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Biotransformation of danazol by *Fusarium solani* and *Gibberella fujikuorii*, and prolyl endopeptidase inhibition studies of transformed products

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Biotransformation of danazol (17β-hydroxy-17α-pregna-2,4-dien-20-yno-[2,3-*d*] isoxazole) (**1**) on fermentation with *Fusarium solani* yielded 17β-hydroxy-2-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (**2**) and 17β-hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (**3**), while the fermentation of **1** with *Gibberella fujikuorii* yielded compound **2** only. The structures of these compounds were deduced on the basis of modern spectroscopic techniques. Prolyl endopeptidase inhibition activities of danazol (**1**) and its transformed products **2** and **3** are also studied.

Key Words: Biotransformation, danazol, *Fusarium solani*, *Gibberella fujikuorii*, prolyl endopeptidase (PEP) inhibition assay

Introduction

Biotransformations of steroids exhibit a vast biochemical potential for the synthesis of new secondary metabolites. Various kinds of steroid modifications, such as hydroxylation, epoxidation, dehydrogenation, double bond formation, oxidation,[1,2] reduction, glycosylation, isomerization, hydrolysis, and acetylation[3,4] are now routinely performed at the industrial level using a wide variety of microorganisms. Through these microbial reactions, many novel intermediates for the synthesis of new steroidal pharmaceuticals have become available.

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Danazol (**1**) is a heterocyclic compound with an isoxazole ring fused to ring-A of a steroidal nucleus. It is an orally effective, pituitary gonadotropin inhibitor, and is effectively used in the treatment of endometriosis, benign fibrocystic mastitis, and precocious puberty.[5]

Prolyl endopeptidase (PEP) or post-proline cleaving enzyme is now called "proly oligopeptidase" (EC. 3.4.21.26). It catalyzes the cleavage of the peptide bonds at the carbonyl side of proline residue.[6,7] This enzyme is widely distributed in various organs, particularly in the human brain. PEP activity has been reported to be significantly higher in Alzheimer's patients than in normal persons. In addition, the abnormal PEP levels may be related to neuropathological disorders, such as major depression, mania, schizophrenia, and senile dementia of Alzheimer's type. PEP also plays a key role in the regulation of blood pressure by participating in the rennin-angiotensin system through metabolism of bradykinin and angitensins II and I. Thus specific inhibitors of PEP are expected to have anti-amnesic and other effects. Many PEP inhibitors have been synthesized as candidates for the treatment of neuropathological disorders.

In continuation of our studies on biotransformation of bioactive compounds,[8–10] danazol (**1**) was incubated with *Fusarium solani* and *Gibberella fujikuorii.* Fermentation of danazol (**1**) with *Fusarium solani* yielded compounds **2** and **3**. Fermentation of **1** with *Gibberella fujikuorii* yielded only **2** (Table 1). They were reported for the first time from these fungal strains. The structures of compounds **2** and **3** were established with the help of spectroscopic studies and reported data.[8] In the present study, we were also interested in examining the prolyl endopeptidase inhibition activities of danazol (**1**) and its transformed products **2** and **3**.

Table 1. % Yields of metabolites of danazol (**1**) by fermentation with *Fusarium solani* and *Gibberella fujikuorii* after 12 days.

Microorganisms	% Yield of 2	% Yield of 3
Fusarium solani	10.8	9.6
Gibberella fujikuorii	12.6	-

Experimental

General Methods: The ^{1}H-NMR spectrum was recorded in $CDCl_3$ on a Bruker AM-400 NMR spectrometer with TMS as an internal standard using the UNIX operating system at 400 MHz. The ^{13}C-NMR spectra were recorded in $CDCl_3$ at 100 MHz on a Bruker AM-400 NMR spectrometer. The HREIMS was recorded on Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000. The IR spectra were recorded on a Jasco A-302 spectrophotometer. The UV spectra were recorded on a Hitachi U-3200 spectrophotometer. The optical rotations were measured on a JASCO DIP-360 digital polarimeter. The melting point was determined on a Buchi 510 apparatus. Column chromatography (CC) was carried on a silica gel column (70-230 mesh). Purity of the samples was checked by TLC on pre-coated silica gel GF-254 preparative plates (20 × 20 cm, 0.25 mm thick, Merck), detected under UV light (254 and 366 nm), while ceric sulfate was used as the spraying reagent. Danazol (**1**) was purified from tablets of Danocrine® (200 mg each, Searle Pharmaceutical Company). Prolyl endopeptidase (*Flavobacterium meningosepticum* origin) was purchased from Seikagaku Corporation (Tokyo, Japan), *N*-benzyloxycarbonyl-Gly-Pro-*p*NA was procured from Bachem Fine Chemicals Co., and bacitracin was purchased from Sigma Co., Ltd. Dr. Hideaki Shimizu (Yakult Central Institute for Microbiological Research,

Tokyo, Japan) kindly gifted a specific inhibitor of PEP, *N*-benzyloxycarbonyl-pro-prolinal.

