THE STRUCTURE OF TRIDECAPTIN A

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

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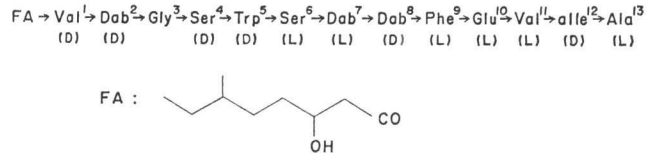
On examining the structure of the antibiotic tridecaptin A, the constituent amino acids were determined to be: 2,4-diaminobutyric acid(2D, 1L), Ser(1D, 1L), Glu(1L), Gly(1), Ala(1L), Val(1D, 1L), aIle(1D), Phe(1L) and Trp(1D). The constituent fatty acid was identified as β-hydroxy anteisononanoic acid by gas chromatography and mass spectrometry. Cleavage reaction with N-bromosuccinimide, sequential analysis by EDMAN degradation, partial acid hydrolysis and some additional evidences clarified the structure of tridecaptin A.

Tridecaptin A, a member of the tridecaptin group of antibiotics produced by strains of *Bacillus polymyxa* is active against Gram-negative and Gram-positive bacteria *in vitro* and *in vivo*[1]. The antibiotic is an acyl peptide, yielding on acid hydrolysis Dab*(3), Ser(2), Glu(1), Gly(1), Ala(1), Val(2), aIle(1), Phe(1), Trp(1) and a fatty acid[1]. Degradation studies described here elucidated the structure of tridecaptin A as in Fig. 1.

Fig. 1. The structure of tridecaptin A.



For preliminary analysis of the constituent fatty acid, the ethereal extracts of the acid hydrolyzate from 1-hour and 20-hour hydrolysis were methylated and analyzed by gas chromatography. The preparation from the 1-hour hydrolyzate gave essentially one peak, whereas the preparation from the 20-hour hydrolyzate gave four major peaks. This is a common phenomenon in acid hydrolysis of β-hydroxy fatty acyl residues[2]. In the chromatogram of the hydrogenated product from the 20-hour hydrolyzate, a peak of identical retention time with methyl anteisononanoate was observed. Furthermore, when the preparation from the 1-hour hydrolyzate was compared with methyl β-hydroxy anteisononanoate prepared from octapeptin C_1[3], a good agreement in retention time was observed. The fatty acid methyl ester was isolated from the 1-hour hydrolyzate by preparative thin-layer chromatography and compared with the preparation from octapeptin C_1 in n.m.r. and mass spectrometry. Essentially the same results were obtained with both specimens.

The constituent amino acids of tridecaptin A were isolated from the acid hydrolyzate by a combination of preparative paper chromatography and the use of a porous polymer Amberlite XAD-2. From their molecular rotations and ORD curves, Glu, Ala and Phe were deduced to be L-forms, aIle and Trp to be D-forms, Ser and Val to be a mixture of one D-form and one L-form and Dab to be a mixture of two D-forms and one L-form (Table 1).

* 2,4-Diaminobutyric acid.

Table 1. Comparison of molecular rotations and ORD curves.

Amino acids	$[M]_D$ in 5 N HCl	COTTON effect in ORD or CD	
Isolated Ser	0.0		1D, 1L
L-Ser	+15.9		
Isolated Glu	+46.5±2.0	positive	L
L-Glu	+47.3		
Isolated Ala	+14.6±2.3	positive	L
L-Ala	+13.1		
Isolated Val	0.0		1D, 1L
L-Val	+33.1		
Isolated aIle	−29.7±2.5	negative	D
L-aIle	+53.1		
Isolated Phe	−8.1±3.3	positive	L
L-Phe	−7.4		
Isolated Dab	−6.8±0.6		2D, 1L
L-Dab	+39.0±1.1		
Calcd. for a mixture of D-Dab(2)+L-Dab(1)	−13		
Isolated Trp		negative	D
L-Trp		positive	

Table 2. Elemental analysis of hydrochloric acid salt and sodium salt of tridecaptin A.

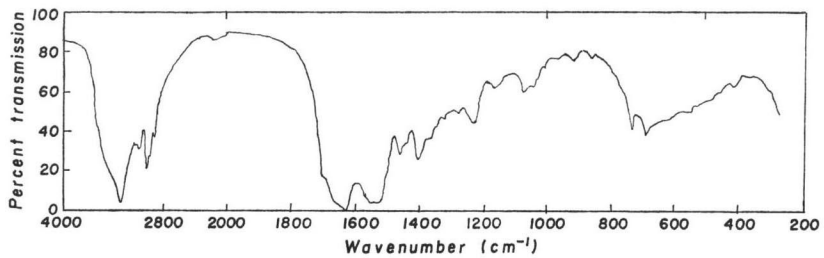
Hydrochloric acid salt	Found	C; 52.07	H; 7.03	N; 13.69	Cl; 6.73
	Calcd. for $C_{73}H_{115}N_{17}O_{20}\cdot 3HCl\cdot 5H_2O$				
		C; 52.24	H; 7.21	N; 14.19	Cl; 6.34
Sodium salt	Found	C; 53.63	H; 7.46	N; 14.48	Na; 1.16
	Calcd. for $C_{73}H_{114}N_{17}O_{20}Na\cdot 4H_2O$				
		C; 53.30	H; 7.48	N; 14.49	Na; 1.40

A molecular formula, $C_{73}H_{115}N_{17}O_{20}$, is anticipated for tridecaptin A from the above constituents, and the calculations for this formula essentially agreed with the elemental analysis of the hydrochloric acid salt and the sodium salt (Table 2).

