The Spore-Surface Depsipeptide of *Pithomyces sacchari*

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SUMMARY

The spore-surface spicules of *Pithomyces sacchari* contain the cyclotetra-depsipeptide, angolide.

INTRODUCTION

Spores of species of the genus *Pithomyces* (Ellis, 1960, 1965) are covered with minute easily detached spicules. In *P. chartarum*, *P. maydicus* and *P. cynodontis* (IMI101184; Ellis, 1965) these spicules are composed largely or entirely of cyclo-depsipeptides (Russell, 1966), the chief components being sporidesmolide I, sporidesmolide IV, and angolide, respectively (Bertaud, Morice, Russell & Taylor, 1963; Bishop *et al.* 1965). The spore-surface spicules of *P. sacchari* (Speg.) M. B. Ellis have not hitherto been examined chemically, because the isolate available, IMI02686, formed very few spores when grown under the same conditions as the other three species (Bishop *et al.* 1965). We now report experiments, with this and two other isolates of *P. sacchari*, which show that the spicules in this species contain angolide. A preliminary account has already been given (Riches & Russell, 1965).

METHODS

Organisms

Isolate IMI02686 of *Pithomyces sacchari* was obtained from the Commonwealth Mycological Institute. Dr M. O. Moss (Tropical Products Institute, Grays Inn Road, London, W.C.1) sent us an isolate, TP122, which was also disposed (Dr M. B. Ellis, personal communication) as *P. sacchari*. This isolate, which spored sparsely on potato glucose agar at 25°, was designated TP122a. From it, by single-spore isolation from a vigorously sporing sector, was obtained TP122b (see p. 83). The two isolates were distinguishable only by their different sporulation intensities. Mycelial colonies growing on potato glucose agar at 25° are superficial, olive green when young becoming black later. Hyphae are of two types, those in contact with the substratum consisting of main branches with laterals radiating as if from a common origin, and aerial hyphae forming a loose irregular network. Conidiophores form from modified aerial hyphae, usually where several of these approximate and form many anastomoses. Aerial hyphal elements are $3.1 \times 19.5 \mu$; conidiophores, 2–5 per hyphal element, are born randomly; conidia are born singly, with 2–5 transverse septa; the distal septum is occasionally oblique. Spores with 2 septa are 14–18 μ long; with 3, 18–22 μ ; with 4, 22–28 μ ; with 5, 27–36 μ ; all spores are 6–8 μ wide at the widest point.

The *Pithomyces chartarum* used was a new isolate, obtained from a high-sporing

sector of a culture of isolate 'c' (Done, Mortimer, Taylor & Russell, 1961) which during repeated subculture on potato glucose agar had lost its capacity to spore freely. Grown in surface culture on potato + carrot broth at 25° it spored profusely and produced the typical mixture of sporidesmolides (Bishop *et al.* 1965) in a yield of more than 300 mg./l.

Cultivation of organisms

The isolates were maintained by subculture on potato glucose agar plates at 25°.

Agar cultures. Each isolate of *Pithomyces sacchari* was grown on the salts + glucose + asparagine medium of Ross (Ross & Thornton, 1962; Butler, Russell & Clarke, 1962) to which was added 2% (w/v) agar. This medium, in 100 ml. portions, was distributed into 1 pint milk bottles (44 mm. internal neck diameter) which were plugged with cottonwool, autoclaved (20 min. at 120°), allowed to cool in a horizontal position and inoculated with a mycelial suspension (2 ml.) prepared as described for *P. chartarum* (Russell, Sturgeon & Ward, 1964). Cultures were incubated at 25° under a standard light regime (Bishop *et al.* 1965) for 2–3 weeks.

Liquid surface cultures were grown in 1 l. Roux bottles as previously described (Bishop *et al.* 1965) on Ross medium containing 0.1% (w/v) yeast extract (Difco).

Shake-flask cultures were grown in plugged 2 l. conical flasks each containing sterile Ross + yeast extract medium (200 ml.), which were inoculated with a spore suspension (0.1 ml. containing 10⁵ spores) and incubated on a Gyrotory Incubator Shaker (New Brunswick Scientific Inc., New Brunswick, N.J.) at 26° and 250 strokes/min.

