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Alpha-glucosidase and tyrosinase inhibitors from fungal hydroxylation of tibolone and hydroxytibolones

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ABSTRACT

Sixteen new and one known metabolites **4**–**20** were obtained by incubation of tibolone (**1**) and hydroxytibolones (**2** and **3**) with various fungi. Their structures were elucidated by means of a homo and heteronuclear 2D NMR and by HREI-MS techniques. The relative stereochemistry was deduced by 2D NOESY experiment. Metabolites of tibolone (**1**) exhibited significant inhibitory activities against α-glucosidase and tyrosinase enzymes. Hydroxylations at C-6, C-10, C-11, C-15 positions and α,β-unsaturation at C-1/C-2, C-4/C-5 showed potent inhibitory activities against these enzymes.

1. Introduction

Microbial transformation is an effective tool to synthesize many steroidal drugs with potential biological activities [1–9]. Such studies are primarily useful in the generation of hydoxylated metabolites for drug toxicity studies [11,12]. Fungi, bacteria and yeast have been utilized successfully as *in vitro* models to mimic and predicts the metabolic fate of drugs and other xenobiotics in mammalian systems [10,12–14]. Previously, many biotransform studies on various 17α-ethynyl steroids had been carried out with various fungal and bacterial strains, which afforded hydroxylation at various positions [9,15–17].

Tibolone (**1**) is a synthetic steroid that combines oestrogenic and progestogenic properties with androgenic property, which mimic the action of a male sex hormone [18]. The *in vivo* metabolism of tibolone (**1**) in human had been studied and three metabolites, 3β-hydroxytibolone (**2**), 3α-hydroxytibolone (**3**), and Δ^4-tibolone (**6**) were identified [19–24].

In the current study, tibolone (**1**) was subjected to microbial transformation, and difference between microbial transformation and human metabolism was studied. The fungal transformation of **1** led to novel hydroxylation at various positions (metabolites **4**–**20**), in which only metabolite **6** was reported as a metabolite in human metabolism [19–24]. Our research group has been focusing on the structural modifications of bioactive natural products by using microorganisms, in order to obtain potent inhibitors of various enzymes [1–9,25–37]. Tibolone (**1**) is used effectively for the treatment of menopausal symptoms and in the prevention of osteoporosis, as a hormone replacement therapy (HRT). The hormone replacement therapy (HRT) effects on glucose metabolism in non-diabetic obese postmenopausal women [38–43]. Compound **1** when incubated with *Rhizopus stolonifer*, *Fusarium lini*, *Cunninghamella elegans* and *Gibberella fujikuroi*, resulted in the formation of a library of hydroxyl derivatives. These hydroxytibolones were then evaluated for inhibition of α-glucosidase and tyrosinase enzymes.α-Glucosidase (EC 3.2.1.20) is a typical *exo*-type glycosidase that catalyze the releases of α-glucosides from the no reducing end side of the substrates [44]. Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels. Tyrosinase (EC 1.14.18.1) is a multifunctional, copper-containing enzyme widely distributed in plants and animals. Tyrosinase inhibitors are clinically useful for the treatment of some dermatological disorders, associated with melanin hyper pigmentation [45].

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We are reporting here a new class of tyrosinase and α-glucosidase inhibitors, i.e. 17α-ethynyl steroids, as transformed products of compound **1**. These compounds exhibited various level of activity against the enzymes, tyrosinase and α-glucosidase in comparison to the standard inhibitors.

2. Experimental

2.1. General

Melting points were determined on a Yanaco MP-S3 apparatus. UV spectra were measured on a Shimadzu UV 240 spectrophotometer. IR spectra were recorded on a JASCO A-302 spectrophotometer in $CHCl_3$. ^1H- and ^{13}C NMR spectra were recorded on a Bruker Avance AM-400 spectrometer with tetramethylsilane (TMS) as an internal standard. 2D NMR spectra were recorded on a Bruker Avance AMX 500 NMR spectrometer. Optical rotations were measured on JASCO DIP-360 digital polarimeter by using 10 cm cell tube. Mass spectra (EI and HREI-MS) were measured in an electron impact mode on Varian MAT 12 or MAT 312 spectrometers and ions are given in *m/z* (%). TLC was performed on a pre-coated silica gel card (E. Merck), spots were viewed with ultraviolet light at 254 nm for florescence quenching spots and at 366 nm for fluorescent spot and stained by spraying with a solution of ceric sulphate in 10% H_2SO_4. For column chromatography, silica gel (E. Merck, 230–400 mesh). Tibolone (**1**) was extracted from Livial-Organon using dichloromethane.

2.2. Fungi and culture conditions

Microbial cultures of the *F. lini* (NRRL 68751), *R. stolonifer* (TSY 0471), *C. elegans* (TSY 0865) and *G. fujikuroi* were grown on Sabouraud-4% glucose-agar (Merck) at 25 °C and stored at 4 °C. *R. stolonifer* (TSY 0471) medium was prepared by adding glucose (100 g), peptone (25 g), KH_2PO_4 (25 g) and yeast extract (15 g) into distilled water (4 L) and pH was maintained at 5.6. *F. lini* (NRRL 68751) and *C. elegans* (TSY 0865) media were prepared by mixing the following ingredients into distilled H_2O (3.0 L) in each case: glucose (30.0 g), glycerol (30.0 g), peptone (15.0 g), yeast extract (15.0 g), KH_2PO_4 (15.0 g), and NaCl (15.0 g). *G. fujikuroi* medium was prepared by adding the following ingredients into distilled H_2O (3.0 L): 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). The Gibberella trace element solution 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 NH_4 molybdate (0.01 g) into distilled water (100 mL).

2.3. General fermentation and extraction conditions

The fungal media were transferred into 250 mL conical flasks (100 mL each) and autoclaved at 121° C. Seed flasks were prepared from 3-day-old slant and fermentation was allowed for 2 days on a shaker at 25° C. The remaining flasks were inoculated from seed flasks. After 2 days, tibolone (**1**) was dissolved in acetone and transferred in each flask (15 mg/0.5 mL) and the flasks were placed on a rotatory shaker (128 rpm) at 22° C for fermentation period. The time course study was carried out after 2 days and the transformation was analyzed on TLC. The culture media were filtered and extracted with CH_2Cl_2. The extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure and the brown gummy crude was analyzed by thin layer chromatography.

2.4. Fermentation of tibolone (**1**) with R. stolonifer (TSY-0471)

Compound **1** (500 mg), dissolved in 15 mL acetone and distributed among 40 flasks, and kept for fermentation. All the media were filtered after 3 days and extracted with dichloromethane and evaporated under reduced pressure to finally obtain a brown thick extract (0.90 g), and the transformed metabolites were isolated by using column chromatography. Metabolite **4** (20 mg) was eluted with pet. ether and EtOAc (60:40), compound **5** (17 mg) with pet. ether–EtOAc (58:42) and compound **6** (40 mg) with pet. ether–EtOAc (55:45). There were more transformed products in crude mixture but due to the insufficient quantities, we did not able to determine the structure of these metabolites.

2.4.1. 6β-Hydroxytibolone (**4**)

White amorphous solid (20 mg); mp 186–188 °C; $[\alpha]^{25}_D$ −17 (*c* 0.35, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204 (3.7) nm; IR ($CHCl_3$) ν_{max} 3381, 2150, 1705, 1668, 1043 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 3; EI MS *m/z* (rel. int. %) 328 (M^+, 6), 309 (5), 241 (16), 226 (9), 169 (14), 149 (23), 138 (28), 121 (28), 109 (23), 107 (100), 97 (20), 93 (21), 81 (26), 71 (22), 69 (41), 55 (64); HREI MS *m/z* 328.2171 (calcd for $C_{21}H_{28}O_3$, 328.2143).

2.4.2. 15β-Hydroxytibolone (**5**)

White solid (17 mg); mp 202–205 °C; $[\alpha]^{25}_D$ +16 (*c* 0.31, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203.4 (3.4) nm; IR ($CHCl_3$) ν_{max} 3383, 2162, 1708, 1663, 1050 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 3; EI MS *m/z* (rel. int. %) 328 (M^+, 3), 312 (100), 245 (27), 229 (36), 203 (17), 189 (14), 187 (17), 174 (24), 161 (28), 149 (26), 135 (24), 121 (25), 96 (38), 81 (23), 69 (21), 67 (24), 55 (59); HREI MS *m/z* 328.2070 (calcd for $C_{21}H_{28}O_3$, 328.2038).

2.4.3. Δ^4-Tibolone (**6**)

Crystalline solid (40 mg); mp 206–208 °C; $[\alpha]^{25}_D$ −145 (*c* 0.21, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 235 (3.2) nm; IR ($CHCl_3$) ν_{max} 3402, 2150, 1687, 1667, 1017 cm^{-1}; ^1H NMR data in $CDCl_3$, Table 1; ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.2 (C-1), 38.3 (C-2), 198.1 (C-3), 126.4 (C-4), 161.4 (C-5), 47.2 (C-8), 45.4 (C-9), 38.2 (C-10), 26.2 (C-11), 79.1 (C-17), 87.5 (C-20), 74.3 (C-21); EI MS *m/z* (rel. int. %) 312 (M^+, 34,), 245 (53), 229 (33), 187 (17), 173 (18), 161 (20), 147 (28), 135 (56), 121 (23), 109 (32), 107 (43), 105 (39), 95 (24), 91 (62), 81 (34), 79 (59), 67 (58), 55 (100); HREI MS *m/z* 312.2023 (calcd for $C_{21}H_{28}O_2$, 312.2089).

