Isolation and Structure of Sporidesmolide IV, a Cyclohexadepsipeptide from *Pithomyces maydicus*

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The cyclodepsipeptides produced by *Pithomyces maydicus* contain a major component, sporidesmolide IV, which has the structure cyclo-(L-α-hydroxyisovaleryl-D-valyl-D-leucyl-L-α-hydroxyisocaproyl-L-valyl-N-methyl-L-leucyl).

FUNGI of the genus *Pithomyces* are remarkable in that their spores are covered with minute spicules of cyclodepsipeptides.[1,2] The first such compound to be isolated was sporidesmolide I (I; $x = 0, y = z = 1$; $R^1 = R^3 = H$, $R^2 = Me$).[3] This is produced by *P. chartarum*, which also produces the lower homologue, sporidesmolide III[4] (I; $x = 0$, $y = z = 1$; $R^1 = R^2 = R^3 = H$). When the growth medium is supplemented with isoleucine, and possibly without such supplementation,[3a] the same species also forms sporidesmolide II[5] (I; $x = 0, y = z =$

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[1] W. S. Bertaud, I. M. Morice, D. W. Russell, and A. Taylor, *J. Gen. Microbiol.*, 1963, **32**, 385.

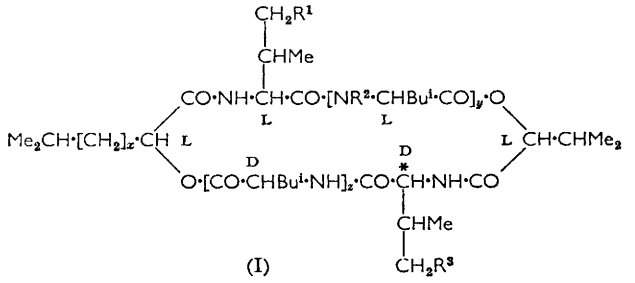
[2] E. Bishop, H. Griffiths, D. W. Russell, V. Ward, and R. N. Gartside, *J. Gen. Microbiol.*, 1965, **38**, 289.

[3] (*a*) D. W. Russell, *J. Chem. Soc.*, 1962, 753; (*b*) M. M. Shemyakin, Yu. A. Ovchinnikov, V. T. Ivanov, and A. A. Kiryushkin, *Tetrahedron*, 1963, **19**, 995.

[4] D. W. Russell, C. G. Macdonald, and J. S. Shannon, *Tetrahedron Letters*, 1964, 2759; Yu. A. Ovchinnikov, A. A. Kiryushkin, and M. M. Shemyakin, *ibid.*, 1965, 1111.

[5] W. S. Bertaud, M. C. Probine, J. S. Shannon, and A. Taylor, *Tetrahedron*, 1965, **21**, 677; M. M. Shemyakin, Yu. A. Ovchinnikov, V. T. Ivanov, A. A. Kiryushkin, and K. Kh. Khalilulina, *Zhur. obshchei Khim.*, 1965, **35**, 1399; M. M. Shemyakin, Yu. A. Ovchinnikov, V. T. Ivanov, and A. A. Kiryushkin, *Tetrahedron Letters*, 1963, 1927.

I; $R^1 = H$, $R^2 = R^3 = Me$; * indicates that the isoleucine residue has the *allo* configuration).



(I)

Another species, *P. cynodontis*,[6] forms a related compound, the cyclotetradepsipeptide angolide [2,7] (I; $x = y = z = 0$, $R^1 = R^3 = Me$; * indicates that this isoleucine residue has the *allo* configuration). Angolide is also produced by *P. sacchari*.[8]

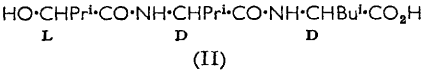
The close morphological similarity between *P. chartarum* and *P. maydicus* (Sacc.) M. B. Ellis [9] having led to the suggestion [10] that they were in fact members of the same species, we undertook an investigation into the spore-surface cyclodepsipeptides of *P. maydicus* IMI98084. It was shown that this isolate produces cyclodepsipeptides which could be readily distinguished from those of *P. chartarum* by their physical properties. On the basis of preliminary chemical and chromatographic studies it was reported that *P. maydicus* IMI98084 produced only one cyclodepsipeptide, which was named sporidesmolide IV.[2] Chemical degradation gave results which led us to assign to this new cyclodepsipeptide the structure (I; $x = y = z = 1$, $R^1 = R^3 = H$, $R^2 = Me$), which is a position isomer of sporidesmolide II, and differs from sporidesmolide I in containing one residue of L-α-hydroxyisocaproic acid in place of L-α-hydroxyisovaleric acid.[11] A compound with this structure was later synthesised by Shemyakin and his colleagues, who reported its identity with the natural product.[12]

