)	-H	-CH_2CH_2NHCOCH$_3$
Carpetimycin D (**2**)	-SO_3H	-CH_2CH_2NHCOCH$_3$

The eluate containing carpetimycin D was concentrated, and desalted on a column (7×80 cm) of Diaion HP-20. The eluate was extracted with 3% trioctylmethylammonium chloride in dichloromethane, followed by back extraction with 6% sodium iodide. The sodium iodide extract was charged on a column (3.5×40 cm) of DEAE-Sephadex A-25 and eluted by gradient elution between 0.6% NaCl and 2.0% NaCl. The active eluate was subjected to succesive column chromatography with Diaion HP-20 and Sephadex G-10. Lyophilization of active fractions from the last column gave a crude powder (38 mg) of carpetimycin D. Further purification of the crude powder was accomplished by preparative HPLC (Bondapak C_{18}/porasil B) eluted with 0.2 M phosphate buffer pH 6.8 followed by charcoal column chromatography eluted with 50% aqueous acetone. The eluate was lyophilized to give pure carpetimycin D as the disodium salt (5.5 mg).

Physico-chemical Properties

Carpetimycin C

Carpetimycin C sodium salt is a colorless powder melting above 145°C with decomposition. It is soluble in water, methanol, dimethylformamide and dimethyl sulfoxide but insoluble in other common organic solvents. The antibiotic is fairly stable in the pH range of 6.0~7.0.

Chromatographic behaviors are presented in Table 1. The antibiotic was detected on chromatograms by bioautography on agar plates containing 125 μg/ml of ampicillin and seeded with *Escherichia coli* ML 1410 R EC-1. By high-voltage paper electrophoresis with 3,300 V for 20 minutes in 10 mM phosphate buffer pH 7.0, the antibiotic moved to the anode with Rm (relative mobility to penicillin N) 1.0.

Carpetimycin C possessed the following constants, $[\alpha]_D^{23.0}$ −43° (*c* 0.11, H_2O); UV (H_2O) λ_{max} 285 nm (ε 9,900); CD $[\theta]_{218}$ −140,400, $[\theta]_{240}$ −78,700, $[\theta]_{256}$ 0, $[\theta]_{277}$ +58,200, $[\theta]_{360}$ 0 (*c* 0.052, H_2O).

The IR band at 1770 cm^{-1} in the spectrum (Fig. 2) was attributed to a β-lactam carbonyl group. The ^1H NMR spectrum indicated two methyl groups and one acetyl group (Table 2).

Fig. 2. IR spectrum of carpetimycin C sodium salt (KBr).

Table 1. Chromatographic properties of carpetimycins C and D.

Chromatographic medium	Solvent system	Rf	
		Carpetimycin C	Carpetimycin D
TLC	1-Butanol - 2-propanol - water, 7: 7: 6	0.55	0.23
Cellulose F_{254}	EtOH - water, 7: 3	0.75	0.60
PPC (ascending)	Acetonitrile - water, 8: 2	0.27	0.14
Toyo Roshi (No. 51)			
HPLC	MeOH - 0.05 M phosphate buffer,		
Radial Pak C_{18}	pH 6.8, 11: 89 at 1 ml/minute	11.8*	5.2*
(0.8×10 cm)			

* Retention time (minutes).

Table 2. Chemical shifts and coupling constants of ^1H NMR spectra of carpetimycins C and D in D_2O.

Proton	Carpetimycin C		Carpetimycin D	
	ppm	J (Hz)	ppm	J (Hz)
8-CH_3	1.37		1.63	
8-CH_3	1.48		1.72	
6-CH	3.85	5.9	3.94	6.1
5-CH	4.53		4.52	
4-CH_2	3.10	10.0, 17.6	3.07	10.7, 18.1
	3.93	8.8, 17.6	3.87	8.8, 18.1
N-$COCH_3$	2.06		2.02	
N-CH_2	3.32*		3.30*	
S-CH_2	3.67*		3.64*	

* Tentative assignments.

Carpetimycin D

Carpetimycin D sodium salt is a colorless powder melting above 145°C with decomposition. It is similar to carpetimycin C in solubility and stability. The chromatographic behaviors are different from those of carpetimycin C (Table 1). By high-voltage paper electrophoresis as described before, the antibiotic moved to the anode with Rm 2.4.

Carpetimycin D possessed the following constants, $[\alpha]_D^{28.0}$ −34° (c 0.64, H_2O); UV (H_2O) λ_{max} 285 nm (12,600); CD $[\theta]_{219}$ −188,900, $[\theta]_{240}$ −108,800, $[\theta]_{256}$ 0, $[\theta]_{278}$ +72,500, $[\theta]_{360}$ 0 (c 0.049, H_2O).

