RESOLUTION OF PEPTIDE ANTIBIOTICS, CEREXINS AND TRIDECAPTINS, BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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

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By high performance liquid chromatography, cerexin B was separated into four components (B_1, B_2, B_3 and B_4), cerexin D into four components (D_1, D_2, D_3 and D_4), tridecaptin A into two components (A_α and A_β), tridecaptin B into four components (B_α, B_β, B_γ and B_δ) and tridecaptin C into three components (C_{α_1}, C_{α_2} and C_{β_1}). All components were preparatively isolated, and their fatty acid and amino acid compositions determined for structural elucidation.

A major problem in the chemistry of peptide antibiotics is the fine resolution of complexes that can not be separated by current separation techniques. Among the peptide antibiotics which we isolated and whose structures we determined, cerexins B and D[2,3,6,7], and tridecaptins B and C[1,8] have been reported to be complexes. Cerexins B and D are heterogeneous with respect to fatty acyl residues, tridecaptin B is heterogeneous in amino acid residues and tridecaptin C is heterogeneous in both fatty acyl and amino acid residues. Recently, a new separation technique, high performance liquid chromatography, has been employed for separation and analysis of various kinds of chemical compounds involving peptide antibiotics[11,12]. The purpose of this study is the complete resolution of the above complexes by high performance liquid chromatography and elucidation of the structures of the separated components.

Materials and Methods

Antibiotics

All antibiotics investigated in this study (cerexins A, B, C and D; and tridecaptins A, B and C) were prepared in the manner described in previous papers.[2,7,8]

High performance liquid chromatography (HPLC)

HPLC was carried out using a Waters Model 6000 A pump with a Waters Model U6K injector throughout this experiment. Chromatography was monitored by a UV detector, Shimadzu SPD-1, at 220 nm. For analytical purpose, a steel column (4 mm inside diameter, 250 mm length) packed with Nucleosil $5C_{18}$ (Macherey-Nagel) was used at a flow rate of 1.0 ml/min and a sample charge of approx.2 mcg. In preparative experiments, a larger column (10 mm inside diameter, 250 mm length) packed with Nucleosil $5C_{18}$ was used at a rate of 4.0 ml/min and a sample charge of 500 ~ 1,000 mcg. Mobile phases used were: (a) a mixture of acetonitrile and 5 mM phosphate buffer, pH 7.0; (b) a mixture of acetonitrile and 10 mM ammonium sulfate, adjusted to pH 7.0; and (c) a mixture of acetonitrile and 5 mM sodium tartarate buffer, pH 3.0, containing 5 mM sodium *n*-butanesulfonate and 50 mM sodium sulfate. The content of acetonitrile in the mobile phase was selected according to the nature of the

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sample to obtain better resolution.

Analyses of fatty acids and amino acids

Each preparation of the components isolated by HPLC was hydrolyzed with constant boiling hydrochloric acid at 110°C for 1 hour and the liberated fatty acids extracted with ethyl ether. The ethereal extract was methylated and analyzed with a Shimadzu Gas Chromatograph GC-7AG equipped with a hydrogen flame detector and a glass column (3.2 mm inside diameter, 1.6 m length) packed with 15% diethylene glycol succinate polymer on Chromosorb W 80～100 mesh under conditions of carrier gas (N_2), 45 ml/min; temperature, 156°C. The procedures for ether extraction and methylation were essentially the same as described in our previous papers[5,9].

The aqueous solution after ether extraction was evaporated to dryness and hydrolyzed with constant boiling hydrochloric acid at 110°C for 24 hours. After evaporation of hydrochloric acid, the hydrolyzate was analyzed with an automatic amino acid analyzer, Hitachi KLA-5. In some cases, another portion of an intact preparation was hydrolysed with 4% thioglycolic acid-containing constant boiling hydrochloric acid in a vacuum-sealed tube for the estimation of Trp residue[4].

Results and Discussion

Cerexin Group of Antibiotics

Cerexins A and C have been reported to be single entities, whereas cerexins B and D have been reported to be complexes of four acylpeptides which differ only in a fatty acyl residue[5~7].

Good resolution of this group of antibiotics was obtained with mobile phase (a) or (b), whose acetonitrile content was approximately 35%. Typical chromatograms are illustrated in Fig. 1. As expected, a main peak was observed with cerexins A and C, and essentially four peaks were observed with cerexins B and D, respectively. The components, separated according to the difference in their fatty acyl residues, were named cerexins B_1, B_2, B_3 and B_4; and cerexins D_1, D_2, D_3 and D_4.

