Gramicidin. VIII. The Structure of Valine- and Isoleucine–Gramicidin C*

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ABSTRACT: By desformylation with hydrogen chloride in methanol, gramicidin C was converted to desformylgramicidin C, which was subjected to fourteen consecutive (modified) Edman degradations. Amino acid analyses of the hydrolysates of the purified peptides resulting from this degradation together with vapor-phase chromatography of the phenylthiohydantoin derivatives of the NH_2-terminal amino acids suggest the following sequence for valine–gramicidin C: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-Tyr-D-Leu-L-Try-D-Leu-L-Try-NH-CH_2CH_2OH. In the gramicidin C sample used in this investigation 5.5% isoleucine–gramicidin C was present.

Gramicidin C is a minor component of the peptide antibiotic gramicidin, which was isolated by Hotchkiss and Dubos (1941) and shown to be heterogeneous by Gregory and Craig (1948). By countercurrent distribution gramicidin was fractionated into gramicidin A (the major component), B, and C (Craig *et al.*, 1949; Ramachandran, 1963). The structure of gramicidin A, a mixture of valine– (compound I) and isoleucine–gramicidin A (compound II) (Ramachandran, 1963; Ishii and Witkop, 1963) was recently elucidated (Sarges and Witkop, 1964a,b, 1965a) and confirmed by synthesis (Sarges and Witkop, 1965b). Gramicidin B, a mixture of the valine (compound III) and isoleucine congeners (compound IV), differs from compounds I and II in that L-tryptophan in position 11 of gramicidin A is substituted by L-*phenylalanine* (Table I) (Sarges and Witkop, 1965c).

Again, the amino acid composition of gramicidin C is very similar to gramicidin A, except that one tryptophan is replaced by *tyrosine*. On thin layer chromatography in the solvent system pyridine–methyl ethyl ketone (3:7), gramicidin C shows the same multiplicity of spots as gramicidin A and B with identical R_F values. In the solvent system chloroform–acetic acid (1:2), all gramicidins give only one spot with R_F values of 0.35 (A), 0.42 (B), and 0.38 (C).

The sequence of gramicidin C was determined in a way analogous to that described for gramicidin A and B (Sarges and Witkop, 1965a,c). Hydrolysis with 50% sulfuric acid released formic acid, which, after distillation and reduction to formaldehyde, was identified by the chromotropic acid test (Grant, 1948). By treatment with hydrogen chloride in methanol, neutral gramicidin C is converted to the basic, ninhydrin-positive desformylgramicidin C (Sarges and Witkop, 1964a, 1965a; Ishii and Witkop, 1964), which has valine and isoleucine as NH_2-terminal amino acids. This was established by dinitrophenylation and by Edman degradation (Edman, 1953). *N*-Bromosuccinimide oxidation (Patchornik *et al.*, 1960) of gramicidin C leads to the liberation of 2-aminoethanol. These results indicate the partial structure: HCO-Val(Ileu)-. . .-Try(or Tyr)-NHCH$_2$-CH$_2$OH. The results of the Edman degradation unequivocally assign to tyrosine position 11.

A fourteen-step Edman degradation was carried out on desformylgramicidin C in the same way as described earlier for gramicidin B (Sarges and Witkop, 1965c). The amino acid composition of hydrolysates of aliquots of the peptides was determined before and after each degradation step (Table II). The evaluation of the results led to the sequence: Val(Ileu)-Gly-Ala-Leu-Ala-Val-Val-Val-Try-Leu-Tyr-Leu-Try-Leu-(Try, 2-aminoethanol). Confirmatory evidence came from gas chromatographic examination of the phenylthiohydantoins of the liberated amino acids (Pisano *et al.*, 1962). Tryptophan does not survive the repeated acid treatments required for the cyclization steps. The position of tryptophan was deduced from the fact that all other amino acids remained constant during the ninth and thirteenth degradation steps. The gramicidin C sample examined consisted of 5.5% isoleucine–gramicidin C and 94.5% valine–gramicidin C.

The optical configuration of the amino acid residues of gramicidin C was determined by incubation of a hydrolysate with L-amino acid oxidase from *Crotalus adamanteus* (Ishii and Witkop, 1963; Wellner and Meister, 1960). Comparison of the amino acid analyses before and after the incubation (Table III) indicates the following composition: 4 D-Leu, 2 D-Val, 2 (L-Val + L-Ileu), 3 L-Try, 2 L-Ala, 1 L-Tyr, Gly, 2-aminoethanol. The deviations (Table III) are due to the differences in rates of oxidation and to the incomplete hydrolysis of Val-Val bonds.

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TABLE I: Composition of Gramicidin.

