



## Microbial transformation of danazol with *Cunninghamella blakesleeana* and anti-cancer activity of danazol and its transformed products



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### ABSTRACT

Biotransformation of danazol (**1**) (17 $\beta$ -hydroxy-17 $\alpha$ -pregna-2,4-dien-20-yno-[2,3-d]-isoxazole) with *Cunninghamella blakesleeana* yielded three new metabolites **2–4** and a known metabolite **5**. These metabolites were identified as 14 $\beta$ ,17 $\beta$ -dihydroxy-2-(hydroxymethyl)-17 $\alpha$ -pregn-4-en-20-yn-3-one (**2**), 1 $\alpha$ ,17 $\beta$ -dihydroxy-17 $\alpha$ -pregna-2,4-dien-20-yno-[2,3-d]-isoxazole (**3**), 6 $\beta$ ,17 $\beta$ -dihydroxy-17 $\alpha$ -pregna-2,4-dien-20-yno-[2,3-d]-isoxazole (**4**), and 17 $\beta$ -hydroxy-2-(hydroxymethyl)-17 $\alpha$ -pregn-1,4-dien-20-yn-3-one (**5**). Danazol (**1**) and its derivatives were evaluated against cervical cancer cell line (HeLa). Compound **1** showed a potent cytotoxicity with IC<sub>50</sub> = 0.283  $\pm$  0.013  $\mu$ M, as compared to doxorubicin (IC<sub>50</sub> = 0.506  $\pm$  0.015  $\mu$ M), where compound **3** was also found to be significantly active with IC<sub>50</sub> = 13.427  $\pm$  0.819  $\mu$ M.

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### 1. Introduction

Biotransformation is an important tool for the synthesis of medicinally important organic compounds. The use of biocatalysis for the stereoselective synthesis of chiral molecules has several advantages over classical chemical synthesis. Biocatalyzed reactions are generally regio- and stereo-selective, cost effective and environmental friendly [1–6]. Microbial cultures are frequently used for biocatalysis. Microorganisms contain a large variety of enzymes which catalyze many chemical reactions, including oxidation, hydroxylation, and reduction. Fungi can also serve as microbial models of mammalian steroid drug metabolism. Microbial cytochrome P450 monooxygenase system facilitates stereoselective hydroxylation at multiple sites of steroidal skeleton. Stereoselective hydroxylation of steroids at non activated position have been achieved by using various fungi [7–9].

Danazol (**1**) is a heterocyclic steroidal drug in which an isoxazole ring is fused with ring-A of a steroidal nucleus. It is a synthetic

analogue of 17 $\alpha$ -ethinyltestosterone. Danazol (**1**) inhibits endometrial tumor cell migration and invasive activity [10]. Compound **1** is orally effective, and used for the treatment of endometriosis, and for the inhibition of pituitary gonadotropin. It is also used in the treatment of precocious puberty, and benign fibrocystic mastitis [11–14].

Cancer is a major cause of human mortality. Many current chemotherapeutic agents against cancers, such as doxorubicin, function by highly toxic, untargeted DNA damage mechanisms. Their efficacy is now seriously challenged by emerging multidrug-resistant in cancer cells [15–17]. However, hormonal therapies targeting cancer do not rely on DNA damage. The HeLa cell line is derived from a human cervical cancer and is a common model to study preliminary anti-cancer potential of chemical compounds [18]. Initially, we evaluated danazol (**1**) against the HeLa cell line, where it showed an excellent cytotoxic effect. Therefore, we decided to evaluate the transformed products of **1** against this cell line. Interestingly, only compound **3** showed some level of cytotoxicity against HeLa cell line.

This manuscript is in the continuation of our studies on the biotransformation of bioactive steroidal compounds [19–23]. We subjected danazol (**1**) to biotransformation with *C. blakesleeana*, which yielded three new metabolites **2–4**, and a known metabolite **5** (Fig. 1).

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and test compound (substrate). The first control flask contained only fungal culture, and the second contained fungal medium (without fungal culture) along with substrate. Substrate was incubated in four flasks containing fully grown fungal cultures. Every 4<sup>th</sup> day a sample of each flask was removed, filtered, and extracted with dichloromethane. The solvent was evaporated and degree of transformation was compared to controls on TLC. After 12 days of incubation, a number of transformation were observed through TLC analysis, during small scale screening. Therefore, the experiment continued toward preparative scale.

