Two new antifungal metabolites produced by *Sparassis crispa* in culture and in decayed trees

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The basidiomycete fungus *Sparassis crispa* produced three antifungal compounds during submerged culture in 2% malt broth. One compound appeared to be sparassol (methyl-2-hydroxy-4-methoxy-6-methylbenzoate), first characterized in 1924. The other two, termed ScI and ScII, exhibited considerably greater antifungal activity than did sparassol against *Cladosporium cucumerinum*, and were characterized as methyl-2,4-dihydroxy-6-methylbenzoate (methyl orsellinate) and an incompletely determined methyl-dihydroxy-methoxy-methylbenzoate, respectively. Both compounds were found in the decayed wood of trees, where their presence was diagnostic of *S. crispa* infection. The possible ecological role of these compounds is discussed.

Introduction

Although largely neglected, the first reported isolation and characterization of a microbially produced antibiotic, antedating comparable work on penicillin (Hüttner, 1987), was from the wood-rotting basidiomycete *Sparassis crispa* (formerly *S. ramosa*). Falck (1923) observed that, unlike cultures of other species, *S. crispa* did not become contaminated with other fungi, and isolated a compound that occurred as crystals in and on the medium of old cultures. This compound exhibited mycocidal activity, although full data on its biological action remained unpublished (cf. Cwielong, 1986; Hüttner, 1987). The compound, given the trivial name sparassol, was shown to be methyl-2-hydroxy-4-methoxy-6-methylbenzoate (Wedekind & Fleischer, 1923, 1924). In subsequent screenings for antibiotic production, *S. crispa* has been noted as moderately active (Wilkins & Harris, 1944; Wilkins, 1945, 1946; Robbins et al., 1945; Mathieson, 1946; Hervey, 1947), and suppression of growth of *Bacillus subtilis* on agar media pre-inoculated with *S. crispa* has been attributed to sparassol (Stiepmann, 1987).

*S. crispa* causes a brown root- and butt-rot in living conifers, closely similar to damage caused by *Phaeolus schweinitzii*. Both fungi can cause significant losses in managed conifer forest, but without basidiocarps or cultures, it is difficult to determine which species is responsible for the damage (Phillips & Burdekin, 1982). Although *S. crispa* has been less intensively studied than *P. schweinitzii*, it appears to resemble the latter in having little or no ability to invade the living tissues of its host (Rayner & Boddy, 1988), and is generally restricted to the older, dead wood of standing trees.

Antibacterial and antifungal compounds have been detected in fruit bodies and cultures of many wood-inhabiting fungi (Wilkins & Harris, 1944; Wilkins, 1945, 1946; Robbins et al., 1945; Mathieson, 1946; Hervey, 1947; Brian, 1951; Broadbent, 1968). However, the significance of these compounds in natural environments is uncertain. Uncharacterized antifungal activity from the aquatic (non-basidiomycete) fungus *Massarina aquatica* has been reported in colonized oak wood blocks, and in culture filtrates (Fisher & Anson, 1983). The colonized blocks inhibited the growth of several other fungi which are found in the same habitat, suggesting that production of the antifungal substances may confer an ecological advantage on *M. aquatica* under natural conditions. The root- and butt-rot pathogen *Heterobasidion annosum* produces inhibitory compounds in the presence of plant cells or antagonistic fungi (Sonnenbichler et al., 1989), but the inference that these are ‘ecological metabolites’ was based only on cultural studies.
This paper describes the characterization of two further antifungal metabolites from *S. crispa*, and their production both in culture and in naturally decayed wood.

**Methods**

Organisms. *Sparassis crispa* isolates 5B and 5C were from laboratory stocks, obtained from decayed conifer trees some years previous to this study, and maintained by repeated subculture on 2% malt agar (2%, w/v, Oxoid malt; 1.5%, w/v, Oxoid agar no. 3) with storage at 4 °C. Additional isolates, 5E–5I, were isolated from decayed wood of European larch (*Larix decidua*) trees from Bagley Wood, Oxfordshire, UK, by plating small pieces (about 5 mm²) of wood removed aseptically from near the margin of brown rot lesions on 3% malt agar. Decay fungi isolated in this way were identified on the basis of their characteristics in culture (Stalpers, 1978).

