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Enduracidin, a New Antibiotic. VII.[1] Primary Structure of the Peptide Moiety

MASATAKE HORI, HIDESUKE IWASAKI, SATOSHI HORII, ISAMU YOSHIDA,
and TOSHIO HONGO

Chemical Research Laboratories and Microbiological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd.[2]

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The primary structure of enduracidin, a cyclodepsipeptide antibiotic, was estimated by the determination of the amino acid sequence, optical isomers of each amino acid, and lactone structure. The result is that the enduracidin has a macro-ring lactone structure which is composed of 16 amino acids, and has a side chain aspartic acid which is connected to a fatty acid by an amide bond. The tentative structure is shown in Fig. 7.

Enduracidin includes 12 kinds of amino acids and it was separated into two main components, enduracidin A (I) and enduracidin B (II), chromatographically though the amino acid compositions of I and II are the same.[1]

This report deals with the determination of the primary structure of the peptide moiety of enduracidins:

1) The amino acid sequence was settled by the analysis of the amino acid composition of the peptide fragments obtained by ion exchange chromatography from partially-hydrolyzed enduracidin, by the elucidation of the N-terminal[3] and C-terminal amino acids[4,5] and by Edman's degradation method.[6]

2) Optical isomers of the amino acids were determined enzymatically.[7,8]

3) The lactone structure between the C-terminal amino acid and the hydroxyl group of threonine was decided by the reduction of the lactone to an alcohol with lithium borohydride.[5]

4) The carboxyl group in aspartic acid which participated in the peptide bond was determined by the reduction with lithium borohydride after methylation of the free carboxyl group.

1. Comparison of the Peptide Moieties of Enduracidin A (I) and B (II)

To compare the amino acid sequences of enduracidins I and II, they were partially hydrolyzed with concentrated hydrochloric acid at 37°, for 72 hr and the peptide fragments obtained were separated on a Dowex 50, X-2 column. From the results shown in Fig. 1a and b, and Table I, the amino acid sequences of these peptides were considered to be the same. Consequently, the mixture of I and II was used for the investigations of amino acid sequence.

2. Amino Acid Composition of Enduracidins

Enduracidins were completely hydrolyzed and the hydrolyzate was analyzed by an automatic amino acid analyzer. Molecular weight of enduracidins was determined by X-ray

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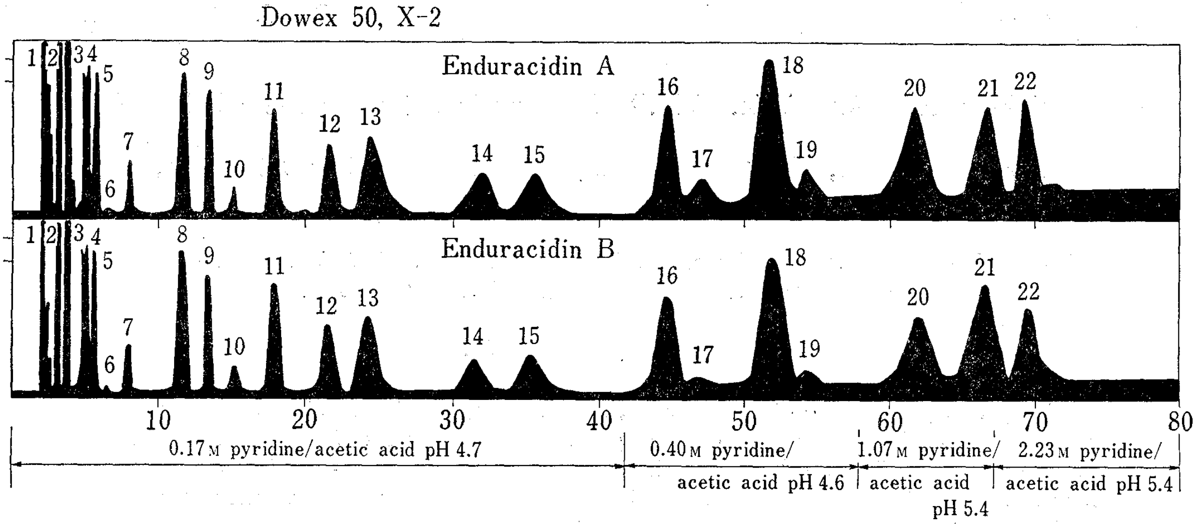

Fig. 1. Comparison of the Peptide Fragments obtained from Enduracidin A and Enduracidin B

Table I. Amino Acid Sequence of Peptide Fragments obtained from Enduracidin A and B

Peak number	Amino acid sequence	Peak number	Amino acid sequence
1	Asp, Gly	12	Ser-K_2-Gly
2	aThr-Cit	13	Y_1
3	K_1	14	Gly-Y_1
4	Thr-K_1, aThr-K_1	15	aThr-Cit-Y_2
5	Ala-K_1	16	Y_1-Ala
6	Asp-Thr-K_1	17	Gly-Y_1-Ala
7	K_2	18	Thr-K_1-Orn
8	Ser-K_2	19	Asp-Thr-K_1-Orn
9	Orn	20	aThr-Cit-Y_2-K_1
10	K_2-Gly	21	Y_2-K_1
11	aThr-K_1-K_1	22	Y_1-Ala-K_1

analysis (about 2350). From these results, the amino acid composition of enduracidin was thought to be as shown in Table II.

