THE STRUCTURE OF CEREXIN B

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

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The structure of cerexin B was examined. The constituent fatty acids were elucidated by gas chromatography and mass spectrometry to be β-hydroxy isodecanoic acid, β-hydroxy decanoic acid, β-hydroxy isoundecanoic acid and β-hydroxy anteisoundecanoic acid. The configurations of constituent amino acids were determined as asparagine (2D, 1L), valine (D), phenylalanine (D), *allo*-threonine (D), tryptophan (D), and *allo*-isoleucine (D) from their optical activities. Treatment with conc. hydrochloric acid cleaved at the γ-hydroxylysine residue to give two peptide fragments, one of which (the N-terminal side) was then deacylated with Polymyxin Acylase. Their amino acid sequences were examined by EDMAN degradation. From the results and analogy to cerexin A, the structure of cerexin B was deduced.

Cerexin B is an antibiotic active against Gram-positive bacteria produced by *Bacillus cereus* Gp-3.[2] The antibiotic is an amphoteric acylpeptide yielding on hydrolysis Asp (3), Val (1), Phe (1), γ-Hyl* (1), aThr** (1), Gly (1), Trp (1), aIle (1) and ammonia (3), with an approximate empirical formula $C_{69}H_{107}N_{15}O_{17}$.[3] Cerexin B is closely related to cerexin A: The essential difference between them has been pointed out[3] to be replacement of serine and one valine residue in cerexin A by glycine and phenylalanine in cerexin B.

The fatty acid constituent of cerexin B was examined by the method described in the preceding paper[1]; *i.e.* the ethereal extracts of the acid hydrolyzates obtained by hydrolysis for 1 hour or 20 hours were methylated and analyzed with gas chromatography. The hydrogenated product of the 20-hour hydrolytic preparation was also analyzed. The preparation from 1-hour hydrolyzate gave essentially four peaks on the chromatogram shown in Fig. 1, whereas the chromatogram obtained from 20-hour hydrolyzate was complicated by formation of α,β-unsaturated fatty acids and butyrolactones resulting from destruction of β-hydroxy fatty acids.[1,4] From the chromatogram of the hydrogenated product, methyl isodecanoate, methyl decanoate, methyl isoundecanoate and methyl anteisoundecanoate were tentatively identified. Thus, the presence of β-hydroxy isodecanoic acid (abbreviated as *i*-$C_{10}h^3$)***, β-hydroxy decanoic acid (*n*-$C_{10}h^3$), β-hydroxy isoundecanoic acid (*i*-$C_{11}h^3$) and β-hydroxy anteisoundecanoic acid (*a*-$C_{11}h^3$) in cerexin B was assumed. Moreover, when the chromatogram of the preparation obtained by 1-hour hydrolysis of cerexin B was compared with that of antibiotic EM49, which has been reported to contain *i*-$C_{10}h^3$, *n*-$C_{11}h^3$ and *a*-$C_{11}h^3$,[4] a good agreement in retention times with the three peaks was observed (Fig. 1). The identity of these fatty acids was further confirmed with gas chromatography-mass spectrometry. The spectra from the four peaks of the preparation from a 1-hour hydrolyzate of cerexin B are illustrated in Fig. 2. In each spectrum, the peak of M−18(H_2O) and a base peak

* γ-Hyl: L-*threo*-γ-Hydroxylysine.

** It has been cited as Thr in the previous report.[3]

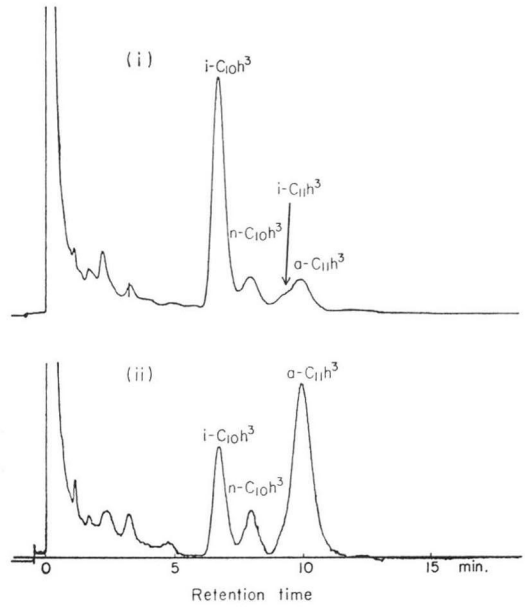
*** h^3 means β-hydroxylated fatty acid.