The wood-decaying basidiomycetes *Armillaria gallica*, *A. mellea*, *A. ostoyae*, *A. tabescens*, *Coriolus versicolor*, *Heterobasidion annosum*, *Hypholoma fasciculare*, *Inonotus dryadybus*, *Phaeolus schweinitzii*, *Rigidopus umarius* and *Stereum sanguinolentum*, and the ascomycete root pathogen *Rhizina undulata*, were from laboratory stocks, maintained and grown as above. *Botrytis cinerea* and *Cladosporium cucumerinum*, also laboratory stock isolates, were maintained and sporulated on *Botrytis* medium X (Last & Hanley, 1956).

Antibiotic production in culture. *S. crispa*, strains 5B and 5C, were grown as stationary submerged cultures in 2% malt extract (Oxoid) broth (50 ml) in 250 ml Erlenmeyer flasks, at 25 °C in darkness. Samples (0.5 ml) were withdrawn from culture flasks, mixed with methanol (1.2 ml) and stored at -20 °C prior to HPLC analysis. Three replicate flasks were used at each sampling time, and the mycelial dry weight of these cultures was determined. Fungal mycelium was collected by filtration through muslin, rinsed with water and dried to constant weight at 80 °C.

Isolation of antibiotic compounds produced by *S. crispa*. Culture filtrates from 28-d-old cultures of *S. crispa* 5B, grown as above, were partitioned twice against 50 ml vols of redistilled chloroform. Combined chloroform phases were evaporated to dryness under reduced pressure at 30 °C, and the residue redissolved in a small volume of ethyl acetate.

Antifungal compounds were detected in these extracts by direct bioassay with *Cladosporium cucumerinum* on developed TLC plates (Homans & Fuchs, 1970). Aluminium-backed TLC plates (silica gel 60F, 0.2 mm thick) were loaded with the ethyl acetate extract (0.5–2.5 ml culture volume equivalent) and developed. Two alternative solvent systems were used: A, chloroform/methanol (96:4, v/v); B, cyclohexane/ethyl acetate (1:1, v/v). Air-dried plates were sprayed with dense conidial suspensions of *C. cucumerinum* in half-strength Czapek Dox salts containing 15 g sucrose l⁻¹.

The two most active antifungal compounds detected on bioassays were purified by sequential preparative TLC on silica gel 60F plates (0.5 mm thick), using the two solvent systems. Compounds were eluted from the silica gel with methanol and crystallized by evaporation under reduced pressure.

Characterization of antibiotic compounds. UV absorption spectra were obtained for the purified antifungal compounds. These compounds were further characterized by mass spectrometry and ¹H nuclear magnetic resonance.

Antifungal activity of pure compounds. Spore germination tests against *Botrytis cinerea* were done as described by Rosell & Mansfield (1978), using the active compounds at a concentration of 100 µg ml⁻¹ in the assay solution. Assays against mycelial growth of *Heterobasidion annosum* were done in flat-bottomed glass tubes (25 mm diam.) containing 2.5 ml 2% malt extract (Oxoid) broth (Woodward & Pearce, 1988), with 100 µg of active compound incorporated.

Interactions of *S. crispa* with other fungi. Interactions between *S. crispa* and other wood-inhabiting fungi were examined in dual culture on Malt Extract Agar (Difco). Petri dishes (90 mm diam.) containing approximately 25 ml of medium were inoculated eccentrically with *S. crispa* and, except with *Armillaria* spp., incubated for 14 d (22 °C, in darkness) before inoculation with other fungi 60 mm from the *Sparassis* inoculation site. Effects on the growth of these fungi were assessed after further incubation for 14 d. In tests with *A. gallica*, *A. mellea*, *A. ostoyae* and *A. tabescens*, the fungi were inoculated simultaneously and examined after 28 d.

