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De novo production of (+)-aristolochene by sporulated surface cultures of *Penicillium roqueforti*

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Abstract

The *de novo* production of the fungal metabolite, (+)-aristolochene by sporulated surface cultures of *Penicillium roqueforti* is reported for the first time. The biosynthesis of fungal volatiles by various sporulated surface cultures was monitored by solid phase micro-extraction (SPME). When comparing malt extract agar with sabouraud dextrose agar, the highest yield of the fungal metabolite (0.04 mg/ml of culture) was obtained with the latter medium. The biosynthesis of (+)-aristolochene showed a maximum during the fourth day after inoculation.

Keywords: *Penicillium roqueforti*; (+)-Aristolochene; Sesquiterpene hydrocarbon; Sporulated surface cultures; Fungal volatiles; Aristolochene synthase; PR-toxin; SPME

1. Introduction

Fungi are known to produce a wide range of secondary metabolites, some of which are volatile (Larsen and Frisvad, 1995a). Fungal volatiles have been studied for several reasons, such as detection of undesired fungal growth on cereals (Börjesson et al., 1989), detection of off-flavours caused by fungi (Börjesson et al., 1993), and the possible relation between fungal volatiles and the 'sick building syndrome' (Nielsen et al., 1998a,b). Volatile sesquiterpenes can be used for taxonomic classification and species identification in *Penicillium*, as well as to indicate mycotoxin formation in *Fusarium* and *Aspergillus* (Jeleń et al., 1995). The correlation between biosynthesis of sesquiterpenes and production of mycotoxins has also been described by Pasanen et al. (1996) and Zeringue et al. (1993) and recently discussed in an excellent review by Jeleń and Wasowicz (1998).

One of these fungal sesquiterpenes is aristolochene [1,2,3,4,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-naphthalene], a member of the group of eremophilane-type sesquiterpenes (see Fig. 1). Aristolochene was first isolated from the roots of the plant *Aristolochia indica* (Govindachari et al., 1970), while later, it was also isolated from *Bixa orellana* leaf oil (Lawrence and Hogg, 1973) and from the liverworts *Porella arboris-vitae* (Fricke, 1999) and *Dumortiera hirsuta* (Saritas et al., 1998). It is important to stress that the configuration of aristolochene depends on its biological source. Indeed, whereas (−)-aristolochene was isolated from *Aristolochia indica* and *Dumortiera hirsuta*, its (+)-enantiomer was described as a constituent of *Dipterocarpus alatus* Roxb. (gurjun balsam oil) (Klein and Rojahn, 1970) and *Porella arboris-vitae* (Fricke, 1999). The occurrence of (−)-aristolochene was also reported in the frontal gland secretion of the *Syntermes* soldier termites (Baker et al., 1981).

More recently, (+)-aristolochene was isolated for the first time as a fungal metabolite of *Aspergillus terreus*, together with (−)-γ-cadinene (Cane et al., 1987). The absolute configuration of the fungal aristolochene was later confirmed by comparison with semi-synthetic (−)-aristolochene, prepared from (+)-valencene (Cane et al., 1990). To the best of our knowledge, the biosynthesis of this metabolite by other fungi has not been published to date.

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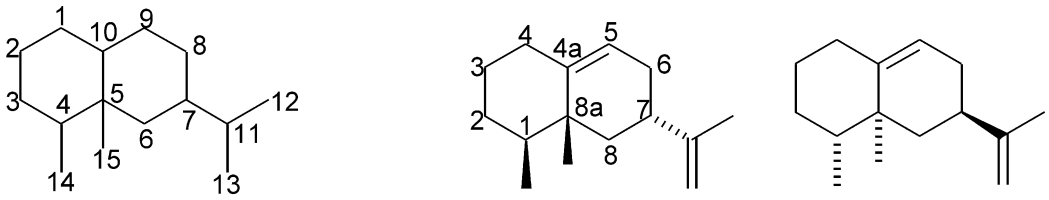

Fig. 1. Structural formulae of the eremophilane type sesquiterpenes (+)- and (−)-aristolochene.