2.5. Fermentation of tibolone (**1**) by F. lini (NRRL 68751) and C. elegans (TSY 0865)

Compound **1** (600 mg), dissolved in 18 mL acetone and distributed among 50 flasks, was kept for fermentation, for 6 days and then filtrates were extracted with dichloromethane and evaporated under reduced pressure to afford a brown thick extract (1.02 g). Column chromatography was used for the separation of metabolites **7**–**10** from *C. elegans* crude, while *F. lini* yielded one major metabolite **6**. Compound **7** (6.2 mg) was eluted with pet. ether–EtOAc (42:58), compound **8** (15.5 mg) with pet. ether–EtOAc (38:62), **9** (5.2 mg) with pet. ether–EtOAc (40:60), whereas compound **10** (10.2 mg) with pet. ether–EtOAc (30:70).

2.5.1. $\Delta^{1,4}$-Tibolone (**7**)

Amorphous solid (6.2 mg); mp 196–200 °C; $[\alpha]^{25}_D$ −172 (*c* 0.32, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 241.5 (4.1) nm; IR ($CHCl_3$) ν_{max} 3303, 2154, 1691, 1666, 1044 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 3; EI MS *m/z* (rel. int. %) 310 (M^+, 42), 241 (100), 230 (11), 199 (17), 187 (27), 161 (22), 149 (34), 145 (17), 119 (18), 109

Table 1
^{1}H NMR data of compounds **4–12** (400 MHz; $CDCl_3$).

No.	4	5	6	7	8	9	10	11	12
1	2.26, 2.32 m	2.16, 2.24 m	1.86, 2.02 m	7.12, dd (8.4, 4.7)	2.36, 2.29 m	2.33, 2.27 m	2.33, 2.27 m	1.82, 2.02 m	1.89, 2.04 m
2	2.28, 2.36 m	2.26, 2.30 m	2.21, 2.28 m	6.62 dd (8.4, 2.6)	2.25, 2.17 m	2.24, 2.20 m	2.24, 2.20 m	2.21, 2.29 m	2.20, 2.27 m
3	–	–	–	–	–	–	–	–	–
4	2.72, 2.70 m	2.69, 2.71 m	5.82 *s*	6.52 d (2.6)	5.77 *s*	2.70, 2.69 m	2.06, 2.14 m	2.48, 2.50 m	5.87 *s*
5	–	–	–	–	–	–	–	–	–
6	4.04 d (4.0)	2.01, 2.12 m	2.18, 2.26 m	2.15, 2.25	2.08, 2.14 m	2.06, 2.14 m	5.42 d (4.2)	5.33 d (4.7)	4.05 d (4.04)
7	1.99 m	1.54 m	1.61 m	1.60 m	1.59 m	1.61 m	1.83 m	1.85 m	1.99 m
8	1.71 m	1.69 m	1.64 m	1.62 m	1.67 m	1.59 m	1.59 m	1.61 m	1.83 m
9	1.66 m	1.48 m	1.51 m	1.59 m	1.72 m	1.62 m	1.62 m	1.45 m	1.68 m
10	–	–	2.31 m	2.42 m	–	–	2.46 m	2.34 m	2.45 m
11	1.81, 2.24 m	1.89, 2.22 m	1.84, 2.23 m	1.82, 2.26 m	1.88, 2.28 m	3.43 ddd (15.1, 11.1, 5.04)	3.40 ddd (15.3, 11.04, 4.57)	1.96, 2.26 m	1.89, 2.24 m
12	1.51, 1.73 m	1.54, 1.71 m	1.59, 1.75 m	1.55, 1.71 m	1.63, 1.70 m	2.05, 1.51 m	2.01, 1.51 m	1.64, 1.65 m	1.54, 1.68 m
13	–	–	–	–	–	–	–	–	–
14	1.67 m	1.85 m	1.66 m	1.65 m	1.66 m	1.80 m	1.85 m	1.62 m	1.65 m
15	1.48, 1.58 m	4.06 m ($W_{1/2}$ ~ 10.8)	1.40, 1.61 m	1.36, 1.68 m	1.38, 1.65 m	4.10 m ($W_{1/2}$ ~ 8.82)	3.91 m ($W_{1/2}$ ~ 9.9)	1.39, 1.66 m	1.46, 1.56 m
16	2.22, 2.25 m	1.7, 2.24 m	2.37, 1.87 m	2.23, 1.80 m	1.99, 2.28 m	2.01, 1.55 m	2.01, 1.55 m	1.79, 2.27 m	1.99, 2.29 m
17	–	–	–	–	–	–	–	–	–
18	0.84 *s*	1.08 *s*	0.90 *s*	0.86 *s*	0.90 *s*	0.90 *s*	0.94 *s*	0.99 *s*	0.93 *s*
19	0.76 d (7.01)	0.86 d (7.02)	0.77 d (7.1)	0.82 d (7.01)	0.75 d (7.19)	0.75 d (7.2)	0.73 d (7.3)	0.86 d (7.1)	0.75 d (7.1)
20	–	–	–	–	–	–	–	–	–
21	2.55 *s*	2.57 *s*	2.56 *s*	2.58 *s*	2.56 *s*	2.56 *s*	2.56 *s*	2.53 *s*	2.56 *s*

(14), 91 (62), 67 (62), 55 (100); HREI MS *m*/*z* 310.2004 (calcd for $C_{21}H_{26}O_2$, 310.2011).

2.5.2. 10β-Hydroxy-Δ^4-tibolone (**8**)

White powdered solid (15.5 mg); mp 198–201 °C; $[\alpha]^{25}_D$ +12 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 239 (2.9) nm; IR ($CHCl_3$) ν_{max} 3345, 2149, 1698, 1649, 1018 cm^{-1}; ^{1}H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 3; EI MS *m*/*z* (rel. int. %) 328 (M^+, 13), 310 (14), 229 (20), 187 (25), 171 (26), 161 (25), 149 (48), 136 (32), 124 (55), 109 (44), 107 (43), 91 (55), 83 (28), 67 (47), 57 (50), 55 (100); HREI MS *m*/*z* 328.2090 (calcd for $C_{21}H_{28}O_3$, 328.2123).

2.5.3. 11α,15β-Dihydroxytibolone (**9**)

White powdered solid (5.2 mg); mp 208–210 °C; $[\alpha]^{25}_D$ −81 (*c* 0.24, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203 (3.3) nm; IR ($CHCl_3$) ν_{max} 3342, 2142, 1718, 1636, 1028 cm^{-1}; ^{1}H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 3; EI MS *m*/*z* (rel. int. %) 344 (M^+, 13), 310 (14), 229 (20), 187 (25), 171 (26), 161 (25), 149 (48), 136 (32), 124 (55), 109 (44), 107 (43), 91 (55), 83 (28), 67 (47), 57 (50), 55 (100); HREI MS *m*/*z* 344.2212 (calcd for $C_{21}H_{28}O_4$, 344.2234).

2.5.4. 11α,15β-Dihydroxy–Δ^5–tibolone (**10**)

White powdered solid (10.2 mg); mp 207–211 °C; $[\alpha]^{25}_D$ +27 (*c* 0.28, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 202.4 (3.4) nm; IR ($CHCl_3$) ν_{max} 3312, 2102, 1722, 1652, 1057 cm^{-1}; ^{1}H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 3; EI MS *m*/*z* (rel. int. %) 344 (M^+, 8), 310 (14), 229 (20), 187 (25), 171 (26), 161 (54), 149 (87), 136 (32), 124 (55), 109 (44), 107 (43), 91 (55), 83 (28), 67 (74), 57 (50), 55 (100); HREI MS *m*/*z* 344.2341 (calcd for $C_{21}H_{28}O_4$, 344.2316).

2.6. Fermentation of tibolone (**1**) by G. fujikuroi (ATCC 10704)

Compound **1** (850 mg) was dissolved in 20 mL acetone and distributed among 30 flasks for fermentation for 12 days. After fermentation, media was extracted with dichloromethane and evaporated to get a crude extract (1.22 g). Column chromatography was used for the separation of metabolites **11**–**16** from crude extract. There were more transformed products in crude mixture but due to the insufficient quantities, we did not able to determine the structure of these metabolites. Metabolite **11** (5.6 mg) was eluted with pet. ether–EtOAc (54:46), compound **12** (10.2 mg) with pet. ether–EtOAc (50:50), compound **13** (11.2 mg) with pet. ether–EtOAc (45:55), compound **14** (8.3 mg) with pet. ether–EtOAc (38:58), compound **15** (9.5 mg) with pet. ether–EtOAc (30:70) and compound **16** (10.3 mg) with pet. ether–EtOAc (35:75).

