Studies concerning the Antibiotic Actinonin. Part I. The Constitution of Actinonin. A Natural Hydroxamic Acid with Antibiotic Activity

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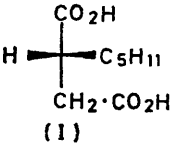
The antibiotic, actinonin, has been shown to have the novel pseudopeptide structure (XII) associated with residues derived from L-prolinol, L-valine, D-pentylsuccinic acid, and hydroxylamine.

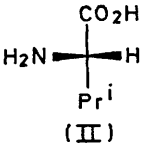
ACTINONIN was first isolated by R. Green and R. Bhagwan Singh from a Malayan strain of *Actinomyces* which was designated *Streptomyces* sp. Cutter C12 (N.C.I.B. 8845).[1] Methods for the culture of the micro-organism and the isolation of the active principle have been reported [1] and a collaborative investigation (J. P. D., A. J. E., W. D. O., and I. O. S.) of the constitution of actinonin was initiated.[2] A substance subsequently shown to be identical with actinonin was independently isolated (L. N.) from cultures of a species designated as *Streptomyces roseopallidus*, DS 40562, which is probably similar to *Streptomyces* sp. Cutter C12; its chemical examination (D. E. W.) provided results complementary to those already reported.[2] The spectrum of biological activity of actinonin against a number of Gram-positive and Gram-negative bacteria encouraged further investigations (Parts II—VIII). These studies include the synthesis and the examination of the biological properties of structural analogues of actinonin.

Actinonin, m.p. 148°, was shown to have the molecular formula $C_{19}H_{35}N_3O_5$ on the basis of its composition and high resolution mass spectrum. The antibiotic was weakly acidic (pK_a 9·32 in water) and optically active, $[\alpha]_D^{20}$ −65° (in water). The u.v. spectrum showed only end-absorption but its i.r. spectrum in dioxan solution indicated the presence of amide groups ($\nu_{max.}$ 1665, 1640, and 1525 cm^{-1}) in addition to OH and/or NH groups ($\nu_{max.}$ 3500 and 3300 cm^{-1}). Vigorous acidic hydrolysis of actinonin with hydrochloric acid followed by fractionation yielded four hydrolysis products: an ether-extractable acid, an amino-acid, and two basic substances isolated as crystalline hydrochlorides.

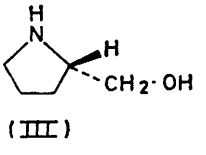
The acid $C_9H_{16}O_4$, directly extracted from the acidic hydrolysate by ether, was identified as a dicarboxylic acid by titration. Its dehydration with hot acetic anhydride yielded an anhydride with an i.r. spectrum ($\nu_{max.}$ 1875 and 1795 cm^{-1}) typical of a five-membered cyclic anhydride. Kuhn–Roth oxidation results suggested the presence of one CMe group. The dicarboxylic acid was optically active, $[\alpha]_D^{25}$ +24° (in ethanol). When its dimethyl ester was heated with methanolic sodium methoxide and the product subjected to alkaline hydrolysis, (±)-pentylsuccinic acid was obtained.[3] The (+)-enantiomer of pentylsuccinic acid has been shown to have the D(≡ *R*)‡-configuration (I).



(I)



(II)



(III)

The amino-acid obtained from the hydrolysis of actinonin was separated by ion-exchange chromatography. It was shown to be (+)-L-valine (II) by direct comparison of its *N*-2,4-dinitrophenyl derivative with an authentic specimen.[5] The mixture of hydrochlorides obtained from the hydrolysis was separated by trituration with chloroform. The chloroform-insoluble fraction was identified as hydroxylamine hydrochloride by its reaction with nickel chloride–biacetyl mono-oxime [6] and by its reaction with *m*-nitrobenzaldehyde yielding *m*-nitrobenzaldoxime. The chloroform-soluble fraction was identified as the hydrochloride of L-prolinol (III) by comparison of its *N*-dinitrophenyl and *NO*-dibenzoyl derivatives with the corresponding compounds prepared from authentic L-prolinol.[7,8] The L-prolinol (III) was synthesised from 2-ethoxycarbonyl-5-pyrrolidone obtained from L-glutamic acid.

These results required that actinonin, $C_{19}H_{35}N_3O_5$, was formally derived from D-pentylsuccinic acid (I), L-valine (II), L-prolinol (III), and hydroxylamine with loss of three molecules of water. The absence of ester-type absorption in the i.r. spectrum of actinonin required that L-prolinol (III) was only *N*-acylated and it therefore provided one terminal residue. The association of the other terminal residue with hydroxylamine was based upon the following evidence which identified actinonin

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‡ The terms L- and D- are used rather than the *RS*-nomenclature in this series of papers in order to follow the more common practice [4] used in the designation of amino-acid configurations.

[1] J. J. Gordon, B. K. Kelly, and G. A. Miller, *Nature*, 1962, **195**, 701.

[2] A. J. East, W. D. Ollis, and I. O. Sutherland, in 'Chemistry of Microbial Products,' Institute of Applied Microbiology Symposium No. 6, University of Tokyo, 1964, p. 204.