Five liters of culture medium was prepared and transferred to 50 Erlenmeyer flasks of 250 mL (each flask containing 100 mL of medium), and autoclaved at 121 °C. Flasks were inoculated with fungal culture and placed on a shaker (121 rpm) at 26 ± 2 °C for incubation. When suitable growth was observed, 500 mg of substrate **1** was dissolved in 50 mL of acetone, evenly distributed over 50 flasks and then left on a rotary shaker for 12 days at 26 °C. After 12 days, contents of all flasks were combined, fungal cultures were filtered, and the aqueous layer was extracted with dichloromethane (18 L). The extraction process was repeated three times. The organic layer was made moisture free with anhydrous sodium sulfate and concentrated over reduced pressure rotary evaporator. This resulted a gum like material. A comparative TLC of small scale and large scale experiments were checked, and similar spots were observed. The gum was fractionated over silica gel column chromatography by using hexanes-acetone as mobile phase. Four main fractions (1–4) were obtained as a result of column chromatography. Further purification of metabolites was carried out by using reverse phase preparative HPLC equipped with L-80 column. Compound **2** (methanol:water; 70:30,  $t_R = 22$  min) was obtained from fraction-1 through reverse phase recycling HPLC. Compound **3** (methanol:water; 70:30,  $t_R = 20$  min) was purified from fraction-2 with reversed phase HPLC. Similarly, fraction-3 yielded metabolite **4** (methanol:water; 70:30,  $t_R = 21$  min) on purification with reversed phase HPLC. Whereas, metabolite **5** (methanol:water; 70:30,  $t_R = 21$  min) was isolated from fraction-4 through reverse phase recycling HPLC by using methanol–water as a solvent system. During purification of metabolites, we also recovered some unconsumed danazol (**1**) along with several minor transformed products, which we could not purified due to their very low quantities.

#### 2.3.1. 14 $\beta$ ,17 $\beta$ -Dihydroxy-2-(hydroxymethyl)-17 $\alpha$ -pregn-4-en-20-yn-3-one (**2**)

White solid; m. p. 228–230 °C; % yield: 1%;  $[\alpha]_D^{25} = 49.3$  (c 0.01, MeOH); UV (MeOH):  $\lambda_{max}$  (log  $\epsilon$ ) = 230 nm (3.1), 248 nm (3.0); IR (CHCl<sub>3</sub>):  $\nu_{max}$  3281 cm<sup>-1</sup> (O–H), 1670 cm<sup>-1</sup> (enone); HREI-MS  $m/z$  358.2152 [M]<sup>+</sup> (mol. Formula, C<sub>22</sub>H<sub>31</sub>O<sub>4</sub>, calcd. 358.2144); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz); Table 1; <sup>13</sup>C NMR (CD<sub>3</sub>OD, 150 MHz); Table 2.

#### 2.3.2. 1 $\alpha$ ,17 $\beta$ -Dihydroxy-17 $\alpha$ -pregna-2,4-dien-20-yno-[2,3-d]-isoxazole (**3**)

White solid; m. p. 231–233 °C; % yield: 1.2%;  $[\alpha]_D^{25} = -144.6$  (c 0.025, MeOH); UV (MeOH):  $\lambda_{max}$  (log  $\epsilon$ ) = 278 nm (2.8); IR (CHCl<sub>3</sub>):  $\nu_{max}$  3414 (O–H), 1624 cm<sup>-1</sup> (C=C); HREI-MS  $m/z$  353.1974 [M]<sup>+</sup> (mol. Formula C<sub>22</sub>H<sub>27</sub>NO<sub>3</sub>, calcd. 353.1991); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz) Table 1; <sup>13</sup>C NMR (CD<sub>3</sub>OD, 150 MHz) Table 2.

