144. *Depsipeptides of* Pithomyces chartarum: *the Structure of Sporidesmolide I.*

By D. W. RUSSELL.

A metabolic product, $C_{33}H_{58}N_4O_8$, sporidesmolide I, has been isolated from cultures of the mould *Pithomyces chartarum* (Berk. & Curt.) M. B. Ellis. It is a neutral compound, yielding on acid hydrolysis valine, leucine, and *N*-methyl-leucine, together with L-α-hydroxy-β-methylbutyric acid. Alkaline hydrolysis furnishes sporidesmolic acid A, $C_{16}H_{30}N_2O_5$, and B, $C_{17}H_{32}N_2O_5$, two complex acids neither of which possesses a free amino-group. Acid hydrolysis of sporidesmolic acid A liberates D-valine, D-leucine, and L-α-hydroxy-β-methylbutyric acid; sporidesmolic acid B furnishes similarly L-valine, *N*-methyl-L-leucine, and L-α-hydroxy-β-methylbutyric acid. Structure (I) is proposed for sporidesmolic acid A, structure (III) for sporidesmolic acid B, and the cyclic depsipeptide structure (V) for sporidesmolide I. The presence of a second, isoleucine-containing depsipeptide in the mixture produced by *P. chartarum* is inferred, and a small yield of a third, sporidesmolide III, is reported.

A discrepancy between observed and published values for the specific rotation of L-valine in glacial acetic acid is recorded.

DURING an examination of the metabolic products of the pasture fungus *Pithomyces chartarum* (Berk. & Curt.) M. B. Ellis (synonymous with *Sporidesmium bakeri* Sydow, Commonwealth Mycological Institute Herbarium no. 74473), a fraction rich in neutral derivatives of amino-acids was obtained.[1] From this mixture of related substances the major component, sporidesmolide I, has been isolated by fractional crystallization. This paper reports investigations into the structure of sporidesmolide I, a brief account of which has appeared elsewhere.[2]

Sporidesmolide I was a colourless, crystalline, optically active compound, $C_{33}H_{58}N_4O_8$, the unusual solubility properties of which considerably facilitated its isolation. In the ultraviolet region it had only general absorption below 270 mμ. From the infrared spectrum ($\nu_{max.}$ 3350, 2968, 2930, 2869, 1753, 1679, 1646, 1529, 1464, 1410, 1381, 1363 cm.$^{-1}$) the presence of ester and amide functions and of isopropyl and *N*-methyl[3] groups was inferred. Although acid hydrolysis of sporidesmolide I liberated amino-acids, the compound was neither acidic nor basic.

After vigorous acid hydrolysis of the compound, four products were detected in the hydrolysate. One was an ether-soluble acid, identified by paper-chromatographic comparison as α-hydroxy-β-methylbutyric acid. The cyclohexylammonium salt, obtained in an overall yield of 1·58 mol., was identical with synthetic cyclohexylammonium L-α-hydroxy-β-methylbutyrate. The other three components were amino-acids. Two of these were identified as valine and leucine by comparison on paper chromatograms. The third reacted only weakly with ninhydrin in the presence of acetic acid, but strongly when the chromatogram was prepared with a solvent containing ammonia and sprayed with a ninhydrin solution of pH *ca.* 7.[4] It also responded to the Waser–Edlbacher test.[5,6] It is thus an *N*-methylamino-acid, and its behaviour in paper chromatography and electrophoresis was indistinguishable from that of synthetic *N*-methyl-leucine.[7] By quantitative

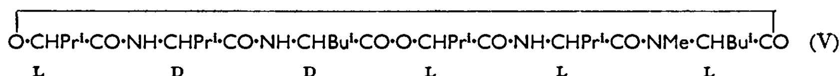
[1] Done, Mortimer, Taylor, and Russell, *J. Gen. Microbiol.*, 1961, **26**, 207.
[2] Russell, *Biochim. Biophys. Acta*, 1960, **45**, 411.
[3] Watson, *Spectrochim. Acta*, 1960, **16**, 1322.
[4] Russell, *J. Chromatog.*, 1960, **4**, 251.
[5] Waser, *Mitt. Lebensm. Hyg.*, 1929, **20**, 260; Edlbacher and Litvan, *Z. physiol. Chem.*, 1940, **265**, 241.
[6] Plattner and Nager, *Helv. Chim. Acta*, 1948, **31**, 2203.
[7] Blanchard, Green, Nocito, and Ratner, *J. Biol. Chem.*, 1944, **155**, 421; Fischer and von Mechel, *Ber.*, 1916, **49**, 1358.

paper chromatography [4] the valine : leucine : *N*-methyl-leucine ratio was shown to be 2 : 1 : 1.

Sporidesmolide I was readily degraded by alkali, two equivalents of base being consumed and two new compounds resulting. The first, sporidesmolic acid A, was a colourless, crystalline, optically active carboxylic acid, $C_{16}H_{30}N_2O_5$, in which no free amino-group could be demonstrated. On hydrolysis with mineral acid, sporidesmolic acid A yielded L-α-hydroxy-β-methylbutyric acid, D-valine, and D-leucine. We conclude that sporidesmolic acid A is a dipeptide of D-valine and D-leucine, in which the terminal amino-group is acylated by the butyric acid. Alternative structures in which the hydroxyacyl residue occupies some other position are inconsistent with the facts that sporidesmolic acid A is not a dipolar ion and that it is stable to alkali. Two structures are possible, according to whether the dipeptide sequence is valyl-leucine (I) or leucyl-valine (II). Clear evidence in favour of the former was obtained by partial acid hydrolysis: leucine was fairly rapidly liberated when sporidesmolic acid A was incubated in concentrated hydrochloric acid at 37°, but free valine was not detected even after four days. That leucine occupied the *C*-terminal position was confirmed by Dakin–West degradation [8] followed by acid hydrolysis: [8,9] leucine was absent from the hydrolysate, but paper chromatograms showed a strong spot corresponding to valine.