[3] A. Fredga, *Arkiv Kemi*, 1953, **6**, 277.

[4] 'Amino-acids, Peptides, and Proteins,' *Chem. Soc. Special Periodical Report*, 1971, **3**; I.U.P.A.C. Information Bulletin No. 23, 'Symbols for Amino-acids and Peptides,' 1972.

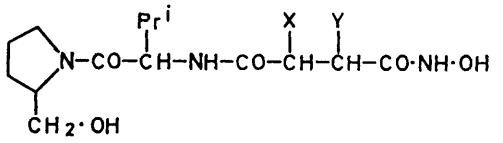
[5] K. R. Rao and H. A. Sober, *J. Amer. Chem. Soc.*, 1954, **76**, 1328.

[6] A. Fiegl, 'Spot Tests in Inorganic Chemistry,' 5th edn., 1958, p. 242.

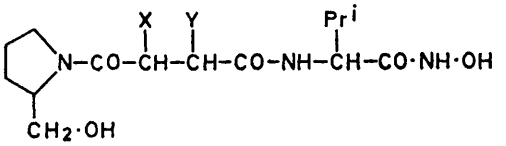
[7] P. Karrer and P. Portmann, *Helv. Chim. Acta*, 1948, **31**, 2088; F. F. Blicke and C.-J. Lu, *J. Amer. Chem. Soc.*, 1955, **77**, 29; F. P. Doyle, M. O. Mehta, G. S. Sach, and J. L. Pearson, *J. Chem. Soc.*, 1958, 4458; R. Buyle, *Chem. and Ind.*, 1966, 195, 380.

[8] Part II, N. H. Anderson, W. D. Ollis, J. E. Thorpe, and A. D. Ward, following paper.

as a hydroxamic acid: (i) actinonin was weakly acidic (Table), (ii) it gave a purple colouration with methanolic iron(III) chloride, and (iii) periodate oxiation,[9] which is associated with the transformation $RCO{\cdot}NH{\cdot}OH \longrightarrow RCO_2H$, gave actinonic acid, $C_{19}H_{34}N_2O_5$. Four constitutions [(IVa and b) and (Va and b)] could now be considered for actinonin.



(IV) a; X = *n*-C_5H_{11}, Y = H
b; X = H, Y = *n*-C_5H_{11}



(V) a; X = *n*-C_5H_{11}, Y = H
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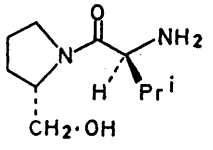
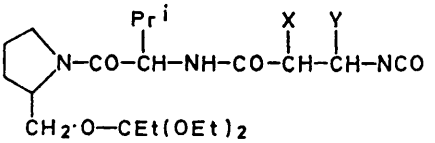


(VI)



(VII) a; X = *n*-C_5H_{11}, Y = H
b; X = H, Y = *n*-C_5H_{11}

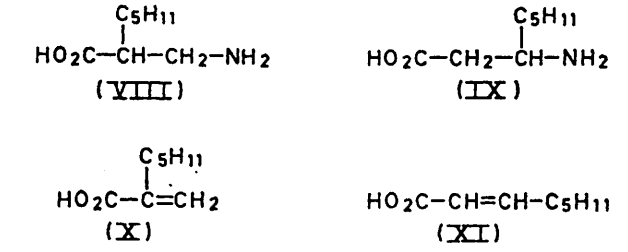
A preference for two (IVa and b) of these was indicated by the following approach. The acidity of actinonin was compared with that of other hydroxamic acids [10,11] and the acidity of actinonic acid with other model carboxylic acids (Table). These comparisons suggested that actinonin did not contain a terminal *N*-acylvalyl-hydroxamic acid residue (Va or b) so that the constitutions (IVa and b) were favoured. This opinion was substantiated by the result of mild acidic hydrolysis of actinonin to yield L-valyl-L-prolinol (VI) and D-pentylsuccinic acid (I). This reduced the number of constitutional possibilities for actinonin to two (IVa and b).

pK_a Values † for actinonin and related compounds

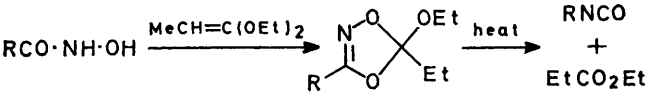
Actinonin (XII)	9·32	Actinonic acid (XVIII)	4·65
$PhCH_2{\cdot}CO{\cdot}NH{\cdot}OH$	9·09	$PhNH{\cdot}CO{\cdot}CH_2{\cdot}CH_2{\cdot}CO_2H$	4·65
$AcNH{\cdot}CHPr^i{\cdot}CO{\cdot}NH{\cdot}OH$	8·88	$AcNH{\cdot}CHPr^i{\cdot}CO_2H$	3·70
$PhCO{\cdot}NH{\cdot}OH$	8·82		

† Determined in water under standard conditions.

