Corymbiferan Lactones from *Penicillium hordei*: Stimulation of Novel Phenolic Metabolites Using Plant Tissue Media

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Received August 24, 2004

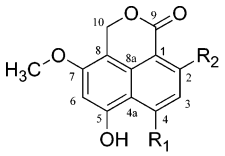
Cultivation of a strain of *Penicillium hordei* on macerated tulip tissue agar resulted in the stimulated production of a series of four novel hydroxymethyl naphthalene carboxylic acid lactones from the fungus. The naphthalene derivatives were isolated using a combination of vacuum liquid chromatography and preparative HPLC. Their structures were determined by 1D and 2D NMR techniques in conjunction with high-resolution electrospray mass spectrometry (HRESIMS). These metabolites were given the trivial names corymbiferan lactones A−D (**1**−**4**).

Penicillium hordei Stolk is a member of the *Penicillium* series *Corymbifera,*[1] *Penicillia* that are pathogenic on various root vegetables and flower bulbs causing a storage rot, generically referred to as blue mold rot.[2−4] Although categorized within the *Corymbifera* series based on similarities in micromorphology and secondary metabolite production, *P. hordei* is more commonly associated with cereals such as wheat and barley.[5] As one means of defense against biotic stresses such as fungal invasion, plant cells initiate an "oxidative burst" releasing reactive oxygen species into the cellular environment surrounding the point of infection. These reactive oxygen species have been hypothesized to act as protective agents against invasive pathogens and to elicit plant defense reactions such as the hypersensitive response (self-initiated death of cells adjacent to the site of infection).[6,7] True plant pathogens can grow extensively through the plant by delaying or not evoking host necrosis.[6] The antioxidant properties of phenolic metabolites are reputed for scavenging oxygen radicals and inhibiting peroxidation.[8] Production and excretion of phenolic metabolites by fungal invaders may act to curtail the "oxidative burst" defensive process and prove advantageous during infection.

During metabolite screening of terverticillate *Penicillia* grown on generic laboratory media formulations (Czapek yeast autolysate (CYA), malt extract (MEA), and yeast extract sucrose (YES) agars; for formulations refer to Samson et al.[9]), Frisvad and Filtenborg[5] identified and confirmed the production of the alkaloids cyclopenol and roquefortine C, the polyketide atrovenetin, and the TCA cycle intermediate-derived metabolite terrestric acid from strains of *P. hordei*. In the present investigation of a *P. hordei* strain grown on plant tissue-based, solid agar media, RP-HPLC analysis of culture extracts demonstrated the stimulated production of a series of phenolic metabolites in addition to the secondary metabolites previously observed by Frisvad and Filtenborg.

Results and Discussion

Comparison of HPLC extract profiles of a *P. hordei* strain (IBT 21039) grown on CYA, YES, oatmeal, and tulip agar demonstrated differences in secondary metabolite production between the strain grown on the standard media (CYA and YES) with those produced on plant-derived oatmeal and tulip agar. Four unknown metabolites (**1**−**4**) produced by *P. hordei* on oatmeal and tulip agar, having similar, characteristic absorption spectra yet differing in retention times, were targeted for further structural elucidation investigation. As these four metabolites were not present in the media controls (see Figure 1) and are produced on two separate media, it can be assumed that these stimulated metabolites are of fungal origin. Large-scale cultivation and extraction of *P. hordei* (strain IBT 21039) followed by subsequent fractionation of the extract by solid-phase extraction (vacuum liquid chromatography) and purification by RP-semipreparative HPLC were carried out to yield five new phenolic, naphthalene derivatives. The structures of these compounds (**1**−**4**) were elucidated by analysis of their respective spectroscopic data (ESIMS and 1D and 2D NMR), and the compounds were given the trival names corymbiferan lactones A−D.



1 R_1=CH_2OH R_2=OH
2 R_1=CH_2OCHO R_2=OH
3 R_1=CH_2OH R_2=OCH_3
4 R_1=CH_3 R_2=OCH_3

The ESIMS spectra of corymbiferan lactone A (**1**) in negative ion mode displayed a peak at *m/z* 275.0548, supporting the molecular formula $C_{14}H_{12}O_6$. The ^{1}H NMR spectrum (DMSO-d_6) showed two aromatic protons at δ_H 6.65 (s) and 7.17 (s), two hydroxymethyls at δ_H 5.13 (s) and 5.62 (s), and a methoxyl at δ_H 3.86 (s). From the number of double-bond equivalents (9) and a single carbonyl resonance at δ_C 170.3, we inferred the molecular skeleton to be a naphthalene ring system having a lactone bridge. Positioning of the functional substituents of ring A could be inferred by the long-range coupling of the C_6 and C_3 aromatic protons (δ_H 6.65 and 7.17) and the C_{4-R1} and C_{10} hydroxymethyl protons (δ_H 5.13 and 5.62) to the aromatic