The second compound resulting from alkaline hydrolysis of sporidesmolide I was sporidesmolic acid B, $C_{17}H_{32}N_2O_5$.[10] This also was a monobasic carboxylic acid, in which no free amino-group could be demonstrated, and was stable to alkali. On vigorous acid hydrolysis it furnished L-α-hydroxy-β-methylbutyric acid (isolated and identified as its cyclohexylammonium salt), L-valine, and *N*-methyl-L-leucine. It was inferred that sporidesmolic acid B was a homologue, (III) or (IV), of sporidesmolic acid A. The latter structure, in which the dipeptide sequence is *N*-methyl-leucyl-valine, was excluded by partial acid hydrolysis and Dakin–West degradation, both of which showed a *C*-terminal *N*-methyl-leucine residue. The compound is therefore (*N*-L-α-hydroxy-β-methylbutyryl-L-valyl)-*N*-methyl-L-leucine (III).

The structure of sporidesmolide I may now be completely defined. Alkaline hydrolysis of the compound is accompanied by consumption of two equivalents of base and disappearance of the ester-carbonyl absorption ($\nu_{max.}$ 1753 cm.$^{-1}$). The two complex hydroxy-acids produced must therefore have resulted from hydrolysis of ester bonds involving the carboxyl and hydroxyl groups of both. The stoicheiometric relations show that sporidesmolide I consists of one residue each of sporidesmolic acids A and B condensed together in a macrocyclic system (V). This interpretation is supported by the mass spectrum of sporidesmolide I, kindly measured by Dr. R. I. Reed, from which a molecular weight of 638 was derived. Some fragment ions consistent with the presence of CH·CO·NH·CH, and some with the presence of $CHMe_2$ or higher homologues, were observed.



Some confirmation for structure (V) was adduced as follows. Such a compound should in principle be susceptible to partial alkaline hydrolysis, with formation of a linear molecule containing only one ester group. When sporidesmolide I was treated with very dilute

[8] Dakin and West, *J. Biol. Chem.*, 1928, **78**, 91, 745; Cleland and Niemann, *J. Amer. Chem. Soc.*, 1949, **71**, 841; Turner and Schmerzler, *J. Amer. Chem. Soc.*, 1954, **76**, 950.
[9] Shepherd, Wilson, Bell, Davis, and Shakespeare, *J. Amer. Chem. Soc.*, 1956, **78**, 5067.
[10] Russell and Brown, *Biochim. Biophys. Acta*, 1960, **38**, 382.

alkali, one equivalent was rapidly consumed, whereas further consumption of alkali was very slow. Treatment with only one equivalent of very dilute sodium hydroxide furnished a new compound, $C_{33}H_{60}N_4O_9$, which with hydrazine gave sporidesmolic acid A. Although the structure of this compound has not been further defined, such behaviour is consistent with a structure in which a molecule of sporidesmolic acid A is acylated with a residue of sporidesmolic acid B.

Sporidesmolide I is a new example of the group of mould and bacterial metabolites known as depsipeptides.[11] Its occurrence in an organism taxonomically distant from those in which other depsipeptides have been found supports the suggestion [12] that such compounds may be common products of micro-organisms. Sporidesmolide I possesses certain interesting structural features. In common with other depsipeptides [11-15] it contains residues of D-amino-acids, but it is unique in that both residues of α-hydroxy-β-methylbutyric acid belong to the L-series: the L-acid has not previously been isolated from natural sources, the fungal depsipeptides hitherto described containing D-residues although one of them, valinomycin, yields also L-lactic acid on hydrolysis.[13]

Several *N*-methylamino-acids have been observed as products of the hydrolysis of microbial metabolites.[16] In particular, *N*-methyl-leucine has been identified by paper chromatography in hydrolysates of enniatin C; [14] the present work represents the first isolation of this amino-acid from a natural source. Its configuration is the same in this case as that of *N*-methylvaline and *N*-methylisoleucine isolated from hydrolysates of enniatin A and enniatin B, respectively.[14]

The most striking difference in structure between sporidesmolide I and other known fungal depsipeptides lies in the arrangement of the hydroxy- and amino-acid residues. In the enniatins,[14] valinomycin,[13] and amidomycin [12] residues of hydroxy- and amino-acids occur alternately disposed in the cyclic structure, and no true peptide bond is present. Sporidesmolide I, on the other hand, contains two true dipeptide sequences which are, moreover, structurally and stereochemically different. It therefore lacks the complete regularity of the fungal depsipeptides hitherto described, while retaining some element of regularity which is lacking from the bacterial depsipeptide esperin.[15]

