

## Isopulegol from Liquid Cultures of the Fungus *Ceratocystis coerulescens* (Ascomycotina)

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*Ceratocystis coerulescens* RWD 451 (Ascomycetes) was cultivated on defined synthetic culture medium containing glucose (3.0%), asparagine (0.1%), mineral salts and thiamine. 6 weeks old cultures produced methyl heptenyl compounds, citronellol, citronellyl acetate, dihydro-farnesol and a hitherto unknown monoterpene alcohol. By GC/MS,  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy, this alcohol could be identified as (–)isopulegol. So far, this monocyclic monoterpene alcohol has not been described as a fungal metabolite.

### Introduction

Volatile terpenes have been isolated from various fungi [1, 2]. In this respect, the ascomycete genus *Ceratocystis* Ellis and Halst. has been investigated very intensively [3, 4]. Besides acyclic and cyclic sesquiterpenoids, mainly acyclic monoterpenes, have been isolated from *Ceratocystis* strains [5–7].

Only sporadically, cyclic monoterpene alcohols and hydrocarbons have been found in *Ceratocystis* species as well as in other fungi [2, 5, 7]. There is some doubt as to whether these cyclic monoterpenes are true products of fungal metabolism. At least some of the described metabolites may originate non-enzymatically from acyclic precursors. It has also been suggested that – concerning the monoterpene formation in *Cronartium fusiforme* [8] – they might be taken up from the host [2].

**Abbreviations:** GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography;  $R_F$ , retention factor; NMR, nuclear magnetic resonance; TMS, tetramethylsilane.

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This paper describes the isolation and identification of the cyclic monoterpene alcohol (–)isopulegol from liquid cultures of *Ceratocystis coerulescens* (Münch) Bakshi strain RWD 451, grown on a defined synthetic culture medium.

### Material and Methods

*Ceratocystis coerulescens* RWD 451 has been isolated as *C. virescens* from *Fagus* sp. (New Hampshire, U.S.A., 1964) [9]. The fungus was cultivated for two months on a defined synthetic liquid medium containing glucose (3.0%), asparagine (0.1%), thiamine and mineral salts [10] in 1 l Fernbach flasks with 250 ml of culture medium. The maximum yield of volatiles was obtained after 43 days by circulation steam distillation [11] in pentane. The total distillate (10 ml from 200 Fernbach flasks) was further separated into five fractions by HPLC, using a Waters M-45 model with a differential refractometer R 401, a LiChrosorb Si 60 column (10  $\mu\text{m}$ , length 30 cm) and *n*-hexane:ethylacetate (8:2) as solvent. The fourth fraction contained 6-methyl-5-hepten-2-one (10%) and isopulegol (90%) which was further purified (99%) by a second HPLC step using the same conditions.

GC analyses were performed with a Perkin Elmer PE F 22 model equipped with a flame ionization detector (FID; range 1, attenuation 1:4; split 1:30) and a computing integrator Perkin Elmer PE M-1.  $\text{N}_2$  was used as carrier gas at a flow rate of 1 ml/min. The WG 11 (FFAP) capillary column (WGA; i.d. 0.3 mm) had a length of 22 m. For identification a temperature programme (70–200 °C/min) was applied. Injection port temperature: 180 °C. Detector temperature: 180 °C. Injection volume: 1.0  $\mu\text{l}$ . Quantities of volatiles were determined using citronellol as an internal standard and FID-specific substance factors.

MS analyses were performed on a Hewlett-Packard Quadrapol HP 5985 A mass spectrometer at 70 eV using a 25 m OV 101 silica column and a temperature programme (80–200 °C; rate 5 °C/min).

The identification of isopulegol resulted from comparison of retention time  $R_F$  values, GC/MS data compared with those of the literature [12] and those of authentic reference substances.

For NMR analyses, the solvent was removed with  $\text{N}_2$  at –196 °C. NMR spectra were recorded on a Bruker WM 400.



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## Results and Discussion

The blue stain fungus *Ceratocystis coerulescens* RWD 451 was cultivated on a defined synthetic liquid medium. The distillate from 200 Fernbach flasks consisted of 1.10 mg/l short chain alcohols and esters (3.7%), 20.48 mg/l methyl heptenyl compounds (68.3%), 5.06 mg/l of an unknown substance (16.9%) and 2.56 mg/l of an isomeric form of this unknown substance (8.5%). Furthermore, 0.12 mg/l citronellol (0.4%), 0.52 mg/l citronellyl acetate (1.7%) and 0.14 mg/l dihydro-farnesol (0.5%), could be identified. Most of these constituents have also been isolated from other *Ceratocystis coerulescens* strains [5].

After HPLC-separation and further purification (99%) the unknown substance could be identified as a monoterpene alcohol by GC/MS analysis. Literature data [12] pointed to an isomeric form of isopulegol. By means of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy the configuration of (–)isopulegol was identified [13].

Comparison of retention time  $R_F$  values with the authentic reference substance confirmed our results. In fraction III we found a further isomeric form of isopulegol (8.5%). The configuration elucidation of this compound is under way.

It has been assumed that cyclic monoterpenoids isolated from *Ceratocystis* cultures might be artifacts [4]. So it should be mentioned that isopulegol may originate from acyclic monoterpene aldehydes [14]. On the other hand, no acyclic monoterpene aldehyde could be traced in the cultures of our strain.

Using higher concentrations of the C-source (glucose 5.0%) and other N-sources (aspartic acid, glutaminic acid and glutamine) the yields of volatiles were distinctly lower compared to the culture conditions described above.

Table I.  $^1\text{H}$  NMR spectroscopy of isopulegol ( $\delta$ -data; 400.13 MHz; in  $[\text{D}_1]\text{chloroform}:[\text{D}_6]\text{benzol}$  3:2 added one drop  $[\text{D}_4]\text{methanol}$ ; TMS as an internal standard).

Chemical shifts	Coupling constants $J$ [Hz]
0.87 = 6'-H	$J_{1,2} = 3.0$
0.90 = 7-CH <sub>3</sub>	$J_{1,2'} = 10.0$
0.95 = 2'-H	$J_{1,7} = 5.5$
1.21 = 5'-H	$J_{2,2'} = 10.3$
1.38 = 1-H	$J_{2,3} = 3.4$
1.58 = 6-H	$J_{2,6} = 1.6$
1.60 = 5-H	$J_{2',3} = 8.5$
1.62 = 10-CH <sub>3</sub>	$J_{3,4} = 8.4$
1.80 = 4-H	$J_{4,5} = 3.6$
1.99 = 2-H	$J_{4,5'} = 10.2$
3.32 = 3-H	$J_{9,9'} = 2.2$
4.80 = 9'-H	$J_{9,10} = 1.2$
4.83 = 9-H	$J_{9',10} = 0.7$

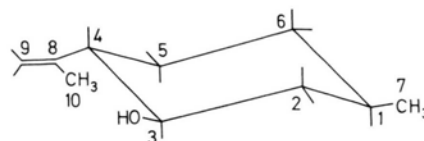


Fig. 1.

In higher plants, cyclic monoterpenes have a widespread distribution. So far, isopulegol and related compounds have been traced in a number of species, e.g. in *Baeckea citridora* [15], East African geranium and *Eucalyptus citridora* [16, 17], *Mentha piperita* L. [18], *Pinus radiata* [19] and *Citrus jambhiri* Lush. [20].

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