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10.1021/np049723c CCC: $27.50
Published on Web 11/03/2004

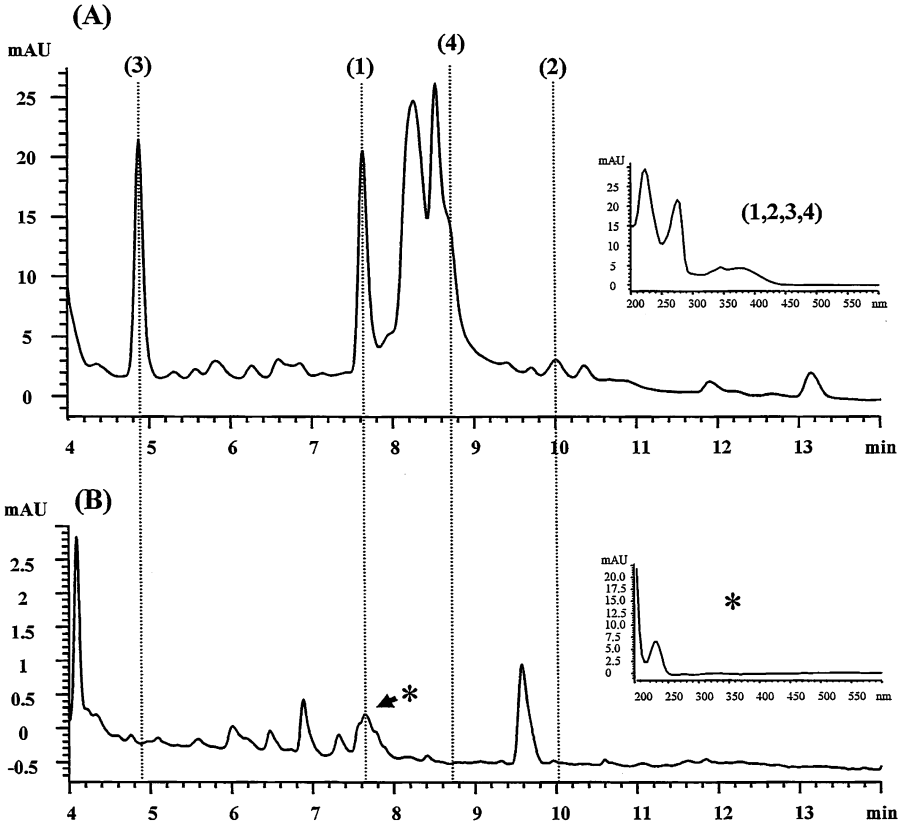

Figure 1. HPLC analysis comparison of extracts representing (A) *P. hordei* grown on tulip agar and (B) tulip agar control. HPLC analyses were performed on an Agilent Hypersil BDS-C18 column (125 × 2 mm, 3 μm) using the following gradient: 15%−100% MeCN in 40 min at a flow rate of 1 mL/min. Traces have been truncated to a window of 4−14 min. *A tulip agar metabolite with a retention time similar to corymbiferan lactone A (**1**).

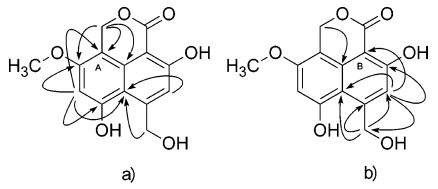

Figure 2. Selected HMBC connectivities in corymbiferan lactone A (**1**): (a) naphthalene ring A; (b) naphthalene ring B.

quaternary carbons at C_{4a}−C_{8a} (δ_C 110.2, 157.5, 96.8, 154.5, 100.0, 132.0), as demonstrated in Figure 2a. The positioning of the C_7 methoxyl substituent was inferred from the three-bond coupling of the methyl ester protons (δ_H 3.86) to the C_7 quaternary carbon resonance at δ 154.5 of ring A. Positioning of the functional substituents of ring B could be inferred by the long-range coupling of the C_3 aromatic proton (δ_H 7.17) and the C_{4-R1} and C_{10} hydroxymethyl protons (δ_H 5.13 and 5.62) to the aromatic quaternary carbons at C_1−C_{4a}, C_8 (δ_C 96.8, 162.3, 110.2, 147.3, 111.7, 132.0), as demonstrated in Figure 2b. The downfield shift of C_{10} (δ_C 67.2) coupled with a proton shift of δ_H 5.62 indicated the aliphatic methyl was adjacent to an oxygen. Long-range coupling of the C_{10} hydroxymethyl protons (δ_H 5.62) and the C_3 aromatic proton (δ_H 7.17) to the C_9 carbonyl group (δ_C 170.3) was used to infer the positioning of the hydroxymethyl lactone bridge.

