THE STRUCTURE OF BREVISTIN

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

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Acid hydrolysis revealed the constituent amino acids of brevistin as follows: aspartic acid (D-form 2, L-form 1), L-threonine (1), glycine (1), sum of L-valine and L-isoleucine (1), [L-phenylalanine (1), L-tryptophan (1), and 2, 4-diaminobutyric acid (D-form 1, L-form 1). The constituent fatty acid was elucidated to be anteisononanoic acid by gas chromatography-mass spectrometry. A lactone linkage was proved between phenylalanine and the hydroxy group of threonine. Opening the lactone ring with dilute alkali afforded brevistinic acid. Deacylation with an enzyme preparation, Polymyxin Acylase, gave deacyl brevistinic acid. Cleavage reaction with N-bromosuccinimide, sequential analysis by EDMAN degradation and some additional evidences clarified the total structure of brevistin.

Brevistin is a new peptide antibiotic produced by *Bacillus brevis* 342-14, active against Gram-positive bacteria *in vitro* and *in vivo*.[1] Degradative studies described here elucidated the structure of brevistin as in Fig. 1.

When an acid hydrolyzate of brevistin dihydrochloride was analyzed with an amino acid analyzer, the amino acids listed in Table 1 were found. This result means that the antibiotic

Fig. 1. Structure of brevistin.

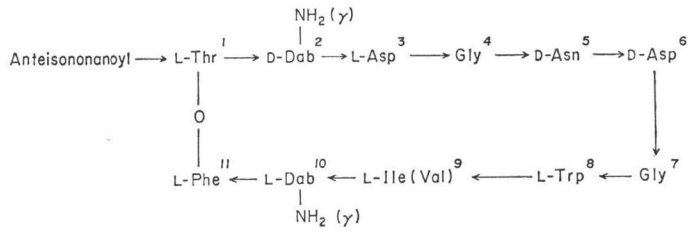


Table 1. Amino acid analysis of brevistin dihydrochloride.

	Asp	Thr	Gly	Val	Ile	Phe	Trp	Dab*	Amm
Found (μmoles/mg)	1.92	0.62	1.23	0.12	0.53	0.65	0.77	1.30	0.76
(moles/mole)	(3)	(1)	(2)	(1)		(1)	(1)	(2)	(1)

* 2,4-Diaminobutyric acid.

is a complex of undecapeptides with an approximate molecular weight of 1,550, consisting of a major peptide (*ca.* 80 %) containing isoleucine and a minor peptide (*ca.* 20 %) containing valine: isoleucine or valine is present in the same position of the sequence. This assumption was verified in the course of sequential studies, which will be described later.

From the acid hydrolyzate, phenylalanine and tryptophan were isolated by chromatography on a porous polymer, Amberlite XAD-2 column, and aspartic acid was separated using an anion-exchange resin, Amberlite IR-4B column. The other amino acids were isolated by preparative manner of paper chromatography. Threonine, isoleucine, phenylalanine and tryptophan were deduced to be L-forms from their plus COTTON effects in the ORD curves.[2,3] Valine, a constituent of the minor peptide, was obtained only in a small amount. From the $[M]_D$ value of its dinitrophenyl derivative, it was deduced to be L-form.[4] The molecular rotations of aspartic acid and its dinitrophenyl derivative were nearly one third of those of the pure D-isomers. This suggested that two D-forms and one L-form of three aspartic acid residues are present in the antibiotic. The 2,4-diaminobutyric acid isolated was optically inactive, indicating the presence of one mole each of the D- and L-forms.

When an ethereal extract of the acid hydrolyzate of brevistin was methylated and analyzed by gas chromatography, a peak of identical retention time with methyl anteisononanoate was observed. Furthermore, when the methyl ester was subjected to GC-MS, the following peaks were observed: *m/e*, 172 (M^+), 157 (M−15), 143 (M−29), 141 (M−31), 129 (M−43), 87 and 74. The base peak at *m/e* 74 and a moderately intense peak at *m/e* 87 are indicative of typical cleavage of saturated fatty acid methyl esters. In this spectrum, the peak at *m/e* 143 (M−29) was relatively high in comparing with the peak at *m/e* 141 (M−31) attributable to the acylium ion, indicating the characteristic cleavage of anteiso acid esters.

