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Microbial transformation of anti-cancer steroid exemestane and cytotoxicity of its metabolites against cancer cell lines

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Abstract

Background: Microbial transformation of steroids has been extensively used for the synthesis of steroidal drugs, that often yield novel analogues, not easy to obtain by chemical synthesis. We report here fungal transformation of a synthetic steroidal drug, exemestane, used for the treatment of breast cancer and function through inhibition of aromatase enzyme.

Results: Microbial transformation of anti-cancer steroid, exemestane (**1**), was investigated by using two filamentous fungi. Incubation of **1** with fungi *Macrophomina phaseolina*, and *Fusarium lini* afforded three new, 11α-hydroxy-6-methylene-androsta-1, 4-diene-3,17-dione (**2**), 16β, 17β-dihydroxy-6-methylene-androsta-1, 4-diene-3-one (**3**), and 17β-hydroxy-6-methylene-androsta-1, 4-diene-3, 16-dione (**4**), and one known metabolites, 17β-hydroxy-6-methylene-androsta-1, 4-diene-3-one (**5**). Their structures were deduced spectroscopically. Compared to **1** (steroidal aromatase inactivator), the transformed metabolites were also evaluated for cytotoxic activity by using a cell viability assay against cancer cell lines (HeLa and PC3). Metabolite **2** was found to be moderately active against both the cell lines.

Conclusions: Biotransformation of exemestane (**1**) provides an efficient method for the synthesis of new analogues of **1**. The metabolites were obtained as a result of reduction of double bond and hydroxylation. The transformed product **2** exhibited a moderate activity against cancer cell lines (HeLa and PC3). These transformed products can be studied for their potential as drug candidates.

Keywords: Steroid, Exemestane, Anti-cancer activity, Cancer cell lines (HeLa, PC3), *Fusarium lini*, *Macrophomina phaseolina*, Microbial transformation

Background

Microbial transformation of steroids has been extensively employed for the synthesis of steroidal drugs, both at laboratory and industrial levels [1-7]. In modern drug discovery process, generation of libraries of bioactive compounds with diverse structures plays an important role [8].

Exemstane (trade name aromasin) is a steroidal irreversible aromatase inhibitor, used for the treatment of breast cancer. Breast cancers have estrogen receptors (ER-positive) and their growth depends on aromatase activity. Therefore, inhibition of aromatase enzyme reduces the estrogen levels and thus slows the growth of breast cancer [9-12].

Interestingly exemestane not only increases the testosterone level and lowers estrogen, but it also increases the levels of insulin-like growth factor (IGF) [10]. The large reduction in estrogen levels combined with a rise in IGF, makes exemestane an effective breast cancer medication [13-15]. Based on the importance of exemestane in the treatment of breast cancer, a number of exemestane derivatives were previously synthesized involving modification of C-6 methylene and reduction

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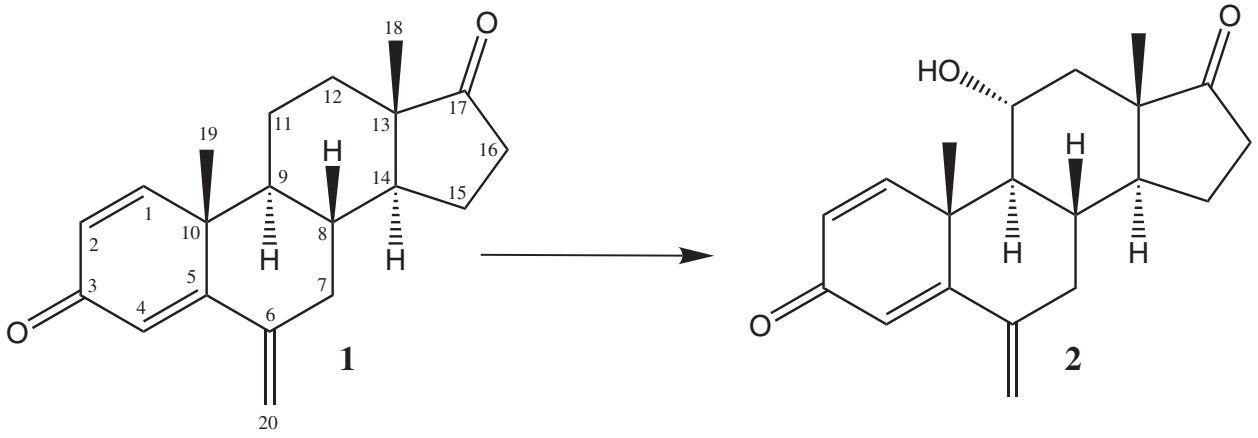

