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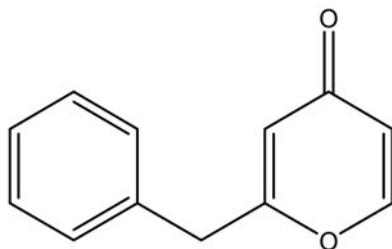
Antimicrobial secondary metabolites of an endolichenic *Aspergillus niger* isolated from lichen thallus of *Parmotrema ravum*

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ABSTRACT

A new 6-benzyl- γ -pyrone (**1**), named aspergyllone was isolated from the culture filtrates of an endolichenic fungus *Aspergillus niger* Tiegh, obtained from lichen thallus *Parmotrema ravum* (Krog & Swinscow) Serus, collected in India. **1** was isolated for the first time from an endolichenic fungus together with six other known metabolites identified as aurasperones A (**2**) and D (**3**), asperpyrone A (**4**), fonsecinone A (**5**), carbonarone A (**6**) and pyrophen (**7**). The compounds were tested against a panel of human, plant, food borne and fish pathogens. Aspergyllone showed strong selective antifungal activity against *Candida parapsilosis* (Ashford) Langeron & Talice, with an IC₅₀ of 52 μ g/mL. Aurasperone A and pyrophen showed moderate to strong antimicrobial activity inhibiting seven different test pathogens, being pyrophen active with IC₅₀ ranging from 35 to 97 μ g/mL.



1, Aspergyllone

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1. Introduction

Lichen thalli are known to be colonized by numerous asymptomatic and cryptic micro fungi that live in close association with the photobiont. These diverse groups of fungi,

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which reside in the interior of a lichen thallus, have been termed as 'endolichenic fungi' (Li et al. 2007; Paranagama et al. 2007). Thus, endolichenic fungi are similar to plant endophytes and established themselves as endosymbiont. Although they have been characterized from a limited number of lichen species and geographic areas, current evidence suggests that like endophytes, endolichenic fungi are horizontally transmitted, form highly localized infections, and include abundant taxa belonging to diverse classes, orders and families within the Ascomycota (Pezizomycotina) (Arnold et al. 2009; Shaaban et al. 2012; Tripathi et al. 2014). In the recent years, endolichenic fungi have been identified as new sources of bioactive secondary metabolites (Kellogg and Raja 2017, Cimmino et al. 2018). In fact, several metabolites obtained from endolichenic fungi have shown promising antibacterial, antifungal and anticancer properties (Ding et al. 2009; Wang et al. 2010; Wang et al. 2013; Wu et al. 2015). Although there are reports on endolichenic fungi from India (Suryanarayan et al. 2005; Suryanarayanan and Thirunavukkarasu 2017) nevertheless, studies on bioactivity and chemistry of their secondary metabolites have not been thoroughly explored and still remain unstudied.

In this manuscript the endolichenic fungus from lichen thallus of *Parmotrema ravum* (Krog & Swinscow) Serus collected from Similipal Biosphere Reserve, India, was investigated and an endolichenic fungus *Aspergillus niger* Tiegh, representing fungal taxa of class Ascomycetes, was also isolated. Similar studies were made from various lichen taxa resulting into isolation of several endolichenic fungi especially of the fungi belonging to phylum Ascomycota (Petrini et al. 1990; Tripathi and Joshi 2015). In many instances researchers working on these fungi focused of secondary metabolites as they have been identified as a new avenue for discovery of bioactive secondary metabolite chemistry in natural products research (Kellogg and Raja 2017).

In our earlier investigation the occurrence of endolichenic fungi from lichen thalli of Similipal Biosphere Reserve India and their antimicrobial potentials (Padhi and Tayung 2015; Padhi et al. 2017; Padhi et al. 2018) were reported. This paper reports the isolation, antimicrobial activity and chemical characterization of a new 6-benzyl- γ -pyrone, named aspergyllone, as well as on the identification of other two known 6-benzyl- γ -pyrones and four known bi[benzo[g]chromenyl]-4,4'-diones.