(103), which is characteristic for β-hydroxy fatty acids,[1] were observed. The relative abundance of these fatty acids in cerexin B was approximately estimated as *i*-$C_{10}h^3$ (66%), *n*-$C_{10}h^3$ (12%), *i*-$C_{11}h^3$ (8%) and *a*-$C_{11}h^3$ (14%) from the peak areas, though it varied somewhat in preparations from different fermentation media.

Thus, cerexin B is heterogeneous with respect to the fatty acid constituents and is considered to be a complex of four acylpeptides, whose peptide parts are common. A molecular formula, $C_{65}H_{90}N_{15}O_{18}$, is anticipated for the acylpeptide with *i*-$C_{10}h^3$ or *n*-$C_{10}h^3$ (which represent *ca.* 80% of the complex), and the calculations for this formula nearly agreed with the elemental analyses of cerexin B and the hydrochloride,[3] as shown in Table 1.

Fig. 1. Gas chromatograms of fatty acid methyl esters from cerexin B and antibiotic EM49

i) Cerexin B
ii) Antibiotic EM49



The constituent amino acids of cerexin B were isolated from the hydrolyzate by combination of preparative paper chromatography, the use of a porous polymer Amberlite XAD-2 column and an anion-exchange resin Amberlite IR-4B column. Valine, phenylalanine, *allo*-threonine, tryptophan, and *allo*-isoleucine were deduced to be D-forms from their ORD curves. As the molecular rotation of the aspartic acid isolated was about one third of that of the pure D-isomer the three asparagine residues contained were deduced to be two D-forms and one L-form.

Treatment with conc. hydrochloric acid[5] selectively cleaved the bond at C-terminal side of the γ-hydroxylysine residue in cerexin B. The C-terminal peptide fragment was elucidated to be aThr→Gly→Trp→aIle by EDMAN degradation. Thus, half of the peptide antibiotic was deduced to be γ-Hyl→aThr→Gly→Trp→aIle as in the case of cerexin A.[5] However, deacylation of cerexin B with Polymyxin Acylase[5,6] was unsuccessful, probably because the solubility of cerexin B in the buffer solution used in the enzyme reaction was lower than that of cerexin A. The use of Tween-80 or carboxylmethyl cellulose to disperse the substrate (cerexin B) did not give any improvement for the enzyme reaction. Moreover, N-succinyl cerexin B, which was soluble in the buffer solution, was unsusceptible to the acylase. Therefore, we tried the enzyme reaction on the N-terminal peptide fragment from the above-described cleavage reaction and obtained the deacylated product in an approximate 10% yield. EDMAN degradation with this peptide proceeded up to third step, indicating a sequence Asn→Val→Phe.

Table 1. Calculations for molecular formulas and analytical data of cerexin B and the hydrochloride.[3]

Cerexin B (free form). *Anal.* Found	C. 55.36; H, 7.28; N, 14.39
$C_{65}H_{99}N_{15}O_{18}\cdot 2H_2O$	C, 55.18; H, 7.34; N, 14.86
Hydrochloride. *Anal.* Found	C, 54.18; H, 7.12; N, 13.98; Cl, 3.14
$C_{65}H_{99}N_{15}O_{18}\cdot HCl\cdot 2H_2O$	C, 53.80; H, 7.22; N, 14.48; Cl, 2.44

Thus, N-terminal side of cerexin B was deduced to be FA*→Asn→Val→Phe. The presence of two Asn residues between the above two sequences can be assumed for the same reason as in the case of cerexin A.[5]

These experiments provided the evidence for concluding the structure of cerexin B, except for the chiralities of individual residues of the three Asn residues and the γ-Hyl residue. However, it can be assumed that their chiralities are the same as those in cerexin A. From this consideration, we propose the structure of cerexin B as shown in Fig. 3.

