458. *Antibiotics of the* E129 (*Ostreogrycin* *) *Complex. Part I. The Structure of* E129B.

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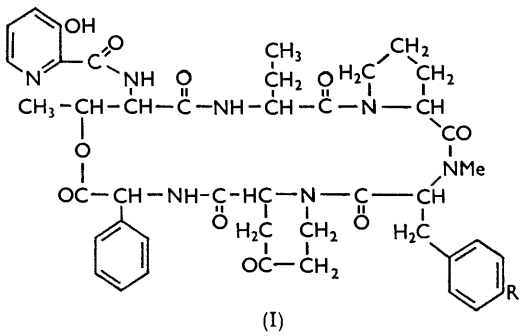
The antibiotic E129B is shown to have structure (I; R = NMe_2), and thus to be closely related to staphylomycin, from which it differs by containing in place of *N*-methyl-phenylalanine the hitherto unknown amino-acid, *p*-dimethylamino-*N*-methyl-phenylalanine.

We have been for some time engaged on an investigation of a mixture of synergistic antibiotics isolated by workers at Glaxo Laboratories, Ltd., from cultures of a soil streptomycete, *Streptomyces ostreogriseus*, and described by them [1] as the "E129 complex." The present paper describes the structural elucidation of one member of this complex, the colourless, crystalline antibiotic E129B, which has been reported to be identical with the antibiotic described in the literature as PA114B.[1,2] Our results indicate that E129B has structure (I; R = NMe_2). It is thus a member of the growing group of peptide antibiotics containing a macrocyclic lactone ring of which etamycin [3,4] was the first example, and it is closely related to staphylomycin, whose structure was announced [5] as (I; R = H) when most of the work described in this paper had been completed.



(I)

Analysis of E129B gave values consistent with a molecular formula $C_{45}H_{54}O_{10}N_8$ containing three *N*-Me groups. It was an optically active diacid base, giving no ninhydrin reaction. It was phenolic, yielded a crystalline oxime, and was evidently a lactone, since it was converted by cold aqueous alkali into an acid having the same ultraviolet absorption as the parent antibiotic and giving the same products as the latter on hydrolytic degradation. Hydrolysis of E129B with 6N-hydrochloric acid yielded seven compounds which were severally isolated by a combination of adsorption and ion-exchange chromatography. One of these compounds had strong ultraviolet absorption, but was optically inactive and ninhydrin-negative; it was identified by direct comparison as 3-hydroxypicolinic acid. Of the remaining six, all of which were ninhydrin-positive, four were readily identified as L(−)-threonine, L(−)-proline, D(−)-α-aminobutyric acid, and L(+)-phenylglycine,[6] the last-named being partially racemised during the hydrolysis and working-up processes. The fifth acid, $C_{12}H_{18}O_2N_2$, contained three *N*-Me groups and, since it gave a red colour

* Ostreogrycin is the name which has been approved by the General Medical Council for E129. We propose to adopt this nomenclature and, by agreement with the discoverers, will in future publications describe E129B as ostreogrycin B.

[1] Ball, Boothroyd, Lees, Raper, and Smith, *Biochem. J.*, 1958, **68**, 24P.
[2] Celmer and Sobin, "Antibiotics Annual," 1955–56, p. 437.
[3] Sheehan, Zachau, and Lawson, *J. Amer. Chem. Soc.*, 1958, **80**, 3349.
[4] Arnold, Johnson, and Mauger, *J.*, 1958, 4466.
[5] Vanderhaeghe and Parmentier, XVIIth Internat. Congress Pure Appl. Chem., Munich, 1959, Abstracts, Vol. II, p. 56; *Bull. Soc. chim. belges*, 1959, **68**, 716.
[6] Rudman, Meister, and Greenstein, *J. Amer. Chem. Soc.*, 1952, **74**, 551.

