PRODUCTION AND ISOLATION OF CEREXINS C AND D

(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. XVIII[1])

JUN'ICHI SHOJI, TOSHIYUKI KATO, KOICHI MATSUMOTO,
YASUO TAKAHASHI and MIKAO MAYAMA

Shionogi Research Laboratory, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka 553, Japan

(Received for publication September 2, 1976)

From the culture broth of *Bacillus cereus* 60-6, in which antibiotic production was improved, a new antibiotic, named cerexin C, was isolated. Similarly, a new antibiotic, cerexin D, was isolated from the culture broth of *Bacillus cereus* Gp-3. Cerexins C and D are closely related to cerexins A and B in their physico-chemical and antimicrobial properties. In fact cerexins C and D are peptides essentially identical with cerexins A and B except for the presence of a lysine residue in place of the γ-hydroxylysine residue.

The bacillus strains, 60-6 and Gp-3, identified as *B. cereus* have been reported to be the producers of cerexins A and B, respectively.[2] Improvement of the fermentation medium was attempted in order to increase the antibiotic production. When the strain 60-6 was cultured in an improved medium, production of a new antibiotic component in addition to cerexin A was recognized accompanying the increased antibiotic production. A similar phenomenon was observed also with the strain Gp-3. The new antibiotic components were isolated and named cerexins C and D, respectively.

In this paper, the production, isolation and characterization of cerexins C and D are discribed.

Production

Spores of the strain 60-6 were inoculated into 120 ml of a medium consisting of glucose 1.0%, peptone 1.0%, meat extract 0.5% and NaCl 0.3%, pH 7.0, in a SAKAGUCHI flask, and cultured for 1 day at 27°C on a reciprocal shaker. Then, 4 ml of the above culture was transferred to a medium consisting of starch 4.5%, soy bean meal 6.0% and $(NH_4)_2SO_4$ 0.3%, pH 7.0, in a SAKAGUCHI flask, which was cultured for 1 day at 27°C on a reciprocal shaker. When the antibiotic produced was measured by pulp diffusion method on a *B. subtilis* assay plate using cerexin A as a standard material, an antibiotic titre of approximate 800 mcg/ml was observed.

Fermentation of the strain Gp-3 was carried out in the same manner, and the production of the antibiotic was assayed as approximately 1,000 mcg/ml using cerexin B as a standard material.

To estimate relative abundance of each antibiotic component, the crude extract of the antibiotics produced was chromatographed on TLC in the manner described in the following section. The separated antibiotics were extracted and their acid hydrolyzates were analyzed with an automatic amino acid analyzer. From the quantities of the amino acids found, the ratio of cerexins A and C in the product of the strain 60-6 was approximately estimated as 4: 1. Similarly, the ratio of cerexins B and D in the product of the strain Gp-3 was estimated as approximately 3: 2.

Isolation

Isolation of cerexins A and C as well as cerexins B and D was carried out in essentially the same manner as described for cerexin A or B,[2] but one example is described here.

About 7 liters of the culture broth of the strain Gp-3 obtained as above was filtered by the aid of Hyflo Super-Cel (100 g) at pH 3.0. The filter cake was extracted with 500 ml of 80% aqueous acetone and then with 500 ml of a mixture of chloroform, methanol and water (3: 7: 2) three times. The extract was concentrated under reduced pressure to a practically aqueous solution, from which the antibiotics were extracted with 500 ml of *n*-butanol three times. The culture filtrate containing the antibiotics in a small amount was discarded. The *n*-butanol extract was washed with dilute sodium bicarbonate, water, dilute hydrochloric acid and water, successively. Concentration of the *n*-butanol solution to a small volume (*ca.* 100 ml) resulted in the formation of a gel-like precipitate. To this, ethanol (*ca.* 100 ml) was added and the mixture was allowed to stand overnight at 4°C to complete the precipitation of the antibiotics. The precipitate was then collected by filtration and washed with ethanol and acetone. The crude mixture of cerexins B and D thus obtained was a colorless powder (4.0 g) with an antibiotic potency of about 90%.

