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Medium dependant production of corymbiferone a novel product from *Penicillium hordei* cultured on plant tissue agar

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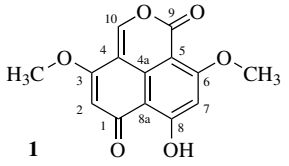
Abstract—Medium dependant production and the structure elucidation of corymbiferone (**1**) from the fungus *Penicillium hordei* grown on oatmeal and macerated tulip, yellow onion and red onion agars are reported. Compound **1** possesses an unusual oxygenated aromatic structure with a lactone bridge preventing full aromatization of the molecule, resulting in several unusual chemical shifts.

Traditionally, conventional natural product screening programs use standard laboratory media during routine secondary metabolite screening of fungi. Wildman has stated that the inclusion of multiple standard media during screening can increase the variety of secondary metabolites recorded for a given fungus.[1] Defined laboratory media do not represent the complexity of a natural substrate and therefore the use of multiple varieties of standardized, defined media is required to elucidate a more complete secondary metabolite spectrum as produced by an organism. Due to genetic variation, not all fungi will behave exactly the same to different environmental stimuli; therefore, in the case of hemi-biotrophic and necrotrophic fungi, the use of host-plant derived media can simulate (to a moderate degree) associated environmental conditions. These conditions can potentially support a more complete secondary metabolic expression from the organism that is more representative of secondary metabolite production in a natural environment.

Penicillium hordei Stolk is a member of the *Penicillium* series *Corymbifera*;[2] penicillia that are responsible for blue mold rot in various flower and vegetable bulbs,[3,4] yet this species also has an affinity for various cereals.[3] When cultured upon standard, defined media, *P. hordei* only produces a small number of secondary metabolites, however when cultured on plant based media this number increases significantly. Cultivation of a strain of *P. hordei* upon tulip agar broadened the overall secondary metabolite expression of the fungus,[5] suggesting that the application of plant derived media during routine fungal screening will improve the chances for the discovery of novel natural products that would not normally have been detected or produced upon standard screening media. We now report the isolation and structure elucidation of the predominant, medium dependant, novel secondary metabolite produced by a strain of *P. hordei* when cultivated upon macerated tulip agar; an undescribed compound with an unusual UV–vis absorption



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spectra. Corymbiferone (**1**) was not detected when cultivated upon defined laboratory media; however the metabolite was expressed by the strain when grown on all of the host-plant derived media.

P. hordei strain IBT 21039[6] was selected for cultivation trials to compare the differences in fungal secondary metabolite production between defined, standard laboratory media and host-plant tissue based media. Czapek yeast extract autolysate (CYA), malt extract (MEA) and yeast extract sucrose (YES) agars were selected to represent defined laboratory media and oatmeal (OAT), tulip (TUL), yellow onion (YO) and red onion (RO) represented the host-plant derived media (for CYA, MEA, YES and OAT recipes refer to Samson et al.;[7] the remaining plant derived media were prepared according to Overy and Blunt[5]). Strain IBT 21039 was then 3 point inoculated on each agar type (9 cm dia. Petri dishes), in triplicate, and incubated in darkness at 25 °C for 14 days.

Following incubation, plugs were removed from the plates (including a set of uninoculated media plates, used as controls) and extracted with EtOAc according to Smedsgaard.[8] Extracts were analyzed by LCMS/DAD according to Nielsen and Smedsgaard.[9] The extract profiles of *P. hordei* demonstrated distinct differences between *P. hordei* grown on defined media (CYA, MEA and YES) and *P. hordei* grown on host-plant derived media. Compound **1** was not detected in the media controls, indicating that it is of fungal origin, and was not produced when strain IBT 21039 was cultured on defined laboratory media.

Extraction of 200 tulip agar plates (containing 3 point inoculated, 14-day old cultures of strain IBT 21039) yielded a dark brown solid (9.2 g). The extract was defatted (partitioned twice, between MeOH/H_2O (1:9) and petroleum ether) and the aqueous methanolic fraction subsequently partitioned against EtOAc, to yield a dark brown solid residue (5.6 g). This residue was fractionated on a C_{18} cartridge (135 g) using a Phenomenex flash system, then by high speed counter current chromatography (HSCCC) and finally purified by repeated MeOH washes[10] to a combined yield of 506.7 mg of **1**.