Despite this confirmation, we suspected that "sporidesmolide IV" might be a mixture, the most telling evidence being that its molecular rotation in chloroform was 8% lower than that of sporidesmolide I. The addition of one methylene group outside the macrocycle would not be expected to produce such a large change. Accordingly, although "sporidesmolide IV" had separated successively from three different solvents without increase in melting point, further fractionation was attempted. The bulk of the material was soluble in carbon tetrachloride, and after the insoluble portion (which had a much lower specific rotation) had been removed, recrystallisation of the soluble material gave a compound with the same molecular rotation in chloroform as sporidesmolide I; $[M]_D$ −1390°. The name sporidesmolide IV is retained for this compound.

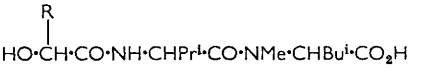
Sporidesmolide IV, $C_{34}H_{60}N_4O_8$, was a neutral compound whose physical properties were similar to those of sporidesmolide I. The two compounds could be readily distinguished by thin-layer chromatography,[2] and sporidesmolide IV was generally the more soluble in organic solvents. There were small differences in their infrared spectra, notably in the region 800—1100 cm.$^{-1}$, the spectra being otherwise almost identical.

Sporidesmolide IV was hydrolysed by acid under vigorous conditions. Paper chromatography of the product permitted tentative identification of the amino-acids valine, leucine, and *N*-methyl-leucine in the proportion 2 : 1 : 1, and of α-hydroxyisovaleric and α-hydroxyisocaproic acid.

On alkaline hydrolysis, sporidesmolide IV consumed two equivalents of base and furnished a mixture of two complex acids. The first of these, $C_{16}H_{30}N_2O_5$, was insoluble in chloroform, and was identified as sporidesmolic acid A (II), previously isolated from alkaline hydrolysates of sporidesmolide I.[3a] This demonstrated the presence in sporidesmolide IV of one residue each of L-α-hydroxyisovaleric acid, D-valine, and D-leucine, and established their sequence.



(II)



(III)

The other acid, $C_{18}H_{34}N_2O_5$, liberated on alkaline hydrolysis of sporidesmolide IV was named sporidesmolic acid C. It was very soluble in chloroform, had no free amino group, and was monobasic. On vigorous acid hydrolysis it gave three products. Two were amino-acids, namely valine and *N*-methyl-leucine; 1 mole of each was obtained by hydrolysis of one mole of sporidesmolic acid C, and both possessed the L configuration. The only other product detected was a hydroxycaproic acid, $C_6H_{12}O_3$. This was identified as α-hydroxyisocaproic acid by vapour phase chromatography of its methyl ester, and by oxidation with sodium bismuthate [13] to isovaleraldehyde. The L configuration of the α-hydroxyisocaproic acid was established polarimetrically.

These findings were consistent with a structure for sporidesmolic acid C in which a dipeptide of L-valine and *N*-methyl-L-leucine was *N*-acylated with L-α-hydroxyisocaproic acid. When sporidesmolic acid C was subjected to Dakin–West reaction,[3a,14] and the product

[6] M. B. Ellis, *Mycol. Pap.*, 1965, no. 103.
[7] D. W. Russell, *J. Chem. Soc.*, 1965, 4664; A. A. Kiryushkin, Yu. A. Ovchinnikov, and M. M. Shemyakin, *Khim. prirod. Soedinenii*, 1965, 58; *Tetrahedron Letters*, 1964, 3313; C. G. Macdonald and J. S. Shannon, *ibid.*, p. 3113.
[8] P. Riches and D. W. Russell, *Biochem. J.*, 1965, **98**, 8P; P. Riches, A. Rothwell, and D. W. Russell, *J. Gen. Microbiol.*, 1967, **46**, 77.
[9] M. B. Ellis, *Mycol. Pap.*, 1960, no. 76.
[10] J. M. Dingley, *N.Z. J. Agric. Res.*, 1962, **5**, 49.
[11] E. Bishop and D. W. Russell, *Biochem. J.*, 1964, **92**, 19P.
[12] A. A. Kiryushkin, Yu. A. Ovchinnikov, and M. M. Shemyakin, *Tetrahedron Letters*, 1965, 143.
[13] W. Rigby, *J. Chem. Soc.*, 1950, 1907.
[14] H. D. Dakin and R. West, *J. Biol. Chem.*, 1928, **78**, 91, 745.