The ESIMS spectra of corymbiferan lactone B (**2**) in negative ion mode displayed a peak at *m/z* 303.0525 corresponding to the molecular formula $C_{15}H_{12}O_7$. By comparison of the long-range coupling of the C_6 and C_3 aromatic protons (δ_H 6.70 and 6.94), the C_{4-R1} and C_{10} hydroxymethyl protons (δ_H 5.64 and 5.79), and the C_7 methoxyl protons (δ_H 3.88), carbon assignments were possible, establishing a structural moiety nearly identical to corymbiferan lactone A (**1**). The presence of a formyl substituent was deduced from a carbon signal at δ 160.9 coupled to a proton singlet at δ 8.46. Long-range coupling of the C_{4-R1} hydroxymethyl protons (δ_H 5.79) to the formyl carbon (δ_C 160.9) was used to infer an ester linkage between the formyl and hydroxymethyl substituents.

The ESIMS spectra of corymbiferan lactone C (**3**) in negative ion mode displayed a peak at *m/z* 289.0743 corresponding to the molecular formula $C_{15}H_{14}O_6$. Again, comparison of the long-range coupling of the C_6 and C_3 aromatic protons (δ_H 6.66 and 7.54), C_{4-R1} and C_{10} hydroxymethyl protons (δ_H 5.19 and 5.37), and the C_7 methoxyl protons (δ_H 3.86) established a structural moiety nearly identical to corymbiferan lactone A (**1**). The presence of the C_{2-R2} methoxyl substituent was inferred from the three-bond coupling of the methyl ester protons δ_H 3.99 to the C_2 quaternary carbon δ_C 162.0.

The ESIMS spectra of corymbiferan lactone D (**4**) in negative ion mode displayed a peak at *m/z* 273.0794 corresponding to the molecular formula $C_{15}H_{14}O_5$. The skeletal structure of corymbiferan lactone D (**4**) was identical to that of corymbiferan lactone A (**1**); however, corymbiferan lactone D (**4**) had two different functional substituents. The C_{4-R1} hydroxymethyl functional group was replaced by a methyl group (δ_H 2.88, δ_C 25.6), as confirmed by the long-range couplings of the methyl protons (δ_H 2.88) to the aromatic quaternary carbons at C_3 and C_4 (δ_C 113.6 and 146.6) and the C_3 aromatic protons (δ_H 7.04) to the C_{4-R1} methyl carbon (δ_C 25.6). The presence of an additional methoxyl group (C_{2-R2}, δ_C 56.7) was inferred from the sole three-bond coupling of the methyl ether protons δ_H 3.97 to the C_2 quaternary carbon δ_C 161.9.

The motivation for this work was to establish if a novel approach to fungal cultivation could result in the stimula-

tion of novel natural products. Due to the pathogenic association of this fungus with cereals and flower bulbs,[5,3] oatmeal and tulip agars were selected for use in stimulation experiments. Production of the corymbiferan lactones A−D was not detected using standard laboratory media (CYA and YES); therefore the implementation of plant tissue extract agars during routine fungal screening would prove advantageous to the discovery of novel natural products. The biological activity of the corymbiferan lactones is currently unknown; however, the antioxidant nature of these phenolic products might work to quench or curtail the "oxidative burst" by scavenging oxygen radical species released as a plant defensive response to pathogen invasion. The complex nature of these naphthalene derivatives suggests that these metabolites might also have a role at the plant/fungal interface as potential phytotoxins or internal signal molecules essential for fungal metabolism during infection. Therefore further bioactivity studies are recommended to determine the functional role of these stimulated metabolites.

