The Free Amino Group of Gramicidin S

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Synge (1945) has recently shown that gramicidin S is a polypeptide composed of equimolecular proportions of the five amino-acids, *l*-valine, *l*-proline, *l*-leucine, *l*-ornithine and *d*-phenylalanine, and that each stoichiometric unit contains one free amino group. Belozersky & Paschina (1944*a*, *b*, 1945), on the other hand, maintain that both amino and carboxyl groups are present and that the δ-amino group of the ornithine residue is not free. Their evidence for this latter point, nevertheless, is not very convincing and the problem was clearly one which could be readily settled by applying the new dinitrofluorobenzene (DNFB) method (Sanger, 1945). Accordingly at Dr Synge's suggestion, the present author has made appropriate experiments, which show that the free amino group is undoubtedly the δ-amino group of the ornithine residue. The finding provides additional support for Synge's contention that gramicidin S is a *cyclo*peptide composed of the five amino-acids linked through their α-amino and carboxyl groups. It is interesting to note that similar results for tyrocidin have recently been obtained by Christensen (1945) using a different technique.

EXPERIMENTAL

Preparation of compounds

δ-2:4-*dinitrophenyl*-l-*ornithine* was prepared by treatment of the copper complex of *l*-ornithine with DNFB as follows. 0·1 g. *l*-ornithine monohydrochloride, dissolved in 5 ml. hot water, was treated with excess $CuCO_3$. After filtration the solution was evaporated to about 2 ml., and 0·3 g. $NaHCO_3$ was added followed by a solution of 0·2 ml. DNFB in 4 ml. ethanol. The mixture was shaken for 2 hr. at room temperature, which brought about the separation of the copper complex of δ-DNP-ornithine as a green powder. This was filtered off, dissolved in dilute HCl, treated with H_2S and filtered with charcoal. The solution was then taken to a small volume *in vacuo*, which brought about the separation of the *hydrochloride of* δ-2:4-*dinitrophenyl*-l-*ornithine* as crystals containing one molecule of water of crystallization. It was recrystallized from N-HCl; yield 0·09 g., m.p. 223° (decomp.). (Found: C, 38·0; H, 5·0; N, 10·0; Cl, 16·4%. $C_{11}H_{14}O_6N_4.HCl.H_2O$ requires C, 37·5; H, 4·9; N, 10·1; Cl, 15·9%.) It lost 5·0% moisture at 100° over P_2O_5 (calc. 5·1%).

When an acid solution of the above hydrochloride was neutralized with pyridine in an attempt to prepare the free amino-acid, there was a slow separation of an oil which would not crystallize, although with the corresponding *dl*-compound the free amino-acid could readily be prepared crystalline in this way. A similar difficulty was encountered in the preparation of ε-DNP-*l*-lysine; ε-DNP-*dl*-lysine has not yet been studied.

α-2:4-*dinitrophenyl*-dl-*ornithine* was prepared by treatment of δ-benzoyl-*dl*-ornithine with DNFB, followed by hydrolysis of the benzoyl group. 0·3 g. *dl*-ornithine monohydrochloride was converted to the copper complex, and the cooled solution benzoylated in the usual manner with 0·32 ml. benzoyl chloride and 6 ml. N-NaOH. The insoluble benzoyl compound was filtered off, suspended in 2 ml. water and treated with H_2S. This solution was brought to the boil and filtered hot. On evaporation of the filtrate to about 5 ml., 0·14 g. δ-benzoyl-*dl*-ornithine crystallized. This was suspended in 2 ml. water containing 0·3 g. $NaHCO_3$ and shaken for 2 hr. with a solution of 0·2 ml. DNFB in 4 ml. ethanol. After removal of the ethanol *in vacuo*, the solution was extracted with ether to remove excess DNFB and acidified. The α-DNP-δ-benzoyl-*dl*-ornithine immediately separated as an amorphous solid; yield 0·2 g. For the removal of the benzoyl group it was heated for 4 days at 105° in an evacuated sealed tube with a mixture of 2 ml. 10N-HCl and 2 ml. acetic acid. After cooling, the solution was taken to dryness and the residue dissolved in water. Unhydrolyzed material, of which there was still a considerable amount, was then extracted with ethyl acetate, and on neutralization of the aqueous solution with pyridine 0·05 g. α-2:4-*dinitrophenyl*-dl-*ornithine* crystallized out; m.p. 227° (decomp.). (Found: C, 44·1; H, 4·9%. $C_{11}H_{14}O_6N_4$ requires C, 44·3; H, 4·7%.)

2:4-*dinitrophenyl-gramicidin S*. 0·1 g. Gramicidin S (specimen 1 of Synge, 1945) was dissolved in 4 ml. ethanol. 0·4 g. $NaHCO_3$, 1 ml. water and 0·2 ml. DNFB were added and the mixture shaken for 3 hr. The DNP derivative of gramicidin S crystallized as the reaction proceeded. It was recrystallized from ethanol water; yield 0·1 g.