Figure 1 Biotransformation of exemestane (1) with *Fusarium lini* yielded metabolite 2.

of C-17 keto group, and evaluated for their aromatase inhibitory potential [16-19].

During current study, we synthesized new analogues of 1 by biotransformation techniques. Screening experiments showed that *Macrophomina phaseolina* and *Fusarium lini* were able to efficiently transform **1** into several metabolites. Subsequent large scale fermentations produced three new metabolites **2**–**4** along with a known metabolite **5**. The structures of metabolites were unambiguously established through detailed spectral analysis.

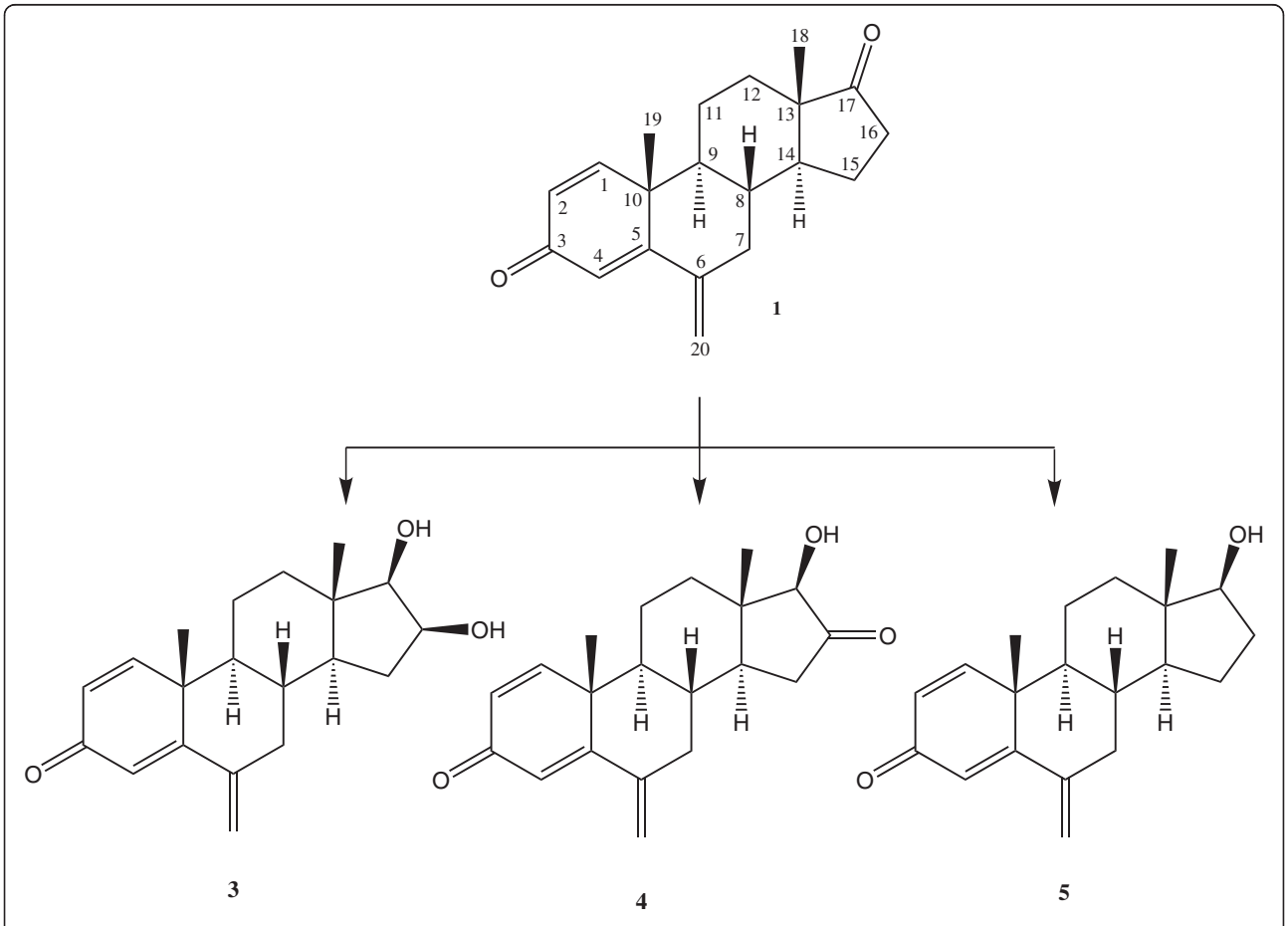

Figure 2 Biotransformation of exemestane (1) with *Macrophomina phaseolina* yielded metabolites 3-5.

Table 1 ^{1}H-NMR data of compounds 1–5 in ppm, *J* in Hz

	COMPOUNDS				
Carbon	**1**[a]	**2**[a]	**3**[b]	**4**[b]	**5**[b]
1	7.21 d (10.0)	7.21 d (10.5)	7.31 d (10.2)	7.34 d (10.5)	7.31 d (10.2)
2	6.14 dd (10.5, 2.0)	6.12 dd (10.5, 2.0)	6.21 dd (10.2, 1.8)	6.42, dd (10.5, 2.0)	6.22 dd (10.2, 1.8)
3	-	-	-	-	-
4	5.99, d (2.0)	6.00 d (2.0)	6.08 d (1.8)	6.11 d (2.0)	6.09 d (1.8)
5	-	-	-	-	-
6	-	-	-	-	-
7	2.69, 1.97 m	2.13, 1.28 m	2.56 d (9.0), 1.87 m	2.57, 1.96 m	2.66, 1.82 m
8	2.03 m	1.98 m	1.86 m	1.98 m	1.83 m
9	1.34 m	1.62 m	1.35 m	1.48 m	1.05 m
10	-	-	-	-	-
11	1.92, 1.76 m	4.30 m	1.91, 1.31 m,	1.93, 1.84 m	1.75, 1.83 m