Table 1. ^{1}H and ^{13}C Chemical Shifts for Corymbiferan Lactones A−D (**1**−**4**) Recorded in DMSO-d_6 at 499.9 MHz for ^{1}H and 125 MHz for ^{13}C

	1		**2**		**3**		**4**	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		96.8		97.8		101.7		101.7
2		162.3		162.2		162		162
3	7.17	111.7	6.94	113.3	7.54	107.9	7.04	113.6
4		147.3		144.3		151		146.6
4a		110.2		110		111.2		113.2
5		157.5		157		157.2		158
6	6.65	95.9	6.70	96.1	6.66	95.7	6.66	95.6
7		154.5		154.8		154.1		154.2
8		100		100.2		101.3		101.2
8a		132		132		134.4		134.6
9		170.3		170.1		162		161.9
10	5.62	67.2	5.64	67.3	5.37	64.2	5.35	64
7-OCH_3	3.86	56.1	3.88	56.1	3.86	56.1	3.87	56.1
2-R_2					3.99	56.5	3.97	56.7
4-R_1	5.13	63	5.79	64.7	5.19	63.2	2.88	25.6
4-CH_2OCHO			8.46	160.9				

Experimental Section

General Experimental Procedures. UV spectra were recorded in MeOH on a Hewlett-Packard 8452A diode array spectrophotometer. ^{1}H, HSQC, and CIGAR NMR spectra were recorded in DMSO-d_6 using a Varian INOVA500 FT-NMR spectrometer at 499.9 MHz for ^{1}H and 125 MHz for ^{13}C. LCMS analyses were carried out on an Agilent HP 1100 liquid chromatograph with a DAD (Waldbronn, Germany) coupled to a LCT oaTOF mass spectrometer (Micromass, Manchester, UK) with a Z-spray ESI source and a Lock Spray probe. LCMS chromatography was performed on an Agilent HypersilBDS-C18 column (125 × 2 mm, 3 μm) according to Nielsen and Smedsgaard.[10] Analytical HPLC was performed on a HP1090M HPLC with a diode array detector (Hewlett-Packard, Germany) using a (100 × 4 mm, 3 μm) HP Hypersil BDS-C_{18} cartridge column (Hewlett-Packard) according to Frisvad and Thrane.[11] Semipreparative HPLC for the purification of corymbiferan lactones A−D (**1**−**4**) was carried out on a Shimadzu LC-4A HPLC with a spectrophotometric detector (SPD-2AS) using a Phenomenex Luna C18 column (250 × 10 mm, 5 μm). Compounds **1**−**4** were eluted using a gradient of 30%−45% CH_3CN/H_2O over 30 min with a flow rate of 5 mL/min.

Media Formulation. Tulip agar was created by removing the outer skin of *Tulipa gesneriana* bulbs prior to maceration in a blender. Macerated material was transferred to 2 L screw cap bottles and weighed, then sterile, deionized water was added to yield a 40% tulip slurry (mass:volume). Agar was added to the mixture (2%; m/v), and the mixture was autoclaved at 121 °C for 20 min. Following sterilization the tulip agar was poured into 9 mm Petri dishes (approx 15 mL/dish). CYA, YES, and oatmeal agars were prepared according to Samson et al.[9]

Strain Cultivation and Extraction. *P. hordei* (strain IBT 21039) was three point inoculated onto three plates of CYA, YES, oatmeal, and tulip agar (9 cm dia. Petri dishes) and incubated in darkness at 25 °C for 14 days (strain IBT 21039 is currently stored and available from the IBT culture collection, BioCentrum-Technical University of Denmark). Plugs were then removed from the plates (including a set of uninoculated media plates to be used as a control) and extracted according to Smedsgaard[12] (using EtOAc as the extraction solvent). Extracts were subsequently analyzed by HPLC, and profiles were compared to assess differences in metabolite production.

Large-scale cultivation of the *P. hordei* strain was carried out by three-point inoculation onto 200 plates of tulip agar (9 cm Petri dishes) and incubated in darkness at 25 °C for 14 days. Following incubation, the agar and fungal colonies were macerated in a stomacher and extracted with an equal volume of EtOAc (solvent:agar). The mixture was left to stand for 1 day, decanted, and then filtered by gravity through filter paper (Whatman #4). The EtOAc was removed under vacuum and residual H_2O removed by freeze-drying to leave a dark brown, porous, amorphous solid (9.2 g).