2. Results and discussion

The organic extract obtained from the *Aspergillus* sp. culture filtrates was purified by bioassay-guided fractionation as reported in Experimental section, yielding seven pure metabolites (1–7, Figure 1). Metabolites 2–7 were identified comparing their spectroscopic data (essentially $^1\text{H-NMR}$ and ESI-MS) with those reported in literature as aurasperones A (Fang et al. 2016) and D (Ghosal et al. 1979), asperpyrone A, fonsecinone A (Fang et al. 2016), carbonarone A (Zhang et al. 2007) and pyrophen (Zhang et al. 2010). Furthermore the absolute configuration of pyrophen was confirmed comparing its specific optical rotation data ($[\alpha]^{25}_{\text{D}}$: -14.2 ($c = 0.1 \text{ CHCl}_3$) with that reported in literature ($[\alpha]^{25}_{\text{D}}$: -13.8 ($c = 0.1 \text{ CHCl}_3$)) (Zhang et al. 2010).

Also a new benzyl- γ -pyrone, named aspergyllone (1, Figure 1) was isolated as an amorphous solid from the same fungal organic extract. Its molecular formula was determined as $\text{C}_{12}\text{H}_{10}\text{O}_2$ based on the HRESIMS and was consistent with eight

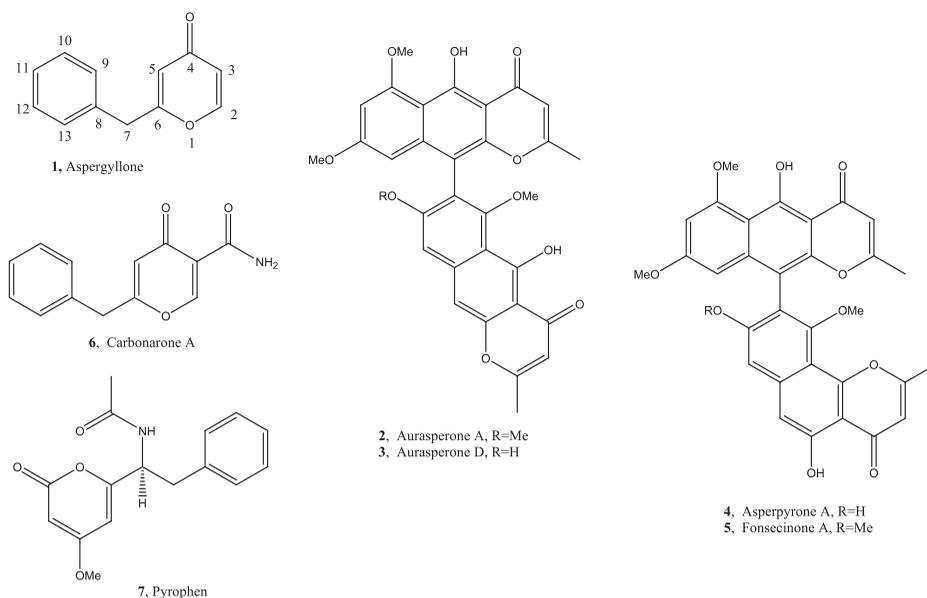


Figure 1. Structures of aspergyllone, aurasperones A and D, asperpyrone A, fonsecinone A, carbonarone A and pyrophen (1–7, respectively).

Table 1. ^1H , ^{13}C NMR and HMBC data of aspergyllone (1) in CDCl_3 ^{a,b}.

Position	δ^c	δH (J in Hz)	
2	154.7 CH	7.66 d (5.8)	
3	117.0 CH	6.26 dd (5.8, 2.5)	
4	178.8 C		H-2
5	115.1 CH	6.13 d (2.5)	H-2, H-7
6	168.8 C		H-2, H-7
7	40.1 CH_2	3.81 s	
8	134.9 C		H-7
9,13	129.5 CH	7.26 dd (7.5, 1.6)	H-7, H-10,12
10,12	129.5 CH	7.35 t (7.5)	H-9,13
11	128.0 CH	7.33 tt (7.5, 1.6)	

^aThe chemical shifts are in δ values (ppm) from TMS.

^b2D ^1H , ^1H (COSY) ^{13}C , ^1H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons.