The infrared absorption spectrum of the sodium salt of tridecaptin A indicated the presence of a peptide bond and suggested the absence of ester or lactone linkage (Fig. 2).

When the hydrochloric acid salt of tridecaptin A was titrated with sodium hydroxide in 80% aqueous dimethylsulfoxide, pK_1' 7.5 (neutralization equivalent 912) and pK_2' 10.5 (neutralization equivalent 553) were observed. This indicated the presence of two carboxyl groups and three amino groups in the molecule.

Fig. 2. Infrared absorption spectra of sodium salt of tridecaptin A (KBr).



When an acid hydrolyzate of dinitrophenylated tridecaptin A was analyzed with an amino acid analyzer, about 3 moles of γ-dinitrophenyl-2,4-diaminobutyric acid (abbreviated as γ-DNP-Dab hereafter) was found and no free Dab. The above data indicates three γ-amino groups of Dab are free and the N-terminal amino group is acylated. The result of titration may account for the carboxyl groups of the Glu residue and a C-terminal amino acid. Thus, tridecaptin A is assumed to be a linear acyl tridecapeptide.

Oxidation with N-bromosuccinimide cleaved the antibiotic into two peptide fragments at the Trp residue. An octapeptide composed of one mole each of Ser, Phe, Glu, Val, aIle and Ala and two moles of Dab were isolated. It was supposed to be a fragment of C-terminal side, because it has no UV absorption depending on the Trp residue. EDMAN degradation of this fragment clarified the sequence to be Ser → Dab → Dab → Phe → Glu → Val → aIle → Ala. Thus the amino acid sequence of the C-terminal side from the Trp residue of tridecaptin A was clarified.

To clarify the amino acid sequence of the N-terminal side, we first attempted deacylation of tridecaptin A with polymyxin acylase, by whose action we have previously succeeded in deacylating some acyl peptide antibiotics, cerexins[4], brevistin[5], polymyxins S_1[6] and T_1[7]. However this enzymic reaction on tridecaptin A was not successful, even various experimental conditions were tried. Then we tried partial acid hydrolysis.

Tridecaptin A was partially hydrolyzed with a mixture of formic acid and conc. hydrochloric acid. From the hydrolyzate, a tripeptide, composed of Val, Dab and Gly was isolated by TLC. When the tripeptide was dinitrophenylated and hydrolyzed, only γ-DNP-Dab was detected as dinitrophenylated amino acid. This result indicated the tripeptide was an N-acylated peptide fragment. When the tripeptide was further partially hydrolyzed, two new fragments were obtained. Since one is negative to ninhydrin reaction and yields Val on hydrolysis, it is deduced to be FA → Val. The other is positive to ninhydrin coloration and yields Val and Dab on hydrolysis, so it was considered to be FA → Val → Dab. Therefore the amino acid sequence of the tripeptide was determined to be FA → Val → Dab → Gly.

From the partial hydrolyzate two other fragments were also obtained. One yields only Ser on hydrolysis and it has UV absorption. Thus this fragment was assumed to be a product due to Ser → Trp fragment, whose Trp residue was degenerated during the hydrolysis. Another is the same one as the C-terminal fragment obtained by NBS-cleavage in the amino acid composition. Thus the sequence of all the amino acid residues were determined as in Fig. 1.

Now elucidation of the chiralities of individual Ser, Val and Dab residues remains to be clarified. The three peptide fragments by partial acid hydrolysis mentioned above were preparatively isolated. Val^1 was isolated from the acid hydrolyzate of the N-terminal fragment. Similarly, Ser^4 was obtained from the fragment of Ser → Trp* (*Trp was degenerated), and Ser^6 and Val^{11} were obtained from the C-terminal fragment. Their ORD curves were measured and Val^1 and Ser^4 were deduced to be D-forms and Val^{11} and Ser^6 to be L-forms.