In order to prove that the assignment of the sequence (IV) was well based and to distinguish between the two possible constitutions (IVa and b), the Lossen degradation [11–15] was selected. It was expected that this reaction might yield the corresponding isocyanate (VIIa or b), which by acidic hydrolysis was expected to give either the β-amino-acid (VIII) or its isomer (IX). Many different methods [16] for carrying out the Lossen degradation were investigated but reactions involving the attempted thermal or base-catalysed transformation of various acyl derivatives of actinonin did not give useful results. This was disappointing particularly as the successful Lossen degradation of several hydroxamic acids containing peptide linkages has been reported.[14,17] However, application of the following method [18] effected the Lossen transformation of actinonin in excellent yield.



(VIII) (IX) (X) (XI)



Actinonin and methylketen diethyl acetal [19] in boiling ether yielded an intermediate which is believed to be a 1,3,4-dioxazole because further heating in boiling benzene gave the isocyanate (VIIa). Acidic hydrolysis of the isocyanate gave L-valine and a β-amino-acid which was

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[13] F. Mathis, *Bull. Soc. chim. France*, 1953, D9.

[14] 'Molecular Rearrangements,' ed. P. de Mayo, Interscience, New York, 1963, vol. 1, p. 548; vol. 2, p. 988.

[15] P. A. S. Smith, 'Open-chain Nitrogen Compounds,' Benjamin, New York, 1966, vol. 2, (*a*) p. 68; (*b*) p. 81.

[16] G. Dougherty and L. W. Jones, *J. Amer. Chem. Soc.*, 1924, **46**, 1535; A. W. Scott and J. H. Mote, *ibid.*, 1927, **49**, 2545; R. D. Bright and C. R. Hauser, *ibid.*, 1939, **61**, 618; A. W. Scott and W. O. Kearse, *J. Org. Chem.*, 1940, **5**, 598; C. D. Hurd, C. M. Buess, and L. Bauer, *ibid.*, 1954, **19**, 1140; C. D. Hurd and L. Bauer, *J. Amer. Chem. Soc.*, 1954, **76**, 2791; L. Bauer, *ibid.*, 1956, **78**, 1945; C. D. Hurd and A. G. Prapas, *ibid.*, 1958, **80**, 6053; L. Bauer and S. Miarka, *J. Org. Chem.*, 1959, **24**, 1293; D. G. Hoare, A. Olson, and D. E. Koshland, *J. Amer. Chem. Soc.*, 1968, **90**, 1638.

[17] T. Wieland and H. Fritz, *Chem. Ber.*, 1953, **86**, 1186; P. M. Gallop, S. Seifter, M. Lukin, and E. Meilman, *J. Biol. Chem.*, 1960, **235**, 2619.

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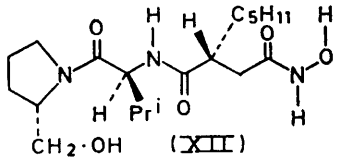
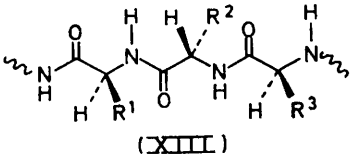
[19] S. M. McElvain and P. M. Walters, *J. Amer. Chem. Soc.*, 1940, **62**, 1482.

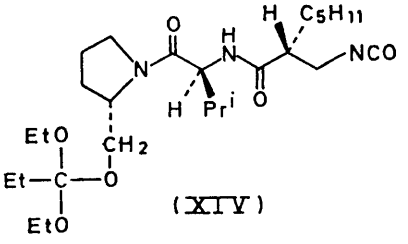
identical with synthetic 2-(aminomethyl)heptanoic acid (VIII) and different from 3-amino-octanoic acid (IX).[20]



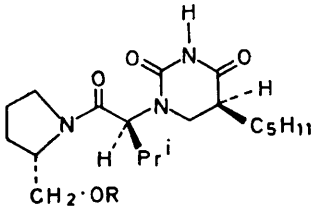
(XII)



(XIII)



(XIV)

