either by reduction to formaldehyde (Mg, HCl) and subsequent reaction with chromotropic acid,[8] or by gas chromatography[9] of the distillate after evaporation of the sodium salt solution and acidification with dilute phosphoric acid (6-ft. column packed with 20% Tween 80 and 2% phosphoric acid on chromosorb W, argon detector). No fatty acid other than formic acid could be detected by gas chromatography, except acetic acid (7%) in a sample of gramicidin A which had been freeze-dried from acetic acid. However, acetic acid was no longer found when gramicidin A was either dried at 100° and $10^{-2}$ mm. over KOH for 25 hr. or recrystallized from ethanol–water.

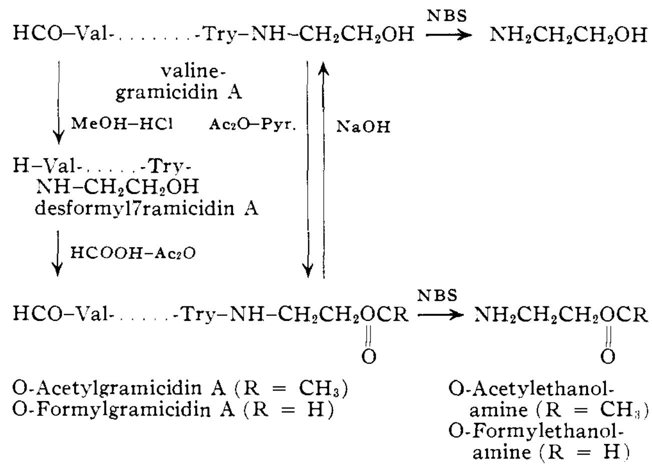
**Desformylgramicidin A** or tryptophan in control experiments did not liberate any trace of formic acid under the same hydrolytic conditions.

Quantitative results obtained by comparison with standard solutions of formic acid indicate that 0.6–0.7 mole of HCOOH (chromotropic acid reaction, extinction at 575 mμ) or 0.8–1.0 mole of HCOOH (gas chromatography) per 1850 g. of gramicidin A are liberated.

The n.m.r. spectrum of gramicidin A in $D_3COD$[10] shows a broad peak at 8.45 p.p.m. (internal standard TMS), the peak area being $^1/_{12}$ to $^1/_{14}$ as large as that of the aromatic indole protons at 6.0–5.2 p.p.m. Addition of formic acid to the solution of gramicidin gives rise to a sharp peak at 8.32 p.p.m. By comparison, the formyl proton of formyl-L-valine appears at 8.40 p.p.m.

In the light of these new results, the gentle methanolysis of gramicidin[1] to the so-called *seco*-gramicidin A, a compound with one new $NH_2$-terminal group per mole of compound (1850 g.), is simply the removal of the N-formyl group to yield desformylgramicidin A, a reaction that was demonstrated to proceed with the comparable N-formyl-L-valine in more than 95% yield under the same conditions. Gramicidin A was treated with 1.5 $N$ HCl in methanol at room temperature for 1 hr. After evaporation, the desformylgramicidin A was separated from 6% unreacted gramicidin A by fractionation of the methanolic solution of the reaction product on a Dowex W50 X2 column. The neutral gramicidin A passes through the column, while the basic desformylgramicidin A is eluted with 1.0 $N$ $NH_4OH$ in methanol. Treatment of desformylgramicidin A, $[\alpha]^{20}D$ +5.4° (propionic acid), with formic acid–acetic anhydride[11] at room temperature for 4 hr. leads to a ninhydrin-negative substance, presumably O-formylgramicidin, which migrates on thin layer chromatography in two different solvent systems (acetic acid–chloroform, 2:1; pyridine–2-butanone, 3:7) like O-acetylgramicidin A, acetylated with $Ac_2O$ in pyridine.[12] Base treatment of this ester leads in 80% over-all yield to a compound which, on thin layer chromatography, is identical with gramicidin A, $[\alpha]^{20}D$ −5° (propionic acid).

While N-bromosuccinimide (NBS) oxidation of gramicidin A leads to the liberation of aminoethanol,[13] acetylated gramicidin A releases O-acetylethanolamine on treatment with NBS. These compounds were identified by paper electrophoresis and by gas chromatography after trifluoroacetylation (4% neopentylglycol succinate on chromosorb W, flame ionization detector).




In the hydrolysate of O-$n$-butylated gramicidin A (butylation with $n$-BuI–$Ag_2O$, hydrolysis in 0.3 ml. of AcOH–1.5 ml. of 6.0 $N$ HCl at 100° for 20 hr.), 2-$n$-butoxyethylamine, aminoethanol, and aminoethanol acetate are found. 2-$n$-Butoxyethylamine is not stable under these hydrolytic conditions and is partially converted to aminoethanol and 2-aminoethanol acetate.

These results support a linear peptide structure such as HCO–Val-. . . . . .-Try–NH–$CH_2CH_2OH$ for valine-gramicidin A and the scheme presented in the chart.

The occurrence of an N-formylamino acid[14] in a peptide raises interesting biosynthetic questions.[15]

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## Gramicidin A. IV. Primary Sequence of Valine and Isoleucine Gramicidin A

*Sir:*

The key to the structure of gramicidin A[1] was the selective removal[2] and identification[3] of the formyl group which blocks the $NH_2$-terminal valine (or isoleucine). This made possible the application of successive Edman degradations and established directly the sequence of the first ten amino acids and indirectly the total sequence of gramicidin A. The solubility of gramicidin A and its degradation products in organic solvents, and their insolubility in water, necessitated a modification of the customary Edman procedure.[4]

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(10) We are greatly obliged to Dr. J. W. Daly for the determination and interpretation of the n.m.r. data.