The chiralities of Dab^2, Dab^7 and Dab^8 were determined as follows: Tri(DNP)-tridecaptin A was subjected to the cleavage reaction of peptides by mild oxidation followed by treatment with hydroxylamine, which was developed by DIBELLO *et al.* to cleave peptides selectively at Thr or Ser residue[8]. Tri(DNP)-tridecaptin A might be cleaved at the N-terminal sides of β-hydroxy acyl residue and the two Ser residues by this reaction. From the reaction mixture, two peptide fragments were isolated

by TLC. One was assumed to be $Val^1 \rightarrow \gamma$-DNP-$Dab^2 \rightarrow Gly^3 \rightarrow Ser^4$* (*Ser is converted to its isooxazolone form) and the other to be γ-DNP-$Dab^7 \rightarrow \gamma$-DNP-$Dab^8 \rightarrow Phe^9 \rightarrow Glu^{10} \rightarrow Val^{11} \rightarrow aIle^{12} \rightarrow Ala^{13}$ from the amino acid compositions and the established amino acid sequence of tridecaptin A. When the latter peptide fragment was dinitrophenylated and then hydrolyzed, α,γ-bis(DNP)-Dab^7 and γ-DNP-Dab^8 were liberated. They were easily separated and the latter was converted to α,γ-bis-DNP-Dab^8. From the N-terminal fragment α,γ-bis(DNP)-Dab^2 was similary isolated. Comparison of optical rotations of these bis(DNP)-Dab specimens clarified that Dab^2 and Dab^8 are in D-forms and Dab^7 in L-form.

From these results we concluded the total structure of tridecaptin A as shown in Fig. 1, except for the configurations of the fatty acyl residue.

Experimental

Amino acid analysis was carried out with an amino acid analyzer Hitachi KLA-5 under the normal conditions directed for the instrument except for the following case. When quantitative analysis of γ-DNP-Dab was needed, the procedure was modified as follows: 0.35 M sodium citrate buffer solution, pH 5.28 for the short column was replaced by 0.525 M sodium citrate buffer solution, pH 5.28 by the reason described in our previous paper[6]. The peptides to be analyzed were hydrolyzed with constant boiling hydrochloric acid for 20 hours at 110°C, except where stated otherwise. ORD and CD curves were recorded with spectropolarimeter JASCO Model ORD/UV-5. TLC was performed on silica gel plates (Kieselgel $60F_{254}$, Merck) using the solvent systems shown below, chloroform - ethanol - 14% ammoniacal water (4: 7: 2, abbreviated as CEN hereafter), *n*-butanol - acetic acid - water (4: 1: 2, abbreviated as BAW hereafter) and *n*-butanol - pyridine - acetic acid - water (10: 6: 1: 4, abbreviated as BPAW hereafter).

Gas chromatographic examination

For analytical purpose a Perkin-Elmer Model 881 equipped with a hydrogen flame detector and a steel column (6 feet, 1/8 inch diameter) packed with 15% diethylene glycol succinate polymer on Chromosorb W 80~100 mesh was used. Conditions, which was essentially same as the case of octapeptin C_1[3], were as follows; carrier gas (N_2), 5.0 kg/cm^2, approximately 30 ml/min, temperature 160°C or 120°C.

Some 10 mg each of tridecaptin A was hydrolyzed with constant boiling hydrochloric acid at 110°C for 1.0 hour or 20 hours. The ethereal extracts of the 1-hour hydrolyzate was methylated with diazomethane. When it was analyzed by GC at 160°C, a main peak of a retention time of 5.8 minutes were observed. The reference compound, methyl β-hydroxy anteisononanoate prepared from octapeptin C_1, showed the same retention time. The ethereal extract of the 20-hour hydrolyzate was hydrogenated for 2 hours in the presence of platinum oxide. The hydrogenated product was converted to the methyl ester. When it was analyzed by GC at 120°C, a main peak of 2.5 minutes was observed. It was identified with the peak of methyl anteisononanoate prepared from the hydrolyzate of polymyxin E.

Isolation of constituent fatty acid methyl ester

Some 400 mg of tridecaptin A dissolved in 8 ml of constant boiling hydrochloric acid in a sealed tube was heated at 110°C for 1.5 hours. The hydrolyzate was diluted with 20 ml of water and extracted with 8 ml of ethyl acetate three times. The ethyl acetate extracts were combined, washed with 0.1 N hydrochloric acid and water, dried over Na_2SO_4 and concentrated. The residue was dissolved in ethyl ether and the insoluble material was filtered off. To this solution an ethereal solution of diazomethane was added and then carefully concentrated by a stream of nitrogen. The residue was subjected to TLC on silica gel with ethyl ether - hexane (7: 3). Several zones with hydrophobic nature were visualized by spraying with water. A zone at Rf 0.41 was extracted with ethyl acetate. The ethyl acetate extracts was evaporated carefully by a stream of nitrogen. The obtained residue was dissolved in

ether and the insoluble residue was filtered off. When the ether was evaporated, the fatty acid methyl ester was obtained as a colorless oil (16 mg). It showed essentially the same n.m.r. (Fig. 3) and mass spectra (Fig. 4) with methyl β-hydroxyanteisononanoate from octapeptin C_1[3].

Fig. 3. N.m.r. spectrum of methyl β-hydroxy anteisononanoate from tridecaptin A.

The spectrum was recorded with a Varian A-60 spectrometer on solution in $CDCl_3$ containing TMS.