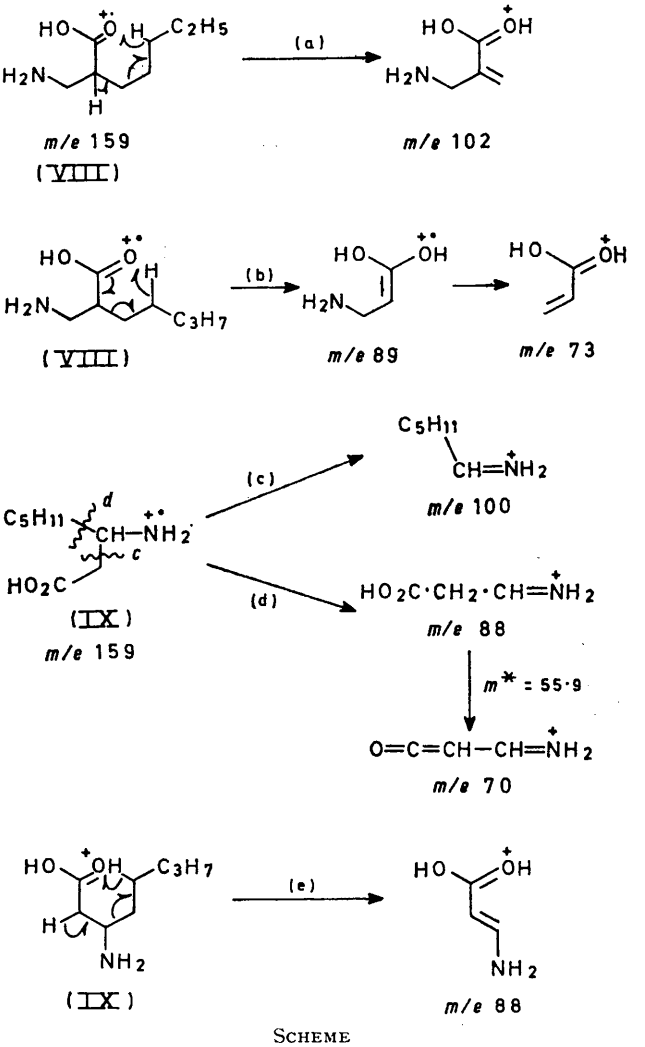


(XV) a; R = CEt(OEt)$_2$
b; R = H

Pyrolysis of the β-amino-acid (VIII) obtained from isocyanate (VIIa) gave 2-methyleneheptanoic acid[21] (X). These results established the configurational formula (XII) for actinonin which was compatible with its n.m.r. (Experimental section) and mass spectra.[22] Neither of these physical methods could be successfully applied to distinguish between the constitutional possibilities (IVa and b) for actinonin, whereas the isomeric β-amino-acids (VIII) and (IX) gave highly characteristic and structurally informative mass spectral fragmentation patterns (Scheme). The formulae of the fragmentions were established by high resolution measurements.

'Mechanisms' of formation of the fragment ions are given in the Scheme. The β-amino-acid (VIII) exhibits (a) a γ-cleavage or (b) a McLafferty rearrangement followed by elimination of $\dot{N}H_2$. In contrast, the mass spectral behaviour of the β-amino-acid (IX) is dominated by two α-cleavages [(c) and (d)], and route (d) gives the ion $[C_3H_6NO_2]^+$ which by dehydration gives the ion $[C_3H_4NO]^+$. An alternative route to an ion $[C_3H_6NO_2]^+$ by a γ-cleavage (e) is also possible.

The isocyanate ($\nu_{max.}$ 2295 cm^{-1}) formed from actinonin and methylketen diethyl acetal was considered to have the constitution (XIV) with an orthoester grouping ($\nu_{max.}$ 1220, 1160, 1060, and 990 cm^{-1}). When this isocyanate (XIV) was heated, it yielded a product identical with that formed from actinonin and methylketen diethyl acetal in boiling *NN*-dimethylformamide. This product is the dihydrouracil (XVa) formed by intramolecular cyclisation of the orthoester isocyanate (XIV); its mild hydrolysis gave the dihydrouracil (XVb). This compound (XVb) showed i.r. bands ($\nu_{max.}$ 1722 and 1680 cm^{-1}) highly characteristic of dihydrouracils[23-25] and its



SCHEME

formulation was clearly supported by its mass spectral fragmentation pattern.

[20] K. Lang and F. Adickes, *Z. physiol. Chem.*, 1941, **269**, 236; Ya. L. Gol'dfarb, B. P. Fabrichynyi, and I. A. Shalavina, *Zhur. obschei. Khim.*, 1958, **28**, 213 (*Chem. Abs.*, 1958, **52**, 12,838).

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[23] K. Schlögl, *Monatsh.*, 1958, **89**, 61.

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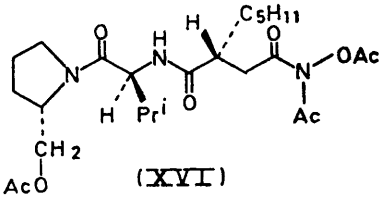
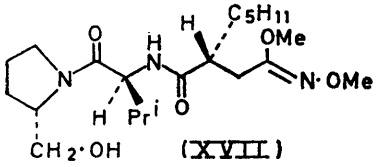
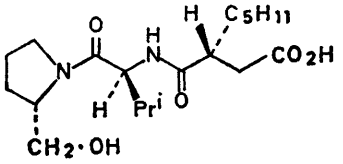
The acylation of hydroxamic acids [12,13,15,26,27] has been associated with considerable uncertainty about the nature of the products; actinonin with acetic anhydride–pyridine yielded a triacetyl derivative (XVI). Actinonin and diazomethane gave the *OO'*-dimethyl derivative (XVII). Actinonic acid formed by periodate oxidation of actinonin must have the structure (XVIII).



(XVI)



(XVII)



(XVIII)

The isolation of L-valyl-L-prolinol (VI) from the acidic hydrolysis of actinonin (XII) demonstrates that one peptide linkage is hydrolysed much more easily than the other. This presumably involves intramolecular assistance in the cleavage of the amide bond by the protonated hydroxamic acid residue. Rather unexpectedly, L-valyl-L-prolinol (VI) is also produced by hydrolysis of the same amide bond by *aqueous sodium hydrogen carbonate*. This reaction presumably involves intramolecular base-catalysed cleavage of the amide bond by the hydroxamic acid residue.