The IR spectrum (Fig. 3) indicated the presence of sulfate (1270~1220 and 1050 cm^{-1}), which was not observed in that of carpetimycin C. Two methyl signals in the ^1H NMR spectrum shifted approximately 0.2 ppm to lower field compared with that of carpetimycin C (Table 2).

Fig. 3. IR spectrum of carpetimycin D sodium salt (KBr).

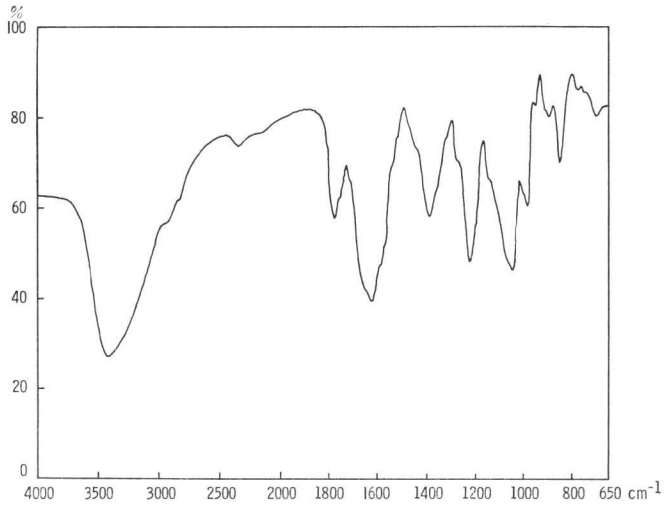


Table 3. Antibacterial activity of carpetimycins C and D compared to that of carpetimycins A and B.

Organisms	MIC (μg/ml)**			
	Carpetimycin C	Carpetimycin D	Carpetimycin A	Carpetimycin B
Staphylococcus aureus 209P JC-1	0.78	6.25	0.39	6.25
Bacillus subtilis ATCC 6633	0.78	6.25	0.2	6.25
Escherichia coli NIHJ JC-2	0.05	1.56	0.05	1.56
E. coli ML 1410 R EC-1*	0.1	3.13	0.2	3.13
Klebsiella pneumoniae PCI 602	0.2	6.25	0.2	6.25
Enterobacter cloacae IID 977	0.2	6.25	0.78	6.25
E. cloacae 3*	1.56	12.5	3.13	12.5
Citrobacter freundii 24*	1.56	6.25	1.56	12.5
Serratia marcescens NHL	0.78	6.25	0.2	6.25
S. marcescens 4*	3.13	12.5	3.13	25
Pseudomonas aeruginosa NCTC 10490	3.13	6.25	6.25	25

* β-Lactamase producing organism.
** 10^6 cells/ml were cultivated in Mueller-Hinton agar.

From these properties carpetimycins C and D were considered to be new carbapenem antibiotics.

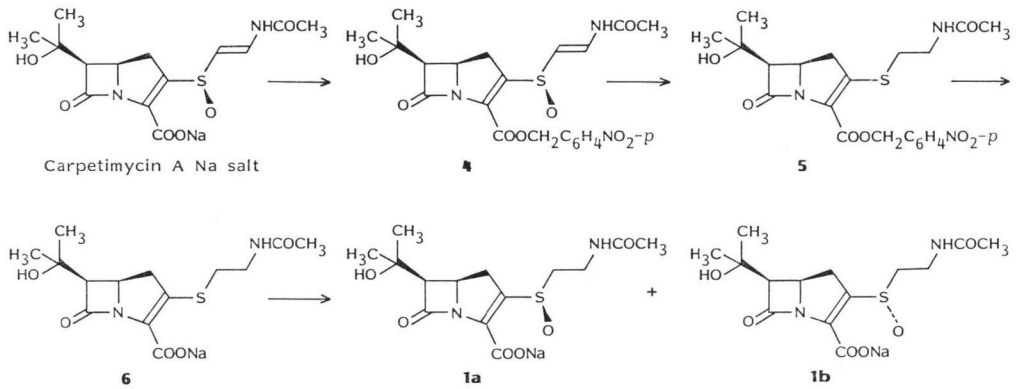
Antibacterial Activity

Antimicrobial activity of carpetimycins C and D was assayed by conventional agar dilution method using Mueller-Hinton agar; carpetimycins A and B were used as control compounds (Table 3). They have strong activity against Gram-positive and Gram-negative bacteria including β-lactamase producing strains. Carpetimycins C and D showed activity comparable to carpetimycins A and B, respectively.