2.6.1. Δ^5-Tibolone (**11**)

White powdered solid (5.6 mg); mp 201–205 °C; $[\alpha]^{25}_D$ − 17 (*c* 0.20, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 203.6 (3.9) nm; IR ($CHCl_3$) ν_{max} 3341, 2157, 1728, 1642, 1088 cm^{-1}; ^{1}H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 3; EI MS *m*/*z* (rel. int. %) 312 (M^+, 42), 242 (100), 227 (63), 187 (27), 161 (22), 149 (34), 124 (26), 91 (21), 67 (23), 55 (100); HREI MS *m*/*z* 312.1456 (calcd for $C_{21}H_{28}O_2$, 312.1513).

2.6.2. 6β-Hydroxy-Δ^4-tibolone (**12**)

White powdered solid (10.2 mg); mp 189–93 °C; $[\alpha]^{25}_D$ −101 (*c* 0.41, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 239.5 (2.1) nm; IR ($CHCl_3$) ν_{max} 3332, 2128, 1698, 1651, 1063 cm^{-1}; ^{1}H and ^{13}C NMR data in $CDCl_3$, Tables 1 and 3; EI MS *m*/*z* (rel. int. %) 328 (M^+, 13), 312 (13), 245 (17), 229 (34), 189 (17), 187 (17), 161 (28), 149 (49), 121 (45), 91 (38), 69 (35), 67 (36), 55 (100); HREI MS *m*/*z* 328.2176 (calcd for $C_{21}H_{28}O_3$, 328.2116).

2.6.3. 6α-Hydroxy-Δ^4-tibolone (**13**)

White powdered solid (11.2 mg); mp 187–190 °C; $[\alpha]^{25}_D$ + 66.2 (*c* 0.35, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 240.1 (3.7) nm; IR ($CHCl_3$)

ν_{max} 3342, 2106, 1683, 1659, 1061 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, Tables 2 and 3; EI MS *m/z* (rel. int. %) 328 (M^+, 56), 312 (11), 245 (45), 229 (6), 201 (17), 187 (14), 171 (20), 161 (34), 149 (57), 135 (24), 121 (46), 91 (34), 81 (34), 67 (4), 55 (59); HREI MS *m/z* 328.2132 (calcd for $C_{21}H_{28}O_3$, 328.2205).

2.6.4. 15α-Hydroxy-Δ^4-tibolone (**14**)

White powdered solid (8.3 mg); mp 203–205 °C; $[\alpha]^{25}_D$ −21.3 (*c* 0.34, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 237.6 (2.6) nm; IR ($CHCl_3$) ν_{max} 3401, 2176, 1691, 1662, 1060 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, Tables 2 and 4; EI MS *m/z* (rel. int. %) 328 (M^+, 3), 312 (100), 245 (27), 229 (36), 203 (17), 189 (10), 187 (17), 174 (14), 161 (56), 149 (26), 135 (5), 121 (67), 96 (38), 81 (23), 69 (15), 67 (34), 55 (51); HREI MS *m/z* 328.2212 (calcd for $C_{21}H_{28}O_3$, 328.2283).

2.6.5. 6α-Hydroxy-Δ^{1,4}-tibolone (**15**)

White powdered solid (9.5 mg); mp 193–196 °C; $[\alpha]^{25}_D$ +44 (*c* 0.25, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 244 (3.7) nm; IR ($CHCl_3$) ν_{max} 3441, 2123, 1680, 1637, 1045 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, Tables 2 and 4; EI MS *m/z* (rel. int. %) 326 (M^+, 13), 312 (34), 245 (27), 229 (67), 203 (5), 189 (14), 187 (14), 174 (24), 161 (56), 149 (26), 135 (24), 121 (100), 96 (45), 81 (23), 69 (12), 67 (24), 55 (34); HREI MS *m/z* 326.2231 (calcd for $C_{21}H_{28}O_3$, 326.2292).

2.6.6. 6β-Methoxy-Δ^4-tibolone (**16**)

White powdered solid (10.3 mg); mp 206–209 °C; $[\alpha]^{25}_D$ −18 (*c* 0.42, $CHCl_3$,); UV (MeOH) λ_{max} (log ε) 242.1 (3.1) nm; IR ($CHCl_3$) ν_{max} 3323, 2153, 1691, 1661, 1101 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, Tables 2 and 4; EI MS *m/z* (rel. int. %) 343 (M^+, 6), 312 (17), 245 (27), 229 (32), 203 (17), 189 (14), 187 (17), 174 (25), 161 (28), 149 (26), 135 (24), 121 (25), 91 (100), 81 (23), 69 (21), 67 (23), 55 (45); HREI MS *m/z* 343.2356 (calcd for $C_{22}H_{30}O_3$, 343.2338).

2.7. Reduction of tibolone (**1**) with sodium borohydride

Compound **1** (1 g) was dissolved in dry dichloromethane (100 mL) and cooled in an ice bath to 0 °C. Sodium borohydride (500 mg) was added to the solution in portions whilst stirring. The mixture was stirred at room temperature for 5 h. Acetic acid (50 mL) was by destroyed the excess sodium borohydride ($NaBH_4$) and the dichloromethane was removed in *vacuo*. Water (100 mL) was added to the resulting oily product, and the suspension was extracted with ethyl acetate (1 L). The ethyl acetate extract was washed with sodium hydrogen carbonate solution (300 mL), water and brine. The removal of the solvent on a rotary evaporator afforded crude extract (1.2 g). Column chromatography was used for the separation of these hydroxytibolones **2** and **3** [47]. Compound **2** (300 mg) was eluted with pet. ether–EtOAc (50:50) and compound **3** (400 mg) was eluted with pet. ether–EtOAc (48:52).

2.8. Fermentation of 3β-hydroxytibolone (**2**) by C. elegans (TSY 0865)

Compound **2** (300 mg), dissolved in 15 mL acetone and distributed among 40 flasks, was kept for fermentation. Fermentation was continued for 12 days and then filtrates were extracted with CH_2Cl_2 and evaporated under reduced pressure to afford brown thick crude (0.81 g). Column chromatography was used for the separation of one hydroxyl metabolites **17** from crude extract. Compound **17** (4.2 mg) was eluted with pet. ether–EtOAc (40:60).

Table 2
^1H NMR data of compounds **13–20** (400 MHz; $CDCl_3$).

No.	13	14	15	16	17	18	19	20
1	1.96, 2.08 m	2.15, 2.26 m	7.08 dd (8.18, 4.2)	2.04, 2.16 m	1.82, 2.02 m	1.89, 2.04 m	1.96, 2.08 m	1.82, 2.02 m
2	2.11, 2.35 m	2.25, 2.28 m	6.76 d (8.22)	2.27, 2.36 m	2.21, 2.29 m	2.20, 2.27 m	2.11, 2.35 m	2.21, 2.29 m
3	–	–	–	–	3.86 m ($W_{1/2}$ = 18.6)	4.04 m ($W_{1/2}$ = 10.7)	4.08 m ($W_{1/2}$ = 7.1)	4.2 m ($W_{1/2}$ = 10.0)
4	5.77 *s*	5.81 *s*	5.77 *s*	5.77 *s*	2.48, 2.50 m	2.40, 2.52 m	5.56 br.*s*	5.38 br.*s*
5	–	–	–	–	–	–	–	–
6	3.60 d (6.4)	1.97, 2.14 m	3.81 d (6.4),	3.93 d (3.9), 3.47 *s* (OCH_3)	3.90 d (3.5)	5.38 d (4.5)	4.13 br.*s*	1.97, 2.14 m
7	1.98 m	1.96 m	1.95 m	1.91 m	1.98 m	1.9 m	1.91 m	1.85 m
8	1.70 m	1.76 m	1.65m	1.73 m	1.61 m	1.83 m	1.70 m	1.61 m
9	1.66 m	1.68 m	1.70 m	1.67 m	1.45 m	1.68 m	1.66 m	1.45 m
10	2.55 m	2.46 m	2.43 m	2.41 m	2.34 m	2.45 m	2.55 m	2.34 m
11	1.88, 2.25 m	1.86, 2.27 m	1.87, 2.25 m	1.82, 2.28 m	1.96, 2.26 m	1.89, 2.24 m	1.88, 2.25 m	3.80 ddd (13.5, 9.5, 3.3)
12	1.61, 1.65 m	1.64, 1.66 m	1.61, 1.74 m	1.62, 1.69 m	1.64, 1.65 m	1.54, 1.68 m	1.61, 1.65 m	1.64, 2.1 m
13	–	–	–	–	–	–	–	
14	1.67 m	1.72 m	1.64 m	1.65 m	1.62 m	1.65 m	1.67 m	1.62 m
15	1.40, 1.55 m	4.02 m ($W_{1/2}$ ~ 14.9)	1.38, 1.55 m	1.44, 1.54 m	1.39, 1.66 m	1.46, 1.56 m	1.40, 1.55 m	1.39, 1.66 m
16	1.92, 2.28 m	1.72, 2.21 m	1.99, 2.27 m	1.98, 2.31 m	1.79, 2.27 m	1.99, 2.29 m	1.92, 2.28 m	1.79, 2.27 m
17	–	–	–	–	–	–	–	–
18	0.90 *s*	0.84 *s*	0.90 *s*	0.90 *s*	0.87 *s*	1.04 *s*	1.06 *s*	1.04 *s*
19	0.77 d (7)	0.72 d (7.2)	0.77 d (7.02)	0.77 d (7.0)	0.73 d (7.1)	0.89 d (8.4)	0.87 d (7.0)	0.88 d (7.2)
20	–	–	–	–	–	–	–	
21	2.55 *s*	2.55 *s*	2.57 *s*	2.55 *s*	2.55 *s*	2.58 *s*	2.56 *s*	2.57 *s*

Table 3
^{13}C NMR data of compounds **4**–**13** (100 and 125 MHz; $CDCl_3$).

