Tulasnein and Podospirone from the Coprophilous Xylariaceous Fungus *Podosordaria tulasnei*

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Tulasnein (**1**), a new metabolite with strong antimicrobial and weaker cytotoxic and phytotoxic activity, was isolated from culture filtrates of three strains of the xylariaceous coprophilous fungus *Podosordaria tulasnei.* The producing strains were identified by their rhizomorphs and by ITS rDNA sequence analysis. A second new metabolite, podospirone (**2**), was also produced by all three strains whereas the weakly cytotoxic (+)-3,4-anhydroshikimic acid methyl ester (**3**) was detected in only one strain.

Key words: Podosordaria tulasnei, Podospirone, Tulasnein

Introduction

One promising approach in the search for fungi producing biologically active secondary metabolites is to focus on species capable of colonizing rich and highly contested substrates. One of the richest natural substrates is the dung of herbivores which is inhabited by a wide range of specialized coprophilous fungi, in addition to opportunistic colonizers from the surrounding soil (Dix and Webster, 1995; Richardson, 2001). A succession of different groups of fungi on fresh dung has been characterized by the order of appearance of their fruit-bodies (Webster, 1970). Early colonizers (mostly zygomycetes) grow and fruit rapidly but soon give way to the more slowly-growing species of asco- and basidiomycetes. We are interested especially in these late colonizers because they must displace the mycota already present on the dung and are therefore more likely producers of bioactive secondary metabolites. Some coprophilous fungi have already been shown to produce interesting secondary metabolites (Gloer, 1995), but many remain to be examined. Although data on the production of antibiotic substances *in situ* are still sparse, antibiosis is thought to be an important determinant of the succession on dung.

A small number of species of Xylariaceae (Ascomycota) have adopted a coprophilous lifestyle, and among them *Poronia punctata* has been shown to produce a range of biologically active sesquiterpenes (Poyser *et al.*, 1985; Edwards *et al.*, 1988). Here we focus on a hitherto unexamined species, *Podosordaria tulasnei*, which is associated with rabbit dung in dry habitats such as sand dunes, spreading into the soil and colonizing new pellets by means of rhizomorphs (Webster and Weber, 2000).

In the following we describe the isolation and characterization of tulasnein (**1**), a new antibiotic with activity against fungi, bacteria, plants and mammalian cells, which was produced by submerged cultures of three strains of *P. tulasnei.* In addition, podospirone (**2**), a new antibiotically inactive compound, and (+)-3,4-anhydroshikimic acid methyl ester (**3**), a known compound, are described (Fig. 1).

Materials and Methods

Producing organisms

Three strains of *P. tulasnei* were isolated as dark stout rhizomorphs from wild rabbit dung pellets incubated on moist sand. D99069 was isolated from a sample collected at Dawlish Warren (Devon, UK), D01029 from Braunton Burrows (Devon, UK) and D02001 from Stonebarrow Hill (Charmouth, Dorset, UK). Mycelial cultures were obtained by plating out clean pieces of rhizomorph material onto 2% malt extract agar augmented with 200 mg/l each of penicillin G and streptomycin sulphate (both from Serva, Heidelberg, Germany). YM medium (malt extract 10 g/l, glucose

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4 g/l, yeast extract 4 g/l) was used for maintenance on agar (20 g agar/l) and fermentation in liquid medium. Mycelial cultures of all strains are maintained in the culture collection of the Dept. of Biotechnology, University of Kaiserslautern.

In order to compare the features of strains D01029 and D02001 with the description of strain D99069 by Webster and Weber (2000), autoclaved rabbit pellets were placed on sterile sea sand and inoculated with mycelial plugs. Growing rhizomorphs (4 weeks old) were buried in sterilized garden soil and incubated for several weeks at 18 °C. For molecular identification, the internal transcribed spacer (ITS) region of the ribosomal DNA gene cluster was amplified, sequenced and analyzed, using the methods described by Köpcke *et al.* (2002).

