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1078. *Chemistry of Fungi. Part I. Pithomycolide, A Novel Depsipeptide from* Pithomyces chartarum.

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A minor fraction of the metabolites of *Pithomyces chartarum* has been shown to contain a new depsipeptide, pithomycolide, possessing the structure (III). Pithomycolide is unusual in having a seventeen-membered macrocyclic ring and aromatic hydroxy-acid residues.

The pasture fungus *Pithomyces chartarum* (Berk. and Curt.) M. B. Ellis (= *Sporidesmium bakeri* Sydow) causes facial eczema in sheep in New Zealand, which frequently results in severe economic loss. By systematic fractionation[1] of the metabolic products of the cultured fungus, the toxic compounds sporidesmin and sporidesmin B have been isolated, to which the respective structures (I; R = OH) and (I; R = H) have been assigned.[2,3] The chief non-toxic metabolite is the cyclohexadepsipeptide, sporidesmolide I (II; R = Pr^i),[4] which is accompanied by small proportions of sporidesmolide II (II; R = Bu^s)[5] and sporidesmolide III.[4] During the chromatography of fractions containing sporidesmin B,[1b,d] a crystalline fraction, m. p. 242—244°, named "Compound 242," was isolated. Its properties resembled those of the sporidesmolides, but it was considerably less polar, and gave alanine on acid hydrolysis. "Compound 242" was generously made available to us by Dr. E. P. White, of the Ruakura Animal Research Station, Hamilton, New Zealand.

"Compound 242" was inhomogeneous, acid hydrolysis giving alanine, *N*-methylalanine, valine, leucine, and *N*-methyl-leucine, detected by paper chromatography. The last three

[1] (*a*) Synge and White, *Chem. and Ind.*, 1959, 1546; (*b*) Synge and White, *New Zealand J. Agric. Res.*, 1960, **3**, 907; (*c*) Done, Mortimer, Taylor, and Russell, *J. Gen. Microbiol.*, 1961, **26**, 207; (*d*) Ronaldson, Taylor, White, and Abraham, *J.*, 1963, 3172.

[2] Fridrichsons and Mathieson, *Tetrahedron Letters*, 1962, 1265.

[3] Hodges, Ronaldson, Taylor, and White, *Chem. and Ind.*, 1963, 42.

[4] (*a*) Russell, *J.*, 1962, 753; (*b*) Shemyakin, Ovchinnikov, Ivanov, and Kiryushkin, *Izvest. Akad. Nauk S.S.S.R., Otdel. Khim. Nauk*, 1962, 1699; *Tetrahedron*, 1963, **19**, 995.

[5] Taylor and Shannon, unpublished work; Shemyakin, Ovchinnikov, Ivanov, and Kiryushkin, *Tetrahedron Letters*, 1963, 1927.

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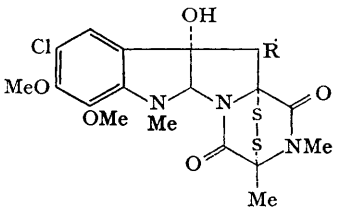
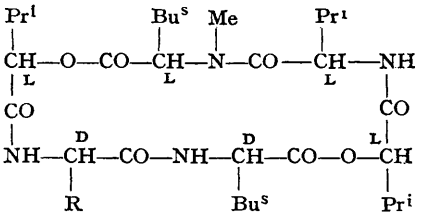
amino-acids are probably derived from sporidesmolide I (II; R = Pr^{i}), as this compound has been separated from "Compound 242" by sublimation.[6] A new depsipeptide, pithomycolide, was finally obtained by fractional crystallisation of "Compound 242". It gave only two amino-acids, L-alanine and *N*-methyl-L-alanine, on hydrolysis.



(I)



(II)

