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Galiellalactone and its biogenetic precursors as chemotaxonomic markers of the Sarcosomataceae (Ascomycota)

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Abstract

(−)-Galiellalactone is a hexaketide metabolite with interesting pharmacological activities which was detected in four strains of *Galiella rufa* (Sarcosomataceae, Ascomycota) and in two unidentified fungi shown by their 18S rDNA sequences also to belong to the Sarcosomataceae. These were a wood-inhabiting apothecial species from Chile and an endophytic isolate from *Cistus salviifolius* (Sardinia). Other members of the family (*Urnula helvelloides*, one *Strumella coryneoidea* isolate) produced no galiellalactone but merely hexaketides structurally related to galiellalactone precursors, whereas a third group of species (*Sarcosoma latahensis*, *Strumella griseola*, one *S. coryneoidea* isolate) lacked hexaketide production altogether. Despite thorough screening programmes, galiellalactone and its precursors have not yet been found in any fungus outside the Sarcosomataceae and may thus be a chemotaxonomic marker of the family, supporting its current phylogenetic definition. Two pentaketide derivatives of the 6-pentyl-α-pyrone type were found in all *G. rufa* strains as well as in A111-95 and the hexaketide-producing *S. coryneoidea* isolate. © 2002 Published by Elsevier Science Ltd.

Keywords: *Galiella*; *Sarcosoma*; *Strumella*; *Urnula*; Sarcosomataceae; *Cistus*; Chemotaxonomy; Phylogeny; Endophyte; Polyketides; Galiellalactone

1. Introduction

(−)-Galiellalactone was first isolated from submerged cultures of the ascomycete *Galiella rufa* (Schweinitz) Nannfeldt & Korf as an inhibitor of gibberellic acid-induced de novo synthesis of hydrolytic enzymes in embryoless seeds of *Triticum aestivum* (Hautzel and Anke, 1990; Steglich et al., 1993). Subsequently, galiellalactone was described as the first fungal metabolite interfering at very low concentrations with interleukin-6-signalling in mammalian cells (Weidler et al., 2000), pointing at its considerable pharmacological potential. Further, some of its biogenetic precursors and structurally related compounds have nematicidal activity (Anke and Sterner, 2002; Köpcke et al., 2002; Johansson et al., 2002a).

G. rufa occurs on dead wood especially in North America (Farr et al., 1989). Other *Galiella* spp. have been described from the tropics (Cao et al., 1992; Zhuang and Wang, 1998; Pant, 2001) whereas related genera such as *Plectania*, *Urnula*, *Sarcosoma* and the anamorphic *Strumella* also occur in subarctic and temperate zones of Europe (Dissing, 1981, 2000; Spooner, 2002). Phylogenetic comparisons of 18S rDNA sequences have placed these genera within the order Pezizales in the family Sarcosomataceae, sister family to the better-known and more widely distributed Sarcoscyphaceae (Landvik et al., 1997; Harrington et al., 1999).

In the course of our screening for biologically active products from fungi, galiellalactone (**7**), deoxygaliellalactone (**6**), pregaliellalactone (**5**) and structurally related compounds (**1**–**4**) have recently been isolated from a Chilean ascomycete (A111-95) with morphological features similar to *Urnula craterium* (Schweinitz) Fries (Johansson et al., 2002a; Köpcke et al., 2002). In addition, the same compounds were detected in an endophytic fungus (E99297) isolated from living twigs of *Cistus salviifolius* L. from Sardinia (B. Köpcke and O. Sterner, unpublished). The repeated occurrence of these interesting fungal metabolites prompted us to determine the phylogenetic position of the two recently discovered producers by sequencing the internal transcribed spacer (ITS) and 18S regions of rDNA, and to

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compare their capacity to produce hexaketide compounds with that of available cultures of other members of the Sarcosomataceae.

2. Results and discussion

Cultures of 11 sarcosomataceous fungi were examined for the production of compounds derived from the hexaketide pathway and could be placed in three groups according to their metabolic spectrum. (−)-Galiellalactone (**7**; Hautzel and Anke, 1990) and its putative biogenetic precursors **2**–**6** (see Fig. 1) were detected in all four *G. rufa* strains as well as in our isolates A111-95 and E99297 (Table 1). Whereas *Sarcosoma latahensis* Paden & Tylutki, *Strumella griseola* von Höhnel and *Strumella coryneoidea* Saccardo & Winter CBS 438.51 did not produce any of the above substances, *S. coryneoidea* CBS 233.91 and *Urnula helvelloides* Donadini et al. produced compounds **2**–**4**, thereby occupying an intermediate position among the strains tested. Additionally, the pentaketides **8** and **9** were detected. Their production was weakly correlated with the occurrence of hexaketides in that they were found in five of six galiellalactone producers, one of two members of the intermediate group, and none of the three non-hexaketide producers (Table 1).