Table II. Amino Acid Composition of Enduracidin

Amino acid	Asp	Thr	aThr	Ser	Cit	Gly	Ala	K_1	K_2	Orn	Y_1	Y_2
mole/molecule	1	1	2	1	1	1	1	5	1	1	1	1

3. Amino Acid Sequence

Partial hydrolysis of enduracidins was performed with concentrated hydrochloric acid at 37°, for 72 hr. The hydrolyzate was separated chromatographically by means of a Dowex 50, X-2 column and pyridine/acetic acid buffer under the conditions described in Fig. 2. The amino acid components of the peptides corresponding to the peaks obtained were analyzed. The N-terminal amino acids of these peptides were determined by thin-layer chromatography after conversion into dansyl derivatives and hydrolysis of the resulting compound. The results could be arranged into four groups as shown in Table III.

Partial hydrolysis of enduracidins was also performed under milder conditions, that is with 0.05 N hydrochloric acid, at 120°, for 24 hr. The hydrolyzate was separated chromato-

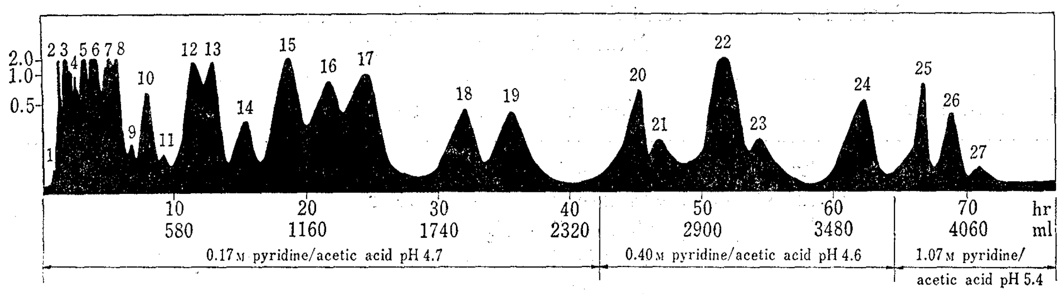

Fig. 2. Chromatogram of partially Hydrolyzed Peptide Fragments of Enduracidin

sample: enduracidin hydrolyzate (conc. HCl, 37°, 72 hr)
column: Dowex 50, X-2 (1.8 × 56.5 cm), 37°
flow rate: 58 ml/hr

TABLE III. Amino Acid Sequence of Peptide Fragments obtained by Column Chromatography

Asp→Thr→K_1→Orn	aThr→K_1→K_1	aThr→Cit→Y_2→K_1	Ser→K_2→Gly→Y_1→Ala→K_1
Asp(2)	aThr→K_1(7)	aThr→Cit(5)	Ser→K_2(12)
Asp→Thr→K_1(9)	aThr→K_1→K_1(15)	aThr→Cit→Y_2(19)	Ser→K_2→Gly(16)
Asp→Thr→K_1→Orn(23)	K_1→K_1(6)	aThr→Cit→Y_2→K_1(24)	K_2(10)
Thr→K_1→Orn(22)		Y_2→K_1(25)	K_2→Gly(14)
Thr→K_1(7)		K_1(6)	Gly(3)
K_1(6)			Gly→Y_1(18)
Orn(13)			Gly→Y_1→Ala(21)
			Y_1(17)
			Y_1→Ala(20)
			Y_1→Ala→K_1(26)
			Ala→K_1(8)
			K_1(6)

