

Fungal Biotransformation of Cyclademol and Antimicrobial Activities of Its Metabolites

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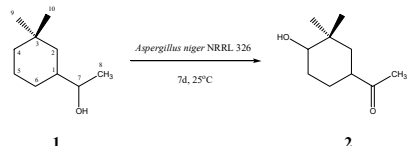
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Cyclademol (**1**) was converted for the first time to 1-(4-hydroxy-3,3-dimethylcyclohexyl) ethanone (**2**) and 4-(1-hydroxyethyl)-2,2-dimethylcyclohexanol (**3**) with 31.2 and 15.1% yields by *Aspergillus niger* and *Neurospora crassa*, respectively. The resulting metabolite structures were established by FT-IR, MS and NMR spectroscopic studies, respectively. In addition, the *in vitro* antimicrobial activities of the substrates and metabolites were evaluated comparatively both by using agar dilution and microdilution methods. The minimum inhibitory concentrations (MIC) of the tested compounds against a panel of pathogenic bacterial strains ranged from 1000 - 4000 µg/mL, whereas the MIC values against fungal strains were between 500 -1000 µg/mL.

Keywords: Antimicrobial evaluation, *Aspergillus niger*, Biotransformation, Cyclademol, *Neurospora crassa*.

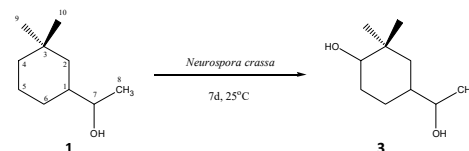
Cyclademol [1-(3,3-dimethylcyclohexyl)ethanol] (**1**) is a monoterpene synthesized by treating dihydromyrcene with formic acid alone or with a carboxylic acid or water in the presence of a Lewis acid such as sulfuric acid or a sulfonic acid resin [1,2]. It has a value in perfume industries due to its fragrance and pleasant odor [2-4]. In this present study, the biotransformation of **1** by 15 different microbial strains were carried out with the aim of producing new compounds for flavor and fragrance use, as well as antimicrobial agents. To the best of our knowledge this is the first report on the biotransformation of cyclademol and resulting metabolites.

Figure 1: Biotransformation of cyclademol (**1**) by *A. niger* NRRL 326.

From pre-biotransformation screening experiment of **1**, it was found that its biotransformation with *A. niger* NRRL 326 and *N. crassa* showed the formation of a fragrant compound. These biotransformation reactions were repeated on semi-preparative scale to elucidate the structures of the metabolites formed. The microbial transformation of **1** by *A. niger* NRRL 326 for 7 days afforded the metabolite **2**; 1-(4-hydroxy-3,3-dimethylcyclohexyl) ethanone, by eluting with 25% (v/v) ethyl acetate in petroleum ether (60-80°C) as seen in Figure 1. The mass spectrum of the metabolite showed a molecular ion at m/z 170 corresponding to the molecular formula of $C_{10}H_{18}O_2$. The IR spectrum displayed a broad absorption at 3422 cm^{-1} , indicating the presence of hydroxyl groups. The ^{13}C -NMR spectrum indicated a new low field hydroxyl-bearing carbon and carbonyl signals at δ_C 75.9 and 211.6 ppm, respectively, whereas a corresponding carbon signal resonating at 28.6 ppm disappeared. This indicated that a hydroxyl group was inserted to the ring, whereas the hydroxyl group at the side chain was oxidized to a ketone group in cyclademol. The newly introduced hydroxyl group position was established by HMBC spectrum which indicated

the connectivity of proton signal of the methyl group at 1.05 (3H, s, 10- CH_3) to the carbon signals at δ_C 17.8 (C-9), 35.5 (C-3), 41.0 (C-2) and 75.9 (C-4) confirming the hydroxylation on C-4.

The biotransformation of **1** by *N. crassa* for 7 days yielded the metabolite **3**, 4-(1-hydroxyethyl)-2,2-dimethylcyclohexanol, by eluting with 35% (v/v) ethyl acetate in petroleum ether (60-80°C) as seen in Figure 2. The mass spectrum of **3** showed a molecular ion at m/z 172 corresponding to the molecular formula of $C_{10}H_{20}O_2$. The IR spectrum showed carbonyl and hydroxyl group absorptions at 1703 and 3430 cm^{-1} , respectively. The ^{13}C -NMR spectrum indicated a new low field hydroxyl-bearing carbon signal at δ_C 77.8 ppm, whereas a carbon signal resonated at 28.6 ppm, which was absent in the cyclademol skeleton. Consequently, this indicated that a new hydroxyl group was added to the cyclademol structure. The newly introduced hydroxyl group position was established by HMBC spectrum which showed the connectivity of proton signal of the methyl group at 1.03 (3H, s, 10- CH_3) to the carbon signals at δ_C 18.0 (C-9), 35.4 (C-2), 41.2 (C-3) and 77.8 (C-4), confirming that the hydroxylation occurred on C-4.

Figure 2: Biotransformation of cyclademol (**1**) by *N. crassa*.Table 1: ^{13}C NMR data for compounds **1**, **2** and **3**.

C	1	2	3
1	40.8	46.7	40.0
2	41.4	41.0	41.2
3	36.8	35.5	35.6
4	39.3	76.9	77.8
5	22.2	29.8	29.3
6	28.6	27.1	27.4
7	72.4	211.6	71.9
8	20.5	28.2	20.8
9	24.6	17.8	18.0
10	33.5	28.9	30.3

Furthermore, the antimicrobial activities of the substrate cyclademol (1) and two biotransformation metabolites (2 and 3) were carried out against 6 pathogenic bacterial strains [*Staphylococcus aureus* ATCC 6538, *Proteus vulgaris* NRRL B-123, *Salmonella typhimurium* ATCC 13311, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* NRRL B-4378 and *Escherichia coli* ATCC 8739] and 3 pathogenic fungi [*Candida albicans* ATCC 90028, *C. glabrata* ATCC 2001, and *C. krusei* ATCC 6258]. The MIC values of the tested compounds against all bacterial strains ranged from 1000 to 4000 µg/mL, whereas the MIC values against fungal pathogenic strains were between 500 and 1000 µg/mL, suggesting rather moderate bioactivity levels.

