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Biotransformation of (20*S*)-20-hydroxymethylpregna-1,4-dien-3-one by four filamentous fungi

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ABSTRACT

Microbial transformation of (20*S*)-20-hydroxymethylpregna-1,4-dien-3-one (**1**) by four filamentous fungi, *Cunninghamella elegans*, *Macrophomina phaseolina*, *Rhizopus stolonifer*, and *Gibberella fujikuroi*, afforded nine new, and two known metabolites **2**–**12**. The structures of these metabolites were characterized through detailed spectroscopic analysis. These metabolites were obtained as a result of biohydroxylation of **1** at C-6β, -7β, -11α, -14α, -15β, -16β, and -17α positions, except metabolite **2** which contain an *O*-acetyl group at C-22. These fungal strains demonstrated to be efficient biocatalysts for 11α-hydroxylation. Compound **1**, and its metabolites were evaluated for the first time for their cytotoxicity against the HeLa cancer cell lines, and some interesting results were obtained.

1. Introduction

Steroidal compounds are reported to have numerous physiological activities, depending on the functional groups present in their skeleton [1]. Structural transformation of steroidal compounds through microorganisms has emerged as an important application in steroidal drug industry [1–3]. Microbial conversions of steroids generally involve dehydrogenation, hydroxylation, esterification, halogenation, isomerization, methoxylation, and side-chain modification of steroidal skeleton [1,4–7]. Extensive investigations have been carried out to get mechanistic insight about the bioconversion process catalyzed by microorganisms. Several studies have also been focused on the use of microorganisms as an *in vitro* model of mammalian drug metabolism [8,9].

(20*S*)-20-Hydroxymethylpregna-1,4-dien-3-one (**1**) is a neurochemical, obtained from the degradation of sterols by microorganisms [10]. It is used as an intermediate in steroidal drug synthesis [11], as well as in the synthesis of vitamin D metabolites [12]. Previously few reports on the microbial transformation of **1** with various fungi have been reported [13,14]. In continuation of our biotransformational studies on various bioactive steroids [2,3,15–17], substrate **1** was initially screened for its cytotoxicity against various cancer cell lines. As compound **1** showed a strong cytotoxicity against HeLa cell lines, we decided to synthesize libraries of the lead compound **1**, for SAR studies by employing microbial transformation.

Cytotoxicity of bioactive compounds can be used against several disorders such as AIDS, cancer, infection, inflammation, etc. Through a bioassay-guided isolation strategy, several classes of compounds had been identified with potent cytotoxicity [18,19].

We are reporting here for the first time the metabolism of **1** with *Cunninghamella elegans*, *Macrophomina phaseolina*, *Rhizopus stolonifer*, and *Gibberella fujikuroi*, which resulted in various mono-, di-, and tri-hydroxylated, as well as acetylated derivatives. Metabolites obtained by microbial transformation were screened for their cytotoxic effects against HeLa cell lines, and some interesting results were obtained.

2. Experimental

2.1. General

(20*S*)-20-Hydroxymethylpregna-1,4-dien-3-one (**1**) was purchased from Acros Chemicals (USA). Column chromatography

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was carried out on silica gel (70–230 mesh, Merck). For TLC precoated plates with silica gel (20 × 20, 0.25 mm thick PF_{254}, Merck) were used and stained by spraying with ceric sulfate solution (10% solution in sulfuric acid). JEOL JMS-600H mass spectrometer was used to record the mass spectra (EI- and HREI-MS) in *m/z* (rel.%). A Buchi-535 apparatus was used for determining the melting points. UV spectra (nm) were measured in methanol on a Hitachi U-3200 spectrophotometer. FT-IR-8900 spectrophotometer was used to record IR spectra (cm^{-1}). Optical rotations were measured in chloroform or methanol with a digital polarimeter JASCO P-2000 (JASCO International Co. Ltd., Japan) by using 10 cm cell tube. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance NMR spectrometer at 300–600 and 75–150 MHz in $CDCl_3$ or CD_3OD, respectively. Standard pulse sequences were used for DEPT, and 2D-NMR experiments. Recycling preparative HPLC separation was performed on a JAI LC-908W instrument, equipped with YMC L-80 (4–5 μm, 20 × 250 mm i.d.) using MeOH–H_2O (2:1) as mobile phase, and UV detection at 254 nm.