( ): peak number in Fig. 2

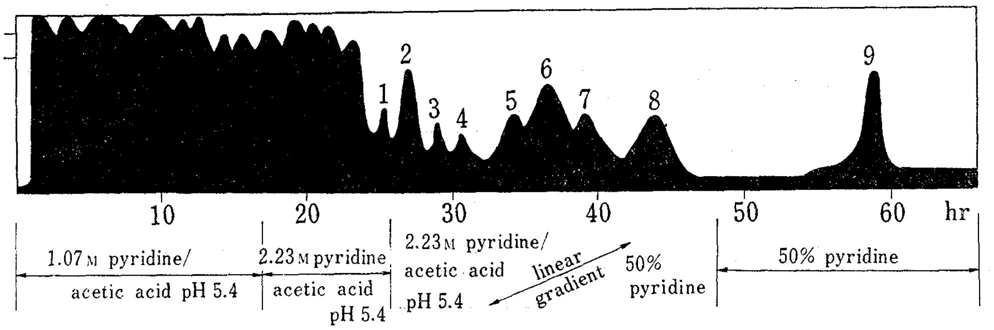

Fig. 3. Chromatogram of Partially Hydrolyzed Peptide Fragments of Enduracidin

sample: enduracidin hydrolyzate (0.05 N HCl, 120°, 24 hr)
column: Dowex50, X-2 (1.8 × 58.5 cm), 37°
flow rate: 58 ml/hr

graphically by the use of the same column as above, with the exception that a more concentrated eluent was used (Fig. 3). Fragments corresponding to five peaks (peaks 2, 4, 6, 7 and 8) among the separated peaks were isolated and the amino acid sequences of them were assigned as shown in Table IV.

When the hydrolyzate obtained under the same mild conditions was extracted with ethyl ether, a substance composed of a fatty acid⁹⁾ and aspartic acid was obtained, and it was ascertained by gas chromatography and amino acid analysis after separation by paper

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9) Fatty acid component will be reported in the No. VIII of this series.

Table IV. Amino Acid Sequence of Peptide Fragments obtained by Column Chromatography

Peak number	Amino acid sequence
8	aThr→K_1→K_1→aThr→Cit→Y_2→K_1→Ser→K_2→Gly
7	aThr→K_1→K_1→aThr→Cit→Y_2→K_1→Ser
6	aThr→K_1→K_1→aThr→Cit→Y_2
4	aThr→Cit→Y_2→K_1→Ser→K_2→Gly→Y_1→Ala→K_1
2	aThr→Cit→Y_2

chromatography and subsequent alkaline hydrolysis. Furthermore, a hexadecapeptide was obtained from the aqueous layer of the above reactant by chromatography on a Sephadex G-25 column using 30 per cent acetic acid as the solvent. The N-terminal amino acid sequence of the hexadecapeptide was clarified by Edman's degradation to be Thr→K_1→Orn→aThr→K_1⋯.

From the results described above the total amino acid sequence of the peptide moiety of enduracidins was determined to be as follows:

$$\text{Asp}\longrightarrow\text{Thr}\longrightarrow K_1\longrightarrow\text{Orn}\longrightarrow\text{aThr}\longrightarrow K_1\longrightarrow K_1\longrightarrow\text{aThr}\longrightarrow\text{Cit}\longrightarrow Y_2\longrightarrow K_1\longrightarrow$$
$$\text{Ser}\longrightarrow K_2\longrightarrow\text{Gly}\longrightarrow Y_1\longrightarrow\text{Ala}\longrightarrow K_1$$

4. Free Carboxyl Group of Aspartic Acid

Since aspartic acid has two carboxyl groups, it must be determined which is involved in the peptide bond. To solve this question, the free carboxyl group of enduracidins was methylated, and then reduced to the alcohol with lithium borohydride. The reduced enduracidins were hydrolyzed and analyzed by an amino acid analyzer. Fig. 4 shows the principle of this method.

In our experiment, enduracidins gave homoserine and, therefore, the α-carboxyl group was considered to be involved in the peptide bond.

