Microbial Transformation of Mestranol by *Cunninghamella elegans*

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The microbial transformation of an oral contraceptive, mestranol (1) by *Cunninghamella elegans* yielded two hydroxylated metabolites, 6β-hydroxymestranol (2) and 6β,12β-dihydroxymestranol (3). Metabolite 3 was found to be a new compound. These metabolites were structurally characterized on the basis of spectroscopic techniques.

Key words mestranol; 17β-methoxymestranol; microbial transformation; *Cunninghamella elegans*

The importance of mestranol (**1**) as an oral contraceptive is well established. It is widely used in combination with other estrogens and progestogens in antifertility medicines.[1] Mestranol (**1**) and 17β-methoxymestranol (**4**) are the mono- and di-*O*-alkylated derivatives of 17α-ethenylestradiol (**5**), respectively.

During our earlier study, the biotransformation of compound **5** with *Cunninghamella elegans* afforded five transformed products, with hydroxylations at C-4, C-7β, C-11α, C-6β, and methoxylation at C-6β.[2]

In continuation of our biotransformational studies on bioactive compounds,[2—14] and in order to study the effects of substitution on metabolism, compounds **1** and **4** were now incubated with *Cunninghamella elegans*. The 3-*O*-methylated derivative of compound **5**, *i.e.* compound **1**, afforded two hydroxylated metabolites, 6β-hydroxymestranol (**2**) and 6β,12β-dihydroxymestranol (**3**). The C-3 methoxy group in compound **1** has apparently reduced the number of transformations and introduced hydroxylations only at C-6β and C-12β, resulting in two transformed products, **2** and **3**. Compound **4** (3,17-*O*-dimethylated derivative of compound **5**) remained unchanged, indicating that the methoxy substitutions at C-3 and C-17 successively reduce the number of transformed products, in comparison to compound **5** (Table 1).

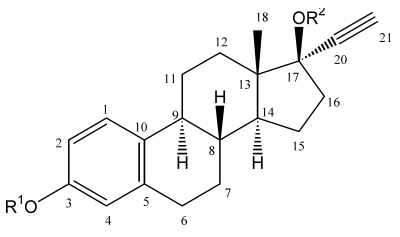
Results and Discussion

Screening scale experiments have shown that *Cunninghamella elegans* is capable of converting compound **1** into polar metabolites. Preparative scale fermentation was thus carried out to produce the sufficient quantities of metabolites for structural elucidation. The structures of known metabolites were identified through comparison of their reported data, while the structure of new metabolite was deduced through comparative spectroscopic studies with the substrate **1**.

Compound **2** was isolated as a crystalline solid and identified as 6β-hydroxymestranol.[15]

The HR-EI-MS of compound **3** exhibited the M^+ at *m*/*z* 342.1394 corresponding to the formula $C_{21}H_{26}O_4$, 32 a.m.u. greater than the parent compound **1**. The IR spectrum exhibited absorption at 3423 cm^{-1}, indicating the presence of a hydroxyl group. The ^{1}H-NMR spectrum of compound **3** showed two additional downfield signals at δ 4.32 (dd, $J_{12ax,11ax}$=11.2 Hz, $J_{12ax,11eq}$=4.6 Hz) and 4.78 (t, J=2.1 Hz, H-6), indicating this to be a dihydroxy derivative of substrate **1**. Furthermore, it showed a downfield shift of an H-4 signal at δ 6.91 (d, $J_{4,2}$=2.7 Hz) compared to substrate **1** (δ 6.58, d, $J_{4,2}$=2.6 Hz), and indicated that the newly introduced hydroxyl group may present at C-6. The ^{13}C-NMR spectrum (Broad-band decoupled and DEPT) showed the presence of 21 carbons, including nine methine, four methylene, two methyl and six quaternary carbons. Two methine carbons appeared at δ 74.8 (C-12) and 68.2 (C-6). A hydroxyl group at C-12 caused a significant γ-upfield shift at the C-18 methyl carbon which appeared at δ 7.38 (Table 2). The COSY 45° spectrum showed couplings between H-6 (δ 4.78) and C-7 methylene protons (δ 2.13, 1.97), and allylic coupling between H-6 and H-4 (δ 6.89). The H-12 showed interactions with H_2-11 (δ 2.48, 2.21). The HMBC spectrum showed heteronuclear interactions of H-6 (δ 4.78) with C-5 (δ 139.0), C-l0 (δ 131.4) and C-8 (δ 32.8), while H-12 showed interactions with C-14 (δ 47.8) and C-9 (δ 42.4), further supporting the position of hydroxyl groups at C-6 and C-12. The splitting pattern of H-6 signal suggested a β (axial) configuration of its geminal hydroxyl group and was found to be similar to compound **2**. The large coupling constants of the H-12 methine signal (δ 4.32, dd, $J_{12ax,11ax}$=11.2 Hz, $J_{12ax,11eq}$=4.6 Hz) indicated a β (equatorial) configuration of its geminal hydroxyl group. Compound **3** may be formed by the C-12 β-hydroxylation of compound **2** (Chart 1).

