THE STRUCTURE OF OCTAPEPTIN D

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

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Amino acid analysis of the acid hydrolyzate of octapeptin D revealed the amino acid composition. These amino acids were converted to L-leucyl-derivatives and analyzed by high performance liquid chromatography to clarify their chiralities. These were determined to be: 2,4-diaminobutyric acid (4L), Ser (D), Leu (2L, 1D). Deacylation with polymyxin acylase afforded deacyl octapeptin D. EDMAN degradation on deacyl octapeptin D revealed the N-terminal amino acid. Application of the chemical cleavage reaction for fragmentation of seryl peptides on tri (DNP)-octapeptin D afforded a DNP-heptapeptide, whose sequence was clarified by EDMAN degradation. Octapeptin D was separated into four components (D_1, D_2, D_3 and D_4) by high performance liquid chromatography. All the components were examined for their amino acid and fatty acid compositions. From the results, the structures of octapeptins D_1, D_2, D_3 and D_4 were determined.

Octapeptin D is a new member of the octapeptin group of antibiotics[2], produced by *Bacillus* strain JP-301[1]. It is a basic substance, soluble in water and is active against both Gram-positive and Gram-negative bacteria. The molecular formula $C_{47}H_{88}N_{12}O_{11}\cdot 3HCl$ has been assigned to the hydrochloride, the IR spectrum indicates the presence of a peptide bond but the absence of either a lactone or an ester linkage[1].

The amino acid analysis performed on the acid hydrolyzate indicated the amino acid composition of octapeptin D to be Dab* (4), Ser (1) and Leu (3). 2,4-Dinitrophenylation of the antibiotic gave a 2,4-dinitrophenyl derivative, which on hydrolysis released one mole of free Dab and three moles of γ-DNP-Dab in addition to the other amino acids.

Thus octapeptin D appears to be a octapeptide, in which the γ-amino groups of the three Dab residues were uncovered and the N-terminal amino groups were masked. Furthermore, a ring structure with a branched chain was also suggested in which one Dab residue was present at the branching point.

When the methyl esters of the constituent fatty acids were examined by gas chromatography, four main peaks with retention times identical with methyl β-hydroxy isodecanoate (i-$C_{10}h^3$), methyl β-hydroxy decanoate (n-$C_{10}h^3$), methyl β-hydroxy isoundecanoate (i-$C_{11}h^3$) and methyl β-hydroxy anteisoundecanoate (a-$C_{11}h^3$) were observed[1].

Thus an acyl octapeptide of a cyclic structure with a branched chain, commonly found in the octapeptin group of antibiotics, was suggested.

To determine the chirality of the amino acids, several methods have been developed using gas chromatography[3] or amino acid analysis[4]. More recently high performance liquid chromatography (HPLC) has been applied in the separation of diastereoisomers of a number of dipeptides[5]. We separated the diastereoisomers of the L-leucyl derivatives of the constituent amino acids of octapeptin

* Dab; 2,4-Diaminobutyric acid.

D by HPLC to determine their chiralities.

The acid hydrolyzate of octapeptin D was reacted with Boc-L-Leu-OSu and the Boc-L-leucyl amino acids thus obtained were treated with trifluoroacetic acid to give the L-leucyl amino acids. The mixtures of L-leucyl amino acids were analyzed by HPLC without further purification. From the chromatogram, the chirality of the constituent amino acids were determined as follows; Dab (4L), Ser (D) and Leu (the mixture of L-form (69%) and D-form (31%)).

Polymyxin acylase from the cells of *Pseudomonas* sp. M-6-3 has previously been used to deaclylate cerexin A[6], brevistin[7], polymyxins[8,9] and tridecaptin B[10]. Octapeptin D was easily deacylated by the action of the enzyme to give deacyl octapeptin D. EDMAN degradation of the deacyl octapeptin D showed the N-terminal amino acid to be Ser, but the degradation reaction could not clarify the sequence further.

It is noteworthy for the structure elucidation of the antibiotic that it contains only one Ser residue, which is present at the 1st position of the N-terminus. Several methods for selective cleavage of peptides depending on Thr or Ser residues are known. We have previously succeeded in the selective cleavage of polymyxin T_1 at the Thr residue[9] by the method developed by DIBELLOW *et al.*[11] We applied this method to tri(DNP)-octapeptin D, and a DNP-heptapeptide, formed by cleavage at the C-terminal side of the Ser residue, was obtained.

When this DNP-heptapeptide was dinitrophenylated and then hydrolyzed, approximately one mole of α-DNP-Dab was found in addition to the other residues. This clarified the mode of branching in the peptide ring. EDMAN degradation on this DNP-heptapeptide was carried out with the same modification in our investigation on tetra(DNP)-deacyl octapeptin C_1[12]. This reaction proceeded well, and the result indicated the sequence of this DNP-heptapeptide as shown in Fig. 2.

