

Biotransformation of α -Cedrol and Caryophyllene Oxide by the Fungus *Neurospora crassa*

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Incubation of α -cedrol and caryophyllene oxide with *Neurospora crassa* afforded 12 β -hydroxy cedrol, 10 α -hydroxy cedrol, and 3 β -hydroxy cedrol, and 12 β -hydroxy caryophyllene oxide as major metabolites, respectively. The antibacterial and radical scavenging activities of the metabolites were evaluated *in vitro* using broth microdilution and bioautographic techniques. However, no significant antibacterial and antioxidant activities were observed when compared with those of standard substances.

Keywords: *Neurospora crassa*, biotransformation, caryophyllene oxide, α -cedrol, antibacterial activity, radical scavenging activity.

Caryophyllene oxide and α -cedrol are two of the oxygenated sesquiterpenes found in essential oils. They are important compounds because not only are they well known as preservatives in food, drugs and cosmetics, but also for their diverse biological activities. For example, caryophyllene oxide has been tested *in vitro* as an antifungal agent against dermatophytes and its antifungal activity has been compared with cyclopiroxolamine and sulconazole, commonly used drugs in onychomycosis treatment. Therefore, their biotransformation with various fungi has been carried out with the aim of producing new metabolites with a variety of biological properties besides their use as flavouring agents [1].

There are many reports on the biotransformation of cedrol by a variety of microorganisms, some of which include *Cephalosporium aphidicola*, *Glomerella cingulata*, *Botrytis cinerea*, *Curvularia lunata* and *Staphylococcus epidermidis*. The results showed that the C-3 atom seemed to be the preferred site of the oxidative attack, along with oxidation at various other positions [C-2, C-4, C-6 and C-15 (methyl)] [2]. A few studies have been made on the biotransformation of caryophyllene oxide by microorganisms, plant cell cultures, and mammals, which include *Catharanthus*

roseus, *Eucalyptus perriniana* and rabbits. As a result of these biotransformation reactions, mono- and di-hydroxylated metabolites have been obtained [3].

Neurospora crassa is a red, bread mold and one of the fast growing filamentous fungi (doubling time under optimal conditions of 2 h) that produces no toxic secondary metabolites. The fungus has been subjected to many biological tests and assays, not only because it has well established DNA transformation systems, but also because it serves as a source of several industrially important enzymes, such as alkaline phosphatase, isocitrate lyase and xylitol dehydrogenase [4]. *N. crassa* has also proved to be a useful organism for hydroxylation of various steroids yielding mono- and poly-hydroxy derivatives at different positions on the steroid nucleus. These include the biotransformation of deoxycorticosterone, progesterone, estradiol and, in recent years, nandrolone decanoate and androst-1,4-dien-3,17-dione [5].

In our systematic assessment of the biotransformation potential of *N. crassa* on sesquiterpenes, we previously reported the biotransformation of racemic diisophorone by this fungus [6a]. Continuing our investigations on this subject, we report here the biotransformation of the

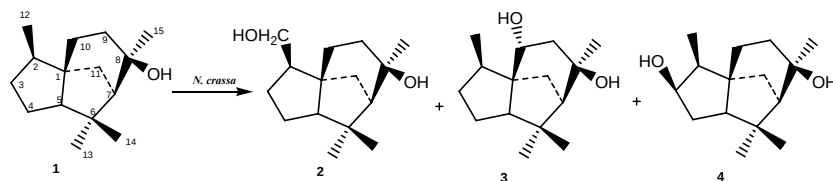


Figure 1: Microbial hydroxylation metabolites of **1** by *N. crassa*.

two oxygenated sesquiterpenes caryophyllene oxide and α -cedrol. In addition, the *in vitro* antimicrobial and antioxidant activities of the substrates and metabolites were investigated using a broth microdilution and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, respectively.

Microbial biotransformation of α -cedrol (**1**) by *N. crassa* on a Czapeck-Dox medium for ten days afforded compounds **2-4** (see Fig. 1). Their structures were elucidated as follows: The EIMS of the first metabolite showed a molecular ion at m/z 238 consistent with the formula $C_{15}H_{26}O_2$. The IR spectrum showed broad hydroxyl absorption at 3285 cm^{-1} . The ^1H NMR spectrum of **2** indicated the appearance of new hydroxyl-bearing methylene protons at δ_{H} 3.46 (1H, dd, $J = 7.7$ and 10.4) and 3.65 (1H, dd, $J = 6.9$ and 10.4 MHz) ppm. A signal at δ_{C} 16.0 assigned to one of the methyl carbons (possibly C-12) in α -cedrol was not present in the ^{13}C NMR spectrum of the metabolite, which showed signals for 15 carbon atoms, while the DEPT spectrum indicated the presence of three methyl, five methylene, one hydroxy-bearing methylene, three methine and quaternary carbon signals. These data suggested the introduction of a hydroxyl group at one of the methyl groups (C-12, C-13, C-14 or C-15). The metabolite was assigned as 12 β -hydroxycedrol (**2**) by comparing its ^{13}C NMR and DEPT spectra with those of α -cedrol and its known metabolites reported in the literature [2a].

