642. *Actinomycin. Part V.* The Structure of Actinomycin D.*

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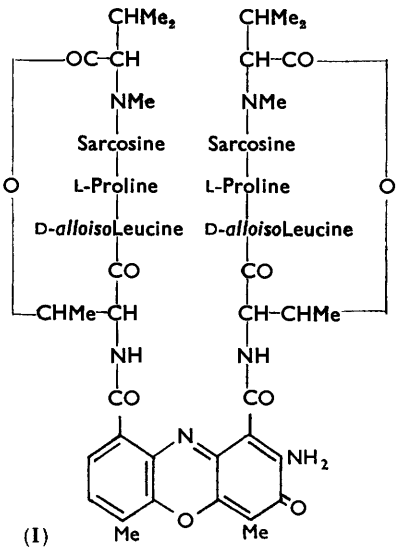
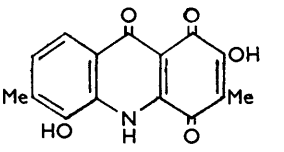
The structure of actinomycin D is shown to be identical with that proposed by Brockmann *et al.*[1] for actinomycin C_3 except that the *alloiso*-leucine components of the peptides are replaced by valine in actinomycin D. A method is described for the separation of the two peptide groups of actinomycin.

Although many of the actinomycin-producing strains of *Streptomyces* which have been studied yield a mixture of actinomycins, Manaker, Gregory, Vining, and Waksman[2] recently described a culture which gives essentially a single actinomycin, referred to as actinomycin D.[3,4] The chemical structure of one component, actinomycin C_3 (I), of the



(I)



(II)

actinomycin C mixture[5] has been determined by Brockmann and his co-workers,[1] and it was suggested[6] that the 2-aminophenoxazin-3-one chromophore was common to all of

* Part IV, *J.*, 1957, 1602.

[1] Brockmann, Bohnsack, Franck, Gröne, Muxfeldt, and Süling, *Angew. Chem.*, 1956, **68**, 70.
[2] Manaker, Gregory, Vining, and Waksman, "Antibiotics Annual," Medical Encyclopedia, New York, 1955, p. 853.
[3] Vining and Waksman, *Science*, 1954, **120**, 389.
[4] Roussos and Vining, *J.*, 1956, 2469.
[5] Brockmann and Gröne, *Chem. Ber.*, 1954, **87**, 1036.
[6] Brockmann and Vohwinkel, *Chem. Ber.*, 1956, **89**, 1373.

the actinomycins examined (not including actinomycin D) which therefore differ only in the nature of the peptide chains. In view of the greatly simplified purification procedure required to obtain actinomycin D, we have examined the structure of this substance. Hydrolysis with aqueous barium hydroxide gave actinomycinol [7] (depeptidoactinomycin [8]) (II) identical with the product formed in this manner fron actinomycin B; and in view of this and the similarity of physical properties of actinomycin D, particularly the light absorption of neutral, acid, and alkaline solutions, to those of all of the other actinomycins,[4] it can be assumed that it also contains the 2-aminophenoxazin-3-one nucleus. Amino-acid analysis of actinomycin D gave [4] values of threonine 1·2 mol., sarcosine 2·0 mol., proline 2·1 mol., valine 2·1 mol., and *N*-methylvaline 2·1 mol. per mol. of actinomycin. Threonine is known to be partly destroyed under conditions of vigorous acid hydrolysis; [9] more reliable results may be obtained after a preliminary reduction of the threonine to α-aminobutyric acid. It seems probable therefore that there are 2 units of threonine present in the molecule of actinomycin D and that the hydroxyl group of each is lactonised with the terminal carboxyl group of the peptide because of the overall similarity of the infrared spectra of the actinomycins.[4] The lactone groupings of actinomycin D, like those of actinomycin C_3, can be hydrolysed with dilute methanolic sodium hydroxide, and actinomycinic acid D, in which the nuclear amino-group is replaced by hydroxyl, is thereby produced.

