from natural gramicidin A) in 5 ml. of ethyl acetate was added 2 ml. of 5% aqueous bicarbonate solution and 2 drops (20 mg.) of carbobenzyloxy chloride. The heterogeneous mixture was stirred vigorously at room temperature for 1 hr. The layers were separated and the ethyl acetate extract was washed with water, dried, and evaporated. The residue was dissolved in 2 ml. of dioxane and treated for 1 hr. at room temperature with 0.5 ml. of 1.0 *N* alkali. The mixture was evaporated, the residue was dissolved in ethyl acetate, washed with water, and dried, and the solvent was removed by evaporation. The residue was precipitated from ethyl acetate–petroleum ether (b.p. 40–60°) to give 50 mg. (86%) of carbobenzyloxydesformylgramicidin A (ninhydrin negative, R_f 0.57 (A) and 0.81 (C)). Hydrogenolysis in methanol containing a few drops of acetic acid over palladium black gave desformylgramicidin A.

Gramicidin. VII. The Structure of Valine- and Isoleucine-gramicidin B

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Contribution from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Maryland. *Received December 9, 1964*

The volatile acidic fragment in the hydrolysate of gramicidin B was identified as formic acid which, after reduction to formaldehyde, was assayed by the chromotropic acid test. The primary sequence of gramicidin B, which in the sample examined consisted of 89% of valine-gramicidin B and 11% of isoleucine-gramicidin B, was determined by a 14-step Edman degradation on desformylgramicidin B (obtained from gramicidin B by mild methanolysis) as HCO-L-Val(L-Ileu)-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-Phe-D-Leu-L-Try-D-Leu-L-Try-NHCH₂CH₂OH.

The peptide antibiotic gramicidin, isolated by Hotchkiss and Dubos,[2] was shown by Gregory and Craig[3] to be heterogeneous. By countercurrent distribution in the solvent system methanol–water–benzene–chloroform (23:7:15:15) three fractions were obtained, the major fraction designated gramicidin A (distribution coefficient K = 0.61), and two minor fractions designated gramicidin B (K = 0.31) and gramicidin C (K = 1.32).[3-5]

The structure of gramicidin A, a mixture of valine- (I) and isoleucine-gramicidin A (II),[5,6] was recently elucidated[7-9] and confirmed by synthesis.[10] Gramicidin B is composed of very similar acids except that one tryptophan is replaced by phenylalanine. Analogously, in gramicidin C one tryptophan is substituted by tyrosine.[5,11]

Gramicidin B shows the same multiplicity of spots on thin layer chromatography as gramicidin A in the solvent system methyl ethyl ketone–pyridine (7:3), and the two congeners cannot be distinguished in this system. However, in the system acetic acid–chloroform (2:1) the R_f value of gramicidin B (one spot, 0.42) is higher than that of gramicidin A (one spot, 0.35).

First we established that gramicidin B, too, is a formyl-peptide ethanolamide. On hydrolysis of gramicidin B with 50% sulfuric acid formic acid is released, which after distillation and reduction to formaldehyde was identified by the chromotropic acid reaction.[7,9,12] By treatment with hydrogen chloride in methanol under the same mild conditions that lead to deformylation of gramicidin A,[7,9,13] neutral gramicidin B is converted to a ninhydrin-positive basic peptide, desformylgramicidin B, in a yield of 95% after purification on Dowex 50W-X2. By N-bromosuccinimide oxidation[14] with 6 moles of NBS per 1845 g. of gramicidin B, *i.e.* 2 moles of NBS per mole of tryptophan, ethanolamine is liberated to the same extent as in gramicidin A. The liberation of ethanolamine was followed by paper electrophoresis and gas chromatography. In gramicidin B the carboxyl group of COOH-terminal tryptophan is connected to 2-aminoethanol in the fashion . . . .-Try-NHCH₂CH₂OH, as is the case in gramicidin A.[7,9]

An improved and extended 14-step Edman degradation[15] was carried out with desformylgramicidin B in the same way as described earlier for desformylgramicidin A.[8,9] Although this time the cyclization step with trifluoroacetic acid was carried out under nitrogen and at room temperature for only 1 hr., the gradual destruction of tryptophan could not be prevented even under such mild conditions. However, the yield in each cyclization step was increased to such an extent that a 14-step degradation was possible.

