901. *The Structure of Viridogrisein (Etamycin).*

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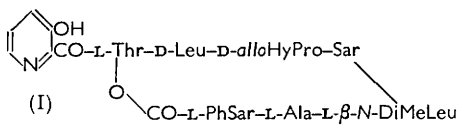
The results of degradative experiments support the structure (I) assigned recently to viridogrisein (etamycin) by Sheehan *et al.* The biogenesis of peptides containing macrocyclic lactone rings is discussed.

Viridogrisein [1] (etamycin [2]) is an antibiotic isolated from strains of *Streptomyces griseus* almost simultaneously in two American laboratories. Preliminary studies [2,3] of its hydrolysis showed it to be a peptide comprising 3-hydroxypicolinic acid and seven compounds giving colours with ninhydrin, including the amino-acids L-alanine, D-leucine, and D-*allo*hydroxyproline. Ingenious degradative studies by Sheehan and his co-workers [4] have recently revealed the total structure of viridogrisein as (I), a peptide which contains three *N*-methylamino-acids (sarcosine, L-phenylsarcosine, and L-β-*N*-dimethyl-leucine) and in which the hydroxyl group of L-threonine is esterified by the carboxyl group of the



(I)

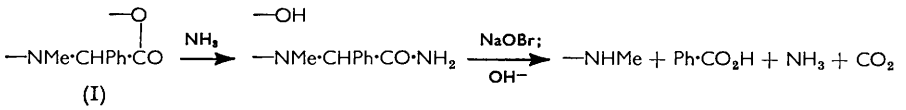
C-terminal amino-acid. The position of L-threonine and its participation in a macrolactone ring recalls the structure of the actinomycins,[5,6] and it is possible that pyridomycin [7] and echinomycin [8] (serine instead of threonine) may contain similar features.

Through the courtesy of Drs. R. E. Bowman and Q. Bartz of Parke Davis & Co., we

[1] Bartz, Standiford, Mold, Johannessen, Ryder, Maretzki, and Haskell, *Antibiotics Annual*, 1954—55, 777.
[2] Heinemann, Gourevitch, Lein, Johnson, Kaplan, Vanas, and Hooper, *ibid.*, p. 728.
[3] Haskell, Maretzki, and Bartz, *ibid.*, p. 784.
[4] Sheehan, Zachau, and Lawson, *J. Amer. Chem. Soc.*, 1957, **79**, 3933.
[5] Brockmann, Bohnsack, Franck, Gröne, Muxfeldt, and Süling, *Angew. Chem.*, 1956, **68**, 70.
[6] Bullock and Johnson, *J.*, 1957, 3280.
[7] Maeda, Kosaka, Okami, and Umezawa, *J. Antibiotics*, 1953, *A*, **6**, 140; Yagashita, *ibid.*, 1957, *A*, **10**, 5.
[8] Corbaz, Ettlinger, Gäumann, Keller-Schierlein, Kradolfer, Neipp, Prelog, Reusser, and Zähner, *Helv. Chim. Acta*, 1957, **40**, 199; Keller-Schierlein and Prelog, *ibid.*, p. 205.

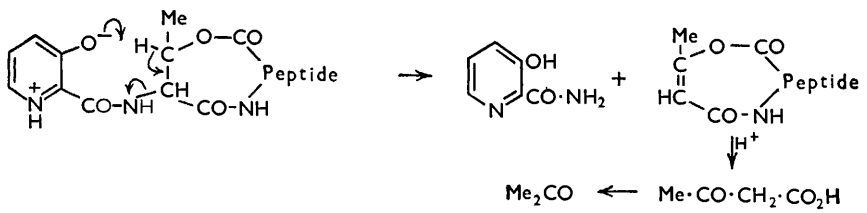
have been able to examine a sample of viridogrisein, and the results of our degradative experiments, most of which were carried out before appearance of the communication of Sheehan *et al.*,[4] are in accord with his structure (I). The antibiotic has not yet been obtained crystalline, but it has been purified both by counter-current distribution [1] and through the crystalline hydrochloride.[2] In our experience, viridogrisein can be purified conveniently by precipitation of the colourless crystalline ammonium salt which separates rapidly from solutions of viridogrisein in ethanolic ammonia, leaving the impurities in solution. The salt is unstable in the dry state and by allowing the decomposition to proceed *in vacuo* the pure antibiotic can be obtained. The formation of the ammonium salt was somewhat unexpected in the absence of a carboxylic acid group but it has since been shown that both ethyl 3-hydroxypicolinate and 3-hydroxypicolinamide form similar unstable ammonium salts. The acidity of the phenolic function is evidently enhanced by the electron-attracting carbonyl group and the heterocyclic nitrogen atom in these compounds (pK_a of viridogrisein,[3] 7·3).