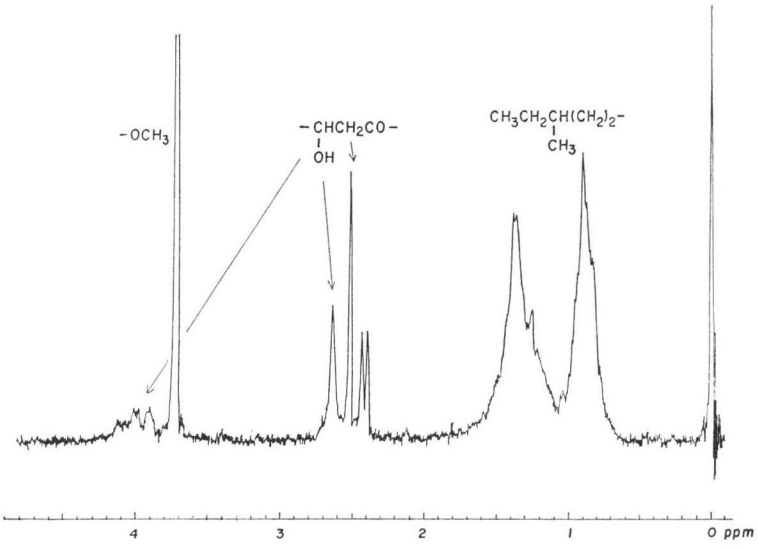
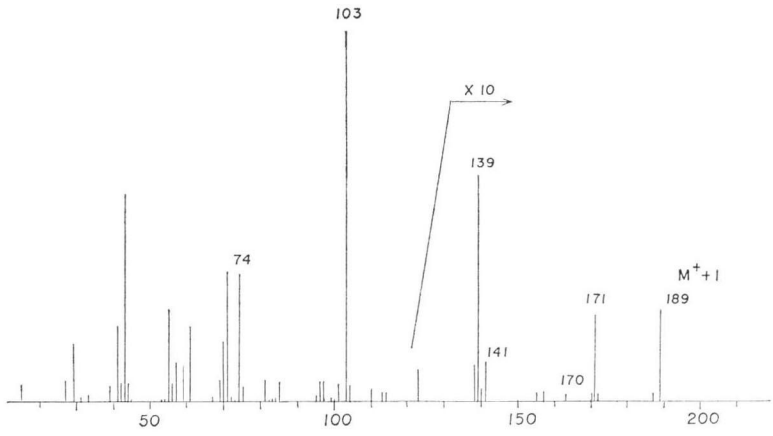


Fig. 4. Mass spectrum of methyl β-hydroxy anteisononanoate from tridecaptin A.



Isolation of constituent amino acids

The residual part of ethyl acetate extraction with the acid hydrolyzate of tridecaptin A (400 mg), described in the former section, was further hydrolyzed at 110°C for 20 hours. The hydrolyzate was concentrated to dryness and subjected to preparative paper chromatography carried out on four sheets of Toyo *Roshi No. 51*, 60×60 cm with BAW in descending manner for 20 hours. Five ninhydrin positive zones (Rf 0.72, 0.64, 0.43, 0.31 and 0.16) were cut out and extracted with water acidified with hydrochloric acid. Each zones were followed as below.

(1) The fastest moving zone (Rf 0.72) containing aIle, Phe and a *trace* amount of Val was dissolved in 2 ml of water, neutralized to pH 7.0 and placed on a small column of a porous polymer Amberlite XAD-2 (1.6×30 cm). Upon washing the column with water, aIle and a trace amount of Val passed through the column and appeared in the fraction of 40~95 ml, whereas Phe was slightly retarded by the resin and appeared in the fraction of 150~350 ml.

From the fraction of aIle and a trace amount of Val, aIle was completely separated by the repeated paper chromatography in the same system. The extract was adsorbed on a small column of Dowex 50×8 (NH_4), which was washed with water and then eluted with 0.3 N NH_4OH. Lyophilization of the eluate and crystallization of the residue from water and acetone gave aIle as colorless crystals (4.5 mg).

Anal. Found: C, 54.93; H, 9.85; N, 10.65

Calcd. for $C_6H_{13}NO_2$: C, 54.93; H, 9.99; N, 10.68
$[\alpha]_D$ $-22.6\pm1.9°$ (*c* 0.328, 5 N HCl)
ORD $[\phi]_{300}$ -350 $[\phi]_{250}$ -980 $[\phi]_{225}$ -3190 $[\phi]_{215}$ -1590 (*c* 0.0909, 0.5 N HCl)

The fraction of Phe was treated with a small column of Dowex 50×8 in the same manner as above. Lyophilization of the eluate and crystallization of the residue from water and acetone gave Phe as colorless crystals (24 mg).

Anal. Found: C, 65.68; H, 6.72; N, 8.27
Calcd. for $C_9H_{11}NO_2$: C, 65.43; H, 6.71; N, 8.48
$[\alpha]_D^{23.0}$ $-4.9\pm2.0°$ (*c* 0.223, 5 N HCl)
ORD $[\phi]_{300}$ $+80$ $[\phi]_{250}$ $+820$ $[\phi]_{225}$ $+5320$ $[\phi]_{210}$ $+3190$ (*c* 0.2375, 0.5 N HCl)

(2) The second zone (Rf 0.64) containing Val and a trace amount of Phe was treated with a small column of a porous polymer Amberlite XAD-2 (1.0×20 cm) and then the fraction of Val (25~70 ml) was treated with a small column of Dowex 50×8. Lyophilization of the eluate and crystallization of the residue from water and acetone gave Val as colorless crystals (24.5 mg).