12	1.78, 1.28 m	2.13, 1.28 m	1.90, 1.16 m	2.01, 1.45 m	1.13, 1.92 m
13	-	-	-	-	-
14	1.43 m	1.37 m	0.93 m	1.63 m	1.27 m
15	1.99, 1.67 m	1.93, 1.80 m	2.20, 1.30 m	2.29, 1.95 m	1.65, 1.38 m
16	2.41, 1.95 m	2.66, 1.95 m	4.07 m	-	2.00, 1.51 m
17	-	-	3.30 d (7.5)	3.77 s	3.55 t (8.7)
18	0.92 s	0.99 s	0.99 s	0.81 s	0.81 s
19	1.19 s	1.18 s	1.18 s	1.21 s	1.17 s
20	5.03, 5.01 s	5.04, 5.02 s	5.02 t (1.9)	5.06, 5.04 s	4.99, 5.01 s

[a] 500 MHz $(CD_3)_2CO$.
[b] 300 MHz CD_3OD.

The microbial transformed metabolites **2** and **4** of exemestane showed a moderate anti-cancer effect against PC3 and/or Hela cancer cell lines. This successful attempt to synthesize new derivatives of an anti-cancer steroid may lead to the discovery of new cancer therapeutic agents.

Results and discussion

Four microbial metabolites were generated by the selected fungal strains, i.e. *Macrophomina phaseolina* and *Fusarium lini* (Figures 1 and 2). *M. phaseolina* is previously reported to catalyze the introduction of double bond between C-1 and C-2, hydroxyl groups at C-6, C-15, C-16 and C-17, and carbonyl group at C-17 of the steroidal skeleton [1,20]. *F. lini* is also reported to catalyze the oxidation at C-1, C-2, C-6, and C-11 of steroidal skeleton [21]. The chemical structures of the metabolites **2**–**4** are reported here for the first time along with their NMR data (Tables 1 and 2).