Compounds **3** and **4** could not be separated under the chromatographic conditions chosen, and were therefore characterized as a mixture. Likewise, the identification of compound **2** was ambiguous because HPLC-MS analysis revealed a family of substances with molecular masses of 176 or 178 Da and almost identical UV spectra whose absorption maxima varied slightly. Compound **1** was often detectable only as traces which did not always permit unequivocal identification of mass peaks (see Table 1). Production of hexaketide metabolites occurred in both growth media tested (MYG and YMG), the levels of galiellalactone (**7**) ranging from 40 to 350 mg l^{-1} culture fluid. Galiellalactone was the predominant metabolite of all four *G. rufa* strains, whereas compounds **2** and **3**+**4** were prevalent in extracts of A111-95 and E99297.

Hexaketides such as galiellalactone are rare among fungi (Turner, 1971; Turner and Aldridge, 1983). In contrast, pentaketides of the 6-pentyl-α-pyrone-type such as substances **8** and **9** are commonly found among ascomycetes and their related anamorphs (Kimura et al., 1978; Turner and Aldridge, 1983; Dictionary of Natural Products, 2002). Compound **9** has already been described from *U. craterium* (Ayer et al., 2000), although it was absent from *U. helvelloides* in our investigations.

The biosynthetic pathway of (−)-galiellalactone has not been fully elucidated yet, but results from incorporation experiments using [1-^{13}C]- and [2-^{13}C]-glucose are in agreement with a polyketide pathway, with