Fermentation and isolation

Submerged cultures were grown in YM medium in a 20 l fermenter (C6, Biolafitte, Paris) at 22 °C with aeration (3 l/min) and stirring (120 rpm). A shaken flask culture (250 ml, 5 d, 120 rpm) in the same medium was used as inoculum. Samples (100 ml) were taken daily, and the mycelium was separated by filtration. The culture fluid was extracted with 100 ml ethyl acetate, and the mycelium with 100 ml ethanol/acetone 1:1 (v/v). After evaporation *in vacuo* to dryness (40 °C), the residues were redissolved in 1 ml MeOH, and 10 μl aliquots on filter paper disks (6 mm diameter) were tested for antifungal activity by incubation for 24 h at 27 °C on YM plates containing cell suspensions of *Nematospora coryli* ATCC 10647.

Compounds **1** and **2** were isolated from D01029, and **3** from D99069. The fermentation of D01029 was terminated after 10 d when the glucose had been used up and the biomass and inhibition zone diameters had reached a plateau. The culture fluid of D01029 (16 l) was separated from the mycelium which contained no activity against *N. coryli* and was discarded. A crude extract was prepared by passing the culture fluid onto Mitsubishi Diaion HP 21 resin (column size 50 × 6.5 cm) followed by washing with 1.5 l aliquots of water and water/methanol (1:1, v/v), and elution of the fraction containing compounds **1** and **2** with 1.5 l methanol. The crude extract (1.0 g) was applied onto a silica gel column (Merck 60, 63–200 μm particle size; 25 × 2.5 cm) and eluted with cyclohexane/ethyl acetate 9:1 (fraction enriched in **1**) and cyclohexane/ethyl acetate 1:1 (fraction enriched in **2**). Final purification was achieved using a Gilson Model 302 preparative HPLC with a variable wavelength detector set to 210 nm (Abimed, Langenfeld, Germany) and fitted with a Merck (Darmstadt, Germany) LiChrosorb® RP-18 column (7 μm particle size; 250 × 25 mm) at a flow rate of 5 ml/min. The gradient was from water to methanol in 90 min. Compounds **1** (9 mg) and **2** (20 mg) eluted as pure peaks at 87% and 44% MeOH, respectively. Fermentation of D99069 was as for D01029 except that the fermenter was harvested after 8 d, and 1.93 g crude extract was obtained from the HP-21 column with water/methanol 1:1 as eluant. Elution from the silica gel column yielded an enriched fraction of **3** in cyclohexane/ethyl acetate 3:1, and 25 mg pure compound **3** was obtained after preparative HPLC (elution in 39% MeOH; conditions as above).

Spectroscopy

IR spectra of pure compounds were recorded in KBr using an IFS-48 FT-IR spectrometer (Bruker, Karlsruhe, Germany). ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^{1}J_{CH}$ = 145 Hz and $^{n}J_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra (HREI, HRFAB) were recorded with a JEOL SX102 spectrometer, and with a HP Series 1100 LCD-MSD (Hewlett-Packard, Waldbronn, Germany). The HPLC gradient was from water to acetonitrile in 20 min at a flow rate of 0.8 ml/min, using a LiChroCART Superspher 100 RP-18 column (125 × 2 mm; 4 μm particle size; Merck). Fragmentation was attempted in the positive and negative APCI modes using a fragmentor voltage of 140 V. The optical rotations were measured with a Perkin-Elmer 141 polarimeter at 22 °C.