#### 2.3.3. 6 $\beta$ ,17 $\beta$ -Dihydroxy-17 $\alpha$ -pregna-2,4-dien-20-yno-[2,3-d]-isoxazole (**4**)

White solid; m. p. 232–234 °C; % yield: 0.8%;  $[\alpha]_D^{25} = -67.5$  (c 0.008, MeOH); UV (MeOH):  $\lambda_{max}$  (log  $\epsilon$ ) = 286 nm (2.9); IR (KBr):  $\nu_{max}$  3400 (O–H s), 1625 (C=C); HREI-MS  $m/z$  353.2000 [M]<sup>+</sup>,

(mol. formula, C<sub>22</sub>H<sub>27</sub>NO<sub>3</sub>, calcd. 353.1991); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 300 MHz) Table 1; <sup>13</sup>C NMR (CD<sub>3</sub>OD, 150 MHz) Table 2.

#### 2.3.4. 17 $\beta$ -Hydroxy-2-(hydroxymethyl)-17 $\alpha$ -pregn-1,4-dien-20-yn-3-one (**5**)

White solid; m. p. 224–226 °C; % yield: 1.2;  $[\alpha]_D^{25} = -69.0$  (c 0.01, MeOH); UV (MeOH):  $\lambda_{max}$  (log  $\epsilon$ ) = 248 nm (4.2); IR (CHCl<sub>3</sub>):  $\nu_{max}$  3305 (O–H), 1663, and 1619 cm<sup>-1</sup> (cross conjugated enone); HRESI-MS  $m/z$  340.2042 [M + H]<sup>+</sup> (mol. formula, C<sub>22</sub>H<sub>28</sub>O<sub>3</sub>, calcd. 340.2038); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz); Table 1; <sup>13</sup>C NMR (CD<sub>3</sub>OD, 125 MHz); Table 2.

### 2.4. Experimental protocol for cytotoxicity assay

MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric method was used for cytotoxicity evaluation against a human cervical carcinoma (HeLa) cell line. The cells (10<sup>5</sup> cells/mL) were grown in tissue culture flask using Minimum Essential Eagle's Medium (MEM), supplemented with fetal bovine serum (FBS, 5%), penicillin (100 IU/ mL) and streptomycin (100 µg/mL). The cells were then plated onto 96-well plates in the concentration of 100 µL/well, and incubated overnight in 5% CO<sub>2</sub> incubator at 37 °C. The next day medium was replaced with the freshly prepared 200 µL medium, along with different concentrations of test compounds (1–50 µM). The 96-well plate was again incubated for 72 h. After incubation, 2 µg/mL of MTT was added to each well. The plate was then incubated for next 4 h. The reaction was ceased by adding 100 µL DMSO to each well. The extent of MMT reduction to formazan within cells was determined by measuring the absorbance at 570 nm using an ELISA Reader (Spectra Max Plus, Molecular Devices, CA, USA). The cytotoxicity was determined at a concentration on which 50% cell growth was inhibited (IC<sub>50</sub>) [24].

### 3. Results and discussion

Three new **2–4** and one known **5** metabolites were obtained from the microbial transformation of danazol (**1**).

Metabolite **2** was obtained as a white powder from the biotransformation of dianazol (**1**) (C<sub>22</sub>H<sub>27</sub>NO<sub>2</sub>). The HREI-MS of metabolite **2** displayed the [M]<sup>+</sup> at  $m/z$  358.2152 (calcd. 358.2144), consistent with the formula C<sub>22</sub>H<sub>30</sub>O<sub>4</sub>, 20 amu greater than substrate **1**, which suggested the loss of a nitrogen atom and addition of two oxygen and two hydrogen atoms. UV  $\lambda_{max}$  at 230 nm suggested the presence of an enone system. The IR spectrum showed absorption for OH (3281 cm<sup>-1</sup>), and  $\alpha$ ,  $\beta$ -unsaturated ketone (1670 cm<sup>-1</sup>). In the <sup>1</sup>H NMR spectrum of metabolite **2**, absence of the most downfield olefinic proton ( $\delta$  8.11) was noted as compared to substrate **1**. Whereas, three additional proton signals at  $\delta$  4.29 (methine), and 3.83 and 3.76 (methylene) were also observed in the <sup>1</sup>H NMR spectrum (Table 1). This suggested reduction of C=C bonds, as well as hydroxylation. Four olefinic carbon signals were found missing in the <sup>13</sup>C NMR spectrum which indicated the reduction of two C=C bonds. In place of these, two additional downfield carbon signals were found resonating at  $\delta$  69.0 and 202.1 in the <sup>13</sup>C NMR spectrum of compound **2**, which suggested hydroxylation and oxidation, respectively (Table 2). The COSY-45° spectrum showed cross-peaks between newly formed methine proton at  $\delta$  4.20 and signals at  $\delta$  2.71, 2.03 (H<sub>2</sub>-16) and 1.41 (H-14). This suggested hydroxylation at C-15. Similarly, H-22 ( $\delta$  3.83) showed geminal coupling with H-22 ( $\delta$  3.83) and vicinal coupling with H-1 ( $\delta$  2.56) in COSY spectrum. H<sub>2</sub>-22 appeared as AB doublets at  $\delta$  3.83 ( $J_{22a,22b} = 11.0$  Hz,  $J_{22a,1a} = 4.5$  Hz), and 3.76 ( $J_{22b,22a} = 11.0$  Hz,  $J_{22b,1a} = 4.5$  Hz), indicating an OH at C-22. The structure of metabolite **2** was further deduced on the