The chiralities of individual Leu residues were determined as follows: the remaining peptide of the 3rd step of the EDMAN degradation on the DNP-heptapeptide, which contains Leu^5 and Leu^8, were hydrolyzed and the hydrolyzates were converted to L-leucyl peptides and analyzed by HPLC. The results (Table 1) indicated that Leu^4 was D-form and Leu^5 and Leu^8 were L-form.

From these results we concluded the total structure of octapeptin D, except for the configuration of the fatty acyl residues, to be shown in Fig. 1.

Table 1. Ratio of L-Leu and D-Leu in the peptide.

	L-Leu	D-Leu
Octapeptin D	2.00	0.99
Remaining peptide at the 3rd step	2.00	0.25

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

Octapeptin D component	Fatty acid found	Amino acids found (in ratio)		
		Dab	Ser	Leu
D_1	a-$C_{11}h^3$	4.34	0.93	3.00
D_2	i-$C_{10}h^3$	4.30	0.92	3.00
D_3	n-$C_{10}h^3$	4.42	0.92	3.00
D_4	i-$C_{11}h^3$	4.10	0.92	3.00

Fig. 1. Structure of octapeptin D.

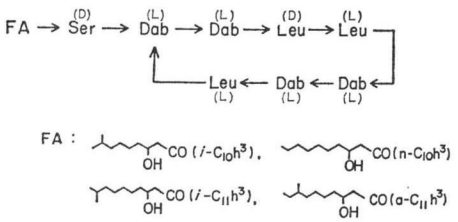
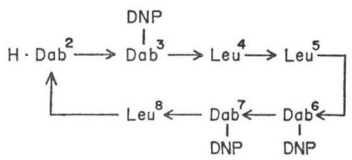
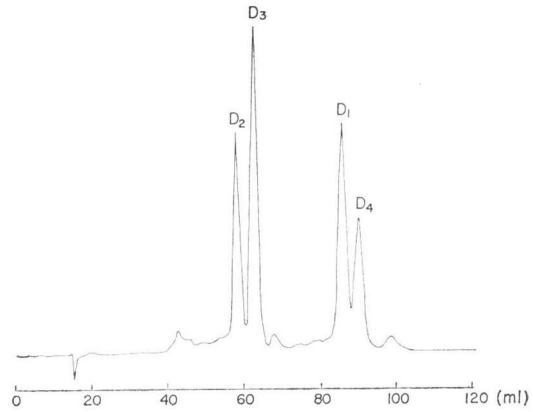


Fig. 2. Structure of DNP-heptapeptide.



Octapeptin D is assumed to be a complex of components which differ at the fatty acyl residue. Recently we have succeeded in the resolution of peptide antibiotic complexes by HPLC[13,14]. When octapeptin D was analyzed by HPLC by the same condition, the complete resolution of the complex was obtained (Fig. 3) and the fatty acid analysis and amino acid analyses elucidated the structures of the separated components (D_1, D_2, D_3 and D_4)*.

Fig. 3. HPLC of octapeptin D.



Experimental

The experimental procedures including amino acid analysis are the same as those described in the preceding paper[8]. HPLC was carried out using a Waters 6000 A pump and Model U6K injector. Chromatography was monitored by a UVIDEC, UV detector at 220 nm.

Tri(DNP)-octapeptin D

Octapeptin D trihydrochloride (21 mg) and $NaHCO_3$ (30 mg) were dissolved in water (0.2 ml). To the solution 4 ml of 10% 2,4-dinitrofluorobenzene ethanol solution was added and the mixture was stirred in the dark. After 30 minutes 1 ml of ethyl acetate was added to obtain a clear solution and stirred for an additional 16 hours. The reaction mixture was evaporated *in vacuo*. To the residue, water and ethyl ether were added and the resulting yellow precipitate was filtered. The precipitate was washed with ether and dried, resulting in a yellow powder of tri(DNP)-octapeptin D (22 mg). Acid hydrolysis released the following amino acids (in ratio): Dab (0.96), γ-DNP-Dab** (2.30), Ser (0.87) and Leu (3.00).

Synthesis of L-Leucyl Amino Acids

L-Leucyl amino acids were prepared from Boc-L-Leu-OSu and the amino acids by the procedure described by A. R. MITCHELL *et al.*[4] After the removal of Boc group by trifluoroacetic acid (TFA), the residue was adsorbed on a Dowex 50×8 column and eluted with 0.3 N ammoniacal water to remove N-hydroxy-succinimide. The eluates were concentrated to dryness and the resulting residues were used for HPLC.