Anal. Found: C, 51.02; H, 9.67; N, 11.79
Calcd. for $C_5H_{11}NO_2$: C, 51.27; H, 9.46; N, 11.96
$[\alpha]_D^{22.5}$ 0.0 (*c* 0.208, 5 N HCl)

(3) The third zone (Rf 0.43) containing Ala and Glu was applied to two sheets of Toyo Roshi No. 51, 60×60 cm, which was developed with BPAW in descending manner for 48 hours. Two separated zones were detected by ninhydrin coloration and extracted with acidified water. The extracts of the upper zone (Rf 0.16) was treated with a small column of Dowex 50×8 (NH_4). Lyophilization of the eluate and crystallization of the residue from water and acetone gave Ala as colorless crystals (13 mg).

Anal. Found: C, 40.72; H, 8.02; N, 15.33
Calcd. for $C_3H_7NO_2$: C, 40.44; H, 7.92; N, 15.72
$[\alpha]_D^{23.0}$ $+16.4\pm2.6°$ (*c* 0.220, 5 N HCl)
ORD $[\phi]_{300}$ $+110$ $[\phi]_{250}$ $+440$ $[\phi]_{224}$ $+1620$ $[\phi]_{212}$ 0 (*c* 0.2380, 0.5 N HCl)

The extract of the lower zone (Rf 0.08) was treated with a small column of Dowex 50×8 (H). Lyophilization of the eluate and crystallization of the residue from water and methanol gave Glu as colorless crystals (17 mg).

Anal. Found: C, 38.91; H, 6.60; N, 8.96
Calcd. for $C_5H_9NO_4 \cdot 1/2H_2O$: C, 38.46; H, 6.46; N, 8.97
$[\alpha]_D^{23.0}$ $+29.8\pm1.3°$ (*c* 0.520, 5 N HCl)
ORD $[\phi]_{300}$ $+310$ $[\phi]_{250}$ $+730$ $[\phi]_{222}$ $+2340$ $[\phi]_{208}$ 0 (*c* 0.2828, 0.5 N HCl)

(4) The fourth zone (Rf 0.31) containing Ser and Gly was applied to two sheets of Toyo Roshi No. 51, 60×60 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 extracted with acidified water. Each of the extract was lyophilized and the residue was repeated by the paper chromatography in the same system.

The extract of the upper zone (Rf 0.45) was treated with a small column of Dowex 50×8 (H). Lyophilization of the eluate and crystallization of the residue from water and ethanol gave serine as colorless crystals (6.1 mg).

Anal. Found: C, 34.08; H, 6.85; N, 13.33
Calcd. for $C_3H_7NO_3$: C, 34.28; H, 6.71; N, 13.33
$[\alpha]_D^{22.5}$ 0.0° (*c* 0.200, 5 N HCl)

The extract of the lower zone (Rf 0.35) was treated with a small column of Dowex 50×8 (NH_4). Lyophilization of the eluate and crystallization of the residue from water and ethanol gave Gly as colorless crystals (6.1 mg).

Anal. Found: C, 31.96; H, 6.91; N, 18.92
Calcd. for $C_2H_5NO_2$: C, 32.00; H, 6.71; N, 18.66

(5) The last zone (Rf 0.16) containing 2,4-diaminobutyric acid was treated with a small column

of Dowex 50×8 (NH_4). After the eluate was concentrated to dryness, the residue was dissolved in 1 N hydrochloric acid and then lyophilized. Crystallization of the residue from water and ethanol gave Dab monohydrochloride as colorless crystals (50 mg).

Anal. Found: C, 31.14; H, 7.24; N, 18.22; Cl, 22.55
Calcd. for $C_4H_{11}N_2O_2Cl$: C, 31.07; H, 7.17; N, 18.12; Cl, 22.93
$[\alpha]_D$ −4.4±0.4° (*c* 0.1012, 5 N HCl)

For the isolation of tryptophan, some 150 mg of tridecaptin A was hydrolyzed with constant boiling hydrochloric acid containing 4% thioglycolic acid in a vacuum sealed tube at 110°C for 20 hours. The hydrolyzate was diluted with water and extracted with ethyl acetate to remove thioglycolic acid and concentrated to dryness. The residue was dissolved in 2 ml of water, neutralized to pH 7.5 and placed on an Amberlite XAD-2 column (1.7×20 cm), which was washed with water slowly. Tryptophan was retarded by the resin and eluted in the fraction of 280~680 ml. Concentration followed by lyophilization of the eluate and crystallization of the residue from water and acetone gave tryptophan as colorless crystals (3 mg).