Thus, all the constituents of brevistin were determined. A molecular formula, $C_{63}H_{91}N_{15}O_{18}$, calculated for the isoleucine peptide from these constituents, is in agreement with that previously obtained from elemental analytical data.[1]

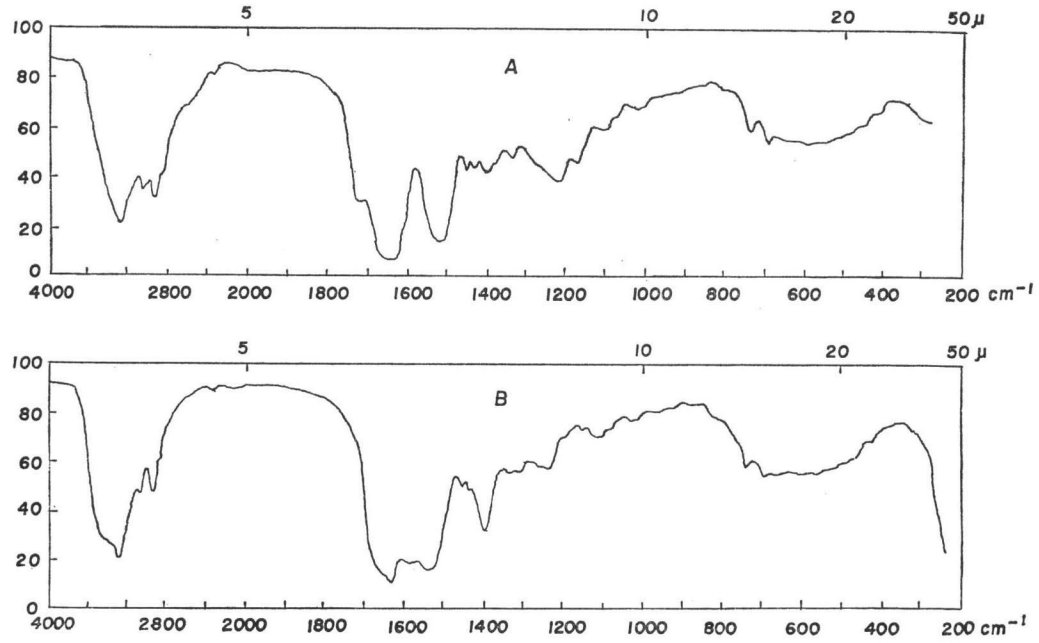
When brevistin dihydrochloride was titrated with sodium hydroxide in 67% aqueous dimethylsulfoxide, pK_1' 6.0 (neutralization equivalent: 787) and pK_2' 9.9 (neutralization equivalent: 666) were observed. This indicated the presence of two carboxyl groups and two amino groups in the molecule.

The presence of a lactone linkage was suggested by an absorption at 1740 cm^{-1} in the IR spectrum of brevistin.[1] In most peptide lactone antibiotics,[5] the carbonyl stretching bands of lactone linkages are located in the neighborhood of 1740 cm^{-1}. When brevistin was treated with dilute sodium hydroxide at room temperature, it was easily converted into a product named brevistinic acid. The hydrochloric acid salt of brevistinic acid shows an absorption at 1725 cm^{-1}, which shifts to lower than 1700 cm^{-1} in the sodium salt (Fig. 2). Elemental analysis indicated an empirical formula of $C_{63}H_{93}N_{15}O_{19}\cdot 2HCl\cdot 5H_2O$ for the hydrochloric acid salt, and amino acid analysis with the acid hydrolyzate revealed the presence of all the amino acid residues found in the intact antibiotic. These data indicates that brevistinic acid is the product of lactone ring opening of brevistin.

Brevistin was reduced with sodium borohydride, and the product was hydrolyzed and analyzed with an automatic amino acid analyzer. The reduced amino acid was phenylalanine, which therefore, was indicated to be that linked as the lactone linkage.

The threonine residue of the intact antibiotic was not destroyed by chromic acid oxidation, whereas that of brevistinic acid was easily destroyed. This is an evidence that the hydroxy group of the threonine residue is involved in the lactone linkage.

Fig. 2. Infrared absorption spectra measured on KBr tablet.
A. Hydrochloric acid salt of brevistinic acid, B. Sodium salt of brevistinic acid.



Since the lactone structure of this peptide antibiotic was established, the two carboxyl groups indicated by titration must be accounted for by two of the three aspartic acid residues. Therefore, one aspartic acid residue must be present as an amide form. Liberation of an equivalent mole of ammonia by acid hydrolysis coincides well with the above.

The two amino groups of brevistin indicated by titration were proved to be the γ-amino groups of the two diaminobutyric acid residues. When the antibiotic was dinitrophenylated and hydrolyzed, only γ-DNP-Dab was found as dinitrophenyl derivatives.