As that of a natural antibiotic, the structure (XII) for actinonin is of considerable interest. Actinonin is the first known naturally occurring derivative of L-prolinol (III). In this connection it may be noted that biological activity is retained [28] in a synthetic analogue of a nonadecapeptide related to adrenocorticotrophic hormone in which an L-proline residue is replaced by an L-prolinol residue. Although the number of known naturally occurring hydroxamic acids [11,29] is increasing, actinonin is the first of the type, RCO·NH·OH, to be identified.

Polypeptide antibiotics [30] are often associated with D-amino-acid residues.[31] This has been an important formulation for the topochemical investigation of biologically active peptides [30,31] and structure–activity correlation.[32] Actinonin is therefore unusual [2] as an antibiotic in that it contains only L-amino-acid residues. The pentyl side-chain of the D-pentylsuccinic acid residue of actinonin (XII) is isosteric with the side-chain R^2 (XIII) of an L-amino acid residue. The structural correspondence between actinonin (XII) and a polypeptide containing only L-amino-acid residues permits its description as a pseudopeptide.

The mode of biological action of actinonin (XII) has been investigated [33] and it is proposed that its site of action is associated with ribonucleic acid synthesis. This opinion may be compared with the inhibitory action of certain hydroxamic acids on deoxyribonucleic acid synthesis.[34]

EXPERIMENTAL

Unless otherwise stated, i.r. spectra were measured in chloroform, u.v. spectra in ethanol, and 60 and 100 MHz n.m.r. spectra in deuteriochloroform (tetramethylsilane as internal reference). Only significant bands from these spectra are quoted. Mass spectra were determined with an A.E.I. MS9 high resolution spectrometer. M.p.s were determined with a Kofler hot-stage apparatus. Evaporation refers to evaporation under diminished pressure. Light petroleum refers to the fraction with b.p. 60—80° unless otherwise stated.

Separations by column chromatography were carried out with Hopkin and Williams MFC grade silica and Mackerey, Nagel & Co. MN-polyamide. Paper chromatography was performed with Whatman No. 1 paper and the following solvent systems: EAW [ethanol–ammonia(*d* 0·88)–water (20 : 1 : 4)], BAW [n-butanol–acetic acid–water (2 : 1 : 1)], PAB [light petroleum–acetic acid–n-butanol (15 : 10 : 12)], and CAA [cyclohexane–acetic acid–isopentyl alcohol (15 : 10 : 2)]. Merck Kieselgel G was used for thick- and thin-layer chromatography. Chromatograms were developed by spraying with (*a*) a solution (0·5%) of ninhydrin in n-butanol, (*b*) ethanolic iron(III) chloride (1%), (*c*) iodine in carbon tetrachloride (5%), and (*d*) cerium(IV) sulphate (2%) in 2N-sulphuric acid solution followed by heating, or by examination under u.v. illumination. Unless otherwise stated, Dowex 50W-X8 resin was used for ion-exchange chromatography. During isolation processes, the appropriate combination of fractions was determined by examination of their i.r. spectra and t.l.c. behaviour.

When substances are stated to be identical, their identity

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[27] M. T. W. Hearn and A. D. Ward, *Austral. J. Chem.*, 1969, **22**, 161.

[28] W. Oelofsen and C. H. Li, *J. Amer. Chem. Soc.*, 1966, **88**, 4254.

[29] H. Maehr, *Pure Appl. Chem.*, 1971, **28**, 603.

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has been established by comparison of m.p. and mixed m.p. determination, and, where appropriate, comparison of i.r., n.m.r., and mass spectra and behaviour on paper and thin-layer chromatography.

Culture of Streptomyces roseopallidus *DS* 40562 *and Isolation of Actinonin* (with M. Dubost, D. Mancy, S. Pinnert, and J. Preud'homme).—The organism was cultured on maltose–tryptone agar and incubated for 15 days at 26° with subsequent inoculation into a liquid medium which contained corn steep liquor (5 g; 50% dry extract), sucrose (7·5 g), ammonium sulphate (0·5 g), calcium carbonate (1·9 g), and water (to 250 ml). The culture was maintained for 2 days at 26° with continuous agitation and added to a second batch (120 l) of this medium with further incubation for 2 days at 27° with continuous agitation.