with *p*-nitrobenzoyl chloride in pyridine, was evidently an α-*N*-methylamino-acid.[3] In alkaline solution the compound showed intense light absorption at 245 mμ (ε 11,900), while in acid solution it showed typical benzenoid absorption; this behaviour is characteristic of aniline derivatives[7] and suggested that the acid might be *p*-dimethylamino-*N*-methylphenylalanine. That this suggestion was correct was shown by condensing *p*-dimethylaminobenzaldehyde with creatinine in presence of acetic anhydride and acetic acid, conversion of the product into 5-*p*-dimethylaminobenzylcreatinine, and hydrolysis of the latter with barium hydroxide; the resulting *p*-dimethylamino-*N*-methyl-DL-phenylalanine had the same ultraviolet absorption and chromatographic behaviour as the acid obtained from the E129B hydrolysate. *p*-Dimethylamino-*N*-methyl-phenylalanine has not hitherto been found in Nature; in E129B it is probably present as the L(−)-isomer, since the isolated material showed a positive change in rotation on passing from neutral to acid solution.[8]

The remaining amino-acid in the hydrolysate, which gave an orange-yellow ninhydrin reaction, was rather unstable in the free state and was isolated and examined as its hydrochloride, $C_6H_{10}O_3NCl$. The hydrochloride contained neither *N*-Me nor *C*-Me groups, but showed two infrared carbonyl bands, at 1746 (carboxyl) and 1732 (ketone) cm.$^{-1}$, while the free base showed no selective carbonyl absorption above 1628 cm.$^{-1}$. At this point in our examination of the substance it was reported that 4-oxo-L(−)-pipecolic acid has been obtained from staphylomycin,[5] and it seemed not unlikely that we were dealing with the same acid. That this surmise was correct was proved by catalytic hydrogenation of our product in acidic solution, which gave L(−)-pipecolic acid, and by its breakdown with alkali to give acetone and formaldehyde; these findings establish conclusively that the seventh amino-acid obtained from E129B was 4-oxo-L(−)-pipecolic acid.

Estimation of threonine, α-aminobutyric acid, phenylglycine, and proline in the E129B hydrolysate after separation on ion-exchange columns showed them to be present in equimolar amounts. 3-Hydroxypicolinic acid and *p*-dimethylamino-*N*-methyl-phenylalanine were also present in equimolar amounts as determined directly from the absorption spectrum of the antibiotic. 4-Oxopipecolic acid was not directly estimated, but consideration of the analytical data for E129B shows that it, too, must be present in molar amount.

The seven components of the acid hydrolysate having been identified, it remained to determine their sequence in order to define completely the structure of E129B. Since the antibiotic itself contains a free phenolic group, it was evident that the hydroxyl group of threonine must be involved in forming the lactone ring of E129B. This was confirmed by careful chromic acid oxidation of E129B and of the parent acid followed by hydrolysis of the products and examination by paper chromatography; only in the oxidation of the parent acid was threonine destroyed (cf. ref. 3). Moreover, the antibiotic, like etamycin,[4] gave 3-hydroxypicolinamide on pyrolysis, showing that the terminal 3-hydroxypicolinic acid was probably attached to threonine. The *C*-terminal amino-acid in the parent acid of E129B was determined by methylation, reduction of the terminal ester grouping with lithium borohydride, and acid-hydrolysis. Neither 4-oxopipecolic acid nor phenylglycine was found in the hydrolysate, but since it contained 2-amino-2-phenylethanol it is clear that the *C*-terminal amino-acid must be phenylglycine. The remainder of the amino-acid sequence was determined by partial hydrolysis of the antibiotic to a series of smaller polypeptides, which were then separately hydrolysed to their constituent amino-acids. The three acidic peptides, 3-hydroxypicolinylthreonine, 3-hydroxypicolinylthreonyl-α-aminobutyric acid, and 3-hydroxypicolinylthreonyl-α-aminobutyrylproline, together with a dipeptide hydrolysed to 4-oxopipecolic acid and phenylglycine, were isolated by using an ion-exchange column. In another experiment a pentapeptide was

[7] Gilham and Stern, " Electronic Absorption Spectroscopy," Edward Arnold, Ltd., London, 1957.
[8] Lutz and Jirgensons, *Ber.*, 1930, **63**, 448; 1931, **64**, 1221; Plattner and Nager, *Helv. Chim. Acta*, 1948, **31**, 2192; Balenovic in " Amino Acids and Peptides with Antimetabolic Activity," Ciba Foundation Symposium, Churchill, Ltd., London, 1958, p. 5.

separated by paper chromatography and shown to be 3-hydroxypicolinylthreonyl-α-aminobutyrylprolyl-*p*-dimethylamino-*N*-methyl-phenylalanine.

