THE AMINO ACID SEQUENCE OF CEREXIN A

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

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N-Bromosuccinimide cleavage reaction on cerexin A liberated *allo*-isoleucine. Treatment with conc. hydrochloric acid cleaved the antibiotic into two peptide fragments selectively at a γ-hydroxylysine residue. Deacylation with an enzyme preparation from *Pseudomonas* sp. afforded deacyl cerexin A. The amino acid sequences of these peptide fragments were examined by EDMAN degradation. From all the results, the entire amino acid sequence of cerexin A was deduced.

Cerexin A is an antibiotic active against gram-positive bacteria produced by *Bacillus cereus* 60-6[2]. It has already been reported that the antibiotic is an amphoteric acylpeptide with an approximate empirical formula of $C_{66}H_{109}N_{15}O_{18}$ containing Asp (3), D-Thr (1), L-Ser (1), D-Val (2), aIle (1), L-γHyl* (1), Trp (1), Amm (3), and a variety of fatty acid residues[3].

It has been well known that threonine and *allo*-threonine behave quite similarly in the usual chromatographic procedures. Re-examination of the preparation reported as D-threonine in our preceding paper[3] by paper chromatography with the solvent system[4] which has been reported to be able to separate threonine and *allo*-threonine, revealed it to be the *allo*-isomer. We have previously confirmed that threonine and *allo*-threonine could not be distinguished, by both retention time and color yield, under the experimental conditions of automatic amino acid analysis and paper chromatography used in preceding work. Therefore, that referred as to D-threonine in our preceding paper[3] is actually D-*allo*-threonine.

The *allo*-isoleucine and tryptophan residues whose chirality had not yet been reported were isolated from the hydrolyzate of cerexin A hydrolyzed with 4% thioglycolic acid-added hydrochloric acid[5].

From the ORD measurement, *allo*-isoleucine was deduced to be D-form according to the empirical rule for the relationship between COTTON effect and configuration of α-amino acids[6]. As simple adoption of the rule in the case of tryptophan was somewhat suspected, direct comparison with a reference specimen was carried out. L-Tryptophan showed positive COTTON effects at 290 nm and 235 nm in 0.5 N HCl, whereas that derived from cerexin A showed opposite signs, indicating it to be D-form.

Cerexin A was partially hydrolyzed with a mixture of formic acid and conc. hydrochloric acid at 37°C, and the hydrolyzate was extracted with ethyl ether. The extract was fractionated on a silica gel plate with chloroform - methanol (9 : 1). A fraction which liberated aspartic acid by acid hydrolysis was isolated. It was hydrophobic in nature and ninhydrin-negative before the hydrolysis, indicating the presence of a fatty acylated amino group. This strongly

* L-*threo*-γ-Hydroxylysine is abbreviated as L-γHyl hereafter.

suggested that the N-terminus of the antibiotic is FA* → Asp.

By the cleavage reaction with N-bromosuccinimide[7], cerexin A liberated *allo*-isoleucine itself. This means that the C-terminal of the antibiotic is Trp→aIle-OH.

In addition to the elucidation of both terminal ends, the following facts lead to the conclusion that the three residues of aspartic acid are present as amide form in the intact antibiotic. Three moles of ammonia have been estimated[3] with the hydrolyzate of the antibiotic, and titration[2] of cerexin A hydrochloride with dilute sodium hydroxide in aqueous dimethylsulfoxide has indicated the existence of only one carboxyl group which accounts for the C-terminal carboxyl group.

Recently, SHIBA and his co-workers[8] have reported selective cleavage of tuberactinomycin into γ-hydroxy-β-lysine lactone and tuberactinamine by conc. hydrochloric acid at room temperature. They postulated a mechanism *via* a cyclic carbinolamine intermediate.

