867. *Angolide, a Naturally-occurring Cyclotetradepsipeptide with a Twelve-membered Ring*

By D. W. Russell

Angolide, $C_{22}H_{38}N_2O_6$, isolated from *Pithomyces* IMI 101184, yields L-α-hydroxy-β-methylbutyric acid and an equimolar mixture of L-isoleucine and D-*allo*isoleucine on vigorous acid hydrolysis. Mild treatment with hydrazine gives L-α-hydroxy-β-methylbutyryl-D-*allo*isoleucylhydrazine (IV) and L-α-hydroxy-β-methylbutyryl-L-isoleucylhydrazine (V). Angolide accordingly has structure (I).

A new spore-surface depsipeptide, angolide, was isolated from dried felts of a species of *Pithomyces*, IMI 101184.[1,2] Angolide was a crystalline, neutral, optically active compound with the empirical formula $C_{11}H_{19}NO_3$. Its infrared spectrum ($\nu_{max.}$ 3300, 1750, 1670, and 1545 cm.$^{-1}$) was very similar to the spectra of amidomycin [3] and valinomycin.[4] These

[1] D. W. Russell and V. Ward, *Abstr. 1st Meeting Fed. European Biochem. Soc.*, 1964, no. A 97.
[2] E. Bishop, H. Griffiths, D. W. Russell, V. Ward, and R. N. Gartside, *J. Gen. Microbiol.*, 1965, **38**, 289.
[3] L. C. Vining and W. A. Taber, *Canad. J. Chem.*, 1957, **35**, 1109.
[4] H. Brockmann and H. Geeren, *Annalen*, 1957, **603**, 216.

are cyclodepsipeptides containing alternate α-amino- and α-hydroxy-acid residues. In particular, all three compounds had only one resolved amide I band in the infrared spectrum. The spectra of sporidesmolides I [5] and III,[6] cyclodepsipeptides containing hydroxyacyldipeptide sequences, have two well-resolved bands in this region.

Vigorous acid hydrolysis of angolide yielded, in the ether-soluble fraction, L-α-hydroxy-β-methylbutyric acid in an overall yield of 0·74 mol., and no other ether-soluble acid was revealed by paper chromatography. The ether-insoluble portion of the hydrolysate gave a positive ninhydrin reaction. Chromatography on paper in several solvent systems revealed the presence of a single amino-acid corresponding in position to isoleucine–*allo*isoleucine, and in a system which separated diastereoisomeric pairs of amino-acids,[7] both isoleucine and *allo*isoleucine were shown to be present in about equal amount. The amino-acid, $C_6H_{13}NO_2$, isolated from the hydrolysate, was weakly dextrorotatory in 6N-hydrochloric acid and in glacial acetic acid, suggesting that it was an equimolar mixture of the epimers, L-isoleucine and D-*allo*isoleucine.[8] This was confirmed by ninhydrin oxidation of the amino-acid which yielded (+)-α-methylbutyraldehyde, as the 2,4-dinitrophenylhydrazone.[9]

Erroneous results have previously been obtained for the molecular weights of cyclodepsipeptides.[10] The molecular weight of angolide was determined by the Kofler method in two different solvents (Dr. F. Pascher), by the thermoelectric method in trifluoroacetic acid (Dr. M. Yu. Feigina) and, most convincingly, by mass spectrometry (Dr. J. S. Shannon). The last method gave the molecular weight as 426, and results obtained by the other methods agreed closely with this figure. Angolide therefore possessed the molecular formula $C_{22}H_{38}N_2O_6$.

Angolide was accordingly a cyclotetradepsipeptide, for which three structures were possible. Of these, (I) has a regular alternate arrangement of amino- and hydroxy-acid residues usually found in naturally-occurring cyclodepsipeptides. The other structures consistent with the results of degradation so far described, namely (II) and (III), have non-alternating arrangements. A decision in favour of (I) was made as follows. Angolide treated with hydrazine under mild conditions gave a mixture of two acyl hydrazides, separable by thin-layer chromatography.[11] On paper electrophoresis at pH 2 the mixture gave a *single* band, the mobility of which, compared with the mobilities of acyl hydrazides of known structures,[11] suggested that the molecular weight of both the products was *ca.* 256.