We have previously succeeded in deacylating an acylpeptide antibiotic, cerexin A, with an enzyme preparation prepared from *Pseudomonas* sp. M-6-3 (Polymyxin Acylase).[3,6] This enzymic reaction was applied with success to deacylating brevistinic acid. Consequently, deacyl brevistinic acid was obtained, and EDMAN degradation revealed its amino acid sequence up to the tryptophan residue to be Thr→Dab→Asp→Gly→Asn→Asp→Gly→(Trp).

That the fatty acyl group is attached to the N-terminal amino acid, threonine, was also supported by isolation of an acyl dipeptide, FA*→(Thr, $\overset{\overset{NH_2(\gamma)}{|}}{Dab}$), from the partial hydrolyzate of brevistin with 70% acetic acid. In this partial hydrolysis, a pentapeptide, Gly→(Trp, Ile(Val), Dab, Phe) was also isolated.

Oxidation with N-bromosuccinimide cleaved bis(dinitrophenyl)brevistinic acid into two peptide fragments at the tryptophan residue. A mixture of tripeptides, composed of Ile (or Val), γ-DNP-Dab and Phe was isolated, as the fragment of the C-terminal side. EDMAN degradation carried out with this mixture clarified the sequence of the tripeptides to be Ile (or Val)

*FA represents fatty acyl groups.

→γ-DNP-Dab→Phe. It must be noted that the Ile and Val residues showed similar behaviors in this sequential analysis.

Thus, the sequence of all the amino acid residues of brevistin was determined. Now, elucidation of the chiralities of individual residues of three Asp and two Dab residues remains to clarify completely the total structure of brevistin.

The amino acid residues are numbered from the N-terminus as shown in Fig. 1. The nonapeptide with Asp^3 at the N-terminus was prepared by two-step process of EDMAN degradation from deacyl brevistinic acid. Dinitrophenylation and hydrolysis afforded DNP-Asp^3, which was isolated by TLC. Similarly, the heptapeptide with Asn^5 at the N-terminus was prepared. Dinitrophenylation and hydrolysis gave DNP-Asp^5 and non-dinitrophenylated Asp^6, which were easily separable. CD curves were measured with these DNP-Asp specimens isolated and compared with those of synthesized DNP-D-Asp and DNP-L-Asp. From the comparison, Asp^3 was deduced to be L-form, and Asn^5 and Asp^6 were deduced to be D-form.

As already described, cleavage reaction with N-bromosuccinimide carried out with bis-(dinitrophenyl)brevistinic acid gave two peptide fragments. One, the N-terminal side, contained Dab^2, whereas the other, the C-terminal side, contained Dab^{10}. From each of the fragments, Dab^2 and Dab^{10} were isolated as their bis(dinitrophenyl)derivatives. Comparison of their CD curves with that of synthesized bis(DNP)-L-Dab clarified that Dab^2 is D-form and Dab^{10} L-form.

From all the results, we concluded the total structure of brevistin as shown in Fig. 1.

Experimental

Amino acid analysis was carried out with an amino acid analyzer Hitachi KLA-5 under the normal conditions directed for the instrument throughout this experiment. The peptides to be analyzed were hydrolyzed with constant boiling hydrochloric acid for 20 hours at 110°C except where described otherwise. α-DNP-Dab was synthesized through 3-aminopyrolidone[8] and γ-DNP-Dab was prepared from the hydrolyzate of perdinitrophenylated polymyxin E as reference samples for amino acid analysis. These two isomers were well separated by the above analytical conditions.

EDMAN degradation was carried out with peptide samples (*ca.* 1.0 μmoles) by the published procedure[9] except where stated otherwise. PTH-Amino acid was identified by the TLC,[9] but quantitation of the PTH-amino acid by OD measurement was omitted.

Amino acid composition of brevistin

A few mg of brevistin dihydrochloride was hydrolyzed with 4% thioglycolic acid-added[7] constant boiling hydrochloric acid in a vacuum-sealed tube at 110°C for 40 hours. The hydrolyzate was analyzed with an amino acid analyzer Hitachi KLA-5. The amino acids found are listed in Table 1.