About 3 mg of cerexin B was separated by use of the larger column with mobile phase (a) (acetonitrile content, 35%), and the four components were separately collected. Each fraction was concentrated under reduced pressure, and extracted with *n*-butanol at neutral pH. The extract was concentrated to dryness and analyzed for fatty acids and amino acids. The results are shown in Table 1. The results of a similar experiment on cerexin D are shown in Table 2.

These results show that the separated components differ only in their fatty acyl residues. Since the structures of cerexins B and D have been established as complexes, the individual components were assigned the structures shown in Fig. 2.

As far as the effects of substituents of these acylpeptide analogues on their behaviors in the HPLC are concerned, the following facts were observed. Cerexins C, D_1, D_2, D_3 and D_4, containing a lysine residue, had longer retention times than the corresponding peptides, *i.e.*, cerexins A, B_1, B_2, B_3 and B_4, containing a γ-hydroxylysine residue. With respect to the fatty acid residue, the retention times of these acyl peptides increased in the order of i-$C_{10}h^3$, n-$C_{10}h^3$, a-$C_{11}h^3$ and i-$C_{11}h^3$. Note that the retention time of the acylpeptide having an *iso*-structure acyl residue is longer than that having an *anteiso*-structure one. Similar behavior has been observed in our study of a group of acylpeptide antibiotics, octapeptins[10]. It is well-known that the retention times of homologous fatty acid esters in gas chromatography increase in the order, *iso*- < *anteiso*- < *normal*-structure. This relationship is illustrated in the chromatogram of the fatty acid methyl esters derived from cerexin B (Fig. 3).

Abbreviations used are: γHyl: γ-hydroxylysine; a-C_9: anteisononanoic acid; a-C_9h^3: β-hydroxy anteisononanoic acid; 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; and a-$C_{11}h^3$: β-hydroxy anteisoundecanoic acid.

Table 1. Analyses of cerexins B_1, B_2, B_3 and B_4.

Cerexin B component	Fatty acid found	Amino acids found (in ratio)					
		Asp	aThr	Gly	Val	aIle	Phe
B_1	i-$C_{10}h^3$	2.82 (3)	1.04 (1)	1.00 (1)	1.15 (1)	0.97 (1)	1.01 (1)
B_2	n-$C_{10}h^3$	2.58 (3)	0.95 (1)	1.00 (1)	1.11 (1)	0.84 (1)	0.93 (1)
B_3	a-$C_{11}h^3$	2.66 (3)	0.95 (1)	1.00 (1)	1.11 (1)	0.85 (1)	0.79 (1)
B_4	i-$C_{11}h^3$	2.86 (3)	0.92 (1)	1.00 (1)	0.94 (1)	0.90 (1)	0.92 (1)

Tryptophane and γ-hydroxylysine could not be detected, because tryptophane was destroyed and γ-hydroxylysine was converted to its lactone under the conditions of hydrolysis[3].

Fig. 1. HPLC of cerexins A, B, C and D.

Mobile phase (b) (acetonitrile content, 36%) was used for cerexins A and C, and mobile phase (b) (acetonitrile content, 35%) was used for cerexins B and D.

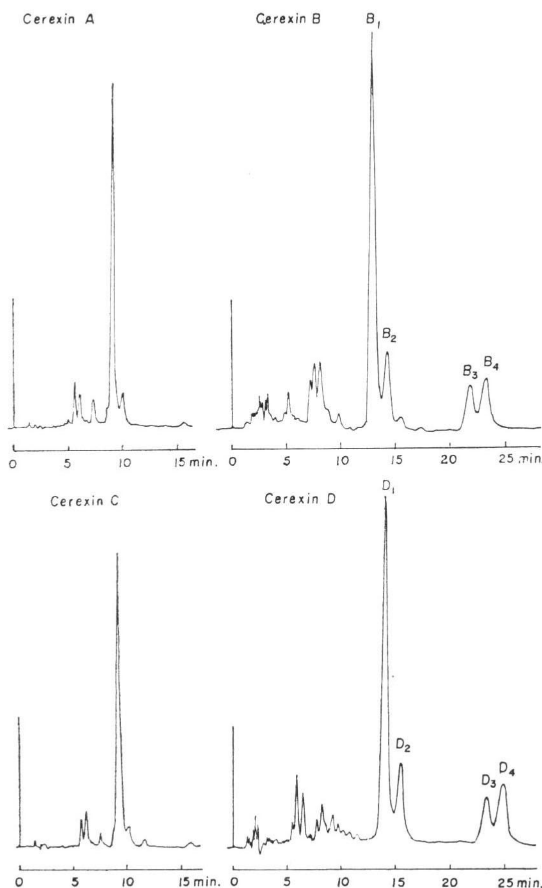
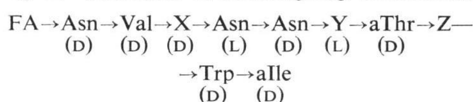