Combustion analyses of pithomycolide and an *X*-ray-crystallographic molecular-weight determination (*M*, 535 ± 20) did not conclusively distinguish between the formulae $C_{29}H_{34}N_2O_8$ (538·6) and $C_{30}H_{36}N_2O_8$ (552·6), but the latter was established from the mass spectrum (molecular ion peak at *m/e* 552) and from the integral of the nuclear magnetic resonance (n.m.r.) spectrum. Kuhn–Roth determination, using a method applicable in the presence of aromatic rings,[7] disclosed 2·5 *C*-Me groups, whilst 1 *N*-Me group was revealed in the Herzig–Meyer estimation. One residue each of alanine and *N*-methylalanine in pithomycolide accounts for 2 *C*-Me groups, the *N*-Me group, and both nitrogen atoms. Pithomycolide was neutral and unreactive towards phenolic and carbonyl reagents. Since it gave negative ninhydrin and Dakin–West reactions neither amino-acid is *C*- or *N*-terminal. Pithomycolide was recovered unchanged after treatment with acetic anhydride and pyridine at room temperature, showing the absence of primary and secondary alcoholic groups. The presence of a sterically hindered secondary amide group in pithomycolide was inferred from the infrared spectrum (N–H stretching at 3356 cm.$^{-1}$) and its failure to react with t-butyl hypochlorite.[8] Infrared peaks at 1669, 1642, and 1534 cm.$^{-1}$ were assigned to secondary and tertiary amide groups, whilst peaks at 1739 and 1718 cm.$^{-1}$ were assigned to ester groups, three of which are required by the molecular formula. The ultraviolet spectrum showed the presence of aromatic functions (ε_{220} 13,000 and plateau ε_{275} 835, cf. ref. 9) and bands in the infrared spectrum at 755 and 700 cm.$^{-1}$ suggested the presence of phenyl rings. Peaks at 1379 and 1372 cm.$^{-1}$ were assigned to a *gem*-dimethyl group. The rotatory dispersion of pithomycolide was negative and plain to 250 mμ.

The products of total acid hydrolysis of pithomycolide were partitioned between water and ether, the amino-acids remaining in the aqueous phase, in which alanine and *N*-methylalanine were detected by paper chromatography. The occurrence of alanine in depsipeptides has hitherto been limited to isariin,[10] whilst *N*-methylalanine appears to have been detected only rarely in natural products, in actinomycin Z,[11] actinomycin H,[12] and destruxin B.[13] The L-configurations of these amino-acids were determined by enzymic oxidation on paper chromatograms,[14] using stereospecific hog kidney (D-) and snake venom (L-)amino-acid oxidases.[15]

[6] Dr. E. P. White, private communication.
[7] Brandenburger, Maas, and Dvoretzky, *Analyt. Chem.*, 1961, **33**, 453.
[8] Mazur, Ellis, and Cammarata, *J. Biol. Chem.*, 1962, **237**, 1619.
[9] Schröder and Lübke, *Experientia*, 1963, **19**, 57.
[10] Vining and Taber, *Canad. J. Chem.*, 1962, **40**, 1579.
[11] Bossi, Hütter, Keller-Schierlein, Neipp, and Zähner, *Helv. Chim. Acta*, 1958, **41**, 1645.
[12] Brockmann, Manegold, and Frommer, G. P. 1,060,091/1959; (*Chem. Abs.*, 1961, **55**, 8774).
[13] Tamura, Kuyama, Kodaira, and Higashikawa, *Agric. Biol. Chem. (Tokyo)*, 1964, **28**, 137.
[14] Auclair and Patton, *Rev. Canad. Biol.*, 1950, **9**, 3; (*Chem. Abs.*, 1950, **44**, 6975); cf. Bonetti and Dent, *Biochem. J.*, 1954, **57**, 77.
[15] Greenstein and Winitz, "Chemistry of the Amino Acids," Wiley, New York, 1961, Vol. 2, pp. 1782—1793.

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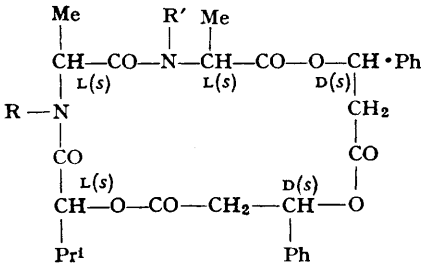
The ethereal layer, on paper-strip chromatography, disclosed only one acidic component, cinnamic acid, isolated in a yield of 1·52 moles per mole of pithomycolide. Pithomycolide was rapidly hydrolysed in dilute alkaline solution. Treatment with N-potassium hydroxide solution at room temperature gave cinnamic acid (0·7—1·0 mole) and (−)-β-hydroxy-β-phenylpropionic acid (0·4—0·7 mole), known to possess the D-(*S*)-configuration.[16] The known natural occurrence of this acid has hitherto been restricted to the plant *Lobelia inflata* where the same enantiomer occurs,[17] whilst a similar L-(*R*)-residue is incorporated in certain of the *Lobelia* alkaloids.[18] Since β-hydroxy-β-phenylpropionic acid is known to give cinnamic acid by dehydration under acid and alkaline conditions (also confirmed in this case under the same conditions as those used in the hydrolyses), and taking into account the combined yields of cinnamic and β-hydroxy-β-phenylpropionic acids, the presence of two residues of the latter acid in pithomycolide is inferred.