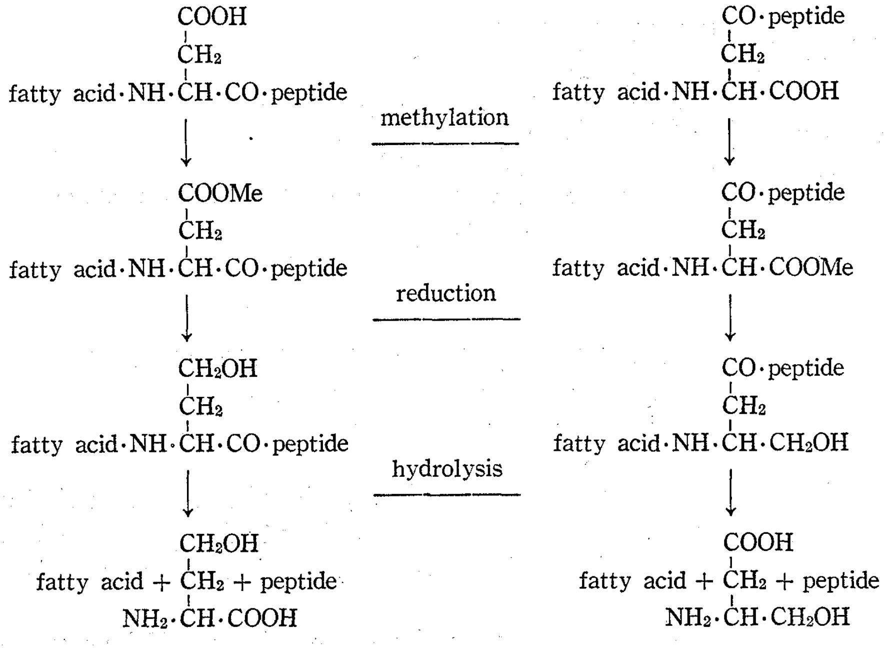

Fig. 4. Free Carboxyl Group of Aspartic Acid

5. Lactone Ring

The infrared absorption at 1750 cm^{-1} and the result of reduction with lithium borohydride described later suggested that the C-terminal carboxyl group of enduracidins forms a lactone ring. Consequently, the elucidation of the hydroxyl group contributing to the lactone was required. Phenylisocyanate was reacted with the hydroxyl group of enduracidins before and after cleavage of the lactone ring by hydrolysis in 1 N sodium hydroxide at room temperature for 30 min. By this reaction, the free hydroxyl groups, amino groups, *etc.* were converted into urethane, urea, *etc.* Then these derivatives were hydrolyzed, and the amino acid composition was analyzed. As shown in Table V, among hydroxy amino acids, only threonine did not decrease in this treatment. On the other hand after cleavage of the lactone ring, threonine also decreased together with the other hydroxy amino acids. This fact means the hydroxyl group of threonine was bonded with the C-terminal carboxyl group to make the lactone ring. During this reaction, Y_1 and Y_2 completely disappeared.

TABLE V. Variation of the Amino Acid Composition in Enduracidin by Protecting the Hydroxyl Groups with Phenylisocyanate

Sample	Amino acid	Molar–ratio of amino acid (Ala=1)										
		Asp	Thr	aThr	Ser	Cit	Gly	Ala	K_1	K_2	Orn[a]	Y_1+Y_2
Lactone	—	0.9	0.9	2.0	0.9	0.4	1.1	1.0	4.0	1.0	1.3	1.7
Lactone	ϕ·NCO	0.7	1.0	0.3↓	0.2↓	0.2	1.2[b]	1.0	2.0	0.6	1.0	0
Acid	—	1.1	1.1	2.0	0.9	0.4	1.1	1.0	4.6	1.2	1.4	1.7
Acid	ϕ·NCO	1.1	0.6↓	0.5↓	0.4↓	0.4	1.6[b]	1.0	2.5	0.9	1.1	0

a) Cit is hydrolyzed to Orn by acid hydrolysis.
b) Gly is always increased by alkaline hydrolysis.

The values for K_1 and K_2 obtained in this method were not reliable due to their poor reactivity with phenylisocyanate or lability of the reaction product, therefore the state of the phenolic hydroxyl group of these substances was determined by measuring the ultraviolet (UV) absorption before and after cleavage of the lactone ring. The UV absorbance at 245 nm (phenolate ion) in diluted alkaline solution corresponded to the sum of five moles of K_1 and one mole of K_2, and it did not change with the cleavage of the lactone ring; hence these phenolic hydroxyl groups were considered to be free in enduracidins.

6. Carboxyl-terminal Sequence of Enduracidins

In order to confirm the above sequence, the C-terminal sequence was also determined. Enduracidins contain five K_1 s in a molecule and, moreover, three of them were found at

