CHEMICAL CHARACTERIZATION OF NEW ANTIBIOTICS, CEREXINS A AND B

(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. II)

JUN'ICHI SHOJI and HIROSHI HINOO

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

(Received for publication July 12, 1974)

Acid hydrolysis revealed that the antibiotic cerexin A is constructed with aspartic acid (3), threonine (1), serine (1), valine (2), *allo*-isoleucine (1), γ-hydroxylysine (1), tryptophan (1), and a variety of fatty acid residues. The essential difference between cerexins A and B is concluded to be replacement of serine and one valine residue in cerexin A by glycine and phenylalanine in cerexin B. Isolation of a new amino acid L-*threo*-γ-hydroxylysine is also described.

In the preceding paper,[1] we reported the isolation of new antibiotics cerexins A and B, which were suggested to be peptides containing tryptophan residuces by IR and UV spectra.

As these antibiotics are amphoteric substances,[1] several salts such as sodium salt, hydrochloride and bisulfate, can be prepared by the usual way. The elemental analytical data of these preparations including free forms[1] (Table 1) indicated approximate empirical formulas of $C_{66}H_{109}N_{15}O_{18}$ for cerexin A and $C_{69}H_{107}N_{15}O_{17}$ for cerexin B.

Table 1. Elemental analyses

Cerexin A free form *Anal.* Found	C, 53.31; H, 7.72; N, 14.27
$C_{66}H_{109}N_{15}O_{18}\cdot 4H_2O$	C, 53.82; H, 8.01; N, 14.27
Cerexin A hydrochloride *Anal.* Found	C, 51.77; H, 7.84; N, 14.32; Cl, 2.64
$C_{66}H_{109}N_{15}O_{18}HCl\cdot 4H_2O$	C, 52.52; H, 7.88; N, 13.92; Cl, 2.35
Cerexin A bisulfate *Anal.* Found	C, 50.25; H, 7.52; N, 13.63; S, 2.38
$C_{66}H_{109}N_{15}O_{18}H_2SO_4\cdot 4H_2O$	C, 50.46; H, 7.64; N, 13.38; S, 2.04
Cerexin A sodium salt *Anal.* Found	C, 54.35; H, 6.26; N, 13.86; Na, 2.96
$C_{66}H_{109}N_{15}O_{18}Na\cdot 2H_2O$	C, 54.34; H, 7.74; N, 14.40; Na, 1.58
Cerexin B free form *Anal.* Found	C, 55.36; H, 7.28; N, 14.39
$C_{69}H_{107}N_{15}O_{17}\cdot 4H_2O$	C, 55.59; H, 7.78; N, 14.10
Cerexin B hydrochloride *Anal.* Found	C, 54.18; H, 7.12; N, 13.98; Cl, 3.14
$C_{69}H_{107}N_{15}O_{17}HCl\cdot 4H_2O$	C, 54.28; H, 7.62; N, 13.76; Cl, 2.32

When these antibiotics were hydrolyzed with hydrochloric acid in the presence of thioglycolic acid[2] and analyzed by an automatic amino acid analyzer, aspartic acid, threonine, serine, valine, *allo*-isoleucine, tryptophan, and an unknown basic amino acid were found from cerexin A. Similarly, aspartic acid, threonine, glycine, valine, *allo*-isoleucine, phenylalanine, tryptophan and the same unknown amino acid as above were shown with cerexin B.

The unknown amino acid was isolated from a hydrolyzate of cerexin A by preparative paper chromatography. An empirical formula $C_6H_{14}N_2O_3\cdot HCl$ was indicated for the amino acid by elemental analysis. Since the nmr spectrum and the easily lactonizable nature of the

amino acid suggested it to be γ-hydroxylysine, the amino acid was compared with a synthetic specimen of L-*threo*-γ-hydroxylysine*. Chromatographic data involving an ion-exchanger as well as ir, nmr and CD spectral data indicated that the amino acid was indeed L-*threo*-γ-hydroxylysine. This is the first case of the isolation of this amino acid as a building stone of a natural product, though *threo*-γ-hydroxy-L-homoarginine has been isolated from *Lathyrus* species.[3,4]