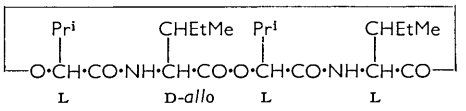
These results were consistent with structure (I) for angolide, which would give with hydrazine a mixture of diastereoisomers, (IV) and (V) for which $M = 245$. On the other hand, hydrazine with (II) or (III) would give α-hydroxy-β-methylbutyrylhydrazine (VIII) and an hydroxyacyldipeptide hydrazide (VI) or (VII). Paper electrophoretic examination of the reaction product of angolide with hydrazine showed that (VIII) was absent.

Methyl sporidesmolate B,[5] treated with hydrazine in the same way, yielded a hydrazide, which moved considerably more slowly on paper electrophoresis than the mixture of hydrazides from angolide. Since the hydrazide of sporidesmolic acid B is isomeric with (VI) and (VII), this was additional evidence for the absence from angolide of a hydroxyacyldipeptide sequence.

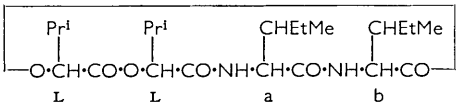
It therefore appeared that the action of hydrazine on angolide gave the diastereoisomeric hydrazides, (IV) and (V). When the mixture was crystallised from ethanol, the crystals

[5] D. W. Russell, *J.*, 1962, 753.
[6] D. W. Russell, C. G. Macdonald, and J. S. Shannon, *Tetrahedron Letters*, 1964, 2759.
[7] D. O. Gray, J. Blake, D. H. Brown, and L. Fowden, *J. Chromatog.*, 1964, **13**, 276.
[8] J. P. Greenstein and M. Winitz, " Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, 1961, vol. III, p. 2044.
[9] W. S. Fones, *J. Amer. Chem. Soc.*, 1954, **76**, 1377.
[10] Yu. A. Ovchinnikov, V. T. Ivanov, A. A. Kiryushkin, and M. M. Shemyakin, in " Peptides," ed. G. T. Young, Pergamon Press, London, 1963, pp. 207 *et seq.*
[11] D. W. Russell, *J. Chromatog.*, 1965, **18**, in the press.

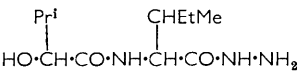
separating were shown by paper chromatography of an acid hydrolysate to contain *allo*-isoleucine but only traces of isoleucine. The more soluble fraction, hydrolysed by acid, gave mainly isoleucine together with some *allo*isoleucine. These results, together with those of hydrazinolysis, excluded structures (II) and (III) and confirmed that angolide had the regular structure (I).



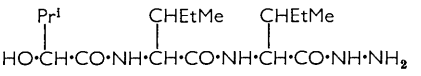
(I)



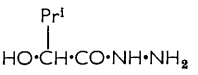
(II); a = D-*allo*, b = L
(III); a = L, b = D-*allo*



(IV); a = D-*allo*
(V); a = L



(VI); a = D-*allo*, b = L
(VII); a = L, b = D-*allo*



(VIII)

These results were communicated to Dr. J. S. Shannon, who has reported the results of a mass-spectrometric investigation of angolide.[12] Professor M.M. Shemyakin was also informed of the structure inferred from the degradative work, and accordingly he and his colleagues synthesised a compound with structure (I) which proved to be identical with natural angolide.[13] Further degradative evidence was therefore superfluous. However, the two diastereoisomeric hydrazides (IV) and (V) were subsequently separated by fractional crystallisation as described in the Experimental section.