was hydrolysed by acid, examination of the hydrolysate showed that *N*-methyl-leucine but not valine had been destroyed. The alkylamino-acid must accordingly occupy the *C*-terminal position, and sporidesmolic acid C could be formulated unequivocally as (III; R = Bu^{i}), a homologue of sporidesmolic acid B (III; R = Pr^{i}) similarly isolated from alkaline hydrolysates of sporidesmolide I.[3a]

The results of degradation lead to the cyclohexadepsipeptide structure (I; $x = y = z = 1$, $R^1 = R^3 = H$, $R^2 = Me$) for sporidesmolide IV, as had previously been deduced by examination of the impure material.[11] Further purification of the synthetic compound (I; $x = y = z = 1$, $R^1 = R^3 = H$, $R^2 = Me$) has been reported to yield a product with the same physical properties as sporidesmolide IV, whose structure may therefore be regarded as confirmed by synthesis.[15]

The portion of the impure "sporidesmolide IV" which was insoluble in carbon tetrachloride gave on repeated recrystallisation a product with a very small positive specific rotation for the sodium D-line in chloroform solution. The infrared spectrum showed features characteristic of cyclodepsipeptides, and an acid hydrolysate contained amino- and hydroxy-acids. This minor component of *P. maydicus* IMI98084 cyclodepsipeptides was not obtained pure.

Previously it was reported that *P. maydicus* IMI46232 produced cyclodepsipeptides similar to those of IMI98084 but with a lower specific rotation.[2] When these were extracted with carbon tetrachloride, sporidesmolide IV was isolated from the soluble fraction. Both available isolates of the species therefore produce the same major cyclodepsipeptide.

EXPERIMENTAL

Melting points were determined on a Kofler block. Infrared spectra were obtained with a Perkin-Elmer Infracord spectrophotometer. Except where otherwise stated, paper chromatography was performed under the conditions, and with the solvents, used previously for investigating the degradation products of sporidesmolide I.[3a]

Sporidesmolide IV from Pithomyces maydicus (*Sacc.*) *M.B. Ellis*.—Dried sporing felts[2] of *P. maydicus* IMI98084 or IMI46232 were exhaustively extracted with chloroform (Soxhlet). The dark brown extract from 100 g. of fungal tissue was stirred overnight with decolorising charcoal (Norit K; 10 g.), filtered, and the solvent evaporated *in vacuo*. The oily, yellow residue (4—10 g.) was allowed to stand with ether (400 ml.) overnight; the insoluble material was collected, washed with ether, allowed to dry in air, and dissolved in chloroform (100 ml.). The solution was passed through charcoal (10 g.) and then through aluminium oxide (10 g.; B.D.H. "for chromatographic analysis"), and the adsorbents were further washed with chloroform (3 × 50 ml.). The effluents were mixed and the solvent was evaporated, to yield colourless material (2—4 g.), m. p. 226—228°, $[\alpha]_D$ −177 to −189° (*c* 2 in chloroform). Other methods of extraction[2,16] were equally suitable, but that described above is the most economical of time spent in manipulation.

The product thus isolated separated in amorphous form from ethanol, 70% (v/v) acetic acid, or 70% (v/v) pyridine in water, m. p. 227—228°, $[\alpha]_D$ −183 to −195° (*c* 2 in chloroform). A sample, $[\alpha]_D$ −191° (4·0 g.), obtained from IMI98084 was extracted with carbon tetrachloride (Soxhet); the extract (100 ml.) was set aside at 0° for 24 hr. and filtered. The filtrate on evaporation of the solvent left a residue that was recrystallised from 95% (v/v) ethanol. The product (2·8 g.), m. p. 232—233°, had $[\alpha]_D$ −213° (*c* 3 in chloroform). It was recrystallised twice more, to give *sporidesmolide IV* (2·2 g.) as a colourless solid which crystallised in small needles when heated above 200°, m. p. 232—233°, $[\alpha]_D$ −212° (*c* 2 in chloroform), −93° (*c* 2 in acetic acid), $\nu_{max.}$ (Nujol) 3360s, 3270m (NH), 1745s (ester C=O), 1685s, 1640s (amide I), 1535s (amide II), 1000m (C–O–C), 857w cm.$^{-1}$, (hexachlorobutadiene) 1470s (*C*-Me), 1385s, 1365s (Pr^{i}) cm.$^{-1}$ [Found: C, 62·8, 62·8; H, 9·3, 9·1; N, 8·7, 8·65; O, 19·7%; *M* (Rast), 677. $C_{34}H_{60}N_4O_8$ requires C, 62·6; H, 9·2; N, 8·6; O, 19·6%; *M*, 653].

