

# Microbial Transformation of (-)-Carvone<sup>‡</sup>

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The cyclic monoterpene ketone (-)-carvone was metabolized by the plant pathogenic fungus *Absidia glauca*. After 4 days of incubation, the diol 10-hydroxy-(+)-neodihydrocarveol was formed. The absolute configuration and structure of the crystalline substance was identified by means of X-ray diffraction and by spectroscopic techniques (MS, IR and NMR). The antimicrobial activity of the substrate and metabolite was assayed with human pathogenic microorganisms.

*Key words:* (-)-Carvone, Microbial Transformation, Antimicrobial Activity

## Introduction

Research on microbial and enzymatic biotransformations of commonly available monoterpenoids into more value added derivatives has always been of interest because of their economical potential to perfume, food, and pharmaceutical industries (Kieslich, 1976; Berger, 1995; van der Werf *et al.*, 1997; Demmyttenaere, 2000; Noma and Asakawa, 2000; Shimoda and Hirata, 2000).

Carvone enantiomers present an important and abundant natural monoterpene resource. (+)-Carvone occurs naturally in caraway, dill seeds and in several essential oils and is used as flavour, medicine and cosmetic ingredient. Whereas, (-)-carvone is present in spearmint and is responsible for its characteristic flavour and fragrance (Bauer *et al.*, 2001).

Carvone biotransformations using various cell types were recently investigated (Shimoda and Hirata, 2000; Engel, 2001). Our group has also previously reported on the microbial transformation and headspace-SPME analysis of carvone enantiomers (Demirci *et al.*, 2001).

In this study, the fungal biotransformation of (-)-carvone is presented. Isolation and subsequent structure elucidation of the newly trans-

formed metabolite 10-hydroxy-(+)-neodihydrocarveol (**4**) is given. For the structure elucidation spectroscopic techniques like GC-MS, HREIMS, <sup>1</sup>H and <sup>13</sup>C NMR, GC-FT/IR and X-ray diffraction were used. Furthermore, antimicrobial activity of carvone enantiomers (**1** and **2**) and the metabolite **4** were evaluated in a microdilution assay against human pathogenic bacteria and fungi (Isican *et al.*, 2002).

## Experimental

### General

Optical rotation was measured on a JASCO DIP-1000 polarimeter. The IR spectrum was measured using a Perkin Elmer Spectrum 2000 GC-FT/IR system. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Bruker AM-400 system at 400 and 100 MHz, respectively;  $\delta$  values were given in Hz. Tetramethylsilane (TMS) at 0.0 ppm was used as internal standard in CDCl<sub>3</sub>. 1D- and 2D-NMR data were also obtained using the same system; the spectra were measured and reported in ppm. HR-MS analyses were measured on a JOEL JMS-AX 500 system. The X-ray diffractometer was a Mac Science MXC 18 instrument, and maXus SIR92 software was used to solve the crystal structure.

(-)-Carvone (Aldrich 12493-1, Taufkirchen, Germany) was obtained from commercial sources. Routine analyses were performed on pre-coated silica gel G-25 UV254 plates (0.25 mm, Macherey-

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Nagel, Düren, Germany) using *n*-hexane/acetone (75:25, v/v) as solvent system. Visualization was under UV (254/366 nm) and/or by spraying with anisaldehyde/H<sub>2</sub>SO<sub>4</sub> spray reagent.

#### Microorganisms and biotransformation

Stock cultures were maintained on agar slants (SDA, Acumedia, Baltimore, Maryland, USA), stored at 4 °C and refreshed every 6 months.