About 250 mg of the crude mixture of cerexins B and D was dissolved in 40 ml of a mixture of chloroform, methanol and 28% aqueous ammonia (2: 2: 1) and applied to four plates of silica gel GF (thickness 750 μ, 20×100 cm), which were developed with chloroform - ethanol - 14% aqueous ammonia (4: 7: 2). Separated zones of cerexins B and D were detected by a UV lamp and extracted with the above solvent mixture. Each extract was evaporated and the antibiotic was extracted with *n*-butanol at pH 2.0 (HCl). The *n*-butanol solution was washed with neutralized water (pH 7.0) and then distilled water, and concentrated to dryness. Cerexins B (70 mg) and D (100 mg) were obtained as colorless amorphous powders.

The same procedure with the culture broth of the strain 60-6 resulted in the preparation of cerexins A and C.

Characterization

Approximate Rf values of cerexins A, C, B and D on TLC with three solvent systems are listed in Table 1. Separation of cerexins A and C as well as that of cerexins B and D was obtained in only one solvent system.

Cerexins C and D are colorless amorphous powders which decompose above *ca.* 185°C. They are soluble in dimethylsulfoxide, dimethylformamide, slightly soluble in aqueous alcohols and aqueous acetone, but insoluble in other organic solvents. In dilute alkaline water, cerexins C and D are less soluble than cerexins A and B. Ninhydrin, DRAGENDORFF's and EHRLICH's reactions are positive.

Table 1. Rf values of cerexins A, C, B and D on thin-layer chromatography (Merck, Silica gel GF)

Solvent system	Rf value			
	A	C	B	D
n-Butanol - acetic acid - water (3: 1: 1)	0.51	0.51	0.58	0.58
Chloroform - ethanol-water (4: 7: 2)	0.51	0.51	0.55	0.55
Chloroform - ethanol - 14% ammonia (4: 7: 2)	0.37	0.41	0.40	0.46

Table 2. Physico-chemical properties of cerexins C and D

	Cerexin C (free form)	Cerexin D (free form)
Anal. Found	C, 52.86; H, 7.54; N, 14.33	C, 54.00; H, 7.27; N, 14.38
Calcd.	C, 52.80; H, 7.82; N, 14.68	C, 53.74; H, 7.56; N, 14.47
	$(C_{63}H_{103}N_{15}O_{18}\cdot 4H_2O)$	$(C_{65}H_{99}N_{15}O_{17}\cdot 2H_2O)$
$[\alpha]_D^{24.5°}$	$+16.4\pm2.6°$ (c 0.214, DMF)	$+11.7\pm1.8°$ (c 0.231, DMF)
$\lambda_{max}^{CH_3OH}$	276 nm ($E_{1cm}^{1\%}$ 35.1); 283 nm ($E_{1cm}^{1\%}$ 38.1) 290.5 nm ($E_{1cm}^{1\%}$ 32.9)	275 nm ($E_{1cm}^{1\%}$ 33.3); 283 nm ($E_{1cm}^{1\%}$ 36.1) 290.5 nm ($E_{1cm}^{1\%}$ 31.3)

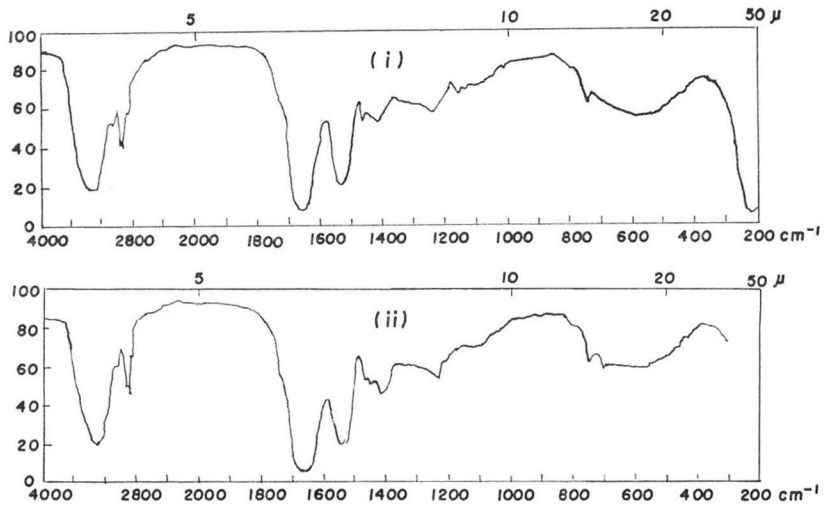
Table 3. Antimicrobial spectra of cerexins C and D compared with cerexin A