The phenylthiohydantoins of the amino acids were

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Table I. Amino Acid Composition of Desformylgramicidin B and of the Residual Peptides P_1 to P_{14}[a]

	Gly	Ala	Val	Ileu	Leu	Phe	Try	Amino-ethanol	NH_3
Desformyl-gramicidin B	1.02	2.02	3.49	0.11	4.00	0.98	1.78	0.76	...
P_1	1.00	2.06	2.74	0.00	4.00	1.00	...	0.91	2.81
P_2	0.02	2.02	2.66		4.00	0.97	2.31	0.80	...
P_3	0.01	1.01	2.61		4.00	0.95	2.04	0.90	1.07
P_4		0.98	2.60		3.10	1.00	1.55	0.84	1.17
P_5		0.09	2.61		3.00	0.97	1.66	0.86	1.53
P_6		0.05	1.75		3.00	1.00	1.15	0.84	1.75
P_7			1.01		3.00	0.96	1.15	0.83	2.01
P_8			0.22		3.00	0.98	0.82	0.81	2.16
P_9			0.14		3.00	0.98		0.86	3.54
P_{10}			0.05		2.38	0.98	0.46	0.71	3.65
P_{11}					2.20	0.37		0.85	4.47
P_{12}					1.40	0.21		0.78	3.37
P_{13}					1.20	0.19	0.21	0.84	4.77
P_{14}					0.80	0.17	0.10	0.80	4.30

[a] After 1 to 14 Edman degradations of desformylgramicidin B.

identified by gas chromatography,[16] and aliquots of the residual peptides were hydrolyzed and analyzed for amino acid composition. Both methods gave consistent results, revealing the sequence Val(Ileu)-Gly-Ala-Leu-Ala-Val-Val-Val-Try-Leu-Phe-Leu-Try-Leu-(Try,-2-aminoethanol). The location of tryptophan in position 9 and 13 was concluded from the lack of disappearance of any other amino acid during these steps (Table I, Figure 1). This sequence very closely resembles that of desformylgramicidin A except for phenylalanine in position 11 which substitutes for a tryptophan. Again L-valine and L-isoleucine in position 1 substitute each other. Of the gramicidin B sample examined, 11% consisted of isoleucine-gramicidin B.

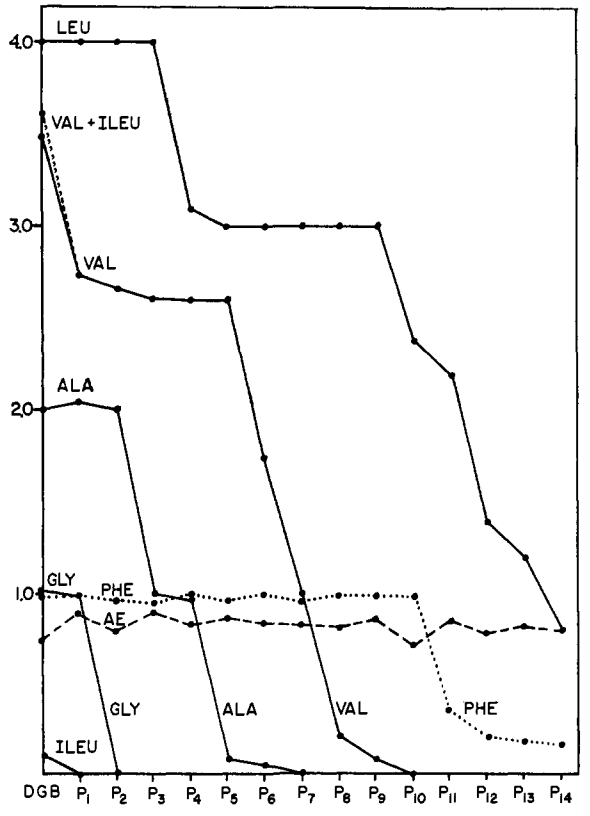

Figure 1. Amino acid composition of desformylgramicidin B (DGB) and the peptides P_1–P_{14} obtained in the course of the 14 Edman degradations.