**Table 1**  
<sup>1</sup>H NMR (CD<sub>3</sub>OD) chemical shift assignments (δ in ppm, J in Hz) of compounds 1–5.

Position	1	2	3	4	5
1	2.81 d ( $J_{a,e} = 15.9$ ), 2.50 d ( $J_{a,e} = 15.9$ )	2.20 overlap, 1.78 overlap	4.52 s	2.82 d ( $J_{a,e} = 15.9$ ), 2.49 d ( $J_{a,e} = 15.9$ )	7.20 s
2	–	2.56 m	–	–	–
3	–	–	–	–	–
4	6.21 s	5.69 s	6.29 s	6.41 s	6.05 s
5	–	–	–	–	–
6	1.78 m, 1.67 m	1.58 m, 1.51 m	1.85 overlap, 1.64 overlap	4.49 br. s	2.53 ddd, ( $J_{6a,7a} = 18.5$ , $J_{6a,6e} = 12.5$ , $J_{6a,7e} = 5.0$ ), 1.77 m
7	2.43 m, 1.38 m	2.20 m, 1.10 m	2.42 m, 1.93 m	1.95 overlap, 1.21 overlap	2.01 overlap, 1.01 m
8	1.54 overlap	2.01 m	1.34 m	1.95 m	1.79 m
9	1.16 m	1.40 m	1.84 overlap	1.31 m	0.98 m
10	–	–	–	–	–
11	1.68 m, 1.53 m	1.68 m, 1.50 m	1.96 m, 1.80 m	1.70 m, 1.62 m	1.89 m, 1.78 overlap
12	1.76 m, 1.67 m	1.63 m, 1.73 m	1.84 overlap, 1.30 m	1.79 m, 1.69 m	2.35 m, 1.69 m
13	–	–	–	–	–
14	1.54 overlap	0.99 m	1.01 m	1.57 m	1.49 m
15	1.74 m, 1.40 m	4.20 m	1.36 m, 2.45 m	1.56 overlap, 1.40 m	1.66 overlap, 1.39 m
16	2.23 m, 1.98 ddd ( $J_{16e,16a} = 21.6$ , $J_{16e,15a} = 13.5$ , $J_{16e,15e} = 3.9$ )	2.71 dd ( $J_{16a,16e} = 14.5$ , $J_{16a,15e} = 7.5$ ), 2.03 overlap	2.23 m, 2.01 m	2.72 m, 1.97 m	2.22 m, 1.96 overlap
17	–	–	–	–	–
18	0.89 s	1.09 s	0.61 s	0.90 s	0.91 s
19	1.04 s	1.29 s	0.91 s	1.19 s	1.25 s
20	–	–	–	–	–
21	2.87 s	2.84 s	2.87 s	2.90 s	2.85 s
22	8.11 s	3.83 dd ( $J_{22a,22b} = 11.0$ , $J_{22a,1a} = 4.5$ ), 3.76 dd ( $J_{22b,22a} = 11.0$ , $J_{22b,1a} = 4.5$ )	8.20 s	8.15 s	4.34 dd ( $J_{22a,22b} = 15.0$ , $J_{22a,1} = 1.5$ ), 4.34 dd ( $J_{22b,22a} = 14.5$ , $J_{22a,1} = 1.5$ )