The evidence for the existence of the lactone ring lies in the infrared spectrum [3] (band at 1751 cm.$^{-1}$) and the observation that saponification at room temperature releases a carboxylic acid group in forming viridogriseic acid [3] (etamycin acid [4]). Further, whereas three of the products, L-threonine, D-*allo*hydroxyproline, and 3-hydroxypicolinic acid, obtained from the acid hydrolysis of viridogrisein, contain hydroxyl groups, we have found that viridogrisein itself yields a diacetate and consequently one of the hydroxyl groups which appears in the degradation products is either sterically hindered in the original antibiotic or is freed only on hydrolysis. The lactone ring can also be opened by ammonolysis, giving a *C*-terminal amide, and when this product was subjected to Hofmann degradation, followed by acid hydrolysis, it gave a small yield of benzoic acid. This confirms the view that the aromatic phenylsarcosine is the *C*-terminal unit of the peptide, the course of the degradation being visualised as follows:



(I)

Pyrolysis of viridogrisein alone at 220° or in pyridine at 160° gives 3-hydroxypicolinamide, which can be sublimed from the reaction mixture. For comparison with the degradation product, 3-hydroxypicolinamide was obtained from the corresponding acid [9] by esterification and ammonolysis. The pyrolysis is interpreted as an olefin-forming elimination, especially as acetone can be isolated by vigorous hydrolysis of the pyrolysis residue. The acetone arises from the peptide threonyl grouping which is converted into an acetoacetic acid enol residue as a result of the amide elimination. This observation is confirmatory evidence that 3-hydroxypicolinic acid is attached to threonine and it reveals the amide character of the linkage. As spectroscopic and dipole-moment measurements [10] indicate the presence of dipolar forms in 3-hydroxypyridine, a plausible reaction mechanism for this decomposition can be postulated:



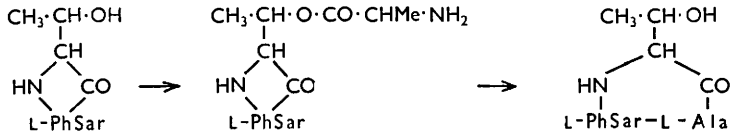
Mycobactin, which gives 2-hydroxy-6-methylbenzamide on pyrolysis, is a similar case,

[9] Kirpal, *Monatsh.*, 1908, **29**, 227; Fibel and Spoerri, *J. Amer. Chem. Soc.*, 1948, **70**, 3908.
[10] Metzler and Snell, *ibid.*, 1955, **77**, 2431; Albert and Phillips, *J.*, 1956, 1294.

although here the serine unit is attached as an oxazoline ring.[11] Antimycin [12] also has an aromatic system attached to an α-amino-β-hydroxy-acid (threonine) and appears to give an amide on thermal treatment though this compound has not been identified. As the actinomycins are comparatively stable to thermal treatment it seems that for amide elimination to occur under fairly mild conditions a phenolic group or the equivalent in the *ortho*-position is probably a structural requirement.

In addition to alanine, leucine, and hydroxyproline reported earlier, we have identified threonine and sarcosine in the products of complete hydrolysis of the antibiotic. Prolonged treatment of viridogrisein with cold aqueous alkali gives some leucylhydroxyproline, identified (tentatively) by hydrolysis and paper chromatography and by the purple colour it gives with ninhydrin which excludes the possibility of an *N*-terminal hydroxyproline residue.[13] The structure of this dipeptide is in accord with the order of the amino-acid units shown in formula (I).

The remarkable similarity between the actinomycins and viridogrisein concerning the position of the threonine unit and the amino-acid sequence and lactonic nature of the peptide raises the question of the biogenesis. Observations by Brenner's school [14] that



amino-acids can be inserted into a peptide containing serine, threonine, or cysteine by esterification of the amino-acid to be inserted with the free hydroxyl or thiol groups invites the suggestion that cyclic peptides containing threonine, serine, etc., could be built up by ring expansion based on the insertion principle starting, in the case of viridogrisein, with the cyclic dipeptide (dioxopiperazine) of L-phenylsarcosine and L-threonine. Insertion of L-alanine would give the cyclic tripeptide, L-phenylsarcosyl-L-threonyl-L-alanyl and so on. The macrocyclic lactone could be obtained from a cyclic threonyl (or seryl) peptide by an *N*- ⟶ *O*-acyl rearrangement, thereby releasing an amino-group for the final condensation with 3-hydroxypicolinic acid. In the actinomycins the aromatic acid is 3-hydroxy-4-methylanthranilic acid and the phenoxazin-3-one nucleus is formed by a final oxidative coupling [15] of the *o*-aminophenol.