For the production of actinonin, a medium containing soya flour (16 kg), distillers soluble (4 kg), soya oil (12 l), sodium chloride (2 kg), and water (to 400 l) was inoculated with the culture described above (40 l) and maintained at 27° for 140 h with continuous agitation and aeration. The culture fluid was acidified to pH 5·0 with hydrochloric acid and filtered. The filtrate was brought to pH 9·0 by addition of sodium carbonate and extracted with n-butanol (120 l). The extract was washed with water (12 l) and evaporated to 12 l under reduced pressure. The concentrated extract was diluted with hexane (36 l) and extracted with water previously acidified to pH 4·0 (3 × 12 l). The combined aqueous extracts were concentrated to 2 l under reduced pressure and lyophilised. The residue (280 g) was dissolved in water (2·8 l) and the solution was extracted with chloroform (4 × 2·8 l). The combined extracts were dried and evaporated to 600 ml; *actinonin* (60 g) separated on cooling. The antibiotic was further purified by dissolution in methanol (500 ml) and dilution of the solution with ether (5 l); *actinonin* was obtained as colourless microprisms (55 g), m.p. 148° (Found: C, 58·9; H, 8·9; N, 11·2%; M^+, 385·2576. $C_{19}H_{35}N_3O_5$ requires C, 59·2; H, 9·2; N, 10·9%; M, 385·2576); $[\alpha]_D^{22}$ −48° (*c* 1·0 in MeOH); $[\alpha]_D^{20}$ −65° (H_2O); pK_a 9·32 (H_2O); $\nu_{max.}$ (dioxan) 3500, 3300, 1665, 1640, and 1525 cm^{-1}; τ (C_5D_5N) 1·84br (s, CO·NH·O*H*), 0·78 (d, Me_2CH·CH·N*H*·CO), 5·16 (t, Me_2CH·C*H*·NHCO), 7·07 (A), 7·43 (B), and *ca.* 7·8 (X) (ABX system with X additionally coupled; J_{AX} 8, J_{BX} 6, J_{AB} 14 Hz).

Acetylation of actinonin in pyridine with acetic anhydride at room temperature (24 h) gave triacetylactinonin (XVI) as a gum after purification on alumina columns and elution with chloroform; $\nu_{max.}$ 1800 and 1720 cm^{-1}; τ 7·63 (3H, s), 7·70 (3H, s), and 7·97 (3H, s).

Acidic Hydrolysis of Actinonin.—(a) *With* 6N-*hydrochloric acid.* (i) *Isolation of* (+)-D-*pentylsuccinic acid* (I). A solution of actinonin (1·05 g) in dilute hydrochloric acid (6N; 90 ml) was heated under reflux (3 h) and extracted with ether. The combined extracts were shaken with aqueous sodium hydrogen carbonate solution (10% w/v) and the aqueous layer was acidified and extracted with ether. Evaporation of the ethereal extract gave a solid (531 mg) which crystallised from light petroleum to give (+)-D-pentylsuccinic acid as needles (450 mg, 88%), m.p. 82·5—83·5° (lit.,[3] 84—86°) (Found: C, 57·4; H, 8·5; CMe, 6·7. Calc. for $C_9H_{16}O_4$: C, 57·4; H, 8·6; CMe, 8·0%), $[\alpha]_D^{25}$ +24° (EtOH). The anhydride (40 mg, 90%), $\nu_{max.}$ 1875 and 1795 cm^{-1}, was prepared by treatment of the acid (49 mg) with boiling acetic anhydride.

(ii) *Racemisation of* (+)-D-*pentylsuccinic acid.* The (+)-acid (45·5 mg) was dissolved in methanol and treated with an excess of ethereal diazomethane. The solution was kept at room temperature for 0·5 h and evaporated to dryness. The residual crude methyl ester was added to a solution of sodium methoxide [from sodium (100 mg) in anhydrous methanol (10 ml)] and the mixture was kept at room temperature for 16 h. The solution was diluted with water (20 ml) and after addition of sodium hydroxide (100 mg) was heated under reflux for 0·5 h and extracted with ether. Acidification of the aqueous layer followed by extraction with ether and evaporation of the ether extract gave (±)-pentylsuccinic acid, m.p. 78—79° [3] (from light petroleum), identical with authentic material.

(iii) *Isolation of hydroxylamine and* L-*prolinol* (III). The original acid hydrolysate of actinonin after ether extraction was evaporated to dryness; examination of the crystalline residue (1·03 g) of mixed hydrochlorides by paper chromatography (system EAW) indicated the presence of two ninhydrin-positive components at R_F 0·5 (purple) and 0·68 (yellow-pink). An aqueous solution of the residue was passed through a column of Amberlite CG 400 ion-exchange resin (HO^- form) and eluted with water. Evaporation of the acidified (hydrochloric acid) eluate gave a residual gum (513 mg) and trituration of this material with anhydrous chloroform gave hydroxylamine hydrochloride as insoluble crystals (186 mg, 98%), identified by the formation of a pink precipitate on treatment with nickel chloride–biacetyl mono-oxime [6] and by its conversion into *m*-nitrobenzaldoxime, m.p. 119—120°, identical with authentic material.[35] The chloroform solution was evaporated and crude L-prolinol hydrochloride was obtained as an oil (308 mg, 82%) which slowly crystallised to a deliquescent solid on prolonged drying *in vacuo*. Paper chromatographic examination (system EAW) of this material showed a yellow-pink spot at R_F 0·69 on development with ninhydrin. The crude hydrochloride (125 mg) was treated with aqueous sodium hydroxide (10% w/v; 2 ml) and benzoyl chloride (380 mg) and the mixture shaken at room temperature for 5 h, then extracted with ether. Evaporation of the extract gave a gum (110 mg) which was purified by column chromatography on silica and elution with benzene and chloroform. The chloroform eluate on evaporation yielded *NO*-dibenzoyl-L-prolinol as a viscous oil (93 mg), $[\alpha]_D^{24·5}$ −153° ($CHCl_3$) which could not be induced to crystallise after microdistillation at 130—150° and 2 × 10^{-4} mmHg. This material was identical with authentic NO-*dibenzoyl*-L-*prolinol* obtained similarly from L-prolinol [8] (Found: C, 73·3; H, 6·2; N, 4·5. $C_{19}H_{19}NO_3$ requires C, 73·8; H, 6·2; N, 4·5%), $[\alpha]_D^{21}$ −154° ($CHCl_3$), $\nu_{max.}$ 1730, 1625, 1610, and 1590 cm^{-1}. The L-prolinol isolated as hydrolysis product was further characterised by its conversion into the *N*-2,4-dinitrophenyl derivative, obtained as a yellow gum (92 mg), $[\alpha]_D^{22}$ −1140° ($CHCl_3$) {lit.,[8] $[\alpha]_D^{24}$ −1275° (MeOH)}. It was identical with authentic *N*-2,4-dinitrophenyl-L-prolinol obtained [28] in a similar manner from L-prolinol [8] as a yellow gum, $[\alpha]_D^{22}$ −1109°, which was purified by microdistillation at 130—150° and 2 × 10^{-4} mmHg (Found: C, 49·3; H, 5·1; N, 15·6. Calc. for $C_{11}H_{13}N_3O_5$: C, 49·4; H, 4·9; N, 15·7%).