In cerexin A, which contains γ-hydroxylysine, an analogous reaction is likely to take place at the residue. In fact, the peptide antibiotic was cleaved into two main peptide fragments. One was revealed to contain Asp, Val, and γHyl by amino acid analysis of the hydrolyzate, which, therefore, was thought to be the fragment of the N-terminal side. The other was considered to be that of the C-terminal side, because it contained aThr, Ser, Trp and aIle. The sequence of the latter peptide fragment was determined to be aThr→Ser→Trp→aIle by EDMAN degradation[9]. Thus, half of the peptide antibiotic was deduced to be γHyl→aThr→Ser→Trp→aIle.

KIMURA and his co-worker have reported that the cell of *Pseudomonas* sp. M-6-3 possessed enzymic activity to deacylate polymixin E and the enzyme (polymixin acylase) was partially purified[10]. They have found also that the enzyme not only acts on polymixin E, but also on other N-fatty acylpeptides and N-fatty acyl amino acids whose fatty acyl groups have relatively long carbon chains (Private communication).

When the enzyme was tried for cerexin A, deacyl cerexin A was produced in an extent of approximately 20 %. The succesive EDMAN degradation reaction carried out on deacyl cerexin A proceeded well up to third step, which indicate a sequence, Asn→Val→Val, but the degradation reaction hardly proceeded at the next step.

From these results, the N-terminal side of cerexin A can be concluded to be FA→Asn→Val→Val. As the C-terminal side has been determined to be γHyl→aThr→Ser→Trp→aIle and all the amino acid residues of this antibiotic have already been elucidated, the remaining two residues, *i.e.* Asn, Asn, must be present between the above two sequences.

That EDMAN degradation hardly proceeded at the step succeeding to Asn→Val→Val in deacyl cerexin A, is suggestive for the assumption that one Asn residue links to the next Asn residue through the β-carboxyl group. Earlier, it had been pointed out that in applying EDMAN degradation to peptides containing Asp or Asn residue, bond migration of the α-carboxyl peptide bond of the Asp residue to β-carboxyl linkage might occur during successive application of the reaction[9b], but this disadvantage has been overcome in the present experimental procedures. However, a suspicion that such a bond migration in the Asn residue of deacyl cerexin A might occur during deacylation procedure can not be completely negated. The problem involving the

* FA represents fatty acyl groups.

position through which the Asn residue in intact cerexin A links to the next should in future be clarified by more certain evidence.

These considerations allow us to draw the amino acid sequence of cerexin A as shown in Fig. 1.

Fig. 1. Amino acid sequence of cerexin A.

$$FA \rightarrow Asn \rightarrow \overset{(D)}{Val} \rightarrow \overset{(D)}{Val} \rightarrow Asp\,(NH_2) \xrightarrow{(\alpha \text{ or } \beta)} Asn \rightarrow \overset{(L)}{\gamma Hyl} \rightarrow \overset{(D)}{aThr} \rightarrow \overset{(L)}{Ser} \rightarrow \overset{(D)}{Trp} \rightarrow \overset{(D)}{aIle}$$

Experimental

ORD curve was recorded with a spectropolarimeter JASCO Model ORD/UV-5.

Amino acid analysis was carried out with an amino acid analyzer Hitachi KLA-5 under the normal conditions directed for the instrument. Peptides to be analyzed were hydrolyzed with 4% thioglycolic acid-added constant boiling hydrochloric acid in a vacuum-sealed tube at 110°C for 20 hours.

EDMAN degradation was carried out with peptide samples (*ca.* 1.0 μmoles) by the published procedures[9a] with slight modification. PTH-Amino acid was identified by the TLC[9a], but quantitation of the PTH-amino acid by OD measurement was omitted. In each step of sequential EDMAN degradation reaction, a portion (1/20~1/10) of the remaining peptide was drawn out for analysis by subtractive mode.

Paper chromatography of *allo*-threonine

The chromatogram was prepared on Toyo Roshi No. 51 with an upper layer of *n*-butanol - acetone - 28% ammonium hydroxide - water (8 : 1 : 1 : 6)[4] by ascending manner. Spots were visualized by the usual ninhydrin coloration.