Angolide is the first cyclodepsipeptide with a twelve-membered ring to be isolated from a natural source. The cyclotetradepsipeptide serratamolide has 14 ring atoms,[14] and the enniatins, formerly thought to possess a twelve-membered ring system, have been shown to possess rings of 18 atoms.[15] Angolide also appears to be the first example of a naturally-occurring compound which contains residues of both L-isoleucine and D-*allo*isoleucine. These isomers are sterically related to each other as are L-valine and D-valine, one residue of each of which is present in sporidesmolide I.[5] In the latter case, both residues are derived *in vivo* to the same extent from exogenous L-valine.[16] A similar situation obtains in the actinomycins[17] and valinomycin.[18] A possible, common explanation is that the " activation " of the L-amino-acids, necessary for the formation of an ester or amide bond, is accompanied by racemisation, a reaction for which there are analogies in peptide chemistry.[19]

Experimental

Melting points were determined on a Kofler hot-stage apparatus. Infrared spectra were measured for paraffin mulls using a Perkin-Elmer Infracord spectrophotometer. Microanalyses and cryoscopic molecular weight determinations were by Dr. F. Pascher, Bonn.

[12] C. G. Macdonald and J. S. Shannon, *Tetrahedron Letters*, 1964, 3113.
[13] A. A. Kiryushkin, Yu. A. Ovchinnikov, and M. M. Shemyakin, *Tetrahedron Letters*, 1964, 3313.
[14] H. H. Wasserman, J. J. Keggi, and J. E. McKeon, *J. Amer. Chem. Soc.*, 1962, **84**, 2978.
[15] M. M. Shemyakin, Yu. A. Ovchinnikov, V. T. Ivanov, and A. A. Kiryushkin, *Tetrahedron*, 1963, **19**, 581; P. Quitt, R. O. Studer, and K. Vogler, *Helv. Chim. Acta*, 1963, **46**, 1715; Pl. A. Plattner, K. Vogler, R. O. Studer, P. Quitt, and W. Keller-Schierlein, *ibid.*, p. 927.
[16] G. W. Butler, D. W. Russell, and R. T. J. Clarke, *Biochim. Biophys. Acta*, 1962, **58**, 507.
[17] L. A. Saltzman, E. Katz, and H. Weissbach, *J. Biol. Chem.*, 1964, **239**, 1864.
[18] J. C. Macdonald, *Canad. J. Microbiol.*, 1960, **6**, 27.
[19] M. W. Williams and G. T. Young, *J.*, 1963, 881.

Angolide (I) *from* Pithomyces *species*, IMI 101184.—Dried sporing felts [2] were coarsely powdered in the fume cupboard. The powder (45 g.) was extracted with light petroleum (b. p. 60—80°; 450 ml.) by stirring for 5 min. at room temperature and filtering. After three such extractions the tissue was similarly extracted with chloroform (3 × 450 ml.) and the filtered chloroform extracts were evaporated *in vacuo* at 50°. The brown residue (0·873 g.) was washed with diethyl ether to give a buff solid (0·392 g.) that, crystallised twice from ethanol (120 ml. per g.), yielded colourless needles of *angolide* (I; 0·303 g.) m. p. 261—262° (decomp.), unaltered by further recrystallisation from ethanol, chloroform, or acetic acid, $[\alpha]_D$ −83° (*c*, 1 in chloroform); $\nu_{max.}$ 3330s (N–H), 1750s (ester C=O), 1670s (amide I), and 1575s (amide II) cm.$^{-1}$ [Found: C, 62·0, 62·1; H, 8·6, 9·1; N, 6·6, 6·55; O, 22·9, 22·55%; *M*, 443 (cryoscopic in camphor), 445 (cryoscopic in naphthalene), 413, 422 (thermoelectric),[13] 426 (mass spectrometric).[12,13] $C_{22}H_{38}N_2O_6$ requires: C, 61·9; H, 9·0; N, 6·6; O, 22·5%; *M*, 426].

Angolide was very insoluble in water, moderately soluble in chloroform, and sparingly soluble in other common organic solvents. Heated above 200° in an open capillary it slowly sublimed. It was not extracted from chloroform by aqueous mineral acid or alkali nor by 70% (v/v) methanol, and it gave no colour reaction with ninhydrin nor with indicators.