Test organism	MIC (mcg/ml)		
	A	C	D
Bacillus subtilis PCI 219	3.13	6.25	6.25
Staphylococcus aureus FDA 209P JC-1 TOHO	3.13	6.25	12.5
Streptococcus pneumoniae type I	0.78	1.56	3.13
Streptococcus pyogenes C-203	3.13	3.13	6.25
Escherichia coli	>50	>50	>50
Klebsiella pneumoniae	>50	>50	>50
Salmonella typhimurium	>50	>50	>50
Pseudomonas aeruginosa	>50	>50	>50

Obtained by the usual agar dilution method.

Elemental analyses, $[\alpha]_D$ values and ultraviolet absorptions are listed in Table 2. The calculations for elemental analyses are based on the molecular formulae anticipated from the constituents found in their acid hydrolyzates. The infrared absorption spectra are shown in Fig. 1.

Fig. 1. Infrared absorption spectra of cerexins C and D (KBr)
i) Cerexin C
ii) Cerexin D



These physico-chemical properties of cerexins C and D are closely similar to those of cerexins A and B.[2]

The antimicrobial spectra of cerexins C and D, and that of cerexin A measured simultaneously are shown in Table 3. Cerexins C and D are active against Gram-positive bacteria, but they are somewhat less active than cerexin A.

The Constituent Amino Acids and Fatty Acids

Cerexins C and D were hydrolyzed with hydrochloric acid and the hydrolyzates were analyzed with an automatic amino acid analyzer as described in the preceding papers.[1,3,4,5] The amino acids found are listed in Table 4. It should be noted that cerexins C and D contain lysine, but not γ-hydroxylysine. All the amino acids except γ-hydroxylysine contained in cerexins A and B are found in cerexins C and D, respectively.

Table 4. Amino acid analyses of cerexins C and D

		Asp	aThr	Ser	Gly	Val	aIle	Phe	Trp	Lys	Amm
Cerexin C	Found (μmoles/mg)	2.08	0.61	0.49		1.21	0.59		0.60	0.65	2.06
	(moles/mole)	(3)	(1)	(1)		(2)	(1)		(1)	(1)	(3)
Cerexin D	Found (μmoles/mg)	1.79	0.52		0.56	0.60	0.61	0.58	0.54	0.59	1.72
	(moles/mole)	(3)	(1)		(1)	(1)	(1)	(1)	(1)	(1)	(3)

Fig. 2. Gas chromatograms of fatty acid methyl esters from 1 hour-hydrolyzates of cerexins A, B, C and D.
Recorded with a Perkin-Elmer Model 881 equipped with a steel column (6 feet, 1/8-inch diameter) packed with 15% diethylene glycol succinate polymer on Chromosorb W 80~100 mesh, at 160°C.

i-$C_{10}h^3$: β-Hydroxy isodecanoic acid
n-$C_{10}h^3$: β-Hydroxy decanoic acid
i-$C_{11}h^3$: β-Hydroxy isoundecanoic acid
a-$C_{11}h^3$: β-Hydroxy anteisoundecanoic acid

