Spore Surface Depsipeptides in Some *Pithomyces* Species

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(Received 23 September 1964)

SUMMARY

Spicules morphologically similar to those present on the spores of *Pithomyces chartarum* invest the spores of several other *Pithomyces* species. Depsipeptides are quantitatively removed from dried mycelial felts by brief chloroform washing, and their amount is proportional to the number of spores present. Three pure spore-coat depsipeptides have no demonstrable antibiotic activity.

INTRODUCTION

The fungus *Pithomyces chartarum* produces a group of metabolic products, the sporidesmolides. These compounds, which have physical properties similar to those of lipids, are cyclic depsipeptides (Russell, 1962). Consideration of analytical data prompted the conjecture (Russell & Brown, 1960) that these materials formed the spiculate spore-coat visible in electron micrographs of spores of this species, a suggestion later confirmed by several independent lines of evidence (Bertaud, Morice, Russell & Taylor, 1963). We have now extended this work by a chemical and electron microscopical examination of some other fungi of the genus *Pithomyces*.

METHODS

Organisms. Not all the species of *Pithomyces* listed by Ellis (1960) could be obtained for this study, but all of those available were examined. Isolate c of *Pithomyces chartarum* (Dingley, Done, Taylor & Russell, 1962) was obtained from Dr Drew Smith, Ruakura Animal Research Station, Hamilton, New Zealand. Dr M. B. Ellis, Commonwealth Mycological Institute, Kew, England, kindly supplied other isolates, which were as follows: *P. maydicus* (IMI 98084, IMI 46232); *P. sacchari* (IMI 102686); *Pithomyces* 'species v' (Dr M. B. Ellis, personal communication; IMI 102682); *Pithomyces* species unknown (IMI 101184). Other *P. chartarum* isolates, kindly supplied as dried mycelial felts by Miss Joan M. Dingley (Plant Diseases Division, D.S.I.R., Auckland, New Zealand), were PDD 36C, PDD 6324 and PDD 6323.

Cultivation of organisms. Reasonably stable high-sporing cultures were obtained by single spore or single hyphal tip transfers from high-sporing sectors of the isolates. These were maintained on plates of potato glucose agar (Oxo Ltd., London)

at 25° in constant light of approximately 3 ft.c. provided by 'Osram' White fluorescent tubes (Osram, (G.E.C.) Ltd., North Wembley, Middlesex) for 8 hr alternating with 16 hr of complete darkness. For electron microscopy, spores were taken from potato glucose agar plates at 10–14 days. For isolation of depsipeptides, the organisms were grown on potato glucose agar slopes, and spores washed from these with sterile 0.05% (v/v) Lissapol N (Imperial Chemical Industries Ltd., Millbank, London) were used for inoculum. The medium for *Pithomyces chartarum* was potato + carrot extract (Done, Mortimer, Taylor & Russell, 1961), and that for IM101184 and *P. maydicus* IM146282 was the salts + glucose + asparagine medium of Ross (Butler, Russell & Clarke, 1962). For *P. maydicus* IM198084 and for IM102682 this medium was enriched with yeast extract (0.1%, w/v; 'Difco' brand, Baird & Tatlock, Ltd., Chadwell Heath, Essex). The medium (100 ml.) was placed in 1 l. Roux bottles or one-pint milk bottles (44 mm. internal neck diameter), plugged with cottonwool. The vessels were autoclaved at 120° for 15 min., cooled, and inoculated with a spore suspension (0.1 ml.) containing 10^5 spores/ml. The bottles were incubated in a horizontal position for 2 weeks at 25°, while being illuminated intermittently as above. At the end of the incubation the fungus was collected, washed and dried as previously described (Done *et al.* 1961).

Electron microscopy. Whole spores were examined by using the technique described to us by Mr W. S. Bertaud (personal communication). A dry specimen of spores was obtained on carbon-coated collodion films on specimen grids by inverting a mature plate culture over the grids, and tapping it until a sparse sprinkling of spores had been released. The preparation was then shadowed at an angle of $\tan^{-1} 1/3$ (with either carbon or gold + palladium wire) and examined in a Philips EM 100 electron microscope.