No. of C	4	5	7	8	9	10	11	12	13
1	25.5 *(t)*	25.2 *(t)*	153.4 *(d)*	30.6 *(t)*	24.7 *(t)*	24.5 *(t)*	24.9 *(t)*	26.7 *(t)*	25.2 *(t)*
2	38.9 *(t)*	38.9 *(t)*	127.0 *(d)*	33.8 *(t)*	38.1 *(t)*	38.1 *(t)*	37.6 *(t)*	36.6 *(t)*	38.8 *(t)*
3	199.5 *(s)*	200.1 *(s)*	199.4 *(d)*	198.7 *(s)*	198.7 *(s)*	198.7 (s)	199.1 *(s)*	199.8 *(s)*	198.5 *(s)*
4	33.1 *(t)*	43.6 *(t)*	125.2 *(d)*	126.7 *(d)*	42.1 *(t)*	41.2 *(t)*	40.9 *(t)*	126.5 *(d)*	126.7 *(d)*
5	122.5 *(s)*	123.0 *(s)*	162.1 *(s)*	162.2 *(s)*	122.5 *(s)*	162.2 *(s)*	162.1 *(s)*	164.8 *(s)*	163.9 *(s)*
6	65.9 *(d)*	31.5 *(t)*	30.7 *(t)*	31.9 *(t)*	31.8 *(t)*	124.1 *(d)*	123.4 *(d)*	65.9 *(d)*	70.0 *(d)*
7	41.6 *(d)*	32.1 *(d)*	32.9 *(d)*	33.5 *(d)*	33.5 *(d)*	33.6 *(d)*	34.6 *(d)*	42.0 *(d)*	40.0 *(d)*
8	39.4 *(d)*	40.4 *(d)*	43.5 *(d)*	38.1 *(d)*	40.0 *(d)*	39.7 *(d)*	44.2 *(d)*	39.1 *(d)*	38.1 *(d)*
9	46.2 *(d)*	46.3 *(d)*	46.4 *(d)*	53.7 *(d)*	58.8 *(d)*	58.1 *(d)*	45.9 *(d)*	46.0 *(d)*	46.2 *(d)*
10	128.4 *(s)*	128.2 *(s)*	43.0 *(d)*	70.3 *(s)*	126.2 *(s)*	38.8 *(d)*	43.4 *(d)*	38.7 *(d)*	39.7 *(d)*
11	29.8 *(t)*	29.7 *(t)*	29.7 *(t)*	22.9 *(t)*	66.1 *(d)*	66.3 *(d)*	31.9 *(t)*	29.8 *(t)*	29.7 *(t)*
12	32.9 *(t)*	32.6 *(t)*	32.4 *(t)*	33.0 *(t)*	47.5 *(t)*	45.1 *(t)*	33.1 *(t)*	32.3 *(t)*	33.6 *(t)*
13	47.4 *(s)*	45.6 *(s)*	46.0 *(s)*	45.9 *(s)*	40.9 *(s)*	40.1 *(s)*	46.2 *(s)*	46.3 *(s)*	46.5 *(s)*
14	48.6 *(d)*	51.2 *(d)*	47.2 *(d)*	49.7 *(d)*	49.7 *(d)*	49.2 *(d)*	48.7 *(d)*	46.9 *(d)*	45.9 *(d)*
15	222.2 *(t)*	65.5 *(d)*	22.6 *(t)*	22.4 *(t)*	65.4 *(d)*	67.0 *(d)*	22.4 *(t)*	22.2 *(t)*	22.4 *(t)*
16	38.9 *(t)*	48.6 *(t)*	39.0 *(t)*	39.2 *(t)*	45.1 *(t)*	44.9 *(t)*	39.8 *(t)*	39.5 *(t)*	39.9 *(t)*
17	79.9 *(s)*	79.1 *(s)*	79.9 *(s)*	79.6 *(s)*	79.6 *(s)*	79.5 *(s)*	79.1 *(s)*	79.7 *(s)*	79.2 *(s)*
18	13.1 *(q)*	13.1 *(q)*	13.2 *(q)*	12.4 *(q)*	12.4 *(q)*	12.4 *(q)*	13.0 *(q)*	12.8 *(q)*	12.4 *(q)*
19	12.5 *(q)*	13.0 *(q)*	12.7 *(q)*	12.2 *(q)*	12.2 *(q)*	12.2 *(q)*	12.5 *(q)*	12.6 *(q)*	12.2 *(q)*
20	87.6 *(s)*	87.4 *(s)*	87.6 *(s)*	87.2 *(s)*	87.6 *(s)*	87.2 *(s)*	87.5 *(s)*	87.3 *(s)*	87.2 *(s)*
21	73.8 *(d)*	74.1 *(d)*	74.0 *(d)*	74.1 *(d)*	74.6 *(d)*	74.1 *(d)*	73.9 *(d)*	74.2 *(d)*	74.1 *(d)*

Multiplicities were assigned from DEPT experiments in parentheses: *s*: quaternary, *d*: CH, *t*: CH_2, and *q*: Me C-atoms.

2.8.1. 3β,6β-Dihydroxytibolone (**17**)

White powdered solid (4.2 mg); mp 206–209 °C; $[\alpha]^{25}_D$ +105 (*c* 0.31, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 201 (3.1) nm; IR ($CHCl_3$) ν_{max} 3353, 2164, 1663, 1078 cm^{-1}; ^{1}H and ^{13}C NMR data in $CDCl_3$, Tables 2 and 4; EI MS *m*/*z* (rel. int. %) 330 (M^+, 7), 312 (17), 245 (27), 229 (32), 203 (17), 189 (14), 187 (17), 174 (25), 161 (28), 149 (26), 135 (24), 121 (25), 91 (100), 81 (23), 69 (21), 67 (23), 55 (45); HREI MS *m*/*z* 330.2346 (calcd for $C_{21}H_{32}O_3$, 328.2374).

2.9. Fermentation of 3α-hydroxytibolone (**3**) by C. elegans (TSY 0865)

Compound **3** (400 mg), dissolved in 18 mL acetone and distributed among 50 flasks, was kept for fermentation. Fermentation was continued for 12 days and then filtrates were extracted with dichloromethane and evaporated under reduced pressure to afford a brown thick crude (0.89 g). Column chromatography was used for the separation of hydroxy metabolites **18**–**20** from crude extract. Compound **18** (4.6 mg) was eluted with pet. ether–EtOAc (40:60). Metabolite **19** (4.2 mg) was eluted with pet. ether–EtOAc (54:46) and compound **20** (5.1 mg) with pet. ether–EtOAc (50:50).

2.9.1. 3α-Hydroxy-Δ^5-tibolone (**18**)

White powdered solid (4.6 mg); mp 206–207 °C; $[\alpha]^{25}_D$ +52.2 (*c* 0.23, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 204.2 (4.0) nm; IR ($CHCl_3$) ν_{max} 3313, 2154, 1646, 1038 cm^{-1}; ^{1}H and ^{13}C NMR data in $CDCl_3$, Tables 2 and 4; EI MS *m*/*z* (rel. int. %) 314 (M^+, 8), 312 (17), 245 (27), 229 (32), 203 (17), 189 (14), 187 (17), 174 (25), 161 (28), 149 (21), 135 (24), 121 (23), 91 (11), 81 (23), 69 (21), 67 (21), 55 (45); HREI MS: *m*/*z* 314.2541 (calcd for $C_{21}H_{32}O_2$, 314.2597).

2.9.2. 3α,6β-Dihydroxy-Δ^4-tibolone (**19**)

White powdered solid (4.2 mg); mp 201–205 °C; $[\alpha]^{25}_D$ −18 (*c* 0.31, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 201.2 (2.9) nm; IR ($CHCl_3$) ν_{max} 3323, 2156, 1665, 1118 cm^{-1}; ^{1}H and ^{13}C NMR data in $CDCl_3$, Tables 2 and 4; EI MS *m*/*z* (rel. int. %) 330 (M^+, 6), 312 (17), 245 (27),

Table 4
^{13}C NMR data of compounds **14**–**20** (100 and 125 MHz; $CDCl_3$).