Table 1. Substitution Effects on the Metabolisms of Compounds **1**, **4** and **5** by *Cunninghamella elegans*



Substrate	Number of transformed products	Transformed products
R^1=R^2=H (**5**)	Five	See ref. 2
R^1=Me, R^2=H (**1**)	Two	6β-Hydroxymestranol (**2**) 6β,12β-Dihydroxymestranol (**3**)
R^1=R^2=Me (**4**)	None	—

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Incubation of 17β-methoxymestranol (**4**) with *Cunninghamella elegans* for 12 d has not afforded any transformed product (Table 1).

Experimental

General Experimental Procedure The IR spectra were recorded in $CHCl_3$ on an FTIR-8900 spectrophotometer. Melting points were determined on a Buchi 535 melting point apparatus. Optical rotations were measured on a Jasco DIP-360 digital polarimeter. UV Spectra were recorded in $CHCl_3$ on a Hitachi U-3200 spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ solutions on Bruker Avance-400 NMR at 400 and 100 MHz, respectively. The EI and HR-EI-MS were measured on a Jeol JMS-600H mass spectrometer. TLC was performed with precoated plates (Silica gel 60, PF_{254}, 0.2 mm, E. Merck). Compounds **1** and **4** were synthesized from compound **5**, while compound **5** was isolated from *Primolut N*, a patent product of Schering AG, Berlin, Germany.

Preparation of Compounds 1 and 4 To 500 mg (1.61 mmol) of compound **5** in 30 ml THF (dried), was added 64.4 mg (1.61 mmol) NaH (60% dispersion in oil), followed by the addition of methyl iodide 0.1 ml (1.61 mmol). The mixture was protected from the atmosphere with a drying-tube, and was stirred at room temperature for 2 h. The solvent was evaporated, the residues were distributed between 20 ml of water and 100 ml of CH_2Cl_2, and the CH_2Cl_2 layer was washed twice with water, dried over Na_2SO_4 and evaporated to obtain compound **1** (413 mg, 82.6%). The single-crystal X-ray data and synthesis of compound **4** was earlier reported by us.[16]

Table 2. ^{13}C-NMR Spectral Data of Compounds **1**—**3**, and ^{1}H-NMR Data of New Metabolite **3** in $CDCl_3$

Position	1	2	3	
	δ_C	δ_C	δ_C	δ_H (J in Hz)
1	126.1, d	126.5, d	126.3, d	7.18, d (8.5)
2	114.2,[a] d	114.4,[a] d	114.4,[a] d	6.74, dd (8.5, 2.8)
3	158.2, s	158.0, s	158.3, s	—
4	114.5,[a] d	114.2,[a] d	114.2,[a] d	6.89, d (2.8)
5	137.8, s	139.0, s	139.0, s	—
6	29.0, t	67.7, d	68.2, d	4.78, t (2.1)
7	27.2, t	34.8, t	34.6, t	2.13, m; 1.97, m
8	39.2, d	32.6, d	32.8, d	1.78, m
9	42.8, d	42.6, d	42.4, d	2.25, m
10	131.3, s	131.5, s	131.4, s	—
11	27.8, t	27.9, t	38.5, t	2.48, dt (4.3, 8.9); 2.21 m
12	33.1, t	34.0, t	74.8, d	4.32, dd (11.2, 4.6)
13	44.3, s	44.0, s	48.4, s	—
14	47.2, d	47.8, d	47.8, d	1.69, m
15	22.1, t	22.5, t	22.6, t	1.81, m; 1.58, m
16	39.0, t	38.9, t	38.9, t	2.14, m; 1.52, m
17	79.5, s	79.8, s	79.9, s	—
18	12.4, q	12.7, q	7.38, q	0.85, s
20	87.6, s	87.4, s	86.9, s	—
21	74.4, d	74.0, d	74.4, d	2.58, s
OCH_3	55.5, q	55.8, q	55.3, q	3.43, s

Carbon multiplicities were determined by DEPT experiments; s=quaternary, d=methine, t=methylene, q=methyl carbons. *a*) May be exchangeable.

Organism and Media Microbial cultures were obtained from the Northern Regional Research Laboratories (NRRL). Cultures were maintained on SDA and stored at 4 °C prior to use. The medium for *Cunninghamella elegans* (NRRL 1392, kingdom: fungi, Phylum: Zygomycota, Class: Zygomycetes, Family: Cunninghamellaceae: Genus: *Cunninghamella*) was prepared by mixing the following ingredients into distilled H_2O (3.0 : 1): glucose (30.0 g), peptone (15.0 g), yeast extract (15.0 g), KH_2PO_4 (15.0 g), and NaCI (15.0 g).