Tulasnein (**1**): White powder. – $[\alpha]_D$ = + 42° (*c* 0.5 in $CHCl_3$). – IR (KBr): ν = 3435, 2950, 1740, 1405, 1280, 1145, 1045, 1020, 1000, 985, 945 and 815 cm^{-1}. – ^1H NMR (500 MHz, $CDCl_3$): δ = 6.33 (s, 13-Ha), 5.89 (s, 13-Hb), 4.45 (dd, *J* = 7.2 and 9.5 Hz, 8-H), 2.11 (d, *J* = 15.2 Hz, 6-Hα), 2.02 (s, 7-OH), 1.90 (m, 9-Hα), 1.78 (ddd, *J* = 4.2, 13.1 and 17.2 Hz, 1-Hβ), 1.68 (m, 9-Hβ), 1.66 (m, 10-H), 1.60 (d, *J* = 15.2 Hz, 6-Hβ), 1.54 (dqd, *J* = 3.8, 6.7 and 11.7 Hz, 4-H), 1.46 (m, 2-Hβ), 1.38 (m, 3-Hα), 1.37 (m, 1-Hα), 1.35 (m, 2-Hα), 1.16 (m, 3-Hβ), 1.00 (s, 14-H_3), 0.80 (d, *J* = 6.7 Hz, 15-H_3). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 169.2 (C-12), 142.6 (C-11), 122.9 (C-13), 85.1 (C-8), 75.1 (C-7), 44.2 (C-6), 37.4 (C-10), 35.5 (C-5), 33.1 (C-4), 32.2 (C-9), 30.4 (C-3), 27.4 (C-1), 22.2 (C-14), 20.9 (C-2), 16.1 (C-15). – HRFABMS: $[M + H]^+$ *m/z* = 251.1636 (required for $C_{15}H_{23}O_3$, 251.1647).

Podospirone (**2**): Light-brown oil. – $[\alpha]_D$ = + 13° (*c* 0.5 in $CHCl_3$). – IR (KBr): ν = 3430, 2950, 1710, 1670, 1625, 1280, 1265, 1135, 1100, 975 and 850 cm^{-1}. – ^1H NMR (500 MHz, $CDCl_3$): δ = 7.07 (d, *J* = 9.9 Hz, 9-H), 6.29 (t, *J* = 7.5 Hz, 12-H), 5.98 (d, *J* = 9.9 Hz; 8-H), 4.79 (d, *J* = 1.7 Hz, 6-H), 4.75 (s, 11-Ha), 4.65 (d, *J* = 6.7 Hz, 1-H), 4.56 (s, 11-Hb), 3.73 (d, *J* = 1.7 Hz, 6-OH), 2.24 (dqd, *J* = 7, 7 and 7 Hz, 13-Ha), 2.03 (dqd, *J* = 7, 7 and 7 Hz, 13-Hb), 1.88 (d, *J* = 6.7 Hz, 1-OH), 1.06 (t, *J* = 7.5 Hz, 14-H_3). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 196.4 (C-7), 167.0 (C-4), 152.0 (C-9), 151.3 (C-12), 143.6 (C-2), 127.8 (C-10), 120.8 (C-8), 86.4 (C-11), 73.6 (C-6), 64.9 (C-1), 57.0 (C-5), 23.2 (C-13), 13.4 (C-14). – FABMS: *m/z* = 154, 136, 107, 89 and 77 (see below).

Biological assays

The phytotoxic effects on germination of *Setaria italica* and *Lepidium sativum* as well as antimicrobial activities were determined as described previously (Anke *et al.*, 1989). For the antimicrobial spectra, an inoculum of 10^5 cells or spores/ml or a fine mycelial suspension of non-sporulating fungi was used. Cytotoxic activities were measured against Colo-320 cells (DSMZ ACC-144, human) and L1210 cells (ATCC CCL 219, mouse) as described by Zapf *et al.* (1995).