Estimation of antibiotics from decayed wood. Wood from a wind-snapped Sitka spruce (*Picea sitchensis*) tree from Bagley Wood, exhibiting an advanced brown cubical rot attributable to *S. crispa* (identified by cultural characteristics after isolation, and from basidiomorphs produced on the stump), was extracted in methanol (5 vols). Extracts were concentrated by evaporation under reduced pressure, and antifungal activity was detected on TLC plates by bioautography with *C. cucumerinum*. Samples equivalent to 1 g fresh wt decayed wood were loaded, and developed in solvent system B.

The compounds in wood extracts were quantified spectrophotometrically (*A₅₆₅*) after methanol elution from preparative TLC plates.

Quantification of antibiotics in culture filtrates using HPLC. Total antibiotics in culture filtrate samples were determined by reverse phase HPLC. Aliquots (20 µl) were separated isoctically on a NovaPak C₈ Radial-Pak cartridge column, 100 mm × 8 mm i.d., protected by a µ-Bondapak C₁₈ Guard-Pak pre-column (Waters). The solvent mixture, 60% (v/v) methanol in water, was pumped at a flow rate of 1.5 ml min⁻¹, and eluting compounds were detected by absorbance at 266 nm. Samples were injected at intervals of 10 min: the column was washed (100% methanol, 10 min) after every 10 determinations. The two antibiotic compounds, which eluted as a single, shouldered, peak, were quantified on a peak area basis against an external standard of the compounds mixed in their naturally occurring proportions.

Detection of antibiotics in decayed wood using HPLC. Samples of brown-rotted wood from felled coniferous trees [mainly, but not exclusively, European larch (*Larix decidua*)] from Bagley Wood were extracted in 2 vols (w/v) methanol for 24 h at 4 °C. Samples (20 µl) of the resulting extract were separated using reverse-phase HPLC as previously, but with gradient elution. The initial solvent was 30% (v/v) aqueous methanol, increasing linearly to 100% methanol over 20 min. This solvent composition was maintained for 5 min before returning to the starting conditions (via 100% water) over a total cycle (including re-equilibration) of 40 min. Purified samples of the antibiotic compounds were used as external standards.

Isolation of sparassol. For use as a chromatography standard for comparison with the antibiotics isolated in this study, a sample of sparassol was obtained from 6–9-month-old cultures of *S. crispa* 5G on 2% malt agar slopes, grown initially at 22 °C for about 1 month, then maintained at 4–8 °C. Crystals, produced abundantly on the surface of these cultures, were harvested mechanically and dissolved in methanol.

**Results**

Antifungal activity produced by *Sparassis crispa* in culture

A large zone of inhibition was observed at R₅ 0.50, and a minor inhibition zone at R₅ 0.67 on TLC plates loaded...
Antifungal metabolites produced by *Sparassis crispa*

With organic phase extracts from submerged cultures of *S. crispa* strain 5B, developed in solvent system A and bioassayed with *Cladosporium cucumerinum*. Solvent system B resolved the large inhibition zone into two major active compounds, with *R*<sub>p</sub> values of 0.44 and 0.53. The minor zone had an *R*<sub>p</sub> of 0.66 in this system. All antifungal zones corresponded to areas of extinction under UV light. Sparassol did not give a strong inhibition zone on bioassay plates at the (undetermined) concentration used. The *R*<sub>p</sub> of sparassol was 0.64 and 0.70 in solvent systems A and B, respectively. The two major antifungal compounds, neither of which could be equated with sparassol, were termed ScI (*R*<sub>p</sub> in B = 0.44) and ScII (*R*<sub>p</sub> in B = 0.53).

When purified from TLC plates, these compounds were obtained as pale yellow crystals. Yields (per litre of culture medium) of 26.5 mg (ScI) and 35.5 mg (ScII) were obtained.

