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ANTIFUNGAL ANTIBIOTICS FROM *PISOLITHUS TINCTORIUS*

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Key Word Index—*Pisolithus tinctorius*; Basidiomycete; antifungal antibiotic; α-keto, α-hydroxy acids; *p*-hydroxybenzoylformic acid; 2-(4′-hydroxyphenyl)-2-oxoethanoic acid; (*R*)-(−)-*p*-hydroxymandelic acid; (*R*)-(−)-2-(4′-hydroxyphenyl)-2-hydroxyethanoic acid; pisolithin A and B.

Abstract—The antibiotic compounds *p*-hydroxybenzoylformic acid [2-(4′-hydroxyphenyl)-2-oxoethanoic acid, pisolithin A] and (*R*)-(−)-*p*-hydroxymandelic acid [(*R*)-(−)-2-(4′-hydroxyphenyl)-2-hydroxyethanoic acid, pisolithin B] were isolated from the growth culture of *Pisolithus tinctorius*. Both of these metabolites, and a few structurally related compounds, were shown to inhibit spore germination and cause hyphal lysis to a significant number of phytopathogenic and dermatogenic fungi. Hence, it was concluded that *P. tinctorius* aids its host plants by providing protection against disease-causing microorganisms.

INTRODUCTION

The mycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker & Couch [=*Pisolithus arhizus* (Scop. pers Pers.) Rauschert] isolated from the roots and surrounding soil of a broad range of host plants [1], is well known for its ability to colonize the roots of trees. Its large network of mycelia spreads for beyond the reach of its host's root system aiding in the transfer of Mg, P, S, K, Ca and other minerals to the host and this process has been proposed as the major factor in the organism's ability to stimulate plant growth in low fertility soils [2–5]. However, accelerated plant development has also been attributed to the ability of the fungus to produce phytohormones. The *in vitro* production of indole acetic acid [6–8], cytokinin and gibberellin [6], or gibberellin-like [9] phytohormones from *P. tinctorius* has been demonstrated. *Pisolithus tinctorius* adapts effectively to mine spoils, sand dunes, and areas of eroded, rocky and shallow soil and its value to agriculture and forestry has been examined by several research groups [10–13].

Ectomycorrhizal Basidiomycete fungi, such as *P. tinctorius*, are also known to provide protection to forest trees against disease-causing microorganisms. Mycelial ensheathed roots of host plants are characteristic of their symbiotic association and are believed to act as a physical barrier against infectious pathogens. The marked resistance of associated trees to aggressive pathogens has been shown to involve fungal production of extracellular metabolites having antibiotic activity. Marx [14–16] was the first to report the antagonistic properties of mycorrhizal fungi against pathogenic fungi and soil bacteria and he has reported [17] the production of antibiotics, *in vitro*, by over 100 Basidiomycete mycorrhizal fungi. Since then, the use of ectomycorrhizal fungi in the protection of roots has been extensively investigated, but very few metabolites with antibiotic activity have been isolated and characterized [18].

Recently, Kope and Fortin [19, 20], demonstrated that the cell free cultures of *P. tinctorius* [Centre de Recherche en Biologie Forestiere (CRBF) strain 0039] exhibit strong antifungal activity causing hyphal lysis to a number of microorganisms. In this paper we wish to report the isolation, structure determination and some aspects of the biological properties of *p*-hydroxybenzoylformic acid [2-(4′-hydroxyphenyl)-2-oxoethanoic acid, **1**] and (*R*)-(−)-*p*-hydroxymandelic acid [(*R*)-(−)2-(4′-hydroxyphenyl)-2-hydroxyethanoic acid, **2**]. The two metabolites were given the trivial names pisolithin A (**1**) and pisolithin B (**2**) respectively, and they were shown to be responsible for the antibiotic activity of *P. tinctorius*. They were both found to exhibit strong antifungal activity against several phytopathogenic and dermatogenic fungi, causing inhibition of mycelial growth comparable (and in some cases superior) to the antifungal antibiotics nystatin and polyoxin D [Kope, H. H., Tsantrizos, Y. S., Fortin, J. A. and Ogilvie, K. K., manuscript in preparation]. Hyphal lysis, characteristic of antifungal antibiotics which inhibit chitin synthesis (e.g. polyoxins [21]) was shown to occur within 12–24 hr of exposure of the test organism to solutions containing low concentrations of pisolithin A or B as well as a few structurally related compounds. It should be noted, that although the chemical structures of pisolithin A and B are known in the literature, to the best of our knowledge, this is the first report on their isolation from a natural source and discovery of their antibiotic properties. Based on the structure–activity relationship observed, and ^{13}C NMR data, a possible mode of action is proposed.