Compd	Positions 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
I	*Valine–gramicidin A:*
	HCO-L-*Val*-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-*Try*-D-Leu-L-Try-$NHCH_2CH_2OH$
II	*Isoleucine–gramicidin A:*
	HCO-L-*Ileu*-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-*Try*-D-Leu-L-Try-$NHCH_2CH_2OH$
III	*Valine–gramicidin B:*
	HCO-L-*Val*-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-*Phe*-D-Leu-L-Try-$NHCH_2CH_2OH$
IV	*Isoleucine–gramicidin B:*
	HCO-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-$NHCH_2CH_2OH$
V	*Valine–gramicidin C:*
	HCO-L-*Val*-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-*Tyr*-D-Leu-L-Try-$NHCH_2CH_2OH$
VI	*Isoleucine–gramicidin C:*
	HCO-L-*Ileu*-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-*Tyr*-D-Leu-L-Try-$NHCH_2CH_2OH$

If one assumes, for reasons of analogy with gramicidin A, that the same D-L-D pattern (Hinman *et al.*, 1950) of the three valine residues in position 6–8 is present in gramicidin C, structures V and VI can be written for valine– and isoleucine–gramicidin C.

A comparison between compounds I–VI shows that, besides the partial substitution of L-valine by L-isoleucine in position 1, which occurs in all samples, the difference between gramicidin A, B, and C is in position 11 where L-tryptophan is substituted by L-phenylalanine and L-tyrosine, respectively. Likewise, in the tyrocidins (Mach and Tatum, 1964; Ruttenberg *et al.*, 1965), which are produced by the same strain of *Bacillus brevis*, tryptophan replaces one or more phenylalanine residues, while the remainder of the molecule is unchanged.

Attempts to utilize mass spectrophotometry for sequence determination of gramicidin A and its congeners have so far not met with the same success as for the peptidolipid fortuitine (Barber *et al.*, 1965).

Experimental Procedure and Results

Desformylgramicidin C. To a solution of 39.0 mg of gramicidin C (Gross and Witkop, 1965) in 30 ml of absolute methanol was added 4 ml of 15.0 N hydrogen chloride in methanol and the mixture was allowed to stand at room temperature for 75 minutes. 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. By elution with 100 ml of methanol, 5.1 mg (13%) of unreacted gramicidin C was recovered. Elution with 140 ml of 2.0 N ammonia in methanol, prepared by mixing 80 ml of concentrated ammonia with 400 ml of methanol, gave 33.9 mg (87%) of (basic) desformylgramicidin C.

Assay of Formic Acid from Gramicidin C. A 150-μg sample of gramicidin C (0.08 μmole) was hydrolyzed in an evacuated, sealed tube with 1 ml of 50% (v/v) sulfuric acid at 110° for 2 hours. The hydrolysate was distilled at room temperature under 10^{-2} mm into a trap, cooled to −80°. To the distillate, filled up to 0.5 ml with water, was added 15 mg of magnesium filings and 2 drops of 12.0 N hydrochloric acid at 0°. After 15 minutes another drop of 12.0 N hydrochloric acid was added and after 30 minutes 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 minutes at 110° the reaction mixture had developed a deep purple color. The extinction (0.17) at 575 mμ, measured against a blank sample, indicated the presence of 0.55 μg (0.018 μmole) of formaldehyde in the mixture.

Determination of NH_2*-Terminal Amino Acids in Desformylgramicidin C.* To a solution of 2 mg of desformylgramicidin C in 31 ml of dimethoxyethane was added 1 ml of water, 20 μl of triethylamine, and 10 mg of 2,4-dinitrofluorobenzene. The mixture was allowed to stand at room temperature for 24 hours and evaporated. The residue was dissolved in ethyl acetate and

TABLE II: Amino Acid Composition of Desformylgramicidin C and of the Residual Peptides P_1 to P_{14} after One to Fourteen Edman Degradation Steps of Desformylgramicidin C.

	Gly	Ala	Val	Ileu	Leu	Tyr	Try	Amino-ethanol	NH_3
Desformyl-gramicidin C	1.16	2.04	3.40	0.06	4.00	0.78	2.72	0.88	2.07
P_1	1.16	1.91	2.34	0.00	4.00	0.72	1.92	0.88	2.45
P_2	0.23	2.02	2.44		4.00	0.76	0.75	0.88	2.71
P_3	0.08	1.07	2.30		4.00	0.75	0.87	0.92	2.46
P_4	0.09	0.98	2.31		3.10	0.76			
P_5	0.06	0.26	2.10		3.00	0.76	0.80	0.77	3.56
P_6			1.35		3.00	0.71		0.76	3.88
P_7		0.13	1.01		3.00	0.65		0.59	5.02
P_8			0.25		3.00	0.76		0.79	5.30
P_9					3.00	0.66		0.70	9.18
P_{10}					2.25	0.66		0.61	10.00
P_{11}					2.10	0.29		0.59	13.9
P_{12}					1.40	0.12		0.62	12.2
P_{13}					1.20	0.11		0.66	14.1
P_{14}					0.75	0.08		0.60	23.3

TABLE III: Amino Acid Composition of Hydrolysates of Gramicidin C before (a) and after (b) Incubation with L-Amino Acid Oxidase (moles/mole of gramicidin C).