These results establish the amino-acid sequence in E129B and indicate that the antibiotic has structure (I). The resemblance between E129B and staphylomycin is striking, the two differing only in that the *N*-methyl-L-phenylalanine residue in staphylomycin is replaced in E129B by *p*-dimethylamino-*N*-methyl-phenylalanine.

Experimental

Paper Chromatography.—Whatman No. 1 paper was employed with the following solvent systems: A, phenol–water (4 : 1) used in presence of hydrogen cyanide; B, 2,6-lutidine–collidine–water (1 : 1 : 1) containing 1% of diethylamine; systems I, L, and O as described by Hardy *et al.*[9]

Antibiotic E129B.—The crude antibiotic (1·88 g.), as supplied by Glaxo Laboratories, Ltd., was purified by countercurrent distribution (100 transfers; 20 ml. phases) in the system toluene–methanol–water (4 : 3 : 1). The material in tubes 63—80 inclusive (1·12 g.), whose distribution corresponded closely to the theoretical $K = 2{\cdot}45$, was combined and recrystallised to yield the material used for all degradative studies.

E129B crystallised from methanol in colourless prisms containing tenaciously held solvent of crystallisation, and from toluene in needles, m. p. 266—268°, $[\alpha]_D^{20}$ −66·8° (*c* 0·5 in MeOH), which were dried at 110° *in vacuo* (loss in wt. 8%) before analysis [Found: C, 62·0; H, 6·3; N, 12·7; NMe 11·2%; *M* 950 (Thermistor in chloroform). $C_{45}H_{54}O_{10}N_8$ requires C, 62·4; H, 6·3; N, 12·9; 3NMe, 10·0%; *M*, 866)]. Titration with tetrabutylammonium hydroxide in dimethylformamide showed the presence of a single phenolic group (pK_a, 8·6; equiv., 895), and with perchloric acid in acetic acid two basic groups of equiv. wt. 415 were detected. Light absorption: in ethanol, max. at 259 (ε 18,280), 305 (ε 8580), and 365 mμ (ε 985); in aqueous-alcoholic 0·1N-sodium hydroxide, max. at 246 (ε 21,600) and 334 mμ (ε 9150); in 5N-hydrochloric acid, max. at 303 mμ (ε 9500). Infrared spectrum (Nujol): bands at 3390, 3330, 3280, 1737, 1718, 1677, 1649, 1624, 1523, 1302, 1249, 1195, 1167, 1132, 1060, 1003, 904, 809, 762, and 696 cm.$^{-1}$.

E129B is soluble in chloroform, sparingly so in methanol, benzene, and toluene, and insoluble in ether and water. It dissolves in dilute acid and in aqueous sodium hydroxide and is reprecipitated on neutralisation. It gives a red colour with ferric chloride, but gives no colour with ninhydrin.

E129B *Oxime.*—Heated with hydroxylamine hydrochloride in aqueous methanol, E129B yields an *oxime* crystallising from benzene in colourless needles, m. p. 193°, $[\alpha]_D^{20}$ −46° (*c* 1·35 in MeOH) (Found: C, 60·9; H, 6·5; N, 14·2. $C_{45}H_{55}O_{10}N_9$ requires C, 61·3; H, 6·3; N, 14·3%).

E129B *Acid.*—A solution of the antibiotic in excess of aqueous 2N-sodium hydroxide was kept at room temperature for 20 hr., neutralised and extracted with chloroform. The dried extract was evaporated and the residue redissolved in ethanol and precipitated with ether. The *acid* was obtained as an apparently amorphous powder, m. p. 153—157° (decomp.), $[\alpha]_D^{20}$ −40·3° (*c* 1·28 in MeOH) (Found: C, 60·4; H, 6·1; N, 13·0. $C_{45}H_{56}O_{11}N_8$ requires C, 61·1; H, 6·4; N, 12·7%). The acid had the same ultraviolet absorption as E129B (pK_a 5·1 and 8·3; equiv., 420).