RESULTS AND DISCUSSION

Pisolithus tinctorius inoculum was incubated in modified Melin Norkans (MMN) liquid medium [15] for

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50–55 days and filtered to remove all vegetative growth. The dark brown liquid culture was evaporated to approximately one-fifth of its original volume and freeze-dried to give a solid. We found that the intensity of the brown colour of the medium was often indicative of the concentration of biologically active metabolites present.

The production of dark-coloured pigments, phenolic, *p*-hydroxybenzoic and humic acid like metabolites had been noted before [22] and in 1985 Gill [23] first reported the isolation of the brightly red pigment norbadion A (**3**) from the sporophores (basidiomata) of *P. tinctorius*. He also suggested that the compounds responsible for the intense pigmentation of the fungus's fruitbodies (basidiomata) could play an important role in its physiology and ecology. The structural similarity between pisolithin A and B and the *p*-hydroxylated pulvinic acid (**4**) side-chain of norbadion A is quite evident and it most likely implies a common biosynthetic scheme for these compounds. Recently, Gill and Watling [24] isolated other structurally related pigments from the fruitbodies of *Pisolithus*. They proposed *p*-hydroxyphenylpyruvic acid as their biosynthetic precurser and suggested that hydroxylated pulvinic acid derivatives may be of taxonomic significance [24].

Soxhlet extraction of the freeze-dried culture of *P. tinctorius* with diethyl ether followed by cellulose column chromatography and repeated paper chromatography of the ether extract, led to the isolation of metabolites **1** and **2**. Both compounds, pisolithin A and B, were shown to arrest hyphal growth of the plant pathogen *Truncatella hartigii* (Tubeuf.) Stey. and cause lysis at the hyphal tips within 24 hr at 50–150 $\mu g\,ml^{-1}$. At the same concentrations, a 0% germination of *T. hartigii* conidia was observed after 48 hr. Both compounds were found to be moderately unstable to light and possibly air. Finally these active metabolities were purified by HPLC, using a C18 reverse phase column with the solvent mixture of 91.8% water, 7.3% methanol and 0.9% acetic acid. At a flow rate of 2 $ml\,min^{-1}$ pisolithin A and B eluted at 5–6 and 8–9 min respectively.

Table 1. Growth inhibition concentration (GIC 50) of pisolithin A

Fungi	GIC 50* ($\mu g\,ml^{-1}$)
Phytopathogenic	
Phytophora sp.	50
Rhizoctonia solani	50
Fusarium solani	250
Pythium debaryanum	50
Pythium ultimum	50
Verticillium dahliae	50
Pyrenochaeta terrestris	50
Cochliobolus sativus	500
Septoria musiva	250
Brunchorstia pinea	125
Dermatopathogenic	
Microsporum gypseum	50
Trichophyton equinum	50

*Changed hyphal morphology was also observed at these concentrations.

The chemical and spectroscopic properties of metabolite **1** suggested a phenol, *para*-substituted with an α-keto acid moiety. Combining all data, including its ^{252}Cf-plasma desorption mass spectra [25, 26], led to the assignment of *p*-hydroxybenzoylformic acid [2-(4'-hydroxyphenyl)-2-oxoethanoic acid] as the structure of pisolithin A. The sodium salt of **1** is commercially available and the natural product (Na^+ salt) as well as its methyl ester (**5**) were identical in all respects to the equivalent derivatives of the commercial sample **1**.