Isolation of the constituent amino acids

Brevistin (200 mg) was hydrolyzed with 4% thioglycolic acid-added constant boiling hydrochloric acid in a vacuum-sealed tube at 110°C for 20 hours. The thioglycolic acid was removed by ethyl ether extraction from the hydrolyzate, which was then freeze-dried. The hydrolyzate was first subjected to preparative paper chromatography on a sheet of Toyo Roshi No. 51, 60×60 cm, with *n*-butanol - acetic acid - water (4:1:2) developed by descending manner for 24 hours. Three separated zones were detected when a strip virtically cut out from the developed paper was visualized with ninhydrin coloration. The most moved zone contained phenylalanine, isoleucine, tryptophan and valine; the second zone contained threonine, glycine and aspartic acid; and the last zone contained only 2, 4-diaminobutyric acid. Each zone was

cut out and extracted with slightly acidified water, respectively. The first and second fractions were followed as described below. The last fraction, containing only 2,4-diaminobutyric acid, was adsorbed on a small column of Dowex 50×8 (NH_4), which was washed with water and eluted with 0.3 N NH_4OH. Lyophilization of the eluate gave a colorless powder (19 mg) of the amino acid.

a) Isolation of isoleucine, valine, phenylalanine and tryptophan: The first fraction from the most moved zone, containing isoleucine, valine, phenylalanine and tryptophan, was dissolved in water (8.0 ml), neutralized to pH 7.0 and placed on top of a column of a porous polymer, Amberlite XAD-2 (1.7×36 cm). Upon washing the column with distilled water, isoleucine and valine passed through without any retardation and appeared in the fraction of 55~105 ml. Phenylalanine was slightly retarded by the resin and eluted in the fraction of 175~320ml. Tryptophan, which was detected by EHRLICH reaction, was more strongly retarded and leaked in the fraction of 490~500 ml. Then, the column was eluted with 20 % aqueous methanol. The leakage of tryptophan continued up to the fraction of 650 ml. The fraction containing phenylalanine was slightly acidified with hydrochloric acid and lyophilized to give phenylalanine hydrochloride as a colorless powder (10 mg). Tryptophan was also obtained by lyophilization of the fraction as a colorless powder (8.5 mg).

The mixture of isoleucine and valine, the effluent fraction, was separated by preparative paper chromatography on Toyo Roshi No. 51, with *n*-butanol - acetic acid - water (4:1:2). The separated zones of respective amino acids were cut out and extracted with slightly acidified water. The extracts were adsorbed on small columns of Dowex 50×8 (NH_4), which were washed with water and eluted with 0.3 N NH_4OH. Lyophilization of the eluates afforded isoleucine (6 mg) and valine as colorless powders. Valine was converted to its dinitrophenyl derivative, because its amount was too small to allow measurement of the optical rotation with itself.

b) Isolation of aspartic acid and threonine: The fraction, prepared from the second zone containing aspartic acid, threonine and glycine, was dissolved in water (5 ml) and passed through a column of Amberlite IR-4B (acetate form) (20 ml) at pH 7.0, and the column was washed with water. Only aspartic acid was adsorbed on the column and eluted with 0.3 N NH_4OH. The eluate containing aspartic acid was then passed through a small column of Dowex 50×8 (NH_4) at acidic pH. After washing the column with water, aspartic acid was eluted with 0.3 N NH_4OH. The eluate was lyophilized to give a gummy residue, which was then dissolved in a little dilute hydrochloric acid and precipitated by addition of ethanol. Aspartic acid hydrochloride was obtained as a colorless crystalline powder (16 mg). A portion of the aspartic acid was converted to its dinitrophenyl derivative by the usual way.

The effluent and washing, containing threonine and glycine, was separated by preparative paper chromatography on Toyo Roshi No. 51 with *t*-butanol - methylethylketone - conc. ammoniacal water - water (4:3:1:2). The separated zone of threonine was cut out, extracted, and treated with a small column of Dowex 50×8 (NH_4) as described above. Some 3 mg of threonine was obtained as a colorless powder. When the sample was compared with *allo*-threonine by paper chromatography with an upper layer of *n*-butanol - acetone - 28 % ammonium hydroxide - water (8:1:1:6),[10] it was clearly differentiated from the *allo*-isomer.

Optical activities of the constituent amino acid

ORD curve was recorded with a spectropolarimeter JASCO Model ORD/UV-5. The optical activities measured with the specimens of the above amino acids and some dinitrophenyl derivatives were as follows:

Threonine: ORD: $[\phi]_{250}+210$, $[\phi]_{225}+1560$, $[\phi]_{214}0$ (*c* 0.0899, 0.5 N HCl)
Isoleucine: ORD: $[\phi]_{250}+970$, $[\phi]_{224}+3080$, $[\phi]_{211}0$ (*c* 0.0908, 0.5 N HCl)
Dinitrophenyl valine: $[M]_D^{20.0}+1301\pm478°$ (*c* 0.032, N NaOH)

As the available amount of this preparation was too small to be weighed, the concentration of the solution used in this measurement was calculated from the optical density at 362 nm. The amplitude of the $[M]_D$ value in this measurement was less reliable, but its plus sign indi-

cated the amino acid to be L-form by comparison with literature.[4]