For isolation of sporidesmolide IV from *P. maydicus* IMI46232 the cyclodepsipeptide mixture (7·6 g.), which had $[\alpha]_D$ −176° (*c* 3 in chloroform), was dissolved in chloroform (50 ml.) and the solvent was distilled off; carbon tetrachloride was added from time to time until all chloroform was removed. The volume was adjusted to 150 ml. with hot carbon tetrachloride, the suspension was set aside at 0° overnight, and the precipitate was removed; the filtrate on evaporation of the solvent gave a colourless residue that was recrystallised from 95% (v/v) ethanol. After three recrystallisations, sporidesmolide IV (3·2 g.) was obtained, m. p. and mixed m. p. with a sample from IMI98084 232—233°, $[\alpha]_D$ −212° (*c* 2 in chloroform), −93° (*c* 2 in acetic acid). The infrared spectra of samples prepared from the two isolates were identical [Found: C, 63·3; H, 8·7; N, 8·6; O, 19·8%; *M* (Rast), 550].

Sporidesmolide IV was very insoluble in water, very soluble in chloroform, and sparingly or moderately soluble in other common organic solvents. When heated in an open capillary above 200° it slowly sublimed, and the sublimate did not depress the m. p. of the unsublimed compound. It was not extracted from chloroform by aqueous or aqueous–methanolic acid or alkali, gave no colour with ninhydrin or indicators, and was unaltered by refluxing with acetic anhydride for 2 hr.

Acid Hydrolysis of Sporidesmolide IV.—An acid hydrolysate of sporidesmolide IV (6·5 mg.) was prepared as described for sporidesmolide I[3a] and similarly examined by paper chromatography. It contained amino-acids corresponding in R_f values to valine, leucine, and *N*-methyl-leucine, the amounts being 20·2, 9·7, and 9·7 μmoles. The ether-soluble components of the hydrolysate were separated from the amino-acids and examined by paper chromatography Two acid spots were detected, with the same R_f values as α-hydroxyisovaleric and α-hydroxyisocaproic acids, respectively; the latter acid was not resolved from α-hydroxy-β-methylvaleric acid.

Alkaline Hydrolysis of Sporidesmolide IV.—(*a*) Samples (*ca.* 0·3 g.) were accurately weighed and dissolved with heating in ethanol (20 ml.). To the cooled solutions was added 2N-sodium hydroxide (2·00 ml.). After various periods the excess of alkali was titrated with 0·5N-hydro-

[15] Yu. A. Ovchinnikov, A. A. Kiryushkin, and M. M. Shemyakin, *Zhur. obshchei Khim.*, 1966, **36**, 620.

[16] J. Done, P. H. Mortimer, A. Taylor, and D. W. Russell, *J. Gen. Microbiol.*, 1961, **26**, 207.

chloric acid (Found: alkali consumed by 1 mmole after 30 min., 1·81; 60 min., 1·93; 120 min., 1·98 mequiv.).