From a further total acid hydrolysis, the remaining component of pithomycolide was shown to be L-α-hydroxyisovaleric acid by paper chromatography and by formation of the cyclohexylammonium salt. The remaining 0·5 *C*-Me group revealed in the Kuhn–Roth determination arises from the isopropyl group of this acid. L-α-Hydroxyisovaleric acid occurs in the sporidesmolides,[4a] but has not been obtained from other natural sources.

The incorporation of one residue each of alanine, *N*-methylalanine, and α-hydroxyisovaleric acid, and two of β-hydroxy-β-phenylpropionic acid into a cyclodepsipeptide with the loss of five molecules of water is consistent with the molecular formula of pithomycolide and the properties described above.

We may now consider the order of the five residues in the macrocyclic ring. Since up to 1·4 moles of aromatic acids were obtained on mild alkaline hydrolysis of pithomycolide, all three ester links, and hence all three hydroxy-acid residues, are adjacent in the ring. It also follows that the two amino-acids are joined together. Moreover, since each aromatic hydroxy-acid must be flanked on either side by ester links, the α-hydroxyisovaleric acid must be linked through its carboxyl group to the amino-group of one of the amino-acids. Therefore pithomycolide must be represented by structure (III) or (IV).



(III): R = Me, R′ = H
(IV): R = H, R′ = Me.

Reduction of pithomycolide with sodium borohydride or lithium borohydride, which reduce ester but not amide groups,[19,20] followed by total acid hydrolysis gave *N*-methylalanine, very small quantities of alanine, and 2-aminopropan-1-ol. This shows that the carboxyl group of alanine must be esterified with a hydroxy-acid, and its amino-group linked to *N*-methylalanine. Hence pithomycolide has the structure and absolute configuration (III).

The n.m.r. spectrum of pithomycolide (see Table) is not only consistent with this structure but, in conjunction with spin decoupling, enables signals to be assigned to every proton.

[16] Lukeš, Bláha, and Kovár, *Chem. and Ind.*, 1958, 527; Schöpf and Wüst, *Annalen*, 1959, **626**, 150.
[17] Wieland, Koschara, Dane, Renz, Schwarze, and Linde, *Annalen*, 1939, **540**, 103.
[18] Schöpf, Kauffmann, Berth, Bundschuh, Dummer, Fett, Habermehl, Wieters, and Wüst, *Annalen*, 1957, **608**, 88.
[19] Bailey, *Biochem. J.*, 1955, **60**, 170.
[20] Keller-Schierlein, Mihailović, and Prelog, *Helv. Chim. Acta*, 1959, **42**, 305.

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Nuclear magnetic resonance spectrum

	Chemical shift (δ, p.p.m.)	Multiplicity*	J(c./sec.)	No. of protons	Assignment
A	7·94†	d	—	1	–N*H*
B	7·34	s	—	5	$-C_6H_5$
C	7·30	s	—	5	$-C_6H_5$
D	6·28	m	—	2	Ph–C*H*–
E	4·74	m	—	3	>C*H*—CO of aliphatic acid
F	2·98	m	—	4	Ph–CH–CH_2–CO
G	2·82	s	—	3	*N*–Me
H	2·19	m	—	1	Me>C*H*–
I	1·47	d	7·4	6	*C*–Me of amino-acids
J	1·11	d	7·0	3	Non-equivalent methyls of Pr^i group
K	0·99	d	7·0	3	(This phenomenon somewhat temperature-dependent)
			TOTAL	36	

Spin decoupling showed that the following protons are coupled: A with one of E, I with two of E, H with J and K, H with one of E, and D with F.

* s, singlet; d, doublet; m, multiplet. † Not exchanged on equilibration with D_2O.

Pithomycolide is unusual in having a 17-membered ring in contrast with the 18-membered-ring sporidesmolides.[21] Examination of a Courtauld model of pithomycolide reveals severe hindrance of the isopropyl and secondary amide groups as well as restricted rotation and different steric environment of the aromatic rings, effects which are reflected in the n.m.r. spectrum. Further, the benzylic protons lie near the planes of the aromatic rings and are thus more deshielded than in the free acid, Ph•CH(OH)•CH_2•CO_2H, δ = 5·15 (cf. the paracyclophanes).[22]

Pithomycolide was hydrogenolysed comparatively slowly over palladium–charcoal to give a mixture of acidic products, shown by paper chromatography to contain hydrocinnamic acid in low yield and a gummy acid as the major product. Hydrocinnamic acid could have arisen only by the hydrogenolysis of two adjacent benzylic ester functions. After Dakin–West degradation of the gummy acid and subsequent acid hydrolysis, alanine was shown by paper chromatography to be present only in traces compared with *N*-methylalanine. Hence, although there are two benzylic ester links in pithomycolide, hydrogenolysis takes place predominantly at the ester link of the alanine residue. The Courtauld model shows that both benzyl–oxygen bonds are severely hindered, with the bond nearer the alanine residue more accessible. This experiment also confirms the order of the amino-acids.