Experimental

Light-absorption figures are given for EtOH solutions except where otherwise stated.

Purification of Viridogrisein.—Crude viridogrisein (4 g. of 90%) was dissolved in saturated ethanolic ammonia (40 c.c.) with shaking. Next day the crystalline precipitate was separated, washed with ethanolic ammonia (10 c.c.), and heated *in vacuo* at 100° for 1 hr., to give viridogrisein (2·84 g.) as a colourless amorphous powder, m. p. 163—165° (decomp.) (Found: C, 59·0; H, 7·05; N, 12·3; NMe, 5·35. Calc. for $C_{44}H_{62}O_{11}N_8,H_2O$: C, 58·9; H, 7·2; N, 12·5; 3NMe, 5·0%), $\lambda_{max.}$ 304 mμ (log ε 3·93).

Di-O-acetylviridogrisein.—Viridogrisein (430 mg.) was dissolved in acetic anhydride (2 c.c.) containing 60% perchloric acid (1 drop). After 24 hr. at room temperature, the solution was poured into water and extracted with chloroform (3 × 50 c.c.). The combined extracts were washed with aqueous sodium hydrogen carbonate and then dried. After removal of the solution, the gummy residue was purified by repeated precipitation from ethyl acetate with light petroleum (b. p. 60—80°) to give the diacetate as a colourless granular solid (Found: C, 58·9; H, 6·95; N, 11·8; Ac, 8·8. $C_{48}H_{66}O_{13}N_8,H_2O$ requires C, 58·8; H, 6·8; N, 11·4; 2Ac, 8·8%), light absorption: $\lambda_{max.}$ in EtOH 270 mμ (log ε 3·55), in N/100-HCl 269 mμ (log ε 3·60), and in N/100-NaOH 338 mμ (log ε 3·82).

[11] Snow, *J.*, 1954, 2588, 4080.
[12] Tener, van Tamelen, and Strong, *J. Amer. Chem. Soc.*, 1953, **75**, 3623.
[13] Johnson and McCaldin, *J.*, 1958, 817.
[14] Brenner, Zimmermann, *et al.*, *Helv. Chim. Acta*, 1957, **40**, 1497, 1933.
[15] Brockmann and Muxfeldt, *Angew. Chem.*, 1956, **68**, 69; Hanger, Howell, Johnson, *et al.*, *J.*, 1957, 1592; 1958, 496.

Viridogriseic Acid.—Viridogrisein (3 g.) was dissolved in 2N-aqueous sodium hydroxide (18 c.c.), kept overnight at 0°, for a further 18 hr. at room temperature, and then neutralised with 2N-hydrochloric acid. The gummy precipitate was extracted with ethylene dichloride (2 × 50 c.c.) and chloroform (50 c.c.). The combined extracts were dried and concentrated to 7 c.c.; addition of ether (150 c.c.) then gave a gum which was reprecipitated from ethanol with ether. The gum so formed slowly solidified (2·25 g.) and was further purified by reprecipitation from ethyl acetate with light petroleum (b. p. 60—80°), being obtained as a colourless amorphous solid, m. p. 96—100° (decomp.) after drying at 20°, m. p. 138—140° (decomp.), after drying at 130° (Found: C, 58·3; H, 7·15; N, 12·1. Calc. for $C_{44}H_{64}O_{12}N_8,H_2O$: C, 58·4; H, 7·35; N, 12·4%), $\lambda_{max.}$ in $CHCl_3$ 307 mμ (log ε 3·92), in N/100-HCl 304 mμ (log ε 3·93), and in N/100-NaOH 333 mμ (log ε 3·93).

Pyrolysis of Viridogrisein.—(i) Viridogrisein (5·0 g.) was heated at 220°/0·1 mm. for 5 hr. and the gummy sublimate which was obtained on a cold finger was dissolved in chloroform (100 c.c.) and extracted with N-sodium hydroxide (3 × 100 c.c.). The combined aqueous extracts were adjusted to pH 6 with hydrochloric acid and extracted with chloroform (4 × 200 c.c.). The chloroform extracts were washed, dried, and evaporated to a pale brown residue (511 mg.) which was sublimed at 100°/0·1 mm.; this yielded colourless crystals (359 mg.) which recrystallised from chloroform as feathery needles, m. p. 193—194°, not depressed when mixed with authentic 3-hydroxypicolinamide (Found: C, 52·2; H, 4·5; N, 20·5. Calc. for $C_6H_6O_2N_2$: C, 52·2; H, 4·4; N, 20·3%), $\lambda_{max.}$ 224 and 302 mμ (log ε 3·83 and 3·84). The infrared spectrum (KBr) showed max. at 777, 807, 866, 1237, 1265, 1302, 1338, 1438, 1453, 1594, 1616, 1691, 3205, and 3406 $cm.^{-1}$.