Experimental

General Experimental Procedures: Cyclademol (1) was obtained from IFF Co, USA. HRMS, Waters SYNAPT G1-MS system; NMR, Varian Mercury Plus 400 spectrometer; CC, Silica gel type 60 (Merck, 230–400 mesh); TLC, silica gel plate (Merck silica gel 60 GF254) in *n*-hexane/ethyl acetate (1:1, v/v). Compounds were visualized by either under UV light (at 254 nm) or by reacting with vanillin/sulfuric acid (1:1) solution and heating.

Microorganisms: Bacterial and fungal cultures used in the present study were obtained from the Agriculture Research Service Culture Collection (NRRL), American Type Culture Collection (ATCC) and the culture collection of the Department of Pharmacognosy at the Faculty of Pharmacy at Anadolu University, Eskişehir, Turkey.

Biotransformation Strains: *Aspergillus parasiticus* NRRL 2999, *A. niger* NRRL 326, *A. niger* ATCC 10549, *A. alliaceus* NRRL 317, *Penicillium claviforme* MR 376, *P. adametzii* NRRL 737, *P. chrysogenum* NRRL 792, *Fusarium solani* ATCC 1284, *F. moniliforme* NRRL 2374, *F. culmorum* Isolate (Anadolu U., Faculty of Sciences), *Saccharomyces cerevisiae* ATCC 9763, *Hansenula anomala* ATCC 20170, *Mucor ramannianus* ATCC 1839, *Neurospora crassa* (WILD Type-Isolate, Regensburg University), *Corynespora cassiicola* DSM 62475

Antimicrobial Evaluation Strains: The bacterial strains were *Staphylococcus aureus* ATCC 6538, *Proteus vulgaris* NRRL B-123, *Salmonella typhimurium* ATCC 13311, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* NRRL B-4378 and *Escherichia coli* ATCC 8739. Tested yeasts were *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 2001 and *Candida krusei* ATCC 6258. All microorganisms were stored at -85°C (New Brunswick) in 15% glycerol and maintained on nutrient agar (Merck, 1.05450) and malt extract agar (Merck) slants at 4°C, respectively. They were sub cultured, prior to use for purity check.

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General Culture Conditions: The cultures were maintained and precultured on potato dextrose agar (PDA) slants at 5°C and 25°C, respectively [5,6]. A medium for growing fungi used in biotransformation experiments was described in our previous study [7].

In vitro Antimicrobial Activity: The antibacterial and antifungal activities of compounds were studied by using the agar diffusion method, according to the National Committee for Clinical Laboratory Standards [8-10]. Ampicillin and chloramphenicol were used as standard antibacterial agents, whereas amphotericin B and ketoconazole were used as standard antifungal agents.

Minimal Inhibitory Concentration: The minimal inhibitory concentration (MIC) of the compounds was determined by the microdilution method using a 96 well plate, according to NCCLS [8-10]. The metabolite concentrations ranged from 4000 to 15.62 µg/mL, whereas concentrations of standard antimicrobial agents were between 0.04 and 40 µg/mL. Stock solutions of the test samples were prepared up to 10% (v/v) dimethyl sulfoxide (Merck).

Biotransformation by *A. niger*: The resulting metabolite 1-(4-hydroxy-3,3-dimethylcyclohexyl)ethanone (2) (170 mg; 31.2% yield), was eluted with 25%, v/v, ethyl acetate in light petroleum (60-80°C) as an oily fragrant compound.

$[\alpha]_D^{20}$: -31.6 (*c* 7.1, Acetone).

FT-IR $\nu_{\max}$ cm^{-1} : 3430 (OH), 1703 (C=O).

^1H NMR (CDCl_3 , 400 MHz): 0.94 (3H, s, 9- CH_3), 1.05 (3H, s, 10- CH_3), 2.16 (3H, s, 8- CH_3), 3.32 (1H, dd, *J* = 3.6, 9.2 Hz, 4- CHOH).

^{13}C NMR: Table 1.

HR-MS $[\text{M}-\text{H}]^+ m/z$ 169.1304 (calcd for $\text{C}_{10}\text{H}_{17}\text{O}_2$, 169.1229).

Biotransformation by *N. crassa*: The metabolite 4-(1-hydroxyethyl)-2,2-dimethylcyclohexanol (3) (83 mg; 15.1% yield), was eluted with 35%, v/v, ethyl acetate in light petroleum (60-80°C) as an oily compound having a characteristic fragrance.

$[\alpha]_D^{20}$: +37.71 (*c* 7, Acetone).

FT-IR $\nu_{\max}$ cm^{-1} : 3422 (OH).

^1H NMR (CDCl_3 , 400 MHz): 0.90 (3H, s, 9- CH_3), 1.03 (3H, s, 10- CH_3), 1.19 and 1.18 (3H, d, *J* = 4.8 Hz, 8- CH_3), 3.32 (1H, dd, *J* = 3.6, 9.6 Hz, 4- CHOH), 3.54 (1H, m, 7- CHOH).

^{13}C NMR: Table 1.

HR-MS: m/z 172.0789 (calcd for $\text{C}_{10}\text{H}_{20}\text{O}_2$, 172.1463).

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