The total sporidesmolide fraction of *P. chartarum* contains at least two other depsipeptides. One was easily isolated because of its low solubility in chloroform. This substance, sporidesmolide III, gave on acid hydrolysis valine, leucine, and α-hydroxy-β-methylbutyric acid. The mixture after removal of sporidesmolide III still contained, in addition to sporidesmolide I, a third substance which yielded isoleucine on acid hydrolysis. The properties of this component were very similar to those of sporidesmolide I, and it was not isolated. However, alkaline hydrolysis of the mixture yielded, in addition to sporidesmolic acid B, impure sporidesmolic acid A, acid hydrolysates of which contained isoleucine. This acid could not be purified by crystallization, its melting point and specific rotation were close to those of pure sporidesmolic acid A, and the isoleucine was not destroyed by Dakin–West degradation, so it is possible that the contaminant was the higher homologue of sporidesmolic acid A containing D-iso-leucinein place of D-valine. Purification was achieved by fractional extraction [17] of a butanol solution with sodium hydroxide, sporidesmolic acid A being extracted preferentially: the difference in this respect was only slight. Although pure sporidesmolic acid A was isolated by this means, the fractionation was not carried to completion. It is nonetheless

[11] Schemjakin, *Angew. Chem.*, 1960, **72**, 342.
[12] Vining and Taber, *Canad. J. Chem.*, 1957, **35**, 1109.
[13] Brockmann and Geeren, *Annalen*, 1957, **603**, 216.
[14] Plattner and Nager, *Helv. Chim. Acta*, 1948, **31**, 665, 2192; Cook, Cox, and Farmer, *J.*, 1949, 1022.
[15] Ito and Ogawa, *Bull. Agric. Chem. Soc. Japan*, 1959, **23**, 536.
[16] Vanderhaege and Parmentier, *J. Amer. Chem. Soc.*, 1960, **82**, 4414; Sheehan, Zachau, and Lawson, *ibid.*, 1958, **80**, 3349; Bullock and Johnson, *J.*, 1957, 3280; Brockmann, Bohnsack, Franck, Gröne, Muxfeldt, and Süling, *Angew. Chem.*, 1956, **68**, 70; Plattner and Nager, refs. 6 and 14; Eastwood, Snell, and Todd, *J.*, 1960, 2286.
[17] Le Quesne and Young, *J.*, 1950, 1954.

clear that a third depsipeptide, containing isoleucine, and of structure similar to that of sporidesmolide I, is present in the total sporidesmolide fraction of *P. chartarum.*

Experimental

M. p.s were determined on a Kofler block. Ultraviolet spectra were measured with a Beckman DU spectrophotometer, and infrared spectra with a Perkin–Elmer model 21 instrument and potassium bromide discs. Microanalyses were by Dr. F. Pascher, Bonn. Determination of the molecular weight of sporidesmolide I by isothermal distillation was by Dr. A. D. Campbell. The mass spectrum was measured by Dr. R. I. Reed using a Metropolitan-Vickers M.S.2. instrument.

Sporidesmolide I (V) *and Sporidesmolide III from the Crude Depsipeptide Fraction of* Pithomyces chartarum (*Berk. & Curt.*) *M. B. Ellis.*—The precipitate (10·0 g.) obtained by cooling a hot methanol extract of the dried mycelium or "ryecorn" culture [1] was shaken with chloroform (200 ml.), water (60 ml.), and methanol (140 ml.).[18] The lower phase was separated and evaporated to dryness *in vacuo*. The residue was extracted with diethyl ether (200 ml.), and the insoluble material filtered off, washed with more ether, and dried *in vacuo* (H_2SO_4). The product (6·2 g.) was dissolved in chloroform (300 ml.) and filtered through decolorizing charcoal (6 g.) contained in a glass tube 1·5 cm. in diameter. The column was washed with chloroform (300 ml.), and the effluents were evaporated to dryness on a steam-bath. The residue (5·4 g.), termed the total sporidesmolide fraction, was redissolved under reflux in chloroform (27 ml.), and the solution was left at 0° for several days. The crystalline precipitate (0·064 g.) was collected and recrystallized from chloroform, to yield *sporidesmolide III*, m. p. 277—278° (Found: C, 60·8, 60·9; H, 8·9, 9·1; N, 8·8, 8·9. $C_{32}H_{56}N_4O_8$ requires C, 61·5; H, 9·0; N, 9·0%). This gave no colour reaction with ninhydrin or with indicators.

The filtrate therefrom was heated to the b. p. and hot methanol (540 ml.) was added. Crystallization was allowed to proceed for several hours at room temperature. Fractional crystallization from 10% (v/v) chloroform in methanol was carried out until an acid hydrolysate of the product, prepared as described below, contained no detectable amount of isoleucine when examined by paper chromatography in t-butyl alcohol–4·25N-ammonia (4 : 1 v/v).[12] Crystallization of the material (1·9 g.), so obtained, from 70% (v/v) acetic acid furnished needles of *sporidesmolide I* (V), m. p. 261—263°, $[\alpha]_D$ −217° (*c* 1·5 in chloroform), −98° (*c* 1·5 in acetic acid), $\nu_{max.}$ 3350s (N–H), 2968s, 2930sh, 2869w (C–H), 1753s (ester C=O), 1679s, 1646s (amide I), 1529s (amide II), 1464m (C–Me), 1410w (N–Me),[3] 1381m, 1363m (Pr^i) $cm.^{-1}$ [Found: C, 62·1, 61·8, 61·8; H, 9·0, 9·1, 9·2; N, 8·6, 8·6, 9·0; O, 20·1, 20·0, 20·3; *N*-Me, 3·1, 3·2%; *M*, 638 (mass spectrometer), 622 (isothermal distillation in chloroform). $C_{33}H_{58}N_4O_8$ requires C, 62·0; H, 9·2; N, 8·8; O, 20·0; 1*N*-Me, 2·4%; *M*, 639].