The anti-cancer effect of exemestane (**1**) [2] and its synthetic analogues on HeLa and PC3 were determined by using MTT assay. Results obtained from these assays are presented in Table 3.

The molecular formula $C_{20}H_{24}O_3$ [M^+, *m/z* 312] of metabolite **2** was deduced from the HREI-MS (M^+ *m/z* 312.1705), suggested the addition of an oxygen in substrate **1**. The ^{1}H-NMR spectral analysis of **2** (Table 1) displayed a downfield methine signal, as compared to the starting material exemestane (**1**), resonating at δ 4.30 (m, $W_{1/2}$ = 15.6 Hz), while its respective carbon signal was at δ 71.5 in ^{13}C-NMR spectrum (Table 2). The HMBC spectrum (Figure 3) displayed long-range couplings of the hydroxyl-bearing methine proton (δ 4.30) with C-9 (δ 48.6), C-10 (δ 44.3), and C-13 (δ 48.1), which suggested the position of the hydroxyl-bearing methine at C-11. H-11 also showed COSY cross peaks with H-9 (δ 1.62) and H_2-12 (δ 1.28, 2.13). The stereochemical assignments were based on NOESY interactions (Figure 3) between H-11 (δ 4.30), H-8 (δ 1.98), and Me-19 (δ 1.18). H-11 was thus deduced as β-oriented. Metabolite **2** was finally identified as 11α-hydroxy-6-methylene-androsta-1,4-diene-3,17-dione.

Molecular composition of metabolite **3** was deduced to be $C_{20}H_{26}O_3$ from the HREI-MS analysis (M^+ = *m/z* 314.1933, calcd 314.1882). The ^{1}H-NMR spectra μm (Table 1) of metabolite **3** showed two hydroxyl-bearing methine proton peaks at δ 3.30 (d, $J_{17,16}$ = 7.5 Hz, H-17) and 4.07 (m, $W_{1/2}$ = 20.0 Hz). The ^{13}C-NMR spectrum of **3** lacks signal for C-17 carbonyl, whereas new methine

Table 2 ^{13}C-NMR data of compounds 1–5 in ppm

	COMPOUNDS				
Carbon	**1**[a]	**2**[b]	**3**[c]	**4**[d]	**5**[d]
1	155.0	154.9	158.1	157.6	158.2
2	128.0	128.0	127.8	127.9	127.7
3	185.8	185.8	188.7	188.6	188.7
4	122.9	122.9	122.7	122.8	122.7
5	167.9	167.9	171.6	171.2	171.7
6	147.1	147.0	147.7	147.3	147.8
7	39.87	32.1	41.4	41.2	41.3
8	36.0	39.6	36.6	35.9	37.2
9	50.9	48.6	51.9	51.4	51.7
10	44.3	44.3	45.6	45.5	45.6
11	22.63	71.5	23.0	23.2	23.6
12	32.0	32.0	38.1	36.6	37.5
13	48.1	48.1	43.7	43.5	44.2
14	51.3	50.8	48.2	45.4	51.8
15	22.3	22.3	35.9	36.6	24.3
16	35.8	39.7	70.6	217.7	30.5
17	218.8	218.0	81.7	86.8	82.1
18	13.9	14.5	12.5	11.9	11.6
19	20.1	20.1	20.1	20.1	20.1
20	112.2	112.2	112.6	112.9	112.4

[a] 125 MHz $(CD_3)_2CO$.
[b] 150 MHz $(CD_3)_2CO$.
[c] 125 MHz CD_3OD.
[d] 75 MHz CD_3OD.

carbon at δ 81.7 suggested the reduction of C-17 ketone into C-17 OH. The proton geminal to the –OH group (δ 4.07) was correlated with C-13 (δ 43.7), C-14 (δ 48.2) and C-17 (δ 81.7) in the HMBC spectrum. The methine C-17 (δ 81.7) showed HMBC correlations with H-14 (δ 0.93, m) and H-18 (δ 0.99, s). Based on the above observations, the hydroxyl-bearing methine carbon was identified as C-16. The H-16 (δ 4.07) showed NOESY cross peaks with H-14 (δ 0.93), but no interaction with H-18 (δ 0.99) (Figure 4). Therefore the C-16 proton was assigned to be α-oriented. The metabolite **3** was thus identified as 16β, 17β-dihydroxy-6-methylene-androsta-1, 4-diene-3-one.