^cMultiplicities were assigned by DEPT spectrum.

hydrogen deficiencies. The first preliminary investigation of its ^1H and ^{13}C NMR (Table 1) showed that **1** was closely related to carbonarone A (**6**) by comparison of their spectroscopic data (Zhang et al. 2007). The IR spectra are very similar but that of **1** lacks the typical amide bands ($\text{C}=\text{O}$ and NH_2) (Nakanishi and Solomon 1977). The ^1H NMR spectrum of **1** differed from that of carbonarone A for the signal system of γ -pyrone ring. In fact, the ^1H NMR spectrum of **1** (Table 1) showed the significant upfield shift ($\Delta\delta$ 1.07) of H-2 which appeared as a doublet ($J=5.8$ Hz) at δ 7.66 and the presence of H-3, which appeared as a double doublet ($J=5.8$ and 2.5 Hz) at δ 6.26, being coupled in the COSY spectrum (Berger and Braun 2004) with both H-2 and H-5. Instead H-2 in carbonarone A was a singlet resonating at δ 8.73 (Pretsch et al. 2000). The ^{13}C NMR spectrum of **1** differed in respect to that of **6** for the absence of the carbonyl of carboamide group and for the significant upfield shift ($\Delta\delta$ 7.3) of C-2

Table 2. Antimicrobial activity of the metabolites isolated.

Name of the Metabolites	IC ₅₀ (µg/mL) values against test pathogens														
	Human pathogens							Plant pathogens			Food borne and fish pathogens				
	<i>Pa</i>	<i>Sa</i>	<i>Ec</i>	<i>Ca</i>	<i>Ck</i>	<i>Cg</i>	<i>Cp</i>	<i>Cu</i>	<i>Ps</i>	<i>Pb</i>	<i>Ds</i>	<i>Ah</i>	<i>Ss</i>	<i>MI</i>	<i>Li</i>
Aspergylone	–	–	–	–	–	–	52	–	–	–	–	–	–	–	–
Aurasperone A	160	135	–	–	373	–	–	–	80	76	63	–	–	–	141
Asperpyrone A	–	112	–	–	–	–	–	–	–	–	–	–	–	–	–
Fonsecinone A	–	120	47	–	–	–	–	–	154	–	–	–	–	–	–
Aurasperone D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Carbonarone A	–	–	–	103	31	–	–	–	–	–	88	–	–	–	–
Pyrophen	–	–	–	74	–	97	35	62	–	–	–	78	–	63	86

**Pa*- *P. aeruginosa* MTCC 424, *Sa* – *S. aureus* MTCC 737, *Ec* – *E. coli* MTCC 443, *Ca*- *C. albicans* MTCC 227, *Ck* – *C. krusei* MTCC 9215, *Cg* - *Candida glabrata* (Anderson) Mey. & Arrow ATCC 2001, *Cp*- *C. parapsilosis* ATCC 22019, *Cu*- *C. utilis* IHEM 400, *Ps*- *P. syringae* pv. *maculicola* I11004, *Pb*-*Pectobacterium* sp. (Waldee) GBBC 1529, *Ds*- *D. solani* GBBC 1502, *Ah*- *A. hydrophila* ATCC 7966, *Ss*- *Shigella sonnei* (Levine) Weldin LMG 10473, *MI*-*M. luteus* DPMB3, *Li*- *L. innocua* LMG11387, '–' indicates no significant activity.

resonating at δ 154.7, while C-3 appeared as a doublet at the similar chemical shift of 117.0 ppm while in **6** is a singlet at 119.3 ppm (Breitmaier and Voelter 1987). The couplings observed in the COSY and HSQC spectrum (Berger and Braun 2004) allowed to assign the chemical shifts to all the protonated carbons while those of the quaternary carbons were assigned by the couplings observed in the HMBC spectrum (Berger and Braun 2004). In particular, the signals at δ 178.8, 168.8 and 134.9, were assigned to C-4, C-6 and C-8 by the coupling of C-4 with H-2, C-6 with H-2 and H-7 and C-8 with H-7 (Breitmaier and Voelter 1987). Consequently, the chemical shifts to all the protons and carbons of **1** were assigned and reported in Table 1 and aspergylone could be formulated as 2-benzyl-4H-pyran-4-one. Thus **1** differs from **6** for the lack of the carboamide group at C-3.