Preparation of Fermentation Media: Two liters of medium for *F. solani* (ATCC 12823) was prepared by mixing glucose (20 g), peptone (10 g), KH_2PO_4 (10 g), yeast extract (10 g), glycerol (20 mL), and NaCl (10 g). Medium of *G. fujikuorii* (ATCC 10704) was prepared by mixing glucose (160 g), NH_4NO_3 (1 g), KH_2PO_4 (10 g), $MgSO_4.7H_2O$ (2 g), and Giberella trace element solution (4 mL) into distilled water (2 L). The fermentation medium thus obtained was distributed equally among 20 flasks of 250 mL capacity (100 mL in each) and autoclaved.

Cultivation of the Microbes: Two-day-old spores of the both microbes were transferred into the broth medium flasks (250 mL) of their respective media containing freshly prepared and autoclaved media (100 mL). The seed flasks of the fungi were incubated on a shake table at 30 °C for 2 days.

Inoculation of the Cultures: Broth cultures (100 mL) from 2-day-old seed flasks of the fungi were equally distributed to 18 media flasks (250 mL) containing the respective media (100 mL). The incubation was continued for a further 2 days for fungi.

Fermentation of Danazol (1): Danazol (**1**) (200 mg) was dissolved in DMSO (10 mL) and the resulting solution was evenly distributed among 18 conical flasks containing shake cultures of microbes, and the fermentation was continued for 12 days.

Extraction and Isolation: Each mycelium was filtered, washed with EtOAc (500 mL), and the broth thus obtained was extracted with EtOAc (6 L). The ethyl extract was dried over anhydrous sodium sulfate and concentrated in vacuo to afford a brown gum (approximately, 1 g for each fungi), which was adsorbed on flash silica gel (3 g), subjected to column chromatography. Elution with EtOAc:pet. ether (5:5) afforded **2** and EtOAc:pet. ether (6:4) afforded **3**.

Prolyl Endopeptidase Inhibition Assay: PEP inhibition activity was assayed by a modified method of Yoshimoto et al.;[11] 100 mM *Tris* (hydroxymethyl)-aminomethane HCl buffer containing 1 mM EDTA (pH 7.0, 247 μL), PEP (0.02 units/well) 15 μL, and a stock solution of the test compound in MeOH (8 μL, diluted to the desired range of concentrations) were mixed in 96-well microplate and preincubated for 10 min at 30 °C. The reaction was initiated by adding 30 μL of 0.2 mM of *N*-benzyloxycarbonyl-Gly-Pro-*p*NA (in 40% 1,4-dioxane) as the substrate. The amount of released *p*-nitroaniline was measured spectrophotometrically, as increase in absorption at 410 nm with a 96-well microplate reader (Molecular Devices, SpectraMax 340, USA). The percentage inhibition was calculated by the following equation:

$$\% \text{ Inhibition} = 100 - [(\text{O.D. of test compound / O.D. of control}) \times 100]$$

The potency of enzyme inhibitory activity was represented by the IC_{50} values, which were defined as the concentration of the test compound that resulted in 50% inhibition of the enzyme with respect to the MeOH control. *Z*-pro-prolinal was used as a positive control.

Results

17β-Hydroxy-2-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (2): Colorless crystalline solid, mp 164-165 °C, $[\alpha]_D^{25}$ –20° (*c* 0.1, $CHCl_3$). UV (MeOH) λ_{max} (log ε): 242 nm (4.16). IR ($CHCI_3$)ν_{max}: 3412 (OH), 3308 (OH), 1654 (C=O), 1620 cm^{-1} (C=C). FDMS *m/z* 342 [M^+, 100%]. EIMS *m/z* (rel. int. %): 342

[M$^+$] (100), 327 (26), 275 (47), 189 (17), 121 (89), 91 (65). HREIMS m/z342.2204 (M^+, calcd 342.2195 for $C_{22}H_{30}O_3$). ^{1}H- ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data listed in Tables 2 and 3, respectively.