2.2. Microorganisms and culture conditions

Fungal cultures of *C. elegans* (TSY-0865), *M. phaseolina* (KUCC-730), *R. stolonifer* (TSY-0471), and *G. fujikuroi* (ATCC-10704) were grown on Sabouraud dextrose-agar at 25 °C and stored at 4 °C. Glucose (50.0 g), glycerol (50.0 mL), peptone (25.0 g), yeast extract (25.0 g), KH_2PO_4 (25.0 g), and NaCl (25.0 g) were mixed into distilled H_2O (5.0 L) to prepare the media for *C. elegans* and *M. phaseolina*.

The medium for *R. stolonifer* was prepared by adding glucose (100.0 g), peptone (25.0 g), yeast extract (25.0 g), and KH_2PO_4 (25.0 g) to distilled H_2O (5.0 L), maintaining the pH at 5.6. Glucose (80.0 g), KH_2PO_4 (5.0 g), $MgSO_4{\cdot}2H_2O$ (1.0 g), NH_4NO_3 (0.5 g), and "*Gibberella* trace-element" solution (2 mL) were added into distilled H_2O (5.0 L) to make the medium of *G. fujikuroi*. The solution of *Gibberella* trace-element was prepared by mixing $Co(NO_3)_2{\cdot}6H_2O$ (0.01 g), $FeSO_4{\cdot}7H_2O$ (0.1 g), $CuSO_4{\cdot}5H_2O$ (0.1 g), $ZnSO_4{\cdot}7H_2O$ (0.161 g), $MnSO_4{\cdot}4H_2O$ (0.01 g), and $Mo(NH_4)_3$ (0.01 g) in distilled H_2O (100 mL).

2.3. General fermentation and extraction protocol

Spores from the 3-day-old slant were used to make the seed flasks, which were then incubated for 48 h on rotary shaker at 25 °C. Aliquots (5 mL) from the seed flasks were transferred to the remaining flasks and incubated on rotary shaker (128 rpm) at 25 °C. After 2 days, compound **1** in acetone was evenly distributed among 40–60 flasks, and fermentation was continued on rotary shaker (128 rpm) at 25 °C. An incubation of the fungus without sample **1**, and an incubation of **1** in the medium without fungus were also conducted as parallel control experiments. Time course study was carried out after 2 days, and the degree of transformation was analyzed periodically on TLC. After 10–14 days, the culture medium was filtered, and extracted with dichloromethane in three portions. The extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressures, and the resulting brown gum was analyzed by thin-layer chromatography.

2.4. Fermentation of (20S)-20-hydroxymethylpregna-1,4-dien-3-one with C. elegans (TSY-0865)

Compound **1** (1.5 g/25 mL acetone) was distributed among 50 flasks containing 3-day-old culture of *C. elegans* and kept for fermentation for 12 days. A brown gummy material (3.6 g) obtained after filtration, extraction, and evaporation, was subjected to column chromatography over silica gel for fractionation with increasing polarity of ethyl acetate in pet. ether. Three main fractions (HMCE-1–3) were obtained on the basis of TLC analysis. When subjected to silica gel column chromatography fraction HMCE-1 yielded metabolite **2** (06 mg, EtOAc:pet. ether = 17:83), metabolite **3** (07 mg, EtOAc:pet. ether = 20:80), and metabolite **4** (130 mg, EtOAc:pet. ether 25:75), while fraction HMCE-2 yielded metabolite **5** (09 mg, EtOAc:pet. ether 40:60), and metabolite **6** (30 mg, EtOAc:pet. ether 40:60). For purification fraction HMCE-3 was subjected to repeated RPHPLC with solvent system MeOH:H_2O (2:1) on L-80 column to afford metabolites **7** (06 mg, R_t: 24 min, 4 mL/min), and **8** (06 mg, R_t: 32 min, 3 mL/min).

2.4.1. (20S)-11α-Hydroxy-20-acetoxymethylpregna-1,4-dien-3-one (**2**)

Colorless solid: m.p.: 203–205 °C. $[\alpha]_D^{25}$: +36° (*c* = 0.016, $CHCl_3$). UV (MeOH): λ_{max} nm (log ε) 247 (3.8). IR (KBr); ν_{max}: 3410, 1736, 1658 cm^{-1}. R_f: 0.43 (pet. ether/EtOAc = 9:1). ^1H NMR ($CDCl_3$, 600 MHz): Table 1. ^{13}C NMR ($CDCl_3$, 150 MHz): Table 3. EI-MS: *m/z* 386 [M^+] (10), 265 (11), 173 (8), 147 (29), 135 (20), 134 (27), 133 (12), 122 (100), 121 (82), 91 (25), 69 (22). HREI-MS: *m/z* 386.2464 (M^+, $[C_{24}H_{34}O_4]^+$; calcd 386.2457).