Sporidesmolide I was very insoluble in water, very soluble in chloroform, but only sparingly soluble in other common organic solvents. When it was heated in an open capillary above 200° it slowly sublimed; the sublimate, alone or mixed with the unsublimed compound, had m. p. 261—263°. The compound was not extracted from its chloroform solution by aqueous or aqueous-alcoholic acid or alkali or by 70% (v/v) methanol. It was recovered unchanged after having been refluxed with acetic anhydride for 2 hr. It gave no colour reaction with ninhydrin or with indicators. After it had been subjected to the degradation procedure of Dakin and West [8,9] the product was hydrolysed with acid; the conditions used are described below. The hydrolysate, examined by paper chromatography, contained amino-acids in the same proportions as were present in a control hydrolysate of sporidesmolide I.

L-α-*Hydroxy*-β-*methylbutyric Acid.*—Extraction by diethyl ether of an acidified (sulphuric acid) solution of sodium L-α-hydroxy-β-methylbutyrate [19] in water, followed by drying (Na_2SO_4) of the extract and evaporation, yielded L-α-hydroxy-β-methylbutyric acid,[20] that was purified by repeated sublimation *in vacuo* at 60°. The compound had m. p. 66—67°, $[\alpha]_D$ +20° (*c* 1·1 in chloroform). The *cyclohexylammonium salt* (from toluene) formed plates, m. p. 140—141°, $[\alpha]_D$ −8·9° (*c* 1·5 in water) (Found: C, 60·5; H, 10·7; N, 6·7. $C_{11}H_{23}NO_3$ requires C, 60·8; H, 10·7; N, 6·4%).

[18] Schwyzer and Sieber, *Helv. Chim. Acta*, 1957, **40**, 624.
[19] Winitz, Bloch-Frankenthal, Izumiya, Birnbaum, Baker, and Greenstein, *J. Amer. Chem. Soc.*, 1956, **78**, 2423.
[20] Fischer and Scheibler, *Ber.*, 1908, **41**, 2891.

Acid Hydrolysis of Sporidesmolide I.—The compound was only slowly attacked by boiling 6N-hydrochloric acid. A sample (0·0064 g.) was heated in a sealed tube with concentrated hydrochloric acid (0·5 ml.) and glacial acetic acid (0·5 ml.) for 24 hr. at 110°. The volatile acids were then removed *in vacuo* (KOH). The residue was extracted with ether (1·0 ml.), and portions (0·1 ml.) of the extract were examined by paper chromatography in t-butyl alcohol–4·25N-ammonia (4 : 1),[12] propan-1-ol–17N-ammonia (8 : 2),[21] and butan-1-ol–90% formic acid–water (10 : 3 : 10).[22] The dried chromatograms were sprayed with a 0·04% solution of Chlorophenol Red in ethanol previously neutralized to pH 7.[23] In each case one major and two minor spots were revealed, corresponding in positions and relative intensities to the three spots produced on chromatograms prepared from authentic α-hydroxy-β-methylbutyric acid.

The ether-insoluble residue was dissolved in water (1·0 ml.) and portions (0·01 ml.) were examined by paper chromatography in the t-butyl alcohol–ammonia solvent, in butan-1-ol–acetic acid–water (4 : 1 : 5, upper phase), in phenol–water, and in benzyl alcohol saturated with water.[24] When the chromatograms were sprayed with a 0·1% solution of ninhydrin in butan-1-ol containing 2% of glacial acetic acid and colour was allowed to develop in the cold, two spots were revealed, corresponding in each solvent system to valine and leucine severally. When the papers were heated, a third faint grey-purple spot was revealed. When the papers were sprayed with a 1% solution of ninhydrin in butan-1-ol previously equilibrated with phosphate buffer (pH 7; 0·05M) [25] and heated at 110° for 30 min., the third spot had a more intense colour, particularly on papers run in the solvent containing ammonia. Three strong spots were obtained by using the pyridine acetate-buffered ninhydrin spray previously described.[4] When the papers were sprayed with the *p*-nitrobenzoyl chloride reagent of Plattner and Nager,[14] a transient pink spot corresponding in position with the third ninhydrin-reacting spot appeared. In all solvent systems, this spot was identical in R_F and staining characteristics with that produced by synthetic *N*-methyl-leucine.[7] Its mobility in paper electrophoresis at pH 2·5 (4M-acetic acid) was also identical with that of *N*-methyl-leucine. Semiquantitative estimation [4] showed 1·9 mol. of valine, 1·0 mol. of leucine, and 1·0 mol. of *N*-methyl-leucine.

For preparative isolation of α-hydroxy-β-methylbutyric acid, sporidesmolide I (0·639 g.) was dissolved under reflux in chloroform (4 ml.) and ethanol (64 ml.). 4N-Sodium hydroxide (0·5 ml.) was added to the refluxing solution, which was then evaporated to dryness *in vacuo*. The residue was boiled in 6N-hydrochloric acid (65 ml.) under reflux for 24 hr. After the volume had been reduced to *ca.* 15 ml. by distillation, the solution was diluted with water (100 ml.). Continuous extraction with ether, then drying (Na_2SO_4) and evaporation of the extract, furnished a crystalline residue (0·230 g.), m. p. 60—63°. This material contained an odorous, deliquescent impurity which was not removed by charcoal or by repeated vacuum-sublimation. When treated in ether with a slight excess of a 10% ether solution of cyclohexylamine the substance gave a crystalline product (0·389 g.), m. p. 137—140°. Recrystallized from toluene, this furnished cyclohexylammonium L-α-hydroxy-β-methylbutyrate (0·333 g.), m. p. and mixed m. p. 139—141°, $[\alpha]_D$ −9·0° (*c* 2·3 in water) (Found: C, 60·6; H, 10·6; N, 6·3; O, 22·2. Calc. for $C_{11}H_{23}NO_3$: C, 60·8; H, 10·7; N, 6·4; O, 22·1%).