Table 2. ^{1}H-NMR (400 MHz, $CDCl_3$)[a]) chemical shifts of danazol (**1**) and its metabolites **2** and **3**. δ in ppm and J in Hz.

C. NO.	1	2	3
1	2.50 (d, J = 15.7) 2.72 (d, J = 15.7)	1.52 (m) 1.90 (m)	6.98 (s)
2	-	2.56 (m)	-
3	-	-	-
4	6.17 (d, J = 1.9)	5.71 (d, J = 1.7)	6.07 (d, J = 1.2)
5	-	-	-
6	2.38 (m); 2.43 (m)	2.37 (m); 2.41 (m)	2.37 (m); 2.41 (m)
7	1.04 (m); 1.78 (m)	1.05 (m); 1.81 (m)	1.06 (m); 1.84 (m)
8	1.50 (m)	1.50 (m)	1.50 (m)
9	1.18 (m)	1.15 (m)	1.15 (m)
10	-	-	-
11	1.62 (m); 1.47 (m)	1.62 (m); 1.47 (m)	1.62 (m); 1.47 (m)
12	1.76 (m); 1.67 (m)	1.74 (m); 1.67 (m)	1.72 (m); 1.67 (m)
13	-	-	-
14	1.53 (m)	1.53 (m)	1.53 (m)
15	1.74 (m); 1.36 (m)	1.74 (m); 1.36 (m)	1.74 (m); 1.36 (m)
16	2.30 (ddd, J = 13.9, 9.6, 5.5) 2.00 (ddd, J = 13.9, 11.9, 4.0)	2.30 (ddd, J = 13.9, 9.6, 5.5) 1.98 (ddd, J = 13.9, 11.9, 4.0)	2.28 (ddd, J = 13.9, 9.6, 5.5) 1.98 (ddd, J = 13.9, 11.9, 4.0)
17	-	-	-
18	0.90 (s)	0.88 (s)	0.92 (s)
19	1.02 (s)	1.23 (s)	1.24 (s)
20	-	-	-
21	2.57 (s)	2.55 (s)	2.53 (s)
22	8.00 (s)	3.70 (dd, J = 14.6, 11.3) 3.72 (dd, J = 18.0, 11.3)	4.34 (d, J = 13.2) 4.36 (d, J = 13.2)

[a]Assignments based on COSY and HMQC.

17β-Hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (3): Colorless solid, mp 214-215 °C; $[\alpha]_D^{25}$ -54° (c1.0, $CHCl_3$). UV (MeOH) λ_{max} (log ε): 248 nm (4.22). IR ($CHCl_3$)ν_{max}: 3425 (OH), 3314 (OH), 1667 (C=O), 1622 (C=C), 1608 cm^{-1} (C=C). EIMS m/z (rel. int. %), 340 [M$^+$] (26), 239 (7), 173 (10), 134 (100), 121 (40), 91 (29), 55 (12). HREIMS m/z 340.2102 (M^+, calcd 340.2038 for $C_{22}H_{28}O_3$). ^{1}H- ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data listed in Tables 2 and 3, respectively.

Table 3. ^{13}C-NMR (100 MHz, $CDCl_3$)[a])[b]) chemical shifts of danazol (**1**) and its metabolites **2** and **3**.

C. NO.	**1**	**2**	**3**
1	32.9 (CH_2)	38.9 (CH_2)	151.5 (CH)
2	107.5 (C)	43.7 (CH)	135.8 (C)
3	164.9 (C)	202.6 (C)	187.0 (C)
4	108.1 (CH)	123.6 (CH)	123.7 (CH)
5	155.1 (C)	171.8 (C)	169.9 (C)
6	32.0 (CH_2)	32.4 (CH_2)	32.6 (CH_2)
7	30.4 (CH_2)	31.4 (CH_2)	32.4 (CH_2)
8	36.7 (CH)	36.2 (CH)	36.2 CH)
9	53.7 (CH)	53.9 (CH)	52.2 (CH)
10	40.8 (C)	39.3 (C)	43.6 (C)
11	21.0 (CH_2)	20.7 (CH_2)	22.7 (CH_2)
12	32.9 (CH_2)	32.6 (CH_2)	33.2 (CH_2)
13	46.4 (C)	46.7 (C)	47.0 (C)
14	49.5 (CH)	49.9 (CH)	49.6 (CH)
15	22.9 (CH_2)	23.1 (CH_2)	23.2 (CH_2)
16	38.4 (CH_2)	38.9 (CH_2)	38.9 (CH_2)
17	78.9 (C)	79.6 (C)	79.6 (C)
18	12.3 (CH_3)	12.7 (CH_3)	12.8 (CH_3)
19	18.4 (CH_3)	17.7 (CH_3)	18.8 (CH_3)
20	73.2 (C)	74.2 (C)	74.3 (C)
21	87.2 (CH)	87.3 (CH)	87.1 (CH)
22	148.3 (CH)	63.8 (CH_2)	62.5 (CH_2)