Fig. 2. Structures of cerexin group of antibiotics.



	FA	X	Y	Z
Cerexin A	i-$C_{11}h^3$	Val	γ-Hyl	L-Ser
Cerexin B_1	i-$C_{10}h^3$	Phe	γ-Hyl	Gly
B_2	n-$C_{10}h^3$	〃	〃	〃
B_3	a-$C_{11}h^3$	〃	〃	〃
B_4	i-$C_{11}h^3$	〃	〃	〃
Cerexin C	i-$C_{11}h^3$	Val	Lys	L-Ser
Cerexin D_1	i-$C_{10}h^3$	Phe	Lys	Gly
D_2	n-$C_{10}h^3$	〃	〃	〃
D_3	a-$C_{11}h^3$	〃	〃	〃
D_4	i-$C_{11}h^3$	〃	〃	〃

Tridecaptin A

Though tridecaptin A has been reported to be a single entity[9], this antibiotic gave two peaks on HPLC with mobile phase (b) (acetonitrile content, 33%) (Fig. 4). Isolation of the two components was successfully carried out in a similar fashion; analyses then revealed the following: the major component (named A_α) contains approx. 1 mole of aIle, whereas the minor component (A_β) contains practically no aIle, but approx. 3 moles of Val residues (Table 3). Therefore, it is concluded that tridecaptin A is a complex consisting of a main component A_α, whose structure is that previously reported as tridecaptin A, and a minor component A_β, in which the aIle residue in A_α is replaced by a Val residue as shown in Fig. 5.

Table 2. Analyses of cerexins D_1, D_2, D_3 and D_4.

Cerexin D component	Fatty acid found	Amino acids found (in ratio)						
		Asp	aThr	Gly	Val	aIle	Phe	Lys
D_1	i-$C_{10}h^3$	2.61 (3)	0.90 (1)	1.00 (1)	0.89 (1)	0.84 (1)	0.85 (1)	0.63 (1)
D_2	n-$C_{10}h^3$	2.59 (3)	0.67 (1)	1.00 (1)	0.97 (1)	0.58 (1)	0.69 (1)	0.58 (1)
D_3	a-$C_{11}h^3$	2.71 (3)	0.83 (1)	1.00 (1)	0.95 (1)	0.79 (1)	0.74 (1)	0.57 (1)
D_4	i-$C_{11}h^3$	2.66 (3)	0.90 (1)	1.00 (1)	0.80 (1)	0.84 (1)	0.82 (1)	0.85 (1)

Tryptophane could not be detected, because it was destroyed under the conditions of hydrolysis.

Fig. 3. Gas chromatogram of fatty acid methyl esters from cerexin B.
Column: 15% DEGS, 3.2 mm × 1.6 m.
Carrier gas: N_2, 45 ml/min.
Temperature: 156°C.