Chirality of Constituent Amino Acids

Octapeptin D trihydrochloride (3.5 mg) was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. It was evaporated to dryness. The residue was dissolved in water and evaporated to dryness *in vacuo*. This procedure was repeated three times to remove excess hydrochloric acid. Sodium bicarbonate stock solution (160 μl, 100 μmole) and Boc-L-Leu-OSu stock solution of tetrahydrofuran (210 μl, 70 μmole) was added. The reaction mixture was stirred at room temperature for 2 hours. It was evaporated to dryness. The residue was dissolved in TFA (1 ml) and allowed to stand for 1 hour at room temperature. TFA was evaporated *in vacuo*. The obtained residue was dissolved in water and the insoluble material was removed by centrifugation. The supernatant was adsorbed on a Dowex 50×8 column and then eluted with 0.3 N NH_4OH. The eluate was evaporated *in vacuo* and the residue was analyzed by HPLC.

Analysis of L-Leucyl Amino Acids by HPLC

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. Mobile phases used were: (a) 20 mM phosphate buffer,

* Subscript number was determined by the analogy of octapeptins A and B[13,15].

** γ-DNP-Dab; γ-Dinitrophenyl-2,4-diaminobutyric acid. Recovery of γ-DNP-Dab was low because it was degraded during the hydrolysis.

pH 4.5; (b) a mixture of acetonitrile and 20 mM phosphate buffer (pH 4.5) (15: 85). The retention volume of synthesized L-leucyl amino acids were as follows (Table 3).

Table 3. Retention volume of L-leucyl amino acids.

	Mobile phase	Retention volume (ml)	
		L-Isomer	D-Isomer
L-Leu→(D,L)-Ser	a	4.4	7.2
L-Leu→(D,L)-Leu	b	4.7	12.0
L-Leu→(D,L)-Dab	b	3.3	5.2

Deacyl Octapeptin D

Octapeptin D trihydrochloride (11 mg) was suspended in 50 mM phosphate buffer (pH 7.0, 1.0 ml). Polymyxin acylase (5 mg) was added to the solution which was stirred at 37°C for 20 hours. The reaction mixture was acidified to pH 3.0 with hydrochloric acid and centrifuged. The supernatant was lyophilized. The residue was subjected to paper chromatography on Toyo Roshi No. 51, developed with *n*-butanol - acetic acid - water (4: 1: 2). A ninhydrin positive zone with Rf 0.51 was extracted with acidified 50% aqueous methanol. Lyophilization of the eluate gave colorless powder of deacyl octapeptin D (2.9 mg).

EDMAN Degradation of Deacyl Octapeptin D

Successive EDMAN degradation of deacyl octapeptin D was carried out with the same modification on tetra(DNP)-deacyl octapeptin C_1.[12] The modification was as follows; excess PTC was removed by extracting with cyclohexane twice and then benzene twice, PTC-amino acid was extracted with ethyl ether, and the PTC-peptide at the second step was heated in TFA at 40°C for 180 minutes and further heated at 80°C for 10 minutes in a mixture of acetonitrile - 2 N HCl (1: 1). The product was used for the next step without purification. The results of EDMAN degradation (Table 4) showed the N-terminal amino acid was Ser, but after the 2nd step the result was ambiguous, so that the sequence was not clearly determined.

Cleavage at Ser Residue

Tri(DNP)-octapeptin D (7.3 mg) was oxidized with dimethylsulfoxide (0.25 ml) and dicyclohexylcarbodiimide (40 mg) by the same procedure used for penta(DNP)-polymyxin T_1[9]. The oxidized product was treated with hydroxylamine hydrochloride (20 mg) in acetic acid (1.3 ml), methanol (0.5 ml) and water (0.3 ml) at 70°C for 1 hour. The reaction mixture was diluted with ethyl acetate, succesively washed with saturated $NaHCO_3$ solution, 0.1 N HCl and water, and dried (Na_2SO_4). After evaporation to dryness, the residue was subjected to TLC on silica gel with chloroform - methanol - acetic acid (9: 1: 1). A yellow zone of Rf 0.25 was extracted with a mixture of chloroform and methanol (1: 1), and the extract was evaporated and then dissolved in ethyl acetate. The ethyl acetate solution was washed with 1 N HCl and water, dried (Na_2SO_4), and concentrated to dryness. Trituration of the residue with ethyl ether gave a yellow powder (1.2 mg). The acid hydrolyzate of the above yellow precipitate gave the following amino acids (in ratio); Dab (1.14), γ-DNP-Dab (2.10) and Leu (3.00).