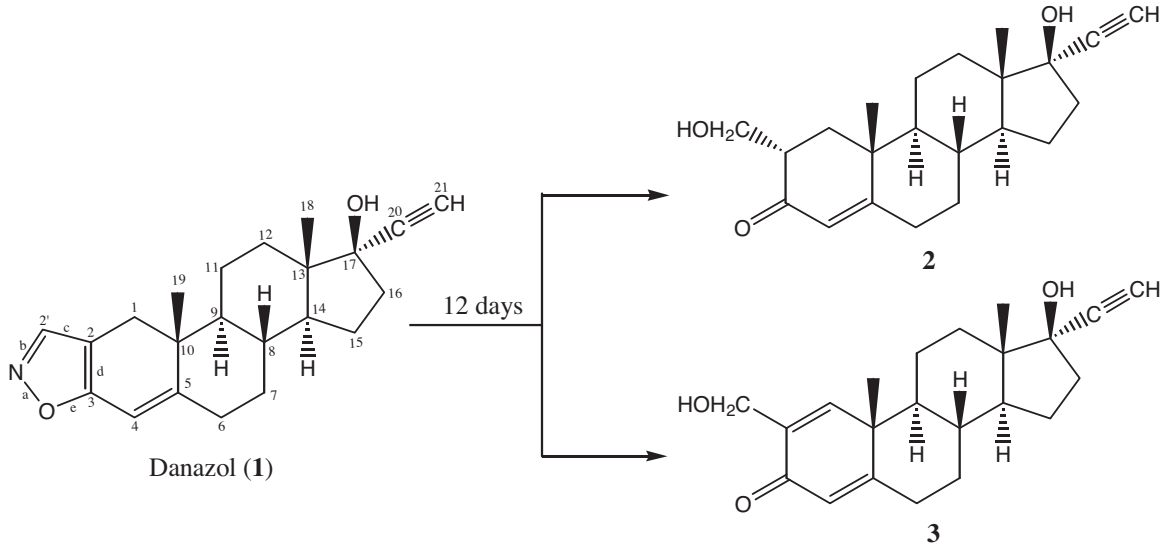
[a]Multiplicities were determined by DEPT experiments.

[b]Assignment based on HMQC and HMBC.

Discussion

Biotransformation of danazol (**1**) using *Fusarium solani* and *Gibberella fujikuorii* yielded compounds **2** and **3** (Scheme). The structures of these compounds were identified on the basis of modern spectroscopic methods and comparison with reported data. They were found to be 17β-hydroxy-2-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (**2**)[8] and 17β-hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (**3**).[8] They were reported for the first time from these fungal strains.

Transformed products **2** and **3** of danazol (**1**) were screened for their enzyme inhibitory activity against prolyl endopeptidase (PEP). These compounds **1-3** displayed inhibitory potential against the PEP enzyme. Danazol (**1**) showed the most potent inhibition against the PEP amongst all the compounds, while its metabolic derivatives **2** and **3** were also found to be significant inhibitors but showing lesser potency than danazol (**1**). The activities of compounds **1-3** are shown in Table 4.



Scheme. Biotransformation of danazol (**1**) by fermentation with *F. solani* and *G. fujikuorii* after 12 days.

Table 4. Prolyl endopeptidase (PEP) inhibition activities by compounds **1**-**3**.

Compounds	IC_{50} (μM) ± S. E. M.
1	57.4 ± 0.002
2	379.3 ± 0.00081
3	827.7 ± 0.04
Z-pro-prolinal *	880 ± 0.001

IC_{50} = Concentration of a test compound

S. E. M. = Standard error of the mean of 5 assays

* = Standard inhibitor used for the inhibition of prolyl endopeptidase

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