*Aspidia glauca* (ATCC 22752) was cultivated at room temperature in 250 ml-culture flasks containing 100 ml sterile (121 °C, 20 min, autoclaved)  $\alpha$ -medium [consisting of 20 g glucose; 5 g NaCl; 5 g K<sub>2</sub>HPO<sub>4</sub>; 5 g yeast extract (Acumedia, Baltimore, Maryland, USA); 5 g peptone (Sigma, St. Louis, MO, USA) per litre of distilled water adjusted to pH 7.0]. Culture flasks were shaken at 140 rpm. After 24–48 h of sufficient growth of the microorganisms, 50  $\mu$ l of substrate [(–)-carvone] was added and incubated for 7 d. The biotransformation products were screened by liquid-liquid extraction of the withdrawn broth (3 ml) which was extracted exhaustively by ethyl acetate, concentrated and evaluated by TLC and GC-MS analysis. Substrate controls were composed of sterile medium to which the substrate (50  $\mu$ l) was added and incubated without the microorganism. Culture controls consisted of fermentation blanks in which the microorganism was grown under identical conditions but without the addition of substrate. After 7 d of incubation, controls were also harvested and analysed by TLC.

#### Gas chromatography mass spectrometry (GC-MS) conditions

The samples were analyzed and screened by GC-MS using a Hewlett Packard GCD system. Innowax FSC column (60 m x 0.25 mm i.d., 0.25  $\mu$ m film thickness) was used with helium as a carrier gas (1.5 ml/min). GC oven temperature was kept at 100 °C for 5 min and programmed to 220 °C at a rate of 20 °C/min, and then kept constant at 220 °C for 11.5 min. Injection was carried out in splitless mode. The injector temperature was at 250 °C. Mass spectra were recorded at 70 eV and mass range was from *m/z* 35 to 425. Library search was carried out using the in-house library “Baser Library of Essential Oil Constituents” and in comparison with authentic samples. Relative percentage amounts were calculated from TIC by the computer.

#### Semi-preparative scale biotransformation and isolation

The substrate (–)-carvone (175 mg) was distributed among three 250 ml-flasks containing 100 ml broth plus the microorganism that was sufficiently pre-grown as described above at room temperature for 4 d. The biotransformation was stopped by adding EtOAc to the flasks and the broths were combined and extracted with equal amounts of EtOAc successively. After filtration of the mycelia, the extracts were dried over anhydrous sodium sulfate and concentrated *in vacuo* to yield a brown gum (*ca.* 250 mg). The biotransformation extract was then subjected to column chromatography (*n*-hexane  $\rightarrow$  EtOAc; 100  $\rightarrow$  0). Compound **4** was eluted in 40% EtOAc to give colourless crystals (10 mg) having a characteristic odour.

*10-Hydroxy-(+)-neodihydrocarveol* [8(9)-*p*-menthene-2,10-diol, C<sub>10</sub>H<sub>18</sub>O<sub>2</sub>] (**4**): [ $\alpha$ ]<sub>D</sub><sup>25</sup> = +25.8° (*c* 0.356, CHCl<sub>3</sub>). – R<sub>f</sub> = 0.3 (*n*-hexane/EtOAc, 6:1, v/v). – GC-FT/IR:  $\nu_{\max}$  = 3659 (OH), 3037, 2934, 2880, 1645, 1454, 1377, 1026 cm<sup>-1</sup>. – <sup>1</sup>H NMR (400 MHz):  $\delta$  = 1.03 (3H, d, *J* = 6.4 Hz, H-7), 3.2 (1H, m, H-2), 4.13 (2H, s, H-10), 4.91 and 5.04 (2H, 2s, H-9). – <sup>13</sup>C NMR (100 MHz):  $\delta$  = 36.1 (C-1), 70.9 (C-2), 39.0 (C-3), 33.1 (C-4), 28.2 (C-5), 31.6 (C-6), 18.3 (C-7), 153.6 (C-8), 108.3 (C-9), 65.2 (C-10). – EI/MS: *m/z* = 170 [M<sup>+</sup>], 152 (3) [M<sup>+</sup>-H<sub>2</sub>O], 134 (87), 119 (100), 106 (77), 105 (55), 95 (82), 93 (60), 81 (61), 79 (74), 67 (87), 55 (82), 41 (98).