Results and Discussion

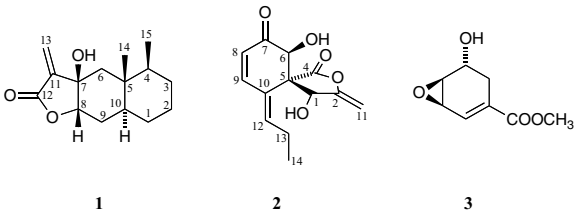
Identification of the producing strains

Both strains D01029 and D02001 were isolated as rhizomorphs very similar in appearance to those of *Podosordaria tulasnei* D99069 described by Webster and Weber (2000). However, it proved impossible to induce fructification and ascospore formation in these two strains using the conditions described for D99069 (Webster and Weber, 2000). In order to confirm that they were sterile isolates of *P. tulasnei*, their ITS sequences were obtained and compared with that of D99069. The ITS-1-5.8S-ITS2 sequences of all three strains were identical over their full length (600 bp from primer ITS5 to ITS4). The sequence has been deposited in GenBank (accession number AY572970). Since the ITS1 and ITS2 regions are transcribed but do not encode any functional gene, they are highly variable regions of the fungal genome, with variations between different isolates of the same species commonly observed (Yurlova *et al.*, 1999; Platas *et al.*, 2004). Therefore, we interpret the sequence identity of our three *P. tulasnei* isolates as a confirmation of species identity.

Rhizomorphs identical to those of *P. tulasnei* are frequently observed when rabbit dung collected from the field is incubated under fairly dry conditions, and this 'unidentified rhizomorphic fungus' is well-known among field mycologists. Our data identify it as *P. tulasnei.*

Structure elucidation

The structure of tulasnein (**1**) was determined by close inspection of the NMR data. The chemical composition suggested by high resolution MS experiments was confirmed by the presence of signals for 15 carbon atoms and 22 protons in the 1D NMR spectra, and as the NMR data indicate that the compound has one carbon-carbon double bond and one carbonyl group it must consequently have three rings. HMBC correlations from 13-H_2 to C-7, C-11 and C-12 show that the exomethylenic double bond is part of an α,β-unsaturated ester/lactone, and HMBC correlations from 7-OH to C-8 and from 8-H to C-12 show that it is in fact a lactone and closes the first of the rings. The ^1H-^1H spin system starting with 8-H and proceeding *via* 9-H_2, 10-H, 1-H_2, 2-H_2, 3-H_2 and 4-H to 15-H_3 observed in the COSY spectrum determines a large part of the structure, and HMBC correlations from 14-H_3 to C-4, C-5, C-6 and C-10 close



1 **2** **3**

Fig. 1. Structures of tulasnein (**1**), podospirone (**2**) and (+)-3,4-anhydroshikimic acid methyl ester (**3**).

another ring and leave only one possibility to close the third (C-6 to C-7). Tulasnein (**1**) is consequently an eremophilane sesquiterpene. The relative stereostructure was suggested by data from a NOESY spectrum. Correlations from 14-H_3 to 1-Hβ, 3-Hβ, 6-Hβ and 9-Hβ as well as from 10-H to 2-Hα and 6-Hα suggest that C-14 and 10-H are on opposite sides of the molecule, while correlations from 13-Hβ to both 6-Hα and 6-Hβ as well as from 8-H to 7-OH support the position of 7-OH and 8-H as shown in Fig. 1.