The NMR and IR spectral analysis of metabolite **2**, and the similarities to those of metabolite **1**, clearly suggested the reduction of the α-keto group of **1** to an alcohol in **2**. The mass spectral data was consistent showing only two mass units difference between their respective molecular ions. Hence, the structure of *p*-hydroxymandelic acid [2-(4'-hydroxyphenyl)-2-hydroxyethanoic acid, **2**) was assigned to pisolithin B. A commercial sample of D,L-*p*-hydroxymandelic acid was compared with **2** and the spectral data were identical in all respects. In a separate study the chirality of pisolithin B was determined to be of the absolute *R* configuration [27].

The antifungal antibiotic properties of pisolithin A and B, and some of their structural analogues

Isolation of the antibiotics pisolithin A and B from the liquid culture of *P. tinctorius* strongly suggests that this microorganism may provide protection to its host plants against disease causing fungi. Throughout our investigation of these compounds, the biological activity (hyphal lysis and inhibition of spore germination) was followed using the phytopathogenic fungus *Truncatella hartigii* as the test organism. This microorganism was chosen for its relatively high sensitivity to the antibiotics and its ease of handling.

Through the course of the isolation of the two compounds, only extracts which caused mycelial growth to be significantly reduced or completely arrested in addition to lysis of the newly formed hyphae were further purified. All tests were done in multiple runs of 10 for concentrations ranging from 250 to 8 $\mu g\,ml^{-1}$ (6 dilutions). Bioassays on spore-germination inhibition were carried out in runs of 10 samples with 10 conidia each (100 conidia total) and the average number of germinated conidia was compared with that of the control. The controls were also run in 10 conidia per 10 samples; since the biology of germination is not absolute, variations in the control were taken into consideration. In a typical control the average number of germinated conidia was 9.3–9.5 (i.e. 93–95%). Growth inhibition concentration 50 (GIC 50) was defined as the minimum concentration at which the average number of germinated conidia in a test solution was less than half of the average of the control (i.e. less than ~4.5–4.7 or ~45–47%) after an incubation period of 5–7 days. Plugs (5 mm^2) of actively growing colonies of *Truncatella hartigii*, cultured on potato dextrose agar (PDA) medium, were excised and placed into wells of a multiwell tissue culture plate to which 300 μl of potato dextrose broth (PDB) at 50% dilution was added. The cultures were incubated at 25° and after 24 hr were inspected for good growth. The PDB was then removed and a sterile water solution (300 μl) of the test sample was added. Overall growth, based on the final mycelial mass, mycelial protein content and hyphal morphology, was assessed after an incubation period of 7 days. Details on

the effects of pisolithin A and B on the mycelial growth of different fungal pathogens, mycelial protein content and a comparison of these effects to those of polyoxin D [21] and nystatin [28] will be published elsewhere (Kope, H. H., Tsantrizos, Y. S., Fortin, J. A. and Ogilvie, K. K., manuscript in preparation).

Some representative examples of plant and animal pathogens which are adversely affected by the active metabolites of *P. tictorius* are given in Table 1, along with the concentrations at which less than 50% growth (as compared to the control) and changed hyphal morphology was observed. Especially noteworthy are the plant pathogens *Rhizoctonia solani* Kotila and members of the species *Pythium*, *Phytophthora*, and *Fusarium*. These fungi are responsible for large economic losses to nurseries as they cause damping off diseases (seed rotting and hypocotyl or root rotting of seedlings) which represent the most important diseases of tree seedlings. In addition the fungi of the species *Microsporum* and *Trichophyton* are known for their ability to infect animals and sometimes humans, causing dermatitis.

In order to gain some insight as to the mode of action of pisolithin A and B and the structural requirements for their biological activity, a number of structurally related compounds were also investigated. Pisolithin A and B were shown to cause hyphal lysis and inhibit spore germination of *T. hartigii at concentrations of* ~ 60–$100\ \mu g\ ml^{-1}$ (within 4 hr, GIC 50 value). However, even at $31\ \mu g\ ml^{-1}$ the extent of spore germination was only 50–60% of the control. Interestingly, the synthetic (*S*) enantiomer of pisolithin B proved to have stronger antifungal activity than the natural product itself (Table 2), possibly due to its higher resistance to enzymatic degradation by the test organisms. When the commercially available compounds sodium *p*-hydroxybenzoylformate, racemic *p*-hydroxymandelic acid, benzoylformic acid, and both the (*R*) and (*S*) enantiomers of mandelic acid were subjected to the same biological testing as the natural products **1** and **2**, GIC 50 concentrations for spore germination of *T. hartigii* very similar to the natural products (Table 2) were observed.