No. of C	14	15	16	17	18	19	20
1	25.3 *(t)*	154.2 *(d)*	25.0 *(t)*	25.1 *(t)*	25.1 *(t)*	25.1 *(t)*	25.2 *(t)*
2	38.8 *(t)*	126.5 *(d)*	38.7 *(t)*	38.8 *(t)*	38.4 *(t)*	38.2 *(t)*	38.8 *(t)*
3	199.5 *(s)*	198.9 *(s)*	198.7 *(s)*	67.0 *(d)*	78.0 *(d)*	78.0 *(d)*	78.1 *(d)*
4	126.5 *(d)*	125.7 *(d)*	126.7 *(d)*	40.2 *(t)*	40.4 *(d)*	124.0 *(t)*	126.0 *(t)*
5	164.9 *(s)*	161.9 *(s)*	162.2 *(s)*	122 *(s)*	147.0 *(s)*	147.0 *(s)*	147.0 *(s)*
6	30.7 *(t)*	69.8 *(d)*	70.2 *(d)*, 57.6 (OMe)	74.2 *(d)*	132.0 *(d)*	74.0 *(d)*	31.9 *(t)*
7	33.1 *(d)*	39.0 *(d)*	38.7 *(d)*	43.3 *(d)*	39.1 *(d)*	43.2 *(d)*	33.2 *(d)*
8	39.7 *(d)*	38.1 *(d)*	39.1 *(d)*	38.3 *(d)*	43.2 *(d)*	39.1 *(d)*	41.2 *(d)*
9	46.0 *(d)*	45.8 *(d)*	45.9 *(d)*	46.2 *(d)*	46.2 *(d)*	46.2 *(d)*	51.0 *(d)*
10	43.5 *(d)*	39.3 *(d)*	44.7 *(d)*	127 *(d)*	43.0 *(d)*	44.2 *(s)*	44.2 *(d)*
11	29.9 *(t)*	29.2 *(t)*	29.6 *(t)*	29.7 *(t)*	29.0 *(t)*	29.7 *(d)*	66.3 *(d)*
12	32.3 *(t)*	31.9 *(t)*	32.9 *(t)*	33.6 *(t)*	33.6 *(t)*	33.6 *(t)*	36.6 *(t)*
13	45.2 *(s)*	46.3 *(s)*	46.6 *(s)*	46.5 *(s)*	46.5 *(s)*	46.1 *(s)*	46.5 *(s)*
14	52.3 *(d)*	46.5 *(d)*	46.2 *(d)*	45.9 *(d)*	45.9 *(d)*	45.9 *(d)*	45.9 *(d)*
15	65.9 *(d)*	22.0 *(t)*	22.4 *(t)*	22.4 *(t)*	22.4 *(t)*	22.5 *(d)*	22.4 *(d)*
16	46.9 *(t)*	38.9 *(t)*	39.6 *(t)*	39.9 *(t)*	39.9 *(t)*	39.9 *(t)*	39.9 *(t)*
17	79.9 *(s)*	79.3 *(s)*	79.6 *(s)*	79.2 *(s)*	79.1 *(s)*	79.1 *(s)*	79.1 *(s)*
18	13.0 *(q)*	12.9 *(q)*	12.4 *(q)*	15.1 *(q)*	15.1 *(q)*	15.1 *(q)*	15.2 *(q)*
19	12.8 *(q)*	12.2 *(q)*	12.2 *(q)*	14.2 *(q)*	14.2 *(q)*	14.1 *(q)*	13.9 *(q)*
20	87.7 *(s)*	87.8 *(s)*	87.2 *(s)*	87.2 *(s)*	87.3 *(s)*	87.0 *(s)*	87.4 *(s)*
21	73.8 *(d)*	73.7 *(d)*	74.2 *(d)*	74.0 *(d)*	74.2 *(d)*	74.2 *(d)*	74.1 *(d)*

Multiplicities were assigned from DEPT experiments in parentheses: *s*: quaternary, *d*: CH, *t*: CH_2, and *q*: Me C-atoms.

229 (32), 203 (17), 189 (14), 187 (14), 174 (25), 161 (21), 149 (26), 135 (22), 121 (25), 91 (100), 81 (23), 69 (21), 67 (23), 55 (45); HREI MS *m/z* 330.2356 (calcd for $C_{21}H_{32}O_3$, 330.2338).

*2.9.3. 3α,11α-Dihydroxy-Δ⁴-tibolone (**20**)*

White powdered solid (5.1 mg); mp 199–201 °C; $[\alpha]^{25}_D$ +61 (*c* 0.45, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 202.6 (3.5) nm; IR ($CHCl_3$) ν_{max} 3323, 2143, 1668, 1105 cm^{-1}; ^1H and ^{13}C NMR data in $CDCl_3$, Tables 2 and 4; EI MS *m/z* (rel. int. %) 330 (M^+, 4), 312 (14), 245 (27), 229 (42), 203 (17), 189 (34), 187 (17), 174 (19), 161 (28), 149 (26), 135 (35), 121 (25), 91 (98), 81 (23), 69 (67), 67 (23), 55 (51); HREI MS *m/z* 330.2256 (calcd for $C_{21}H_{32}O_3$, 330.2238).

2.10. Material and methods for tyrosinase inhibition assay

Tyrosinase inhibition assays were performed in 96-well microplate format using the SpectraMax 340 microplate reader (Molecular Devices, CA, USA) according to the method developed by Hearing (1987) [48]. Briefly, first the compounds were screened for the *o*-diphenolase inhibitory activity of tyrosinase using L-DOPA as the substrate. All the active inhibitors from the preliminary screening were subjected to IC_{50} studies. Compounds were dissolved in methanol to a concentration of 2.5%. 30 units of mushroom tyrosinase (28 nM from Sigma Chemical Co., USA), first preincubated with the compounds in 50 nM Na-phosphate buffer (pH 6.8) for 10 min at 25 °C. Then, the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm (at 37 °C) due to the formation of the DOPA chrome for 10 min. The percent inhibition of the enzyme was calculated as follows, by using MS Excel®™ 2000 (Microsoft Corp., USA) based program developed for this purpose:

$$\text{Percent inhibition} = \left[\frac{B-S}{S}\right] \times 100$$

where *B* and *S* are absorbances for the blank and samples, respectively. After screening, the compounds median inhibitory concentration (IC_{50}) was also calculated. All the studies have been carried out at least in triplicates and the results represent the mean ± S.E.M. (standard error of the mean). Kojic acid and L-mimosine were used as standard inhibitors for the tyrosinase, and both of them were purchased from Sigma Chem. Co., USA [48].

2.11. α-Glucosidase enzyme inhibition assay

α-Glucosidase (E.C.3.2.1.20) enzyme inhibition assay has been performed according to the slightly modified method of Matsui et al. α-Glucosidase (E.C.3.2.1.20) from *Saccharomyces* sp. was purchased from Wako Pure Chemical Industries Ltd. (Wako 076-02841). The inhibition was measured spectrophotometrically at pH 6.9 and at 37 °C using 0.7 mM *p*-nitrophenyl α-D glucopyranoside (PNP-G) as a substrate and 250 m units/mL enzyme in 50 mM sodium phosphate buffer containing 100 mM NaCl. 1-Deoxynojirimycin (0.425 mM) and acarbose (0.78 mM) were used as positive controls. The increment in absorption at 400 nm due to the hydrolysis of PNP-G by α-glucosidase was monitored continuously with the spectrophotometer (Molecular Devices, CA, USA) [49].

2.11.1. Estimation of IC_{50} values

The concentrations of the test compounds, which inhibited the hydrolysis of PNP-G by α-glucosidase by 50% (IC_{50}), were determined by monitoring the effect of increasing the concentration of these compounds on the inhibition values. The IC_{50} values were then calculated by using EZ-Fit enzyme kinetics program (Perrella Scientific Inc., Amherst, MA, USA).

3. Results and discussion

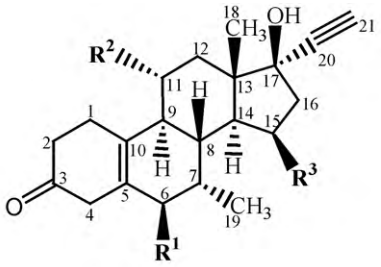
Fermentation of tibolone (**1**) with *R. stolonifer* (TSY 0471) yielded two new mono-hydroxylated metabolites, **4** and **5**, and a known metabolite **6** (Fig. 1). The HREI MS of metabolite **4** exhibited the molecular ion (M^+) at *m/z* 328.2171, corresponding to the formula $C_{21}H_{28}O_3$, which indicated that a new oxygen functionality was introduced during fermentation period. The IR absorptions were attributed to hydroxyl (3381 cm^{-1}) and carbonyl (1705 cm^{-1}) functionalities. The ^1H NMR spectrum, when compared with the substrate (Table 1), showed a new signal of OH-bearing methine proton at δ 4.04, resonating as a doublet ($J_{6e,7e}$ = 4.0 Hz) with its corresponding carbon at δ 65.9 in ^{13}C NMR spectrum, which was assigned to C-6 on the basis of HMBC correlations of H-6 (δ 4.04) with C-5 (δ 122.5) and C-10 (δ 128.4) (Table 3). In the COSY 45° spectrum, the aforementioned methine proton showed correlation with the C-7 methine proton (δ_H 2.0). The stereochemistry of C-6 hydroxyl group was determined to be *axial* by the NOESY correlations between H-6 (δ 4.04) and H-19 (δ 0.76). The above spectral data indicated that new metabolite **4** has an –OH group at C-6 position, as compared to compound **1**.