Table II. Amino Acid Composition of Hydrolysates of Gramicidin B[a]

	DGB	GB	I	II	RR
Gly	1.02	1.02	1.00	1.00	
Ala	2.02	2.08	1.82	0.05	0.4
Val	3.49	3.28	2.94	1.58	4
Ileu	0.11	0.13	0.00	0.00	31
Leu	4.00	4.00	4.00	3.67	100
Phe	0.98	0.96	0.00	0.00	82
Try	1.78	2.70	0.00	0.00	89
AE	0.76	1.11	0.89	1.06	
NH_3	..	0.40	2.48	5.40	

[a] Before (GB) and after incubation with 1 mg. (I) and 10 mg. of L-amino acid oxidase (II). For comparison, the amino acid composition of a desformylgramicidin hydrolysate (DGB) and the relative rates of reaction of the enzyme toward the L-amino acids (RR)[18] are included.

The optical configurations of the amino acids were determined by incubation of a hydrolysate of gramicidin B with crystalline L-amino acid oxidase from *Crotalus adamanteus*.[6,17] Amino acid analyses of the hydrolysate before and after incubation with the enzyme clearly show the disappearance of tryptophan, phenylalanine, and isoleucine, which therefore have the L-configuration. Alanine and valine showed only a slight decrease when the hydrolysate of 0.35 μmole of gramicidin B was incubated with 1 mg. of the enzyme for 10 hr. This is in agreement with the relative reactivity of the enzyme toward these amino acids[18] (Table II). However, after incubation with 10 mg. of the enzyme alanine disappeared, and only 2 moles of valine was left. This then indicates the following amino acid composition for gramicidin B: 4 D-Leu, 2 D-Val, 2 (L-Val + L-Ileu), 3 L-Try, 2 L-Ala, 1 L-Phe, Gly, 2-aminoethanol. This composition is very similar to that of gramicidin A, and the only question remain-

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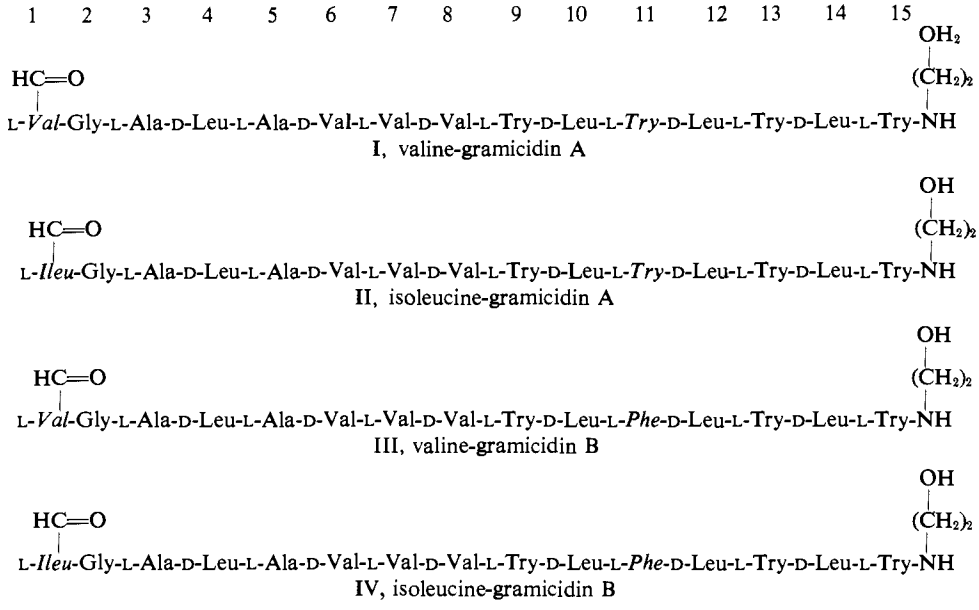
ing is the distribution of the L- and D-isomers of the valine residues.

For reasons of analogy it is assumed that the same alternation of D-L-D[19] for the sequence of three valines in position 6–8 occurs both in gramicidin A (I and II) and B. For valine- and isoleucine-gramicidin B structures III and IV are suggested.