Anal. Found: C, 60.63; H, 5.74; N, 12.80
Calcd. for $C_{11}H_{12}N_2O_2 \cdot 3/4H_2O$: C, 60.67; H, 6.25; N, 12.87
ORD $[\phi]_{300}$ −380 $[\phi]_{290}$ −1100 $[\phi]_{255}$ −550 $[\phi]_{235}$ −2500 (*c* 0.0748, 0.5 N HCl)
CD $[\theta]_{308}$ 0 $[\theta]_{289.5}$ −1370 $[\theta]_{281}$ −1260 $[\theta]_{250}$ −320 $[\theta]_{225.5}$ −10600 $[\theta]_{216.5}$ 0(*c* 0.0748, 0.5 N HCl)

Tri(DNP)-tridecaptin A

Hydrochloric acid salt of tridecaptin A (53 mg) was dissolved in a mixture of water (2 ml), *n*-butanol (2 ml), and ethanol (1 ml). To this solution sodium bicarbonate (30 mg) and 5% dinitrofluorobenzene in ethanol (1.0 ml) was added. The solution was stirred for 4 hours and allowed to stand for 16 hours. Ethanol and *n*-butanol was evaporated *in vacuo* and from the aqueous solution excess dinitrofluorobenzene was removed by ether-extraction. Then the solution was acidified by 1 N HCl to give a yellow oil, which was separated by centrifugation. It was washed with ether and then triturated with water, giving a yellow powder (48 mg). When a portion of the powder was hydrolyzed for amino acid analysis, the following amino acids were found (in ratio); Ser (1.76), Glu (1.00), Gly (1.00), Ala (0.87), Val (1.89), aIle (0.60), Phe (0.91), Trp (0.80) and γ-DNP-Dab (2.14).

Cleavage reaction with N-bromosuccinimide

Tridecaptin A (5 mg, 2.8 μmole) was dissolved in 70% aqueous acetic acid (1.0 ml). N-Bromosuccinimide (5 mg) was added to the solution, which was then allowed to stand for 10 minutes at room temperature. To decompose excess N-bromosuccinimide, a few drops of formic acid was added to the solution and allowed to stand for 5 minutes, which was then lyophilized. When the residue was chromatographed on a silica gel GF with BAW, three ninhydrin positive spots were observed (Rf 0.51, 0.30 and 0.20). Two zones (Rf 0.51 and 0.20) were separated by preparative manner of the TLC and extracted with a slightly acidified aqueous methanol. The extracts were concentrated to dryness. The residue from the zone of Rf 0.20 was further purified by paper chromatography carried out on one sheet of Toyo Roshi No. 51, 20×60 cm, developed with BAW in descending manner for 20 hours. The zone (Rf 0.48) detected by ninhydrin coloration was extracted with slightly acidified aqueous methanol. The extracts were concentrated to dryness. When small portions of these preparations were hydrolyzed for amino acid analysis, the following amino acids were found (in ratio); Rf 0.51 substance: Dab (0.99), Ser (0.82), Gly (1.0), Val (1.03); and Rf 0.20 substance: Dab (2.24), Ser (0.97), Glu (1.20), Ala (1.0), Val (1.24), aIle (0.85), Phe (1.08). The substance of Rf 0.20 was thought to be a C-terminal fragment, because this fragment showed no UV absorption depending on the Trp residue. In several runs the yield of this fragment was about 10~16%.

EDMAN degradation of the C-terminal octapeptide obtained by NBS cleavage

Successive EDMAN degradation of the C-terminal octapeptide described in the former section was achieved with some modifications to diminish the loss of the remaining peptide[6]. Excess phenylisothiocyanate was removed by extraction with cyclohexane twice and then a mixture of cyclohexane and benzene (1: 1), and phenyl thiocarbamyl amino acids were extracted with ethyl ether three times. The result showed the sequence of the octapeptide to be Ser → Dab → Dab → Phe → Glu → Val →

Table 3. EDMAN degradation of C-terminal octapeptide.

	PTH-Amino acid	Amino acids found (in ratio)						
		Ser	Dab	Phe	Glu	Val	aIle	Ala
Original peptide		1.1	2.0	1.2	1.1	1.2	0.6	1.0
Step 1	not detected	**0.0**	1.5	0.8	0.8	1.2	0.6	1.0
Step 2	Dab*	0.1	**1.1**	1.1	1.2	1.3	0.8	1.0
Step 3	Dab*	0.0	**0.0**	0.9	1.0	1.1	0.7	1.0
Step 4	Phe	0.1	0.0	**0.1**	1.0	1.2	0.8	1.0
Step 5	Glu	0.0	0.0	0.0	**0.0**	1.0	0.8	1.0
Step 6	Val	0.0	0.0	0.0	0.0	**0.3**	0.8	1.0
Step 7	aIle	—	—	—	—	—	—	+**

* Phenylthiohydantoin of γ-phenylthiocarbamyl-2,4-diaminobutyric acid
** Analyzed before hydrolysis

aIle → Ala.