Phenylalanine hydrochloride: ORD: $[\phi]_{300}0$, $[\phi]_{225}+2800$, $[\phi]_{218}0$ (*c* 0.0724, 0.5 N HCl)
Tryptophan: ORD: $[\phi]_{300}+860$, $[\phi]_{280}+1120$, $[\phi]_{260}+430$, $[\phi]_{234}+3870$, $[\phi]_{229}0$ (*c* 0.0949, 0.5 N HCl)

The figures measured with L-tryptophan are cited in our previous report.[3]

Aspartic acid hydrochloride: $[M]_D^{24.0}-7.1\pm1.3°$ (*c* 0.547, 5 N HCl)
A commercial sample of L-aspartic acid showed: $[M]_D^{26.0}+32.1\pm2.0°$ (*c* 0.519, 5 N HCl)
Dinitrophenyl aspartic acid: $[M]_D^{25.0}-74.8\pm15.3°$ (*c* 0.128, N NaOH)
A synthesized specimen of dinitrophenyl-D-aspartic acid showed: $[M]_D^{22.0}-292\pm47°$ (*c* 0.088, N NaOH)
2, 4-Diaminobutyric acid: $[M]_D^{26.0}$ 0 (*c* 0.447, 5 N HCl)

Elucidation of the constituent fatty acid

For gas chromatographic analysis, a Perkin-Elmer Model 881 equipped with a hydrogen flame detector and a steel column (six feet, 1/8-inch diameter) packed with 15 % diethylene glycol succinate polymer on Chromosorb W 80~100 mesh was used. Mass spectrum was measured with a Hitachi RMU-6 gas chromatograph-mass spectrometer.

Some 10 mg of brevistin was hydrolyzed with constant boiling hydrochloric acid at 110°C for 20 hours. The hydrolyzate was extracted with 2 ml of ethyl ether three times. The ethereal extract was dried with anhydrous sodium sulfate and filtered into a 10-ml glass stoppered centrifuge tube. The tube was dipped in a cold water bath (*ca.* 15°C), and the ether was evaporated by a stream of nitrogen. To the concentrate, a few drops of ethereal solution of diazomethane was added, and the solution was allowed to stand for a few minutes at room temperature. Excess diazomethane was removed by a stream of nitrogen before GC analysis. When the sample was analyzed under the following conditions: carrier gas (N_2), 5.0 kg/cm^2, approximately 30 ml/min; temperature, 110°C, a peak with a retention time of 3.0 minutes was observed. An authentic specimen of methyl anteisononanoate prepared from polymyxin E showed an identical peak in the analysis. Then, the sample was subjected to GC-MS. The result and interpretation of the mass spectrum are described in the text.

Titration of brevistin dihydrochloride

A solution of brevistin dihydrochloride (33 mg) in 3 ml of 67 % aqueous dimethylsulfoxide was titrated with 0.1 N sodium hydroxide solution in the same solvent. Titration curve was recorded with a Metrohm Herisan potentiograph E-430 equipped with a glass caromel combine electrode. From the titration curve obtained, pK_1' 6.0 (neutralization equivalent 787) and pK_2' 9.9 (neutralization equivalent 667) were calculated. The neutralization equivalent calculated for pK_2' was slightly smaller than that for pK_1', probably because lactone ring opening was caused to a slight extent near the end point of the titration, resulting in consumption of slight excess of the alkaline solution.

Brevistinic acid

Some 30 mg of brevistin was dissolved in 2 ml of 0.1 N sodium hydroxide, and allowed to stand for 20 minutes at room temperature. It was then acidified with hydrochloric acid and extracted with 2 ml of *n*-butanol twice. The *n*-butanol solution was washed with water and then freeze-dried to give the dihydrochloride of brevistinic acid as a colorless powder (28 mg), which gave a single spot on a silica gel GF plate with chloroform - ethanol - 14 % ammoniacal water (4:7:2) (this solvent is abbreviated as CEN hereafter) with an Rf of 0.06, lower than that of brevistin. It was dissolved in methanol and precipitated by ethyl acetate before analysis.

Anal. Found: C, 49.47; H, 6.74; N, 13.47; Cl, 4.80.
$C_{63}H_{93}N_{15}O_{19}\cdot 2HCl\cdot 5H_2O$ requires: C, 49.53; H, 6.93; N, 13.76; Cl, 4.64.
Amino acid found (μmoles per mg): Asp (1.81), Thr (0.56), Gly (1.13), Ile (0.41), Val (0.10), Phe (0.61), Trp (0.44), Dab (1.16).