Acid-hydrolysis of E129B.—Hydrolysis of either E129B or the parent acid (above) at 105° for 16 hr. with 6N-hydrochloric acid and working-up in the usual way gave a mixture of acids which, examined by two-dimensional paper chromatography, was found to contain six ninhydrin-positive amino-acids and a ninhydrin-negative acid with marked ultraviolet absorption. Prolonged heating with acid did not change the composition of this mixture. The R_F values of the components in the solvent systems A and B were as follows (for convenience the names of the compounds as subsequently identified are employed): threonine (0·45, 0·24); α-aminobutyric acid (0·62, 0·31); 4-oxopipecolic acid (0·52, 0·43); proline (0·81, 0·30); phenylglycine (0·73, 0·52); *p*-dimethylamino-*N*-methyl-phenylalanine (0·93, 0·75); 3-hydroxypicolinic acid (0·79, 0·91). When the paper chromatograms were developed with ninhydrin the α-aminobutyric acid, phenylglycine, and threonine spots were purple, the proline

[9] Hardy, Holland, and Nayler, *Analyt. Chem.*, 1955, **27**, 971.

yellow, the 4-oxopipecolic acid orange-yellow, and the *p*-dimethylamino-*N*-methyl-phenylalanine spot gave a brown colour when developed above 80°. The last spot also gave a red colour with *p*-nitrobenzoyl chloride–pyridine.[3]

Separation and Identification of Amino-acids from Hydrolysates.—(I) The acid hydrolysate of the antibiotic (380 mg.) was put on a column of Amberlite CG 120 (2 × 150 cm.; H^+ form) at 25° and the column eluted initially with N-hydrochloric acid. Amino-acids were detected in the eluate fractions (4 ml.) by spotting on ninhydrin-impregnated paper. 3-Hydroxypicolinic acid was eluted first, but was rather impure. 4-Oxopipecolic acid (fractions 200—240) and threonine (fractions 250—280) were eluted before the column temperature was raised to 50° and gradient elution begun (exponential; 4N added to N-hydrochloric acid in a 1 l. mixing chamber). α-Aminobutyric acid (fractions 430—465) was then eluted, followed by proline (fractions 500—540), phenylglycine (fractions 750—840), and *p*-dimethylamino-*N*-methyl-phenylalanine (fractions 1050—1150).

4-Oxopipecolic acid. This was isolated from appropriate fractions as its *hydrochloride*, crystallising from propan-2-ol–ether in plates, decomp. 175—180°, $[\alpha]_D^{20}$ +3·8° (*c* 2 in H_2O) (Found: C, 40·1; H, 5·9; N, 7·9; Cl^-, 19·7. $C_6H_{10}O_3NCl$ requires C, 40·1; H, 5·6; N, 7·8; Cl, 19·7%), $\lambda_{max.}$ in H_2O 225 (ε 169) and 275 mμ (ε 29), $\nu_{max.}$ (in KBr) 3400, 2910, 2770, 2690, 2480, 1746, 1732, 1630, 1481, 1402, 1345, 1240, 1186, 1107, 1084, 1049, 1008, 946, 917, 820, 789, 762, and 684 $cm.^{-1}$. Its proton resonance spectrum (8% in water) had bands at +57, +102, and +114 c./s. with respect to water at 40 Mc (chemical shifts +1·4, +2·5, +2·8). Treated with Reinecke acid it gave a *reineckate* which darkened on heating to 170—180° [Found: C, 26·1; H, 3·7; N, 21·6. $C_6H_9O_3N,Cr(NH_3)_3(SCN)_4$ requires C, 26·0; H, 3·5; N, 21·2%]. The free amino-acid had $[\alpha]_D^{20}$ −14° (*c* 1 in H_2O); it formed colourless prisms, but was rather unstable.