The dark brown residue from the pyrolysis was treated with boiling 4N-hydrochloric acid for 4 hr., the distillate being collected in a solution of 2 : 4-dinitrophenylhydrazine (750 mg.) in 4N-hydrochloric acid (500 c.c.). The resulting cloudy, orange solution was extracted with chloroform (6 × 100 c.c.), and the combined chloroform extracts were washed with water (100 c.c.) and dried. After removal of the solvent, the residue was chromatographed in benzene on alumina (Spence type H) (40 × 3·5 cm.). The main band was eluted with benzene and after removal of the solvent, the 2 : 4-dinitrophenylhydrazone was obtained as orange plates (283 mg.) which after crystallisation from methanol had m. p. 124—125° alone or when mixed with the authentic derivative of acetone (Found: C, 45·4; H, 4·3; N, 23·6. Calc. for $C_9H_{10}O_4N_4$: C, 45·4; H, 4·2; N, 23·5%).

(ii) Viridogrisein (162 mg.) was heated in dry pyridine (2 c.c.) in a sealed tube at 160° for 14 hr. After removal of the solvent, the residue was sublimed at 120°/1 mm. to give 3-hydroxypicolinamide (9 mg.) which crystallised from chloroform as colourless needles, m. p. and mixed m. p. 193—194°.

Ethyl 3-Hydroxypicolinate.—3-Hydroxypicolinic acid [9] (508 mg.) was heated in ethanol (25 c.c.) containing sulphuric acid (1 c.c.) for 6 hr. The product was concentrated to 10 c.c., diluted with water (to 75 c.c.), adjusted to pH 6 with aqueous sodium hydroxide, and extracted with chloroform (3 × 50 c.c.). The chloroform extracts were washed with water (2 × 50 c.c.), then dried and the solvent was removed. The residual oil was distilled to give the *ester* as a colourless liquid (300 mg.) (Found: C, 57·2; H, 5·1; N, 8·4. $C_8H_9O_3N$ requires C, 57·5; H, 5·4; N, 8·4%), $\lambda_{max.}$ 226 and 302 mμ (log ε 3·96 and 4·05).

3-Hydroxypicolinamide.—The foregoing ester (101 mg.) was added to saturated ethanolic ammonia (2·5 c.c.), a white precipitate of the ammonium salt of the ester being formed immediately. A sample of the precipitate was removed but decomposed rapidly, leaving an oily residue. The precipitate dissolved when the reaction mixture was heated in a sealed tube; heating was continued at 110° for 16 hr., after which the solvent was removed and the colourless solid residue sublimed at 100°/0·1 mm. to give the *product* (67 mg.) which crystallised from chloroform as feathery needles, m. p. 192—194° (Found: C, 52·1; H, 4·55; N, 20·1%), $\lambda_{max.}$ 224 and 302 mμ (log ε 3·81 and 3·84).

Ammonolysis of Viridogrisein.—Viridogrisein (5·43 g.) was dissolved in ethanol (10 c.c.), and aqueous ammonia (*d* 0·88; 100 c.c.) was added. The clear solution was kept in a stoppered flask at room temperature for 51 hr., then boiled briefly to expel ammonia, adjusted to pH 6 with hydrochloric acid, and extracted with chloroform (3 × 75 c.c.). The combined chloroform extracts were shaken with saturated aqueous sodium hydrogen carbonate (2 × 150 c.c.). The aqueous layers were combined, adjusted to pH 6 with hydrochloric acid, and extracted with chloroform (3 × 75 c.c.). These chloroform extracts were washed with water (100 c.c.), dried

(Na_2SO_4), and evaporated, giving viridogriseic acid monohydrate, as a white amorphous powder (1·49 g.), m. p. 96—100° (decomp.) after drying *in vacuo* at 20°, m. p. 137—140° (decomp.), after drying *in vacuo* at 130°.

The original chloroform extracts, when washed with water (2 × 75 c.c.), dried (Na_2SO_4), and evaporated, gave a pale yellow amorphous powder (viridogriseamide) (3·09 g.). The product was purified through the crystalline ammonium salt by the method used for viridogrisein, giving the *amide* as a white amorphous powder, m. p. 165—170° (decomp.) (Found, in a sample dried at 140° *in vacuo*: C, 59·1; H, 7·15; N, 14·1. $C_{44}H_{65}O_{11}N_9$ requires C, 58·9; H, 7·3; N, 14·1%), $\lambda_{max.}$ 304 mμ (log ε 3·92).