The biosynthesis of (+)-aristolochene takes place by the cyclization of *E,E*-farnesyldiphosphate (FPP) to germacrene A, through the action of aristolochene synthase. Two distinct aristolochene synthases, acting by identical mechanisms have been isolated. A first aristolochene synthase has been isolated from *Penicillium roqueforti* (Hohn and Plattner, 1989), while a second aristolochene synthase was isolated from *Aspergillus terreus* (Cane et al., 1989). The formation of (+)-aristolochene is believed to be the first step in the biosynthesis of a number of fungal toxins. The most important of these toxins are the PR-toxin of *Penicillium roqueforti* and sporogen-AO1 of *Aspergillus oryzae* (Hohn et al., 1992; Proctor and Hohn, 1993).

Different methods have been used for the collection of fungal volatiles (Larsen and Frisvad, 1995b). This paper describes the use of solid phase micro-extraction as monitoring technique for the collection and detection of the fungal volatile metabolite (+)-aristolochene by sporulated surface cultures of *Penicillium roqueforti*. Although (+)-aristolochene has been isolated from mycelial cultures of *Aspergillus terreus* and aristolochene synthase has been isolated from *Penicillium roqueforti* (Cane et al., 1989), and although the conversion to the above mentioned toxins is believed to occur, the production of this fungal metabolite by sporulated surface cultures of *P. roqueforti* has not been reported previously. As such, in this paper this missing link was established in *P. roqueforti*. In addition, it allows the isolation of (+)-aristolochene in substantial amounts, free of side products, by a preparative procedure. This result contrasts dramatically with the usual production of sesquiterpenes as mixtures of difficultly separated terpene compounds. Furthermore, these volatile markers can be used for the early detection of food spoilage by mycotoxin producing fungi.

2. Results and discussion

2.1. Screening of sporulated surface cultures by SPME

More than 60 fungal strains, grown as sporulated surface cultures, were screened for their capacity to produce volatile metabolites, such as hydrocarbon terpenes by *de novo* biosynthesis. The headspace of the surface cultures was sampled, using SPME as the monitoring technique. Typical fungal odour compounds, such as 3-octanone, 3-octanol and 1-octene-3-ol were produced by a *Penicillium* sp. isolated from cheese and by *Aspergillus versicolor*. *Penicillium* sp., isolated from geraniol containing media, produced (*Z*)-3-hexen-1-ol, 1-hexanol, ethyl 2-hexenoate, α-terpineol and linalool. Other strains also produced eucalyptol (1,8-cineol) and β-myrcene. *Penicillium digitatum*, on the other hand, was able to produce a wide variety of fungal metabolites, such as ethyl 2-hexenoate, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl isovalerate, linalool, (*E*)-β-caryophyllene, α-(*E,E*)-farnesene and γ-selinene.

The most interesting strains, however, were the *Penicillium roqueforti* aggr., isolated from citronellol containing contaminated media, marked CZW, CCV and CDG, as well as *Penicillium digitatum*, marked CMC (in order of decreasing concentration of metabolites). The most important compounds in the headspace profile of these cultures were (+)-aristolochene (70–79% of the headspace profile) and valencene (up to 11% of the headspace profile). This remarkably high production of (+)-aristolochene is worked out here as a method to obtain this sesquiterpene in a preparative way.

The relative contribution of the volatile metabolites in the SPME headspace extracts (average of two measurements) of the four different fungal strains, marked CCV, CZW, CDG, and CMC is displayed in Table 1.

The structures of the fungal metabolites obtained from the headspace samples of these *Penicillium* cultures by SPME are displayed in Fig. 2.

2.2. Production of volatile metabolites by sporulated surface cultures of Penicillium roqueforti

Three strains of *Penicillium roqueforti*, marked CZW, CDG and CCV were cultivated as sporulated surface cultures on three media, namely MEA, PDA and SAB (see Experimental). After 2 days, the surfaces were completely covered with a green sporulated mat, and headspace samples of the cultures were taken and analysed by GC/MS. From the analyses, it could be concluded that the

Table 1
Relative contribution (%) of each *de novo* fungal metabolite in the headspace SPME extracts (avg. of two measurements) of different fungal strains