In a molecule such as (I) which contains two separate peptides, there is the possibility that these may not be identical and consequently the results of normal peptide degradations must be interpreted with caution. Thus the isolation of a peptide degradation product is not in itself sufficient to warrant assumption (cf., *e.g.*, ref. 1) that the product is derived from both peptides. However, as an example of a peptide degradation applied to the whole actinomycin molecule to yield significant results, the Dakin–West reaction might be mentioned. It is known [10,11] that treatment of a peptide with acetic anhydride and pyridine destroys the carboxy-terminal amino-acid although actinomycinic acid C was the first example in which a terminal *N*-methylamino-acid was removed by this degradation.

$$\text{–NH·CHR′·CO·NH·CHR·CO}_2\text{H} \xrightarrow{1} \text{CO}_2 + \text{–NH·CHR′·CO·NH·CHR·CO·CH}_3$$

$$\text{–NH·CHR′·CO·NH·CHR·CO·CH}_3 \xrightarrow{2} \text{–NH·CHR′·CO}_2\text{H} + \text{NH}_2\text{·CHR·CO·CH}_3$$

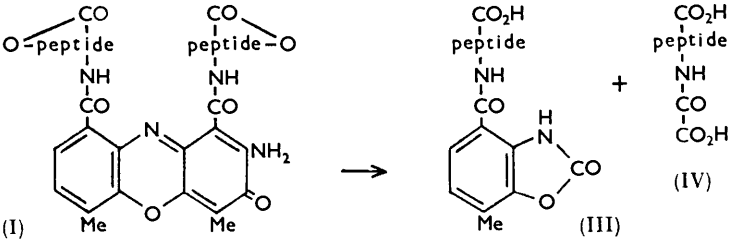
[1] $(CH_3·CO)_2O$–C_5H_5N. [2] H_2O.

When actinomycin D was hydrolysed under mild conditions to open the lactone groups, the dibasic actinomycinic acid D (cf. ref. 11) was obtained and this, when subjected to treatment with acetic anhydride and pyridine followed by vigorous acid hydrolysis, gave all of the constituent amino-acids except *N*-methylvaline. As the reaction involves only the carboxy-terminal amino-acids and as each of the two units of *N*-methylvaline is destroyed, it can be assumed that each of the peptides of actinomycinic acid D is terminated at the carboxy end (*i.e.*, removed from the chromophore) by *N*-methylvaline.

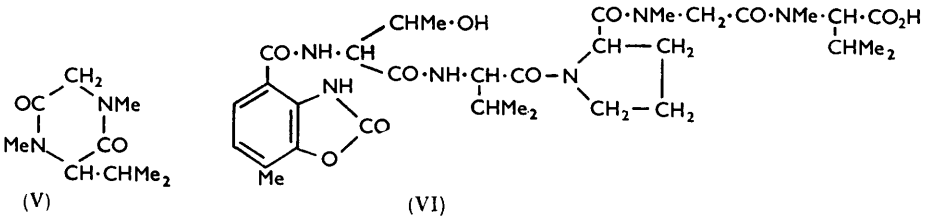
The oxidation of actinomycin with hydrogen peroxide [12] is a method whereby the two peptide chains can be separated and investigated independently, and it should be stressed that this type of degradation is essential for the investigation of actinomycins where the peptides are not known to be identical. A cold alkaline solution of hydrogen peroxide cleaves the chromophore of actinomycin and the two peptide-containing fragments are readily separated by partition between ethyl acetate and water, and can be purified by counter-current distribution. Each of the products is thus obtained as a hygroscopic

[7] Johnson, Todd, and Vining, *J.*, 1952, 2672.
[8] Brockmann and Grubhofer, *Chem. Ber.*, 1953, **86**, 1407.
[9] Brockmann, Gröne, and Timm, *Naturwiss.*, 1955, **42**, 125.
[10] Turner and Schmerzler, *J. Amer. Chem. Soc.*, 1954, **76**, 949.
[11] Brockmann and Franck, *Angew. Chem.*, 1956, **68**, 68.
[12] Bullock and Johnson, *J.*, 1957, 1602.

amorphous solid containing all five of the constituent amino-acids, which provides independent confirmation of a general structure of type (I) for the actinomycins. Both peptides contain a non-amino-acid fragment; in the first (peptide A; III) this had been shown to be 7-methylbenzoxazolone-4-carboxylic acid [12] and in the second (peptide B; IV) it is oxalic acid.