General Fermentation and Extraction Protocol The fermentation media was distributed among 30 flasks of 250 ml capacity (100 ml in each), which were autoclaved. The fermentation was carried out according to a standard two-stage protocol.[17] Substrates were dissolved in DMSO and were evenly distributed into 30 flasks (20 mg/0.5 ml in each flask) containing 24 h-old stage II cultures. Fermentation was continued for additional 12 d on a rotatory shaker (200 rpm) at 29 °C. During the fermentation period, aliquots from flasks were drawn daily and analyzed by TLC in order to determine the degree of transformation of the substrate. In all experiments, one control flask without biomass (for checking substrate stability) and one flask without substrate (for checking the endogenous metabolite) were used. The culture media and mycelium were separated by filtration. The mycelium were washed with CH_2Cl_2 (1 l) and the filtrate was extracted with CH_2Cl_2 (3×1.5 l). The combined organic extract was washed with brine and dried over anhydrous Na_2SO_4, evaporated under reduced pressure, and analyzed by thin layer chromatography. Control flasks were also harvested and checked by TLC to detect the bio-transformed products.

Fermentation of Mestranol (1) with *Cunninghamella elegans* (NRRL 1392) Compound **1** (600 mg) was dissolved in DMSO (15 ml) and distributed among 30 flasks containing stage II cultures. Fermentation was continued for 12 d. Culture filtrates were extracted with CH_2Cl_2. The resulting organic extract was dried to afford a brown gum (3.5 g). The crude extract was subjected to column chromatography (silica gel). Elution with a gradient of petroleum ether and ethyl acetate afforded unchanged substrate **1** (121 mg, petroleum ether–EtOAc, 81 : 19), and metabolites **2** (16.7 mg, petroleum ether–EtOAc, 76 : 24) and **3** (21.7 mg, petroleum ether–EtOAc, 71 : 29).

6β-Hydroxymestranol (**2**): Colorless crystalline solid, mp 181—182 °C. $[\alpha]_D^{25}$ −107° (c=0.1, MeOH). IR ($CHCl_3$) ν_{max}: 3421, 2927 and 2869 cm^{-1}. UV ($CHCl_3$) λ_{max} nm (log ε): 202 (2.9). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.24 (1H, d, $J_{1,2}$=8.5 Hz, H-1), 6.82 (1H, dd, $J_{2,1}$=8.5 Hz, $J_{2,4}$=2.9 Hz, H-2), 6.89 (1H, d, $J_{4,2}$=2.9 Hz, H-4), 4.76 (1H, t, J=2.2 Hz, H-6), 0.89 (3H, s, Me-18), 2.58 (1H, s, H-21), 3.79 (3H, s, OMe). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: Table 2. MS (EI, 70 eV): m/z (%): 326 (12) [M$^+$], 308 (7), 273 (5), 243 (16), 225 (54), 197 (18), 171 (56), 128 (33), 115 (56), 91 (53), 83 (100). HR-EI-MS m/z: 326.1739 (Calcd for $C_{21}H_{26}O_3$: 326.1766).

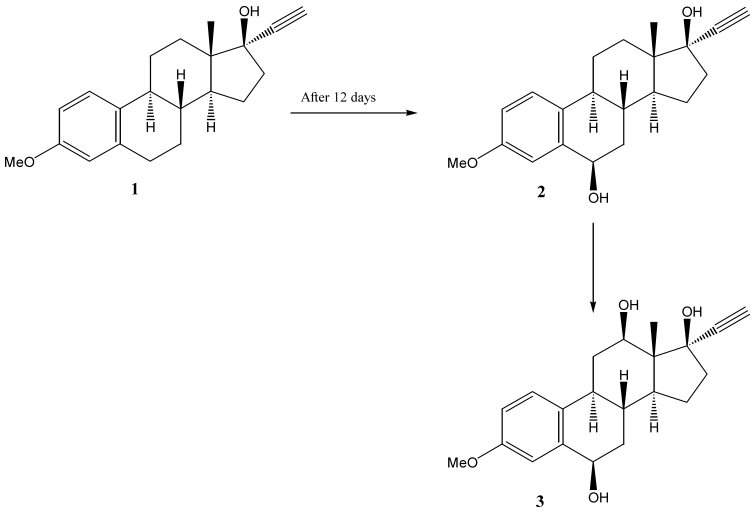

Chart 1. Metabolism of Compound **1** by *Cunninghamella elegans*

6β,12β-Dihydroxymestranol (**3**): Colorless crystalline solid, mp 204—205 °C. $[\alpha]_D^{25}$ −71° (c=0.2, MeOH). IR ($CHCl_3$) ν_{max}: 3423, 2974, 2816 cm^{-1}. UV ($CHCl_3$) λ_{max} nm (log ε): 205 (3.0). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: Table 2. ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: Table 2. MS (EI, 70 eV) m/z (%): 342 (12) [M^+], 324 (18), 243 (8), 239 (33), 223 (7), 171 (100), 149 (84), 115 (63), 91 (77), 41 (69). HR-EI-MS m/z: 342.1394 ($C_{21}H_{26}O_4$, Calcd 342.1321).

Fermentation of 17β-Methoxymestranol (4) with *Cunninghamella elegans* (NRRL 1392) Compound **4** was fermented in the same way as compound **1**, and afforded an organic extract (2.9 g) which gave an unchanged compound **4** (512 mg, petroleum ether–EtOAc, 90 : 10).

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