Partial acid hydrolysis

Hydrochloric acid salt of tridecaptin A (47 mg, 26 μmole) was dissolved in a mixture of formic acid (0.5 ml) and conc. hydrochloric acid (0.5 ml). The solution was allowed to stand at 37°C for 20 hours and then concentrated to dryness. When the residue was subjected to TLC with BAW, three main ninhydrin positive spots (Rf 0.38, 0.30 and 0.20) and several minor ninhydrin positive spots were observed. Three main products were separated by preparative manner of the TLC and extracted with slightly acidified aqueous methanol. The extracts were evaporated to dryness and each residue was further purified if necessary and then characterized as described below.

(1) When a small portion of the Rf 0.38 substance was hydrolyzed and analyzed with an amino acid analyzer, Val (1.0), Gly (0.96) and Dab (0.98) were found (in ratio). The other portion was dinitrophenylated by the usual way. When the dinitrophenylated product was hydrolyzed and analyzed, Val (1.0), Gly (0.88) and γ-DNP-Dab (0.66) were found (in ratio). Thus this tripeptide was thought to be an acylated N-terminal fragment. Its amino acid sequence was determined as described in the next section.

(2) The residue of Rf 0.30 was applied to paper chromatography on one sheet of Toyo Roshi No. 51, 30×60 cm, developed with BAW in descending manner for 20 hours. A zone which showed yellow color with ninhydrin (Rf 0.75) was extracted with slightly acidified aqueous methanol. When a portion of this substance was hydrolyzed and analyzed, only Ser was found. This fragment has UV absorption, presumably caused by partially degenerated Trp residue ($\lambda_{max}^{0.01\,N\ HCl}$ 243 nm, 292 nm, 300 nm). Thus this fragment was assumed to be a product due to Ser → Trp fragment, whose Trp residue was degenerated during the hydrolysis.

(3) The residue of Rf 0.20 was applied to paper chromatography on one sheet of Toyo Roshi No. 51, 60×60 cm, developed with BAW in descending manner for 20 hours. A zone (Rf 0.51) detected by ninhydrin coloration was extracted with acidified aqueous methanol. When a small portion of this fragment was hydrolyzed and analyzed, Ser (1.01), Glu (1.00), Ala (0.82), Val (0.93), aIle (0.71) and Dab (1.95) were found (in ratio). From the amino acid composition, this fragment was thought to be the same fragment as the C-terminal fragment obtained by NBS-cleavage.

The amino acid sequence of the N-terminal tripeptide

The N-terminal tripeptide (2.5 μmoles), as described in the former section, obtained by the partial acid hydrolysis was dissolved in a mixture of formic acid (0.1 ml) and conc. hydrochloric acid (0.1 ml) and allowed to stand at 37°C for 40 hours. The solution was evaporated to dryness. When the residue was chromatographed on a silica gel plate with CEN and sprayed water, two new spots (Rf 0.41 and 0.32) other than the starting material (Rf 0.27) were visualized. They were separated by the preparative manner of the TLC and the zone of Rf 0.41 was extracted with methanol and the zone of Rf 0.32 was

extracted with slightly acidified aqueous methanol. The extracts were evaporated to dryness and the residue was hydrolyzed for amino acid analysis.

The residue of Rf 0.41 was negative to ninhydrin coloration and gave only Val on hydrolysis. Thus this fragment was assumed to be FA → Val.

The residue of Rf 0.32 was positive to ninhydrin coloration and gave Val (1.0) and Dab (0.87) on hydrolysis (in ratio). Thus this fragment was assumed to be FA → Val → Dab.

Therefore the amino acid sequence of the original N-terminal tripeptide was determined to be FA → Val → Dab → Gly.

The chiralities of Val^1, Val^{11}, Ser^4 and Ser^6 residues

The three peptide fragments by the partial acid hydrolysis was preparatively separated. The Rf 0.38 substance (11 μmoles), which contains Val^1, was hydrolyzed and Val^1 was isolated by the paper chromatography. Approximately 740 mcg (6.2 μmoles) of Val^1, which was estimated by the amino acid analyzer, was obtained.

Similarly from the Rf 0.30 substance (12 μmoles), which contains Ser^4, about 989 mcg (9.4 μmoles) of Ser^4 was obtained. From the Rf 0.20 substance (15 μmoles), which contains Ser^6 and Val^{11}, about 985 mcg (9.4 μmoles) of Ser^6 and about 450 mcg (3.8 μmoles) of Val^{11} were obtained. These isolated amino acids were subjected to ORD measurement.

Val^1 : $[\phi]_{250}$ −720 $[\phi]_{225}$ −2290 $[\phi]_{220}$ −1750 (*c* 0.0703, 0.5 N HCl)
Val^{11}: $[\phi]_{250}$ +460 $[\phi]_{224}$ +2660 $[\phi]_{215}$ 0 (*c* 0.0203, 0.5 N HCl)
Ser^4 : $[\phi]_{250}$ −570 $[\phi]_{226}$ −1650 $[\phi]_{218}$ −970 (*c* 0.0890, 0.5 N HCl)
Ser^6 : $[\phi]_{250}$ +470 $[\phi]_{223}$ +1900 $[\phi]_{212}$ 0 (*c* 0.0886, 0.5 N HCl)

The chiralities of Dab^2, Dab^7 and Dab^8 residues

Tri(DNP)-tridecaptin A (*ca.* 200 mg) was dissolved in anhydrous dimethylsulfoxide (3.0 ml) and dicyclohexylcarbodiimide (250 mg), pyridine (30 μl) and trifluoroacetic acid (15 μl) were added to the solution. It was stirred for 1 hour and then allowed to stand for 16 hours at room temperature. Then acetic acid (200 μl) was added to the solution to destroy excess dicyclohexylcarbodiimide and the solution was stirred for 1.5 hours. The white precipitate of dicyclohexylurea was filtered off and washed with 1 ml of dimethylsulfoxide twice. To the filtrate, water was added and the resulted yellow precipitate of the oxidized product was filtered.