The above hydrochloride (49 mg.) in acetic acid (34 ml.) containing concentrated hydrochloric acid (2 ml.) was hydrogenated (4 hr.) at a platinum oxide catalyst (absorbed, 12·5 ml.; $2H_2$, 13·5 ml.). Recrystallisation of the product from ethanol–ether gave colourless needles of L(−)-pipecolic acid hydrochloride, $[\alpha]_D^{20}$ −7·8° (*c* 1·28 in H_2O), m. p. 256° (Found: C, 44·0; H, 7·0; N, 8·8; Cl^-, 21·2. Calc. for $C_6H_{12}O_2NCl$: C, 43·5; H, 7·3; N, 8·5; Cl, 21·4%). Identity was confirmed by the infrared spectrum and by proton magnetic resonance spectrum (8% in water) which showed one band at +122 c./sec. with respect to water at 40 Mc. (chemical shift +3·05).

The hydrochloride (10 mg.) obtained from the acid hydrolysate was dissolved in 20% aqueous potassium hydroxide (1·5 ml.) and slowly distilled in a stream of nitrogen (water being added to keep the volume constant) into a solution of 2,4-dinitrophenylhydrazine in aqueous perchloric acid. Separation of the products on a kieselguhr–bentonite (1 : 4 w/w) column (1 × 10 cm.) by elution first with chloroform and then chloroform–ethanol gave the 2,4-dinitrophenylhydrazones of acetone and formaldehyde undepressed in m. p. by authentic specimens.

Threonine. The free amino-acid was obtained from the appropriate fractions of the Amberlite column eluate by passage through Dowex 1 × 2 resin (acetate form). It crystallised from aqueous ethanol in colourless plates, $[\alpha]_D^{22}$ −34·8° (*c* 0·4 in H_2O) (Found: C, 40·3; H, 7·7; N, 11·5. Calc. for $C_4H_9O_3N$: C, 40·3; H, 7·6; N, 11·8%). The infrared spectrum and chromatographic behaviour were indistinguishable from those of L(−)-threonine.

α-Aminobutyric acid. Appropriate Amberlite column fractions (430—465), treated with Dowex 1 × 2 (acetate form), gave an amino-acid crystallising in needles, $[\alpha]_D^{20}$ −6·7° (*c* 2·68 in H_2O) (Found: C, 46·9; H, 8·7; N, 13·3%; equiv., 102. Calc. for $C_4H_9O_2N$: C, 46·7; H, 8·7; N, 13·6%; equiv., 103). It was identical with D-(−)-α-amino-n-butyric acid in infrared spectrum and chromatographic behaviour.

Proline. Isolated in similar fashion from eluate fractions 500—540, L(−)-proline was obtained in needles, $[\alpha]_D^{20}$ −87·5° (*c* 0·5 in H_2O), identical in infrared spectrum and chromatographic behaviour with an authentic specimen (Found: C, 52·4; H, 8·0; N, 11·9. Calc. for $C_5H_9O_2N$: C, 52·2; H, 7·9; N, 12·2%).

(II) For the isolation and identification of the other products of acid hydrolysis of E129B, the hydrolysate (from 1 g. of antibiotic) was applied to a column of Dowex 1 × 2 resin (4 × 35 cm.; acetate form), and the mixed amino-acids were eluted with 0·5M-acetic acid. On further washing with N-hydrochloric acid an acid was eluted which crystallised from methanol (charcoal) in colourless needles, m. p. 216° with gas evolution (Found: C, 52·1; H, 3·9; N, 10·1. Calc. for $C_6H_5O_3N$: C, 51·8; H, 3·6; N, 10·1%). It was identified as 3-hydroxypicolinic acid by m. p., mixed m. p., and ultraviolet and infrared spectra.

The mixture of amino-acids (900 mg.) eluted from the column as described above was dissolved in acetic acid (50 ml. of 5%) and put on a column of Darco G 6 charcoal (2 × 30 cm.); the column was eluted with 5% acetic acid, and the fractions (5 ml.) were examined by ultraviolet absorption and ninhydrin response. Fractions 15—45 contained α-aminobutyric acid, 4-oxopipecolic acid, proline, and threonine. Pure phenylglycine and *p*-dimethylamino-*N*-methyl-phenylalanine were obtained from fractions 70—95 and 105—145 respectively, there being some overlap in the intermediate fractions.