Table 3 *In vitro* cytotoxicity of compounds 1–5

Compound Codes	HeLa (Cervical cancer) (IC_{50}± S.D.) μM	PC-3 (Prostate cancer) (IC_{50} ±S.D.) μM
1	>50	>50
2	16.83±0.96	24.87±0.72
3	>50	>50
4	37.20 ± 0.88	>50
5	>50	>50
Doxorubicin	**3.10 ± 0.20**	**0.91 ± 0.12**

Molecular formula $C_{20}H_{24}O_3$ (M^+ *m/z* 312.1725, calcd 312.1720) was deduced from the HREI-MS of metabolite **4**. A distinct downfield methine proton signal appeared at δ 3.77 (br. s, $W_{1/2}$ = 9.3 Hz) in the ^{1}H-NMR spectrum of **4**. The ^{13}C-NMR spectrum showed a saturated ketone carbon signal at δ 217.7. The rest of the spectrum was distinctly similar to metabolite **2**. The deshielded methine proton was HMBC correlated with this ketonic carbon, while its corresponding methine carbon at δ 86.8 showed the HMBC correlations with H_2-15 (δ 1.95, 2.29), and CH_3-18 (δ 0.81). These interactions, along with appearance of a downfield proton (δ 3.77), indicated that the ketone at C-17 has been reduced into an –OH. Geminal H-17 (δ 3.77) showed NOESY correlations with H-14 (δ 1.63), indicating it to be *axially* (α-) oriented. The saturated ketone carbon (δ 217.7) was place at C-16, based on the above mentioned HMBC correlations (Figure 5). The structure of metabolite **4** was finally identified as 17β-hydroxy-6-methylene-androsta-1, 4-diene-3, 16-dione.

Metabolite **5** has a molecular composition $C_{20}H_{26}O_2$ (HREI-MS, M^+ *m/z* 298.1730, calcd 298.1733). Based on ^{1}H- and ^{13}C-NMR spectral data (Tables 1 and 2), compound **5** was identified as 17β-hydroxy-6-methylene-androsta-1, 4-diene-3-one. It has previously been reported as an *in-vitro* cytochrome P_{450}-mediated transformed product of exemestane [22].

The cytotoxic effect of the compounds **1**-**5** against two tumor cell lines, PC-3 (prostate cancer cell) and Hela (cervical cancer cell), was evaluated (Table 3) using the MTT assay. Compound **2** showed a moderate cytotoxicity against both the cancer cell line with IC_{50} = 16.83 ± 0.96 and 24.87 ± 0.72 μM, respectively, as compared to the standard drug, doxorubicin. Compound **4** exhibited a moderate activity against HeLa cell line.

Conclusion

In conclusion, the biotransformation of exemestane (**1**) with *F. lini* and *M. phaseolina* were investigated for the first time which provided an efficient route towards the synthesis of several new metabolites **2**–**5**. Metabolite **2** was found to be moderately active against both cancer cell lines (HeLa and PC3). The work presented here can be helpful for the study of *in vivo* metabolism of exemestane (**1**), as well as for the discovery of new anticancer drugs

Experimental

Substrate and chemicals

Exemestane (**1**) was purchased from local market as drug (Pfizer Canada Inc., Brand name Aromasin), extracted and further purified by flash chromatography. Thin layer chromatography (TLC) was carried out on silica gel precoated plates (PF_{254}; Merck). Column chromatography (CC) was performed by using silica gel (E. Merck, Germany). Optical rotations were measured in methanol with a