The results obtained from all α-hydroxy acid samples consistently indicate that the (*S*) enantiomer is more active than the (*R*). Surprisingly, the sodium and potassium salts of pisolithin A and B (as well as the sodium salt of mandelic acid and benzoylformic acid) were found to be biologically inactive even at high concentration ($500\ \mu g\ ml^{-1}$). However, when the compounds were converted to their free acids, via an ion exchange column, the same biological results as with the original natural products were obtained. It was then speculated that 'the mode of action' of these compounds may be similar to that of ionophore-type antibiotics involving the chelation of metal ions essential to the normal growth of the susceptible microorganisms. In such an event, the carboxylate ion would play a key role in the metal binding process. The presence of sufficient metal ions (e.g. Na^+) could 'associate' all of the carboxylate ions leading to a loss of antibiotic effects. Ionophore-type activity would not be observed when the carboxylate ion is unavailable for binding with the metal(s) involved in the microbial growth. While a thorough proof for this speculation requires further testing some NMR data was obtained which, along with literature information, supports the notion that chelation of metal ions may be involved.

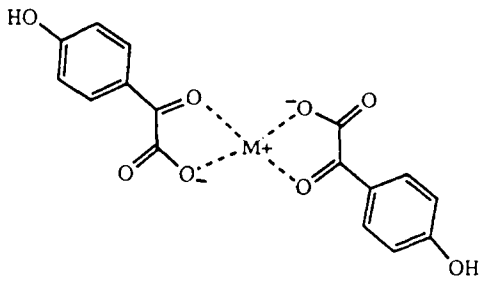
NMR spectroscopy has been used in the past for the investigation of metal–ligand bonding formation [29]. The ^{13}C NMR chemical shifts of pisolithin A in the free acid form and as the lithium, sodium and potassium salts were compared (Table 3). A downfield shift of ~ 4 ppm was observed for the formation of the carboxylate ion as expected. However, a significant downfield shift (~ 8 ppm) of the α-carbon (C-2) was also observed, possibly due to the formation of a bimolecular metal–ligand complex (**6**) which would decrease the electron density of C-2. In addition, it was noted that among all the salt compounds, there was very little difference in the chemical shifts except for the aromatic carbons C-1′ and C-4′ of the potassium salt which showed a smaller and larger change in shift (respectively) from the corresponding carbons of the other salts. These results suggested the formation of a stronger metal–ligand chelate between the carboxylate ion of pisolithin A and the potassium ion. The absence of any significant difference between all *ortho* (C-2′ and C-6′) carbons suggested a minimal contribution of electron density from the phenol to the C-2 carbon *via* resonance. This conclusion is supported by molecular modeling calculations indicating that the α-ketone and the carboxylate ion are co-planar and at a 53° angle from the plane of the aromatic ring (Fig. 1), which would ensure very little resonance of the ring with the C-2 carbonyl. However, the existence of a dipole inductive effect through the ring would be most pronounced on carbons C-1′ (increased electron density) and C-4′ (decreased electron density). The strong electron-withdrawing effect of the metal ions on the C-2 carbonyl was evident even when the ^{13}C NMR was observed in fairly dilute D_2O solutions (Table 3). Similar results were also observed when the ^{13}C NMR of pisolithin B in the free acid form was compared to that of its metal salts.