Phenylglycine. The colourless crystalline amino-acid from fractions 70—95 had m. p. 239°, $[\alpha]_D^{20}$ +67·5° (*c* 0·6 in N-HCl) (Found: C, 63·2; H, 6·0; N, ·2.9. Calc for $C_8H_9O_2N$: C, 63·5; H, 6·0; N, 9·3%). This product was fully racemised by heating it in glacial acetic acid. It then formed needles which sublimed at 267° and was identical with DL-phenylglycine (infrared spectrum).

p-*Dimethylamino*-N-*methyl-phenylalanine.* The product from fractions 105—145 crystallised from aqueous ethanol in colourless needles, m. p. 208°, containing water of crystallisation (Found: C, 62·7; H, 8·2. $C_{12}H_{18}O_2N_2,\frac{1}{2}H_2O$ requires C, 62·4; H, 8·3) which was only partially removed at 60° *in vacuo* (Found: C, 63·6; H, 7·6; N, 12·2. Calc. for $C_{12}H_{18}O_2N_2$: C, 64·8; H, 8·2; N, 12·6%). It had $[\alpha]_D^{20}$ +22·6° (*c* 0·5 in N-HCl) and $[\alpha]_D^{20}$ −3·2° (*c* 1·07 in H_2O). Ultraviolet absorption: (*a*) in water, max. at 249 mμ (ε 10,000); (*b*) in aqueous 0·4N-sodium hydroxide, max. at 245 mμ (ε 11,900). Infrared absorption in Nujol: principal bands, 3370, 3070, 2790, 2370, 1622, 1568, 1526, 1442, 1388, 1320, 1227, 1156, 1065, 948, 883, 870, and 818 $cm.^{-1}$. The product formed a *dihydrochloride* (Found: Cl^-, 23·8%; equiv., 97·8. $C_{12}H_{20}O_2N_2Cl_2$ requires Cl, 24·0%; equiv., 98·4), $\lambda_{max.}$ in N-HCl 251 (ε 198), 257 (ε 232), 262 (ε 190), and 266 mμ (ε 120).

To confirm that this product was p-*dimethylamino*-N-*methyl-phenylalanine*, the racemic form of this compound was synthesised as follows:

Acetyl-5-(p-*dimethylaminobenzylidene*)*creatinine.*—A mixture of creatinine (2 g.) and *p*-dimethylaminobenzaldehyde (3 g.) in acetic anhydride (20 ml.) and acetic acid (8 ml.) was heated under reflux for 30 min., then set aside at 0° overnight, and the precipitated *product* was collected and recrystallised from ethanol. It formed red needles (2·66 g., 68%), m. p. 247° (Found: C, 62·8; H, 6·3; N, 19·7. $C_{15}H_{18}O_2N_4$ requires C, 62·9; H, 6·3; N, 19·6%), $\lambda_{max.}$ in EtOH 252 (ε 6875), 301 (ε 7175), and 442 mμ (ε 1800).

5-(p-*Dimethylaminobenzyl*)*creatinine Dihydrochloride.*—Hydriodic acid (6·25 ml.; 1·7) was added with stirring to a mixture of the above product (1·45 g.), red phosphorus (1 g.), and acetic anhydride (6·25 ml.) during 1 hr. and the whole heated under reflux for 3 hr., then cooled, filtered from phosphorus, and evaporated to dryness *in vacuo*. The residue was taken up in water (5 ml.), extracted with ether to remove impurities, then boiled with charcoal, and filtered. On neutralisation with 15% ammonia solution and cooling, the product separated as colourless plates (1·1 g., 88%). The substance slowly decomposed and for analysis it was converted into its *dihydrochloride*, colourless plates, m. p. 252° (decomp.) (from ethanol–ether) (Found: C, 49·3; H, 6·4; N, 17·7; Cl^-, 21·9. $C_{13}H_{20}ON_4Cl_2$ requires C, 48·9; H, 6·3; N, 17·6; Cl, 22·2%), $\lambda_{max.}$ in EtOH 258 mμ (ε 16,100), infl. at 300 mμ (ε 1980).