Hydrogenolysis with sodium borohydride–boron trifluoride,[23] which is little affected by steric factors, gave cinnamic acid rather than hydrocinnamic acid, in accord with the ready dehydration of benzhydrol derivatives by the reagent.

On treatment with 1 mole of 0·0076N-potassium hydroxide solution at room temperature for 5 hr., pithomycolide was almost quantitatively hydrolysed to give a gummy acid from which both amino-acids were obtained on total acid hydrolysis. After Dakin–West degradation of this material, followed by total acid hydrolysis, alanine was shown by paper-strip chromatography to be present only in traces compared with *N*-methylalanine. This hydrolysis, like the catalytic hydrogenolysis, therefore takes place predominantly at the alanine ester group.

Although pithomycolide is formally related to the sporidesmolides in its possession of a hydroxyacyldipeptide unit, its structure suggests a different biogenesis. It is likely that the

[21] Cf. Dale, *J.*, 1963, 93.
[22] Waugh and Fessenden, *J. Amer. Chem. Soc.*, 1957, **79**, 846.
[23] Pettit, Green, Hofer, Ayres, and Pauwels, *Proc. Chem. Soc.*, 1962, 357.

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peptide portion has a common origin with the dioxopiperazine ring of sporidesmin (I; R = OH) and sporidesmin B (I; R = H). This supposition is strengthened by the conversion of hydroxy-*N*-acyl lactams into cyclodepsipeptides by way of cyclol intermediates.[24] The L-α-hydroxyisovaleric acid residue undoubtedly has the same origin as those of the sporidesmolides, and the β-hydroxy-β-phenylpropionic acid may arise from phenyl pyruvate, which is known[25] to be the precursor of naturally occurring cinnamyl alcohols.

Finally, the pithomycolide model shows that all the carbonyl oxygen atoms of the ester and amide groups are on one side and the non-polar side-chains on the other, a fact which may be of biological significance.

Experimental

Microanalyses were by Dr. A. D. Campbell and associates, University of Otago, New Zealand. Ultraviolet and infrared spectra were measured with Perkin–Elmer 137 UV and Infracord instruments, respectively. N.m.r. spectra were measured in deuterochloroform solutions on a Varian V-4300B spectrometer operating at 60 Mc./sec. Major peaks in the spectra were calibrated with respect to tetramethylsilane as internal reference by the audiofrequency side-band method. Spin-decoupling experiments were carried out using a lock-in detector, and the spin-decoupled spectra recorded using a fixed 600 c./sec. side-band.

Paper Chromatography.—Four solvent systems were used: (A) butan-1-ol–acetic acid–water (70:30:11);[26] (B) phenol saturated with water (HCN atmosphere);[27] (C) t-butyl alcohol–4·25N-aqueous ammonia (4:1);[28] (D) butan-1-ol–formic acid–water (10:1:5).[29] Whatman's No. 1 paper was used. Amino-acids were detected with ninhydrin (0·25% solution in butan-1-ol) followed by heating (cf. ref. 30) and carboxylic acids with a permanganate-indicator reagent.[31]

"*Compound* 242."—"Compound 242" was obtained [16,d] as stout, friable rods, which softened above 220° and melted at 235—244°. A mixed melting point with the beaker test substance[4] (sporidesmolides I, II, and III) was undepressed. Acid hydrolysis, performed as described below, gave rise to alanine, *N*-methylalanine, valine, leucine, and *N*-methyl-leucine, but no isoleucine, as shown by paper chromatography.