(+)-*trans*-Dihydrocarvone (**2**) (C<sub>10</sub>H<sub>16</sub>O): GC-FT/IR:  $\nu_{\max}$  = 3342, 3085, 2941, 2879, 1729, 1648, 1451, 1375, 1210 cm<sup>-1</sup>. – EI/MS: *m/z* = 152 (17) [M<sup>+</sup>], 137 (14), 123 (5), 109 (36), 95 (69), 82 (42), 81 (43), 68 (50), 67 (100), 55 (31), 41 (57).

(+)-*Neodihydrocarveol* (**3**) (C<sub>10</sub>H<sub>18</sub>O): GC-FT/IR:  $\nu_{\max}$  = 3660 (OH), 3083, 2935, 2878, 1644, 1454, 1377, 1222, 993 cm<sup>-1</sup>. – EI/MS: *m/z* = 154 (1) [M<sup>+</sup>], 136 (73), 121 (86), 107 (100), 93 (87), 79 (98), 68 (39), 67 (58), 55 (48), 41 (75).

#### Antimicrobial assay

Microdilution broth susceptibility assay (Iscan *et al.*, 2002) was used for the antimicrobial evaluation of the substrate and metabolite. Stock solution was prepared in dimethylsulfoxide (DMSO) and dilution series were prepared up to 0.97  $\mu$ g/ml using sterile distilled water in 96-well microtiter plates. Overnight grown microbial suspensions in double strength Mueller-Hinton broth and suspen-

sion of *Candida albicans* in yeast medium were standardized to approximately  $10^8$  CFU/ml (using McFarland No: 0.5). 100  $\mu$ l of each microbial suspension was then added to each well. The last row containing only the serial dilutions of antimicrobial agent without microorganism was used as negative control. Sterile distilled water and medium served as a positive growth control. After incubation at 37 °C for 24 h the first well without turbidity was determined as the minimal inhibition concentration (MIC) expressed both in  $\mu$ g/ml and mM, as seen in Table I. Chloramphenicol was used as standard antibacterial agent for the bacteria, whereas ketoconazole was used for *C. albicans*. Microorganisms were kindly supplied by the Microbiology Department of Osmangazi University (O.G.Ü), Medical Faculty (see Table I for the results).

### Results and Discussion

(-)-Carvone (**1**) was incubated with the plant pathogenic fungus *Absidia glauca* for 7 d. Preliminary TLC screening showed a new metabolite at the fourth day; it was confirmed by GC-MS analysis along with known major metabolites such as (+)-*trans*-dihydrocarvone (**2**) and (+)-neodihydrocarveol (**3**) which already were published in our previous work (Demirci *et al.*, 2001). These metabolites could be expected due to the reaction mechanism and stages (Fig. 1). Regarding the unknown new metabolite, although the molecular ion peak was absent, another peak [152 + H<sub>2</sub>O] having the characteristic carveol spectrum led to a previously reported (Tahara and Sakuda, 1976) synthetic diol after a detailed literature and structure search. Very recently, the carveone-diol was also described by the work of Matsumura *et al.* (2002), however, as a hydrolyzed product from a glucoside which was isolated from a methanolic extract of *Carum carvi* fruits. We have also pre-

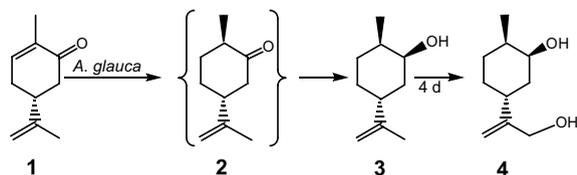


Fig. 1. The biotransformation of (-)-carvone (**1**) and its metabolism to the diol 10-hydroxy-(+)-neodihydrocarveol (**4**) via (+)-*trans*-dihydrocarvone (**2**) and (+)-neodihydrocarveol (**3**) in 4 days.

viously reported on several enantiomers as biotransformation products of carvone, however, this compound could not be detected by means of headspace-SPME at that time (Demirci *et al.*, 2001).