Strain→	*Penicillium roqueforti*				*P. digitatum*
Component↓	CCV[b]	CZW[b]	CDG[b]	average	CMC[b]
β-Myrcene	2.5	4	1.9	2.8	1.0
Limonene	4.9	1	0.3	2.1	0.9
β-Elemene	5.9	6	5.8	5.9	6.3
Caryophyllene	0.4	0.7	0.2	0.4	0.2
(+)-Aristolochene	67.4	70	79	72.1	71.2
Valencene	10.3	11	7.0	9.4	7.8
α-Selinene	3.5	4	2.9	3.5	4.1
α-Panasinsen	0.6	0.7	0.0	0.4	0.6
γ-Patchoulene	nd[a]	nd	nd	nd	0.2

[a] nd = Not detected.
[b] CCV, CZW and CDG = three strains of *Penicillium roqueforti*, CMC = *P. digitatum*.

production of the volatiles was higher when the SAB medium was used. However, no difference in production of the metabolites was observed between the different strains.

The headspace profile of the fungal metabolites is a mixture of the main compound (+)-aristolochene (up to 80%), β-elemene (9%), and valencene (7%).

2.3. Dynamic study of the production of (+)-aristolochene during time course

The production of the fungal sesquiterpene, (+)-aristolochene, by the three strains of *Penicillium roqueforti* was investigated during time course, using two media, MEA and SAB (Fig. 3).

From Fig. 3 it can be concluded that a maximum production of the fungal metabolite occurred during the second headspace period, i.e. 84–108 h after inoculation. The production on medium SAB is also significantly higher than on medium MEA.

In Table 2, the total production of (+)-aristolochene by the various cultures is displayed. The medium SAB is responsible for a 4–5 times better production of the fungal metabolite (+)-aristolochene than the medium MEA (up to 2 mg/50 ml culture). The strain CCV produces significantly lower levels of the metabolite than the other two strains.

The difference between aristolochene production from the medium SAB and its yield from the other two media is possibly due to the influence of the medium composition on the aristolochene biosynthesis, i.e. the Mg^{2+} content, necessary for the aristolochene synthase (Caruthers et al., 2000). The cyclization to aristolochene is induced by the formation of a carbenium ion and is dependent on Mg^{2+}.

The effect of the pH on the aristolochene synthase activity has also been investigated (Hohn and Plattner, 1989). In relation to this fact, it has been established by the same authors that a broad peak of activity occurred between a pH of 5.5 and 8.5, with a maximum activity between pH 6.25 and 7.5. The pH of both media MEA and SAB are very similar (5.4±0.2 and 5.6±0.2, respectively) and are situated at the lower threshold of the pH limits for high enzyme activity. It is expected that increasing the pH and buffering the culture media would enhance the fungal biosynthesis of (+)-aristolochene.

2.4. Conclusions

The *de novo* production of the fungal metabolite, (+)-aristolochene by sporulated surface cultures of *P. roqueforti* was reported here for the first time. As such,

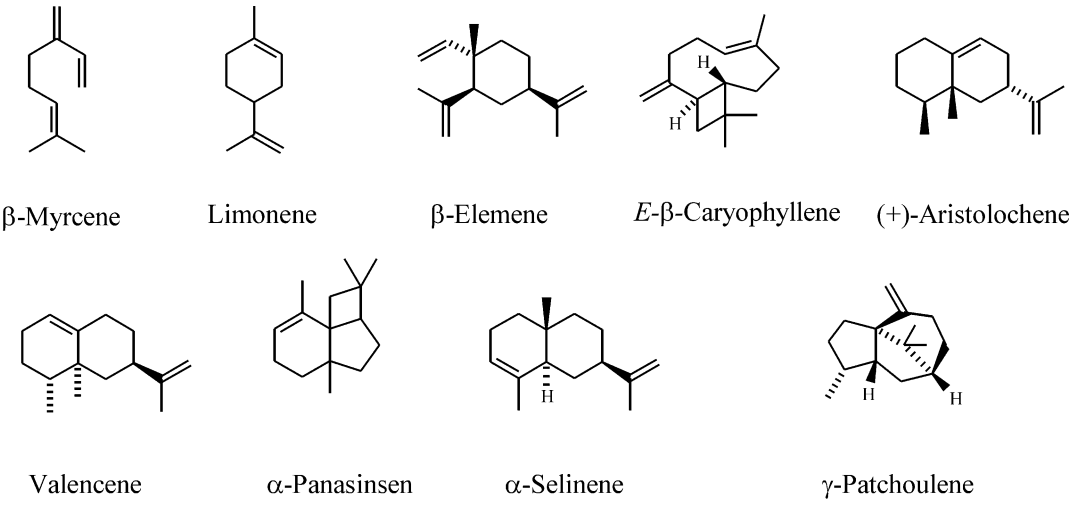

Fig. 2. Structures of the fungal metabolites obtained from the headspaces of *Penicillium roqueforti* cultures by headspace SPME (the absolute configuration of β-elemene, valencene, α-selinene and γ-patchoulene is tentatively shown, since these metabolites were detected and identified by GC/MS).