(*b*) The cyclodepsipeptide (7·25 g.) was warmed with N-potassium hydroxide in 90% (v/v) ethanol (500 ml.). After a few minutes dissolution was complete, and water (500 ml.) was added. The solution was allowed to stand at room temperature for 1 hr., the volume was reduced to 500 ml. by distillation *in vacuo* below 50°, and 2·5N-hydrochloric acid (250 ml.) was added. After 16 hr. at 0° the precipitate was collected, washed with water, and dried at 100°. The product (6·7 g.) was stirred with chloroform (67 ml.) for 1 hr., and the insoluble material (2·65 g.) was thrice recrystallised from 25% (v/v) acetic acid, to give sporidesmolic acid A [3a,17] (1·75 g.), m. p. and mixed m. p. 200—202°, $[\alpha]_D$ +60° (*c* 4 in acetic acid). The infrared spectrum (Nujol) was identical with those of reference samples obtained from sporidesmolide I or by synthesis [17] (Found: C, 58·35; H, 8·9; N, 8·3%; Equiv., 332. Calc. for $C_{16}H_{30}N_2O_5$: C, 58·2; H, 9·15; N, 8·5%; Equiv., 330). An acid hydrolysate (6N-hydrochloric acid; 110°; 24 hr.), examined by paper chromatography, contained valine, leucine, and α-hydroxyisovaleric acid. Valine, but no leucine, was detected in the hydrolysate of a sample previously heated with pyridine and acetic anhydride by the Dakin–West procedure.[3a,14] Treated with diazomethane, the acid gave methyl sporidesmolate A, m. p. 161—162°, mixed m. p. 160—161°, $[\alpha]_D$ +30·6° (*c* 2·5 in chloroform). The reference sample [3a] had $[\alpha]_D$ +29·5° under the same conditions, and the infrared spectra (Nujol) of the two samples were identical.

The chloroform solution of the remainder of the product obtained by alkaline hydrolysis of sporidesmolide IV gave, on evaporation, a residue (4·05 g.) that was crystallised from 25% (v/v) acetic acid. The product (3·48 g.) had m. p. 171—173° and $[\alpha]_D$ −103° (*c* 0·4 in acetic acid). Repeated recrystallisation of this material from 25% (v/v) acetic acid gave colourless needles of *sporidesmolic acid C* (III; R = Bu^i) (0·33 g.), m. p. 173—174°, $[\alpha]_D$ −107° (*c* 1 in acetic acid), $\nu_{max.}$ (Nujol) 3475s (OH), 3360s (NH), 1730s (CO_2H), 1645s, 1605s (amide I), 1545s (amide II) cm.$^{-1}$, (hexachlorobutadiene) 1470s (*C*-Me), 1415s (*N*-Me), 1385sh, 1365s (Pr^i) cm.$^{-1}$ (Found: C, 60·5, 60·35; H, 9·3, 9·8; N, 7·6, 7·8%; Equiv., 357, 358. $C_{18}H_{34}N_2O_5$ requires C, 60·3; H, 9·6; N, 7·8%; Equiv., 358).

Sporidesmolic acid C was sparingly soluble in water or dilute mineral acids and gave no colour with ninhydrin; it dissolved readily in aqueous sodium hydrogen carbonate or dilute sodium hydroxide, and was reprecipitated by acid. When it was hydrolysed by acid (6N-hydrochloric acid; 110°; 24 hr.), valine and *N*-methyl-leucine (0·96 and 0·97 mole/mole, respectively) were the only ninhydrin-positive compounds detected among the products of hydrolysis. When, before hydrolysis, it was heated with pyridine and acetic anhydride under the conditions of the Dakin–West reaction,[3a,14] valine but no *N*-methyl-leucine was detected in the hydrolysate.

Acid Hydrolysis of Sporidesmolic Acid C.—The acid (362 mg.) was boiled with 6N-hydrochloric acid (40 ml.) for 48 hr. The cooled solution was diluted with water and continuously extracted with ether. Evaporation of the dried (Na_2SO_4) and decolourised (Norit K) extract gave a colourless acidic oil (131 mg.), vacuum-sublimation of which yielded crystals (115 mg.), m. p. 78—79°. A portion of the acid was esterified with diazomethane and investigated by vapour phase chromatography. Samples of α-hydroxyisocaproic, α-hydroxy-n-caproic, and *erythro-* and *threo*-α-hydroxy-β-methylvaleric acids, prepared by nitrous acid deamination of leucine, norleucine, isoleucine, and *allo*-isoleucine, respectively, were similarly esterified, and the esters were used as reference standards. Using a support of acid- and alkali-washed Celite with 10% polyethylene glycol adipate as stationary phase, the retention volumes at 100°, relative to the retention volume of methyl n-octanoate, were: methyl α-hydroxy-n-caproate, 2·60; methyl α-hydroxyisocaproate, 1·99; methyl α-hydroxy-β-methylvalerate: *erythro*, 1·90; *threo*, 1·76; methyl ester of the acid from sporidesmolic acid C, 1·99.