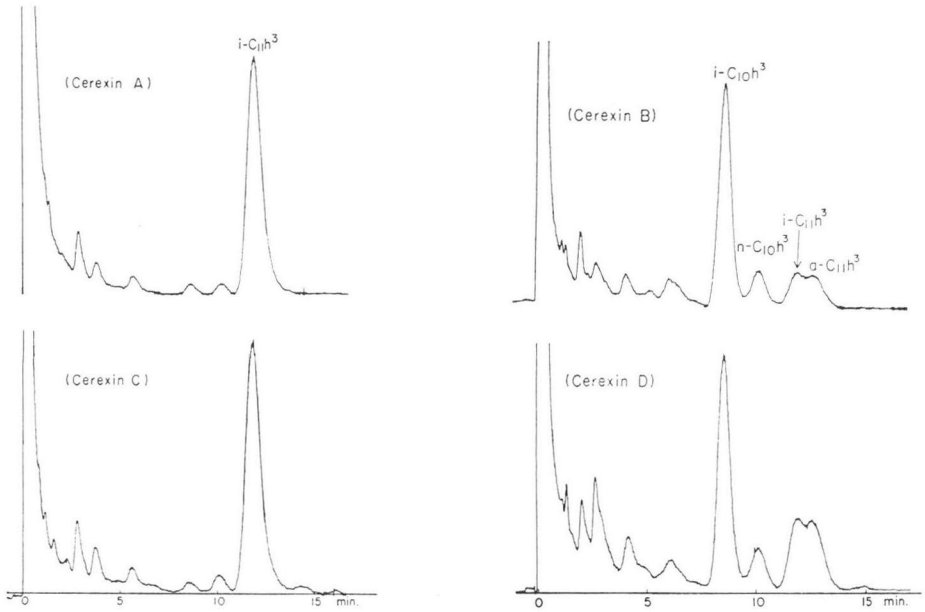
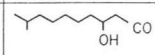
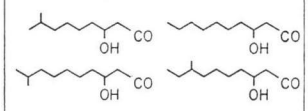
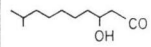
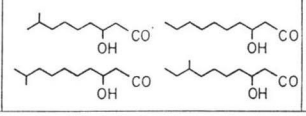


Fig. 3. Structures of cerexins A, B, C and D

FA → Asn → Val → [X] → Asn → Asn → [Y] → αThr → [Z] → Trp → Ile

	FA	X	Y	Z
Cerexin A		Val	γHyl	Ser
Cerexin B		Phe	γHyl	Gly
Cerexin C		Val	Lys	Ser
Cerexin D		Phe	Lys	Gly

The ethereal extracts from 1-hour hydrolysis of cerexins C and D were analyzed by gas chromatography as described in the previous papers.[1,5] When the gas chromatograms were compared with those of cerexins A and B, close similarities were observed between cerexins A and C, and also between cerexins B and D as illustrated in Fig. 2. This means that the fatty acid constituents of cerexins A and C, and also of cerexins B and D, are essentially the same.

From the above, it was concluded that cerexins C and D are the acylpeptides analogous to cerexins A and B, respectively, in which the γ-hydroxylysine residue is replaced by a lysine residue. The structures of cerexins A, B, C and D can be summarized as shown in Fig. 3.

References

1) Shoji, J. & T. Kato: The structure of cerexin B (Studies on antibiotics from the genus *Bacillus*. XVII). J. Antibiotics 29: 1275~1280, 1976
2) Shoji, J.; H. Hinoo, Y. Wakisaka, K. Koizumi, M. Mayama, S. Matsuura & K. Matsumoto: Isolation of two new related peptide antibiotics, cerexins A and B (Studies on antibiotics from the genus *Bacillus*. I). J. Antibiotics 28: 56~59, 1975
3) Shoji, J. & H. Hinoo: Chemical characterization of new antibiotics, cerexins A and B (Studies on antibiotics from the genus *Bacillus*. II). J. Antibiotics 28: 60~63, 1975
4) Shoji, J. & T. Kato: The amino acid sequence of cerexin A (Studies on antibiotics from the genus *Bacillus*. VII) J. Antibiotics 28: 764~769, 1975
5) Shoji, J.; T. Kato & R. Sakazaki: The total structure of cerexin A (Studies on antibiotics from the genus *Bacillus*. XVI). J. Antibiotics 29: 1268~1274, 1976