Structural Studies

The molecular formula $C_{14}H_{20}N_2O_6S$ for carpetimycin C (**1**) was derived from the FD mass spectrum of the methyl ester (**3**) and the ^1H NMR spectrum. The ^1H NMR spectral data (Table 2) were essentially correlative to the respective signals of carpetimycin A except that it showed no vinyl protons but four me-

Fig. 4. Synthesis of carpetimycin C from carpetimycin A.



thylene protons. Accordingly, it was suggested that **1** differed from carpetimycin A in side chain at C-3 and had a saturated substituent at the position. Since the signals of the two methylene groups underwent a low-field shift, same as asparenomycin B[4], a sulfoxide group attached to the saturated substituent should be present in **1**. The orientation of β-lactam hydrogens was determined to be *cis* by virtue of the vicinal coupling constants ($J_{5,6}$=5.9 Hz). Both configurations of C-5 and sulfoxide were probably *R* from biogenetic point of view.

From the results mentioned above, the structure of **1** was proposed as shown in Fig. 1.

This assignment was confirmed by synthesis of **1** from carpetimycin A. Carpetimycin A *p*-nitrobenzyl ester (**4**), which was prepared by the same method as described in previous paper[3], was treated with *N*-acetylcysteamine in the presence of sodium hydride to give a *N*-acetylcysteaminyl derivative (**5**). The derivative (**5**) could be converted to the sodium salt (**6**) by catalytic reduction. Oxidation of **6** with *m*-chloroperbenzoic acid gave a sulfoxide (**1a**) and its stereoisomer (**1b**). The former compound was identical with naturally occurring carpetimycin C in all respects. The configuration of sulfoxide in **1** was determined to be *R* by the CD spectrum compared with that of **1b**.

The molecular formula of carpetimycin D (**2**) was derived as $C_{14}H_{20}N_2O_9S_2$ from the elemental analysis and the ^{13}C NMR spectrum. Mild acid hydrolysis of **2** gave **1**. It was suggested from this evidence that the chemical relationship between **1** and **2** was similar to that between carpetimycins A and B, as shown in Fig. 1.

Thus, the structures of carpetimycins C and D have been determined to be (5*R*,6*R*)-3-[2-acetamidoethyl-(*R*)-sulfinyl]-6-(1-hydroxy-1-methylethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid and (5*R*,6*R*)-3-[2-acetamidoethyl-(*R*)-sulfinyl]-6-(1-hydroxysulfonyloxy-1-methylethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, respectively.

Experimental

IR and UV spectra were recorded on Jasco-403G and Hitachi 200-20 spectrophotometer, respectively. ^1H NMR and ^{13}C NMR spectra were measured at 200 MHz on a JEOL FX-200 instrument with Me_4Si as internal standard. CD spectra were recorded on a Jasco J-500A spectropolarimeter. FD mass spectrum was obtained with a JEOL JMS 01SG spectrometer.

Carpetimycin C Methyl Ester (**3**)

To a solution of 1.5 mg of **1** in 0.5 ml of dimethylformamide, 20 μl of methyl iodide was added.

The solution was stirred for 3 hours at room temperature. The reaction mixture was evaporated to dryness under reduced pressure. The residue was chromatographed on a silica gel plate in dichloromethane - methanol (9: 1) to give 0.8 mg of **3**: UV (MeOH) λ_{max} 305 nm; ^{1}H NMR ($CDCl_3$) δ 1.25 (3H, s), 1.53 (3H, s), 2.00 (3H, s), 2.88~3.20 (6H, m), 3.64 (1H, d, J=5.6 Hz), 3.88 (3H, s), 4.26 (1H, m); FD mass m/z 359 (MH^+).

(5*R*,6*R*)-3-(2-Acetamidoethylthio)-6-(1-hydroxy-1-methylethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid *p*-Nitrobenzyl Ester (**5**)

A stirred solution of 70 mg of **4** and 34 mg of *N*-acetylcysteamine in 10 ml of dry dimethylformamide was treated with 5.8 mg of sodium hydride at −40°C under a nitrogen atmosphere for 40 minutes. The reaction mixture was poured into 50 ml of ethyl acetate and washed with 50 ml of 0.1 M phosphate buffer, pH 6.8. The organic layer was dried over magnesium sulfate and evaporated to dryness. The residue was chromatographed on silica gel plates in benzene - acetone (1: 2) to give 29 mg of **5**: $[\alpha]_D^{27}$ −37° (*c* 1, $CHCl_3$); UV (MeOH) λ_{max} 265 nm (18,100), 318 nm (16,400); ^{1}H NMR ($CDCl_3$) δ 1.32 (3H, s), 1.53 (3H, s), 1.99 (3H, s), 5.23 (1H, d, J=13.9 Hz), 5.51 (1H, d, J=13.9 Hz), 7.66 (2H, d, J=8.9 Hz), 8.23 (2H, d, J=8.9 Hz).

