THE STRUCTURE OF CEREXIN B
(STUDIES ON ANTIBIOTICS FROM THE GENUS BACILLUS. XVII)

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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 (1),
phenylalanine (1), allo-threonine (1), tryptophan (1), and allo-isoleucine (1) 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), alle (1) and ammonia (3), with an approximate empirical formula C_{69}H_{107}-
N_{13}O_{17}.3) Cerexin B is closely related to cerexin A: The essential difference between them has been
pointed out to be replacement of serine and one valine residue in cerexin A by glycine and phenylala-
nine in cerexin B.

The fatty acid constituent of cerexin B was examined by the method described in the preceding
paper; 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 anteisoundeco-
nanoic 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_{10}h^{3} and a-C_{11}h^{3}, 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_{2}O) and a base peak

* γ-Hyl: L-threo-γ-Hydroxylysine.
** It has been cited as Thr in the previous report.8)
*** h^{3} means β-hydroxylated fatty acid.
(103), which is characteristic for $\beta$-hydroxy fatty acids,\(^1\) were observed. The relative abundance of these fatty acids in cerexin B was approximately estimated as $i\text{-C}_{10}h^3$ (66%), $n\text{-C}_{10}h^3$ (12%), $i\text{-C}_{11}h^3$ (8%) and $\alpha\text{-C}_{11}h^3$ (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, $C_{65}H_{99}N_{15}O_{18}$, is anticipated for the acylpeptide with $i\text{-C}_{10}h^3$ or $n\text{-C}_{10}h^3$ (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.

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\(^4\) selectively cleaved the bond at C-terminal side of the $\gamma$-hydroxylysine residue in cerexin B. The C-terminal peptide fragment was elucidated to be $\alpha\text{-Thr}\rightarrow\text{Gly}\rightarrow\text{Trp}\rightarrow\text{alle}$ by EDMAN degradation. Thus, half of the peptide antibiotic was deduced to be $\beta\text{-Hyl}\rightarrow\alpha\text{-Thr}\rightarrow\text{Gly}\rightarrow\text{Trp}\rightarrow\text{alle}$ as in the case of cerexin A.\(^5\) However, deacylation of cerexin B with Polymyxin Acylase\(^6\) 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 carboxymethyl 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$\rightarrow$Val$\rightarrow$Phe.

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**Table 1. Calculations for molecular formulas and analytical data of cerexin B and the hydrochloride.\(^3\)**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Molar Formula</th>
<th>C.</th>
<th>H.</th>
<th>N.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerexin B (free form)</td>
<td>$C_{65}H_{99}N_{15}O_{18}\cdot2H_2O$</td>
<td>55.36</td>
<td>7.28</td>
<td>14.39</td>
</tr>
<tr>
<td>Hydrochloride. Cerexin B</td>
<td>$C_{65}H_{99}N_{15}O_{18}\cdotHCl\cdot2H_2O$</td>
<td>55.18</td>
<td>7.34</td>
<td>14.86</td>
</tr>
<tr>
<td>Cerexin B (free form)</td>
<td>$C_{65}H_{99}N_{15}O_{18}\cdot2H_2O$</td>
<td>54.18</td>
<td>7.12</td>
<td>13.98</td>
</tr>
<tr>
<td>Hydrochloride. Cerexin B</td>
<td>$C_{65}H_{99}N_{15}O_{18}\cdotHCl\cdot2H_2O$</td>
<td>53.80</td>
<td>7.22</td>
<td>14.48</td>
</tr>
</tbody>
</table>

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

i) Cerexin B

ii) Antibiotic EM49
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 \(\gamma\)-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 hydrolysates were methylated and analyzed by described procedures.\(^3\) 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

\[\text{FA means fatty acyl groups.}\]
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 isoctanoate, 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 X 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 x 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 x 8 (NH₄), which was washed with water and then eluted with 0.3 N NH₄OH. 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 x 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 x 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)9 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 x 8 to give glycine as a colorless powder (14 mg). The aspartic acid adsorbed on the column was eluted with 0.3 N NH₄OH. 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 x 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: \([\phi]_{250}^{250} = 780, [\phi]_{269}^{269} = 2720, [\phi]_{289}^{289} = 0\) (c 0.2268, 0.5 N HCl)

allo-Isoleucine: ORD: \([\phi]_{250}^{250} = 1030, [\phi]_{269}^{269} = 3280, [\phi]_{289}^{289} = 1900\) (c 0.2345, 0.5 N HCl)

Phenylalanine: ORD: \([\phi]_{250}^{250} = 890, [\phi]_{269}^{269} = 4770, [\phi]_{289}^{289} = 2670\) (c 0.2082, 0.5 N HCl)

allo-Threonine: ORD: \([\phi]_{250}^{250} = 810, [\phi]_{269}^{269} = 2400, [\phi]_{289}^{289} = 940\) (c 0.2035, 0.5 N HCl)

Tryptophan: ORD: \([\phi]_{350}^{350} = 730, [\phi]_{269}^{269} = 1300, [\phi]_{289}^{289} = 0, [\phi]_{289}^{289} = 3400, [\phi]_{289}^{289} = 0\) (c 0.2196, 0.5 N HCl)

Aspartic acid hydrochloride: \([M]_{D}^{20} = -7.3 \pm 1.4^\circ\) (c 0.533, 0.5 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->alle, was revealed as follows:

<table>
<thead>
<tr>
<th>PTH-Amino acid</th>
<th>Amino acid found (in ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aThr</td>
<td>1.15</td>
</tr>
<tr>
<td>Step 1</td>
<td>aThr,* ΔThr</td>
</tr>
<tr>
<td>Step 2</td>
<td>Gly</td>
</tr>
<tr>
<td>Step 3</td>
<td>—</td>
</tr>
</tbody>
</table>

* 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 examined by Edman degradation, and the sequence, Asn->Val->Phe as follows:

<table>
<thead>
<tr>
<th>PTH-Amino acid</th>
<th>Amino acid found (in ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>Val</td>
</tr>
<tr>
<td>Original peptide</td>
<td>3.01</td>
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<tr>
<td>Step 1</td>
<td>Asn</td>
</tr>
<tr>
<td>Step 2</td>
<td>Val</td>
</tr>
<tr>
<td>Step 3</td>
<td>Phe</td>
</tr>
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Acknowledgement

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References