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(4) *Cf.* P. Edman, *Acta Chem. Scand.*, **7**, 700 (1953).

The reaction with phenyl isothiocyanate was carried out in pyridine–triethylamine–phenyl isothiocyanate (100:3:1)[5] at 40° for 4 hr. Excess reagent was removed under high vacuum. Trifluoroacetic acid,[6] within 1 hr., effected cyclization at room temperature to the phenylthiohydantoins (PTH) which were separated from the residual peptides by fractionation on a Dowex 50WX2 column with methanol and 1.0 $M$ ammonia in methanol. The PTH-amino acids were identified by thin-layer chromatography[7] in chloroform–formic acid (20:1) and by gas chromatography[8] on 1% SE-30 on Gas-Chrom P. Aliquots of the residual peptides were hydrolyzed (0.3 ml. of AcOH–1.5 ml. of 6.0 $N$ HCl, 110°, 49 hr.) and analyzed on an automatic amino acid analyzer (Phoenix)[9] (Fig. 1).

The combination of these three techniques gave consistent results, suggestive of the sequence of H–Val–Gly–Ala–Leu–Ala–Val–Val–Val–Try–Leu– for desformyl-valine-gramicidin A, and H–Ileu–Gly–. . . . . for desformylisoleucine–gramicidin A.[1,10] After the ninth degradation step, PTH-tryptophan was identified by thin-layer chromatography. After the tenth step, PTH-leucine was found using gas chromatographic analysis. The gradual destruction of tryptophan in the course of the multiple Edman degradation made its quantitative determination impossible. The degradations were therefore not pursued beyond the tenth step.

Oxidation of gramicidin A by N-bromosuccinimide does not lead to the liberation of the 5-bromodioxindole-spirolactone expected from a Try–Try sequence. This was ascertained by comparison of the electrophoretic mobility of the cleavage products as well as by their separation on a Sephadex G-25 column (50% aqueous acetic acid). A sample of authentic spirolactone, prepared by NBS treatment of Cbz–L-Try–L-Try–OH had properties completely different from the oxidation products of gramicidin A. The following structure for valine–gramicidin A at the present time best expresses the published observations, including the earlier findings on the optical configuration of the valine peptides[1,11]: 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–D-Leu–L-Try–NH–$CH_2$–$CH_2$–OH. Of the gramicidin A sample examined, 18% consisted of isoleucine–gramicidin A: HCO–L-Ileu–Gly–. . . . . This structure is in agreement with previously isolated peptides from partial hydrolysates, except for leucyl–glycine, which is probably isoleucyl–glycine.[12] These results also resolve doubts about the molecular weight[13] of gramicidin A and establish mol. wt. 1882 for valine–gramicidin A, a value independently confirmed by ultracentrifuge studies.[14] The unprecedented alternating pattern of L- and D-amino acids, the unusual accumulation of hydrophobic groups, and the complete insolubility in water readily explain the resistance of gramicidin A to attack by enzymes, such as nagarse, pronase, chymotrypsin, and pepsin.[2]

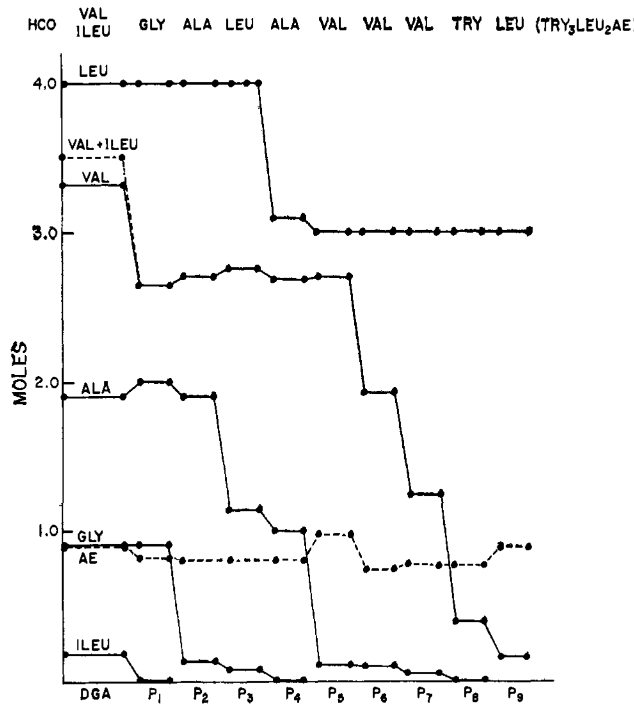


Fig. 1.—Edman degradation of Desformylgramicidin A (DGA). The abscissa lists the residual peptides $P_1$–$P_9$ resulting from 1–9 successive degradative steps; the ordinate shows the decrease in molar composition for the individual amino acids (except tryptophan). Full values for valine are not obtained unless the time of hydrolysis is extended from the practical time, *viz.*, 49 hr., to 72 hr.

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## Furazans and Furazanium Salts[1]

*Sir:*

The utility of 3-unsubstituted isoxazolium salts as peptide-forming reagents has been amply demonstrated.[2] The commercially available reagent, N-ethyl-5-phenylisoxazolium-3′ sulfonate,[2] is expensive, and we entertained the idea of testing N-ethylfurazanium fluoroborate (I) as an economical substitute. We expected I to undergo peptide forming reactions according to eq. 1. The synthesis and characterization of furazan (IV)[3] and

(1) This research was supported by grants from the U. S. Public Health Service (GM-09317) and the Milton Fund of Harvard University. Presented in part at the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept., 1963, p. 63-Q.
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