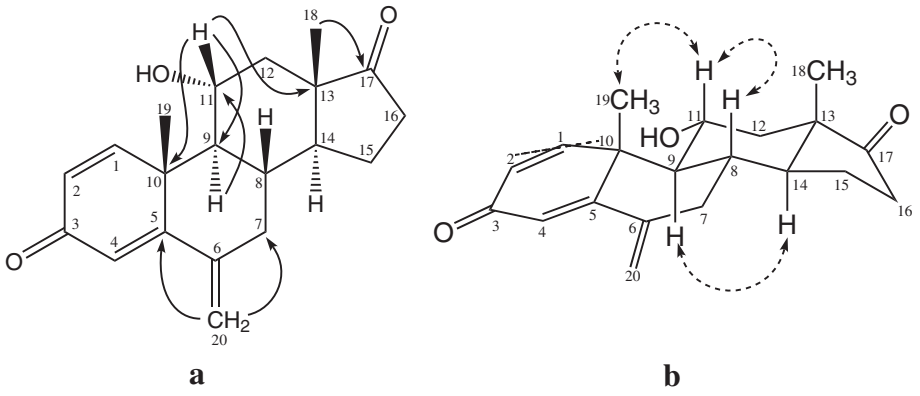

Figure 3 Important HMBC (a) and NOESY (b) correlations in metabolite 2.

JASCO P-2000 polarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded in $(CD_3)_2CO$ and CD_3OD on Bruker Avance spectrometers. The chemical shifts (δ values) are presented in ppm and the coupling constants (*J*) are in Hz. For 1D- and 2D-NMR experiments, standard Bruker pulse sequences were used. UV Spectra (in nm) were recorded in methanol with a Hitachi U-3200 spectrophotometer. Infrared (IR) spectra (in cm^{-1}) were recorded with an FT-IR-8900 spectrophotometer. JEOL (Japan) JMS-600H mass spectrometer was used for recording of EI-MS and high-resolution mass spectra (HREI-MS) in *m/z* (rel. %).

The anticancer activity of compounds **2**-**5** was evaluated in 96-well flat-bottomed micro-titer plates [Iwaki, Japan] by using the standard dye MTT (3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyl-tetrazolium bromide) [Sigma-Aldrich Chemicals, St. Louis, USA] through colorimetric analysis. For this purpose, the cells were cultured in Minimum Essential Medium (MEM), supplemented with 10% Fetal Calf Serum (FCS), 1 mmol/l sodium pyruvate, 1% (v/v) antibiotic / antimycotic and passaged weekly, using 0.25% trypsin / EDTA [Sigma-Aldrich Chemicals, St. Louis, USA] in tissue culture flasks T-75 [Iwaki, Japan]. Absorbance was taken at 540 nm wavelength by using microplate reader (Spectra Max plus, Molecular Devices, USA) using software SoftMax Pro 340 [Molecular Devices, CA, USA]. Dimethyl sulfoxide (DMSO) and doxorubicin (standard inhibitor) were purchased from Sigma-Aldrich Chemicals, [St. Louis, USA].

Microorganisms and culture medium

The fungi were purchased from the Northern Regional Research Laboratories (NRRL), or obtained as gift from the Karachi University Culture Collection (KUCC).

Fusarium lini (NRRL 2204), and *Macrophomina phaseolina* (KUCC 730) were grown in a culture medium prepared by mixing glucose (40.0 g), glycerol (40.0 mL), peptone (20.0 g), yeast extract (20.0 g), KH_2PO_4 (20.0 g), and NaCl (20.0 g) in distilled H_2O (4.0 L).

Cell lines

PC3 (prostate cancer) and HeLa (cervical cancer) cell lines were purchased from the American Type Culture Collection (ATCC) for anticancer activity.