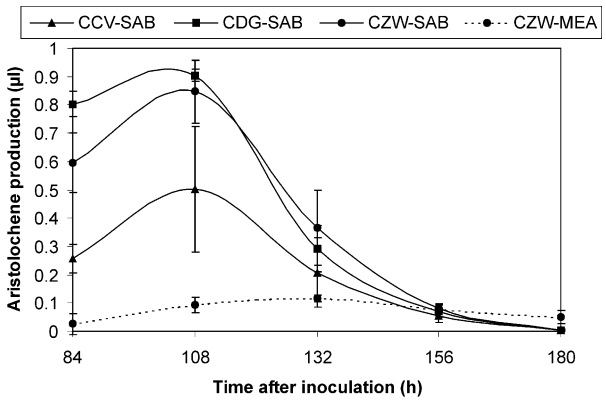

Fig. 3. Production of (+)-aristolochene by sporulated surface cultures of *Penicillium roqueforti* during time course (four headspace samples of 24 h each and one sample of 48 h were taken)—influence of strain and culture mediums; CCV, CZW and CDG = three strains of *Penicillium roqueforti*, SAB = sabouraud dextrose agar, MEA = malt extract agar.

Table 2
Total yield (mg) of (+)-aristolochene produced by sporulated surface cultures of three *Penicillium roqueforti* strains on 50 ml SAB or MEA-medium (sum of headspace samples)

Strain[a]	Medium[b]	Production of (+)-aristolochene (mg)
CCV	SAB	0.98±0.30
CDG	SAB	1.97±0.08
CZW	SAB	1.80±0.35
CZW	MEA	0.35±0.05

[a] CCV, CZW and CDG = three strains of *Penicillium roqueforti*.
[b] SAB = sabouraud dextrose agar, MEA = malt extract agar.

this result yields up a missing link in this area of research. When comparing malt extract agar with sabouraud dextrose agar, the highest yield of the fungal metabolite was obtained with the latter medium. The biosynthesis of (+)-aristolochene showed a maximum during the fourth day after inoculation. Headspace analysis of volatile fungal metabolites by solid phase microextraction is a suitable monitoring technique for fast detection of mycotoxin producing fungi.

3. Experimental

3.1. Micro-organisms and cultivation

More than 60 fungal strains were used for preliminary screening tests and three fungal strains were selected for further study. These strains were isolated from contaminated solid media in Petri dishes, containing 0.1% of citronellol. They were identified by the MUCL (Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium) as *Penicillium roqueforti* aggr., different from *Penicillium roqueforti* var. *roqueforti* and they were marked as follows: CCV, CDG and CZW. The fungi were cultivated and conserved by periodic replications (every 2 weeks) on malt extract agar (MEA: malt extract 2%, bacteriological peptone 0.1%, glucose 2% and agar 2%—pH 5.4±0.2). They were also cultivated on potato dextrose agar (PDA: potato extract 0.4%, glucose 2% and agar 1.5%—pH 5.6±0.2) and Sabouraud dextrose agar (SAB: mycological peptone 1%, glucose 4% and agar 1.5%— pH 5.6±0.2).

3.2. Screening of sporulated surface cultures of fungi by SPME

The fungi were cultivated as small sporulated surface cultures in 40-ml SPME-vials (Supelco Inc., Bellefonte, USA) and the volatile metabolites were extracted by headspace SPME (Demyttenaere et al., 2001).

3.3. Headspace analysis and production of (+)-aristolochene during time course

The sporulated surface cultures were sampled by dynamic headspace purge and trap (Demyttenaere et al., 2000, 2001). For qualitative analysis, no internal standard was applied. For estimation of the amount of volatile metabolites produced, *n*-decane was added to the samples (1 ml of a 0.1% v/v solution in Et_2O), while for quantification, (−)-*E*-β-caryophyllene (99%, Fluka) was used as internal standard. For the dynamic study of the production of (+)-aristolochene during time course, the fungi were cultivated in 500-ml conical flasks with 50 ml of medium (MEA and SAB were used), and inoculation was performed with 1 ml of a spore suspension of $2–5\times10^7$ spores/ml. After 60 h of incubation, five headspace samples were taken (4×24 h and 1×48 h, resp.) and analysed.