Experimental

Desformylgramicidin B. To a solution of 37 mg. of gramicidin B[20] in 30 ml. of absolute methanol was added 4 ml. of 15.0 *N* hydrogen chloride in absolute methanol, and the mixture was allowed to stand at room temperature for 1.5 hr. After evaporation the residue was dissolved in 10 ml. of methanol and applied to a column (equipped with cooling jacket), filled with 20 ml. of Dowex 50W-X2 resin, equilibrated in the H^+ form with methanol. Elution with 130 ml. of methanol gave 2.1 mg. (5%) of unreacted, neutral gramicidin B. By elution with 130 ml. of 2.0 *N* ammonia in methanol, prepared by mixing 80 ml. of concentrated ammonia with 400 ml. of methanol, 35 mg. (95%) of (basic) desformylgramicidin B were obtained. In the solvent system acetic acid–chloroform (2:1) desformylgramicidin B had R_f 0.14, desformylgramicidin A R_f 0.07 (t.l.c.).

Assay of Formic Acid from Gramicidin B. A 150-μg. sample of gramicidin B (0.08 μmole) was hydrolyzed in an evacuated and sealed tube at 110° for 2.5 hr. with 1 ml. of 50% (v./v.) sulfuric acid. The hydrolysate was distilled at room temperature under 10^{-2} mm. into a trap cooled to −80°. To the distillate (0.5 ml.) was added 2 drops of 12.0 *N* hydrochloric acid and 15 mg. of magnesium filings at 0°. After 15 min. another drop of 12.0 *N* hydrochloric acid was added, and after 30 min. 1.5 ml. of chromotropic acid reagent, prepared from 600 mg. of chromotropic acid, 180 ml. of sulfuric acid, and 20 ml. of water. After 45 min. at 110° the solution acquired a deep purple color. The extinction (0.2) at 575 mμ, measured against a blank sample, indicated the presence of 0.6 μg. (0.02 μmole) of formaldehyde in the mixture.

Edman Degradation of Desformylgramicidin B. A 34-mg. sample of desformylgramicidin B was dissolved in 20 ml. of reagent, prepared by mixing 100 ml. of pyridine with 3 ml. of triethylamine and 1 ml. of phenyl isothiocyanate. The reaction mixture was warmed to 40° for 4 hr., the solvent was removed on a rotary evaporator, and the last traces of reagent were removed at 40° at 10^{-2} mm. The residue was dissolved under nitrogen in 5 ml. of anhydrous trifluoroacetic acid and kept at room temperature for 1 hr. The solvent was removed by evaporation and the residue dissolved in methanol and fractionated on a column filled with Dowex 50W-X2 resin (H^+ form, equilibrated with methanol) into a *neutral fraction* (elution with 100 ml. of methanol) containing the phenylthiohydantoins of the NH_2-terminal amino acids, and into a *basic fraction* (elution with 140 ml. of 2.0 *N* ammonia in methanol) containing the degraded peptide. The phenylthiohydantoins of the neutral fractions were identified by thin layer chromatography in the solvent system chloroform–formic acid (20:1)[21] as well as by gas chromatography on a column (6 ft. × 1/6 in.) filled with 1% SE-30 on gaschrom P.[15] At a column temperature of 216° with 19 ml./min. of nitrogen as a carrier gas the phenylthiohydantoins of alanine, valine, leucine, and isoleucine were eluted as sharp peaks after 2.5, 3.1, 4.1, and 4.1 min., respectively. The phenylthiohydantoin of phenylalanine was eluted after 3.8 min. at a column temperature of 257° with 14 ml./min. of nitrogen.

An aliquot of the degraded basic peptide was hydrolyzed with 0.3 ml. of acetic acid containing 1.5 ml. of constant boiling hydrochloric acid in an evacuated and sealed tube at 110° for 24 hr. The hydrolysate was analyzed on an automatic amino acid analyzer.[22] The remainder of the degraded peptide was used for

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the subsequent Edman degradation step as described above. The results are summarized in Table I and Figure 1.