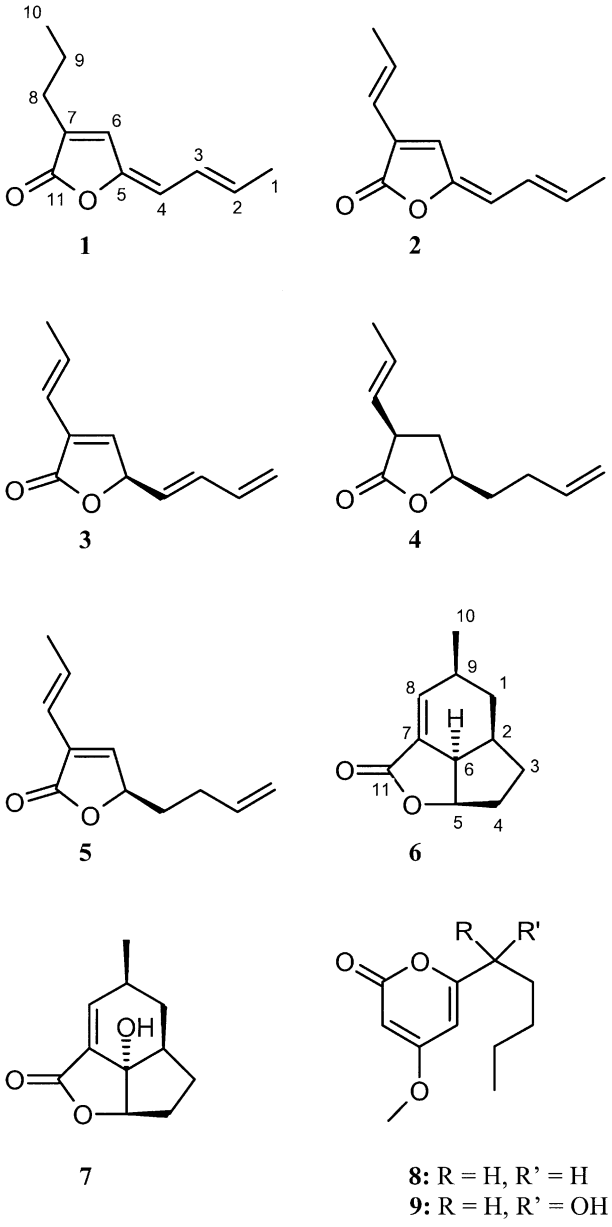

Fig. 1. Structures of secondary metabolites investigated. **1**, 5-(*E*)-but-2-enylidene-3-propyl-5*H*-furan-2-one; **2**, 5-(*E*)-but-2-enylidene-3-(*E*)-propenyl-5*H*-furan-2-one; **3**, 5-(*E*)-buta-1,3-dienyl-3(*E*)-propenyl-5*H*-furan-2-one; **4**, 5-(*E*)-but-3-enyl-3-(*E*)-propenyl-dihydrofuran-2-one; **5**, (−)-pregaliellalactone; **6**, (+)-deoxygaliellalactone; **7**, (−)-galiellalactone; **8**, 6-pentyl-4-methoxy-6-pyran-2-one; **9**, 6-(1-hydroxypentyl)-4-methoxy-6-pyran-2-one.

(−)-pregaliellalactone (**5**) and (+)-deoxygaliellalactone (**6**) being the last two intermediate products (Steglich et al., 1993; Knerr, 1995). There is no experimental evidence as yet that any of the remaining compounds **1**–**4** are direct precursors of (−)-galiellalactone although they show a strong structural similarity to **5**. Compounds **1**–**7** were not present continuously throughout the fermentations. Especially deoxygaliellalactone (**6**) could only be detected at the beginning of the fermentations in all *G. rufa* species, disappearing after several

Table 1
Occurrence of compounds **1**–**9** in cultures of sarcosomataceous fungi

Organism	GenBank accession number	Compound[a]							
		1	**2**	**3**+**4**[b]	**5**	**6**	**7**	**8**	**9**
Ascomycete A111–95	AF485075	+	+	+	+	+	+	+	+
Galiella rufa A75–86	AF485073	+	+	+	+	+	+	+	+
G. rufa ATCC 200370	AF485071	±[c]	+	+	+	+	+	+	+
G. rufa CBS 135.92	AF485070	±[c]	+	+	+	+	+	+	+
G. rufa CBS 762.85	AF485072	±[c]	+	+	+	+	+	+	+
Sarcosoma latahensis CBS 733.68	AF485079	–	–	–	–	–	–	–	–
Strumella coryneoidea CBS 438.51	AF485069	–	–	–	–	–	–	–	–
S. coryneoidea CBS 233.91	AF485076	±[c]	+	+	–	–	–	+	+
S. griseola CBS 433.59	AF485078	–	–	–	–	–	–	–	–
Urnula helvelloides CBS 763.85	AF485077	+	+	+	–	–	–	–	–
Endophyte E99297	AF485074	±[c]	+	+	+	+	+	–	–

[a] The presence (+) or absence (–) of compounds was tested by HPLC and HPLC-MS analysis.
[b] Compounds **3** and **4** could not be separated and were characterized and detected as a mixture.
[c] Compound **1** possibly present in trace amounts.

days. The occurrence of galiellalactone (**7**) was always coupled with the presence of pregaliellalactone (**5**). The prolonged presence of **5** in ongoing fermentations may be explained by the fact that the Diels–Alder cyclization of this molecule to yield compound **6** is a time-limiting step in the biosynthetic pathway (Johansson et al., 2002b). In contrast, the hydroxylation of **6** is probably a very fast reaction once the appropriate enzymes are present, explaining its detection only in the early fermentation phase. This is supported by the efficient and fast conversion of synthetically prepared pregaliellalactone (**5**) via compound **6** to (–)-galiellalactone without an accumulation of **6** (Johansson et al., 2002b; B. Köpcke, unpublished).

The biochemical data presented in Table 1 were partly supported by comparisons of the ITS1–5.8S–ITS2 DNA sequences from the nucleus-encoded ribosomal DNA gene cluster. Thus, the sequences of all four *G. rufa* strains were very similar to each other, their entire alignment of 632 bases showing heterogeneity at only seven positions (data not shown). This degree of variation of ITS sequences is within that which would be expected of different isolates of the same species long-established at geographically discrete localities (e.g. Zambino and Szabo, 1993; Pfunder et al., 2001). Unfortunately, the ITS sequences of the other strains were too variable to permit unequivocal alignment for phylogenetic analysis. This also applied to the two *S. coryneoidea* isolates which, together with their different metabolic spectra, suggests that at least one of them was mistaken for another member of the Sarcosomataceae during identification.