Harvesting and extraction

Agar cultures. Chloroform (50 ml.) was rocked gently over the surface of each culture for 5 min. and decanted through Whatman no. 1 silicone-treated filter paper (H. Reeve Angel and Co. Ltd., London) to remove entrained water. The filtrates from three successive extractions were mixed and evaporated to dryness *in vacuo* at 50°. To the residue was added diethyl ether (2.5 ml. for each culture extracted). The depsipeptides were isolated from the ether-insoluble material and recrystallized from ethanol to constant melting point and specific rotation.

Liquid cultures were harvested at intervals, and spore counts and dry-weight determinations made; depsipeptide analyses were done, using ether saturated with angolide.

In one experiment, five 2-week liquid cultures of *Pithomyces chartarum* were mixed with five similar cultures of *P. sacchari* TP122b in a Waring Blendor, and the chopped felts collected, washed, dried and coarsely powdered. The powder (10.4 g.) was stirred with chloroform (20 ml.) in a sintered-glass Büchner funnel (porosity 4) for 15 sec. and the extract removed by suction. Three more similar extractions were made. The powdered felts were then completely extracted with chloroform in a Soxhlet extractor. From each of the five extracts so obtained, total depsipeptides were isolated by using ether saturated with respect to both angolide and the total sporidesmolide fraction of *P. chartarum* (Done *et al.* 1961).

Spore profiles were examined in the electron microscope as before (Bishop *et al.* 1965).

Analytical methods

Except where otherwise stated, details of or references to the analytical methods used in this work were given by Bishop *et al.* (1965).

Isoleucine + alloisoleucine in cyclodepsipeptide hydrolysates were determined by paper chromatography in a solvent system that did not separate them. Spots (10 μ l.) of an acid hydrolysate of authentic angolide (Russell, 1965*a*) or of a presumptive angolide sample, reconstituted to contain the material from 4.26 mg. cyclodepsipeptide/ml., were applied to Whatman no. 3MM paper. The paper, bearing three spots each of sample and standard arranged alternately, was chromatographed at 25° in the descending sense for 16 hr with the solvent *t*-butyl alcohol + 4.25N-ammonia (4+1, by vol.; Vining & Taber, 1957) and dried overnight in a stream of air at room temperature. It was then dipped in a solution of ninhydrin (0.1%, w/v) in acetone and heated at 105° for 15 min. All samples gave a single spot corresponding exactly with that given by the standard. The spot intensities were measured by reflectance, on both sides of the paper, with a Chromoscan densitometer (Joyce, Loebel and Co. Ltd., Gateshead-on-Tyne), with the dark green filter no. 5025 and the slit no. 5007 supplied for the instrument, which was operated at maximum sensitivity and with a chart speed:strip speed ratio of 1:1. The six readings for the sample were averaged (*T*) as were those for the standard (*S*). The total amount of isoleucine + *alloisoleucine*, in mmoles/426 mg., was $2T/S$, since 1 mole of angolide yields 1 mole of each amino acid on hydrolysis. In view of the close correspondence of the sample and standard readings no standard curve was used. In separate experiments it was established that the densitometer reading was proportional to the amount of amino acid in the range 0.02–0.2 μ mole.

Separation of isoleucine from *alloisoleucine* in the system *t*-amyl alcohol + acetic acid + water (20 + 1 + 20, by vol., lower phase; Gray, Blake, Brown & Fowden, 1964) was insufficient for this method to be used for their separate determination, but enabled a visual estimate of the relative amounts to be made.

Partial hydrazinolysis was performed, and the products examined, as described by Russell (1965*a, b*).

RESULTS

Profile electron micrographs of spores of *Pithomyces sacchari* TP122a and TP122b showed the presence of spore-surface spicules similar to those observed earlier in IM102686 (Bishop *et al.* 1965) (Pl. 1, figs. 1, 2). Spicules were absent from spores which had been rinsed with chloroform on the grids before being shadowed.