Alkaline Hydrolysis of Sporidesmolide I.—(*a*) The compound (V) (0·639 g.) was dissolved in warm dimethylformamide (50 ml.), the solution cooled rapidly to room temperature, and N-sodium hydroxide (10·00 ml.) added. After 30 min. the excess of alkali was titrated with 0·1N-hydrochloric acid. A control determination was performed. The alkali consumption was 1·98 mequiv. When the reaction time was increased to 2 hr. no more alkali was consumed. The neutral solution was freed from solvent in a vacuum-desiccator (KOH, H_2SO_4), the residue extracted with 0·1N-hydrochloric acid, and insoluble material filtered off and dried *in vacuo* (KOH). The crystalline product (0·672 g.) was shaken with chloroform (14 ml.) for 1 hr. and the insoluble portion filtered off, the residue on the filter being washed with chloroform and dried *in vacuo* (H_2SO_4). Recrystallization of the product (0·312 g.; m. p. 199—200°) furnished *sporidesmolic acid A* (I), prisms (from 25% acetic acid), m. p. 200—201°, $[\alpha]_D$ +61° (*c* 4 in acetic acid), $\nu_{max.}$ 3228s (O–H), 1712s (carboxyl C=O), 1655s, 1637s (amide I), 1549s (amide II),

[21] Isherwood and Hanes, *Biochem. J.*, 1953, **55**, 824.
[22] Lugg and Overell, *Austral. J. Sci. Res.*, 1948, **1**, *A*, 98.
[23] Brown, *Nature*, 1951, **167**, 441.
[24] Consden, Gordon, and Martin, *Biochem. J.*, 1944, **39**, 251.
[25] Peart, *Biochem. J.*, 1956, **62**, 520.

1388m and 1372m (Pr^i) cm.$^{-1}$ (Found: C, 58·4; H, 9·1; N, 8·6; O, 24·2%; equiv., 330. $C_{16}H_{30}N_2O_5$ requires C, 58·2; H, 9·2; N, 8·5; O, 24·2%; equiv., 330).

Sporidesmolic acid A was sparingly soluble in water, dilute mineral acids, ether, and chloroform, but dissolved readily in ethanol. It was recovered unchanged by acidification (3N-hydrochloric acid) of its solution in 2N-sodium hydroxide, after the solution had been boiled and cooled. It did not react with ninhydrin, and on paper electrophoresis at pH 2·5 (4M-acetic acid) had zero mobility relative to a glucose marker; at pH 8 (10% pyridine in water) it migrated towards the anode. Titrated potentiometrically (glass electrode) with 0·1N-sodium hydroxide it behaved as a monobasic acid of p*K* *ca.* 4. Prepared with diazomethane, the *methyl ester* formed prisms (from carbon tetrachloride), m. p. 162—162·5°, $\nu_{max.}$ 1759s (ester C=O) cm.$^{-1}$ (Found: C, 59·7; H, 9·7; N, 8·0. $C_{17}H_{32}N_2O_5$ requires C, 59·2; H, 9·4; N, 8·1%). *Dicyclohexylammonium sporidesmolate A* formed prisms (from ether–chloroform), m. p. 171—172° (Found: C, 65·5; H, 10·3. $C_{28}H_{53}N_3O_5$ requires C, 65·7; H, 10·3%).

The chloroform filtrate and washings obtained after removal of sporidesmolic acid A were evaporated *in vacuo*, to yield crystals (0·359 g.), m. p. 155—162°, repeated recrystallization of which furnished *sporidesmolic acid B* (III), needles (from 25% acetic acid), m. p. 165—166°, $[\alpha]_D$ −108° (*c* 4 in acetic acid), $\nu_{max.}$ 3375s (O–H), 1725s (carboxyl C=O), 1637s, 1602s (amide I), 1536s (amide II), 1487w (N–Me),[3] 1388m and 1369m (Pr^i) cm.$^{-1}$ (Found: C, 59·3; H, 9·4; N, 8·1%; equiv., 345. $C_{17}H_{32}N_2O_5$ requires C, 59·2, H, 9·4; N, 8·1%; equiv., 344). This acid was sparingly soluble in water or dilute mineral acids, but readily soluble in most common organic solvents. It was recovered unchanged by acidification of its solution in 2N-sodium hydroxide, after the solution had been boiled and cooled. It did not react with ninhydrin, and in paper electrophoresis and potentiometric titration behaved similarly to sporidesmolic acid A. The *methyl ester*, prepared with diazomethane, formed prisms [from light petroleum (b. p. 60—80°)], m. p. 105—106°, $\nu_{max.}$ 1744s (ester C=O) cm.$^{-1}$ (Found: C, 60·2; H, 9·9; N, 7·6. $C_{18}H_{34}N_2O_5$ requires C, 60·3; H, 9·6; N, 7·8%). The *S-benzylisothiouronium salt* (from dilute ethanol) had m. p. 154—155°, $[\alpha]_D$ −71° (*c* 1·2 in methanol) (Found: C, 59·0; H, 8·5; N, 11·0; S, 6·0. $C_{25}H_{42}N_4O_5S$ requires C, 58·8; H, 8·3; N, 11·0; S, 6·3%).