**Chemical and physical properties of antifungal compounds**

**ScI.** Methyl-2,4-dihydroxy-6-methylbenzoate (methyl orsellinate). Fig. 1(b). C<sub>9</sub>H<sub>10</sub>O<sub>4</sub>; m.p. 137-139 °C; UV λ<sub>max</sub> nm (log ε) 301 (3.51), 264 (3.94), 229 (3.43); precise mass measurement *M*<sup>+</sup> 182.055 1; MS (electron impact 40 eV) *m/z* 182 (M<sup>+</sup>, 46%), 181 (14), 152 (28), 151 (36), 150 (100), 131 (23), 122 (34); ¹H NMR (200 MHz, CDCl<sub>3</sub>, TMS internal standard) δ 2.49 (3H, s, Me), 3.92 (3H, s, MeO-), 6.23 (1H, δ, Ar-H, *J* = 2.9 Hz), 6.28 (1H, δ, Ar-H, *J* = 2.9 Hz), 11.73 (1H, s, chelated Ar-OH).

**ScII.** Methyl-dihydroxy-methoxy-methylbenzoate (substitution pattern not determined except for hydroxy- in the 2-position). Fig. 1(c). C<sub>9</sub>H<sub>12</sub>O<sub>4</sub>; UV λ<sub>max</sub> nm (log ε) 302 (3.36), 268 (3.96), 224 (3.95); precise mass measurement *M*<sup>+</sup> 212.067 1; MS (electron impact 40 eV) *m/z* 212 (M<sup>+</sup>, 49%), 181 (35), 180 (95), 165 (25), 152 (100), 151 (82), 150 (62), 137 (48), 122 (26); ¹H NMR (200 MHz, CDCl<sub>3</sub>, TMS internal standard): δ 2.46 (3H, s, Me), 3.94 (6H, s, 2* MeO- degenerate), 6.36 (1H, s, Ar-H), 11.93 (1H, s, chelated OH).

**Sparassol** (for comparison). Methyl-2-hydroxy-4-methoxy-6-methylbenzoate. Fig. 1(a). C<sub>9</sub>H<sub>12</sub>O<sub>4</sub> (Wedekind & Fleischer, 1924.)

**Antifungal activity of ScI and ScII**

The effects of these compounds on germ-tube growth in...
Fig. 2. Dual culture interactions between *Sparassis crispa* (right on all plates) and (a) *Stereum sanguinolentum* (mutual inhibition), (b) *Heterobasidion annosum* (growth strongly suppressed), (c) *Rhizina undulata* (no obvious inhibition) and (d) *Phaeolus schweinitzii* (interaction line formation).

Table 1. Effects of antibiotic compounds from *Sparassis crispa* on extension of *Botrytis cinerea* germ tubes and mycelial growth of *Heterobasidion annosum*

<table>
<thead>
<tr>
<th>Compound (100 μg ml⁻¹)</th>
<th>Percentage decrease in germ tube length*</th>
<th>LSD†</th>
<th>Percentage decrease in mycelial dry wt‡</th>
<th>LSD‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScI</td>
<td>61.9 ± 2.7</td>
<td>7.0***</td>
<td>8.9 ± 6.3</td>
<td>7.0**</td>
</tr>
<tr>
<td>ScII</td>
<td>37.7 ± 2.7</td>
<td>7.4***</td>
<td>15.1 ± 9.6</td>
<td>4.8***</td>
</tr>
</tbody>
</table>

* Based on means of 120 measurements on three replicate slides per treatment ± SEM. 100% = 351.75 ± 1.12 μm.
† Least significant difference between treatments and control, expressed as a percentage.
** P = 0.01; *** P = 0.001.
‡ Based on means of 10 replicates per treatment ± SEM. 100% = 27.1 ± 1.0 mg.