The HREI MS of metabolite **5** showed the M^+ at *m/z* 328.2070 ($C_{21}H_{28}O_3$), indicating an increment of 16 mass units as compared to compound **1**. The ^1H and ^{13}C NMR data of **5** revealed the presence of a new OH-bearing methine group, that resonated at δ_H 4.06 (m, $W_{1/2} \sim 10.8$ Hz) and δ_C 65.5 and deduced to be C-15 on the basis of HMBC of C-16 protons (δ_H 2.24, 1.7) and C-14 methine proton (δ_H 1.85) with C-15 (δ_C 65.5) (Tables 1 and 3). The stereochemistry of C-15 hydroxyl group was deduced as β on the basis of NOESY correlations between H-15 (δ_H 4.06) and H-14 (δ_H 1.85). From these spectral data, the new compound **5** was identified as 7α-methyl-17α-ethynl-15β,17β-dihydroxy-19-norandrost-5(10)-en-3-one.

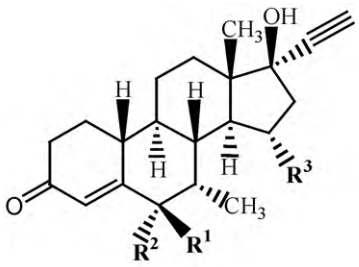
The incubation of compound **1** with *F. lini* (NRRL 68751) for 6 days also led to the isolation of a UV active metabolite **6** with exhibiting the M^+ at *m/z* 312.2023 in HREI MS spectrum ($C_{21}H_{28}O_2$). The ^1H NMR spectrum showed a singlet for an olefinic proton at δ 5.82. Its broad-band decoupled ^{13}C NMR spectrum showed, in comparison with that of the substrate **1**, the disappearance of one quaternary carbon signal resonating at δ 128.2 for C-10 and appearance of an olefinic methine carbon at δ 126.4 which was assigned to the C-4 on the basis of HMQC spectrum, indicating the migration of the C-5/C-10 double bond to C-4/C-5 (Tables 1 and 3). This indicated α,β-unsaturated ketone moiety in metabolite **6**. The *axial* orientation of C-10 methine proton was assigned on the basis of NOESY coupling between H-10 (δ 2.31) and H-8 (δ 1.64) (Fig. 2). The above spectral data supported the structure of metabolite **6** as 7α-methyl-17α-ethynl-17β-hydroxy-19-norandrost-4-en-3-one, previously obtained from human metabolism of tibolone [19].

The incubation of **1** (600 mg) with *C. elegans* (TSY 0865) for 6 days yielded metabolites **7**–**10** (Fig. 1). The HREI MS of compound **7** showed the M^+ at *m/z* 310.2004, in accordance with the formula $C_{21}H_{26}O_2$. Comparison of the ^1H and ^{13}C NMR data of compounds **6** and **7**, metabolite **7** showed the presence of two additional olefinic proton signals at δ 7.12 (dd, $J_{1,2}$ = 8.4 Hz, $J_{1,10}$ = 4.7 Hz, H-1) and 6.62 (dd, $J_{2,1}$ = 8.4 Hz, $J_{2,4}$ = 2.6 Hz, H-2) with the corresponding carbons resonated at δ 153.4 (C-1) and 127.0 (C-2), respectively (Tables 1 and 3). The presence of a double bond between C-1 and C-2 was deduced from COSY 45° correlations between H-1 (δ 7.12), H-2 (δ 6.62) and H-10 (δ 2.42). While HMBC interactions of H-1 with C-2 (δ 127.0) and C-10 (δ 43.0) were observed. The structure of metabolite **7** was finally deduced to be 7α-methyl-17α-ethynl-17β-hydroxy-19-norandrost-1,4-dien-3-one.

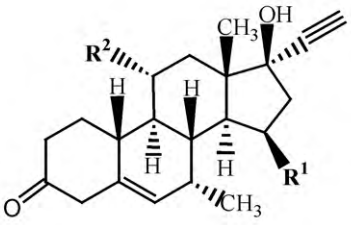
The HREI MS of metabolite **8** showed the M^+ at *m/z* 328.2090, in agreement with the formula $C_{21}H_{28}O_3$ indicating an introduction of a new oxygen in the molecule, probably as OH. The ^{13}C



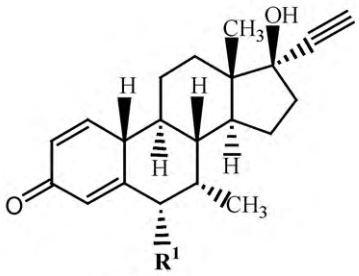
1. $R^1 = R^2 = R^3 = H$
4. $R^1 = OH, R^2 = R^3 = H$
5. $R^1 = R^2 = H, R^3 = OH$
9. $R^1 = H, R^2 = R^3 = OH$



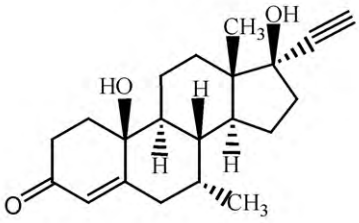
6. $R^1 = R^2 = R^3 = H$
12. $R^1 = OH, R^2 = R^3 = H$
13. $R^1 = R^3 = H, R^2 = OH$
14. $R^1 = R^2 = H, R^3 = OH$
16. $R^1 = OCH_3, R^2 = H, R^3 = H$



10. $R^1 = R^2 = OH$
11. $R^1 = R^2 = H$



7. $R^1 = H$
15. $R^1 = OH$



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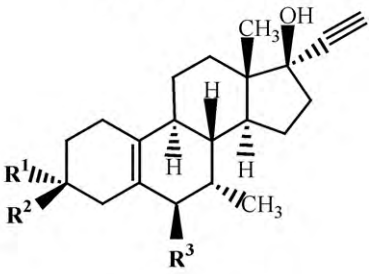
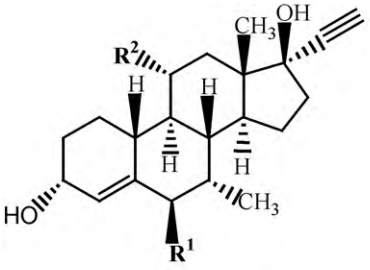


2. $R^1 = R^3 = H, R^2 = OH$
3. $R^1 = OH, R^2 = R^3 = H$
17. $R^1 = H, R^2 = R^3 = OH$



19. $R^1 = OH, R^2 = H$
20. $R^1 = H, R^2 = OH$

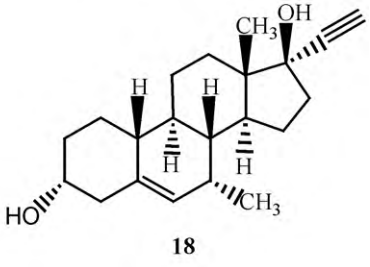


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Fig. 1. Structures of substrates and their metabolites **1**–**20**.

NMR spectrum showed a downfield oxygen-bearing quaternary carbon resonated at δ 70.3, which was assigned to C-10 through its HMBC interactions with H-1 (δ 2.36, 2.29) and H-4 (δ 5.77) (Tables 1 and 3). The 10β-hydroxylation was also deduced by the β-SCS (substituents chemical shift) of −4.5, −9.1 and −5.3 ppm for C-2, C-8 and C-11, respectively, and by the downfield shifts of C-1 and C-9 (+5.4 and +8.3, respectively) with respect to the ^{13}C NMR chemical shifts in compounds **6** and **8** [16]. The spectral data supported the structure of a new metabolite **8** as 7α-methyl-17α-ethynl-10β,17β-dihydroxy-19-norandrost-4-en-3-one.

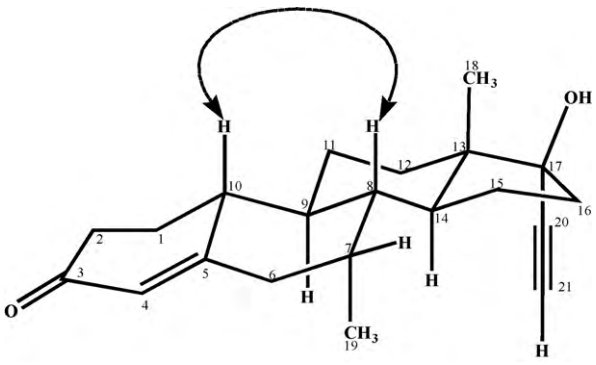

Fig. 2. Key correlations of compound **6** in NOESY spectrum.

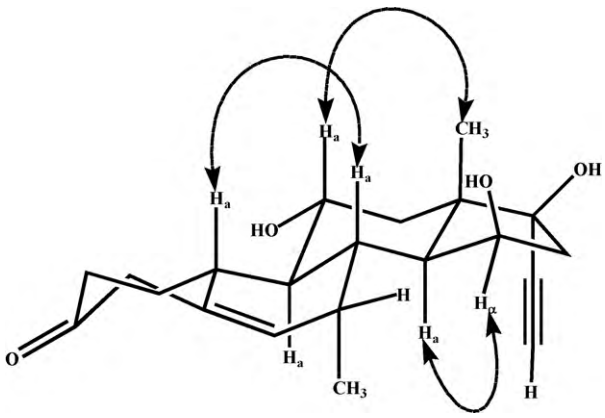

Fig. 4. Key correlations of compound **10** in NOESY spectrum.