The structure assigned to **1** was supported from the other couplings observed in the HMBC spectrum as those observed between C-5 with H-2 and H-7, C-9,13 with H-7 and H-10,12, and C-10,12 with H-9,13, respectively and by the data of its HR ESIMS spectrum. This latter spectrum showed the dimeric-sodiated $[2M+Na]^+$ and the pseudo-molecular dimeric $[2M+H]^+$ forms, the sodium cluster $[M+Na]^+$ and the pseudo-molecular ion $[M+H]^+$ at m/z 395, 373, 209.0682 and 187.0763, respectively.

2-benzyl-4H-pyran-4-one was reported as intermediate in the synthesis of substituted γ -pyrones (Koreeda and Akagi 1980; Zawacki and Crimmins 1996; Crimmins et al. 2000). Natural products containing 2-benzyl-4H-pyran-4-one are uncommon and have been encountered in relatively few fungi outside of the black aspergilli clade (Henrikson et al. 2011).

The purified metabolites were tested for antimicrobial activity against a wide range of pathogenic microorganisms comprising human, plant, fish and food borne pathogens. The results of the antimicrobial activity are presented in Table 2. Aspergylone **1** showed antifungal activity against *Candida parapsilosis* (Ashford) Langeron & Talice (ATCC 22019) with a promising IC₅₀ value 52 µg/mL and no activity was observed against the other test pathogens. Similarly, carbonarone A showed antifungal activity against *Candida albicans* C. Berkhout and *Candida krusei* (Castell.) Berkhout exhibiting IC₅₀ 103 µg/mL and 31 µg/mL respectively. It also showed activity against a plant pathogenic bacterium, *Dickeya solani* van der Wolf with IC₅₀ 88 µg/mL. Aurasperone A

(2) displayed antimicrobial activity against a range of the test pathogens. It showed antibacterial activity against *Pseudomonas aeruginosa* C. Gessard and *Staphylococcus aureus* A. Ogston with IC_{50} of 160 $\mu\text{g/mL}$ and 135 $\mu\text{g/mL}$ respectively, anti-candidal activity against *C. krusei* with IC_{50} of 373 $\mu\text{g/mL}$. However, this compound did not show activity against the other *Candida* species. He et al. (2016) reported that this compound possesses antibacterial activity. However, in the present work its ability to inhibit both bacterial and fungal test pathogens was reported indicating its broad spectrum nature. Similarly, fonsecinone A (5) showed antibacterial activity against few test pathogens. It inhibited *S. aureus* and *Escherichia coli* T. Escherich with IC_{50} of 120 $\mu\text{g/mL}$ and 47 $\mu\text{g/mL}$, respectively. It also showed antibacterial activity against one of the test plant pathogen, *Pseudomonas syringae* pv. *maculicola* McCulloch with IC_{50} of 154 $\mu\text{g/mL}$. Asperpyrone A (4) showed only antibacterial activity against *E. Coli* with IC_{50} of 112 $\mu\text{g/mL}$. Pyrophen (7) showed promising antimicrobial activity inhibiting 50% of the test organisms of human, fish and food borne pathogens. It also exhibited antifungal activity against the entire test *Candida* species except *C. krusei*. In particular, significant activity was observed against *C. parapsilosis* with IC_{50} of 35 $\mu\text{g/mL}$ followed by *Candida utilis* (Henneberg) Lodder & Kreger (IC_{50} 62 $\mu\text{g/mL}$) and *C. albicans* (IC_{50} 74 $\mu\text{g/mL}$). Considerable activity was also observed against *Micrococcus luteus* (Schroeter) Cohn (IC_{50} 63 $\mu\text{g/mL}$) followed by *Aeromonas hydrophila* (Chester) Stanier (IC_{50} 78 $\mu\text{g/mL}$) and *Listeria innocua* Seeliger & Schoofs (IC_{50} 86 $\mu\text{g/mL}$). Earlier researchers reported this compound to possess antifungal activity against *C. albicans* and cytotoxic properties against some cancer cell lines (Shaaban et al. 2012; Astuti et al. 2016). No activity was detected for aurasperone D (3).