Accumulation of ScI and ScII in cultures of *S. crispa*

ScI (methyl orsellinate) comprised the major component of the shouldered peak, with a retention time of approximately 5-18 min. The ScII shoulder appeared at a retention time of 5-83 min. Throughout the incubation period, the peak height ratio ScI:ScII remained essentially constant between 3.8 and 4.1, in both isolates.
Table 2. HPLC detection of antibiotics ScI and ScII in brown-rotted wood of conifers and the decay fungi isolated from these trees
+ - Detected; - , not detected.

<table>
<thead>
<tr>
<th>Tree</th>
<th>ScI + ScII</th>
<th>Fungus isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sitka spruce</td>
<td>-</td>
<td><em>Sparassis crispa</em></td>
</tr>
<tr>
<td>2. Larch</td>
<td>-</td>
<td>Undetermined. White, rapidly growing mycelium</td>
</tr>
<tr>
<td>3. Larch</td>
<td>-</td>
<td>Undetermined. White, rapidly growing mycelium</td>
</tr>
<tr>
<td>4. Corsican pine</td>
<td>-</td>
<td><em>Phaeolus schweinitzii</em></td>
</tr>
<tr>
<td>5. European larch</td>
<td>+</td>
<td><em>S. crispa</em></td>
</tr>
<tr>
<td>6. European larch</td>
<td>+</td>
<td><em>S. crispa</em></td>
</tr>
<tr>
<td>7. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
<tr>
<td>8. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
<tr>
<td>9. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
<tr>
<td>10. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
<tr>
<td>11. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
<tr>
<td>12. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
<tr>
<td>13. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
<tr>
<td>14. Corsican pine</td>
<td>+</td>
<td><em>S. crispa</em></td>
</tr>
<tr>
<td>15. European larch</td>
<td>+</td>
<td><em>S. crispa</em></td>
</tr>
<tr>
<td>16. European larch</td>
<td>+*</td>
<td><em>S. crispa</em></td>
</tr>
<tr>
<td>17. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
<tr>
<td>18. European larch</td>
<td>-</td>
<td><em>P. schweinitzii</em></td>
</tr>
</tbody>
</table>

* Very small peaks at a retention time of about 14 min in this sample.

examined. Total antibiotic levels (ScI + ScII) and fungal biomass production for *S. crispa* isolates 5B and 5C are shown in Fig. 3.

Although growth curves for the two isolates were closely similar, the onset of antibiotic production was later in 5B than 5C: mean concentrations of total antibiotics in culture filtrates after 8 weeks were 39 and 65 μg ml⁻¹, respectively. However, variation between individual replicate flasks was large.

Detection of antibiotics in decayed wood

Zones of antifungal activity were detected at *R*ₚ values of 0·44 and 0·53 in solvent system B, corresponding to ScI and ScII, but not at *R*ₚ 0·65–0·70 (sparassol). Quantitative estimations of the two active compounds were 102±44 μg (g fresh wt)⁻¹ (ScI) and 90±13 μg (g fresh wt)⁻¹ (ScII).

Gradient HPLC of extracts from the decayed Sitka spruce wood revealed a shouldered peak at retention time 14-1 min, corresponding to ScI and ScII. A peak at 18·4 min may relate to sparassol, although the identity was not confirmed (Fig. 4).

Double or merged peaks, eluting with retention times between 13·5 and 14·2 min, were observed in HPLC separations of 6 out of 18 brown rotted wood samples. *S. crispa* was isolated from the same 6 wood samples, whereas other species of decay-causing fungi were cultured from the remaining 12 trees (Table 2).

HPLC of sparassol crystals

Sparassol eluted in the HPLC gradient at about 18·2 min...
as a single peak. Only trace quantities of ScI and ScII (< 1% total A$_{290}$) were present in the methanol solution of these crystals.

Discussion

Three antifungal orsellinic acid derivatives were produced by _S. crispa_ in culture and in wood decayed by this species. The previously described sparassol appears to be the least active, although it is possible that its high vapour pressure (Cwielong, 1986) resulted in loss during sample preparation. The identity of sparassol was inferred from its chromatographic properties, which were identical to those of crystals harvested from old cultures of _S. crispa_, the original source of sparassol (Falck, 1923).