Although ITS sequences have been extensively used in fungal molecular phylogeny, examples of the Sarcosomataceae were unavailable in GenBank and other data bases at the time of the current work. In order to permit future species-specific identification, we have deposited our sequences in GenBank (for accession numbers, see Table 1). Previous phylogenetic analyses of the Sarcosomataceae have been conducted at higher taxonomic levels and were therefore based on the more highly conserved 18S rDNA gene (Landvik et al., 1997; Harrington et al., 1999). In order to assess the affinity of our two strains E99297 and A111-95 with the Sarcosomataceae, we obtained sequences of about 750 bp from the 5′ end of the 18S rRNA gene. A FASTA search placed both species within the Sarcosomataceae in that the eight most similar sequences belonged to members of that family (Table 2). The sequence identity was 98.8–100% for E99297 and 98.7–99.6% for A111-95, *Sarcosoma mexicana* Paden & Tylutki and *U. craterium* being the closest sequenced relatives of both isolates.

In the absence of any reports of the occurrence of galiellalactone or its biogenetic precursors in other fungi, our results confirm that all galiellalactone producers known to date are clustered in the very small ascomycete family Sarcosomataceae sensu stricto, as delimited by 18S rDNA sequencing data (Harrington et al., 1999). This taxonomic concept is therefore supported also by chemotaxonomic arguments. No natural products other than galiellalactone and its precursors, or chemically unrelated pentaketides including substance **9** and hydroxynaphthalenones (Ayer et al., 2000), have been described for Sarcosomataceae in chemical databases.

Sarcosomataceous fungi are known mainly as degraders of wood or as pathogens (Farr et al., 1989; Dissing, 2000), and to our knowledge E99297 represents the first case of an endophytic member of the family, growing from several healthy-looking twigs of *Cistus salviifolius*. No pathogenic Sarcosomataceae appear to be known on this plant genus. The occurrence of galiellalactone, a

Table 2
The 10 18S rDNA GenBank sequences with the highest similarity to those of E99297 and A111–95

Species[a]	GenBank accession number	Number of differences[b] to	
		E99297	A111-95
Sarcosoma mexicana	AF104346	0	3
Urnula craterium	AF104347	0	3
Plectania sp.	AF104344	1	4
Donadinia sp.	AF104342	3	4
Urnula hiemalis Nannfeldt	UHRRNA18S	1	4
Galiella rufa	AF004948	2	4
Pseudoplectania nigrella (Persoon:Fries) Fuckel	AF104345	6	5
Neournula pouchetii (Berthet & Riousset) Paden	AF104666	9	10
Rhizina undulata Fries:Fries	RUU42664	10	11
Strobiloscypha keliae Weber & Denison	AF006310	10	12

[a] With the exception of *Rhizina* and *Strobiloscypha*, all genera are considered to belong to the Sarcosomataceae (Harrington et al., 1999; Kirk et al., 2001).
[b] The length of the sequenced 18S rDNA fragment was 747 bp for E99297 and 752 bp for A111-95.

compound interfering with signal transduction in plants and animal or human cells, invites the speculation that it might have a function in the plant–endophyte relationship, e.g. by inhibiting signal transduction events involved in the induction of defense reaction in the host plant. Clearly, the Sarcosomataceae present a continuum of modes of nutrition, ranging from the mutual, neutral or at worst mildly antagonistic endophytic symbiosis (E99297) over parasitism (*S. coryneoidea*; Fergus, 1951) to pure saprotrophy (*Galiella*, *Sarcosoma*, *Urnula*).

3. Experimental

3.1. Microorganisms and culture conditions

G. rufa A75-86 and its growth conditions have been described by Hautzel and Anke (1990), and strain A111-95 by Köpcke et al. (2002). *G. rufa* ATCC 200370, CBS 135.92 and CBS 762.85, *S. latahensis* CBS 733.68, *U. helvelloides* CBS 763.85, and the anamorphic *S. griseola* CBS 433.59 and *S. coryneoidea* CBS 438.51 and CBS 233.91 were purchased from the American Type Culture Collection (ATCC, Manassas, USA) or Centraalbureau voor Schimmelcultures (CBS, Utrecht, Netherlands). The endophyte E99297 was isolated from a living twig of *C. salviifolius* collected in Sardinia, which had been surface-sterilized in 70% ethanol (1 min), 5% sodium hypochlorite (3 min) and 70% ethanol (30 s), and washed in sterile dist. H_2O prior to plating out on 2% malt extract agar.