3. Experimental

The general experimental procedures, the details of identification, microbial growth, antibiotic activity and their discussion, as well as statistical analysis are available as [supplementary materials](#).

3.1. Fungal material

The fungal strain designated as GYT3 was isolated from a healthy lichen thallus of *P. ravum*, collected from Bhimkund forest division (21° 33' N latitude and 86° 01' E longitudes) of Similipal Biosphere Reserve, located at the Border region of Mayurbhanj and Keonjhar districts of the state Odisha, Country India. The lichen thallus was identified based on its morphology characters and authenticated by Rasananda Kar, Botanical Survey of India. A fragment of the thallus has also been deposited to the Curator, Gauhati University Botanical Herbarium as a voucher specimen (GUBH 0994). The fungus was grown in 10.5 L of Czapek Dox liquid medium in fifteen 1 L Erlenmeyer flasks each containing 700 mL incubated in BOD shaking incubator for 14 days at $29 \pm 1^\circ\text{C}$ with periodic shaking at 150 rpm. The fungus was stained with lactophenol cotton blue and identified as *Aspergillus niger* based on the reproductive structures observed microscopically with reference to standard identification manual (Barnett and Hunter

2004; Gilman 1971) and ITS rDNA sequence. The sequence data has been deposited into the GenBank with an accession number MG062788.

3.2. Purification and characterization of aspergyllone and other metabolites

The fungus used in this study was grown in fifteen 1 L Erlenmeyer flasks containing 700 mL CDB. A total of 10.5 L of the growth medium was exhaustively extracted twice with an equal volume of EtOAc. The organic extracts, were combined, dried with Na_2SO_4 and evaporated under reduced pressure. The brown-red oil residue (332 mg) was purified on silica gel column (75×3 cm) chromatography using 2 L of CHCl_3 -*i*-PrOH (9:1, v/v) as eluent and thirteen homogeneous fraction groups were collected. Bioassay guided fractionation yielded three active fractions (F2, F4 and F5). The residue of second fraction (26.7 mg) was further purified by preparative TLC on silica gel using *n*-hexane-EtOAc (5.5:4.5, v/v) as eluent to yield four different homogeneous solid compounds identified as aurasperone A (**2**, 4.1 mg), aurasperone D (**3**, 1.5 mg), asperpyrone A (**4**, 1.6 mg) and fonsecinone A (**5**, 2.8 mg) as reported below. The residue of fourth fraction (17.6 mg) was purified on an analytical TLC using CHCl_3 -*i*-PrOH (98:2, v/v) as eluent to afford two pure compounds as homogeneous solids. One of them was identified as carbonarone A (**6**, 2.2 mg) while the other, being new as below reported, was named aspergyllone (**1**, 2.6 mg). Similarly, the residue of fifth fraction (20.6 mg) was further purified on analytical TLC eluted with petroleum ether- Me_2CO (7:3, v/v) to yield one homogenous solid identified as pyrophen (**7**, 3.1 mg).

3.3. Aspergyllone

Amorphous solid; UV (MeOH) λ_{max} ($\log \epsilon$) 249 (3.0) nm; IR ν_{max} 1730, 1566 cm^{-1} ; ^1H and ^{13}C NMR data (Table 1); HRESIMS m/z 373 [$2\text{M} + \text{H}$] $^+$, 395 [$2\text{M} + \text{Na}$] $^+$, 209.0582 [$\text{M} + \text{Na}$] $^+$ (calculated for $\text{C}_{12}\text{H}_{10}\text{NaO}_2$, 209.0578), 187.0763, [$\text{M} + \text{H}$] $^+$ (calculated for $\text{C}_{12}\text{H}_{11}\text{O}_2$, 187.0759).

4. Conclusion

In this study, a new 6-benzyl- γ -pyrone (**1**) along with other known compounds (**2–7**) was isolated from EtOAc extract of an endolichenic fungus *A. niger*. All compounds were determined for antimicrobial activity against a range of pathogens, **1** showed significant anticandidal activity against *C. parapsilosis* with an IC_{50} 52 $\mu\text{g}/\text{mL}$.

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Disclosure statement

The authors declare no conflict of interest.

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