Acid Hydrolysis of Angolide.—The depsipeptide (I; 0·386 g.) was dissolved in boiling ethanol (90 ml.), the solution cooled and treated with N-sodium hydroxide (10 ml.). At intervals, portions were titrated to phenolphthalein with N-hydrochloric acid (control titrations were performed) (Found: alkali consumed after 30 min., 1·34; 60 min., 1·61; 105 min., 1·63; 200 min., 1·74 mequiv. Rupture of two ester bonds of $C_{22}H_{38}N_2O_6$ requires 1·81 mequiv.). The solutions after titration were combined and evaporated to dryness *in vacuo*. The residue was boiled with 6N-hydrochloric acid (150 ml.) for 48 hr., and the volume of the resulting solution was reduced by distillation to *ca.* 30 ml. Water (70 ml.) was added and the solution was continuously extracted with diethyl ether for 6 hr. The extract, dried (Na_2SO_4) and evaporated, furnished a crystalline residue (0·220 g., m. p. *ca* 60°) that gave after two vacuum-sublimations at 60° L-α-hydroxy-β-methylbutyric acid (0·158 g.), m. p. and mixed m. p. 65—66°, $[\alpha]_D$ −19·1° (*c*, 1·4 in chloroform). The infrared spectrum was identical with that of the reference sample. On paper chromatography in the system t-butyl alcohol–4·25N-ammonia solution (4 : 1) the crude and purified isolated acids and the reference sample gave spots [5] that had the same R_F values and that stained identically with a permanganate-indicator spray reagent.[20] The cyclohexylammonium salt,[5] $[\alpha]_D$ −7·2° (*c*, 5 in water), had m. p. 140—141° (Found: C, 60·9; H, 10·3. Calc. for $C_{11}H_{23}NO_3$: C, 60·8; H, 10·7%).

The ether-extracted hydrolysate, examined by paper chromatography, gave a single ninhydrin-reacting spot in all four solvent systems used for examining similar hydrolysates of sporidesmolide I,[5] and in each case the R_F and staining characteristics were identical with those of authentic isoleucine or *allo*isoleucine chromatographed on the same paper. In t-amyl alcohol–acetic acid–water (20 : 1 : 20)[7] two spots of approximately equal intensity, corresponding respectively to isoleucine and *allo*isoleucine, were obtained. The total amino-acid present was determined by 2,4,6-trinitrobenzenesulphonic acid, L-isoleucine being used as standard [21] (Found: 0·426 g. of angolide on hydrolysis gave 1·92 mequiv. of amino-acids).

The acid solution of the amino-acids was evaporated to dryness *in vacuo*; the residue was dissolved in a little water and the amino-acids were adsorbed on a short column of Amberlite CG 120 (H form). The column was washed with water until the effluent was neutral (litmus) and then with N-ammonia solution. The eluted amino-acids {0·206 g., $[\alpha]_D$ + 1·0° (*c*, 1 in 6N-hydrochloric acid) and +3·4° (*c*, 2 in acetic acid)} were crystallised once from water by addition of ethanol (Found: C, 54·3; H, 9·8; N, 11·0. Calc. for $C_6H_{13}NO_2$: C, 54·9; H, 10·0; N, 10·7%). An authentic epimeric mixture of L-isoleucine and D-*allo*isoleucine had $[\alpha]_D$ +1·1° (*c*, 2 in 6N-hydrochloric acid) and +3·0° (*c*, 2 in acetic acid.)

Oxidation of Amino-acids from Angolide.—The amino-acids (0·086 g.) were dissolved in water (7 ml.), the solution was boiled, and ninhydrin (0·5 g.) in water (7 ml.) was added. The mixture was steam-distilled into a solution of 2,4-dinitrophenylhydrazine (0·12 g.) in 2N-hydrochloric acid (100 ml.). The precipitate {0·115 g., $[\alpha]_D$ +28·3° (*c*, 2·3 in chloroform)} was crystallised from ethanol to give (+)-α-methylbutyraldehyde 2,4-dinitrophenylhydrazone (0·094 g.), m. p. 135—136°, $[\alpha]_D$ +33·0° (*c*, 1·7 in chloroform). An authentic sample, similarly prepared

[20] J. Pásková and V. Munk, *J. Chromatog.*, 1960, **4**, 241.
[21] K. Satake, T. Okuyama, M. Ohashi, and T. Shinoda, *J. Biochem. Japan*, 1960, **47**, 654.

from an epimeric mixture of L-isoleucine and D-*allo*isoleucine, had m. p. 134—135°.[9] The mixed m. p. was 134—135°, and the infrared spectra of the two samples were identical.