Peptide A, $C_{32}H_{46}O_{10}N_6,2H_2O$, which corresponds to the oxidation product from actinomycin B described earlier [12] (where it was given the erroneous molecular formula $C_{32}H_{44}O_{10}N_6,2H_2O$), is strongly fluorescent in ultraviolet light, and partial hydrolysis studies have shown that the constituent amino-acids can be liberated in a stepwise manner. In addition, thermal cleavage, which was used to advantage in the ergot alkaloid field,[13] has been investigated. Pyrolysis of peptide A at 180—190° in a high vacuum, as well as pyrolysis of actinomycin D itself, yields a volatile product which has been shown to be the dioxopiperazine (V) derived from *N*-methylvaline and sarcosine, and acid hydrolysis of this piperazine has given the two constituent amino-acids. The same product (V) was obtained from actinomycins C_2 and C_3 by treatment with hydrazine.[14]



Treatment of peptide A with 6N-hydrochloric acid at room temperature liberated *N*-methylvaline before any other ninhydrin-reacting substance could be detected, and this observation coupled with the result of the Dakin–West degradation indicates that *N*-methylvaline is the terminal acid at the carboxy-end of peptide A; the result of the pyrolysis suggests that sarcosine is attached to *N*-methylvaline. Hydrolysis of peptide A with dilute hydrochloric acid at 100° liberated *N*-methylvaline, sarcosine, and proline in appreciable amount whereas valine, which normally gives a very intense ninhydrin colour, was obtained only in traces and threonine not at all. Longer hydrolysis liberated valine and some threonine. No ninhydrin-reacting substances other than amino-acids were observed in any of the partial hydrolyses carried out, and hence in view of the molecular formula and the facts that peptide A fails to react itself with ninhydrin and to give any reactions of a free amino-group, this component can be formulated as (VI) with the amino-acids arranged in the order –NH-threonine–valine–proline–sarcosine–*N*-methylvaline-CO_2H.

Peptide B was a stronger acid than peptide A, and examination of an acid hydrolysate by chromatography suggested that the non-amino-acid fragment was oxalic acid. This

[12] Stoll, Hofmann, *et al.*, *Helv. Chim. Acta*, 1943, **26**, 1602; 1951, **34**, 1544; Grob and Meier, *ibid.*, 1956, **39**, 776.
[14] Brockmann, Bohnsack, and Süling, *Angew. Chem.*, 1956, **68**, 66.

was substantiated by the analysis of peptide B which agreed reasonably with the calculated formula, $C_{25}H_{41}O_{10}N_5,H_2O$. The structure of peptide B followed from experiments similar to those applied to peptide A. Pyrolysis at 150—160° yielded the dioxopiperazine, sarcosyl-*N*-methylvalyl (V), and the results of the Dakin–West degradation had suggested that *N*-methylvaline was the carboxy-terminal amino-acid. Furthermore, hydrolysis of the peptide with 6N-hydrochloric acid at room temperature for 4½ days liberated *N*-methylvaline as the only product detectable on paper with ninhydrin. Peptide B, like peptide A, is therefore terminated by sarcosyl-*N*-methylvaline. Hydrolysis of peptide B with N-hydrochloric acid at 100° for 3 hours gave a product which contained all five of the constituent amino-acids although valine and threonine were present only in traces. Hydrolysis of the peptide with 0·5N-hydrochloric acid at 100° for 2½ hours gave a product containing a trace of valine and no detectable threonine. It was thus deduced that in the structure (IV) of peptide B, the arrangement of the constituent amino-acids in the peptide is identical with that of peptide A (VI).

It therefore follows that the structure of actinomycin D is similar to that of actinomycin C_3 except that the D-*alloiso*leucyl units in the latter are replaced by D-valyl groups, the D-configuration being assumed by analogy with the earlier work on actinomycins B [15] and C.[16]

Experimental

The solvent systems used for chromatography on paper of peptide hydrolysates were (i) phenol–water (454 g. of phenol; 100 c.c. of water), (ii) the organic phase of butan-1-ol–acetic acid–water (4 : 1 : 5). Chromatograms were developed by spraying with a 0·1% solution of ninhydrin in butan-1-ol, and the papers heated at 100—110° for 3 min.

Actinomycin D.[4]—Crystallised from methanol–ethanol (1 : 3), this had the following absorption max. (mμ) (log ε in parentheses) : (i) in EtOH, 445 and 240 (4·43 and 4·49); (ii) in EtOH–10N-HCl (1 : 1), 477 (4·21); (iii) in EtOH–0·1N-NaOH, 458, 344, and 285 (3·05, 4·38, and 4·13).