An aqueous solution of the dihydrochloride was neutralized to pH 6.0 with sodium

hydroxide and extracted with *n*-butanol. The *n*-butanol solution was washed with water and after addition of three equivalent amounts of 0.1 N sodium hydroxide, freeze-dried to give the sodium salt of brevistinic acid. The IR spectra of the dihydrochloride and the sodium salt are illustrated in Fig. 2.

Reduction with sodium borohydride

To a suspension of brevistin (2.0 mg) in pyridine (0.1 ml), the minimum amount of water needed to produce a clear solution was added. Sodium borohydride (22 mg) was added to the solution, which was then allowed to stand for 90 hours at room temperature. After decomposition of excess sodium borohydride with a few drops of acetic acid, the solution was freeze-dried. Without any purification, it was hydrolyzed and analyzed with an amino acid analyzer.

Amino acid found (in ratio): Asp (3.16), Thr (1.07), Gly (1.87), Val, Ile (1.00), Phe (0.09), Dab (2.06).

Chromic acid oxidation

Chromic acid (100 mg) was dissolved in a mixture of pyridine (0.10 ml) and acetic acid (3.0 ml), and the small residue was filtered off. Some 2 mg each of brevistin and brevistinic acid was dissolved in the chromic acid solution (0.2 ml) and allowed to stand for 20 hours at room temperature. The reaction mixture was diluted with water and extracted with *n*-butanol. The extract was concentrated to dryness, hydrolyzed and analyzed with an amino acid analyzer.

Preparation	Amino acid found (in ratio)					
	Asp	Thr	Ile, Val	Phe	Dab	Gly
From brevistin	3.35	0.96	0.84	1.00	1.60	1.92
From brevistinic acid	3.66	0.01	0.83	1.00	1.63	2.01

Cleavage reaction with N-bromosuccinimide

The 2, 4-dinitrophenyl derivative of brevistinic acid was prepared by the usual way. The DNP-derivative (4.3 mg, 3 μmoles) was dissolved in 0.5 ml of 0.5 N sodium hydroxide, to which acetic acid was added to prepare a solution of pH 2.2 (*ca.* 0.8 ml). N-Bromosuccinimide (3.1 mg, 18 μmoles) 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 reaction mixture, which was then lyophilized. When the residue was chromatographed on a silica gel GF with CEN, three yellow spots (Rf 0.82, 0.48 and 0.25) were separated. Only the spot of Rf 0.82 was positive to ninhydrin. These were separated by preparative manner of the TLC and extracted with a mixture of methanol and 14 % ammoniacal water (1:1). The extracts were concentrated to dryness, then dissolved in butanol, washed with dil. hydrochloric acid and water, and concentrated to dryness. Small portions of these preparations were hydrolyzed for amino acid analysis. The following amino acids were found (in ratio): Rf 0.82 substance: Ile, Val (0.57, 0.20), Phe (1.00), γ-DNP-Dab (+); Rf 0.25 substance: Asp (3.00), Thr (1.00), Gly (2.02), γ-DNP-Dab (+). The quantity of γ-DNP-Dab could not be estimated because of overlap of the peak with that of ammonia.

EDMAN degradation of a tripeptide obtained by cleavage with N-bromosuccinimide

The substance of Rf 0.82 described in the former section was thought to be a mixture of tripeptides derived from the C-terminal side of brevistin from the amino acid composition. Since the peptides are composed of two lipophilic amino acids and a dinitrophenylated basic amino acid, the procedure of EDMAN degradation was modified as below.

To a solution of the tripeptide (2.3 mg) in dimethylallylamine buffer solution (1.6 ml), phenylisothiocyanate (0.2 ml) was added, and the mixture was kept under nitrogen atmosphere for 60 minutes at 45°C. Excess phenylisothiocyanate was extracted with cyclohexane four times, and the aqueous layer was freeze-dried and followed by drying at 50°C for 60 minutes *in vacuo*. The residue was moistened with trifluoroacetic acid (0.2 ml) and kept for 40 minutes

at 40°C. After removing trifluoroacetic acid by a stream of nitrogen, the residue was dissolved in 0.1 N HCl (1 ml). PTC-Amino acid was extracted with ethyl ether (1 ml) three times from this solution. The ethereal extract was used for detection of PTH-amino acid by the usual manner. The aqueous layer was freeze-dried and used for the next cycle of EDMAN degradation. The result showed the sequence of the tripeptides to be Ile (Val)→γ-DNP-Dab→Phe.