Table 4. Result of EDMAN degradation on deacyl octapeptin D.

	PTH-Amino acid	Amino acids found (in ratio)		
		Ser	Dab	Leu
Original peptide		0.81	3.28	3.00
1st step	trace Ser	0.0	2.91	3.00
2nd step	not detected	0.0	2.31	3.00
3rd step	γ-PTC-Dab*	0.0	2.04	3.00
4th step	Leu	—	—	—**
5th step	Leu	—	—	—

* Phenylthiohydantoin of γ-phenylthiocarbamyl 2,4-diaminobutyric acid.

** No detectable change was observed.

Table 5. Result of EDMAN degradation on DNP-heptapeptide.

	PTH-Amino acid	Amino acids found (in ratio)		
		Dab	γ-DNP-Dab	Leu
Original peptide		1.14	2.10	3.00
1st step	not detected	0.0	2.20	3.00
2nd step	γ-DNP-Dab	0.0	1.66	3.00
3rd step	Leu	0.0	1.47	2.00
4th step	Leu	0.0	1.12	1.00
5th step	γ-DNP-Dab	0.0	0.94	1.00
6th step	γ-DNP-Dab	0.0	0.45	1.00
7th step	Leu	—	—	—

A portion (0.27 μmole) of the above product was dinitrophenylated in a mixture of water (20 μl), ethyl acetate (20 μl), $NaHCO_3$ (1 mg) and 5% 2,4-dinitrofluorobenzene ethanol solution (40 μl) at room temperature for 16 hours. The reaction mixture was diluted with ethyl acetate and then washed with 3% $NaHCO_3$ solution and water. The residue obtained by evaporation of the solvent was repeatedly washed with petroleum ether to remove unreacted 2,4-dinitrofluorobenzene. The dinitrophenylated product released the following amino acids (in ratio) by acid hydrolysis; α-DNP-Dab (0.70), γ-DNP-Dab (2.06) and Leu (3.00).

These results indicated that the above precipitate was a cyclic heptapeptide, formed by the cleavage at the C-terminal side of the Ser residue of tri(DNP)-octapeptin D.

EDMAN Degradation of the DNP-Heptapeptide

Successive EDMAN degradation on the DNP-heptapeptide obtained as above was carried out with the same modification as deacyl octapeptin D. The PTC-peptide at the 1st step was heated in TFA at 40°C for 180 minutes and further heated at 70°C for 10 minutes in a mixture of acetonitrile - 2 N HCl (1: 1). The product was used for the next step without purification.

Approximately 0.9 μmole of the above cyclic heptapeptide was subjected to the reaction and the result shown in Table 5 was obtained.

The result clearly indicated the sequence of the DNP-heptapeptide as shown in Fig. 2.

Chirality of Leu Residues

The remaining peptide of EDMAN degradation at 3rd step (*ca.* 0.1 μmole) of the cyclic heptapeptide described in the former section was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. It was evaporated *in vacuo* and the excess hydrochloric acid was removed by repeated evaporation of water. The hydrolyzates were dissolved in water (10 μl) and sodium bicarbonate stock solution (10 μl, 7 μmole), Boc-L-Leu-OSu stock solution in tetrahydrofuran (20 μl, 6.6 μmole) and acetonitrile (20 μl) were added to the solution. The reaction mixture were stirred at room temperature for 2 hours and then evaporated to dryness. The residue was dissolved in TFA (0.5 ml) and allowed to stand at room temperature for 1 hour to remove Boc group. TFA was evaporated *in vacuo*. Water was added to the residue and the insoluble material was removed by centrifugation. The clear supernatant was adsorbed on a Dowex 50 column. The eluate with 0.3 N ammoniacal water was evaporated to dryness. The residue was dissolved in water and then analyzed by HPLC by the procedure described in the former section.

Separation of Octapeptin D Components by HPLC

A steel column (10 mm inside diameter, 250 mm length) packed with Nucleosil $5C_{18}$ was used at a flow rate of 4.0 ml/min and a sample charge 400 μg. The mobile phase was prepared by mixing acetonitrile and 5 mM tartarate buffer (pH 3.0) containing 5 mM sodium 1-butanesulphonate and 50 mM sodium sulphate in a ratio (35: 65). A total amount of about 4 mg of octapeptin D was injected and fractionated into four portions. After concentration to about 5 ml, the pH of each fraction was

adjusted to pH 9.0 with sodium hydroxide solution and then extracted with *n*-butanol three times. The combined *n*-butanol extract was washed with water and evaporated to dryness. The obtained residue was used for analyses of fatty acids and amino acids. The procedures for analyses of fatty acids and amino acids were essentially the same as described in our previous paper[14].

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