The mass spectrum of the second metabolite showed a molecular ion at m/z 238 consistent with the molecular formula $C_{15}H_{26}O_2$. Broad hydroxyl absorption at 3285 cm^{-1} was observed in the IR spectrum. The ^1H NMR spectrum was similar to that of α -cedrol with a new proton signal appeared as a doublet of doublets at δ_{H} 3.95, attributable to a $\text{CH}(\text{OH})$ group, indicating that hydroxylation had taken place at one of the secondary positions (C-3, C-4, C-9, C-10 or C-11). This was confirmed by the ^{13}C NMR spectrum, which indicated resonances for fifteen carbons, while the DEPT spectrum displayed the presence of four methyl, four methylene, three methine and three quaternary carbon signals. Hydroxylation of a methylene carbon was further indicated by a new low-field, hydroxyl-bearing methine signal at δ_{C} 71.6. This compound was identified as 10 α -hydroxycedrol (**3**) based on the known

metabolites of α -cedrol in the literature. The same metabolite has also been reported previously as a biotransformation product of *G. cingulata* [2b].

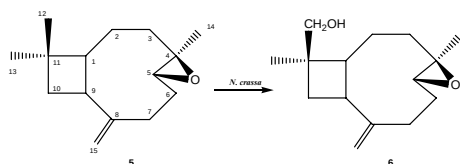
The third metabolite, identified as 3 β -hydroxycedrol (**4**), displayed a molecular ion peak at m/z 238 in the EIMS, corresponding to the molecular formula $C_{15}H_{26}O_2$. A broad, strong absorption at 3285 cm^{-1} was observed in the IR spectrum. The ^1H NMR spectrum of **4** revealed additional hydroxyl-bearing methylene protons (2H, dd) at δ_{H} 4.28. This was further confirmed by the ^{13}C NMR spectrum, which showed a hydroxy-bearing methylene signal at δ_{C} 74.9, which proved the hydroxylation of a methylene carbon of C-3, C-4, C-9, C-10 or C-11. The downfield shifts of the signal assigned to C-12 in **1** suggested that the hydroxyl group was located at C-3. The same metabolite had also been reported previously as a biotransformation product of *Cephalosporium aphidicola* [2a], *G. cingulata* [2b] and *Bacillus cereus* [7a].

The biotransformation of caryophyllene oxide (**5**) with *N. crassa* for seven days afforded compound **6** (see Fig. 2). Its structure was established as follows: The EIMS showed a molecular ion at m/z 236 corresponding to the molecular formula $C_{15}H_{24}O_2$. The IR spectrum showed new broad hydroxyl absorption at 3435 cm^{-1} . The ^1H NMR spectrum of **6** was very similar to that of **5** with the appearance of a new proton signal at δ_{H} 3.33, attributable to a CH_2OH group and the loss of one of the methyl protons, indicating that hydroxylation of one the methyl groups (C-11, C-12 or C-13) had taken place. This was confirmed by the ^{13}C NMR spectrum of **6**, which indicated resonances for 15 carbons, while the DEPT spectrum displayed the presence of two methyl, five methylene, two double-bond bearing methylenes and three methines, as well as two quaternary carbon signals. Hydroxylation of a methylene carbon was further indicated by a low field hydroxyl-bearing methylene signal at δ_{C} 70.8. The structure and position of the newly introduced hydroxyl group were assigned by comparing the NMR spectra of **6** with literature data for the natural product 12 β -(hydroxymethyl)-4,5-epoxycaryophyllene, reported as a biotransformation product of caryophyllene oxide by *B. cinerea* [7b]. The same metabolite was reported by Choudhary *et al.* [3a] and Asakawa *et al.* [3c]. The comparative MIC results of the standard antibacterial agents chloramphenicol and

Table 1: Antibacterial activity results of substrates and metabolites (MIC, µg/mL)

Microorganisms	1	2	3	4	5	6	Chlph	Amp
<i>Escherichia coli</i> -NRRL B-3008	125	500	125	125	500	500	3.9	15.6
<i>Staphylococcus aureus</i> -ATCC 6538	125	250	125	125	500	>1000	31.2	250
<i>Pseudomonas aeruginosa</i> -ATCC 27853	62.2	250	125	250	500	500	7.8	15.6
<i>Enterobacter aerogenes</i> -NRRL 3567	125	125	125	250	250	250	125	-
<i>Proteus vulgaris</i> - NRRL B-123	125	125	125	125	500	500	1.9	7.8
<i>Salmonella typhimurium</i> - ATCC 13311	125	250	125	250	500	500	7.8	-
<i>Staphylococcus epidermidis</i> -ATTC 12228	-	-	-	-	500	500	0.9	-

Chlph: Chloramphenicol, Amp: Ampicillin.

**Figure 2:** Microbial hydroxylation of **5** by *N. crassa*.

ampicillin, and the substrates, as well as the metabolites, towards human pathogenic bacterial strains are summarized in Table 1. As seen in this table, no significant antibacterial activities were observed compared with the substrates. MIC ranges were found from 62.5–500 µg/mL, far less than those of the standard antibacterial agents. Interestingly the substrates were more active than the metabolites.