Experimental

Elucidation of constituent fatty acids

The experimental methods are the same as those described in the preceding paper.[1]

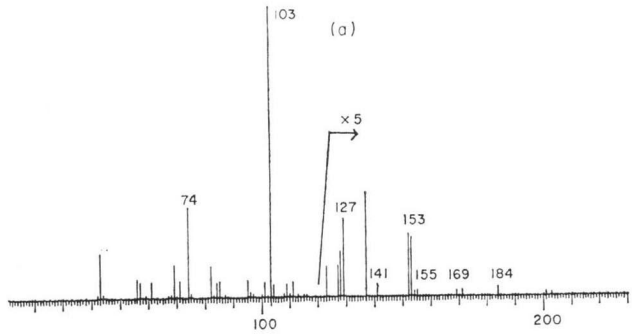
About 10 mg each of cerexin B and antibiotic EM49 was hydrolyzed with constant-boiling hydrochloric acid at 110°C for 1 hour or 20 hours. The ethereal extracts of the hydrolyzates were methylated and analyzed by described procedures.[1] The gas chromatograms of the preparations from 1-hour hydrolysis run at 160°C are shown in Fig. 1. A second preparation from cerexin B after 1-hour hydrolysis was subjected to GC-MS which was measured with a Hitachi RMU-6 gas chromatogram-mass spectrometer. The spectra obtained are illustrated in Fig. 2.

The preparations from 20-hour hydrolysis were hydrogenated and methylated.[1] In the chromatography of the hydrogenated product from cerexin B run at 110°C, four peaks observed at retention times of 6.1, 7.6, 9.7 and 10.2 minutes, were tentatively identified with methyl isodecanoate, methyl

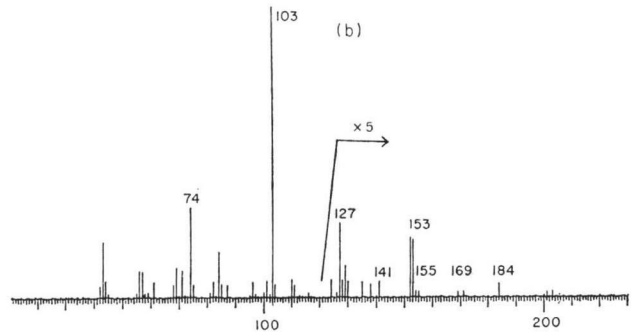
* FA means fatty acyl groups.

Fig. 2. Mass spectra of fatty acid methyl esters from cerexin B

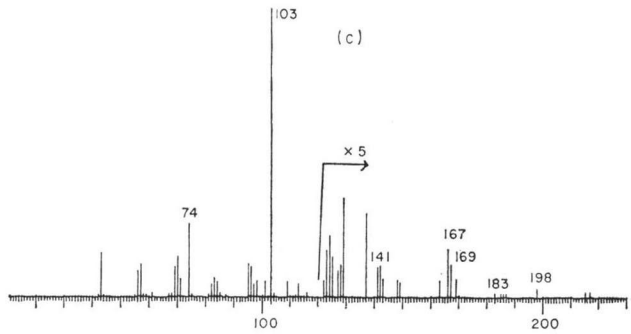
a) Methyl β-hydroxy isodecanoate



b) Methyl β-hydroxy decanoate



c) Methyl β-hydroxy isoundecanoate



d) Methyl β-hydroxy anteisoundecanoate

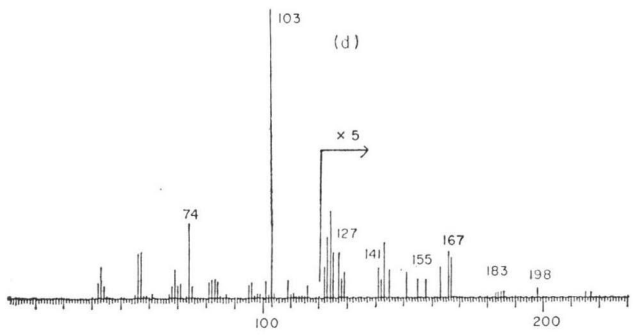
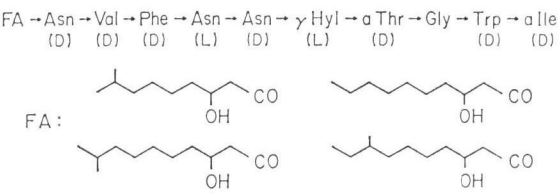


Fig. 3. Structure of cerexin B



decanoate, methyl isoundecanoate and methyl anteisoundecanoate, respectively, by comparison with reference fatty acid methyl esters. A preparation from antibiotic EM49 was used as reference in addition to methyl isooctanoate, methyl anteisononanoate (prepared from polymyxin E), methyl isododecanoate, methyl anteisotridecanoate (prepared from amphomycin), and commercially available normal fatty acid methyl esters.