The HREI MS of metabolite **9** showed the M^+ at *m/z* 344.2212, supporting the formula $C_{21}H_{28}O_4$, indicated that two oxygen had been incorporated into the molecule, as compared to **1**. The ^1H and ^{13}C NMR displayed two OH-bearing methine groups at δ_H 3.43 (ddd, $J_{11e,9a}$ = 15.1 Hz, $J_{11a,12a}$ = 11.1 Hz, $J_{11a,12e}$ = 5.0 Hz); and 4.10 (m, $W_{1/2} \sim 8.82$ Hz) and δ_C 66.1 and 65.4, respectively (Tables 1 and 3). The COSY 45° spectrum showed correlations of H-11 (δ 3.43) with H-9 (δ 1.62) and H_2-12 (δ 2.05, 1.51), and of H-15 (δ 4.10) with H-14 (δ 1.80) and H_2-16 (δ 2.01, 1.55). Hydroxylations at C-11 and C-15 was further supported by HMBC correlations of H_2-12 (δ 2.05, 1.51) and Me-18 (δ 0.90) with C-11 (δ 66.1), and correlation of H-14 (δ 1.80) with δ 65.4 (C-15). The axial orientation of C-11 proton was deduced on the basis of NOESY correlation of H-11 (δ 3.43) with Me-18 (δ 0.90) and multiplicity of H-11 (δ_H 3.43, ddd, $J_{11e,9a}$ = 15.1 Hz, $J_{11a,12a}$ = 11.1 Hz, $J_{11a,12e}$ = 5.0 Hz) [6], while β-stereochemistry of the newly introduced OH group at C-15 was deduced by the NOESY correlations between H-14 (δ 1.80) and H-15 (δ 4.10) (Fig. 3). According to this spectral data, the structure was deduced to be 7α-methyl-17α-ethynl-11α,15β,17β-trihydroxy-19-norandrost-5(10)-en-3-one.

The HREI MS of metabolite **10** showed the M^+ at *m/z* 344.2341, supporting the formula $C_{21}H_{28}O_4$, with an increment of 32 a.m.u. The UV spectrum showed a weak absorption at 202 nm, while IR showed absorptions at 3312 (OH), 1722 (C=O) and 1652 (C=C) cm^{-1}. The ^1H NMR spectrum showed an upfield doublet for an olefinic methine proton at δ 5.42 ($J_{6,7e}$ = 4.2 Hz, H-6), which showed COSY 45° correlations with H-7 (δ 1.83). Two additional OH-bearing methine protons at δ 3.40 (ddd, $J_{11e,9a}$ = 15.3 Hz, $J_{11a,12a}$ = 11.0 Hz, $J_{11a,12e}$ = 4.75 Hz) and 3.91 (m, $W_{1/2} \sim 9.9$ Hz) were unambiguously assigned to H-11 and H-15, respecting through 2D NMR and ^{13}C NMR spectra (Tables 1 and 3). The stereochemistry of C-11 OH was deduced to be α (*equatorial*) on the basis NOESY between H-11 (δ 3.40) and H-18 (δ 0.94) and larger coupling constants ($J_{11e,9a}$ = 15.3 Hz) of H-11 signal [6]. The β-orientation of the OH group at C-15 was deduced on the basis of NOESY between H-14 (δ 1.85) and H-15 (δ 3.91) (Fig. 4). The β-orientation (*axial*) of C-10 proton was deduced through NOESY cross peaks between H-10 (δ 2.46) and H-8 (δ 1.59) (Fig. 4). Based on the above mentioned spectral data, the structure was deduced as 7α-methyl-17α-ethynl-11α,15β,17β-trihydroxy-19-norandrost-5-en-3-one.

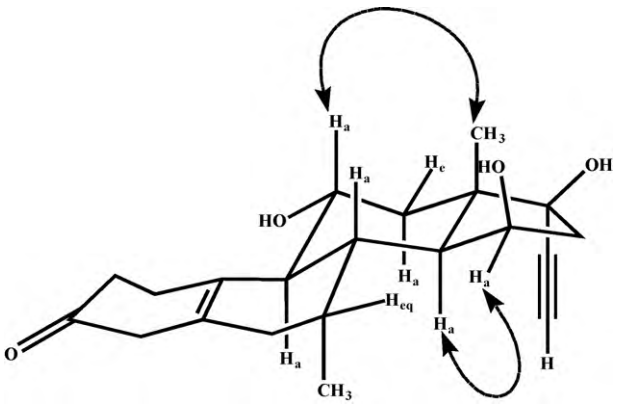

Fig. 3. Key correlations of compound **9** in NOESY spectrum.

Tibolone (**1**) was fermented with *G. fujikuroi* (ATCC 10704) for 12 days yielding six new metabolites **11**–**16** (Fig. 1). The HREI MS of metabolite **11** showed the M^+ at *m/z* 312.1456, with corresponding formula $C_{21}H_{28}O_2$. The ^1H NMR spectrum showed an upfield doublet of olefinic proton at δ 5.33 ($J_{6,7e}$ = 4.7 Hz), which was assigned to H-6 through its COSY 45° correlation with H-7 (δ 1.85). The ^{13}C NMR spectrum showed downfield methine carbon at δ 123.4 (Tables 1 and 3). A double bond between C-5 and C-6 was deduced through HMBC between H-6 (δ 5.33) and C-7 (δ 34.6). The structure was thus deduced as 7α-methyl-17α-ethynl-17β-hydroxy-19-norandrost-5-en-3-one.

Metabolites **12** and **13** were found to be epimers and differentiated on the basis of ^1H NMR and NOESY experiments. The ^1H NMR spectrum of metabolite **12** displayed a doublet at δ 4.05 ($J_{6e,7e}$ = 4.0 Hz), while **13** exhibited a doublet at δ 3.60 ($J_{6a,7e}$ = 6.4 Hz) (Tables 1 and 2). The ^{13}C NMR spectra of both the isomers **12** and **13** showed OH-bearing methine carbons resonating at δ 65.9 and 70.0, respectively (Table 3). The position of the newly introduced hydroxyl at C-6 in both isomers was inferred from the HMBC coupling. The relative configuration of the new hydroxyl group at C-6 in compound **12** was inferred on the basis of coupling pattern and NOESY correlations between H-6 (δ 4.05) and C-19 methyl protons (δ 0.75), while NOESY spectrum of compound **13** displayed correlations between H-6 (δ 3.60) and C-7 methine proton (δ 1.98). The above spectral data concluded that metabolites **12** and **13** have an –OH group at C-6 position with different orientations.

The molecular formula of metabolite **14** was deduced as $C_{21}H_{28}O_3$ by HREI MS (*m/z* 328.2132). The ^1H and ^{13}C NMR spectra of compound **14** exhibited a OH-bearing methine group, resonated at δ_H 4.02 (m, $W_{1/2} \sim 14.9$ Hz); δ_C 65.9 was unambiguously assigned to C-15 on the basis of two-dimensional NMR experiments (Tables 2 and 4). In the COSY 45° spectrum, the aforementioned methine proton showed correlation with the C-14 methine proton (δ_H 1.72). This was further supported by the HMBC of C-14 proton (δ_H 1.72) with C-15 (δ_C 65.9). The α-relative configuration of OH group at C-15 was deduced on the basis of NOESY correlations between H-15 (δ 4.02) and C-18 methyl protons (δ 0.84) (Fig. 5). This spectral data led to the structure **14** as 7α-methyl-17α-ethynl-15α,17β-dihydroxy-19-norandrost-4-en-3-one.

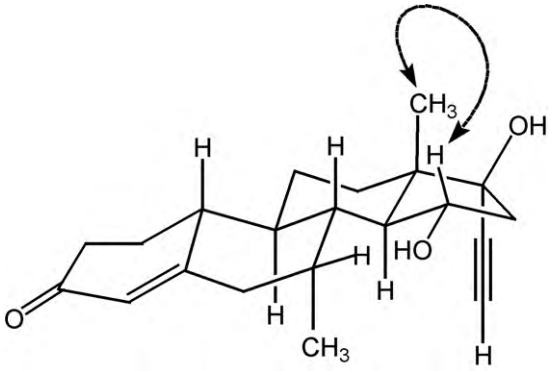

Fig. 5. Key correlations of compound **14** in NOESY spectrum.

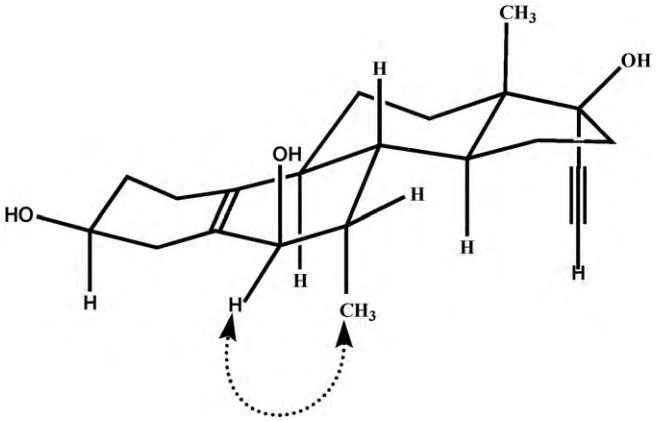

Fig. 6. Key correlations of compound **17** in NOESY spectrum.