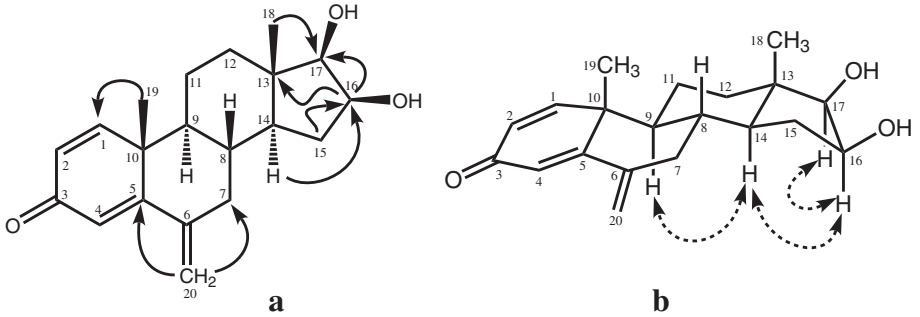

Figure 4 Important HMBC (a) and NOESY (b) correlations in metabolite 3.

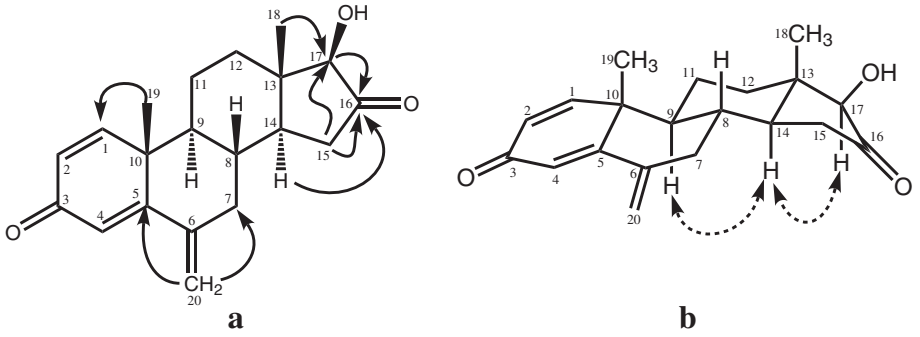

Figure 5 Important HMBC (a) and NOESY (b) correlations in metabolite 4.

General Fermentation and Extraction Conditions

4 Liters fungal media was prepared and distributed into 40 conical flasks (100 mL in each flask). All flasks were then autoclaved at 121°C. The fungal cultures were then inoculated into each flask containing media and incubated at room temperature on shaker for three days. Compound **1** was dissolved in 40 mL methanol and distributed equally to all 40 flasks. All experimental flasks were then kept for fermentation. Two control experiments, i.e. media + compound **1** and media + fungus were also conducted. The transformation was then checked on TLC. After the detection of transformation on TLC, fungal culture from all 40 flasks was filtered and extracted with CH_2Cl_2 (12 L) by using liquid-liquid chromatography. The dichloromethane layer was evaporated *in vaccue*. The obtained gum was analyzed by thin-layer chromatography.

Fermentation and Purification of Exemestane (1) with Fusarium lini

Exemestane (**1**; 1.0 g) was dissolved in 40 mL methanol, and incubated with culture of *F. Lini.* The obtained gum (2.3 g) was fractionated (ARC 1-3) by using silica gel column chromatography. The mobile phase was composed of petroleum ether and acetone with a gradient of 10%. Fraction ARC-2 yielded metabolite **2** (4 mg, pet. ether: acetone = 8:2) after elution through silica gel column.

***11α-Hydroxy-6-methylene-androsta-1,4-diene-3,17-dione* (2)** Amorphous material; $[\alpha]^{25}_D$: +81.4 (*c* = 0.096, MeOH); IR (KBr): ν_{max} 3408, 1657 cm^{-1}; UV (MeOH): λ_{max} nm (log ε) 247 (3.78); ^{1}H- and ^{13}C-NMR: see Tables 1 and 2 (Additional file 1).

Fermentation and Purification of Exemestane (1) with *Macrophomina phaseolina* Incubation of **1** (1.0 g / 40 mL methanol) with 3 days old culture of *M. phaseolina* in 40 flasks for 12 days produced the metabolites **3** (10 mg), **4** (5 mg) and **5** (6 mg).