Podospirone (**2**) did not give any reasonable mass spectra, although a number of different techniques were used. It is obviously a highly sensitive molecule and very easily degrades during the ionization. However, the 1D NMR spectra show that it has 13 carbon atoms and 14 hydrogen atoms, of which two are not attached to carbon atoms. The chemical shifts suggest that the compound contains two hydroxylated carbon atoms, C-1 and C-6, and both hydroxyl protons are visible in the ^{1}H NMR spectrum. The ethyl group comprising C-13 and C-14 is connected to C-12, part of a trisubstituted double bond together with C-10, and strong HMBC correlations could be observed from 12-H to C-5 and C-9. 9-H is part of a *cis* double bond (according to the ^{1}H-^{1}H coupling constant, 9.9 Hz) which is conjugated with what appears to be a keto function (C-7). A HMBC correlation from 8-H past the keto group to C-6 indicates that this is present on the other side, and HMBC correlations from 6-OH to C-5, C-6 and C-7 close the first ring. The extreme chemical shift of C-11 as well as the HMBC correlations observed from 11-H_2 to only two carbon atoms (C-1 and C-2) suggest that the exocyclic double bond is substituted with one heteroatom besides C-1. HMBC correlations from 1-OH to C-1, C-2 and C-5 confirm this, and also link this partial structure to C-5. 1-H also gives a HMBC correlation to C-5, and in addition to C-4 which must be on the other side. The chemical shift of C-4 suggests that it is an ester/lactone carbonyl carbon, and a second ring must be closed with an oxygen between C-2 and C-4. Podospirone (**2**) should therefore have the composition $C_{13}H_{14}O_5$ and a molecular weight of 250. However, from the molecular structure it can be predicted that podospirone should degrade rapidly when ionized, and it was indeed impossible to observe the molecular ion in the mass spectra. The relative stereostructure of podospirone (**2**) was indicated from the NOESY correlations observed between 9-H and 12-H, between 1-H and 6-OH, and between 1-OH and 13-H_2.

3,4-Anhydroshikimic acid methyl ester was identified by comparing the spectral data with those previously reported (Fex *et al.*, 1981).

Biological activities

The biological activities of compounds **1**–**3** are given in Table I. Tulasnein (**1**) showed moderate to high antifungal and antibacterial, and weaker phytotoxic and cytotoxic activities. In contrast, podospirone (**2**) showed no activity in any of the biological tests, and (+)-3,4-anhydroshikimic acid methyl ester (**3**) was weakly cytotoxic and phytotoxic. We are currently investigating whether tulasnein is produced at toxic levels by rhizomorphs or assimilative hyphae of *P. tulasnei* on dung.

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Table I. Minimal inhibitory concentrations (MIC) of tulasnein (**1**), podospirone (**2**) and (+)-3,4-anhydroshikimic acid methyl ester (**3**). MIC values are given in μg/ml, with 2.5 μg/ml (**1**) corresponding to 10 μM, 100 μg/ml (**2**) to 400 μM, and 100 μg/ml (**3**) to 588 μM.

Test organism	**1**	**2**	**3**
Yeasts			
Nadsonia fulvescens Tü 561	10	> 100	> 100
Nematospora coryli ATCC 10647	2.5	> 100	100
Rhodotorula glutinis ATCC 26085	5	> 100	> 100
Saccharomyces cerevisiae S 288c	5	> 100	> 100
S. cerevisiae is1	5	> 100	> 100
Filamentous fungi			
Absidia glauca mating type '+' CBS 101.08	5	> 100	100
A. glauca mating type '–' CBS 102.08	2.5	> 100	50
Alternaria porri CBS 225.76	2.5	> 100	100
Ascochyta pisi CBS 108.26	10	> 100	100
Aspergillus ochraceus DSM 63304	2.5	> 100	100
Cladosporium cladosporioides CBS 109.21	5	> 100	> 100
Fusarium fujikuroi CBS 221.76	5	> 100	100
F. oxysporum f. sp. *cubense* CBS 149.25	10	> 100	> 100
Paecilomyces variotii ETH 114646	10	> 100	100
Penicillium islandicum ATCC 10127	5	> 100	50
P. notatum	10	> 100	100
Zygorhynchus moelleri CBS 111.10	5	> 100	100
Gram-positive bacteria			
Arthrobacter citreus ATCC 11624	10	> 100	> 100
Bacillus licheniformis ATCC 21415	10	> 100	> 100
Mycobacterium phlei	10	> 100	> 100
Gram-negative bacteria			
Escherichia coli K12	5	> 100	> 100
Salmonella typhimurium TA 98	5	> 100	> 100
Plants			
Lepidium sativum	> 100	> 100	100
Setaria italica	25	> 100	50
Cell lines			
Colo-320 DSMZ ACC-144	25	> 100	25
L1210 ATCC CCL 219	25	> 100	25

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