Cleavage of Gramicidin B by N-Bromosuccinimide. A 1.4-mg. (0.76 μmole) sample of gramicidin B was dissolved in 0.5 ml. of 60% aqueous ethanol containing 1 drop of 0.10 *N* sulfuric acid and allowed to react with 0.8 mg. (4.6 μmoles) of N-bromosuccinimide at room temperature. Similarly, 1.1 mg. (0.58 μmole) of gramicidin A was oxidatively cleaved with 0.8 mg. (4.6 μmoles) of N-bromosuccinimide. Aliquots of the reaction mixtures were examined by paper electrophoresis. In both cases a ninhydrin-positive substance, migrating as fast as 2-aminoethanol, was liberated. To the remainder of the reaction mixture was added 100 μl. of an aqueous solution containing 200 μg. of 1-amino-2-hydroxypropane per ml. After the addition of 2 drops of 2.0 *N* hydrochloric acid the samples were evaporated and dried. The residues were dissolved in 500 μl. of 1,2-dimethoxyethane, acylated with 10 μl. of trifluoroacetic anhydride, and analyzed by gas chromatography on a column containing 4% neopentyl glycol succinate on Chromosorb W (6 ft. × 1/6 in., flame ionization detector). At a column temperature of 145° with 36 ml./min. of nitrogen the bistrifluoroacetylated 1-amino-2-hydroxypropane and 2-aminoethanol were eluted after 2.9 and 4.4 min., respectively. By comparison of the peak areas[9] a release of 0.18 mole of 2-aminoethanol per 1882 g. of gramicidin A and of 0.19 mole of 2-aminoethanol per 1845 g. of gramicidin B was calculated.

Determination of the Optical Configuration of the Amino Acids in Gramicidin B. A 2.1-mg. (1.15 μmoles) sample of gramicidin B was hydrolyzed in an evacuated and sealed tube with 0.45 ml. of acetic acid and 2.25 ml. of constant boiling hydrochloric acid at 110° for 24 hr. The hydrolysate was evaporated and kept in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide. The residue was dissolved in 3.3 ml. of water, and 1-ml. aliquots of this solution were used for the enzymatic incubation reactions and for the amino acid analysis. The incubation with L-amino acid oxidase from *Crotalus adamanteus* was carried out at 38° in a Warburg apparatus with oxygen as the gas phase. The main compartment contained 1 ml. of 0.1 *M* 2-amino-2-hydroxymethyl-1,3-dihydroxypropane HCl buffer (pH 7.66 at 24°), 1 ml. of 0.1 *M* KCl solution, and 1 ml. of the hydrolysate. A control flask contained 1 ml. of water instead of the hydrolysate. The side arm contained 1 mg., in a second experiment 10 mg., of the enzyme in 0.5 ml. of 0.1 *M* KCl. The center well contained 0.1 ml. of 5 *N* KOH. After a 10-min. equilibration period the flasks were tipped and readings were taken at 10-min., later 60-min., intervals for 10 hr. until the oxygen uptake had virtually ceased. The reaction mixture was evaporated and analyzed on an amino acid analyzer. The results are given in Table II.

The Stereospecific Synthesis of *threo*-γ-Hydroxyhomo-L-arginine from *Lathyrus* Species

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γ-Hydroxy-L-lysine (III) prepared from L-lysine (I) by photochemical chlorination and subsequent reaction with silver acetate was cyclized with nitrosyl chloride to a mixture of 80% cis-4-hydroxy-L-pipecolic acid (VI) and 20% trans-4-hydroxy-D-pipecolic acid (VII). This establishes the threo configuration (III) for γ-hydroxy-L-lysine (III) which was converted to threo-γ-hydroxy-L-homoarginine (VIII ⇌ IX), identical in all respects with the naturally occurring amino acid from Lathyrus seeds.

The photochlorination of amino acids in strong acid permitted an easy synthesis of several biochemically interesting C-substituted amino acids.[3] The photochlorination of L-lysine (I) is specific both with regard to position and configuration. γ-Hydroxy-L-lysine (III) prepared from the γ-chloro-L-lysine (II) showed a single sharp peak by ion-exchange column chromatography under conditions which easily resolve the diastereoisomers of δ-hydroxylysine as well as of γ-hydroxyornithine.[4]

The configuration of γ-hydroxy-L-lysine is of interest for the mechanism of the photochlorination and for the related γ-hydroxyhomoarginine, a new natural amino acid of unknown configuration recently isolated from *Lathyrus* species.[5,6] In this investigation the configuration of γ-hydroxylysine is shown to be *threo*. It is assumed that γ-chlorolysine has the *erythro* configuration and that its conversion to hydroxylysine involves 100% inversion.

The configuration of the asymmetric centers of γ-hydroxy-L-lysine was determined in the same way as that of γ-hydroxyornithine, the next lower homolog,[4] in which the *erythro* diastereoisomer is sterically

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