Actinomycinol D.—Actinomycin D (2 g.) was dissolved in the minimum quantity (*ca.* 75 c.c.) of boiling ethanol, treated with cold saturated aqueous barium hydroxide, and heated on the steam-bath for 15 min. The purple precipitate was separated and warmed with 2N-hydrochloric acid (350 c.c.), actinomycinol D being obtained as a red precipitate (200 mg.). It was removed and crystallised from 10% aqueous acetic acid, forming red needles which showed an absorption spectrum (in EtOH) identical with that of actinomycinol B : [7] $\lambda_{max.}$ 473, 321, 277, and 245 mμ (log ε 3·51, 3·76, 4·19, and 4·36 respectively).

Actinomycinic Acid D.—Actinomycin D (0·5 g.) was dissolved in 0·1N-methanolic sodium hydroxide (50 c.c.) and kept at 35° for 4 hr. The solution was diluted with water, neutralised with 3N-hydrochloric acid (to 250 c.c.), and extracted with chloroform (3 × 100 c.c.). The solvent was removed from the combined extracts, and the residue crystallised from chloroform–ethyl acetate (1 : 10) to yield *actinomycinic acid D* (410 mg.) as orange needles (Found, on a sample dried for 24 hr. at room temperature : C, 55·2; H, 6·95; N, 11·9. $C_{62}H_{89}O_{19}N_{11},3H_2O$ requires C, 55·3; H, 7·1; N, 11·45%), $\lambda_{max.}$ 420—424 mμ (log ε 3·74), $\lambda_{infl.}$ 315 mμ (log ε 3·97).

Dakin–West Degradation of Actinomycinic Acid D.—Actinomycinic acid D (10 mg.) was heated with pyridine (0·5 c.c.) and acetic anhydride (1·25 c.c.) at 135° for 6 hr. The solvents were then removed by distillation under reduced pressure and the brown residue was heated under reflux with 6N-hydrochloric acid (5 c.c.) for 12 hr. The acid was removed in a vacuum-desiccator containing solid sodium hydroxide, and the residual hydrolysate was examined by chromatography on paper. Four ninhydrin-reacting compounds were detected : (i) phenol–water, R_F 0·88 (yellow), 0·77, 0·74, and 0·44; (ii) butanol–acetic acid, R_F 0·55, 0·40 (yellow), 0·28, and 0·28 corresponding to valine, proline, sarcosine, and threonine.

Similar degradations were carried out on peptides A and B (see below) with exactly similar results.

Oxidation of Actinomycin D.[12]—Actinomycin D (1 g.), dissolved in the minimum quantity of methanol (80 c.c.), was treated with 30% w/v hydrogen peroxide (15 c.c.), 10% aqueous

[15] Dalgliesh, Johnson, Todd, and Vining, *J.*, 1950, 2946.
[16] Brockmann, Grubhofer, Kass, and Kalbe, *Chem. Ber.*, 1951, **84**, 260.

sodium hydroxide (15 c.c.), and water (120 c.c.). After 1 hr. at room temperature, a further quantity of 30% hydrogen peroxide (15 c.c.) was added and the solution kept for an hour. The pH of the resulting solution was adjusted to 3—4, water (400 c.c.) was added, and the product extracted with butan-1-ol (3 × 100 c.c.). The combined butanol extracts were washed with water (100 c.c.), and the aqueous layer was re-extracted with butan-1-ol (50 c.c.). This extract was added to the original butanol extracts, and the solvent removed under reduced pressure. The residue (0·95 g.) was a pale yellow amorphous solid and it was equilibrated between ethyl acetate (100 c.c.) and water (100 c.c.). The phases were separated and the solvents removed under reduced pressure, then the oxidation product was partitioned in the ratio 1·3 : 1. The ethyl acetate-soluble fraction, essentially *peptide A*, was purified as described for the similar product from actinomycin B in an earlier paper,[7] to yield a colourless microcrystalline product (0·598 g., 43·4%, from 1·387 g. of crude oxidation product) which softened at 164—170° and sintered without melting at 172—174° and was very hygroscopic. For analysis, it was precipitated from ethyl acetate by light petroleum (b. p. 60—80°) and dried at 80°/0·1 mm. (P_2O_5) (Found : C, 53·6; H, 7·0; N, 11·8. $C_{32}H_{46}O_{10}N_6,2H_2O$ requires C, 54·1; H, 7·1; N, 11·8%). Its absorption max. in EtOH were at 297 and 206 mμ (log ε 3·61 and 4·43).