Identification of the free amino group of gramicidin S

5 mg. DNP-gramicidin S were heated for 54 hr. at 110° in a sealed evacuated tube with a mixture of 1 ml. 10N-HCl and 1 ml. acetic acid. The hydrolysate was taken to dryness, dissolved in water and extracted with ether. Since the whole of the colour remained in the aqueous solution, the presence in this solution of a mono-DNP derivative of ornithine was indicated. Any other DNP derivative of the amino-acids present in gramicidin S would have been extracted by the ether (Sanger, 1945). It remained only to identify the ornithine derivative present.

It was found that α-DNP-ornithine and δ-DNP-ornithine moved on a 66% methyl ethyl ketone (MEK)-ether column at $R = 0·07$ and 0·12 respectively (Gordon, Martin & Synge, 1943), and thus could be separated from one another. The material

from DNP-gramicidin S moved at $R = 0{\cdot}12$, and when mixed with α-DNP-ornithine, two bands were formed having $R = 0{\cdot}12$ and 0·07 respectively. This shows that δ-DNP-ornithine and no other DNP derivative was present, and that the only free amino group present in gramicidin S is the δ-amino group of the ornithine residue.

In a further quantitative experiment, the δ-DNP-ornithine in a hydrolysate of DNP-gramicidin S was estimated colorimetrically, using synthetic δ-DNP-*l*-ornithine hydrochloride as a standard. A parallel experiment showed that the conditions of hydrolysis lead to no appreciable breakdown, so it was not necessary to make any correction for this. The yield of δ-DNP-ornithine in duplicate experiments was 35·0 and 35·8 % of the DNP-gramicidin S taken. If gramicidin S is a *cyclo*pentapeptide, the theoretical yield would be 40·5 %.

Isolation of δ-DNP-ornithine from DNP-gramicidin S

100 mg. DNP-gramicidin S were hydrolyzed as described above, taken to dryness and dissolved in N-HCl. After extraction with ether and filtration, the solution was allowed to evaporate at first *in vacuo* and then slowly in a desiccator, whereupon the δ-DNP-ornithine hydrochloride crystallized out, and was recrystallized four times from 5N-HCl; yield 5·7 mg. The various mother liquors were then combined and purified on a 66 % MEK-ether column. Only material from the centre dark portion of the band was collected. This solution was taken to dryness and the residue dissolved in a minimum volume of hot 5N-HCl, a trace of charcoal added and the solution filtered hot. On cooling, 6·2 mg. of the hydrochloride crystallized out, and a further 2·9 mg. was obtained from the mother liquors, making a total yield of 14·8 mg. (31 % theoretical).

Crystallographic examination of δ-DNP-l-ornithine hydrochloride

This material and the synthetic δ-DNP-*l*-ornithine hydrochloride were investigated crystallographically by Mr G. M. J. Schmidt of the Department of Chemical Crystallography, Oxford. He found that 'the synthetic preparation consisted of both plates and long thin needles, whereas the product from natural sources was mainly in the form of needles. However, both X-ray and optical examination showed the two preparations to be identical, the difference in crystal habit being presumably due to differences in the composition of the solutions from which the crystals were grown.

'The crystals are yellow orthorhombic needles or plates elongated along [010], the former showing {001} and {101}, whereas the latter show {100} and {001}. The orientation of the three principal refractive indices is $\alpha//c$, $\beta//b$, $\gamma//a$. The unit cell dimensions are $a = 10{\cdot}08$ A., $b = 4{\cdot}88$ A., $c = 32{\cdot}9$ A. Space group $P2_12_12_1$. ρ calculated 1·4 for $n = 4$.'

CO_2 liberated by interaction between ninhydrin and DNP derivatives of amino-acids

In Table 1 are shown the values for the CO_2 liberated by ninhydrin (Van Slyke, Dillon, MacFadyn & Hamilton, 1941) from the above material and from various synthetic derivatives. These analyses were carried out by Dr R. L. M. Synge.

Table 1. *CO_2 liberated by ninhydrin from various DNP derivatives (Synge)*

Compound	CO_2 found (% of 1 m-equiv.)
DNP-glycine	2
α-DNP-*dl*-ornithine	4
δ-DNP-*l*-ornithine.HCl.H_2O (synthetic)	97
Product from gramicidin S	92

The reaction was effected with 50 mg. ninhydrin at pH 2·5 and volume 1·3 ml. They confirm that the derivative from gramicidin S is δ-DNP-ornithine, and not α-DNP-ornithine.

SUMMARY

The free amino group of gramicidin S is the δ-amino group of the ornithine residue, and there are no other free amino groups. Gramicidin S is thus a *cyclo*peptide.

I wish to thank Mr G. M. J. Schmidt for carrying out the crystallographic examinations, and Dr R. L. M. Synge for his help and advice with the work.

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