DL-p-*Dimethylamino*-N-*methyl-phenylalanine.*—The above compound (1 g.) was dissolved in water (15 ml.), and barium hydroxide (12 g.) was added. The mixture was refluxed for 12 hr., cooled, and diluted to 150 ml., and barium was removed by passing in carbon dioxide. The resulting solution was poured on a column of Amberlite CG 120 (1 × 6 cm.; H^+ form), and the column washed with water and eluted with 2N-hydrochloric acid (80 ml.). The hydrochloride so obtained was converted into the free *amino-acid* by passage through a column of Dowex 1 × 2 resin (acetate form). Recrystallised from aqueous ethanol it formed colourless needles, m. p. 214°, which, when dried at 60° *in vacuo* for 4 hr., contained water of crystallisation (Found: C, 63·8, 63·8; H, 8·0, 7·9%), only removed after drying at 60° *in vacuo* for 3 days (Found: C, 64·3; H, 8·3; N, 12·3. $C_{12}H_{18}O_2N_2$ requires C, 64·8; H, 8·2; N, 12·6%). The ultraviolet absorption spectrum was identical with that of the product from E129B and it showed identical chromatographic behaviour in six solvents [R_F values in solvent systems: A, 0·93; B, 0·68; L, 0·81; I, 0·76; O, 0·77; butanol–acetic acid–water (4 : 1 : 5), 0·30].

Amino-acid Content of E129B.—Estimation by the method of Moore and Stein [10] showed that threonine, α-aminobutyric acid, and phenylglycine were present in equimolecular amounts,

[10] Moore and Stein, *J. Amer. Chem. Soc.*, 1954, **76**, 6063; *J. Biol. Chem.*, 1954, **211**, 907.

as was also proline as estimated by Chinard's method.[11] Direct estimation of 3-hydroxypicolinic acid and *p*-dimethylamino-*N*-methyl-phenylalanine from the ultraviolet absorption data showed that the antibiotic contained one molecular proportion of each.

Determination of the C-*Terminal Amino-acid.*—E129B acid was esterified with diazomethane in the usual manner. The amorphous product (which had the expected band at 1742 cm.$^{-1}$) (100 mg.) was dissolved in tetrahydrofuran (250 ml.), cooled to −10°, and treated slowly with powdered lithium borohydride (250 mg.). The mixture was heated under reflux for 40 hr. with exclusion of moisture, then butan-1-ol (100 ml.) was added and the tetrahydrofuran distilled off. Saturated aqueous butanol (50 ml.) was added, the mixture was heated at 60° for 10 min., then filtered, and the filtrate was evaporated to dryness. The amorphous product, which showed no selective carbonyl absorption in the infrared spectrum above 1642 cm.$^{-1}$, was hydrolysed by boiling 6N-hydrochloric acid (5 ml.) for 20 hr. After removal of the mineral acid, the hydrolysate was examined by paper chromatography. It contained all the amino-acids obtained by direct hydrolysis of the E129B acid except 4-oxopipecolic acid and phenylglycine. A new compound was, however, present which, with ninhydrin, gave a brown colour changing to purple, and gave a positive periodate–anisidine reaction.[12] This new compound was indistinguishable from DL-2-amino-2-phenylethanol on paper chromatography or paper electrophoresis.

A portion of the above hydrolysate was neutralised with sodium hydrogen carbonate and shaken with 1-fluoro-2,4-dinitrobenzene in ethanol for 3 hr. Excess of reagent was destroyed by shaking with aqueous glycine for 5 hr.; the product isolated by ether-extraction was a yellow oil which ran as a single spot on paper chromatograms indistinguishable from authentic 2-(2,4-dinitrophenylamino)-2-phenylethanol [R_F in decalin–acetic acid–isopentyl alcohol (15 : 10 : 2), 0·27; in decalin–10% acetic acid–isopentyl alcohol (15 : 10 : 4), 0·86].[13] The latter *derivative* was prepared from the parent synthetic alcohol as an amorphous powder, m. p. *ca.* 42° (Found: C, 55·5; H, 4·6; N, 13·9. $C_{14}H_{13}O_5N_3$ requires C, 55·4; H, 4·3; N, 13·9%).