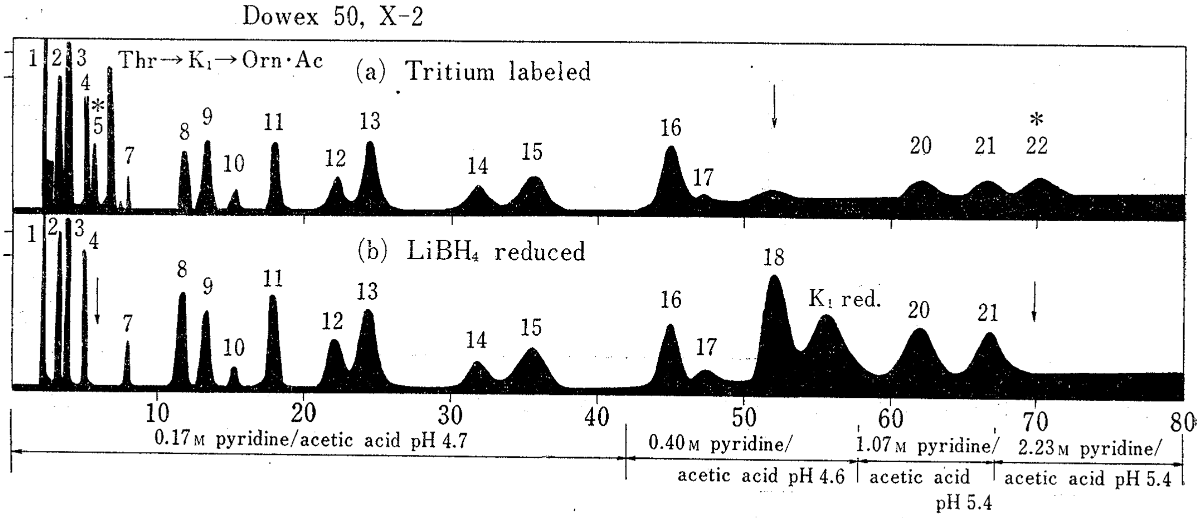

Fig. 5. Chromatograms of Peptide Fragments obtained from Tritium Labeled Enduracidin and from $LiBH_4$ reduced Enduracidin

the C-terminal of the peptide fragmants in Table III. Therefore, at least the second amino acid (Ala) from the C-terminal must also be determined to find out the K_1 corresponding to C-terminal.

As the C-terminal of enduracidins forms a lactone ring, the lactone ring was opened beforehand. The linear peptide (III) obtained was treated with acetic anhydride and tritium water, and the resulting tritiated linear peptide was hydrolyzed partially in concentrated hydrochloric acid at 37°, for 72 hr. Then the reaction mixture was separated on a Dowex 50, X-2 column under the conditions shown in Fig. 5a.

From the result, it was found that the peptides Ala$\rightarrow K_1$ (Fig. 5a, peak 5), and $Y_1\rightarrow$Ala$\rightarrow K_1$ (Fig. 5a, peak 22) were labeled with radio-activity. In this reaction, the peptide Thr$\rightarrow K_1\rightarrow$Orn was also acetylated and the peak of the acetylated peptide was eluted after about 6 hr.

In addition, enduracidins or the methylated linear peptide (Me-III) were reduced with lithium borohydride to convert the C-terminal carboxyl group into a hydroxyl group. The reduced peptides were hydrolyzed partially in the same way as above, and then separated on a Dowex 50, X-2 column as described above (Fig. 5b). As shown in Fig. 5b, the peaks of both Ala$\rightarrow K_1$ and $Y_1\rightarrow$Ala$\rightarrow K_1$ were eliminated and a peak due to reduced K_1 (Fig. 5b, peak K_1 red.) appeared newly.

These results also support the conclusion that the C-terminal sequence of enduracidins is $\cdots Y_1\rightarrow$Ala$\rightarrow K_1$.

7. Optical Isomerism of Amino Acids

Enduracidins were hydrolyzed directly to the component amino acids and the mixture of amino acids was treated with L-amino acid oxidase and D-amino acid oxidase, respectively. The amino acid composition of the resulting mixture was determined and the results are shown in Table VI. Apparently, citrulline was proved to be L-isomer and serine, alanine and ornithine to be D-isomers. Aspartic acid was not digested by both enzymes, therefore the optical rotation was measured after isolation of aspartic acid. It showed that aspartic acid is L-form. The configurations of the three novel amino acids, L-K_2,[10] L-Y_1[11] and D-Y_2,[11] have already been reported.

Table VI. Enzymatic Degradation of the Amino Acids
(Residual molar-ratio for control sample)

	Asp	Thr+aThr	Ser	Cit	Gly	Ala	K_1	K_2	Orn	Y_1+Y_2
Control sample	1	3	1	1	1	1	5	1	1	2
After degrad.										
by L-A.A. oxydase	0.84	1.75	0.95	0	1.17	0.95	2.05	0.78	0.78	1.78
by D-A.A. oxydase	0.63	1.72	0	0.92	0.53	0.17	5.07	0.95	0.30	0.75
Discrimination	?	D+L	D	L	—	D	D+L	?	D	D+L
	optical rotation ↓ L-Asp	↓					↓	X-ray ↓ L-K_2		NMR ↓ L-Y_1 D-Y_2

More than two moles of threonine+allothreonine and K_1 are contained in the mixture of D- and L-forms in one molecule of enduracidins, therefore the positions of each isomer must

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[10] K. Kamiya, M. Nishikawa, H. Matsumaru, M. Asai, and K. Mizuno, *Chem. Pharm. Bull.* (Tokyo), **16**, 2303 (1968).

[11] S. Horii and Y. Kameda, *J. Antibiotics*, **21**, 665 (1968).