	PTH-Amino acid	Amino acid found (in ratio)			
		Ile	Val	γ-DNP-Dab	Phe
Original peptide		0.57	0.20	+	1.00
Step 1	Ile	0.00	0.00	+	+
Step 2	γ-DNP-Dab	0.00	0.00	0.00	+*

* Analyzed before hydrolysis.

Partial hydrolysis with 70 % aqueous acetic acid

Brevistinic acid (19 mg) was dissolved in 70 % aqueous acetic acid (1.2 ml), and heated for 15 hours at 85°C in a sealed test tube. The solution was then concentrated to dryness, and examined by TLC on silica gel GF with CEN. A main spot (Rf *ca.* 0.35) positive to ninhydrin reaction was observed. It was separated by preparative manner of the TLC and extracted with slightly acidified aqueous methanol. When it was examined by paper chromatography with butanol - acetic acid - water (4:1:2), two spots (Rf *ca.* 0.76 and 0.64) were detected by ninhydrin coloration. They were separated by preparative manner of the paper chromatography and extracted with aqueous methanol.

When a portion of the substance of Rf 0.76 was hydrolyzed and analyzed with an amino acid analyzer, Thr (1.00) and Dab (1.04) were found (in ratio). The other portion was dinitrophenylated by the usual way, and the product was hydrolyzed and analyzed with an amino acid analyzer. Thr and γ-DNP-Dab were found.

The substance of Rf 0.64 was subjected to EDMAN degradation by the published procedure.[9] The result showed the substance to be a pentapeptide with Gly at the N-terminal end. The degradation reaction did not proceed at the second cycle and thereafter, suggesting the presence of Trp residue next to the Gly residue.

	PTH-Amino acid	Amino acid found (in ratio)				
		Gly	Trp	Ile, Val	Dab	Phe
Original peptide		1.02	0.67	0.86	0.90	1.00
Step 1	Gly	**0.15**	0.52	0.99	0.50*	1.00
Step 2	—	0.15	0.00	0.97	0.68	1.00

* γ-PTC-Dab is formed before hydrolysis.

Deacylation of brevistinic acid

Brevistinic acid (30 mg, 18 μmoles) was suspended in 3 ml of 0.05 M phosphate buffer, pH 8.0. Fifteen mg of Polymyxin Acylase (acetone powder of *Pseudomonas* sp. M-6-3),[6] which was kindly supplied by Prof. KIMURA of Mukogawa Women's University, was added to the suspension, which was then stirred for 2 days at 37°C.

The reaction mixture was freeze-dried and the residue was extracted with 0.01 N hydrochloric acid. The extract was subjected to preparative paper chromatography on Toyo Roshi No. 51 with propanol - pyridine - acetic acid - water (15:10:3:12). A zone of Rf *ca.* 0.39, positive to ninhydrin reaction, was cut out, and extracted with slightly acidified aqueous methanol. When a portion of the extract was hydrolyzed under the presence of thioglycolic acid and analyzed with an amino acid analyzer, all amino acid residues in brevistin were

found and the yield was estimated as 4.4 μmoles from the analysis.

EDMAN degradation carried out with this preparation by the published procedures[9] clarified the amino acid sequence of deacyl brevistinic acid up to the position before the Trp residue as follows.

	PTH-Amino acid	Amino acid found (in ratio)						
		Thr	Dab	Asp	Gly	Trp	Ile, Val	Phe
Original peptide		1.01	2.20	3.14	2.10	0.91	0.92	1.00
Step 1	Thr+ΔThr	**0.00**	1.02*	2.85	1.99	0.78	0.94	1.00
Step 2	Dab	0.00	**0.56**	3.10	2.07	0.73	0.97	1.00
Step 3	Asp	0.00	0.51	**2.39**	2.22	0.65	0.96	1.00
Step 4	Gly	0.00	0.56	2.38	**1.56**		1.10	1.00
Step 5	Asn	0.00	0.64	**1.86**	1.56		1.05	1.00
Step 6	Asp	0.00	0.50	1.73	1.72		1.02	1.00
Step 7	Gly	0.00	0.58	1.95	1.60	0.56	1.04	1.00
Step 8	—	0.00	0.59	2.08	1.67	0.43	1.04	1.00

* γ-PTC-Dab is formed before hydrolysis.