Pithomycolide from "Compound 242".—"Compound 242" (1·56 g.) was heated to boiling with methanol (90 ml.), chloroform added to complete solution, and the solution set aside at 20°. During 8—10 days stout crystals were deposited, which were crystallized repeatedly from chloroform–methanol and finally from benzene–hexane to give *pithomycolide* as rods, m. p. 242—244° (decomp.) [Found, for sample dried to constant weight at 100° *in vacuo* over P_2O_5: (from methanol–chloroform) C, 64·25, 65·9, 64·7; H, 6·7, 6·9, 6·7; N, 4·65, 5·2; O, 24·2, 22·7, 23·6; (from methanol–dichloromethane) C, 64·7; H, 6·9; N, 5·2; O, 23·9; (from benzene–hexane) C, 65·5; H, 6·8; N, 5·4; O, 23·4; *N*-Me, 2·5; *C*-Me, 7·1%; *M* (mass spectrometer), 552; (crystallographic) 535 ± 20. $C_{30}H_{36}N_2O_8$ requires C, 65·2; H, 6·6; N, 5·1; O, 23·2; 1 *N*-Me, 2·7; 2 *C*-Me, 5·4%; *M*, 552·6]. The mass spectrum also showed small peaks at *m/e* 563 and 580; [α] 589 mμ, −60°; 550, −65°; 500, −100°; 450, −130°; 400, −180°; 350, −260°; 325, −340°; 300, −500°; 275, −900°; 250, −1400° (*c* 0·095 in $CHCl_3$); ε_{300} 0; ε_{275} 835 (plateau), ε_{220} 13,000 (in EtOH); $\nu_{max.}$ (KBr) 3356 (N–H), 3077, 2985 (C–H), 1739, 1718 (ester C=O), 1669, 1642 (amide C=O), 1534 (amide II), 1493 (*N*–Me), 1379, 1372 (Pr^i), 1250—1200 (ester C–O), 755, 700 (phenyl rings) cm.$^{-1}$; $\nu_{max.}$ (CCl_4) 3322, 1757, 1733, 1689, 1658 cm.$^{-1}$. The yield of pithomycolide from "Compound 242" was *ca.* 70%. Pithomycolide is soluble in most organic solvents, except light petroleum, especially in halogenic solvents and hot benzene but is very sparingly soluble in water, and aqueous acids and alkalis. Unlike sporidesmolide I, it did not sublime on heating at atmospheric pressure. Dakin–West degradation of pithomycolide by the simplified technique

[24] Shemyakin, Antonov, Shkrob, Sheinker, and Senyavina, *Tetrahedron Letters*, 1962, 701; Shemyakin, Ovchinnikov, Antonov, Kiryushkin, Ivanov, Shchelokov, and Shkrob, *ibid.*, 1964, 47; Sheppard, *Experientia*, 1963, **19**, 125; Jones, Kenner, and Sheppard, *ibid.*, p. 126; Griot and Frey, *Tetrahedron*, 1963, **19**, 1661.

[25] Acerbo, Schubert, and Nord, *J. Amer. Chem. Soc.*, 1958, **80**, 1990.

[26] Linstead, Elvidge, and Whalley, "A Course in Modern Techniques of Organic Chemistry," Butterworths, London, 1955, p. 16.

[27] Consden, Gordon, and Martin, *Biochem. J.*, 1944, **38**, 224.

[28] Vining and Taber, *Canad. J. Chem.*, 1957, **35**, 1109.

[29] Brockmann and Schmidt-Kastner, *Chem. Ber.*, 1955, **88**, 57.

[30] Russell, *J. Chromatog.*, 1960, **4**, 251.

[31] Pásková and Munk, *J. Chromatog.*, 1960, **4**, 241.

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of Russell[4a] showed no alteration in the ninhydrin-sensitive components of a total acid hydrolysate.

Total Acid Hydrolysis of Pithomycolide.—(*a*) Pithomycolide (6 mg.) in acetic acid (1 ml.) and 25% hydrochloric acid (0·5 ml.) was heated at 100° for 48 hr. Volatile acids were removed at 20° *in vacuo* and an aqueous solution of the residue was extracted with ether. Paper chromatography of the aqueous phase in systems (A), (B), and (C) disclosed only two spots in each, identified by co-chromatography with authentic samples as alanine, R_F 0·20 (C), 0·58 (B), 0·21 (A), and *N*-methylalanine, R_F 0·28 (A), 0·84 (B), 0·28 (C). On treatment with ninhydrin, alanine spots gave a strong purple colour on papers run in all three systems; the spots from *N*-methylalanine, however, were purple-pink on papers run in system (A), purple on those from system (B), and purple-blue in those from system (C). In accord with Russell's observations on other *N*-methylamino-acids,[30] the colours on papers run in the ammonia system were more intense than those obtained with systems (A) and (B). When the papers were treated with 10% pyridine in acetone followed by 0·2% *p*-nitrobenzoyl chloride in benzene[32] the *N*-methylalanine spots gave a transient yellow colour. The ethereal layer gave crystalline acidic material, m. p. 131—131·5° (1 mg.).