Isolation and identification of angolide

All three isolates of *Pithomyces sacchari* spored on the agar medium. Chloroform that had been left for a short time in contact with the surface of such sporing cultures contained depsipeptides, which were isolated from the chloroform extracts by an established technique (Bishop *et al.* 1965) and shown to be homogeneous by thin-layer chromatography. The yields (mg./l. culture fluid) were 48 and 18 for IM102686, 146 for TP122a and 241 for TP122b. The materials so obtained, after being purified by recrystallization, were identified as angolide, the cyclotetradepsipeptide previously (Bishop *et al.* 1965) isolated from *P. cynodontis* IM101184. The physical and chemical

Table 1. *Physical properties of pure depsipeptides from isolates of Pithomyces sacchari*

Property	Value determined for:			
	Depsipeptide isolated from			Angolide
	IMI02686	TPI122 a	TPI122 b	
M.p.	261–262°	261–262°	261–262°	261–262°
Mixed m.p.*	261–262°	261–262°	261–262°	.
R_F †	0.61	0.60	0.61	0.60
$[\alpha]_D$ ‡	–82°	–81°	–82°	–83°
M_s	431	433	409	443

* With authentic angolide.

† In thin-layer chromatography on Kieselgel G, and solvent chloroform + ethyl acetate (4 + 1 by volume).

‡ Specific rotation, for the sodium D line, in chloroform solution.

§ Molecular weight, micro-Rast method. Calculated molecular weight of angolide = 426.

Table 2. *Elemental analysis of depsipeptides from isolates of Pithomyces sacchari*

Element	Percentage found in depsipeptide from:			$C_{22}H_{38}N_2O_6$ requires
	IMI102686	TPI122 a	TPI122 b	
Carbon	61.5	61.6	61.6	61.9
Hydrogen	9.1	8.8	8.8	9.0
Nitrogen	6.7	6.85	6.65	6.6

Table 3. *Properties of the products of chemical degradation of depsipeptides from isolates of Pithomyces sacchari*

Degradation product	Depsipeptide isolated from:			Angolide
	IMI102686	TPI122 a	TPI122 b	
Components of a total acid hydrolysate				
α -Hydroxyisovaleric acid*	+	+	+	+
Isoleucine*	+	+	+	+
Alloisoleucine*	+	+	+	+
Isoleucine + alloisoleucine†	2.02	2.07	2.03	2.00
Properties of hydrazinolysis products‡				
U_{REL}	1.02	0.98	0.98	1.00
R_F (pic)	0.61	0.61	0.61	0.61
R_F (TLC)§	0.41, 0.48	0.39, 0.46	0.40, 0.47	0.40, 0.48

* In the hydrolysate of authentic angolide, the amounts of the degradation products are given arbitrarily as +; in the hydrolysates of the other samples, the symbol + indicates that no difference in the amount of the degradation product could be detected by visual inspection of the chromatograms of standard and sample.

† In millimoles released on hydrolysis of 426 mg. of the sample.

‡ U_{REL} = electrophoretic mobility on paper at pH 2 relative to the products from angolide; R_F (pic) = R_F of the picryl derivatives on Whatman 3 MM paper with solvent 15% (w/v) dipotassium hydrogen phosphate in water; R_F (TLC) = R_F in thin-layer chromatography with Kieselgel G and solvent benzene + ethanol (2 + 1, by vol.).

§ Two spots.

evidence on which this identification is based is presented in Tables 1–3. In addition, the *P. sacchari* depsipeptides and authentic angolide had identical infrared spectra and optical rotatory dispersion curves. The latter were kindly measured for us by Dr V. T. Ivanov as a part of his study (to be published) of conformation in cyclic peptides and cyclodepsipeptides.

Fig. 1. Dry weight, spores and angolide production in *Pithomyces sacchari* TP1122b grown in liquid surface culture at 25°. Results are given for two separate experiments, open symbols being used for one and full symbols for the other. ● and ○, spore count $\times 10^{-5}$ /ml.; ▲ and △, angolide (mg./l.); ■ and □, mycelial dry weight (mg./ml. culture).

Morphological location of angolide in *Pithomyces sacchari*

Having thus established that three isolates of *Pithomyces sacchari* produced angolide, we wished to determine whether, as in other species, the depsipeptide was present in the spore-surface spicules. Only one of the isolates, TP1122b, spored in liquid surface culture. Under such conditions it also formed angolide, the amount formed being approximately proportional to the number of spores present in a culture at any given time, but not to the total dry weight (Fig. 1). The same isolate, grown in submerged liquid culture, formed abundant vegetative mycelium but no spores, and only traces of angolide were isolated from such cultures. Similar results have been recorded for sporidesmolide production by *P. chartarum* (Dingley *et al.* 1962). This evidence, taken with the previous failure to obtain more than traces of depsipeptide from feebly sporing liquid surface cultures of *P. sacchari* IM102686 (Bishop *et al.* 1965), established that the angolide from *P. sacchari* is associated with the spores.