3.4. Preparative separation and identification of the main metabolite

For the isolation of the fungal metabolite, combined headspace extracts (solutions obtained after elution of Tenax tubes) were concentrated under a gentle N_2-flow and injected in a Delsi Intersmat IGC 120 ML gas chromatograph equipped with a 3 m packed column (5% SE-30, Chromosorb W-AW 60–80, 6 mm o.d.) and a thermal conductivity detector (TCD). The carrier gas was H_2 (1 bar). The crude compound (aristolochene) was collected in glass tubes and identified by ^1H, ^{13}C NMR and MS. Proton and carbon-13 NMR-spectra were recorded at $T = 303$ K on a Bruker DRX-500 instrument (500 MHz for ^1H and 125 MHz for ^{13}C) locked to the deuterium resonance of the solvent ($CDCl_3$).

3.5. Analysis of the samples with GC and GC–MS

GC–MS-analyses were performed as described earlier (Demyttenaere et al., 2000, 2001). GC-analyses were performed with a HP 6890 GC Plus, with a

split/splitless-injector and an FID-detector and equipped with either an EC-5 column (Demyttenaere et al., 2001) or a polar EC-WAX column (30 m×0.25 mm i.d.; 0.25 μm df). Isothermal conditions were used (120 °C) with a column flow of 0.9 ml/min (He, split 1/20). For elucidation of the configuration of the metabolite (+)-aristolochene, chiral GC-analyses were performed with the same GC, equipped with a Cydex-B chiral column (SGE: 50 m×0.22 mm i.d.; 0.25 μm df). Isothermal conditions were used (150 °C) with a column flow of 1 ml min^{-1} (split 1/20). For confirmation, co-injections of the fungal (+)-aristolochene with synthetic (−)-enantiomer, prepared from (+)-nootkatone (McMurry et al., 1975; Marshall and Greene, 1972) were carried out on two additional chiral stationary phases: heptakis(6-*O*-*t*.butyldimethylsilyl-2,3-di-*O*-methyl)-β-cyclodextrin (50% in OV1701, w/w) and heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin (50% in OV 1701, w/w) (25 m fused slica capillary, 0.25 mm i.d.; 0.125 μm df). The carrier gas was hydrogen at 0.5 bar inlet pressure. The separations were carried out under isothermal conditions (115 and 120 °C, respectively).

The other fungal metabolites were identified by comparison of their mass spectra and retention indexes (Kováts Indexes) with those of reference substances (where possible) and by comparison with the NIST Mass Spectral Library (Version 1.6d, 1998) and with literature data (Adams, 1995; Joulain and König, 1998).

3.6. (+)-Aristolochene

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.83 (3H, *d*); 0.96 (3H, *s*); 1.15 (1H, *dd*); 1.25–1.7 (4H, *broad*); 1.27 (1H, *broad*); 1.72 (3H, *broad s*); 1.76 (1H, *ddd*); 1.86 (1H, *dddd*); 1.98–2.00 (2H, *m*); 2.12 (1H, *ddm*); 2.20 (1H, *dddd*); 4.68–4.70 (2H, *m*); 5.30 (1H, *ddd*). ^{13}C NMR (125 MHz, $CDCl_3$): δ 15.6 (*q*); 18.0 (*q*); 20.7 (*q*); 27.7 (*t*); 29.6 (*t*); 31.2 (*t*); 32.5 (*t*); 37.7 (*d*); 38.7 (*s*); 43.2 (*t*); 44.1 (*d*); 108.2 (*t*); 118.7 (*d*); 144.5 (*s*); 150.5 (*s*). Kóvats Retention Index (HP-5 MS): 1482. EIMS 70 eV *m*/*z*: (rel. int.): 204 $[M]^+$ (12), 189 (100), 105 (76), 91 (40), 121 (39), 107 (38), 93 (34), 133 (31), 161 (25), 119 (23), 80 (22).

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