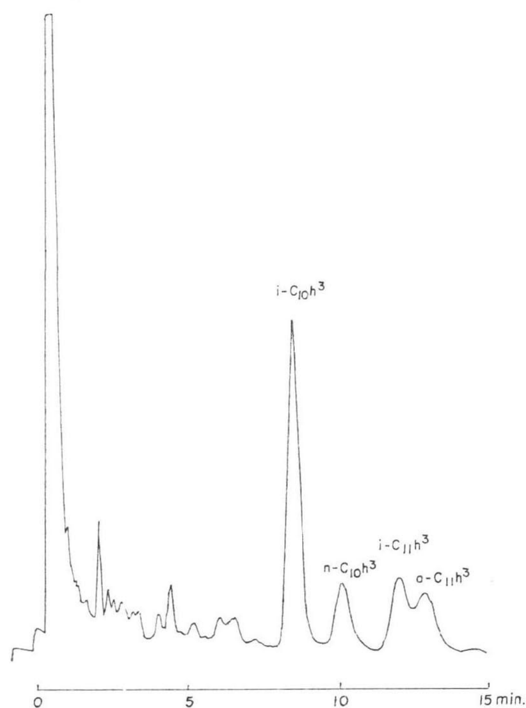
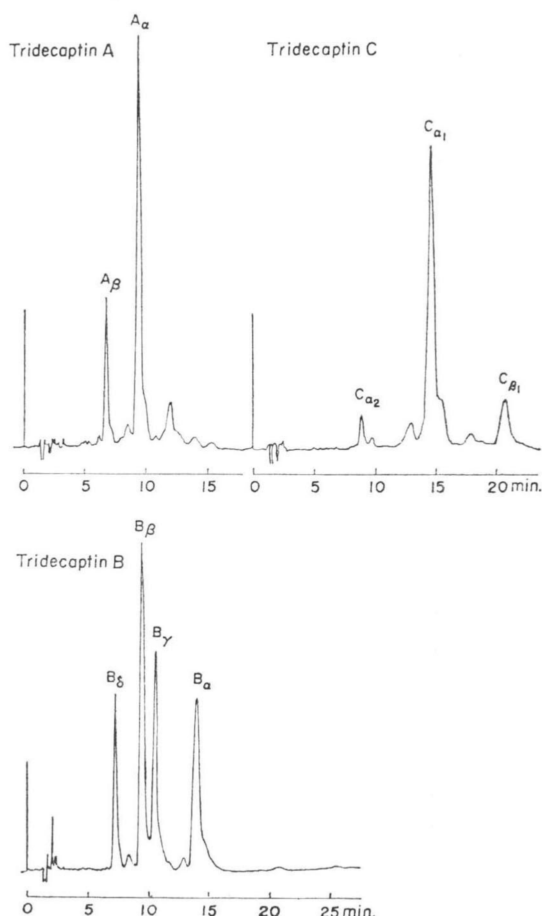


Fig. 4. HPLC of tridecaptins A, B and C.
Mobile phase (b) (acetonitrile content, 33%) was used for tridecaptins A and C, and mobile phase (c) (acetonitrile content, 30%) was used for tridecaptin B.



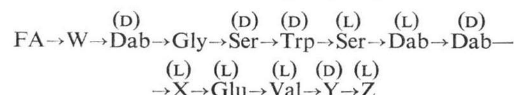
Tridecaptin B

Tridecaptin B has been reported to be a complex of acylpeptides whose amino acid sequences differ in two positions; one is occupied by Ile or Val and the other by aIle or Val[1]. Therefore, the presence of four analogues is anticipated by combination of these residues. When tridecaptin B was analyzed by HPLC with mobile phase (a) (acetonitrile content 38%), only three peaks were

Table 3. Analyses of tridecaptins A_α and A_β.

Tridecaptin A component	Fatty acid found	Amino acids found (in ratio)								
		Ser	Glu	Gly	Ala	Val	aIle	Phe	Trp	Dab
A_α	a-C_9h^3	1.58 (2)	1.00 (1)	1.01 (1)	0.99 (1)	1.92 (2)	0.86 (1)	1.00 (1)	0.86 (1)	3.14 (3)
A_β	a-C_9h^3	1.59 (2)	1.00 (1)	1.01 (1)	0.98 (1)	2.60 (3)	0.15 (0)	0.97 (1)	0.73 (1)	3.08 (3)

Fig. 5. Structures of tridecaptin group of antibiotics.



	FA	W	X	Y	Z
Tridecaptin A_α	a-C_9h^3	D-Val	Phe	aIle	Ala
A_β	〃	〃	〃	Val	〃
Tridecaptin B_α	a-C_9	Gly	Ile	aIle	Ser
B_β	〃	〃	〃	Val	〃
B_γ	〃	〃	Val	aIle	〃
B_δ	〃	〃	〃	Val	〃
Tridecaptin $C_{\alpha1}$	a-$C_{11}h^3$	D-Val	Phe	Val	Ser
$C_{\alpha2}$	i-$C_{10}h^3$	〃	〃	〃	〃
$C_{\beta1}$	a-$C_{11}h^3$	〃	〃	aIle	〃

observed. However, complete resolution with separation of four components was obtained when mobile phase (c) (acetonitrile content 30%) was used. These four components, named B_α, B_β, B_γ and B_δ as in Fig. 4, were isolated and subjected to analyses of fatty acids and amino acids in a similar manner (Table 4). These components differ only in the ratio of Val, aIle and Ile residues present: B_α contains one mole each of Val, aIle and Ile, B_β contains two moles of Val and one mole of Ile, B_γ contains two moles of Val and one mole of aIle, and B_δ contains three moles of Val. Consequently the structures of these components are deduced to be as shown in Fig. 5. The difficulty encountered in the separation of B_β and B_γ is explained by their close structural similarity.