**Table 2**  
<sup>13</sup>C NMR (CD<sub>3</sub>OD) data of compounds 1–5.

Carbon	1	2	3	4	5
1	34.2	40.4	68.1	35.2	153.2
2	109.3	45.4	113.2	111.2	137.1
3	166.3	202.1	166.2	165.8	187.8
4	109.2	124.3	108.6	113.7	123.9
5	157.1	174.2	157.6	155.6	173.0
6	32.0	33.4	33.6	72.6	33.4
7	33.2	32.3	33.7	38.6	34.7
8	38.2	33.0	37.9	31.9	37.3
9	55.6	55.9	44.6	55.3	54.2
10	42.2	40.5	47.1	41.8	44.9
11	22.4	21.7	22.9	22.3	23.7
12	33.9	34.9	31.6	33.5	33.7
13	47.8	47.1	48.1	49.8	48.4
14	51.2	55.7	51.4	51.0	51.0
15	24.0	69.0	24.2	24.0	24.1
16	39.8	52.2	39.8	39.8	39.7
17	80.2	79.9	82.5	80.3	80.0
18	13.2	15.8	13.2	13.3	13.3
19	19.1	17.9	18.4	21.1	19.1
20	73.2	88.3	88.7	80.7	88.4
21	88.6	74.6	74.0	74.8	74.8
22	149.8	62.1	150.0	149.9	59.8

basis of long range HMBC correlations. The HMBC correlations of H-16 (δ 2.03) and H-14 (δ 1.41) with C-15 (δ 69.0), and HMBC of H-15 (δ 4.20) with C-17 (δ 79.9), further indicated an OH at C-15 (Fig. 2). The newly formed oxymethylene protons (δ 3.83, 3.76) showed HMBC correlations with C-1 (δ 40.4), and C-2 (δ 45.4), and with newly formed ketonic carbonyl carbon (δ 202.1). This suggested oxidation at C-3. The relative stereochemistry of newly formed stereogenic centers was deduced on the basis of NOESY correlations. H-15 (δ 4.20) showed NOESY cross peaks with H-14 (δ 1.41). As H-14 is α-oriented in substrate **1**, therefore its NOESY correlation with H-15 suggested a β-hydroxylation at C-15

(Fig. 3). The NOESY correlation of H-2 (δ 2.56) with H<sub>3</sub>-19 (δ 1.29) suggested β-orientation of H-2. Therefore, hydroxymethylene at C-2 was deduced to be α-oriented (*pseudo-equatorially* oriented). The structure of new compound **2** was thus deduced as 14β,17β-dihydroxy-2-(hydroxymethyl)-17α-pregna-4-en-20-yn-3-one.

Metabolite **3** was obtained as a white powder. The [M]<sup>+</sup> at *m/z* 353.1974 (calcd. 353.1991) in the HREI-MS was consistent with the formula C<sub>22</sub>H<sub>27</sub>NO<sub>3</sub>. The 16 amu increase indicated addition of an oxygen atom as a hydroxyl group. The UV absorbance at 278 nm indicated the presence of a conjugated triene system. The IR spectrum showed absorbances for hydroxyl (3414 cm<sup>-1</sup>) and C=C (1624 cm<sup>-1</sup>). The <sup>1</sup>H NMR spectrum of **3** distinctly resembled with the substrate **1**, with an additional methine singlet at δ 4.52 (Table 1) due to hydroxylation. The <sup>13</sup>C NMR spectrum showed an additional downfield methine carbon signal at δ 68.1 (Table 2), as compared to the substrate **1**. The position of OH was deduced on the basis of long-range HMBC correlations between H-19 and C-1 (δ 68.1), and C-5 (δ 157.5), suggesting an OH at C-1 (Fig. 2). The HMBC correlations of H-1 (δ 4.52) with C-3 (δ 166.2), C-5 (δ 157.5), and C-10 (δ 48.5) further supported this inference. The stereochemistry of OH at C-1 was deduced through the NOESY correlations. H-1 showed NOESY correlations with H<sub>3</sub>-19. As CH<sub>3</sub>-19 is β-oriented in substrate **1**, its correlation with H-1 suggested an α-hydroxylation (Fig. 3). The structure of new metabolite **3** was thus deduced as 1α,17β-dihydroxy-17α-pregna-2,4-dien-20-yno-[2,3-*d*]-isoxazole.