The chiralities of Asp[3], Asn[5] and Asp[6]

The CD curve was recorded with a spectropolarimeter JASCO Model ORD/UV-6. Some 8 mg of deacyl brevistinic acid was processed by the procedure of EDMAN degradation up to the second step. The remaining peptide, which is the nonapeptide with Asp[3] at the N-terminus as described in the former section, was dissolved in 1.5 ml of a mixture of ethanol and water (2:1). To the solution, 8 mg of sodium bicarbonate and then 0.2 ml of 5% solution (w/v) of 2, 4-dinitrofluorobenzene in ethanol was added. After 2 hours in the dark, the reaction mixture was evaporated and extracted with ether, before and after acidification with HCl. Then, the DNP-peptide was extracted with butanol and hydrolyzed with hydrochloric acid. The DNP-Asp[3] was extracted with ethyl acetate from the hydrolyzate and isolated by TLC (silica gel GF, CEN, Rf *ca.* 0.32). It was extracted from the plate with a mixture of methanol and 14% ammoniacal water (1:1) and transferred to ethyl acetate, which was washed with dil. HCl and water, dried (Na_2SO_4) and evaporated to dryness. Approximately 150 mcg of DNP-Asp[3], which was estimated from the optical density at 362 nm, was obtained, and subjected to CD measurement.

DNP-Asp[3], CD: $[\theta]_{285}+1400$, $[\theta]_{265}0$, $[\theta]_{229}-16000$, $[\theta]_{217}0$ (*c* 0.0685, N NaOH)

Some 18 mg of deacyl brevistic acid was processed by the procedure of EDMAN degradation up to the 4th step. The remaining peptide, which is the heptapeptide with Asn[5] at the N-terminus, was dinitrophenylated and hydrolyzed in essentially the same manner as above. The ethyl acetate extract of the hydrolyzate contained DNP-Asp[5], which was purified by TLC. The residual aqueous solution, which contained a mixture of amino acids involving Asp[6], was dinitrophenylated and separated by TLC. DNP-Asp[6] was extracted from the plate in the same manner.

DNP-Asp[5], CD: $[\theta]_{270}0$, $[\theta]_{230}+9700$, $[\theta]_{220}+5400$ (*c* 0.0163, N NaOH)
DNP-Asp[6], CD: $[\theta]_{288}-1400$, $[\theta]_{270}0$, $[\theta]_{228}+15400$, $[\theta]_{220}+10700$ (*c* 0.0437, N NaOH)

Synthetic specimens showed the following figures:

DNP-D-Asp, CD: $[\theta]_{285}-1490$, $[\theta]_{267}0$, $[\theta]_{228}+15700$, $[\theta]_{216}0$ (*c* 0.0785, N NaOH)
DNP-L-Asp, CD: $[\theta]_{280}+1070$, $[\theta]_{266}0$, $[\theta]_{230}-12100$, $[\theta]_{216}0$ (*c* 0.0685, N NaOH)

The chiralities of Dab[2] and Dab[10]

Bis (dinitrophenyl) brevistinic acid (21 mg, 13 μmoles) was cleaved by N-bromosuccinimide oxidation to two peptide fragments, Rf 0.82 and 0.25 substances, which were isolated by TLC

as described in the former. Each of the peptide fragments was hydrolyzed. The hydrolyzates were dinitrophenylated, and subjected to TLC on silica gel GF with chloroform - acetic acid (20:1) developed by continuous flow method for three hours. The separated zone of bis(DNP) Dab (Rf *ca.* 0.10 on the system) was extracted with a mixture of methanol and 14 % ammoniacal water (1:1) and transferred to ethyl acetate, which was washed with dil. HCl and water, dried (Na_2SO_4), and evaporated. Bis(DNP)Dab^2 (150 mcg) from the Rf 0.25 substance, and bis(DNP)Dab^{10} (788 mcg) from the Rf 0.82 substance, were obtained. These amounts were estimated from the optical density at 352 nm.

Bis(DNP)Dab^2, CD: $[\theta]_{520}0$, $[\theta]_{450}+1510$, $[\theta]_{405}0$, $[\theta]_{377}-4540$, $[\theta]_{350}0$, $[\theta]_{335}+2390$, $[\theta]_{295}0$ (*c* 0.0270, N NaOH)

Bis(DNP)Dab^{10}, CD: $[\theta]_{520}0$, $[\theta]_{455}-1220$, $[\theta]_{408}0$, $[\theta]_{373}+2500$, $[\theta]_{342}0$, $[\theta]_{330}-680$, $[\theta]_{300}0$ (*c* 0.0750, N NaOH)

A synthetic sample of bis(DNP)-L-Dab showed: CD: $[\theta]_{510}0$, $[\theta]_{450}-810$, $[\theta]_{404}0$, $[\theta]_{373}+1760$, $[\theta]_{350}0$, $[\theta]_{335}-540$, $[\theta]_{295}0$ (*c* 0.1100, N NaOH)

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