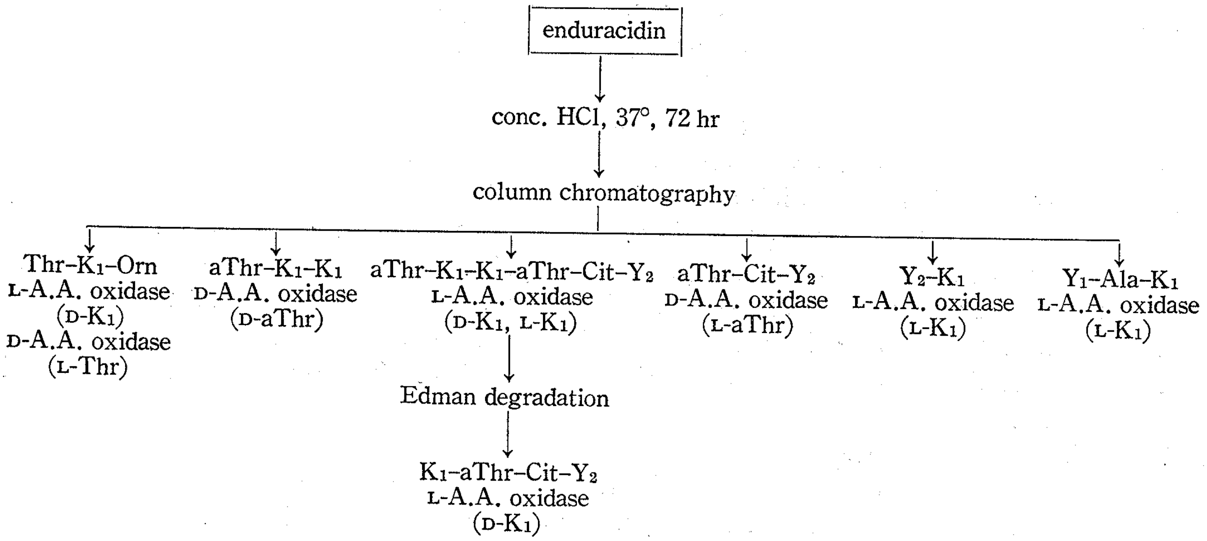

Fig. 6. Determination of the Optical Isomers of Thr, aThr and K_1

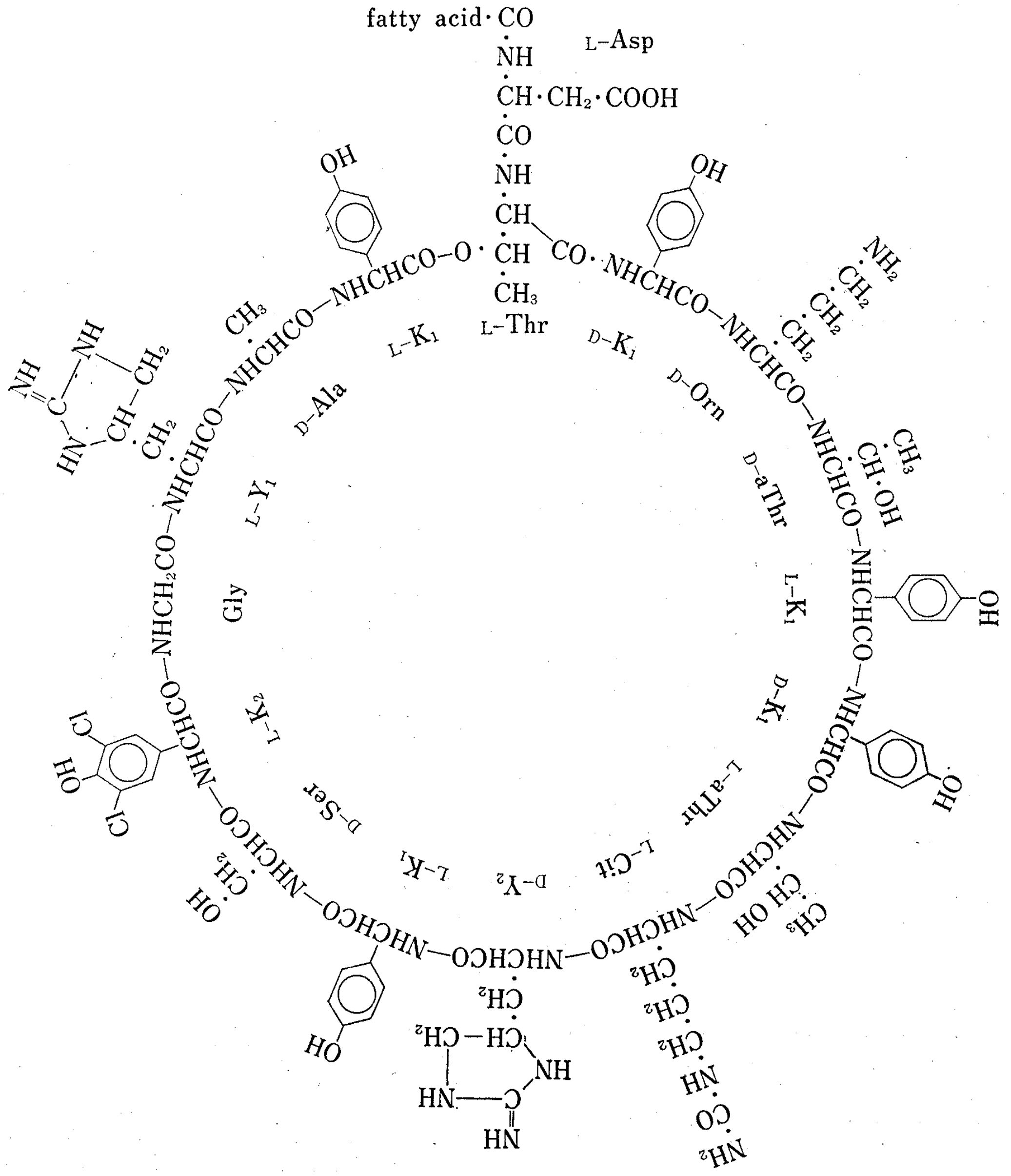

Fig. 7. Structure of Enduracidin

be determined. For this purpose, each of the peptide fragments was completely hydrolyzed and subjected to enzymic oxidation. The processes and the optical isomerism determined by this method are shown in Fig. 6.

The recemization of K_1 during the hydrolysis was possible. To determine the recemization rate, a large amount of K_1 was obtained by the method described in the case of aspartic acid, then treated with the L-amino acid oxidase, and the intact D-K_1 was purified by recrystallization. The crystalline D-K_1 was then treated with 6N hydrochloric acid, at 105°, for 24 hr (the same conditions as the complete hydrolysis). The optical rotations before and after the treatment were found to be $-170.2°$ for nontreated D-K_1 and $-147.6°$ for the treated D-K_1. This fact shows that the racemization is about 10 per cent under these conditions, and is almost negligible for the determination of isomerism. The D-amino acid oxidase did not digest D-K_1.

8. Total Tentative Structure

From all of the results obtained and other reports,[10,11] the total tentative structure of the peptide moiety of the enduracidins, was presented as shown in Fig. 7.

The structure of fatty acid and its bonding position will be reported in the No. VIII of this series.

Experimental

1. Amino Acid Analysis——All amino acid analyses were performed by means of a Hitachi KLA-3B Amino Acid Analyzer with a 50 cm column, except the separation of threonine and allothreonine. The separation of threonine and allo threonine was accomplished by the use of a 150 cm column and pH 5.28 citrate buffer.

