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}H and ^{13}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].

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 µm, 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. 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 µl. Quantities of volatiles were determined using citronellol as an internal standard and FID-specific substance factors.

MS analyses were performed on a Hewlett-Packard Quadrupol 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 N_2 at −196 °C. NMR spectra were recorded on a Bruker WM 400.

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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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}H and ^{13}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}H NMR spectroscopy of isopulegol (δ-data; 400.13 MHz; in [D_1]chloroform:[D_6]benzol 3:2 added one drop [D_4]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_3	$J_{1,2'}$ = 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_3	$J_{3,4}$ = 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

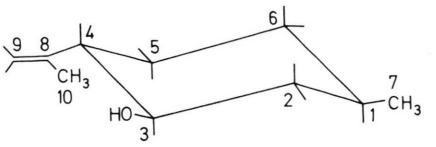

Fig. 1.

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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[1] E. Sprecher, in: Vorkommen und Analytik etherischer Öle (K.-H. Kubeczka, ed.), p. 11, Thieme Verlag, Stuttgart 1979.
[2] W. B. Turner and D. C. Aldridge; Fungal Metabolites II, Academic Press, London 1983.
[3] E. Sprecher and H.-P. Hanssen, Antonie van Leeuwenhoek **49,** 493 (1983).
[4] H.-P. Hanssen, Volatile Terpenes from Sapwood Staining Fungi, Colloque sur la Chimie des Terpènes, Grasse (F), April 1986 (Proc. in press).
[5] E. Sprecher, K.-H. Kubeczka, and M. Ratscho, Arch. Pharm. **308,** 843 (1974).
[6] E. Lanza and J. K. Palmer, Phytochemistry **16,** 1555 (1977).
[7] H.-P. Hanssen and E. Sprecher, Z. Naturforsch. **36c,** 1075 (1981).
[8] J. L. Laseter, J. D. Weete, and C. H. Walkinshaw, Phytochemistry **12,** 387 (1973).
[9] E. Sprecher, Arch. Microbiol. **60,** 24 (1968).
[10] E. Sprecher, Planta **53,** 565 (1959).
[11] E. Sprecher, Dtsch. Apoth. Ztg. **103,** 213 (1963).
[12] J. Iwamura, K. Beppu, and N. Hirao, Anal. Ind. Tokyo **14,** 162 (1976).
[13] F. Bohlmann, R. Zeisberg, and E. Klein, Organic Magn. Reson. **7,** 426 (1975).
[14] T. Oritani and K. Yamashita, Agr. Biol. Chem. **37,** 1687 (1973).
[15] A. R. Penfold, F. R. Morrison, J. L. Willis, H. H. G. McKern, and M. C. Spies, J. Proc. Roy. Soc. N.S. Wales **85,** 123 (1952).
[16] T. E. Furia and N. Bellanca; Fenaroli's Handbook of Flavor Ingredients, Chemical Rubber Co., Cleveland, Ohio 1971.
[17] A. I. Argiro and J. A. Retamar, Arch. Bioquim., Quim. Farm. **18,** 29 (1973).
[18] B. M. Lawrence, J. W. Hogg, and S. J. Terhune, Flavour Ind. **9,** 467 (1972).
[19] R. F. Simpson and R. M. McQuilkin, Phytochemistry **15,** 328 (1976).
[20] E. D. Lund, P. E. Shaw and C. L. Kirkland, J. Agric. Food Chem. **29,** 490 (1981).