L-Threonine and a synthesized sample of D, L-*allo*-threonine, kindly supplied by Prof. T. SHIBA of Osaka University, were used as references. Threonine showed an Rf 0.18 and *allo*-threonine Rf 0.12, and their mixture was clearly separated.

The specimen prepared from the hydrolyzate of cerexin A coincided with *allo*-threonine on the paper chromatogram.

Isolation of *allo*-isoleucine and tryptophan

Cerexin A (140 mg) was hydrolyzed with 4% thioglycolic acid-added constant boiling hydrochloric acid in a vacuum-sealed tube at 110°C for 20 hours. After removing thioglycolic acid with ethyl acetate, the hydrolyzate was evaporated to dryness and subjected to preparative paper chromatography on two sheets of Toyo Roshi No. 51, 60×60 cm with *n*-butanol - acetic acid - water (4 : 1 : 2) in a descending manner for 24 hours. The zones of *allo*-isoleucine, tryptophan and valine were separated being partially overlapped. The zones mainly containing *allo*-isoleucine and tryptophan, respectively, were cut out and extracted with slightly acidified 50% aqueous methanol.

The *allo*-isoleucine-containing fraction was passed through an Amberlite XAD-2 column at pH 7.0 to remove a small amount of contaminating tryptophan, and then adsorbed on a small column of Dowex 50×8 (NH_4^+) at pH 2.0. After washing the column with water, the *allo*-isoleucine was eluted with 0.3 N NH_4OH, and prepared as a colorless amorphous powder (2 mg) by freeze-drying.

ORD: $[\phi]_{250}-700$, $[\phi]_{225}-2140$, $[\phi]_{211}$ 0 (*c* 0.0803, 0.5 N HCl)

From the fraction mainly containing tryptophan, the amino acid was adsorbed on an Amberlite XAD-2 column (5 ml) at pH 7.0 and after being washed with water was eluted with 50% aqueous methanol. The prepapation was further purified by preparative paper chromatography with water-saturated *sec.*-butanol: Toyo Roshi No. 51, 20×60 cm, descending for 40 hours. The separated zone of tryptophan (Rf *ca.* 0.50) was extracted with slightly acidified 50% aqueous methanol, then adsorbed on a small column of Dowex 50×8 (NH_4^+) and eluted

with 0.3 N NH_4OH. By lyophilization of the eluate, the tryptophan preparation was obtained as a colorless amorphous powder (2 mg).

ORD: $[\phi]_{300}-520$, $[\phi]_{290}-1070$, $[\phi]_{260}$ 0, $[\phi]_{235}-3020$, $[\phi]_{230}-1650$ (*c* 0.0990, 0.5 N HCl)

A commercial sample of L-tryptophan showed:

ORD: $[\phi]_{300}+850$, $[\phi]_{290}+1540$, $[\phi]_{257}$ 0, $[\phi]_{235}+3760$, $[\phi]_{230}+2630$ (*c* 0.1085, 0.5 N HCl)

Partial acid hydrolysis

Cerexin A (100 mg) was dissolved in formic acid (5 ml). Approximately 5 ml of conc. hydrochloric acid was added to the solution, which was then allowed to stand for 72 hours at 37°C. The partial hydrolyzate was concentrated to dryness, dissolved in water (5 ml) and extracted with ethyl ether. The ethyl ether extract was applied to a Silica gel GF plate (20×20 cm) which was developed with chloroform - methanol (9 : 1) continuously for 80 minutes. Several zones of hydrophobic nature were visualized by spraying water. The first and second zones, in the order of the mobility, were extracted with a mixture of chloroform and methanol (1 : 1). These fractions, ninhydrin-negative, were completely hydrolyzed with hydrochloric acid and subjected to paper chromatography for detection of amino acids. Only aspartic acid was detected from the former, whereas aspartic acid and valine were found in the latter.

No additional information was obtained from further experiments with the partial hydrolyzate.