Fig. 1. Nmr spectrum of L-*threo*-γ-hydroxylysine monohydrochloride in D_2O

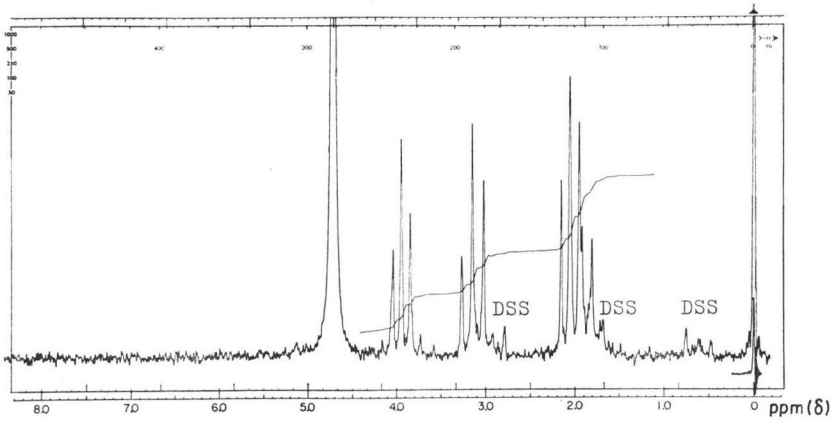
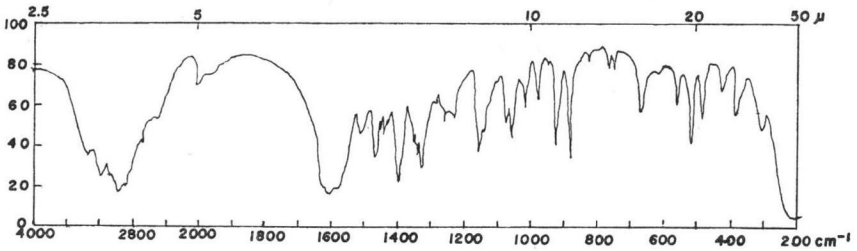


Fig. 2. Infrared absorption spectrum of L-*threo*-γ-hydroxylysine monohydrochloride (KBr)



Aspartic acid, serine, threonine and valine were also isolated from the hydrolyzate, and their optical activities were measured. From these data, aspartic acid was deduced to be a mixture of D and L forms in which the D form slightly predominated. However, stereochemistry of each residue of aspartic acid will be elucidated in the course of sequential studies. It was also deduced that serine was L form, threonine D form and valine D form.

For quantitative analysis of γ-hydroxylysine, the hydrolyzate was treated with silver acetate[5] to open its lactone formed during acid hydrolysis. Results of amino acid analyses of cerexins A and B were shown in Table 2, which are expressed in μ moles of amino acid found per mg of antibiotic. The results show that both cerexins A and B are constructed with 10 amino acid residues and their molecular weights are approximately 1,500. It is also shown that serine and one residue of valine in cerexin A are replaced by glycine and phenylalanine in cerexin B. This is the only difference between the two antibiotics, aside from fatty acids.

When ethereal extracts of the acid hydrolyzates of the antibiotics were methylated and

* The sample of L-*threo*-γ-hydroxylysine used was kindly supplied from Prof. T. SHIBA of Osaka University, who synthesized the amino acid and confirmed its stereochemistry by ORD and nmr.

Table 2. Amino acid analyses* of cerexins A and B

	Asp	Thr	Ser	Gly	Val	*allo*-Ile	Phe	Trp	Hy-Lys**	Amm
Cerexin A	2.05 (3)	0.69 (1)	0.62 (1)		1.38 (2)	0.62 (1)		0.52 (1)	0.60 (1)	2.02 (3)
Cerexin B	2.03 (3)	0.67 (1)		0.64 (1)	0.78 (1)	0.62 (1)	0.68 (1)	0.55 (1)	0.50 (1)	2.03 (3)

* μmoles of amino acid found per mg of antibiotic
** γ-Hydroxylysine

analyzed with gas liquid chromatography, several fatty acids were detected from cerexins A and B.

Structural studies on these fatty acids and sequential studies on the amino acids of these antibiotics are now in progress. The results will be reported elsewhere.

Experimental

Isolation and Identification of L-*threo*-γ-Hydroxylysine

Cerexin A (400 mg) was hydrolyzed with constant-boiling hydrochloric acid at 110°C for 20 hours. The hydrolyzate was extracted with ethyl ether and the aqueous phase was concentrated to dryness. The residue was then subjected to preparative paper chromatography on Toyo Roshi No. 525 with *n*-butanol-acetic acid-water (4:1:2). Zones of γ-hydroxylysine, a mixture of aspartic acid and serine, threonine, valine and *allo*-isoleucine, were separated. The zone of γ-hydroxylysine was extracted with water, and the extract was concentrated to dryness and triturated with ethanol, giving a colorless powder (45 mg) which seemed to be contaminated with ammonium chloride. The preparation was then adsorbed on a short column of Dowex 50 (NH_4^+) and eluted with 0.5 N NH_4OH. The eluate was lyophilized to remove ammonium hydroxide, and the residue was dissolved in water, acidified to pH 2.0 with dilute hydrochloric acid, lyophilized and crystallized from water-ethanol to give colorless needles of the amino acid hydrochloride (8 mg), m.p. 198~209°C (dec.).

Anal. Found: C, 36.47; H, 7.43; N, 13.88; Cl, 17.62.
Calcd. for $C_6H_{14}N_2O_2 \cdot HCl$: C, 36.27; H, 7.56; N, 14.11; Cl, 17.88.
CD: *$[\theta]_{245}$0, $[\theta]_{210}$+4870, $[\theta]_{203}$+3840 (*c* 0.0322, 0.5 N HCl).