Isolation of the constituent amino acids

Cerexin B (250 mg) was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. The hydrolyzate was extracted with ethyl ether. The aqueous residue was concentrated to dryness and then subjected to preparative paper chromatography carried out on two sheets of Toyo Roshi No. 51, 60×60 cm, with *n*-butanol - acetic acid - water (4: 1: 2) developed in descending manner for 20 hours. Four ninhydrin-positive zones were cut out and extracted with water. The fastest moving zone contained *allo*-isoleucine, phenylalanine and a trace amount of tryptophan, the second zone a trace amount of tryptophan and valine, the third zone *allo*-threonine, glycine and aspartic acid, and the last zone γ-hydroxylysine and its γ-lactone.

The first fraction containing *allo*-isoleucine, phenylalanine and a trace amount of tryptophan was dissolved in 5 ml of water, neutralized to pH 7.0 and placed on a column of a porous polymer, Amberlite XAD-2 (1.7×31 cm). Upon washing the column with water, *allo*-isoleucine passed through the column and appeared in the fraction of 42~84 ml, whereas phenylalanine was slightly retarded by the resin and eluted in the fraction of 140~252 ml. Each fraction was adsorbed on a small column of Dowex 50×8 (NH_4), which was washed with water and then eluted with 0.3 N NH_4OH. Lyophilization of the eluates gave phenylalanine (13 mg) and *allo*-isoleucine (16 mg) as colorless powders.

The second zone containing a trace amount of tryptophan and valine was treated with an XAD-2 column in essentially the same manner as above, resulting in the preparation of valine as colorless powder (13 mg).

The third zone containing *allo*-threonine, glycine and aspartic acid was applied to a sheet of Toyo Roshi No. 51, 60×60 cm, which was developed with *t*-butanol - methyl ethyl ketone - 28% ammonium hydroxide - water (4: 3: 1: 2) in descending manner for 20 hours. Two separated zones were detected by ninhydrin coloration and extracted with water. The extract from the upper zone (Rf *ca.* 0.50) was treated with a small column of Dowex 50×8 as above, resulting in the preparation of *allo*-threonine as a colorless powder (7 mg). Paper chromatography with *n*-butanol - acetone - 28% ammonium hydroxide - water (8: 1: 1: 6, upper layer)[8] confirmed this specimen to be the *allo*-isomer. The extract from the lower zone (Rf *ca.* 0.2~0.3) was then adsorbed on an Amberlite IR-4B (acetate) column at pH 7.0. Glycine passed through the column and the effluent was treated with a column of Dowex 50×8 to give glycine as a colorless powder (14 mg). The aspartic acid adsorbed on the column was eluted with 0.3 N NH_4OH. After concentration to dryness, it was dissolved in water slightly acidified with hydrochloric acid. Lyophilization gave aspartic acid hydrochloride as a colorless powder (34 mg).

For the isolation of tryptophan, some 100 mg of cerexin B was hydrolyzed with constant-boiling hydrochloric acid containing 4% thioglycolic acid in a vacuum sealed tube at 100°C for 20 hours. The hydrolyzate was extracted twice with ethyl ether to remove thioglycolic acid and concentrated to dryness. The residue was dissolved in a small amount of water, neutralized to pH 7.0 and placed on an XAD-2 column (0.85×27 cm), which was then washed with distilled water slowly. Tryptophan was

retarded by the resin, but eluted in the fraction of 90~250 ml. Lyophilization of the eluate gave tryptophan as a colorless powder (5 mg).

ORD curves were recorded with a spectropolarimeter JASCO Model ORD/UV-5. With the above specimens, the following values were obtained.