Mild Hydrazinolysis of Angolide.—The depsipeptide (I; 0·408 g.) was boiled gently with ethanol (9 ml.) and hydrazine hydrate (1 ml.) for 30 min. The solution was evaporated to dryness *in vacuo* at 40° and the residue dried *in vacuo* (H_2SO_4) to constant weight. The product (0·466 g.; addition of 2 mol. of hydrazine to $C_{22}H_{38}N_2O_6$ requires 0·469 g.) was examined by paper electrophoresis at pH 2.[11] Crude hydrazides of sporidesmolic acids A and B[5] and of α-hydroxy-β-methylbutyric acid, similarly prepared from the methyl esters, were run on the same paper. The angolide reaction product gave a single band (mobility = 1·00). The other three hydrazides each gave a single band (mobilities 0·88, 0·85, and 1·47, respectively). Bands corresponding in position to sporidesmolic acid A and B hydrazides were also given by the crude hydrazinolysis product of sporidesmolide I, prepared similarly to that of angolide. By graphical interpolation on a curve relating molecular weight to electrophoretic mobility for a number of acyl hydrazides,[11] the molecular weight of the angolide hydrazinolysis product was found to be 256 ($C_{11}H_{23}N_3O_3$ requires 245).

The total reaction product of angolide with hydrazine gave two spots on thin-layer chromatograms.[11] It was crystallised from ethanol. A small portion of the crystalline product (0·177 g.) was hydrolysed in 6N-hydrochloric acid for 24 hr.; the hydrolysate was evaporated to dryness and chromatographed on paper in t-amyl alcohol–acetic acid–water 20 : 1 : 20.[7] Ninhydrin revealed a strong spot of *allo*isoleucine and a very weak spot of isoleucine. A similar hydrolysate of the material remaining in the mother-liquor contained mainly isoleucine with a small amount of *allo*isoleucine. The crystalline product was further crystallised, once from ethyl alcohol and once from isopropyl alcohol, to give L-α-*hydroxy*-β-*methylbutyryl*-D-allo*isoleucylhydrazine* [(IV); 0·099 g.], m. p. 222—223° (Found: C, 53·7; H, 9·15; N, 17·1; O, 19·7. $C_{11}H_{23}N_3O_3$ requires C, 53·9; H, 9·45; N, 17·1; O, 19·6%). This compound on thin-layer chromatograms gave a single spot corresponding to the faster of the two spots given by the total hydrazinolysis product. An acid hydrolysate prepared as described above contained *allo*isoleucine but no detectable amount of isoleucine, and α-hydroxy-β-methylbutyric acid was detected on paper chromatograms prepared in t-butyl alcohol–4·25N-ammonia solution (4 : 1).[3]

In a second experiment, angolide (I; 0·650 g.) was boiled with ethanol (18 ml.) and hydrazine hydrate (2 ml.) for 2 min. and the clear solution set aside at room temperature for 64 hr. The precipitate (0·147 g.; m. p. 219—222°) of almost pure (IV) was removed. The material in the mother-liquor was fractionally crystallised from ethanol to yield fractions (total 0·177 g.) which gave a single spot on thin-layer chromatograms, corresponding to the slower of the two spots given by the total hydrazinolysis product. This material, thrice crystallised from isopropyl alcohol, gave L-α-*hydroxy*-β-*methylbutyryl*-L-*isoleucylhydrazine* (V; 0·0255 g.), m. p. 188—189° (Found: N, 16·7. $C_{11}H_{23}N_3O_3$ requires N, 17·1%). This compound was very much more soluble than its diastereoisomer (IV) in ethanol. An acid hydrolysate, examined by paper chromatography, contained α-hydroxy-β-methylbutyric acid and isoleucine, but no detectable amount of *allo*isoleucine. A mixture of (IV) and (V) in equal amount was indistinguishable from the total hydrazinolysis product from angolide by paper electrophoresis and thin layer and paper chromatography.[11]

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