(*b*) Pithomycolide (152 mg.) in acetic acid (10 ml.) and concentrated hydrochloric acid (3 ml.) was hydrolysed and worked up as above, and the crystalline product partitioned between water and ether. The ethereal layer gave acidic crystals, m. p. 105—115°, which were chromatographed on silica gel coloured with Bromcresol Green,[33] and shown to be essentially homogeneous by paper chromatography in system (D). The acid, on paper, gave a single bright green spot, R_F 0·96 (D), appearing instantaneously on treatment with permanganate-indicator reagent. The chloroform eluate gave plates of cinnamic acid, m. p. and mixed m. p. 133·5—134·5° (from ether then benzene–cyclohexane), identified also by infrared and ultraviolet spectra. Quantitative total acid hydrolysis of pithomycolide (51 mg.) gave cinnamic acid (21·4 mg., 1·52 moles), m. p. 129—130·5°.

L-α-Hydroxyisovaleric acid was detected by warming the crystalline acid hydrolysate from pithomycolide (5·5 mg.), with ether and chromatography of portions of the solution in systems (A), (C), and (D). Co-chromatography of authentic material, and treatment of the strips with permanganate-indicator reagent, disclosed the acid as bright pink-yellow spots, R_F 0·78 (A), 0·48 (C), 0·86 (D) accompanied by weak spots due to artefacts, R_F (A) 0·22, 0·53, 0·65, (C) 0·08, 0·23, 0·31, (D) 0·52, 0·73. For isolation of L-α-hydroxyisovaleric acid, pithomycolide (276 mg.) in acetic acid (10 ml.) and 25% hydrochloric acid (5 ml.) was hydrolysed as usual to give a crystalline product which was partitioned between water and benzene. Chloroform extraction of the aqueous layer for 26 hr. gave a musty-smelling yellow oil which, with a slight excess of cyclohexylamine in dry ether, gave crystalline material, recrystallised from toluene to give cyclohexylammonium L-α-hydroxyisovalerate as nacreous plates (40 mg., 37%), m. p. and mixed m. p. 144—145°, $[\alpha]_D^{25}$ −5° (*c* 0·1 in water) {lit.,[4a] m. p. 140—141°, $[\alpha]_D^{25}$ −9·0° (*c* 2·3 in H_2O)}.

Cyclohexylammonium D-α-*hydroxyisovalerate*, prepared from D-valine by the method of Greenstein and his co-workers,[34] crystallised from toluene as nacreous plates, m. p. 143—145°, depressed to 131—135° on admixture with the L-enantiomer (Found: C, 61·1; H, 10·8. $C_{11}H_{23}NO_3$ requires C, 60·8; H, 10·7%). The rotatory dispersion curves of the salts were plain mirror images. *Cyclohexylammonium* DL-α-*hydroxyisovalerate*, similarly prepared, crystallised from toluene as irregular plates, m. p. 130—131° (Found: C, 61·1; H, 11·0%).

Action of D- *and* L-*Amino-acid Oxidases on Amino-acids from Total Acid Hydrolysis of Pithomycolide.*—1% Solutions of hog kidney D-amino-acid oxidase and snake venom L-amino-acid oxidase (Nutritional Biochemicals Corp., Cleveland, Ohio) were prepared in pH 7·0 buffer (Burroughs Wellcome). Dried spots of the solutions on paper gave only pale brown colours with ninhydrin. Chromatograms of the total acid hydrolysate were run in systems (A), (B), and (C), then dried overnight and cut longitudinally into three sections, which were sprayed with D-oxidase, ninhydrin, and L-oxidase, respectively. After 2½ hr. the amino-acids were almost completely destroyed (ninhydrin) by L-oxidase, but were unaffected by D-oxidase. After 5¾ hr. in the presence of L-oxidase, destruction of the amino-acids was complete.

[32] Plattner and Nager, *Helv. Chim. Acta*, 1948, **31**, 2192.
[33] Brockmann and Geeren, *Annalen*, 1957, **603**, 216.
[34] Winitz, Bloch-Frankenthal, Izumiya, Birnbaum, Baker, and Greenstein, *J. Amer. Chem. Soc.*, 1956, **78**, 2423.

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Action of Acids on Pithomycolide at Room Temperature.—Pithomycolide was unaffected by 2N-hydrochloric acid solutions and could be recovered almost quantitatively. When a solution of pithomycolide in acetic acid–concentrated hydrochloric acid was held at 37°, very weak spots due to both amino-acids were detected after 24 hr.