***16β, 17β-Dihydroxy-6-methylene-androsta-1,4-diene-3-one* (3)** Amorphous material; $[\alpha]^{25}_D$: +181.6 (*c* = 0.032, MeOH); IR (KBr): ν_{max} 3388, 1658 cm^{-1}; UV (MeOH): λ_{max} nm (log ε) 249 (4.03); ^{1}H- and ^{13}C-NMR: see Tables 1, and 2 (Additional file 2).

***17β-Hydroxy-6-methylene-androsta-1,4-diene-3,16-dione* (4)** Amorphous material; $[\alpha]^{25}_D$: -56.0 (*c* = 0.043, MeOH); IR (KBr): ν_{max} 3411, 1749, 1658 cm^{-1}; UV (MeOH): λ_{max} nm (log ε) 247 (4.04); ^{1}H- and ^{13}C-NMR: see Tables 1 and 2 (Additional file 3).

***17β-Hydroxy-6-methylene-androsta-1,4-diene-3-one* (5)** Amorphous material; $[\alpha]^{25}_D$: +174.5 (*c* = 0.046, MeOH); IR (KBr): ν_{max} 3421, 1657, cm^{-1}; UV (MeOH): λ_{max} nm (log ε) 248 (4.24); ^{1}H- and ^{13}C-NMR: see Tables 1 and 2 (Additional file 4).

Cell Viability Assay

The cytotoxicity of metabolites **1**-**5** were determined by using MTT-based colorimetric assay in 96-well plate [23]. Both cell lines (PC-3 and HeLa) were cultured in DMEM and MEM media, respectively, in 25 cm^3 tissue culture flasks. The media were supplemented with FBS (5%), pencillin (100 IU/mL) and streptomycin (100 mg/mL). The flasks were then incubated at 37°C in an incubator containing 5% CO_2. The flask (80% confluence) was processed for MTT-based cytotoxicity assay. The percent viability of the cells was monitored by trypan blue dye. The cells with clear cytoplasm were considered viable. For the assay, the cells (1×10^5) were loaded onto 96-well tissue culture treated plate. The plate was incubated for 24 hours at 37°C. After incubation, the cells were treated with different concentrations (1.56-50 μM dissolved in DMSO) of compounds **1**-**5** and kept in an incubator for 48 hours at 37°C. At the end of the incubation, the MTT dye (50 μL, 2 mg/mL) was added to each well and the plate was incubated for 4 hours at 37°C in an incubator. Following incubation, the insoluble formazan crystals were dissolved by adding DMSO (100 μL).

The following formula was used to analyze the cytotoxic effects of the compounds.

$$\%\ Inhibition = 100 - [(Absorbance\ of\ test\ compound - Absorbance\ of\ blank)/(Absorbance\ of\ control - Absorbance\ of\ blank) \times 100]$$

Additional files

Additional file 1: Spectroscopic data of metabolite 2. Include spectra of EI-MS, UV, IR, ^{1}H-NMR, ^{13}C-NMR (BB, DEPT-135°, DEPT-90°), HMQC, HMBC, COSY-45°, NOESY experiments.

Additional file 2: Spectroscopic data of metabolite 3. Include spectra of EI-MS, UV, IR, ^{1}H-NMR, ^{13}C-NMR (BB, DEPT-135°, DEPT-90°), HSQC, HMBC, COSY-45°, NOESY experiments.

Additional file 3: Spectroscopic data of metabolite 4. Include spectra of EI-MS, UV, IR, ^{1}H-NMR, ^{13}C-NMR (BB, DEPT-135°, DEPT-90°), HMQC, HMBC, COSY-45°, NOESY, experiments.

Additional file 4: Spectroscopic data of metabolite 5. Include spectra of EI-MS, UV, IR, ^{1}H-NMR, ^{13}C-NMR (BB, DEPT-135°, DEPT-90°), HSQC, HMBC, COSY-45°, NOESY experiments.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
EB, MIC, AR, DF, and CM participated in experimental strategy design, supervision and manuscript writing. MB and MAI carried out the experiments. AW performed NMR experiments, while SAS carried out the biological screenings. All authors read and approved the final manuscript.

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