Analytical methods

Spore number, mycelial dry weight and reducing sugars were determined as before (Done *et al.* 1961), and *total nitrogen* was estimated by a micro-Kjeldahl procedure (Chibnall, Rees & Williams, 1943). *Specific rotations* for the D line of the sodium spectrum were measured for 0.5–2.0% (w/v) solutions in chloroform at room temperature in a 1 dm. tube. *Infrared spectra* were determined for paraffin mulls with a Perkin-Elmer 'Infracord' spectrophotometer, and *ultraviolet spectra* were measured for ethanol solutions with a Unicam S.P. 500 spectrophotometer. *Melting points* were determined on a Kofler block and are corrected.

Chromatography. Thin-layer plates (250 μ) of Kieselgel G (E. Merck A. G., Darmstadt, Germany) were washed chromatographically in methanol and concentrated hydrochloric acid (9 + 1, by vol.) to remove traces of iron, and dried for 1 hr at 110°. Depsipeptide solutions (4 μ l.; 2%, w/v, in chloroform) were applied and the solvent allowed to evaporate. The plates were developed in chloroform + ethyl acetate (4 + 1, by volume) and dried for 5 min. at 100°. Spots were revealed by spraying the dried plates with iodine (0.25%, w/v) in diethyl ether, and stabilized by further spraying with an aqueous solution of soluble starch (1%, w/v) and potassium iodide (1%, w/v).

Depsipeptides. The dried fungal tissue (5 g.), coarsely powdered, was stirred with chloroform (50 ml.) for 5 min. and collected by filtration. Two further extractions were made. The combined filtrates were evaporated to dryness *in vacuo* at 50°.

To the residue was added diethyl ether (20 ml.) which had previously been saturated with the appropriate depsipeptide and filtered. After 16 hr the insoluble residue was collected at the centrifuge, washed with depsipeptide-saturated ether (10 ml.) and allowed to dry at room temperature. The crude depsipeptide was dissolved in chloroform (4 ml.) and the solution decolorized by passage through activated carbon as previously described (Done *et al.* 1961). It was advantageous to support the carbon on a similarly sized pad of aluminium oxide (British Drug Houses Ltd., Poole, Dorset; 'for chromatography'). The effluent and washings (3 × 2 ml.) were mixed, the volume adjusted to 10.0 ml. and the optical rotation determined. The chloroform was evaporated on a steam bath and the residue dried to constant weight at 110°. The technique used on the preparative scale (30–40 g. dried mycelial felts) was identical except that pure diethyl ether was used.

Residues obtained in this way were usually electrostatically charged. The charge was completely removed by moistening the solid with diethyl ether and allowing the ether to evaporate.

Tests for antibiotic activity

Depsipeptides were screened for antimicrobial activity by an agar plate technique. They were dissolved in chloroform to give solutions of 10 mg./ml. Paper discs, 7 mm. diameter, cut from Whatman no. 1 filter paper, were moistened with 0.01 ml. of solution by means of a micropipette, and sterilized by dry heat at 160°. The impregnated discs, each containing 100 µg. depsipeptide, were then applied to the surfaces of agar plates previously seeded with *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *Escherichia coli*, *Candida albicans* or *Trichophyton mentagrophytes*. The type and depth of medium used, the size of the inoculum and other conditions were adjusted to afford maximum sensitivity in the tests. After appropriate incubation periods the plates were examined for the presence of zones of partial or complete inhibition around the discs. Discs containing 10 µg. Framycetin sulphate (Laboratoires Roussel, Boulevard des Invalides, Paris 7e, France) were used as positive controls in the antibacterial tests, and discs containing 100 µg. Episol (Crookes Laboratories, Gorst Road, London, N.W. 10) were used as positive controls on the *Candida* and *Trichophyton* test plates.

RESULTS

Spores from all isolates grown on potato glucose agar and examined in the electron microscope were found to bear spicules similar to those present on *Pithomyces chartarum* spores (Bertaud *et al.* 1963; Pl. 1, figs. 1–5). The spicules were apparently easily detached, even by the relatively gentle methods used to prepare the specimens, and frequently the grid around a spore showed numbers of free spicules (Pl. 2, fig. 6). These often looked more obviously crystalline than the undetached spicules, which frequently, particularly in early experiments with carbon shadowing, appeared to have melted partially and then resolidified (Pl. 2, fig. 7). This may perhaps be explained by the greater rate of heat loss from a small crystal when resting on a grid, as compared with that from a spicule surrounded by many others and suspended in a vacuum.