Hofmann Degradation of Viridogriseamide.—Bromine (2·4 g.) was added dropwise with stirring to a solution of sodium hydroxide (3·8 g.) in water (45 c.c.) at 2°. After the bromine had dissolved, viridogriseamide (2·43 g.) was added gradually with stirring. The solution was stirred at 0—2° for 1½ hr., then sodium hydroxide (3·0 g.) was added, and the solution heated on the water-bath for 2 hr. The solution then gave a positive Nessler's test. After being washed with chloroform (2 × 50 c.c.) the solution was acidified with concentrated hydrochloric acid (35 c.c.) and extracted with ether (4 × 50 c.c.). The combined ether extracts were washed with water (50 c.c.) and dried (Na_2SO_4), and the solvent was removed. The brown semi-crystalline residue was sublimed at 60°/0·1 mm., giving benzoic acid (11 mg.), m. p. and mixed m. p. 121°.

Paper-chromatographic Examination of the Acid Hydrolysate of Viridogrisein.—Viridogrisein (20 mg.) was heated in 6N-hydrochloric acid (1 c.c.) in a sealed tube at 120° for 18 hr. After removal of the hydrochloric acid the residue was redissolved in water (2 c.c.), and the solution used for two-dimensional paper chromatography by the ascending technique. The developing solvents were butan-1-ol–acetic acid–water (4 : 1 : 5) and phenol–water. After drying, the papers were sprayed with ninhydrin solution and then heated at 110° for 10 min. Seven coloured spots were observed (R_F values for the butanol solvent given first; colours purple except where otherwise stated): (i) 0·20, 0·70 (yellow, hydroxyproline); (ii) 0·23, 0·75 (pink, sarcosine); (iii) 0·26, 0·47 (threonine); (iv) 0·31, 0·55 (alanine); (v) 0·53, 0·87 (colourless but fluorescent in ultraviolet light before ninhydrin treatment, 3-hydroxypicolinic acid); (vi) 0·58, 0·95; (vii) 0·62, 0·82 (leucine); (viii) 0·72, 0·97. Spots (vi) and (viii) are now known [4] to correspond to *N*-phenylsarcosine and β-*N*-dimethyl-leucine. The R_F values were checked against the appropriate amino-acid standard.

Deamination of Viridogrisein Hydrolysate.—Viridogrisein was hydrolysed as described in the previous experiment, and the solution diluted with water (10 c.c.) and treated with nitrous fumes for 1 hr. The solution was heated under reflux for 30 min. and then after removal of the solvent the residue was redissolved in water (2 c.c.) and used for paper-chromatographic examination (ascending technique) in the butan-1-ol–acetic acid–water solvent. After development with ninhydrin four spots (R_F values given) were observed: (i) 0·12 (yellow, hydroxyproline); (ii) 0·18 (sarcosine); (iii) 0·53; (iv) 0·65. Using methanol–pyridine–water (20 : 5 : 1) also gave four spots: (i) 0·43 (yellow, hydroxyproline); (ii) 0·49 (sarcosine); (iii) 0·68 (brown); and (iv) 0·76.

Partial Hydrolysis of Viridogrisein.—Viridogrisein (2·07 g.) was dissolved in 0·3N-aqueous barium hydroxide (100 c.c.) and kept at room temperature for 8 weeks. Then it was neutralised (pH 7) with sulphuric acid, filtered from barium sulphate, washed with chloroform (3 × 100 c.c.) (to remove viridogriseic acid), and taken to dryness, giving a pale brown amorphous residue (1·60 g.). This was subjected to partition chromatography on powdered cellulose (30 × 4 cm.) in the system butan-1-ol–acetic acid–water (4 : 1 : 5), and fractions (10 c.c.) were collected. Alternate fractions were examined by paper chromatography in the same solvent system: those up to no. 39 contained no ninhydrin-positive substances; fractions 41—61 contained leucine (R_F 0·55); fractions 67—93 contained a substance (purple spot; R_F 0·45) which did not correspond with any of the amino-acids in viridogrisein. These fractions were taken to dryness, giving a pale brown solid residue (40 mg.) which did not crystallise. Hydrolysis with 6N-hydrochloric acid at 120° for 12 hr. gave hydroxyproline (yellow spot; R_F 0·16) and leucine (R_F 0·55) when examined by paper chromatography in the same solvent system.

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