2.4.2. (20S)-17α-Hydroxy-20-hydroxymethylpregna-1,4-dien-3-one (**3**)

Colorless solid: m.p.: 197–198 °C. $[\alpha]_D^{25}$: +32° (*c* = 0.27, $CHCl_3$). UV (MeOH): λ_{max} nm (log ε) 248 (3.3). IR (KBr); ν_{max}: 3348, 2939, 1656, 1610 cm^{-1}. R_f: 0.44 (pet. ether/EtOAc = 8:2). ^1H NMR ($CDCl_3$, 600 MHz): Table 1. ^{13}C NMR ($CDCl_3$, 150 MHz): Table 3. EI-MS: *m/z* 344 [M^+] (20), 327 (23), 326 (10), 225 (11), 223 (32), 209 (13), 167 (66), 161 (24), 122 (100), 95 (32), 91 (37). HREI-MS: *m/z* 344.2319 (M^+, $[C_{22}H_{32}O_3]^+$; calcd 344.2351).

2.4.3. (20S)-11α-Hydroxy-20-hydroxymethylpregna-1,4-dien-3-one (**4**)

Colorless crystalline solid: m.p.: 211–213 °C. $[\alpha]_D^{25}$: +24° (*c* = 0.01, $CHCl_3$). UV (MeOH): λ_{max} nm (log ε) 248 (3.7). IR (KBr); ν_{max}: 3375, 2939, 1656, 1608 cm^{-1}. R_f: 0.45 (Pet. ether/EtOAc = 7.5:2.5). ^1H NMR ($CDCl_3$, 600 MHz): Table 1. ^{13}C NMR ($CDCl_3$, 150 MHz): Table 3. EI-MS: 344 [M^+] (15), 327 (14), 267 (8), 223 (25), 173 (17), 161 (17), 159 (14), 147 (49), 134 (32), 122 (100), 107 (23), 95 (25), 55 (17). HREI-MS: *m/z* 344.2402 (M^+, $[C_{22}H_{32}O_3]^+$; calcd 344.2351).

2.4.4. (20S)-6β,11α-Dihydroxy-20-hydroxymethylpregna-1,4-dien-3-one (**5**)

Colorless solid: m.p.: 197–199 °C. $[\alpha]_D^{25}$: +12° (*c* = 0.02, $CHCl_3$). UV (MeOH): λ_{max} nm (log ε) 248 (3.9). IR (KBr); ν_{max}: 3371, 1657 cm^{-1}. R_f: 0.54 (pet. ether/EtOAc = 6:4). ^1H NMR ($CDCl_3$, 500 MHz): Table 1. ^{13}C NMR ($CDCl_3$, 125 MHz): Table 3. EI-MS: *m/z* 360 [M^+] (12), 342 (6), 324 (2), 314 (4), 283 (6), 241 (2), 224 (6), 222 (8), 202 (2), 173 (10), 167 (24), 150 (17), 147 (40), 145 (24), 137 (99), 122 (28), 109 (100), 105 (33), 95 (48), 91 (50), 79 (67), 55 (87). HREI-MS: *m/z* 360.2320 (M^+, $[C_{22}H_{32}O_4]^+$; calcd 360.2301).

2.4.5. (20S)-11α,15β-Dihydroxy-20-hydroxymethylpregna-1,4-dien-3-one (**6**)

White solid: m.p.: 202–204 °C. $[\alpha]_D^{25}$: +49° (*c* = 0.023, $CHCl_3$). UV (MeOH): λ_{max} nm (log ε) 243 (3.6). IR (KBr); ν_{max}: 3444, 1655 cm^{-1}. R_f: 0.49 (pet. ether/EtOAc = 6:4). ^1H NMR ($CDCl_3$, 600 MHz): Table 1. ^{13}C NMR ($CDCl_3$, 150 MHz): Table 3. EI-MS: *m/z* 360 [M^+] (6), 342 (8), 309 (3), 265 (6), 283 (3), 239 (8), 225 (4), 189 (8), 173 (10), 161 (13), 159 (14), 154 (80), 147 (30), 137 (21), 134 (63), 122 (60), 121 (100), 119 (20), 109 (21), 95 (22), 91 (40), 81