On potato+carrot extract at 25° the degree of sporulation varied widely from isolate to isolate and from batch to batch of the same isolate. It was therefore

necessary to determine, for each isolate, conditions for obtaining satisfactory sporulation. We were thus led to a more general study of environmental effects on sporulation in this genus, the results of which will be described in a later publication. The *Pithomyces sacchari* isolate spored only sparsely under all conditions tried. The other isolates were grown at 25° for 2 weeks on defined or partially defined media, except for the *P. chartarum* isolates which were grown on potato + carrot broth. Early in this work we found that sporulation of *P. chartarum* was affected by light, and thereafter we adopted standard conditions of illumination for all cultures.

Dried felts of each of the other isolates were first analysed by the technique used for determining sporidesmolides in *Pithomyces chartarum* (Done *et al.* 1961), but with the use of pure ether in place of sporidesmolide-saturated ether. In every case but that of *P. sacchari*, materials were isolated whose properties were similar to those of sporidesmolides: they were insoluble in water, very difficult to wet, very sparingly soluble in ether and readily soluble in chloroform. Shaken with chloroform + methanol + water (10 + 7 + 3, by vol.) they were recovered quantitatively from the lower phase, and it was therefore clear that they could be isolated quantitatively in the same way as the sporidesmolides. However, this technique includes a preliminary overnight extraction with hot methanol, and can give no indication of the possible location of the substances extracted. Compounds located on the spore surface and readily soluble in chloroform should be removed from dried mycelial felts by brief washing with this solvent. Sporidesmolides were accordingly extracted from dried *P. chartarum* felts by brief washing with chloroform, and were readily and quantitatively isolated from the extracts in pure form. When the chloroform-washed fungal tissue was dried and further extracted with hot methanol (Done *et al.* 1961), no more sporidesmolides were obtained.

Dried felts of the other isolates were examined in the same way, except that the ether used was saturated with the material isolated from the appropriate organism by the earlier method. Occasionally a final overnight stirring with chloroform replaced the extraction with hot methanol. Crystalline ether-insoluble materials were completely removed by three chloroform washes, and were shown by infrared spectroscopy to be identical with the products isolated previously. Spicules were absent from chloroform-washed spores. The accessibility of the isolated substances, as shown by the results in Table 1, was similar to that of the sporidesmolides, and this was consistent with their being similarly located. Such results would also be expected if three chloroform washes sufficed to extract all chloroform-soluble material completely. This was not the case, however, for, in other routine experiments, stirring the residue overnight with chloroform invariably removed considerable amounts of extractive. This material, which was readily and completely soluble in ether and may have been lipid derived from the cell wall (Russell, Sturgeon & Ward, 1964) or cytoplasm, was not examined further.

The chloroform-soluble ether-insoluble materials isolated in this way were examined by thin-layer chromatography (Table 2). Use of the non-specific detecting reagent iodine allowed the purity of the isolated materials to be assessed. The *Pithomyces* species IM101184 and IM102682 both gave residues which produced a single spot when examined in this way. The R_f values were identical, and recrystallization from ethanol gave, in both cases, the same compound, angolide (Russell

& Ward, 1964), $C_{22}H_{38}N_2O_8$, m.p. 261–262°, $[\alpha]_D -83^\circ$. A single spot was also given by the residue obtained from *P. maydicus* IMI98084, and recrystallization from pyridine and water (7+3, by vol.) furnished a second compound (Bishop & Russell, 1964), $C_{34}H_{60}N_4O_8$, m.p. 227–228°, $[\alpha]_D -195^\circ$, for which we propose the name sporidesmolide IV. The residue from *P. maydicus* IMI46232 was indistinguishable from that obtained from *P. maydicus* IMI98084 in chromatographic behaviour, melting point and infrared spectrum. However, it had a lower specific rotation ($[\alpha]_D = -183^\circ$) even after two recrystallizations, and appears therefore to be a very closely related compound, or a mixture (Table 3).

Table 1. *Removal of depsipeptides by chloroform washing of dried powdered mycelial felts of some Pithomyces species*

Mycelial felts were stirred with chloroform for 5 min. and filtered. After three such extractions, either a fourth overnight extraction was performed, or depsipeptides were determined in an extract prepared by continuous overnight extraction with hot methanol.