Cleavage reaction with N-bromosuccinimide

Cerexin A (7 mg, *ca.* 5 μmoles) was dissolved in 10 drops of glacial acetic acid. After the solution was diluted with 4 drops of water, N-bromosuccinimide (4.5 mg, *ca.* 25 μmole) was added and the mixture was stirred for 20 minutes at roomtemperature. After addition of 2 drops of formic acid and standing for a few minutes, the reaction mixture was freeze-dried.

When the residue was subjected to TLC on Silica gel GF with chloroform - ethanol - 10% acetic acid (4 : 7 : 2), two ninhydrin-positive spots (Rf 0.57 and 0.45) were observed. The latter (Rf 0.45) was separated by preparative manner of the TLC and extracted with aqueous methanol. Examination by paper chromatography with *n*-butanol - acetic acid - water (4 : 1 : 2) and an automatic amino acid analyzer revealed it to be *allo*-isoleucine (*ca.* 1.4 μmoles).

Fragmentation with conc. hydrochloric acid

Cerexin A (29 mg) was dissolved in 2 ml of 4 % thioglycolic acid-added conc. hydrochloric acid in a test tube, which was then evacuated, sealed and allowed to stand for 70 hours at 4°C. The mixture was extracted with ether and the residual solution was freeze-dried.

When the residue was examined by TLC on a Silica gel GF plate with *n*-butanol - acetic acid - water (4 : 1 : 2), two main ninhydrin-positive spots (Rf *ca.* 0.50 and *ca.* 0.30) were observed. Each was separated by the TLC and extracted with aqueous methanol. Small portions of these isolates were hydrolyzed and analyzed with an automatic amino acid analyzer. aThr, Ser, Trp and aIle from the former (Rf 0.50), and Asp, Val and γHyl from the latter (Rf 0.30) were detected.

EDMAN degradation on the former peptide revealed it to be aThr→Ser→Trp→aIle as follows:

	PTH-Amino acid	Amino acid found (in ratio)			
		aThr	Ser	Trp	aIle
Original peptide		1.0	0.9	0.7	1.0
Step 1	aThr*, *d*Thr	0.0	1.3	0.3	1.0
Step 2	Ser**	0.0	0.3	0.2	1.0
Step 3	—				+***

* PTH-Thr was used as reference for the identification.
** Poorly observed.
*** Analyzed before hydrolysis.

Deacyl cerexin A

Cerexin A sodium salt (10 mg) was dissolved in 0.5 ml of water. Approx. 0.5 ml of 0.1 M phosphate buffer, pH 8.0, was added to the solution, resulting in the formation of a fine suspension of the antibiotic. Five mg of polymixin acylase (acetone powder of *Pseudomonas* sp. M-6-3)[10], which was kindly supplied by Prof. KIMURA of Mukogawa Women's University, was added to the suspension, which was then stirred for 4 days at 37°C.

The reaction mixture was centrifuged (3,000 rpm, 10 minutes) to remove the precipitate which contained the enzyme particles and unreacted cerexin A. The supernatant was subjected to preparative paper chromatography on Toyo Roshi No. 51, 20×60 cm, with *n*-butanol - acetic acid - water (4 : 1 : 2). A zone which is positive to ninhydrin and shows Rf *ca.* 0.40 was cut out, and extracted with slightly acidified aqueous methanol. When a portion of the extract was hydrolyzed and analyzed with an automatic amino acid analyzer, all of the amino acid residues contained in cerexin A were detected. The yield of deacyl cerexin A was estimated as approx. 20 % by amino acid analyses in several experiments.

The result of EDMAN degradation on deacyl cerexin A was as follows:

	PTH-Amino acid	Amino acid found (in ratio)						
		Val	Asp	γHyl	aThr	Ser	Trp	aIle
Original peptide		1.7	3.5	0.2	1.2	1.1	0.9	1.0
Step 1	Asn	1.6	2.4	0.1	1.1	1.0	0.9	1.0
Step 2	Val	1.2	2.4	0.0	1.0	1.0	0.9	1.0
Step 3	Val	0.2	2.2	0.0	0.8	0.9	0.7	1.0
Step 4	—	0.2	1.8	0.0	0.9	0.9	0.7	1.0

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