(iv) *Isolation of* (+)-L-*valine* (II).—The Amberlite CG 400 ion-exchange resin employed in the separation of hydroxylamine and L-prolinol from the acid hydrolysate was subsequently eluted with N-hydrochloric acid to give L-valine hydrochloride (409 mg, 97%). The *N*-2,4-dinitrophenyl derivative [5] was obtained as yellow crystals (from ether–

[35] G. Ciamician and P. Silber, *Ber.*, 1903, **36**, 4266.

light petroleum), m.p. 130—131° (Found: C, 46·6; H, 4·8; N, 14·9. Calc. for $C_{11}H_{13}N_3O_6$: C, 46·6; H, 4·6; N, 14·8%), $[\alpha]_D^{24·5}$ −25° ($CHCl_3$), and was identical with authentic *N*-2,4-dinitrophenyl-L-valine, m.p. 131—132° (lit.,[5] 132°).

(b) *With* N-*hydrochloric acid.* A mixture of actinonin (500 mg) and N-hydrochloric acid (10 ml) was heated at 100° for 3 h. The oil which separated was extracted into ether (3 × 10 ml) and the combined extracts were evaporated. The residual oil was triturated with light petroleum and the crystalline solid (180 mg) which separated was crystallised from light petroleum to give prisms (130 mg, 53%) of (+)-D-pentylsuccinic acid, m.p. 79—80°.

Aqueous sodium hydroxide (50% w/v) was added to the acid hydrolysate and the alkaline solution then saturated with sodium chloride and continuously extracted with ether for 16 h. The extract was dried and evaporated giving crude L-valyl-L-prolinol (VI) (220 mg, 85%) as an oil. The *picrate* was obtained as yellow prisms (from methanol–ether), m.p. 193·5—194·5° (Found: C, 44·7; H, 5·5; N, 16·0. $C_{10}H_{20}N_2O_2$ requires C, 44·8; H, 5·4; N, 16·3%), identical with an authentic sample.[8]

Hydrolysis of Actinonin with Sodium Hydrogen Carbonate.—A mixture of actinonin (500 mg), methanol (5 ml), water (1 ml), and sodium hydrogen carbonate (110 mg, 1 equiv.) was heated under reflux for 4 h, then acidified with 2N-hydrochloric acid, saturated with sodium chloride, and extracted with ether. The extract gave a syrup (200 mg) which contained some actinonin (100 mg). The aqueous layer was made alkaline with 2N-sodium hydroxide and continuously extracted with ether for 20 h. The extract was dried and evaporated giving L-valyl-L-prolinol (55 mg, 21%) characterised as its picrate (see above).

Periodic Acid Oxidation of Actinonin. Formation of Actinonic Acid [36] (XVIII).—A suspension of actinonin (200 mg) in water (20 ml) was treated with aqueous periodic acid (1·5N; 5 ml) with stirring. The yellow solid which separated slowly dissolved and the solution was kept at room temperature overnight. The solution was saturated with sodium chloride and extracted with ether. The ethereal solution was thrice extracted with aqueous sodium hydrogen carbonate and the aqueous layer was acidified with 2N-hydrochloric acid, then saturated with sodium chloride and extracted with ether. The ethereal extract was evaporated to dryness to give actinonic acid as a gum (155 mg, 81%), *m/e* 370 (M^+, $C_{19}H_{34}N_2O_5$), $\nu_{max.}$ (film) 3350br, 2950, 1710, 1665, and 1610 cm^{-1}.

DL-2-(*Aminomethyl*)*heptanoic Acid* (VIII).—A mixture of 2-methyleneheptanoic acid [21] (12·5 g) and ammonium hydroxide (*d* 0·88; 250 ml) was heated in an autoclave at 160—170° for 24 h. After filtration and aeration the solution was evaporated to half volume and shaken with ether. The aqueous layer was further concentrated until the crude amino-acid separated as a cream solid (3·7 g, 26%). Crystallisation from water gave prisms (2·3 g) of DL-2-(*aminomethyl*)*heptanoic acid*, m.p. 224—225° (Found: C, 60·3; H, 10·7; N, 8·7. $C_8H_{17}NO_2$ requires C, 60·3; H, 10·8; N, 8·8%).