Batch no.	Isolate	Wt. (g.)	Wt. of depsipeptide (mg.) extracted by				
			chloroform			or	methanol
			1	2	3	4	
2	<i>P. chartarum</i>	21.6	453	98	25	—	0
27	IMI 101184	30.3	206	35	11	—	0
34	IMI 101184	10.9	107	31	0	—	—
29	<i>P. maydicus</i> (IMI 98084)	35.2	236	7	5	—	0
36	IMI 102682	8.4	18		0	—	—
54	IMI 102682	7.2	21		0	—	—
49	IMI 101184	23.8	173		—	0	—
32	<i>P. maydicus</i> (IMI 98084)	35.2	354		—	0	—
55	<i>P. maydicus</i> (IMI 46232)	22.1	962		0	—	—

Table 2. *Behaviour of some Pithomyces depsipeptides on thin-layer chromatography*

The chromatography was done with Kieselgel G, and solvent chloroform+ethyl acetate (4+1, by vol.).

Compound	R_F
Sporidesmolide III	0.26
Sporidesmolide I	0.54
Sporidesmolide IV	0.62
Angolide	0.70

Thin-layer chromatograms prepared from the total sporidesmolide fraction of *Pithomyces chartarum* isolate 'C' revealed with iodine two spots corresponding to the known constituents, sporidesmolide I (Russell, 1962) and sporidesmolide III (Russell, Macdonald & Shannon, 1964). The spot due to sporidesmolide I possessed a 'beard' which may have been due to the presence of a higher homologue of sporidesmolide I since its position coincided approximately with that of sporidesmolide IV. The same chromatographic pattern was also given by residues from other isolates of *P. chartarum*, and the physical and chemical properties of the sporidesmolide mixtures were the same (Table 3).

That the two new compounds were cyclic depsipeptides was suggested by their high melting points and specific rotations, by their neutral character, and in particular by their infrared spectra, in which absorptions due to ester and amide linkages were present. Neither had other than end-absorption in the ultraviolet region. Preliminary chemical studies, briefly reported elsewhere, have confirmed this identification (Russell & Ward, 1964; Bishop & Russell, 1964).

Fig. 1

Fig. 2

Fig. 1. Utilization of medium constituents and production of depsipeptides by *Pithomyces maydicus* IMI 98084 (I) and *Pithomyces* IMI 101184 (II). A, Reducing sugar; B, nitrogen; C, mycelial dry weight (all in mg./ml.); D, spore count $\times 10^{-6}/\text{ml.}$; E, depsipeptides, $\text{mg.} \times 10^{-1}/\text{l.}$

Fig. 2. Spore count/ml. medium as a function of depsipeptide production. ●—●, *Pithomyces maydicus* IMI 98084; ×—×, *Pithomyces* IMI 101184. Figures in parentheses indicate the age of culture (days) at which the particular analysis was made.

Table 3. *Spore-surface depsipeptides of some Pithomyces species*

Isolate	Species	Depsipeptides identified	Yield (mg./l. medium)
C			
PDD 36C	<i>P. chartarum</i>	Sporidesmolides I and III	$\left\{ \begin{array}{l} 100-300 \text{ (15)} \\ 9 \text{ (1)*} \\ 68 \text{ (1)*} \\ 138 \text{ (1)*} \end{array} \right.$
PDD 6324			
PDD 6323			
IMI 98084	<i>P. maydicus</i>	Sporidesmolide IV	77-230 (17)
IMI 46232	<i>P. maydicus</i>	Unknown	481 (1)
IMI 101184	Not assigned	Angolide	20-160 (9)
IMI 102682	'Species v'	Angolide	11-15 (2)

Figures in parentheses refer to the number of experiments performed.

* Identified by physical properties of mixture and thin-layer chromatography only.

The isolate of *Pithomyces sacchari* examined formed few spores and yielded little extractive to chloroform. The extract gave two slow-moving spots on a thin-layer chromatogram, and an acid hydrolysate of the material present was ninhydrin-positive. There was too little of this material for further investigation; it seems very likely that the extract contained peptide-like substances.