(*b*) On a larger scale, preparation of sporidesmolic acids A and B was carried out by hydrolysis of sporidesmolide I in methanolic sodium hydroxide. The compound (5·0 g.) was dissolved under reflux in chloroform (50 ml.), and methanol (450 ml.) added. The solution was immediately cooled, and 5N-sodium hydroxide (125 ml.) added. After 15 min. the clear solution was diluted with water (450 ml.) and most of the methanol and chloroform removed by distillation *in vacuo* at 40°. The residual solution was filtered, if necessary, and acidified with 6N-hydrochloric acid. After the mixture had been left overnight at 0° the precipitated acids were filtered off and dried *in vacuo* (KOH). The mixed acids (4—5 g.) were separated and purified as described under (*a*).

Acid Hydrolysis of Sporidesmolic Acid A.—The acid (I) (3·30 g.) was boiled with 6N-hydrochloric acid (350 ml.) for 24 hr., the volume of the resulting solution reduced by distillation to *ca.* 50 ml., water (150 ml.) added, and the solution extracted continuously with ether for 16 hr. The ether extract, dried (Na_2SO_4) and evaporated, furnished crystalline L-α-hydroxy-β-methylbutyric acid (1·15 g.), m. p. 62—64°, raised by repeated vacuum-sublimation at 60° to67—68°, mixed m. p. 66—68°, $[\alpha]_D$ +20° (*c* 4 in chloroform) (Found: C, 51·1; H, 8·6%; equiv., 118. Calc. for $C_5H_{10}O_3$: C, 50·9; H, 8·5%; equiv., 118). The cyclohexylammonium salt formed plates (from toluene), m. p. and mixed m. p. 140—142°, $[\alpha]_D$ −8·8° (*c* 5 in water) (Found: C, 60·7; H, 10·5; N, 6·5. Calc. for $C_{11}H_{23}NO_3$: C, 60·8; H, 10·7; N, 6·4%).

Paper chromatography [4] of the aqueous solution remaining after ether-extraction showed the presence of valine (*ca.* 1 mol.) and leucine (*ca.* 1 mol.). The amino-acids were adsorbed on a short column of Amberlite CG 120 (H form), and the column was washed with water until the effluent was neutral to litmus, and then with N-ammonia. Evaporation of the ammonia effluent furnished a mixture of the amino-acids (2·41 g.). This was dissolved in the minimum volume of t-butyl alcohol–4·25N-ammonia (4 : 1) and applied to a ChroMax paper-roll chromatographic column (58 × 750 mm.; LKB-Produkter, Sweden). Elution with the same mixture of solvents, followed by evaporation of appropriate fractions, gave chromatographically pure D-leucine (1·08 g.), plates (from dilute ethanol), $[\alpha]_D$ −14·9° (*c* 2·0 in 6N-hydrochloric acid) (Found: C, 54·6; H, 9·9; N, 10·9; O, 24·7. Calc. for $C_6H_{13}NO_2$: C, 54·9; H, 10·0; N, 10·7; O, 24·4%). The naphthalene-2-sulphonate,[26] plates (from water), had m. p. 185—187°, mixed

[26] Bergmann and Stein, *J. Biol. Chem.*, 1939, **129**, 609; Hotchkiss, *ibid.*, 1941, **141**, 171.

m. p. 184—186°, $[\alpha]_D$ −10·0° (*c* 3 in 90% v/v ethanol). An authentic sample, m. p. 184—186°, had $[\alpha]_D$ −9·9° under the same conditions (Found: C, 56·8; H, 6·3; N, 3·9; O, 23·3; S, 9·3. Calc. for $C_{16}H_{21}NO_5S$: C, 56·6; H, 6·2; N, 4·1; O, 23·6; S, 9·5%).

Similarly there was obtained from later fractions chromatographically pure D-valine (0·95 g.), plates (from t-butyl alcohol–water), $[\alpha]_D$ −28·3° (*c* 2 in 6N-hydrochloric acid) (Found: C, 51·3; H, 9·2; N, 12·0; O, 27·2. Calc. for $C_5H_{11}NO_2$: C, 51·3; H, 9·5; N, 12·0; O, 27·2%). The *N*-2,4-dinitrophenyl derivative [27] formed prisms [from ether–light petroleum (b. p. 60—80°)], m. p. and mixed m. p. 129—130° (Found: C, 46·8; H, 4·9; N, 14·6. Calc. for $C_{11}H_{13}N_3O_6$: C, 46·6; H, 4·6; N, 14·8%).

Acid Hydrolysis of Sporidesmolic Acid B.—The acid (III) (3·44 g.) was hydrolysed, and the concentrated, rediluted hydrolysate extracted with ether, as described for sporidesmolic acid A. From the ether extract there was obtained crude L-α-hydroxy-β-methylbutyric acid (1·16 g.), m. p. 60—62°, not raised by repeated vacuum-sublimation. This acid contained an odorous, deliquescent contaminant which persisted through attempted purification techniques. It had $[\alpha]_D$ +15·5° (*c* 2·1 in chloroform) (Found: C, 51·3; H, 8·6; O, 40·4%; equiv., 120). The cyclohexylammonium salt formed plates (from toluene), m. p. and mixed m. p. 140—142°, $[\alpha]_D$ −9·0° (*c* 2·0 in water) (Found: C, 60·7; H, 10·5; N, 6·3. Calc. for $C_{11}H_{23}NO_3$: C, 60·8; H, 10·7; N, 6·4%).