High resolution ESI$^+$ MS on **1**[11] indicated a molecular formula of $C_{14}H_{10}O_6$ (found 275.0530 $[M+H]^+$ calcd 275.0555, 10 double bond equivalents), which was confirmed by ^{13}C NMR spectroscopy. ^1H, ^{13}C and HSQC spectra of **1**[11] (Table 1) were employed in conjunction with 7.2 and 3 Hz optimized HMBC experiments to determine the structure of corymbiferone (**1**). In the 7.2 Hz optimized HMBC experiment, correlations were seen from H-2 to C-1, C-3, C-4, C-8, C-8a and C-10 (w-coupling).[12] W-couplings could only be assigned after the carbon skeleton had been ascertained through HMBC correlations. The H-7 proton also correlated to C-1 (w-coupling), C-8 and C-8a, as well as C-5, C-6 and C-9 (w-coupling). Correlations from the enol H-10 proton at (δ_H 8.57) were seen to C-3, C-4 and C-9 to create a 12-membered ring system (Fig. 1A in bold). The H-10 proton showed a fourth correlation to a quaternary aromatic carbon resonating at δ_C 136.4 (C-4a). The methoxyl protons 3-*O*CH_3 and 6-*O*CH_3 correlated to C-3 and C-6, respectively (Fig. 1A). No further correlations were observed in the 7.2 Hz HMBC experiment. Correlations in the 3 Hz optimized HMBC experiment (Fig. 1B) were seen from the phenolic hydroxyl signal (δ_H 14.83, 8-OH) to C-1, C-6, C-7, C-8 and C-8a thus placing the phenolic group on C-8. Two additional $^4J_{CH}$ correlations from the H-10 proton were also seen in the 3 Hz experiment. The correlation to C-8a could only be made if the C-4a carbon was attached to C-8a. The second correlation from H-10 to C-5 connected C-5 to C-4a closing the macrocyclic lactone ring and thereby satisfying the double bond equivalents, establishing the structure of corymbiferone **1**. To support the structure determined by long-range ^1H–^{13}C correlations, NOESY spectra were recorded with different mixing times (300-, 400-, 500- and 600 ms), and at two field strengths (500 and 600 MHz). The structure derived by

Table 1. ^1H and ^{13}C chemical shifts for corymbiferone (**1**)[a]

Position	δ_H	δ_C	HMBC 7.2 Hz[b,c]	HMBC 3 Hz[b,c]	NOESY[c]
1		188.3			
2	6.07 s	100.7	1*m*,3*m*,4*s*,8*w*,8a*s*,10*w*	1s,3*s*,4*s*,8*w*,8a*s*,10*m*	3-*O*CH_3*s*
3		165.3			
4		107.0			
4a		136.4			
5		98.7			
6		167.4			
7	6.75 s	99.7	1*w*,5*s*,6*m*,8*s*,8a*s*,9*m*	1*w*,5*s*,6*s*,8*s*,8a*s*,9*s*	6-*O*CH_3*s*, 8-OH*w*
8		169.8			
8a		104.0			
9		155.0			
10	8.57 s	154.1	3*m*,4*s*,4a*s*,9*s*	3*s*,4*m*,4a*s*,5*w*,8a*w*,9*m*	3-*O*CH_3*w*
3-*O*CH_3	3.96 s	56.5	2*w*,3*s*	2*w*,3*s*,4*w*	2*s*,10*w*
6-*O*CH_3	3.99 s	56.9	6*s*,7*w*	5*w*,6*s*,7*w*	7*s*
8-OH	14.83 s		6*w*, 7*s*, 8*s*, 8*as*	1*w*,6*m*,7*w*,8*s*,8a*s*	7*w*

[a] Measured in DMSO-d_6 at 500.1 MHz for ^1H and 125.8 MHz for ^{13}C. All signals were referenced to DMSO at 2.50 for ^1H and 39.5 ppm for ^{13}C.
[b] Observed HMBC[18] peaks from proton to the carbon positions indicated. The 7.2 Hz HMBC is measured with a delay for magnetization transfer τ = 69 ms and the 3 Hz with a τ = 167 ms.
[c] Peak intensity represented as: *s* (strong), *m* (medium) and *w* (weak).