Table 4. Analyses of tridecaptin B_α, B_β, B_γ and B_δ.

Tridecaptin B component	Fatty acid found	Amino acids found (in ratio)							
		Ser	Glu	Gly	Val	aIle	Ile	Trp	Dab
B_α	a-C_9	2.63 (3)	1.00 (1)	1.95 (2)	0.95 (1)	0.85 (1)	0.95 (1)	0.78 (1)	2.89 (3)
B_β	a-C_9	2.55 (3)	1.00 (1)	2.01 (2)	1.85 (2)	tr.* (0)	0.94 (1)	0.96 (1)	2.89 (3)
B_γ	a-C_9	2.37 (3)	1.00 (1)	1.99 (2)	1.85 (2)	0.76 (1)	tr.* (0)	0.67 (1)	2.76 (3)
B_δ	a-C_9	2.37 (3)	1.00 (1)	2.01 (2)	2.39 (3)	tr.* (0)	tr.* (0)	0.82 (1)	2.87 (3)

* Present in a trace amount.

Tridecaptin C

Tridecaptin C is known to be a complex of acylpeptides that are heterogeneous with respect to the fatty acyl residue and one position in the amino acid sequence; the former is occupied by a-$C_{11}h^3$ or i-$C_{10}h^3$ acid and the latter by Val or aIle[1]. The presence of four analogues was anticipated. By an HPLC experiment with solvent system (b) (acetonitril content 33%), three components were isolated and named $C_{\alpha1}$, $C_{\alpha2}$ and $C_{\beta1}$ (Fig. 4). Analyses of these components for fatty acids and amino acids showed that $C_{\alpha1}$ contains a-$C_{11}h^3$ acid and three moles of Val, $C_{\alpha2}$ contains i-$C_{10}h^3$ acid and three moles of Val, and $C_{\beta1}$ contains a-$C_{11}h^3$ acid, two moles of Val and one mole of aIle (Table 5).

Table 5. Analyses of tridecaptins C_{α_1}, C_{α_2} and C_{β_1}.

Tridecaptin C component	Fatty acid found	Amino acids found (in ratio)						
		Ser	Glu	Gly	Val	aIle	Phe	Dab
C_{α_1}	a-$C_{11}h^3$	2.63 (3)	1.00 (1)	1.05 (1)	2.67 (3)	0 (0)	0.97 (1)	2.91 (3)
C_{α_2}	i-$C_{10}h^3$	2.37 (3)	1.00 (1)	1.23 (1)	2.61 (3)	0 (0)	1.04 (1)	2.57 (3)
C_{β_1}	a-$C_{11}h^3$	2.32 (3)	1.00 (1)	0.98 (1)	1.92 (2)	0.61 (1)	0.92 (1)	2.51 (3)

Tryptophane could not be detected, because it was destroyed under the conditions of hydrolysis. The structures of the components were then deduced to be as shown in Fig. 5. One of the four anticipated components, that in which the fatty acyl residue is i-$C_{10}h^3$ and position Y in the amino acid sequence (Fig. 5) is occupied by aIle, could not be detected, probably because the content of this component in the complex is extremely low.

Conclusion

Complexes of the peptide antibiotics, cerexins B and D; and tridecaptins A, B and C, that could not be separated previously, were resolved by HPLC in the mode of reversed phase or reversed phase ion-pair chromatography into their constituent components, and their structures were clarified. Separation was achieved even though these relatively large molecules differed minimally in structure, *e.g.* an aIle or Val residue between tridecaptins A_α and A_β; *normal-* or *iso*-structure of a fatty acid residue between cerexins B_1 and B_2; and *anteiso-* or *iso*-structure of a fatty acid residue between cerexins B_3 and B_4. These results demonstrated that HPLC is a useful separation technique for application to the peptide antibiotics.

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