Cultures of IMI 101184 and *Pithomyces maydicus* IMI 98084 were serially harvested and analysed, with results shown in Fig. 1; repetition of the experiments gave similar

results. The two species produced, respectively, angolide and sporidesmolide IV, in amounts proportional to the number of spores present in the cultures at harvest (Fig. 2).

Antibiotic tests. Sporidesmolides I and IV and angolide, tested for antibiotic properties, produced no inhibition zones on the test plates, indicating that they possessed little or no activity. The positive control discs all produced zones of inhibition, ranging in diameter from 12 mm. (*Streptococcus pyogenes*) to > 50 mm. (*Trichophyton mentagrophytes*).

DISCUSSION

Bertaud *et al.* (1963) gave very complete evidence that the spores of *Pithomyces chartarum* were covered with spicules of sporidesmolides. Earlier isolations of sporidesmolides from dried pasture samples (White, 1958; Perrin, 1959; Sandos, Clare & White, 1959; Russell, Synge, Taylor & White, 1962) and their correlation with numbers of *P. chartarum* spores (Thornton & Sinclair, 1960) make it clear that this is not an *in vitro* phenomenon only, but that these compounds are also produced by the organism under natural conditions in the field. The present work provides analytical and electron-microscope evidence for a similar feature in each of the *Pithomyces* isolates examined. We infer that the possession of a crystalline depsipeptide spore coat is a general feature of fungi of the genus.

It has been suggested (Bishop & Russell, 1964) that depsipeptide analysis in this genus is a simple and useful taxonomic aid. Thus, all isolates of *Pithomyces chartarum* examined in the present work gave depsipeptides whose pattern of spots on thin-layer chromatograms was identical, and was readily distinguished from the single spot given by both the isolates of *P. maydicus* examined. Since it has been reported (Dingley, 1962) that spore morphology is not reliable in distinguishing between the two species, the simple technique described here could be helpful in cases where differentiation was important, as in studies on the pathogenesis of facial eczema.

It was suggested (Bertaud *et al.* 1963) that because of their chemical similarity to certain antibiotics the *Pithomyces chartarum* depsipeptides might confer a selective advantage on this organism in its natural habitat by inhibiting the growth of bacteria. To demonstrate conclusively the absence of antibiotic activity is impossible, but neither the present findings nor those of other workers (Shemyakin *et al.* 1963) provide any evidence to support this suggestion. However, as pointed out to us by Dr A. Taylor (personal communication), none of the organisms against which the compounds were tested is a likely neighbour of *Pithomyces* spp. in the field.

It was also suggested earlier that the spicules might contribute to the water-repellent properties of the spores. This effect, if present, might be of more general interest, for spores of *Aspergillus* and *Penicillium* contain cyclic peptides that may be on the surface (Sumiki & Miyao, 1952), and these spores are also water-repellent. The experiments of Davies (1961) suggested that water-repellent properties may be important in spore dispersal; this is supported for *Pithomyces chartarum* by the observations of Crawley, Campbell & Smith (1962). However, spores of this organism also bear a surface layer of lipid (Bertaud *et al.* 1963). Whether the depsipeptide spicules significantly augment water-repellency is therefore open to doubt, particularly as they are so easily detached. There is no compelling reason to suppose

that the spore-surface depsipeptides possess a 'function', i.e. that their presence confers a selective advantage. They may be no more than excretory products formed by an aerial structure. In the absence of more efficient competing forms, the apparent wastefulness of such a situation has no selective significance.

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EXPLANATION OF PLATES

PLATE 1

Electron micrographs of whole spores of *Pithomyces* species shadowed with gold + palladium, all $\times 4950$.

Fig. 1. *Pithomyces maydicus* IMI98084.

Fig. 2. *P. maydicus* IMI46232.

Fig. 3. *P. sacchari* IMI102686.

Fig. 4. *Pithomyces* 'species v', IMI102682, with detached spicules.

Fig. 5. *Pithomyces* IMI101184.

PLATE 2

Fig. 6. Profile electron micrograph of a spore of *P. sacchari* IMI102686, showing detached spicules; $\times 7200$.

Fig. 7. Profile electron micrograph of a spore of *Pithomyces* 'species v', IMI102682, showing partially melted appearance of spicules; $\times 30,000$.