	Gly	Ala	Val	Ileu	Leu	Tyr	Try	Amino-ethanol	NH_3
(a)	1.10	1.78	3.17	0.08	3.93	0.67	2.98	0.76	0.36
(b)	1.12	0.08	1.44	0.00	3.56	0.00	0.00	0.79	3.46

washed with 1.0 N hydrochloric acid, 5% sodium bicarbonate solution, and water. After drying and evaporation the residue was dissolved in chloroform and applied to a 20-ml column of silicic acid. After washing with 40 ml of chloroform the top of the column was mechanically separated and eluted with methanol. The yellow residue of the methanol eluate was hydrolyzed with a mixture of 0.3 ml of acetic acid and 1.5 ml of constant-boiling hydrochloric acid at 110° for 24 hours. The hydrolysate was evaporated and extracted with ether. An aliquot of the ether extract on thin layer chromatography in the solvent system methyl ethyl ketone–benzene–pyridine–acetic acid (120:80:30:3, v/v) revealed the presence of DNP-valine. The remainder of the ether extract was esterified with diazomethane. Gas chromatographic analysis on a 1% SE-30 column showed the presence of DNP-valine methyl ester and a small amount of DNP-isoleucine methyl ester (Ishii and Witkop, 1963).

Edman Degradation of Desformylgramicidin C. A 24-mg sample of desformylgramicidin C was dissolved in 20 ml of reagent, prepared by mixing 100 ml of pyridine with 3 ml of triethylamine and 1 ml of phenylisothiocyanate. The reaction mixture was warmed to 40° for 4 hours, the solvent was removed on a rotary evaporator, and the last traces of reagent were removed at 40° under 10^{-2} mm pressure. The residue was dissolved under nitrogen in 5 ml of anhydrous trifluoroacetic acid and kept at room temperature for 1 hour. The solvent was removed by evaporation and the residue was 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 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 (Brenner *et al.*, 1961), as well as by gas chromatography on a column (183 × 0.38 cm) filled with 1% SE-30 on Gas-Chrom P. The phenylthiohydantoins of tyrosine and tryptophan could not be detected by gas chromatography.

An aliquot of the degraded basic peptide was hydrolyzed in a mixture of 0.3 ml of acetic acid and 1.5 ml of constant-boiling hydrochloric acid at 110° for 24 hours in an evacuated and sealed tube. The hydrolysate

was analyzed on an automatic amino acid analyzer. The remainder of the peptide was used for the subsequent Edman degradation step as described. The results are summarized in Table II.

N-Bromosuccinimide Cleavage of Gramicidin C. A sample of 0.8 mg (0.43 μmole) of gramicidin C was dissolved in 0.5 ml of 60% aqueous ethanol containing one drop of 0.1 N sulfuric acid and allowed to react with 0.8 mg (4.5 μmoles) of *N*-bromosuccinimide at room temperature. Similarly, 1.0 mg (0.53 μmole) of gramicidin A was oxidatively cleaved with 1.0 mg (5.6 μmoles) of *N*-bromosuccinimide. By paper electrophoresis a ninhydrin-positive substance, moving as fast as 2-aminoethanol, was detected in aliquots of both samples. To the remainder of each of the reaction mixtures was added 100 μl of an aqueous solution containing 200 μg of 1-amino-2-hydroxypropane per ml and 2 drops of 2.0 N hydrochloric acid.

The samples were evaporated and dried, and the residues were dissolved in 500 μl of 1,2-dimethoxyethane, acylated with 10 μl of trifluoroacetic anhydride, and analyzed on a column filled with 4% neopentylglycol succinate on Chromosorb W. At a column temperature of 145° with 36 ml of nitrogen per minute as carrier gas, the bitrifluoroacetylated 1-amino-2-hydroxypropane and 2-aminoethanol were eluted after 2.9 and 4.4 minutes, respectively. By comparison of the peak areas a release of 0.6 mole of 2-aminoethanol per 1882 g of gramicidin A and per 1859 g of gramicidin C was calculated (Sarges and Witkop, 1965a).

Determination of the Optical Configuration of the Amino Acids in Gramicidin C. A sample of 2.4 mg (1.29 μmoles) of gramicidin C was hydrolyzed with 0.3 ml of acetic acid and 1.5 ml of constant-boiling hydrochloric acid in an evacuated and sealed tube at 110° for 24 hours. The hydrolysate was evaporated and dried, and the residue was dissolved in 3.3 ml of water. Of this solution 1-ml aliquots were used for enzymatic incubation and amino acid analyses. The incubation with L-amino oxidase was carried out in a Warburg apparatus with oxygen as the gas phase at 38°. 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 10 mg of the enzyme in 0.5 ml of 0.1 M KCl. The center well contained 0.1 ml of 5.0 N KOH. The flasks were tipped after a 10-minute equilibration period, and readings were taken after 10-minute (later 60-minute) intervals until, after 10 hours, the oxygen uptake had virtually ceased. The reaction mixture was evaporated and analyzed on an amino acid analyzer. The results are given in Table III.

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