Metabolite **4** was also obtained as a white powder from the biotransformation of dianazol (**1**). The HREI-MS of compound **4** showed the [M]<sup>+</sup> at *m/z* 353.2000 (calcd. 353.1991), consistent with C<sub>22</sub>H<sub>27</sub>NO<sub>3</sub>, and 16 amu greater than dianazol (**1**). This indicated addition of an oxygen atom in the form of OH. The λ<sub>max</sub> in UV spectrum at 286 nm indicated the presence of a conjugated triene system [25]. The IR spectrum showed absorbance for OH (3400 cm<sup>-1</sup>) and C=C (1625 cm<sup>-1</sup>). The <sup>1</sup>H NMR spectrum of

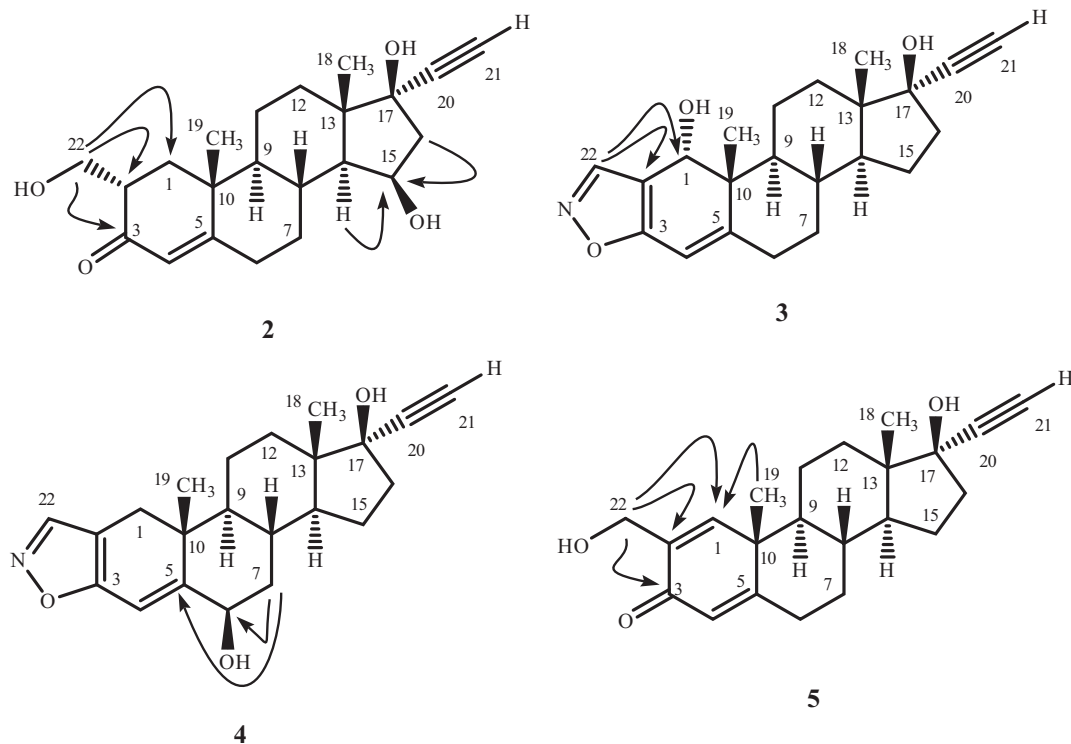


Fig. 2. Key HMBC correlations in compounds 2–5.