By semiquantitative paper chromatography [4] the remainder of the hydrolysate was found to contain valine (*ca.* 0·9 mol.) and *N*-methyl-leucine (*ca.* 1·0 mol.). The amino-acids were separated on a ChroMax paper-roll column in the same way as were the amino-acids from the hydrolysate of sporidesmolic acid A. Chromatographically pure *N*-methyl-L-leucine (1·17 g.) was eluted first, needles (from t-butyl alcohol–water), $[\alpha]_D$ +21·0° (*c* 1·5 in water) (Found: C, 57·9; H, 10·1; N, 10·0; O, 22·4. Calc. for $C_7H_{15}NO_2$: C, 57·9; H, 10·4; N, 9·7; O, 22·0%). The *N*-2,4-dinitrophenyl derivative [6] formed needles (from carbon tetrachloride), m. p. 152—153°, mixed m. p. 151—153°, $\lambda_{max.}$ 388 mμ ($\log_{10} \varepsilon$ 4·26) in N-sodium hydroxide (Found: C, 49·9; H, 5·6; N, 13·4. Calc. for $C_{13}H_{17}N_3O_6$: C, 50·1; H, 5·5; N, 13·5%).

From later fractions there was obtained chromatographically pure L-valine (1·07 g.), needles (from t-butyl alcohol–water), $[\alpha]_D$ +27·2° (*c* 2·2 in 6N-hydrochloric acid) (Found: C, 51·4; H, 9·4; N, 11·95; O, 27·3. Calc. for $C_5H_{11}NO_2$: C, 51·3; H, 9·5; N, 12·0; O, 27·2%). In glacial acetic acid the amino-acid had $[\alpha]_D$ +47·2° (*c* 2·0). Greenstein and his co-workers have recorded a specific rotation of +62° for L-valine in this solvent.[28] However, a sample of authentic L-valine, $[\alpha]_D$ +28·1° (*c* 2·1 in 6N-hydrochloric acid), had $[\alpha]_D$ +47·9° (*c* 2·1) in the same sample of glacial acetic acid. This value was found to be reproducible in different samples of glacial acetic acid, and no explanation can be offered for the discrepancy. The isolated amino-acid gave an *N*-2,4-dinitrophenyl derivative,[27] m. p. and mixed m. p. 129—130° (Found: C, 46·4; H, 5·0; N, 15·2. Calc. for $C_{11}H_{13}N_3O_6$: C, 46·6; H, 4·6; N, 14·8%).

Partial Acid Hydrolysis of Sporidesmolic Acids A (I) *and B* (III).—To the appropriate compound, finely powdered (0·004 g.), was added concentrated hydrochloric acid (0·8 ml.). The solution which resulted on warming was stored at 37°. At daily intervals 0·2 ml. was withdrawn and evaporated to dryness *in vacuo* (KOH). The residue was redissolved in water (0·2 ml.) and examined by paper chromatography; the chromatograms were sprayed with a solution of ninhydrin buffered with pyridine and acetic acid.[4] In the case of sporidesmolic acid A, a weak spot, corresponding in position to leucine, was present on chromatograms prepared from a 1 day hydrolysate. After 4 days, when the experiment was stopped, the leucine spot was strong. No valine was detected at any stage. Similarly, a 1 day partial hydrolysate of sporidesmolic acid B produced a strong spot corresponding to *N*-methyl-leucine. Valine was detected only after 2 days, and then in traces, slightly increased after 3 and 4 days.

Dakin–West Degradation of Sporidesmolic Acids A (I) *and B* (III).—The degradation [8,9] was performed by a simplified technique. The compound (0·003 g.) was heated with pyridine (0·2 ml.) and acetic anhydride (0·3 ml.) in a sealed tube at 110° for 2·5 hr. Volatile material was removed *in vacuo* (KOH, H_2SO_4), and the residue heated in glacial acetic acid (0·1 ml.) and concentrated hydrochloric acid (0·1 ml.) in a sealed tube at 110° for 24 hr., then again evaporated to dryness *in vacuo* (KOH), and this residue was examined in water (0·5 ml.) by paper chromatography. The chromatograms were sprayed with a solution of ninhydrin buffered with pyridine and acetic acid.[4] In each case only valine was detected. No spot attributable

[27] Rao and Sober, *J. Amer. Chem. Soc.*, 1954, **76**, 1328.
[28] Greenstein, Birnbaum, and Otey, *J. Biol. Chem.*, 1953, **204**, 307.

to an amino-ketone was observed, and the appearance of the chromatograms was not altered by adding aqueous ammonia to the reconstituted hydrolysates and passing air through the solutions before applying them to the paper.[9]

The same procedure was used in an unsuccessful attempt to detect a *C*-terminal amino-acid in sporidesmolide I. When it was applied to the crude sporidesmolic acid A obtained by alkaline hydrolysis of the total sporidesmolide fraction (see below), isoleucine was detected in the hydrolysate in addition to valine, but leucine was absent.

Partial Alkaline Hydrolysis of Sporidesmolide I.—The compound (V) (0·480 g.) was dissolved in 1 : 1 chloroform–ethanol (10 ml.) and diluted rapidly to 50·0 ml. with ethanol. Portions (10·0 ml.) were placed in 0·1N-sodium hydroxide (5·0 ml.) and ethanol (10·0 ml.) and after an appropriate interval titrated with 0·1N-hydrochloric acid (control titrations were performed) (Found: alkali consumed after 15 min., 0·96; 30 min., 1·07; 60 min., 1·11 equiv.).