Ionophore-type antibiotics often display a metal-binding 'cavity' of a characteristic size, hence, showing

Table 2. Inhibition of spore germination of *T. hartigii*

Sample	GIC 50 ($\mu g\ ml^{-1}$)	% (S) ($\mu g\ ml^{-1}$)
p-Hydroxybenzoylformic acid	67	—
Benzoylformic acid	72	—
(*R/S*)-*p*-Hydroxymandelic acid	56	28
(*R*)-*p*-Hydroxymandelic acid, 33% ee	71	23
(*S*)-*p*-Hydroxymandelic acid, 60% ee	33	20
(*R*)-Mandelic acid, >95% ee	82	
(*S*) Mandelic acid, >95% ee	31	

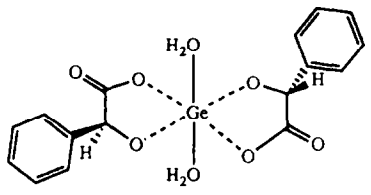
Table 3. ^{13}C NMR shifts* (300 Mz)



metal–ligand complex **6**

In DMSO C-1	C-1	C-2	C-1'	C-2',6'	C-3',5'	C-4'	Ionic radii of $[M]^+$
$-CO_2H$	167	187	123	132	116	164	
$-CO_2^-Li^+$	170	195	126	132	115	162	0.60A
$-CO_2^-Na^+$	171	195	126	132	115	162	0.95A
$-CO_2^-K^+$	171	195	124	132	116	166	1.33A
$-CO_2^-Ag^+$	171	194	125	132	115	163	1.26A
in D_2O							
$-CO_2^-Na^+$	174	196	125	133	117	163	
$-CO_2^-K^+$	175	196	124	134	118	166	

*All chemical shifts are in values of ppm relative to DMSO as internal reference. In the experiments using D_2O, a very small amount of DMSO (10–50 μl) was added as a reference.



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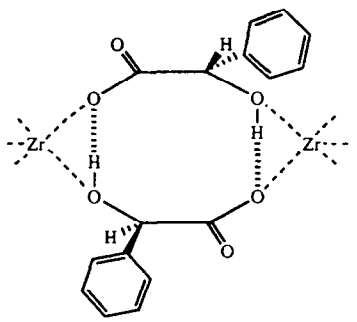

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binding preferences for metal ions of the corresponding atomic radii. In an attempt to further investigate the mode of action of pisolithin A and B the ^{13}C NMR data of the silver salt of pisolithin A was compared with that of the potassium salt. As the charge and atomic radii of K^+ and Ag^+ ions are approximately equal (Table 3) it was anticipated that the ^{13}C NMR shifts would also be very similar, suggesting the formation of a metal–ligand complex of equal strength and stability. However, the results obtained were unreliable due to the occurrence of a redox reaction between the silver ion and the ligand during the course of the NMR experiment, leading to deposition of elemental silver.

Although references to α-keto acid metal chelates are scarce, there is an abundance of literature regarding the formation of metal complexes with α-hydroxy acids [30]. Mandelic acid itself has been shown to form chelates with a large number of metals, including nickel(II) [31], zirconium(IV) and hafnium(IV) [32], several lanthanide ions [33] and transition metals [29, 34, 35]. A number of complexes involving polymeric bridged dimers (**7**) [33] or bidentate ligands coordinating via the deprotonated form of the α-hydroxyl and the carboxylate ion have been proposed (**8**) [30]. It is also noteworthy that a number of α-hydroxy acid biomolecules, such as malic and citric acid [36], are well known for their ability to chelate metals and the latter plays an important role in the transport of inorganic ions in biofluids.

Another class of biomolecules containing an α-hydroxy acid are the iron-chelating siderophores [37]. Iron is an essential element for almost all biosystems. However, the solubility of ferric ions at physiological pH is about 10^{-18} M, which is many orders of magnitude lower than that required by either microbial or plant cells. Hence, through evolution this problem has been circumvented by the production of the chelating agents, the siderophores. The chemical structure of these compounds is very complex, but the parts of the ligand which are known to bind to the iron are almost always α-amino acids, α-hydroxy acids or hydroxamates.

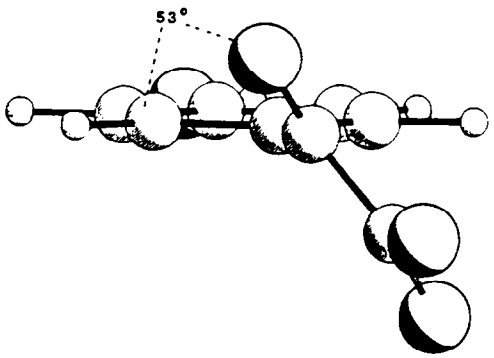

Fig. 1. Conformational analysis of the pisolithin A anion.