The water-soluble peptide fraction, essentially *peptide B*, was purified by repeated extraction of an aqueous solution with ethyl acetate in a counter-current machine. After 50 changes of solvent, the aqueous layers of tubes 0, 1, and 2 were combined, and the solvent was removed to yield a white crystalline residue (0·45 g., 32·4%, from 1·387 g. of crude oxidation product), m. p. 92—94° (decomp.), which was very hygroscopic (Found, in a sample dried at 50°/0·1 mm. : C, 51·4; H, 7·3; N, 11·9. $C_{25}H_{41}O_{10}N_5,H_2O$ requires C, 50·9; H, 7·35; N, 11·9%) and had absorption max. in EtOH at 207 mμ (log ε 4·43).

Hydrolysis of peptide B with 6N-hydrochloric acid in a sealed tube at 110° for 6 hr. gave a product which by chromatography on paper was shown to contain all five of the constituent amino-acids as well as an additional acidic compound. Chromatography in ethanol–aqueous ammonia (*d* 0·88)–water (7 : 1 : 2) showed that this acid had properties identical with those of oxalic acid (cf. ref. 12).

Pyrolysis of Peptide A.—Peptide A (100 mg.) was heated in a long tube at 180—190°/0·2 mm., and after 90 min. the oil (*ca.* 20 mg.) which had distilled was removed and resublimed at 78°/0·1 mm., a copper tube being placed as a jacket over the sublimation tube in order to obtain an extended temperature gradient. The crystalline sublimate was separated and repeatedly sublimed, to give a main crystalline dioxopiperazine fraction (V) (*ca.* 5 mg.), m. p. 116° (lit.,[14] 118°) (Found : C, 58·4; H, 8·45; N, 15·2. Calc. for $C_9H_{16}O_2N_2$: C, 58·65; H, 8·75; N, 15·2%).

This product was hydrolysed with 6N-hydrochloric acid in a sealed tube at 110° for 6 hr., and the hydrolysate examined by two-dimensional paper chromatography. Two spots which gave a positive ninhydrin reaction were observed : (i) phenol–water, R_F 0·68 and 0·93; (ii) butanol–acetic acid, R_F 0·20 and 0·49. These correspond to sarcosine and *N*-methylvaline respectively.

Similar pyrolyses were carried out with actinomycin D and peptide B, and in each case the same dioxopiperazine (V) was isolated.

Partial Hydrolyses.—The peptide (2—3 mg.) was placed in a Pyrex tube (0·3 cm. internal diam.) with hydrochloric acid of the requisite strength (0·3—0·5 c.c.), and the tube was sealed and heated. After cooling, the hydrolysate was evaporated in a vacuum-desiccator containing sodium hydroxide; water (0·5 c.c.) was added to the residue and the whole again evaporated to dryness. The product was dissolved in aqueous ethanol and examined by two-dimensional chromatography on paper (i) in phenol–water and (ii) in butanol–acetic acid.

(1) *Peptide A*. (*a*) 6N-Hydrochloric acid at room temperature for 4½ days liberated only one amino-acid, R_F (i) 0·91, (ii) 0·49 (*N*-methylvaline).

(*b*) N-Hydrochloric acid at 100° for 2 hr. gave three amino-acids with a trace of a fourth; R_F values were (i) 0·93, 0·86 (yellow), 0·70 (trace), 0·65, (ii) 0·49, 0·43 (trace), 0·29 (yellow), 0·20 [*N*-methylvaline, proline, and sarcosine with valine (trace)]. A similar result was obtained after hydrolysis with 0·5N-hydrochloric acid at 100° for 2 hr., except that in this case the valine was barely detectable.

(2) *Peptide B*. (*a*) 6N-Hydrochloric acid at room temperature for 4½ days liberated only one amino-acid, R_F (i) 0·91, (ii) 0·49 (*N*-methylvaline). (*b*) N-Hydrochloric acid at 100° for 3 hr. gave three amino-acids with small amounts of two others : R_F values were (i) 0·94, 0·84 (yellow), 0·70 (weak), 0·67, and 0·44 (weak), (ii) 0·50, 0·43 (weak), 0·29 (yellow), 0·19, and 0·19 (*N*-methylvaline, proline, and sarcosine with small amounts of valine and threonine).

Hydrolysis with 0·5N-hydrochloric acid at 100° for 2½ hr. gave three amino-acids (*N*-methylvaline, proline, and sarcosine) with a trace of valine but no threonine : R_F values were (i) 0·94, 0·86 (yellow), 0·73 (trace), and 0·68, (ii) 0·49, 0·43 (trace), 0·29 (yellow), and 0·18.

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