Alkaline Hydrolysis of Pithomycolide.—(*a*) Pithomycolide (20 mg.) in 0·02N-potassium hydroxide solution in 80% (v/v) aqueous methanol (20 ml.) was held at 37° for 5 hr. and then at 20° overnight. Water was added, and the mixture extracted with ether and acidified. Ether extraction of the acidic fraction gave a partly crystalline residue, which was chromatographed in systems (A), (B), and (C). With ninhydrin the strips gave a single pink spot, R_F 0·79 (A), 0·76 (B), 0·49 (C), due to cinnamic acid. This is an anomalous reaction of ninhydrin. Chromatography in systems (C) and (D), followed by treatment with permanganate-indicator reagent gave three spots in each system, *viz.*, a strong pink spot [R_F 0·26 (C), 0·15 (D)] due to acids from partly hydrolysed pithomycolide; a weak purple spot [R_F 0·49 (C), 0·87 (D)] corresponding to β-hydroxy-β-phenylpropionic acid; a strong bright green spot [R_F 0·55 (C), 0·93 (D)] identified as cinnamic acid by co-chromatography.

(*b*) 5N-Potassium hydroxide solution (6 ml.) was added to pithomycolide (227 mg.) in chloroform (3 ml.) and methanol (11 ml.) and the solution set aside at 20° for 15 min. Water was added, the organic solvents were removed *in vacuo* at 30° and the solution was acidified to yield cinnamic acid (50 mg., 0·68 mole), m. p. and mixed m. p. 130—132°. The filtrate was saturated with potassium chloride and extracted both with ether and chloroform to give partly crystalline gums which were combined and chromatographed in benzene on silica gel. Elution with benzene–ether (4:1 and 2:1) gave needles (from chloroform–hexane) of D-(−)-β-hydroxy-β-phenylpropionic acid (55·2 mg.), m. p. and mixed m. p. 114—116° (Found: C, 65·1; H, 6·1. Calc. for $C_9H_{10}O_3$: C, 65·05; H, 6·0%). The infrared spectrum was identical with that of the authentic hydroxy-acid.[35] Total acid hydrolysis of this compound gave only cinnamic acid.

With L-(+)-β-hydroxy-β-phenylpropionic acid, m. p. 114—116°, prepared similarly to the D-(−)-isomer, the acid from pithomycolide had mixed m. p. 91—104°, whilst the synthetic DL-acid had m. p. 89—91·5° (lit., 92°, 94°, 96°[36]). Further elution of the column gave no identifiable material, whilst mother liquors from recrystallisation of the β-hydroxy-acid contained only this acid and cinnamic acid, identified as its *S*-benzylisothiouronium salt.

The yields of pure aromatic hydroxy-acids isolated were critically dependent on the temperature at which organic solvents were removed from the alkaline hydrolysate before acidification. When the concentration was performed at 40°, 1·0 mole of cinnamic acid and 0·4 mole of β-hydroxy-β-phenylpropionic acid were isolated. The yield of the latter acid was always less than 1·0 mole, but the combined yield of aromatic acids was always *ca.* 1·4 moles.

Borohydride Reductions of Pithomycolide.—(*a*) *With sodium borohydride.* Pithomycolide (27 mg.) and sodium borohydride (123 mg.) in dry tetrahydrofuran (60 ml.) were heated under reflux for 17·5 hr. After normal work-up, the neutral residue was hydrolysed (HCl–HOAc) and the hydrolysate chromatographed in systems (A), (B), and (C), to show *N*-methylalanine, a little alanine, and 2-aminopropan-1-ol, R_F 0·45 (A), 0·95 (B), 0·58 (C) (blue with ninhydrin), identified by co-chromatography with material prepared according to Karrer and his co-workers.[37]

(*b*) *With lithium borohydride.* Pithomycolide (10 mg.), sodium borohydride (67 mg.), and lithium iodide (406 mg.)[38] in dry tetrahydrofuran (20 ml.) were heated under reflux for 3 hr. Treatment, essentially as described for sodium borohydride reduction, showed, in the chromatograms, the presence of 2-aminopropan-1-ol, to the almost complete exclusion of alanine.