For fermentations, all fungi were grown in YMG medium composed of 4 g yeast extract, 10 g malt extract, 10 g glucose and 1 l dist. H_2O. The pH was adjusted to 5.5 before autoclaving. MYG medium was used as an alternative fermentation medium with a higher carbon/nitrogen ratio. It contained 20 g maltose, 10 g glucose, 1 g yeast extract, 0.5 g KH_2PO_4, 1 g $MgSO_4{\cdot}7H_2O$, 10 mg $FeCl_3$, 1 mg $ZnSO_4{\cdot}7H_2O$, 56 mg $CaCl_2$ and 1 l dist. H_2O (pH 5.5).

Fungi were grown in 2-l Erlenmeyer flasks containing 1000 ml culture medium. The flasks were inoculated with 10 disks of growing agar culture (1 cm diam.) and incubated on an orbital shaker (120 rpm) at 24 °C. The fermentation was stopped after 28 days or earlier if the glucose levels in the medium had dropped to zero. Samples (50 ml) were taken every 3–4 days. After separating the culture filtrate from the mycelium by filtration, the culture fluid was extracted with the same volume of EtOAc while the mycelium was extracted with a mixture of MeOH/Me_2CO (1:1). The solvents were evaporated in vacuo and the dried extracts stored at −20 °C. For analytical HPLC the samples were redissolved in 0.5 ml MeOH.

3.2. Analytical methods

The secondary metabolites in the extracts were identified by analytical HPLC/MS using a Hewlett-Packard Series 1100LC-MSD instrument fitted with a LiChroCART Superspher 100 RP-18® analytical column (4 μm; 125 × 2 mm; Merck, Darmstadt, Germany) and a DAD and MS detector. The mobile phase consisted of a linear gradient from H_2O to acetonitrile in 19.5 min at a flow rate of 0.8 ml min^{-1}. Eluting compounds were identified by comparing their retention time and UV/visible and mass spectra (APCI positive and APCI negative) with the reference data of pure substances (Johansson et al., 2002a; Köpcke et al., 2002).

3.3. Determination of rDNA sequences

Mycelium (1–3 g wet weight) was filtered from cultures grown in liquid YMG medium for 28 days, snap-frozen

in liquid nitrogen, stored at −80 °C and ground to a fine powder using a mortar and pestle, adding liquid N_2 as required to keep the material frozen. For DNA extraction, the phenol-chloroform procedure of Sacks et al. (1995) was used.

PCR amplification of the ITS1–5.8S rDNA–ITS2 region of nuclear DNA utilized the universal fungal primers ITS5 (5′GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′TCCTCCGCTTATTGATATGC) which bind to conserved flanking regions of the 18S and 26S rDNA, respectively (White et al., 1990). The PCR mixture (50 μl) contained 2.5 μl of a 10 μM solution of each primer (MWG Biotech, Ebersberg, Germany), 2.5 μl DNA (100 ng ml^{-1}), 2 μl Taq polymerase (1000 U ml^{-1}; Fermentas, St. Leon-Rot, Germany), 5 μl of the appropriate 10-fold concentrated buffer, and 4 μl 25 mM $MgCl_2$; after heating of the tubes to 94 °C, 5 μl dNTP mix (Fermentas) containing 2 mM of each deoxynucleotide was added, followed by a drop of mineral oil. After an initial pre-heat at 94 °C for 3 min, 35 amplification cycles were performed (30 s at 94 °C, 1 min at 45 °C, 1 min at 72 °C), followed by a final extension period of 10 min at 72 °C. A PCR Sprint thermocycler (Hybaid, Maidenhead, UK) was used. Amplified bands of 550–950 bp were separated on a 1% agarose gel, excised, and purified using the Geneclean II® kit (Bio 101, Vista, USA). Independent sequencing of three PCR products for each species was carried out by MWG using the same primers as for amplification. Sequences were assigned and proof-read by eye using Chromas 1.62 (Technelysium, Helensvale, Australia).

For amplification of the 5′ end of the 18S rDNA gene, primers NS1 (5′GTAGTCATATGCTTGTCTC; White et al., 1990) and SR4 (5′AAACCAACAAAATAGAA; http://www.biology.duke.edu/fungi/mycolab) were used; the PCR protocol was as above, except that the annealing temperature was lowered from 45 to 38 °C. Sequencing was carried out by MWG as above.

Searches for matching sequences were performed using the FASTA facility provided by the HUSAR package (DKFZ, Heidelberg, Germany).

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