The experiments of Bertaud *et al.* (1963) showed that the bulk at least of spori-

desmolides in *Pithomyces chartarum* was located on the spore surface, and Bishop *et al.* (1965) found sporidesmolides to be completely removed from dried sporing felts by three successive brief chloroform washes. To compare the rates of removal of sporidesmolides from dried *P. chartarum* felts and of angolide from dried *P. sacchari* felts, liquid surface cultures of the two species were mixed before harvesting. Very brief extractions of the mixed, dried and powdered felts were made with a limited volume of chloroform; after four such extractions the residual depsipeptides were completely removed by a fifth extraction in order to determine the total amount present. The depsipeptides present in each extract were isolated and weighed, and the proportions of sporidesmolides and angolide calculated from the measured specific rotations of the mixtures. Rather more than 60% of both depsipeptides was removed by the first two extractions. The third and fourth extracts, however, contained a rather smaller proportion of angolide, and after the fourth extraction about 20% of the total angolide remained unextracted, as compared with only 10% of the total sporidesmolides (Table 4). It was concluded that most of the angolide in *P. sacchari* was present on the spore surface but that, as compared with the sporidesmolides of *P. chartarum*, a larger proportion was relatively inaccessible. Whether, in either species, any of the depsipeptide is intracellular is uncertain. Conceivably some might be located within the matrix of the thick spore wall.

Table 4. *Extraction of sporidesmolides and angolide from mixed powdered dried felts of Pithomyces chartarum and P. sacchari*

Each extraction was performed by stirring mixed dried powdered felts with chloroform for 15 sec. After four extractions, the remaining depsipeptide was removed by continuous extraction with chloroform.

Extraction no.	Depsipeptide remaining unextracted (% of total)	
	Sporidesmolides	Angolide
1	72.9	67.3
2	37.9	38.3
3	19.3	27.3
4	10.8	21.3

DISCUSSION

Although many species of the genus *Pithomyces* are described and figured by Ellis (1960, 1965), only *P. chartarum*, *P. maydicus*, *P. cynodontis* and *P. sacchari* are available in culture. It may therefore be useful at this stage to summarize the state of our knowledge of the spore-surface spicules. As to their nature, it has been established that a major component in each case is a cyclodepsipeptide (whose chemical nature has been defined and which can serve as a taxonomic character). This does not exclude the possibility that other substances, such as lipids, may enter into the spicule structure. As to the location of the spore-surface spicules, there can be no doubt that the major portion of the spicular depsipeptides is located on the surface of the spores. We cannot exclude the possibility that small numbers of spicules, or small amounts of spicular depsipeptides, may occur elsewhere than on the spore surface. As to the genesis of the spore-surface spicules, it is known (Butler *et al.* 1962) that sporidesmolide I can be derived from exogenous amino acids, but biochemical details of cyclodepsipeptide biosynthesis, where it takes place, and how the insoluble products come

to be located on the spore surface, are all unknown. As to the significance of the surface spicules, they appear unlikely to confer a selective advantage by antimicrobial action; no such action was demonstrable by Bishop *et al.* (1965). Their contribution to spore water-repellancy, and their possible biochemical implication in the process of sporogenesis, remain open questions. Of the outstanding problems, the last appears to be the most fundamental. All the *Pithomyces* spore-surface cyclodepsipeptides are composed of residues which may be derived from branched-chain aliphatic α -keto acids. This suggests that such acids may accumulate at the time of sporulation. Whether such accumulation does occur, and whether it is causally related to sporogenesis, are questions which it is hoped to explore further.

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EXPLANATION OF PLATE

Profile electron micrographs of portions of spores of two *Pithomyces sacchari* isolates, shadowed with carbon; $\times 20,000$. Fig. 1. Strain TPI122a. Fig. 2. Strain TPI122b.

NOTE

Isolates TPI122a and TPI122b have been allocated the accession numbers IMI 120724 and IMI 120725 respectively.