2. Complete Hydrolysis——Hydrolysis of peptides to the amino acid components was carried out by heating with 6N HCl at 105°, for 24 hr in a sealed tube.

3. Partial Hydrolysis——The conditions of partial hydrolysis are described in each experiment.

4. Methylation——Methylation was accomplished by the use of methanolic HCl in the usual manner.

5. Separation of the Peptide Fragments——An automatic column chromatograph was assembled with a Dowex 50, X-2 column (1.8 × 50 cm, 37°), Accu-Flow pump (Beckman), and an Auto-Analyzer system (Technicon). The effluent from the column was divided in the ratio 1: 9 and the smaller part was reacted with ninhydrin reagent, and the chromatogram was recorded photometrically. The larger part was collected every 20 ml by a fraction collector (Toyo Kagaku Sangyo Co., Ltd.).

The solvent used for elution was pyridine/acetic acid buffer and the pH and the concentrations were changed successively. The fractions corresponding to each ninhydrin peak were combined and the solvent was removed under reduced pressure.

6. Paper Chromatography——Whatman No. 1 paper (28 cm in width and 47 cm in length) was used with two kinds of solvents, *i.e.*, pyridine:acetic acid:water = 1: 1: 1 and *n*-butanol:acetic acid:water = 3: 1: 1. The chromatograms were stained with ninhydrin reagent or hypochlorite reagent.

7. Preparation and Observation of Dansyl Amino Acids and Peptides[3]——About 1 mg of sample was dissolved in 0.2 ml of 0.1M sodium bicarbonate and 0.2 ml of a solution (20 mg/ml) of 1-dimethylaminonaphthaline-5-sulfonic chloride in acetone was added. The mixture was shaken for 30 sec, then allowed to stand overnight. In the case of dansyl peptides, the reactants were hydrolyzed with 6N HCl, at 105°, for 15 hr, before thin-layer chromatography.