Valine: ORD: $[\phi]_{250}$–780, $[\phi]_{225}$–2720, $[\phi]_{209}$ 0 (*c* 0.2268, 0.5 N HCl)

allo-Isoleucine: ORD: $[\phi]_{250}$–1030, $[\phi]_{225}$–3280, $[\phi]_{215}$–1900 (*c* 0.2345, 0.5 N HCl)

Phenylalanine: ORD: $[\phi]_{250}$–890, $[\phi]_{230}$–4770, $[\phi]_{220}$–2670 (*c* 0.2082, 0.5 N HCl)

allo-Threonine: ORD: $[\phi]_{250}$–810, $[\phi]_{225}$–2400, $[\phi]_{215}$–940 (*c* 0.2035, 0.5 N HCl)

Tryptophan: ORD: $[\phi]_{300}$–730, $[\phi]_{290}$–1300, $[\phi]_{265}$ 0, $[\phi]_{235}$–3400, $[\phi]_{227}$ 0 (*c* 0.2196, 0.5 N HCl)

Aspartic acid hydrochloride: $[M]_D^{23.5}$–7.3±1.4° (*c* 0.533, 5 N HCl)

The values of L-tryptophan and L-aspartic acid are cited in our previous report.[5]

Amino acid sequence

The experimental methods were the same as those described in our previous report.[5]

Cerexin B (6 mg) was dissolved in 0.4 ml conc.hydrochloric acid containing 4% thioglycolic acid in a test tube, which was then evacuated, sealed and allowed to stand for 90 hours at 4°C. The reaction mixture was extracted with ether and the residual solution was lyophilized. The residue was subjected to TLC on a Silica gel GF plate with *n*-butanol - acetic acid - water (4: 1: 2). Two ninhydrin-positive zones (Rf *ca.* 0.59 and *ca.* 0.39) were cut out and extracted with aqueous methanol. The Rf 0.59 substance (peptide fragment of C-terminal side) was examined by EDMAN degradation, and the sequence, aThr→Gly→Trp→aIle, was revealed as follows:

	PTH-Amino acid	Amino acid found (in ratio)			
		aThr	Gly	Trp	aIle
Original peptide	—	1.15	1.22	0.64	1.00
Step 1	aThr,* ΔThr	0.05	1.17	0.49	1.00
Step 2	Gly	0.05	0.17	0.24	1.00
Step 3	—	0.00	0.00	0.00	+**

* PTH-Thr was used as reference for the identification.

** Analyzed before hydrolysis.

The Rf 0.39 substance (N-acylated peptide fragment of N-terminal side) was heated in 0.1 N NaOH at 80°C for 10 minutes to cleave the lactone linkage possibly formed within the γ-hydroxylysine residue. It was then neutralized with dry ice (CO_2) and diluted with 0.5 ml of water and 1.0 ml of 0.1 M phosphate buffer, pH 8.0. Ten mg of Polymyxin Acylase was added to the solution, which was then stirred for 6 days at 37°C. The reaction mixture was centrifuged (3,000 rpm, 10 minutes) and the supernatant was lyophilized. The residue was subjected to paper chromatography with *n*-butanol - acetic acid - water (4: 1: 2), and a wide ninhydrin-positive zone (Rf *ca.* 0.30) was cut out and extracted with aqueous methanol. EDMAN degradation on this specimen revealed the sequence, Asn→Val→Phe as follows:

	PTH-Amino acid	Amino acid found (in ratio)			
		Asp	Val	Phe	γ-Hyl
Original peptide	—	3.01	1.09	1.00	0.12
Step 1	Asn	2.43	1.01	1.00	0.08
Step 2	Val	2.50	0.13	1.00	0.13
Step 3	Phe	2.00	0.12	0.20	0.08

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References

1) 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
2) SHOJI, J.; H. HINOO, Y. WAKISAKA, K. KOIZUMI, S. MATSUURA & K. MATSUMOTO: Isolation of two 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) PARKER, W. L. & M. L. RATHNUM: EM49, a new peptide antibiotic. II. Chemical characterization. J. Antibiotics 26: 449~456, 1973
5) 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
6) SHOJI, J. & T. KATO: The structure of brevistin (Studies on antibiotics from the genus *Bacillus*. X). J. Antibiotics 29: 380~389, 1976