Sporidesmolide I (V) (0·639 g.) was dissolved under reflux in chloroform (4 ml.) and ethanol (64 ml.). To the rapidly cooled solution was added 4N-sodium hydroxide (0·25 ml.) in ethanol (9·75 ml.). After 16 hr. the clear, neutral solution was evaporated to dryness *in vacuo*, the residue extracted with water (15 ml.), and the filtered extract acidified with N-hydrochloric acid (1·5 ml.). After 24 hr. the precipitate was collected, washed with water, and dried *in vacuo* (KOH). The linear *depsipeptide* (0·555 g.) was completely soluble in 4% sodium hydrogen carbonate solution and in chloroform. It recrystallized from dilute acetic acid. The crystalline product (0·472 g.) collapsed to a glass at 117° and melted completely at 174—179°; on cooling of the melt, needles were obtained, with m. p. 175—177°. Attempted recrystallization from carbon tetrachloride furnished an amorphous product, m. p. 178—180°. The compound, recrystallized from dilute acetic acid, had $[\alpha]_D$ −50° (*c* 1·5 in chloroform) (Found: C, 59·2; H, 9·4; N, 8·1. $C_{33}H_{60}N_4O_9,H_2O$ requires C, 58·7; H, 9·3; N, 8·3. Found, in a sample dried to constant weight at 100°: C, 59·7, 60·0; H, 9·4, 9·4; N, 8·3. $C_{33}H_{60}N_4O_9$ requires C, 60·3; H, 9·2; N, 8·5%).

Reaction of the Linear Depsipeptide with Hydrazine.—The above compound (0·75 g.) was dissolved in ethanol (3 ml.) and hydrazine hydrate (0·3 ml.) added. After 40 hr. the clear solution was evaporated to dryness *in vacuo* at 30°. An aqueous solution of the residue was extracted with ether and with ethyl acetate, and acidified (N-hydrochloric acid). The crystalline precipitate, washed with water and dried *in vacuo* (KOH) (0·15 g.), had $[\alpha]_D$ +58·8° (*c* 1·4 in acetic acid), m. p. 196—199°, mixed m. p. with sporidesmolic acid A 199—200°.

Acid Hydrolysis of Sporidesmolide III.—The compound (0·0064 g.) was hydrolysed in a sealed tube as described for sporidesmolide I. Examination of the dried and reconstituted hydrolysate by paper chromatography [4] revealed valine (*ca.* 1·8 mol.), leucine (*ca.* 2·1 mol), and α-hydroxy-β-methylbutyric acid.

Alkaline Hydrolysis of the Total Sporidesmolide Fraction.—The mixture of depsipeptides (5·0 g.) was freed from sporidesmolide III and hydrolysed as described for sporidesmolide I under (*b*) above. The mixed sporidesmolic acids (3·9—4·2 g.) were separated by extraction with chloroform. The chloroform-soluble fraction (2·0—2·3 g.), on recrystallization from 25% (v/v) acetic acid, yielded sporidesmolic acid B (III) (1·4—1·7 g.), m. p. and mixed m. p. 165—166°, $[\alpha]_D$ −108° (*c* 4 in acetic acid). Recrystallization of the chloroform-insoluble fraction gave an acid (1·3—1·7 g.), m. p. 197—199°, mixed m. p. with sporidesmolic acid A (I) 198—200°, $[\alpha]_D$ +58·6° (*c* 4 in acetic acid) (Found: C, 58·8; H, 8·9; N, 8·4. Calc. for $C_{16}H_{30}N_2O_5$: C, 58·2; H, 9·2; N, 8·5%). An acid hydrolysate of this material, prepared and examined by paper chromatography as described for sporidesmolide I, was found to contain α-hydroxy-β-methylbutyric acid, with valine and leucine in approximately equivalent amount; a weak spot corresponding in position to isoleucine was observed. Repeated recrystallization of the acid from 25% (v/v) acetic acid did not remove the isoleucine-containing substance.

Isolation of Sporidesmolic Acid A (I) *from the Chloroform-insoluble Acid obtained by Alkaline Hydrolysis of the Total Sporidesmolide Fraction.*—The above chloroform-insoluble acid (6·6 g.) was dissolved in butan-1-ol (200 ml.) and extracted with 5 ml. portions of 0·05N-sodium hydroxide containing 1% of sodium chloride. Each extract was collected separately and acidified with N-hydrochloric acid (1 ml.). The precipitates were collected and dried *in vacuo* (KOH) and small portions hydrolysed in 6N-hydrochloric acid (0·2 ml.) for 24 hr. at 110° in sealed tubes. The dried and reconstituted hydrolysates were examined by paper chromatography; the material present in earlier extracts was poorer in isoleucine than that from later ones. The crystalline material from the first 40 extracts was pooled and subjected to

the same fractional extraction procedure twice more. Fractions 1—40 from the final series of extractions were mixed and acidified, and the precipitate (0·97 g.) was collected and recrystallized from 25% (v/v) acetic acid. The crystalline product (0·77 g.) had m. p. 200—201°, mixed m. p. with sporidesmolic acid A from sporidesmolide I 200—201°, $[\alpha]_D$ +60·4° (*c* 4 in acetic acid) (Found: C, 58·5; H, 8·9; N, 8·7; O, 24·3. Calc. for $C_{16}H_{30}N_2O_5$: C, 58·2; H, 9·2; N, 8·5; O; 24·2%). An acid hydrolysate of the acid contained no isoleucine.

I am most grateful to Dr. A. Taylor for supplies of crude *P. chartarum* depsipeptides, as well as for his interest and encouragement; to my wife and to Dr. R. L. M. Synge, F.R.S., for helpful discussions; to Mr. I. R. Macdonald and Mr. C. C. Watson for measuring the infrared spectra; and to Mrs. M. E. Carruthers for able assistance.

Plant Chemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand. [*Received, September 18th,* 1961.]