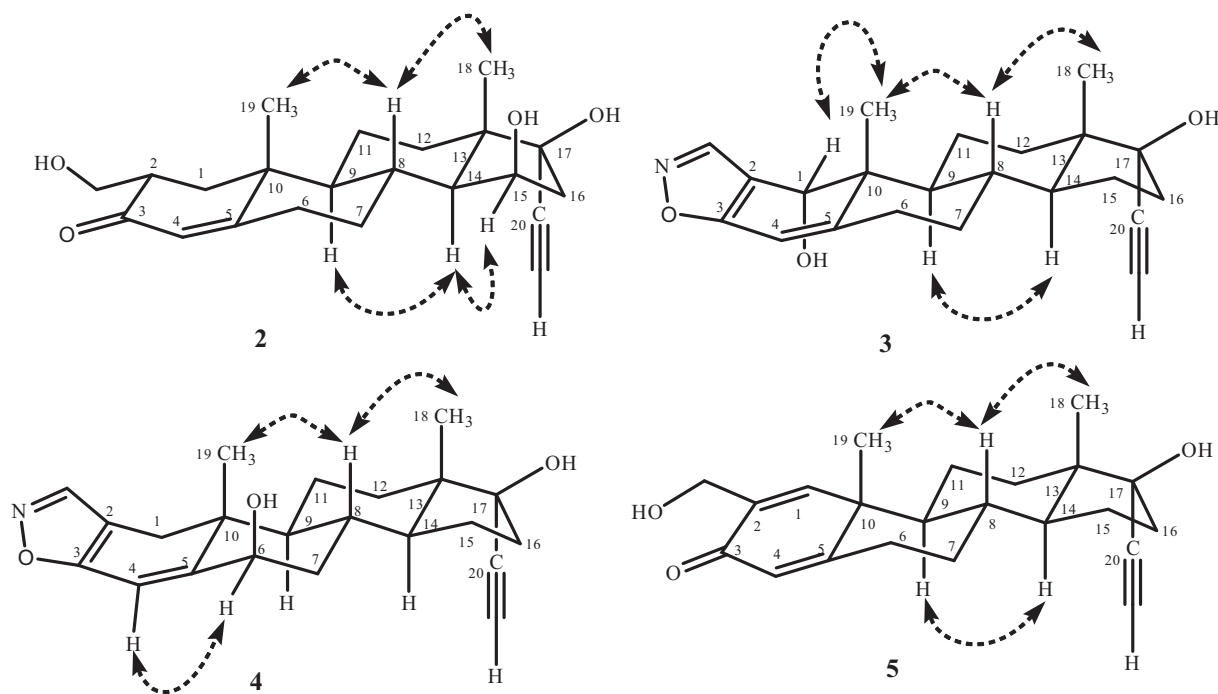


Fig. 3. Key NOESY correlations in compounds 2–5.

metabolite **4** showed an additional methine signal at  $\delta$  4.49, which indicated hydroxylation of danazol (**1**) (Table 1). The  $^{13}\text{C}$  NMR spectrum also showed signals for an oxymethine carbon at  $\delta$  72.6 (Table 2). This supported hydroxylation of substrate **1**. The position of OH at C-6 was deduced through HMBC correlations of  $\text{H}_2$ -7 ( $\delta$  1.95 and 1.21) with C-6 ( $\delta$  72.6) and C-5 ( $\delta$  155.6) (Fig. 2). The stereochemistry of OH at C-6 was deduced through NOESY

correlations between H-6 and H-4. The H-6 can only show correlation with H-4, if it is *equatorially* oriented. Therefore a  $\beta$ -OH was placed at C-6 (Fig. 3). The structure of new compound **4** was thus deduced as  $6\beta,17\beta$ -dihydroxy- $17\alpha$ -pregna-2,4-dien-20-yno-[2,3-*d*]-isoxazole.