Extraction and Isolation. The crude extract (9.2 g) was defatted by resuspending in MeOH/H_2O (200 mL; 9:1) and washing twice with petroleum ether (200 mL). The MeOH in the defatted fraction was removed under vacuum, and the residual water was then washed twice in a separating funnel with ethyl acetate. The ethyl acetate fractions were then removed under vacuum to yield a dark brown solid residue (5.6 g). In preparation for SPE the defatted residue was adsorbed onto Celite (combined weight 7.9 g). A fraction of the Celite/extract mix (1.8 g) was eluted from a Waters Sep-pak C18 (10 g) disposable cartridge under vacuum in five steps with increasing concentrations of MeOH/H_2O (10:90, 25:75, 50:50, 75:25, and 100:0 (+0.05% TFA); 200 mL each). The fractions were taken to dryness and evaluated by RP-HPLC-DAD analysis. This showed that the target compounds eluted in the MeOH/H_2O (50:50 and 75:25) fractions. A portion of the 75:25 fraction (50 mg) was further purified by semipreparative HPLC to afford pure **1** (9.1 mg, eluting at 11.5 min), **2** (3.5 mg, eluting at 24.5 min), **3** (7.6 mg, eluting at 6.5 min), and **4** (3.2 mg, eluting at 16.5 min).

Corymbiferan lactone A (1): orange solid; mp 184−188 °C; UV (MeOH) λ_{max} (log ϵ) 218 (3.95), 225 (3.94), 268sh (3.66), 278 (3.75), 326 (3.14), 341 (3.19), 368 (3.17) nm; NMR data (DMSO-d_6) see Table 1; HRESIMS *m/z* 275.0548 (calcd for $C_{14}H_{11}O_6$, *m/z* 275.0556).

Corymbiferan lactone B (2): yellowish orange solid; mp 202−206 °C; UV (MeOH) λ_{max} (log ϵ) 217 (3.84), 230sh (3.80), 260sh (3.51), 278 (3.62), 325 (3.01), 343 (3.07), 369 (3.06) nm; NMR data (DMSO-d_6), see Table 1; HRESIMS *m/z* 303.0525 (calcd for $C_{15}H_{11}O_7$, *m/z* 303.0505).

Corymbiferan lactone C (3): yellow solid; mp 228−232 °C; UV (MeOH) λ_{max} (log ϵ) 221 (3.95), 260sh (3.75), 277 (3.81), 327 (2.91), 343 (3.06), 369 (3.05) nm; NMR data (DMSO-d_6), see Table 1; HRESIMS *m/z* 289.0743 (calcd for $C_{15}H_{13}O_6$, *m/z* 289.0712).

Corymbiferan lactone D (4): orange solid; mp 163−167 °C; UV (MeOH) λ_{max} (log ϵ) 219 (3.46), 274 (3.32), 304 (2.72), 332 (2.81), 343 (2.84), 366 (2.75) nm; NMR data (DMSO-d_6), see Table 1; HRESIMS *m/z* 273.0794 (calcd for $C_{15}H_{13}O_5$, *m/z* 273.0763).

Acknowledgment. Research activities were funded by the Danish Research Council under the project "Functional biodiversity in *Penicillium* and *Aspergillus*" (grant no. 9901295). The authors would like to acknowledge M. Squire for MS technical support and C. Christophersen and R. Phipps for proofreading the manuscript.

References and Notes

(1) Frisvad, J. C.; Filtenborg, O.; Lund, F.; Samson, R. A. In *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification*; Samson, R. A., Pitt, J. I., Eds.; Harwood Academic Publishers: 2000; pp 265−283.
(2) Filtenborg, O.; Frisvad, J. C.; Thrane, U. *Int. J. Food Microbiol.* **1996**, *33*, 85−102.
(3) Overy, D. P.; Frisvad, J. C.; Steinmeier, U.; Thrane, U. *Postharvest Biol. Technol.* **2004** (in press).
(4) Saaltink, G. J. *Int. Soc. Hortic. Sci*. **1971**, *23*, 235−41.
(5) Frisvad, J. C.; Filtenborg, O. *Mycologia* **1989**, *81*, 837−61.
(6) Mehdy, M. C. *Plant Physiol*. **1994**, *105*, 467−72.
(7) Tenhaken, R.; Levine, A.; Brisson, L. F.; Dixon, R. A.; Lamb, C. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4158−63.
(8) Hanasaki, Y.; Ogawar, S.; Fukui, S. *Free Radical Biol. Med*. **1994**, *16*, 850.
(9) Samson, R. A.; Hoekstra, E. S.; Frisvad, J. C.; Filtenborg, O. *Introduction to food-borne fungi*, sixth 2nd print ed.; Centraal Bureau voor Schimmelcultures: Utrecht, 2002.
(10) Nielsen, K. F.; Smedsgaard, J. *J. Chromatogr., A* **2003**, *1002*, 111−36.
(11) Frisvad, J. C.; Thrane, U. *J. Chromatogr*. **1987**, *404*, 194−214.
(12) Smedsgaard, J. *J. Chromatogr. A* **1997**, *760*, 264−270.

NP049723C