Several more vacuum-sublimations of the acid gave L-α-hydroxyisocaproic acid (100 mg.), m. p. and mixed m. p. 80—81°, $[\alpha]_D$ −12·3° (*c* 1 in water), $[\alpha]_D$ of sodium salt −21·9° (*c* 1 in water). The infrared spectrum was identical with that of a reference sample (Found: C, 54·7; H, 8·8; O, 36·3%; Equiv., 132. Calc. for $C_6H_{12}O_3$: C, 54·5; H, 9·15; O, 36·3%; Equiv., 132).

The acid was further characterised by oxidation using sodium bismuthate [13] to the aldehyde with one less carbon atom. A solution (38·4 mg. in 1 ml. of M-phosphoric acid) was shaken with sodium bismuthate (92 mg.; 87·4% $NaBiO_3$) for 40 min.; the suspension was then cooled in ice and treated with a solution of 2,4-dinitrophenylhydrazine (60 mg.) in 6N-hydrochloric acid (10 ml.). After 30 min. at 0° the precipitate was collected, washed free from soluble bismuth salts with dilute hydrochloric acid, washed with water, and dried at 100°. The orange product (56·5 mg.), m. p. 121—123°, recrystallised from ethanol, gave isovaleraldehyde 2,4-dinitrophenylhydrazone (33·6 mg.), m. p. and mixed m. p. 122—123°, indistinguishable in its infrared spectrum (Nujol) from an authentic sample. In thin-layer chromatography on Silica Gel G, using a triple development with benzene, the hydrazone was indistinguishable from the reference sample but was separated from α-methylbutyraldehyde 2,4-dinitrophenylhydrazone.

The acid hydrolysate of sporidesmolic acid C, after removal of the hydroxy-acid, was evaporated to dryness. The residue of amino-acid hydrochlorides was converted into the free amino-acids with Amberlite CG-120, and the amino-acids were separated by chromatography on a paper roll column, as previously described.[3a] Appropriate fractions were pooled, and the solvents were evaporated, to yield chromatographically pure samples of valine (97 mg.) and *N*-methyl-leucine (126 mg.). The former, after three recrystallisations from aqueous ethanol, had $[\alpha]_D$ +28·3° (*c* 1·7 in 6N-hydrochloric acid), and the infrared spectrum was indistinguishable from that of a reference sample (Nujol) (Found: N, 12·2. Calc. for $C_5H_{11}NO_2$: N, 12·0%). The *N*-methyl-leucine, similarly recrystallised three times, had $[\alpha]_D$ +18·7° (*c* 1·5 in water), and its infrared spectrum was identical with that of the authentic amino-acid (Found: N, 9·66. Calc. for $C_7H_{15}NO_2$: N, 9·65%).

Investigation of the Carbon-tetrachloride-insoluble Cyclodepsipeptides of Pithomyces maydicus IMI98084.—The residue (254 mg.) left after extraction of the total *P. maydicus* cyclodepsipeptide fraction (4·0 g.) with carbon tetrachloride had m. p. 247—250°, $[\alpha]_D$ −9·9° (*c* 1·5 in chloroform). Further extraction with boiling carbon tetrachloride gave an insoluble residue (211 mg.), $[\alpha]_D$ −4·0° (*c* 2 in chloroform), which, after three recrystallisations from ethanol, gave crystals (160 mg.), $[\alpha]_D$ 0·0° (*c* 1·5 in

[17] K. Lübke and E. Schröder, *Z. Naturforsch.*, 1961, **16**b, 847; *Annalen*, 1963, **665**, 205.

chloroform). An acid hydrolysate of this substance (650 μg.) contained amino-acids tentatively identified as valine (1·00 μmole), leucine (1·04 μmoles), and *N*-methylleucine (0·99 μmole), together with an acid mixture identified by vapour phase chromatography of the methyl esters as consisting largely of α-hydroxyisocaproic acid with a trace of α-hydroxyisovaleric acid. Recrystallisation from ethanol gave a product, $[\alpha]_D$ +1·2° (*c* 1·3 in chloroform) (Found: C, 62·1; H, 8·8; N, 6·6; O, 22·55%). One more recrystallisation gave a product, $\nu_{max.}$ (Nujol) 3335m, 3240s, 1740s, 1680s, 1640s, 1515s, 1400m cm.$^{-1}$ (Found: C, 63·35; H, 9·0; N, 6·1; O, 21·5%).

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