When the above amino acids was compared with synthetic L-*threo*-γ-hydroxylysine by PC and TLC, the same mobilities (Rf 0.15 on Toyo Roshi No. 51 with *n*-butanol-acetic acid-water (4:1:2); Rf 0.12 on Avicel plate with water-saturated phenol) were observed. Furthermore, the same retention time (about 2.0 hours) of both the natural and synthetic specimens was observed with ion-exchange chromatography using an automatic amino acid analyzer (conditions: 50-cm column, 0.70 M sodium citrate buffer, pH 5.28) in which condition diastereoisomers of γ-hydroxylysine were thought to be separable. Nmr and ir spectra (Figs. 1 and 2) of both specimens were also identical. δ 1.88 (2H, m, $CH_2-\delta$), 2.04 (2H, *t*, J=6, $CH_2-\beta$), 3.14 (2H, t, J=7.5, $CH_2-\varepsilon$), 3.94 (H, t, J=6, $CH-\alpha$), 3.94 (H, quintet, J=6, $CH-\gamma$).

Optical Rotations of the Aspartic Acid, Serine, Threonine and Valine

From the preparative paper chromatography described above, other zones of amino acids were also extracted. The mixture of aspartic acid and serine was separated by crystallization from water-methanol, resulting in the crystallization of aspartic acid hydrochloride. The mother liquid containing serine and aspartic acid were separated by preparative paper chromatography on Toyo Roshi No. 51 with water-saturated phenol. Serine was extracted with water, lyophilized and crystallized from water-methanol to give the hydrochloride.

From the zones of threonine and valine, the respective hydrochlorides were obtained as

* L-*threo*-γ-Hydroxylysine CD: $[\theta]_{245}$ 0, $[\theta]_{210}$+4850, $[\theta]_{203}$+3650 (*c* 0.0977, 0.5 N HCl).

colorless needles.

Aspartic acid hydrochloride: $[\alpha]_D^{22.0°}-1.6\pm0.4°$ (c, 1.026, 6 N HCl), CD: $[\theta]_{234}$ 0, $[\theta]_{215}-190$, $[\theta]_{250}-120$ (c, 0.1581, 0.5 N HCl).

Serine hydrochloride: ORD: $[\phi]_{250}+700$, $[\phi]_{225}+2500$, $[\phi]_{215}+700$; CD: $[\theta]_{242}$ 0, $[\theta]_{210.5}+5000$, $[\theta]_{200}+2900$, (c, 0.989, 0.5 N HCl).

Threonine hydrochloride: ORD: $[\phi]_{250}-1320$, $[\phi]_{225}-3770$, $[\phi]_{215}-1440$; CD: $[\theta]_{245}$ 0, $[\theta]_{211}-6200$, $[\theta]_{200}-3700$ (c, 0.1109, 0.5 N HCl).

Valine hydrochloride: ORD: $[\phi]_{250}-1100$, $[\phi]_{225}-3360$, $[\phi]_{215}-1560$; CD: $[\theta]_{245}$ 0, $[\theta]_{211}-5100$, $[\theta]_{200}-3100$ (c, 0.0938, 0.5 N HCl).

Amino Acid Analysis

An amino acid analyzer, Hitachi Model KLA-5, was used for the analysis. For analytical purposes, samples were hydrolyzed with 4% thioglycolic acid-added[2] constant boiling hydrochloric acid in a vacuum-sealed tube at 110°C for 48 hours. For the quantitative analysis of γ-hydroxylysine, a portion of the hydrolyzate was treated with silver oxide and silver acetate in the manner described by IZUMIYA *et al.*[5] before analysis. The results are illustrated in Table 2.

Acknowledgement

The authors wish to thank to Dr. TETSUO SHIBA, Professor of Osaka University, for his kind supply of an authentic specimen of L-*threo*-γ-hydroxylysine.

References

1) 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
2) MATSUBARA, H. & R. M. SASAKI: High recovery of tryptophan from acid hydrolyzates of proteins. Biochem. Biophys. Res. Commun. 35: 175~181, 1969
3) BELL, E. A.: A new amino acid, γ-hydroxyhomoarginine, in *Lathyrus*. Nature 199: 70~71, 1963
4) FUJITA, Y.; J. KOLLONITSCH & B. WITKOP: The stereospecific synthesis of *threo*-γ-hydroxyhomo-L-arginine from *Lathyrus* species. J. Amer. Chem. Soc. 87: 2030~2033, 1965
5) IZUMIYA, N.; Y. FUJITA, F. IRREVERRE & B. WITKOP: The synthesis of *erythro*-γ-hydroxy-L-lysine and its non-occurrence in collagen. Biochemistry 4: 2501~2506, 1965