Sodium (5*R*,6*R*)-3-(2-Acetamidoethylthio)-6-(1-hydroxy-1-methylethyl)-7-oxo-1-azabicyclo[3.2.0]-hept-2-ene-2-carboxylate (**6**)

A mixture of 29 mg of **5** and 14 mg of 10% palladium over charcoal in 3 ml of 70% dioxane - 0.01 M phosphate buffer, pH 6.8, was pressurized at 4 kg/cm^2 under a hydrogen atmosphere and shaken for 40 minutes. The catalyst was filtered and washed. The filtrate was concentrated and chromatographed on a column (1.5×20 cm) of Diaion HP-20 with water. The fractions containing **6** were collected and lyophilized to give 10 mg of **6**: $[\alpha]_D^{27}$ −77° (*c* 0.14, H_2O); UV (H_2O) λ_{max} 298 nm (8,750); ^{1}H NMR (D_2O) δ 1.35 (3H, s), 1.47 (3H, s), 2.04 (3H, s), 2.98~3.17 (3H, m), 3.45 (2H, m), 3.76 (1H, d, J=5.8 Hz), 3.87 (1H, m), 5.52 (1H, m).

Sodium (5*R*,6*R*)-3-(2-Acetamidoethylsulfinyl)-6-(1-hydroxy-1-methylethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (**1a**), (**1b**)

To a solution of 10 mg of **6** in 100 ml of water, a solution of 22 mg of *m*-chloroperbenzoic acid in 7 ml of 10% aqueous solution of acetonitrile was added and stirred at 0°C. After 90 minutes, the reaction mixture was treated with 9 mg of sodium thiosulfate and poured into 300 ml of 0.01 M phosphate buffer, pH 6.8. The solution was charged on a column (2.5×20 cm) of QAE-Sephadex A-25 and eluted by gradient elution between 0.1% NaCl and 1.1% NaCl. The eluate containing the product was desalted on Diaion HP-20 and lyophilized to give a crude powder. The crude powder was then applied to a column (0.8×120 cm) of Bondapak C_{18}/porasil B and eluted with 2.5% MeOH in 0.1 M phosphate buffer, pH 6.8. Two bioactive fractions were obtained. The latter fraction was desalted with Diaion HP-20 and lyophilized to give **1a**, which was identical with **1** in all respects: $[\alpha]_D^{28}$ −49° (*c* 0.16, H_2O).

The former fraction was also desalted and lyophilized to give **1b**: $[\alpha]_D^{28}$ −158° (*c* 0.25, H_2O); UV (H_2O) λ_{max} 288 nm (9,880); ^{1}H NMR (D_2O) δ 1.40 (3H, s), 1.52 (3H, s), 2.07 (3H, s), 3.20 (1H, dd, J=11.0, 17.2 Hz), 3.23 (3H, m), 3.53 (1H, m), 3.71 (1H, m), 3.84 (1H, d, J=6.4 Hz), 3.89 (1H, dd, J=8.2, 17.2 Hz), 4.59 (1H, m); CD $[\theta]_{205}$ −52,000, $[\theta]_{215}$ 0, $[\theta]_{219}$ +7,800, $[\theta]_{225}$ 0, $[\theta]_{230}$ −4,300, $[\theta]_{234}$ −3,100, $[\theta]_{245}$ −7,000, $[\theta]_{253}$ 0, $[\theta]_{263}$ +12,500, $[\theta]_{273}$ 0, $[\theta]_{293}$ −30,900, $[\theta]_{360}$ 0 (*c* 0.047, H_2O).

Hydrolysis of Carpetimycin D (**2**)

A solution of 10 mg of **2** in 100 ml of 3 mM phosphate buffer, pH 6.9, was heated at 60°C for 4 hours. The hydrolysate was adsorbed on a column (2×20 cm) of QAE-Sephadex A-25 and eluted by gradient elution between 0.1% NaCl and 1.1% NaCl. The eluate was desalted with Diaion HP-20 and lyophilized to give 0.8 mg of **1**, which was identical with the authentic product obtained from the fermentation in physico-chemical and biological properties.

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