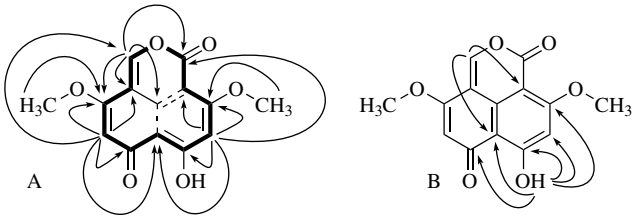
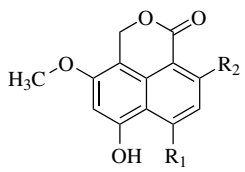


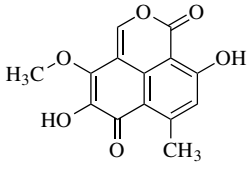
Figure 1. (A) Selected 7.2 Hz optimized HMBC correlations observed for **1**, and (B) phenolic proton correlations and important 3 Hz optimized $^4J_{CH}$ correlations for **1**.

HMBC could be confirmed by the NOEs observed. On the left side of the molecule the following NOEs were observed; between H-2 and 3-*O*CH_3 (strong), and between 3-*O*CH_3 and H-10 (weak) and on the right side; between 8-OH and H-7 (weak), and between H-7 and 6-*O*CH_3 (strong).

Two unusual chemical shift values were noted for some of the carbons resonating in **1**, namely C-8 (δ_C 169.8, Ar-OH), and C-9 (δ_C 155.0, C=O). In the similar compound (**6**), the lactone C-9 carbon shift is almost 10 ppm lower field than that observed in (**1**). The shifts can be explained with different tautomeric forms (Fig. 2). Switching between two tautomeric forms (Fig. 2a and b) would cause a downfield shift of the C-8 carbon signal from that expected for an oxygenated aromatic carbon, as the C-8 carbon would partially exist as a carbonyl group. When in the third tautomeric form (Fig. 2c) the C-9 carbon signal would shift upfield from the expected shift for a carbonyl lactone carbon, as it can also exist as a dioxygenated vinylic carbon resulting in an overall averaging effect. The presence of tautomeric forms is also supported by the UV–vis spectrum of **1**. The spectrum showed a very broad absorbance between 300 and 400 nm containing no defined peaks (Fig. 3a). A class of related compounds, corymbiferan lactones A-D[5] (**2**–**5**) with a similar structure, but lacking the possibility of tautomeric forms show clear defined peaks in the UV spectrum (Fig. 3b).



2 R^1=CH_2OH R^2=OH
3 R^1=CH_2OOCH R^2=OH
4 R^1=CH_2OH R^2=OCH_3
5 R^1=CH_3 R^2=OCH_3



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Corymbiferone (**1**) is most similar in structure to the naphthalene lactones corymbiferan lactone A-D[5] (**2**–**5**), the phenalanone moiety of atrovenetin (produced by several *Penicillium* spp.) and related herqueinone derivatives (from *P. herquei*),[13] the naphthol[1,8-*cd*]pyran derivative simonyellin (**6**) (isolated from the lichen *Simonyella variegata*)[14] and the monomeric units of duclauxin and related metabolites (from *P. duclauxi*).[13]

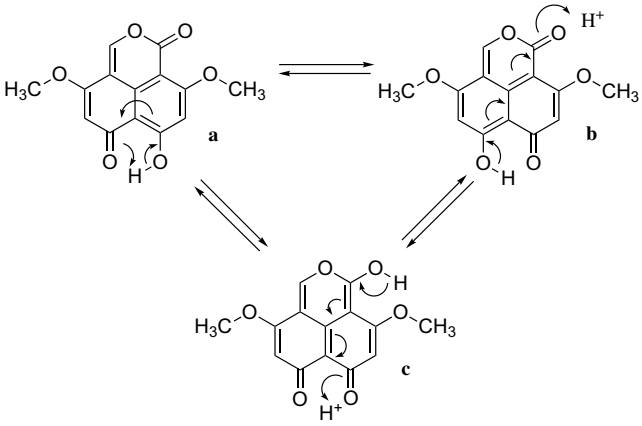

Figure 2. Proposed tautomeric forms for corymbiferone (**1**).