The two more active compounds, ScI and ScII, were purified and characterized in the present study. ScI was identified as methyl-2,4-dihydroxy-6-methylbenzoate (methyl orsellinate) and ScII as a methyl-dihydroxy-methoxy-methyl benzoate. In the latter compound, one of the hydroxyl groups occupies the 2-position: the relative positions of the second hydroxy– group and the methyl– and methoxy– groups remain to be determined. It is likely that a major part of the antifungal activity that Falck (1923) observed preventing the contamination of old cultures was attributable to ScI and ScII, rather than to sparassol.

Antifungal orsellinic acid derivatives are also known in other fungi. A series of sesquiterpene orsellinates has been isolated and characterized from cultures of _Armillaria mellea_ and _A. ostoyae_ (Donnelly et al., 1982, 1984; Medland et al., 1982), and orsellinic acid itself is reported to exhibit antibacterial and antifungal activity (Lennartz, 1947; Fujikawa et al., 1956; 1970; Cwielong, 1986). Although inhibition of other fungi by _S. crispa_ may be due to the effects of these compounds, and growth of _Heterobasidion annosum_, which is strongly inhibited by _S. crispa_ on agar plates, was reduced by purified ScI and ScII, the data available are insufficient for proof.

In an early survey, the rate of extension and antibiotic production among a range of 500 basidiomycetes appeared to be negatively correlated (Hervey, 1947). _S. crispa_ is particularly slow growing in culture compared with other wood decay fungi (Stalpers, 1978). Although self-inhibition provides a possible explanation for this effect, rapid early growth of cultures before compounds ScI and ScII had begun to accumulate was not apparent (Fig. 3). Also, growth of isolates 5B and 5C was closely similar, despite their different patterns of antibiotic accumulation.

Overall, the patterns of accumulation of ScI and ScII observed parallel those of the phytotoxic fomajorins produced by _H. annosum_ (Donnelly et al., 1987). Despite the phytotoxic nature of the fomajorins, the disease syndrome caused by _H. annosum_ does not appear typically toxin-mediated. It has been postulated that the biological role of the fomajorins is in the infection process (Sonnenbichler et al., 1989). However, as they are also produced in interactions with antagonistic fungi (Sonnenbichler et al., 1989) they may be important in the long-term maintenance of the fungus in resources (cf. Rayner & Boddy, 1988) captured initially in the living tree. _H. annosum_ is capable of survival for many years in buried wood (e.g. stumps) (Phillips & Burdekin, 1982).

Other root- and butt-rot fungi, including _Phaeolus schweinitzii_, also persist for prolonged periods in the decayed wood of stumps initially infected when the trees were living (Barrett, 1985). In the living tree, the endogenous defences of the host may serve to exclude other micro-organisms from competition with such decay fungi, but this protection ceases on death of the host (Gramss, 1987) and the residual tissues, comprising the stump, then become available to a wide range of colonizers (cf. Rayner & Boddy, 1988). The present study has demonstrated that ScI and ScII occur naturally in wood decayed by _S. crispa_, at concentrations sufficient to impair the growth of other fungi. It seems possible, therefore, that these compounds may contribute to the suppression of potential competitors, hence allowing _S. crispa_ to persist for an extended period. The production of antifungal compounds by many wood-decay fungi, e.g. _P. schweinitzii_ (S. Woodward, unpublished data) suggests that such effects may be widespread among these fungi.

Detection by HPLC of ScI and ScII in extracts of conifer wood (which in some cases had been exposed to environmental weathering) decayed by _S. crispa_, but not in wood decayed by other brown rot fungi, notably _P. schweinitzii_, may provide the basis for a rapid diagnostic method. This technique would obviate the requirements for basidiome production, which is often sporadic, or isolation of the causal fungi, which can be problematic, especially from wood in an advanced state of decay. Moreover, the widespread production of antibiotic secondary metabolites by wood-inhabiting fungi suggests that this approach may be more generally applicable.

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References