Pyrolysis of E129B.—The antibiotic (300 mg.) was heated at 250°/0·1 mm. during 6 hr. The crystalline compound which sublimed was collected, resublimed, and then recrystallised from chloroform. It formed colourless needles, m. p. 191°, identical in ultraviolet and infrared absorption with 3-hydroxypicolinamide.[4]

Partial Hydrolysis of E129B.—(*A*) A solution of E129B (250 mg.) in acetic acid (5 ml.) and concentrated hydrochloric acid (5 ml.) was kept at 37° and samples were withdrawn at intervals, the acid being removed and the products examined by two-dimensional paper chromatography (solvents A and L). *p*-Dimethylamino-*N*-methyl-phenylalanine, phenylglycine, and 4-oxopipecolic acid appeared simultaneously, as subsequently did α-aminobutyric acid and proline, followed by threonine. A number of peptides giving no ninhydrin colour, but showing fluorescence in ultraviolet light, were shown to be present in the hydrolysates by chromatography in t-pentyl alcohol–phthalate buffer of pH 6.[14]

A sample (1 ml.) of the hydrolysis mixture was withdrawn after 14 days, the acid removed, and the residue dissolved in water (1 ml.) and applied to a column of Dowex 1 × 2 resin (1·5 × 26 cm.; acetate form). The column was eluted with water until 10 fractions (10 ml.) had been collected and then with a linear gradient of acetic acid (1 l. of 5M-acetic acid added to 1 l. of water). The fractions obtained were examined by ultraviolet absorption (303 mμ or by scanning where necessary). The ninhydrin-negative peptides separated in this way were shown to be homogeneous by paper electrophoresis (acetate buffer, pH 6) and chromatography (t-pentyl alcohol–phthalate buffer, pH 6), and their amino-acid contents were determined by total hydrolysis followed by two-dimensional chromatography in systems A and L.

Fractions 1—5 contained all the free amino-acids. Fractions 19—23 contained a peptide yielding 4-oxopipecolic acid and phenylglycine. Fractions 31—42 contained 3-hydroxypicolinic acid, while 44—55 contained a tetrapeptide hydrolysed to 3-hydroxypicolinic acid, α-aminobutyric acid, proline, and threonine. In fractions 65—75 there was a tripeptide yielding 3-hydroxypicolinic acid, α-aminobutyric acid, and threonine, and in fractions 80—96 a dipeptide hydrolysed to 3-hydroxypicolinic acid and threonine. The tri- and the tetra-peptide overlapped in fractions 55—65.

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[12] Bragg and Hough, *J.*, 1958, 4050.
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(*B*) The antibiotic (28 mg.) was dissolved in concentrated hydrochloric acid and acetic acid (0·4 ml. of 1 : 1 v/v) and set aside for 26 days at 20°. Examined in the usual way by two-dimensional paper chromatography in solvents A and B, the hydrolysate gave strong spots of 4-oxopipecolic acid, phenylglycine, and *p*-dimethylamino-*N*-methyl-phenylalanine, and weak spots of proline and α-aminobutyric acid. Chromatography in t-pentyl alcohol–phthalate buffer of pH 6 on Whatman No. 4 paper gave two fluorescent peptide spots of R_F 0·34 and 0·77. These were separately eluted and hydrolysed, and the hydrolysates subjected to two-dimensional chromatography in solvents A and B after prior removal of phthalic acid. The first of these spots proved to be a tetrapeptide yielding 3-hydroxypicolinic acid, α-aminobutyric acid, proline, and threonine, and the second a pentapeptide giving 3-hydroxypicolinic acid, α-aminobutyric acid, proline, threonine, and *p*-dimethylamino-*N*-methyl-phenylalanine.

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