This compound was purified from the microbial transformation broth using the EtOAc extract *via* column chromatography. Apolar to polar gradient elution starting from *n*-hexane allowed the isolation of pure crystals which were subjected to further spectroscopic analyses. The <sup>1</sup>H NMR spectrum showed at  $\delta$  1.03 a methyl group demonstrating that there was no change at position 1, but at position 10, where the methyl was transformed to a methylene group. The broad signal at  $\delta$  4.13 with an integration of 2 H suggested that there were two hydroxyl groups in the molecule which also was supported by mass spectroscopic data. In addition, GC-FT/IR analysis showed a characteristic OH peak at 3659 nm. Furthermore, HREIMS data gave the molecular formula as C<sub>10</sub>H<sub>18</sub>O<sub>2</sub>. Finally, the evidence of the diol **4** was proven by X-ray diffraction [ $\lambda$ (Cu K $\alpha$ ) = 1.54051 Å] as monoclinic; space group *P*2<sub>1</sub>, with *a* = 7.642 (1) Å, *b* = 18.353 (8) Å, *c* = 10.688 (5) Å,  $\beta$  = 92.50 (5)°, *V* = 1497.65 (1) Å<sup>3</sup>, *Z* = 4. Scattering-factors were used according to Waasmaier and Kirfel (1995). The final *R* value was 0.067 for 1741 reflections.

To the best of our knowledge, the isolation and absolute configuration of 10-hydroxy-(+)-neodihydrocarveol (**4**) *via* biotransformation is reported with this work for the first time. As stated above, previous reports on the synthesis (Tahara and Sakuda, 1976) and a glucoside derivative (Matsumura *et al.*, 2002) were found during the structure search. The spectroscopic and physical data of metabolite **4** was comparable to the recent work of Matsumura *et al.* (2002).

The substrate (-)-carvone (**1**) and its (+)-isomer, as well as the metabolite **4** were subjected to a microdilution broth assay (Iscan *et al.*, 2002) against various gram positive and negative human pathogenic bacteria and the yeast *Candida albicans*. Surprisingly, the metabolite showed less inhibitory activity compared to both carvones and the standard antimicrobial agents. Since it is more polar compound it was expected to be more active than its counterparts. However, the inhibitory activity was rather moderate. On the other hand, since metabolites synthesized as a result of a biotransformation process, they may be considered as a detoxification product. This may explain the low

Table I. Antimicrobial activity<sup>#</sup> of carvones and metabolite **4**.

Microorganism	Source	(-)-Carvone ( <b>1</b> )	(+)-Carvone	Diol <b>4</b>	St.
<i>Escherichia coli</i>	ATCC 25922	62.5 (0.416)	62.5 (0.416)	250 (1.468)	62.5 (0.193)
<i>Staphylococcus aureus</i>	ATCC 6538	62.5 (0.416)	250 (1.665)	125 (0.734)	15.62 (0.048)
<i>Pseudomonas aeruginosa</i>	ATCC 27853	250 (1.665)	250 (1.665)	250 (1.468)	250 (0.773)
<i>Enterobacter aerogenes</i>	NRRL 3567	125 (0.832)	250 (1.665)	250 (1.468)	125 (0.386)
<i>Proteus vulgaris</i>	NRRL B123	125 (0.832)	125 (0.832)	250 (1.468)	31.25 (0.096)
<i>Salmonella typhimurium</i>	NRRL B4420	125 (0.832)	125 (0.832)	250 (1.468)	62.5 (0.193)
<i>Candida albicans</i>	O. G. Ü	125 (0.832)	62.5 (0.416)	250 (1.468)	125 (0.235)*

# MIC values are given in both µg/ml and mm.

St.: Chloramphenicol.

\* Ketoconazole.

toxicity of the compound against microorganisms. The tested gram positive and negative bacteria, as well as the yeast with their inhibition values expressed in minimum inhibitory concentrations, compared to standard antimicrobials, can be seen in Table I. Antimicrobial activity of carvone against various pathogens was previously reported (Knobloch *et al.*, 1989; Hinou *et al.*, 1989; Naigre *et al.*, 1996; McGready *et al.*, 2002). A recent inves-

tigation showed that (+)-carvone was effective even in low concentrations against the pathogenic yeast *Candida albicans* (McGready *et al.*, 2002).

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