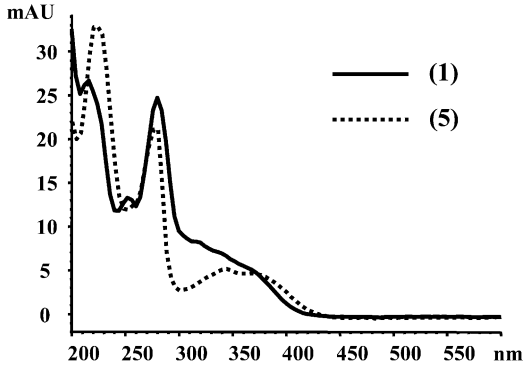

Figure 3. UV absorption spectra overlay from 200–600 nm of: (a) corymbiferone (**1**) and (b) corymbiferan lactone D (**5**).

The biosynthesis of the duclauxin monomer and phenalanone moiety of atrovenetin and related derivatives have been established to arise from a heptaketide precursor[15,16] and corymbiferone (**1**) is proposed here as arising similarly from a polyketide precursor.

As demonstrated here, the implementation of plant tissue agars during routine fungal screening can prove advantageous to the discovery of novel natural products. Production of corymbiferone (**1**) was not detected when using defined laboratory media for fungal culture and only occurred when the fungus was cultured upon plant tissue agar. Medium dependant production of the structurally similar compounds **2**–**5** have also been reported from *P. hordei* cultures grown on tulip agar. The medium dependant production of **1** was not a result of an increase in carbon nutrition, as increasing the sucrose content of the media (from 3% in CYA to 15% in YES) failed to promote production of this metabolite. The selective production of corymbiferone (**1**) on plant tissue media suggests that this metabolite could have a role at the plant/fungal interface. The highly oxygenated aromatic system in corymbiferone (**1**), well known to have antioxidant effects, suggests this molecule might also have antioxidative properties, which may be involved in the repression of the oxidative burst associated with pathogen resistance in plants and/or the detoxification of peroxide saturated necrotic

tissue during colonization.[17] The biological activity of this compound however, currently remains unknown and further bioactivity studies are planned to determine the functional role of this stimulated metabolite.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.03.043.

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10. Solid phase extraction started at 10% MeOH/H_2O, eluting in 15% steps of 500 mL each, at a pressure of 2 bar. Compound **1** eluted in the final four fractions, but was the predominant metabolite in fractions 6 and 7; 85:15 (287.4 mg) and 100:0 (108.8 mg), respectively. Purification of **1** from fraction 5 (276.2 mg) was carried out by HSCCC (PharmaTECH CCC 1000, Waters pump and DAD) using 350 mL coils, a stationary phase of MeOH/H_2O (1:2) and mobile phase of heptane/EtOAc (1:2), in tail position with a coil rotation of 60% obtaining a 90% stationary phase retention at a mobile phase flow rate of 2 mL/min to yield a 201.7 mg fraction of semi-pure **1** eluting between 85 and 260 min. Compound **1** was only slightly soluble in MeOH; therefore the final purification was carried out by sonication and repeated washing of the HSCCC fraction and the SPE fractions 6 and 7.
11. Corymbiferone (**1**): amorphous, off white solid; mp 163–167 °C; UV (MeCN) λ_{max} (log ε): 211 (4.00), 250 (0.621), 279 (3.95), 310sh (3.36), 367sh (3.16) nm; IR (KBr) ν_{max} 3382 br, 1746, 1656, 1597, 1567, 1440, 1383, 1305, 1245, 1218, 1185, 1141, 1111, 1017, 978 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 275.0530 (calcd for $C_{14}H_{11}O_6$, *m/z* 275.0555).
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