Catalytic Hydrogenolysis of Pithomycolide.—Pithomycolide (200 mg.) with 10% palladium–charcoal (200 mg.) in methanol was hydrogenated at 20° and 47 p.s.i. for 24 hr. Catalyst and solvent were removed to give a pale yellow, sweet-smelling gum, $\nu_{max.}$ (film) 3300 (N–H), 1739, and 1700—1640 cm.$^{-1}$ (ester and amide C=O). Partitioning between chloroform and saturated sodium hydrogen carbonate solution gave negligible neutral material and a colourless, acidic gum. Chromatography of this gum showed a little hydrocinnamic acid, R_F 0·67 (C), by co-chromatography with authentic material, as well as the predominant acidic component, of R_F 0·84 (A), 0·78 (C), 0·93 (D). When the hydrogenolysis was performed for 20 hr. at 19 p.s.i., 9% of pithomycolide was recovered.

[35] Noyce and Lane, *J. Amer. Chem. Soc.*, 1962, **84**, 1635, 1641.
[36] "Beilstein's Handbuch der Organischen Chemie," Springer-Verlag, Berlin, 1927, Band X, p. 249.
[37] Karrer, Portmann, and Suter, *Helv. Chim. Acta*, 1948, **31**, 1617.
[38] Sallay, Dutka, and Fodor, *Helv. Chim. Acta*, 1954, **37**, 778.

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Dakin–West Degradation of the Product of Catalytic Hydrogenolysis of Pithomycolide.—The hydrogenolysis product (5·5 mg.), in acetic anhydride (0·5 ml.) and pyridine (1·2 ml.), was heated at 110° for 2·5 hr. Solvent was removed and the residue hydrolysed (HCl–HOAc) to show, on chromatography in systems (A), (B), and (C), strong spots of *N*-methylalanine, but only traces of alanine.

Action of 1 Mole of Base on Pithomycolide.—Potassium hydroxide (33 mg., 1 mol.) in ethanol (40 ml.) was added to pithomycolide (276 mg.) in chloroform (5 ml.) and ethanol (35 ml.) and set aside at 20° for 4 hr. After removal of most of the solvent, the residual gum was partitioned between ether and water. The neutral, ethereal layer gave pithomycolide (5 mg.); the aqueous layer, on acidification and ether extraction gave a gum. Hydrolysis (HCl–HOAc) of this gum gave a product which was partitioned between ether and water. The aqueous and ethereal layer were shown to contain the two amino-acids and cinnamic acid, respectively.

Reactions of the Product of Hydrolysis of Pithomycolide with 1 Mole of Base.—(*a*) *With sodium borohydride.* The gummy acidic product (20 mg.) and sodium borohydride (120 mg.) in tetrahydrofuran (60 ml.) were heated under reflux for 23 hr. The product, obtained in the usual way, was hydrolysed (HCl–HOAc), and the presence of alanine, *N*-methylalanine, and 2-aminopropan-1-ol shown in the hydrolysate by paper chromatography.

(*b*) *Dakin–West degradation.* This was performed as described previously. Only small traces of alanine were detected by paper chromatography.

Attempted Isolation of "Sporidesmolic Acid D." (L-α-*Hydroxyisovaleryl*-N-*methyl*-L-*alanyl*-L-*alanine*).[4a]—The acidified aqueous phase remaining from hydrolysis of pithomycolide with N-potassium hydroxide solution, after removal of cinnamic acid, was extracted with chloroform, and the aqueous phase rapidly taken to dryness. Extraction of the residue with dry methanol gave a gummy product which gave weak reactions for both amino-acids on chromatography, but which on total acid hydrolysis showed strong spots due to alanine and *N*-methylalanine. Attempted separation of "sporidesmolic acid D" by formation of brucine, *S*-benzylisothiouronium, cyclohexylammonium, nitron, or heavy-metal salts was unsuccessful. Alternatively, the aqueous layer was saturated with ammonium sulphate and continuously extracted with butan-1-ol for 24 hr., but with no different results.

Action of Sodium Borohydride–Boron Trifluoride on Pithomycolide.—Nitrogen was passed through an ice-cold suspension of pithomycolide (55 mg.) and sodium borohydride (21 mg.) in ether (20 ml.), and redistilled boron trifluoride etherate (1.3 ml.) added dropwise. The mixture was held at 20° for 75 min., under reflux for 1 hr., and at 0° for 15 hr. After acidification, the aqueous layer was extracted with ether to give a gum which was chromatographed in systems (A), (C), and (D). Treatment of the strips with permanganate-indicator reagent showed: system (A), R_F 0·01, 0·15—0·54 (streak), 0·87 (cinnamic acid); system (C), R_F 0·01, 0·12, 0·47 (pink), 0·49 (cinnamic acid), 0·62, 0·79 (green); system (D) R_F 0·28—0·63 (streak), 0·92 (cinnamic acid). Hydrocinnamic acid was absent.

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