The radical scavenging activities of the metabolites of both cedrol and caryophyllene oxide were rather low at the tested concentration when compared with the standard antioxidant agent vitamin C.

Incubation of α -cedrol and caryophyllene oxide with *N. crassa* afforded monohydroxylated cedrol and caryophyllene oxide derivatives as major metabolites. This indicates that *N. crassa* could be used as an alternative biocatalyst for hydroxylation of α -cedrol and caryophyllene oxide. No significant antibacterial and antioxidant activities were observed for the metabolites when compared with those of the substrates and standard substances.

Experimental

General experimental procedures: Melting point data were obtained using a Gallenkamp MPD350 melting point apparatus and are uncorrected. IR spectra were recorded in KBr pellets with a Bruker Tensor 27 infrared spectrophotometer. GC-MS and EIMS were recorded on a 6890 N network GC system, a 5975 inert mass and VG Autospec Fisons instruments, respectively. The ^1H NMR and ^{13}C NMR spectra were determined by either Bruker DPX 500 or Varian UNITY 600 spectrometers, while DEPT spectra were measured at 125 and 150 MHz in deuteriochloroform (CDCl_3) with tetramethylsilane (TMS) as internal standard. The solvents used for purification were distilled prior to use. Silica gel type 60 (Merck, 230–

400 mesh) was used for column chromatography. Thin-layer chromatography (TLC) was carried out on a 0.25 mm thick silica-gel plate (Merck silica gel 60 GF₂₅₄) using either *n*-hexane-ethyl acetate (4:1, v,v) or ethyl acetate-light petroleum (60–80°C)(1:1,v,v). Compounds and metabolites were detected either under UV light (at 254 nm) or by spraying with either methanol: sulfuric acid (1:1) or vanillin: sulfuric acid (1:1) reagent mixtures. All chemicals, solvents and culture media used were obtained from Sigma-Aldrich-Fluka (Taufkirchen, Germany), Merck (Darmstadt, Germany) or Oxoid (Hampshire, UK).

Microorganisms: Microorganisms used for biotransformations or bioassay studies were *Escherichia coli* NRRL B-3008, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* NRRL 3567, *Proteus vulgaris* NRRL B-123, *Salmonella typhimurium* ATCC 13311, *Staphylococcus epidermidis* ATCC 12228 and *N. crassa* (isolate) obtained from Hacettepe University, Ankara. All microorganisms were stored at -85°C (Ultrafreezer, New Brunswick Sci.) in 15% glycerol, refreshed in Mueller Hinton Broth (Merck, Germany) at $35^\circ\text{--}37^\circ\text{C}$, and afterwards inoculated on Mueller Hinton Agar (MHA, Mast Diagnostics, UK) plates for purity check.

General culture conditions: The cultures were maintained and precultured on potato dextrose agar (PDA) slants at 5°C and 25°C , respectively. The slants were prepared as described in our previous studies [6b].

A medium for growing *N. crassa* was prepared by mixing glucose (15 g), potassium dihydrogen phosphate (1 g), saccharose (15 g), polypeptone (5 g), potassium chloride (500 mg), magnesium sulfate (500 mg) and iron (II) sulfate (10 mg) in distilled water (1 L). The substrate (500 mg) was added after 2 days growth and the fermentations were continued for a further 7 days. The mycelium was filtered off and the broth was acidified with diluted HCl and extracted with ethyl acetate. The extract was dried and the solvent evaporated to give a residue which was chromatographed on silica with increasing concentrations of ethyl acetate in either light petroleum or *n*-hexane.

Biotransformation of α -cedrol by *N. crassa*: After 10 days of incubation, 3 compounds (**2-4**) were isolated. They were assigned as 12 β -hydroxycedrol (**2**) [2a], 10 α -hydroxycedrol (**3**) [2b] and 3 β -hydroxycedrol (**4**) [2a].

Biotransformation of caryophyllene oxide by *N. crassa*: After 7 days of incubation, the metabolite was eluted with 20% ethyl acetate in light petroleum. It was assigned as 12 β -hydroxy caryophyllene oxide (**6**) [3a].

Antibacterial bioassay: A broth micro-dilution susceptibility assay was used as previously described [8a]. Stock solutions of the test samples were prepared in 10% (v/v) dimethylsulfoxide (DMSO; Carlo Erba). Serial dilutions of samples were prepared up to 1.95 μ g/mL by using sterile distilled water in a 96-well micro titer plate. After incubation at 37°C for 24 h the first well without turbidity was determined as the minimum

inhibition concentration (MIC, μ g/mL). Ampicillin and chloramphenicol were used as standard antimicrobials. All experiments were repeated in triplicate and average MICs are given in Table 1.

Free radical scavenging activity using DPPH: The test substances were screened for their radical scavenging activity using a TLC assay to detect active substances in comparison with vitamin C similar as previously reported [8b].

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