The thin-layer chromatography was performed using Silica gel H plates (20 × 20 cm, 0.25 mm thick) with the following three kinds of solvents;

Solvent 1, chloroform:benzyl alcohol:acetic acid = 70: 30: 3
Solvent 2, chloroform:*tert*-butanol:acetic acid = 6: 3: 1
Solvent 3, benzene:pyridine:acetic acid = 16: 4: 1

The chromatograms obtained were observed under a UV light at 254 nm to estimate the yellow fluorescent spots.

8. Edman's Degradation[6]——The sample (1—10 mg) was dissolved in 0.5 ml of 60% aqueous pyridine solution containing 5% of dimethylallylamine, and 25 µl of phenylisothiocyanate was added, then the atmosphere was replaced with nitrogen, and allowed to stand at 40°, for 1 hr. The solvent in the reaction mixture was distilled off at 35° *in vacuo* to dryness, and the residue was extracted and centrifuged 5 times using 2 ml of benzene each time to remove the benzene soluble fraction. Finally the precipitate was dried at 50° under reduced pressure. To the dry material, 50 µl of trifluoroacetic acid was added and the mixture was allowed to stand at 40°, for 20 min under a nitrogen atmosphere. After the reaction 0.3 ml of water and 2 ml of ethylacetate were added, then extraction was performed by centrifuging three times with 2 ml of ethylacetate each time. The precipitate finally obtained was dried again at 35° and successively raised to 50° *in vacuo*. A small part of the dried substance (which corresponds to the peptide which is one amino acid shorter at the N-terminal than the initial peptide) was hydrolyzed and the amino acid composition was determined to define the cleaved amino acid.

9. **Tritium Labeling**[4]——Enduracidin (200 mg) was dissolved in 3 ml of 1N NaOH and saponified for 30 min at room temperature to open the lactone ring. To the reaction mixture, 6 ml of ethanol was added and the pH was acidified to 3.5 with 1N HCl; thus a white precipitate was formed. After further addition of 50 ml of water, the mixture was stored in a refrigerator for several hours and precipitation was completed. The precipitate was collected by filtration, washed with water and dried at 60° *in vacuo*, and a pale yellow substance was obtained. The substance was dissolved in a mixture of pyridine (15 ml), tritium water (1 Ci/ml, 2.5 ml) and water (2.5 ml), and glacial acetic acid (5 ml) was added under cooling with ice, then the mixture was allowed to stand for 15 hr. After the reaction the reaction mixture was evaporated at 60° *in vacuo*, and the residual tritium water was removed by the repeated addition of water (10 ml) and evaporation *in vacuo*. The labeled substance was hydrolyzed partially and separated on a Dowex 50, X-2 column under the conditions described in Fig. 4.

The tritium activity of each fraction was counted with a Gas Flow Counter (Aloka).

10. **Hydrazinolysis**——The sample (1 mg) and anhydrous hydrazine (0.3 ml) were sealed in a Pyrex tube and heated at 100°, for 10 hr, then the reaction mixture was acidified to pH 2 with 6N HCl and the amino acid remaining was analyzed.

11. **Reduction with Lithium Borohydride**[5]——A few mg of the sample was dissolved in methanol (10 ml) and a large excess of crystalline lithium borohydride was added little by little with mixing. The solution was permitted to stand for 6 hr; then a white precipitate was formed. The precipitate was collected by filtration, washed with water, and dried. The dried substance was then hydrolyzed to amino acids and the components were analyzed by an amino acid analyzer.

12. **Digestion by D-Amino Acid Oxidase**[7]——The sample amino acid mixture (1—5 μmole) was dissolved in 2 ml of 0.1M pyrophosphate buffer (pH 8.32) and 0.1 ml of bovine liver catalase (260000 u/ml) and 0.05 ml of porcine kidney D-amino acid oxidase (75 u/ml) were added. The mixture was allowed to stand for 15 hr, at 37°, and then neutralized with 1N HCl (0.15 ml) and added 1 ml of 0.2M citrate buffer (pH 2.2). The mixture was centrifuged at 10000 rpm, (9000 × *g*), and the supernatant was used to measure the amino acid composition. In this digestion, D-amino acids were decomposed.

13. **Digestion by L-Amino Acid Oxidase**[8]——The sample amino acid mixture (0.5—2 μmole) was dissolved in 0.3 ml of 0.2M triethanolamine–HCl buffer (pH 7.6) and 0.1 ml of bovine liver catalase (120000 SIGMA u/ml in water) and 0.1 ml of L-amino acid oxidase from Crotalus terr. terr. (0.5 u/ml in water) were added. The mixture was allowed to stand for 16 hr at 37°, and then neutralized, buffered and centrifuged, and amino acid analysis was performed as above. In this digestion, L-amino acid were decomposed.

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