The involvement of iron in the biological activity of fungal metabolites is not unprecedented in the literature. For instance, Oku and Nakanishi [38] have shown that both the phytotoxic and the antibiotic effects of the β-keto-/β-hydroxy acid ascohitine are antagonized by the presence of an equal molar concentration of ferric ions. The phytotoxicity of fusaric acid, a strong wilting agent from the *Fusarium oxysporum* fungus, can be eliminated [39] by the addition of Fe^{3+}, Cu^{2+} or Mn^{2+} ions. Similarly, Ayer [40] has proposed that the blue complex formed when iron is chelated by the fungal metabolite 2,3-dihydroxybenzoic acid is involved in the staining of the sapwood of lodgepole pines infected with blue stain fungi. However, in the case of the metabolites from *P. tinctorius*, the addition of Fe^{3+} ions to the test solutions of the antifungal activity bioassays had no apparent effect.

In conclusion, we believe that *Pisolithus tinctorius* protects its host plants against pathogenic fungi *via* the production of the antibiotic compounds pisolithin A and B. The total lack of biological activity for both the sodium and potassium salts of these natural products and the salts of their biologically active structural analogues (*R* and *S* mandelate salts, benzoylformate salts) along with the ^{13}C NMR data strongly suggest, but far from prove, that the mechanism of action of these antibiotics may involve the chelation of metal ions.

EXPERIMENTAL

General. Sodium *p*-hydroxybenzoylformate, benzoylformic acid, (*R*)- and (*S*)-mandelic acid and racemic *p*-hydroxymandelic acid were obtained from Aldrich. Multiwell tissue culture plates were obtained from Becton Dickson Labware, Oxnard, CA. Doubly distilled H_2O and HPLC grade MeOH were filtered through a 0.45 μ filter membrane (Millipore) before using them for HPLC. All chromatographic solvents were fractionally distilled prior to use with the exception of HOAc. Silica gel chromatography was performed on Merck Kieselgel 60 (230–400 mesh, #9385) using flash chromatography [41]. TLC was performed on cellulose plates (0.16 mm thickness, Eastman Kodak, #13254), while CF 11 Whatman powder (American Chemicals. Montreal, Que.) was used for CC. Paper chromatography was performed on Whatman paper sheets (3MM). Desalting of metabolites was carried out on an ion exchange column (~2 cm × 100 cm) packed with pre-swollen and washed Dowex 50W-X8 (H^+ form, 20–50 mesh) resin (J. T. Baker Chemical Co., Phillipsburg, N.J.). Ion exchange chromatography was performed using IRC-50 resin purchased from BDH (Montreal, Que.) The column (~2 × 100 cm) was packed with pre-swollen resin, washed and equilibrated with the appropriate cation (Li^+, Na^+, K^+, Ag^+). HPLC analysis and purifications were carried out on two reverse phase C18 columns; Whatman Partisil 5 ODS 3 (10 cm × 9.4 mm i.d., 5μ particles, Chromatographic Specialities Inc.) and CSC-S ODS2 (25 cm × 9.4 mm i.d. 5μ particles, Chromatography Science Company Inc., Montreal, Que.)

Spectra. UV NMR spectra were obtained at 20–22° using 200, 300 and 500 MHz instruments. ^1H and ^{13}C NMR chemical shifts are quoted in ppm and are referenced to the internal deuterated solvent downfield from tetramethylsilane (TMS). The samples used for D_2O–^1H NMR were first dissolved in ~1 ml of 99.8% D_2O and lyophilized twice before their data was recorded in 99.96% D_2O. ^{252}Cf-Plasma desorption MS was performed by Dr C. J. McNeal (Chemistry Department, Texas A&M University). All other mass spectra were obtained by Dr O. Mamer (Biomedical Mass Spectrometry Unit, McGill University).