The above yellow precipitate and hydroxylamine hydrochloride (100 mg) were dissolved in a mixture of acetic acid (5 ml), water (5 ml) and ethanol (10 ml). It was heated at 70°C for 1 hour and then the reaction mixture was evaporated to dryness. Two main products were roughly separated by distributing the residue between *n*-butanol and 0.01 N HCl and followed as below.

(1) The aqueous layer was neutralized to pH 7.5 and then extracted with *n*-butanol. The *n*-butanol layer was evaporated to dryness and the residue was applied to silica gel plate and developed with CEN. A yellow zone (Rf 0.44) was separated and extracted with chloroform - methanol - 14% ammoniacal water (2: 2: 1). The extract was evaporated to dryness. When a portion of the residue was hydrolyzed and analyzed with an amino acid analyzer, Gly (1.0), Val (0.85) and γ-DNP-Dab (0.70) were found. This fragment was positive to ninhydrin coloration. Thus this fragment was assumed to be Val^1 → γ-DNP-Dab^2 → Gly^3 → Ser^4* (*Ser residue was present in the isooxazolone form) from its amino acid composition and the determined amino acid sequence of tridecaptin A.

This N-terminal fragment (10 μmoles) was hydrolyzed and the hydrolyzate was evaporated to dryness. The residue was dinitrophenylated in the usual manner and the product was subjected to TLC on a silica gel with chloroform - methanol - acetic acid (19: 1: 0.5) developed by continuous flow method for 5 hours. The separated zone of bis(DNP)-Dab (Rf 0.25 on the system) was extracted with a mixture of chloroform and methanol (1: 1) and the extract was evaporated to dryness. The residue was dissolved in ethyl acetate and washed with 0.1 N HCl and water, dried (Na_2SO_4) and evaporated. Approximately 3,072 mcg of bis(DNP)-Dab^2, which was estimated from the optical density at 352 nm, was obtained.

(2) The *n*-butanol layer was evaporated to dryness and the residue was applied to silica gel plate and developed with CEN. A yellow zone (Rf 0.43) was separated and extracted with chloroform -

methanol - 14% ammoniacal water (2: 2: 1). The extracts were evaporated to dryness and the residue was applied to silica gel plate again and developed with BPAW. A yellow zone (Rf 0.56) was separated and extracted with the same solvent as above. The extracts were evaporated to dryness and the residue was dissolved in *n*-butanol and then washed with 0.1 N HCl and water. Then it was evaporated to dryness. When a portion of the preparation was hydrolyzed and analyzed with an amino acid analyzer, Glu (1.0), Ala (0.70), Val (0.87), aIle (0.63), Phe (0.73) and γ-DNP-Dab (1.23) were found (in ratio). From the amino acid constituents of this fragment and the determined amino acid sequence of tridecaptin A, this fragment was assumed to be the C-terminal fragment of γ-DNP-Dab^{7} → γ-DNP-Dab^{8} → Phe^{9} → Glu^{10} → Val^{11} → aIle^{12} → Ala^{13}.

The C-terminal fragment (11 μmoles) was dissolved in methanol (1.2 ml), water (0.6 ml) and ethyl acetate (0.3 ml). To this solution 5% dinitrofluorobenzene in ethanol (0.6 ml) and sodium bicarbonate (30 mg) were added and then stirred for 16 hours. It was diluted with water and the excess dinitrofluorobenzene was removed by ether extraction at pH 9.0. The aqueous layer was acidified and extracted with ethyl acetate, which was evaporated to dryness. The residue was hydrolyzed and the hydrolyzate was extracted with ethyl acetate.

The ethyl acetate layer, which contains bis (DNP)-Dab^{7}, was purified by the TLC in the same manner as described above and bis(DNP)-Dab^{7} was obtained as a pure preparation (835 mcg).

The aqueous layer, which contains a mixture of amino acids involving γ-DNP-Dab^{8}, was dinitrophenylated. Bis(DNP)-Dab^{8} was separated by the TLC in the same manner and obtained as a pure preparation (768 mcg).

Table 4. Molecular rotations of bis(DNP)-Dab specimens.

	$[M]_D$ in acetic acid	Configuration
bis(DNP)-L-Dab	−360	
bis(DNP)-Dab^{2}	+383±41	D
bis(DNP)-Dab^{7}	−571±93	L
bis(DNP)-Dab^{8}	+215±57	D

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