Lossen Degradation of Actinonin.—A stirred mixture of actinonin (1·04 g), methylketen diethyl acetal [19] (2 g), and anhydrous ether (30 ml) was heated under reflux for 30 h. The solution was evaporated and the oily residue treated with anhydrous benzene (30 ml) and heated under reflux for 3 h. Evaporation gave the crude isocyanate (XIV) as an oil (1·8 g), $\nu_{max.}$ (film) 2295 cm^{-1}. The isocyanate (XIV) was heated under reflux with dilute hydrochloric acid (6N; 80 ml) for 3 h. The solution was washed with ether and evaporated. Paper chromatography of the residue (1·23 g) (system EAW) indicated the presence of valine (R_F 0·5), prolinol (0·74), and a third ninhydrin-positive component (0·67, blue-violet). Prolinol hydrochloride was separated from this mixture by using Amberlite CG 400 ion-exchange resin (HO^- form) as described above. The remaining mixture of amino-acid hydrochlorides (0·68 g) was subjected to chromatography on thick paper sheets (Whatman 3MM) with the system EAW as developing solvent: zone separation of valine (R_F 0·54) and the second amino-acid (0·64) was detected by marginal application of ninhydrin. Elution of the zones with aqueous ethanol yielded valine (353 mg) and the second amino-acid (113 mg). The latter material after crystallisation from water and methanol gave DL-2-(aminomethyl)heptanoic acid (VIII) as prisms, m.p. 227—229° (Found: C, 60·0; H, 10·7; N, 8·7. Calc. for $C_8H_{17}NO_2$: C, 60·3; H, 10·8; N, 8·8%), identical with the material described above.

Pyrolysis of DL-2-(*Aminomethyl*)*heptanoic Acid.*—The acid (7·5 mg) obtained from the Lossen degradation of actinonin was heated at 250° in a slow stream of nitrogen. The condensate yielded 2-methyleneheptanoic acid (X) (3·7 mg), identical with authentic material.[21] In a similar manner, *trans*-oct-2-enoic acid [37] (XI) was obtained from 3-aminooctanoic acid [20] (IX).

Formation of the Dihydrouracils (XVa *and* b).—A mixture of actinonin (230 mg), methylketen diethyl acetal (1 ml), and *NN*-dimethylformamide (10 ml) was heated under reflux (3 h) and evaporated. The residue was dissolved in cyclohexane (5 ml) and the solution filtered and evaporated to give the dihydrouracil (XVa) as an oil (230 mg), $\nu_{max.}$ (film) 3360, 1722, 1680, 1640, 1160, 1055, and 958 cm^{-1}. This compound (XVa) was dissolved in aqueous methanol and the solution kept at room temperature for 3 days. Evaporation yielded (XVb) as a gum (190 mg), *m/e* 367 (M^+, $C_{19}H_{33}N_3O_4$), $\nu_{max.}$ (film) 3360, 1722, 1680, and 1640 cm^{-1}.

Dimethylactinonin (XVII).—A solution of actinonin (250 mg) in methanol (20 ml) was treated with an excess of diazomethane and kept at room temperature for 12 h. Addition of acetic acid and evaporation gave a residue which was dissolved in chloroform (10 ml) and kept at 0° for 1 h. Filtration and evaporation of the filtrate gave dimethylactinonin as an oil (250 mg), *m/e* 413 (M^+, $C_{21}H_{39}N_3O_5$), $\nu_{max.}$ 3450, 1660, 1630, and 1525 cm^{-1}.

DL-2-*Acetamido*-3-*methylbutyrohydroxamic Acid.*—A solution of *N*-acetyl-DL-valine [38] (1 g) in methanol (20 ml) was treated with an excess of ethereal diazomethane and the mixture was kept at room temperature for 0·5 h and filtered. The filtrate was evaporated and the crude methyl ester (1·2 g) was dissolved in methanol (10 ml). The solution was added to a filtered solution of hydroxylamine in methanol [previously prepared by mixing solutions of potassium hydroxide (0·56 g) in methanol (5 ml) and hydroxylamine hydrochloride (0·35 g) in methanol (5 ml)] and the mixture kept at 0° for 36 h. Evaporation gave a crude potassium salt which was converted into the hydroxamic acid by passage of its aqueous solution through a column of Amberlite CG 50 ion-exchange resin (CO_2^- form) and elution with water. Evaporation of the eluate, trituration of the residue with chloroform, and crystallisation from dioxan gave DL-2-*acetamido*-3-*methylbutyrohydroxamic acid* as plates, m.p. 155°

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(Found: C, 48·4; H, 8·1; N, 15·8%; Equiv., 174. $C_7H_{14}N_2O_3$ requires C, 48·3; H, 8·1; N, 16·1%; Equiv., 172). The compound gave an intense wine-red colouration with ethanolic iron(III) chloride.

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