General extraction and isolation of pisolithin A and B from P. tinctorius. Conical flasks (250 ml) containing 100 ml of MMN medium were inoculated with *Pisolithus tinctorius* disks (4 × 6 mm^2), cut from the edge of a colony growing on MMN agar plates. The solns were buffered to pH 5.5 with 0.1 M Na^+ citrate/citric acid. The flasks were incubated at 25° on a rotary shaker at 100 rpm for 50–55 days. The liquid cultures of *P. tinctorius* were then collected and filtered through several layers of cheese-cloth to remove the mycelia. The reddish-brown liquid obtained was reduced in vol. (*ca* = 1/5) under high vacuum at 40°. The remaining H_2O was removed by freeze-drying to give a solid, the yields of which varied from 2.5 to 4.3 gl^{-1} of original culture.

Soxhlet extraction of the above solid with reagent grade Et_2O (500 ml day^{-1} 15 g^{-1} solid) over a period of 2–3 days gave a light orange oil upon evapn of the solvent. A small amount of glucose often crystallized out of the Et_2O soln, which was removed by filtration. The yields of biologically active material ranged from 7 to 60 mg g^{-1} solid (average = 33 mg g^{-1}), but could be increased if the extraction was allowed to continue for up to 7 days. A longer period of extraction, however, led to the isolation of a much darker crude, whose subsequent purification was much more difficult. After three days, the amounts of active metabolities isolated were minimal whereas the amounts of dark colour pigments increased proportionally with time. These results were further supported by the level of toxicity observed for each of these extracts.

Further purification was carried out on a tightly packed column (using air pressure) of Whatman Cf11 cellulose powder, applied as a slurry in EtOH. Once the crude toxin was applied to the top of the column, a solvent mixt. of 95% *n*-BuOH (saturated with H_2O)–5% HOAc was allowed to filter through by gravity. Cellulose TLC was used to follow the elution of active metabolites (pisolithin A, R_f ~0.28 and pisolithin B, R_f ~0.33 in *iso*-PrOH–0.5M NH_4HCO_3). The majority of the middle frs were combined and rechromatographed.

Paper chromatography (Whatman 3MM) with a solvent mixt. of *iso*-PrOH–0.5M NH_4HCO_3 (4/1) for a total length of *ca* 40 cm (12–15 hr) separated the mixt. into 5 bands at R_f values 0.05–0.1 (yellow), 0.22–0.32, 0.32–0.4, 0.4–0.56 (fluorescent) and at 0.55–0.65. All bands were cut, dried, eluted with H_2O freeze-dried. The active material (band at R_f 0.22–0.32) was obtained as a light brown solid containing both metabolites, pisolithin A and B, average yield 30–35 μg mg^{-1} of crude.

Final purification through descending PC, eluted with 95% *n*-BuOH (satd with H_2O)–5% HOAc for ~15 hr, led to the separation of the two biologically active metabolites. Metabolite **1** (pisolithin A) was found at R_f 0.2–0.6 and **2** (pisolithin B) at R_f

0.6–0.7. The visualization of these bands was difficult and it could only be done in a completely dark room under UV light. The strips of paper impregnated with the antifungal compounds were cut out and dried in a desiccator, under high vacuum, eluted with H_2O and freeze-dried to pure white solids.

Final purification was carried out on a reverse phase C18 HPLC column (CSC-S ODS2, 25 cm × 9.4 mm i.d., 5 μ particles) using a solvent mixt. of 91.8% H_2O, 7.3% MeOH, 0.9% $MeCO_2H$ at a flow rate of 2 ml min^{-1}. *p*-Hydroxybenzoylformic acid (pisolithin A) eluted after 5–6 min while (*R*)-(−)-*p*-hydroxymandelic acid (pisolithin B) eluted after 8–9 min.