Metabolite **5** was obtained as a white powder. The molecular formula  $\text{C}_{22}\text{H}_{28}\text{O}_3$  of compound **5** was deduced on the basis of

**Table 3**  
Cytotoxicity of compounds **1–5** against HeLa cancer cell line.

Compounds	IC <sub>50</sub> ± SD (µg/mL)
1	0.283 ± 0.013
2	>30
3	13.427 ± 0.819
4	>30
5	>30
Standard (doxorubicin)	0.506 ± 0.15

HREI-MS, which showed the [M]<sup>+</sup> at *m/z* 340.2042 (calcd. 340.2038). The λ<sub>max</sub> in UV spectrum at 248 nm indicated the presence of a cross conjugated dienone system [26]. The IR spectrum displayed absorbance for OH (3305 cm<sup>-1</sup>), and a cross conjugated dienone moiety (1663, and 1619 cm<sup>-1</sup>). The <sup>1</sup>H NMR spectrum of metabolite **5** was distinctly similar to substrate **1**, except for a downfield signal at δ 4.30 (Table 1). The signal for one olefinic carbon was also found missing in the <sup>13</sup>C NMR spectrum of metabolite **5**. This indicated the reduction of one C=C bond, out of three. It also showed ketonic carbonyl carbon at δ 187.8 (Table 2). The structure of compound **5** was further deduced based on HMBC correlations. H-19 (δ 1.25) showed HMBC correlations with C-1 (δ 153.2) and C-5 (δ 173.0), indicating the presence of a C=C between C-1/C-2 (Fig. 2). The C-1/C-2 C=C resulted from the isomerization of C-2/C-3 double bond. Whereas, isoxazole ring was hydrolytically cleaved leaving an OH at C-22. The newly formed oxymethylene protons (δ 4.29 and 4.34) showed HMBC correlations with C-1 (δ 153.2), and C-2 (δ 137.1), further supporting isomerization of C=C, and hydroxylation at C-22. The stereochemistry of compound **5** was found to be similar to substrate **1** (Fig. 3). The structure of compound **5** was thus deduced as 17β-hydroxy-2-(hydroxymethyl)-17α-pregn-1,4-dien-20-yn-3-one. It was previously reported by Choudhary et al., from the biotransformation of substrate **1** with *Fusarium lini*, *Cephalosporium aphidicola*, and *Aspergillus niger* [27].

### 3.1. Cytotoxicity against cancer cell line

Danazol (**1**) showed strong cytotoxicity against the human cervical cancer cell line (HeLa) with IC<sub>50</sub> value of 0.283 ± 0.013 µM in preliminary screening, more cytotoxic than the anticancer drug doxorubicin (standard) (IC<sub>50</sub> = 0.506 ± 0.015 µM). We therefore, decided to evaluate the transformed products of danazol (**1**) against HeLa cell line. Interestingly only metabolite **3** was found to be active with IC<sub>50</sub> = 13.427 ± 0.819 µM (Table 3).

Danazol (**1**) has an isoxazole ring fused with the ring-A of steroidal skeleton. The cytotoxic potential of danazol (**1**) may be due to isoxazole ring. It showed no cytotoxic potential due to loss of isoxazole ring, as observed in compounds **2** and **5**. Hydroxylation at various positions of danazol (**1**) also decreased its activity or even made it inactive. C-1 hydroxylation (compound **3**) decreased cytotoxic potential of danazol (**1**) (IC<sub>50</sub> = 13.427 ± 0.819 µM). Similarly, β-hydroxylation at C-6 (as in compound **4**) made it inactive against HeLa cancer cell lines.

## 4. Conclusion

In conclusion, the microbial transformation of danazol (**1**) with *C. blakesleeana* yielded three new **2–4** and a known metabolite **5**. This involves hydroxylation at C-1, C-6 and C-15, whereas oxidation at C-3, and N–O bond cleavage was also occurred. Danazol (**1**) exhibited a strong cytotoxicity against cervical cancer cell line (HeLa) with IC<sub>50</sub> value of 0.283 ± 0.013 µM, two-fold stronger than the standard anticancer drug doxorubicin (IC<sub>50</sub> = 0.506 ± 0.015 µM). One of the transformed product *i.e.*, metabolite **3** also showed

some activity with IC<sub>50</sub> value of 13.427 ± 0.819 µM. Whereas, compounds **2**, **4**, and **5** were found to be inactive. Apparently hydrolytic cleavage of isoxazole ring and hydroxylation of steroidal skeleton decrease the cytotoxicity. Strong cytotoxicity of danazol (**1**) against cervical cancer cell line (HeLa) is an interesting finding which deserves further research.

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## A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.steroids.2015.11.010>.

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