Spectral data of 2-(4′-*hydroxyphenyl*)-2-*oxoethanoic acid* (p-*hydroxybenzoylformic acid, pisolithin A*, **1**). IR ν^{KBr} cm^{-1}: 3150–3380, 1735, 1654, 1608, 1586, 1443, 1400, 1252, 1173, 979, 855, 621. UV $\lambda^{0.1\,HCl}$ nm: max 296, min 226 and 206; 0.1M NaOH: max 334, min 240. ^1H NMR (500 MHz, DMSO, ppm) δ: 6.88 (2H, *dd*, $J = 8$ and 0.9 Hz, H-3′ and H-5′), 7.75 (2H, *dd*, $J = 8$ and 0.9 Hz, H-2′ and H-6′), 10.74 (*s*, 1H, $-CO_2H$). ^{13}C NMR (free acid) (300 MHz, DMSO + D_2O, ppm) δ: 116.0 (C-3′ and C-5′), 123.3 (C-1′), 132.3 (C-2′ and 6′), 163.8 (C-4′), 166.8 (C-1), 187.0 (C-2). *Na salt* (300 MHz, DMSO, ppm) δ: 115.2 (C-3′ and C-5′), 125.7 (C-1′), 131.5 (C-2′ and C-6′), 162.2 (C-4′), 171 (*br*, C-1) 195 (*br*, C-2) ^{252}Cf-plasma desorption MS: electrosprayed sample[26] *m/z* 211.2 $[M^{2-} + H^+ + 2Na^+]^+$, 165.1 $[M^{2-} + H^+]$, tridodecylmethylammonium chloride salt[25] *m/z* 1774.7 $[(TDMA^+)_3(M^{2-})]^+$, 866.7 $[(TDMA^+)(M^{2-} + H)_2]^-$, 165.1 $[M^{2-} + H^+]$, 121.1 $[M^{2-} + H^+ - CO_2]$.

Spectral data of (*R*)-(−)-p-*hydroxymandelic acid* (*pisolithin B*, **2**). UV $\nu^{0.1M\,HCl}$ nm: max 228, min 206 and 274; 0.1M NaOH: max 248, min 292. ^1H NMR (300 MHz, D_2O, ppm) δ: 5.0 (1H, *s*, H-2), 6.7 (2H, *d*, $J = 8.2$ Hz, H-3′, and H-5′), 7.1 (2H, *d*, $J = 8.2$ Hz, H-2′ and H-6′). ^{13}C NMR (300 MHz, DMSO + D_2O, ppm) δ: 74.4 (C-2), 117.3 (C-3′ and C-5′), 130.4 (C-2′ and C-6′), 132.3 (C-1′), 157.5 (C-4′), 178 (C-1). ^{252}Cf-plasma desorption MS: electrosprayed sample[26] *m/z* 213.2 $(M^{2-} + H^+ + 2Na^+)^+$, 167.1 $(M^{2-} + H^+)^+$; tridodecylmethylammonium chloride salt[25] *m/z* 167.1 $(M^{2-} + H^+)^+$, 869.9 $[(TDMA^+)(M^{2-} + H^+)_2^-]^-$, 1776.6 $[(TDMA^+)_3M^{2-}]$, 121.1 $[M^{2-} - HCO_2H]^-$.

Methyl p-*hydroxybenzoylformate* (**5**). *p*-Hydroxybenzoylformic acid (*ca* 5 mg) and catalytic amounts of *p*-toluenesulphonic acid were dissolved in 10 ml MeOH and refluxed overnight. Evaporation to dryness of the reaction mixt. followed by flash chromatography on silica gel, with CH_2Cl_2, gave quantitative amounts of the Me ester as a clear oil. TLC: EtOAc, R_f 0.56 (on silica compound has a bright yellow colour). ^1H NMR (200 MHz, $CDCl_3$, ppm) δ: 3.9 (3H, *s*, Me), 6.3 (1H, *s*, OH-4′), 6.9 (2H, *d* $J = 9.0$ Hz, H-3′ and H-5′), 7.9 (2H, *d*, $J = 9.0$ Hz, H-2′ and H-6′). ^{13}C NMR (300 MHz, $CDCl_3$, ppm) δ: 42.9 (Me), 116.1 (C-3′ and C-5′), 124.9 (C-1′), 133.3 (C-2′ and C-6′), 162.8 (C-4′), 164.6 (C-1), 185.0 (C-2). MS [C.I.(NH_3), direct inlet ~105°], *m/z* (% rel. int., assignment): 181 $[M + 1]^+$, 198 $[M + NH_4]^+$, 121 $[HO - C_6H_4 - CO]^+$

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