The HREI MS of metabolite **15** showed the M^+ at *m/z* 326.2231 ($C_{21}H_{28}O_3$). Two additional doublets in the ^1H NMR spectrum for two olefinic protons appeared at δ 7.08 (dd, $J_{1,2}$ = 8.2 Hz, $J_{1,10}$ = 4.2 Hz) and 6.76 (d, $J_{2,1}$ = 8.2 Hz) and one new hydroxymethine proton (δ 3.81, d, $J_{6a,7e}$ = 6.4 Hz). The DEPT spectrum of compound **15** showed two olefinic methine carbon signals at δ 154.2 and 126.5, corresponding to a double bond between C-1/C-2, as deduced on the basis of HMBC of H-1 (δ 7.08) with C-2 (δ 126.5) and of H-2 (δ 6.76) with C-1 (δ 154.2) and C-4 (δ 125.7) (Tables 2 and 4). One OH-bearing methine carbon resonated at δ 69.8 was identified as C-6 based on ^3J correlations between H-6 (δ 3.81) and C-4 (δ 125.7). The α-orientation of newly hydroxyl group at C-6 was deduced to be *equatorial* on the basis of NOESY correlations between H-6 (δ 3.81) and H-7 (δ 1.95). According to these spectral studies, the structure of metabolite **15** was deduced as 7α−methyl-17α-ethynl-6α,17β-dihydroxy-19-norandrost-1,4-dien-3-one.

The HREI MS of metabolite **16** showed the M^+ at *m/z* 343.2356 corresponding to the formula $C_{22}H_{30}O_3$. The ^1H NMR spectrum showed the presence of a methoxy singlet at δ 3.47, while geminal methoxy protons were resonated at δ 3.93 (d, $J_{6e,7e}$ = 3.9 Hz). The ^{13}C NMR spectrum showed a methoxy carbon signal at δ 57.6 and a methoxy-bearing carbon at δ 70.2 (Tables 2 and 4). The position of the methoxy group at C-6 was deduced through HMBC of H-6 (δ 3.93) with C-4 (δ 126.7). The β-orientation of the newly introduced OCH_3 group at C-6 was deduced on the basis of NOESY correlations between H-6 (δ 3.93) and C-19 methyl protons (δ 0.77). The metabolite **16** was thus identified as 7α-methyl-17α-ethynl-6β-methoxy-17β-hydroxy-19-norandrost-4-en-3-one.

In order to check the effect on hydroxylation at various positions in presence of C-3 hydroxyl group (α and β isomers) tibolone (**1**) (1 g) was reduced with $NaBH_4$ in dichloromethane yielded reduced products, i.e. 3β-hydroxytibolone (**2**) and 3α-hydroxytibolone (**3**) [47]. The hydroxyl group at C-3 promoted the hydroxylation at C-6 as in case of metabolites **17** and **19** and better yield of these metabolites was produced as compared to tibolone (**1**). The ^1H NMR spectra of both the isomers **2** and **3** showed OH-bearing geminal methine protons at δ 3.80 (m, $W_{1/2} \sim 21.5$ Hz) and 4.04 (m, $W_{1/2} \sim 10.7$ Hz), which indicated the reduction of the C-3 ketonic group. The orientation of C-3 methine proton in the both isomers **2** and **3** was deduced from the multiplicity of C-3 proton signals resonated at δ 3.80 (m, $W_{1/2} \sim 21.5$ Hz, H-3α) and 4.04 (m, $W_{1/2} \sim 10.7$ Hz, H-3β), respectively [46].

Incubation of 3β-hydroxytibolone (**2**) (300 mg) with *C. elegans* for 12 days yielded a hydroxyl-bearing metabolite **17** (Fig. 1). Metabolite **17** showed the M^+ at *m/z* 330.2346 corresponding to the formula $C_{21}H_{30}O_3$ by HREI MS. The ^1H NMR spectrum showed a resonance for OH-bearing methine proton at δ 3.90 (d, $J_{6e,7e}$ = 3.5 Hz), with corresponding carbon at δ 74.2 (Tables 2 and 4). The position of the new hydroxyl group at C-6 was deduced through HMBC interactions. The α (*equatorial*) orientation of C-6 proton, geminal to the hydroxyl group, was deduced on the basis of NOESY between H-6 (δ 3.90) and C-19 methyl protons (δ 0.73) (Fig. 6). The structure of metabolite **17** was identified as 7α-methyl-17α-ethynl-3β,6β,17β-trihydroxy-19-norandrost-5(10)-en-3-one.

Fermentation of 3α-hydroxytibolone (**3**) (400 mg) with *C. elegans* for 12 days yielded three polar metabolites **18**–**20** (Fig. 1). Metabolite **18** showed the M^+ at *m/z* 314.2541 ($C_{21}H_{30}O_2$) in HREI MS, while the ^1H and ^{13}C NMR spectra showed an olefinic proton at δ 5.38 (d, $J_{6,7e}$ = 4.5 Hz) with corresponding carbon at δ 132.0, as compared to substrate **3**, and was assigned to C-6 methine carbon (Tables 2 and 4). The β (*axial*) orientation of C-10 proton was deduced on the basis of NOESY correlations. From these data, the new compound **18** was identified as 7α-methyl-17α-ethynl-3α,17β-dihydroxy-19-norandrost-5-en-3-one.

The HREI MS of metabolite **19** showed the M^+ at *m/z* 330.2356 corresponding to the formula $C_{21}H_{30}O_3$. The ^1H NMR spectrum of **19** showed an additional OH-bearing methine proton at δ 4.13 (br. s), with corresponding methine carbon at δ 74.0 (C-6) (Tables 2 and 4). The COSY 45° spectrum also showed an allylic coupling between H-4 and H-6 (δ 4.13). The α (*equatorial*) orientation of C-6 proton, geminal to OH, was deduced on the basis NOESY correlations between H-6 and Me-19 protons (δ 0.87). The structure of metabolite **19** was identified as 7α-methyl-17α-ethynl-3α,6β,17β-trihydroxy-19-norandrost-4-en-3-one.

Metabolite **20** showed the M^+ at *m/z* 330.2256 in HREI MS in agreement with the formula $C_{21}H_{30}O_3$. The ^1H and ^{13}C NMR spectra of **20** showed an OH-bearing methine proton at δ_H 3.80 (ddd, $J_{11e,9a}$ = 13.5 Hz, $J_{11a,12a}$ = 9.5 Hz, $J_{11a,12e}$ = 3.3 Hz) and δ_C 66.3 and assigned C-11 through COSY between H-11 (δ 3.80), H-18 (δ 1.04) and H_2-12 (δ 2.1, 1.64). The HMBC correlations revealed the connectivities between H-11, H-18 (δ 1.04) and C-11 (δ 66.3) (Tables 2 and 4). The β (*axial*) orientation of C-11 proton, geminal to an OH, was deduced on the basis of larger coupling constant of H-11 signal (δ_H 3.82, ddd, $J_{11e,9a}$ = 13.5 Hz, $J_{11a,12a}$ = 9.5 Hz, $J_{11a,12e}$ = 3.3 Hz) and NOESY correlations between H-11 and Me-18 protons (δ 0.88) (Fig. 7) [6]. The structure of metabolite **20** was proposed as 7α-methyl-17α-ethynl-3α,11α,17β-trihydroxy-19-norandrost-4-en-3-one.

Some hydroxy metabolites of tibolone (**1**) showed significant inhibitory activity against enzyme tyrosinase, the OH at C-6, C-11 and C-15 as in metabolites **4**, **9** and **10**, respectively showed pronounced inhibitory activity the against enzyme, while α,β-unsaturated system (C-4/C-5) in metabolite **6** also showed a good activity (Table 6).

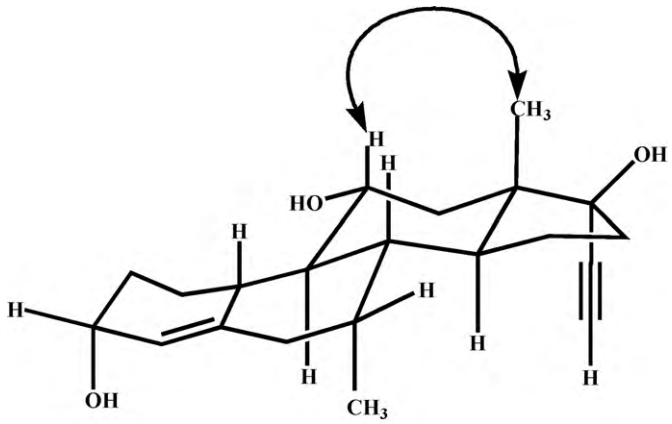

Fig. 7. Key correlations of compound **20** in NOESY spectrum.

Table 5
α-Glucosidase inhibitory activities of tibolone (1) and its analogs **4**–**16**, as compared to the reference inhibitors.

Compounds	$IC_{50} \pm$ S.E.M.[a] (in μM)
1	NA[b]
4	225.0 ± 0.00
5	NA[b]
6	343.0 ± 0.00
7	227.0 ± 0.02
8	877.0 ± 0.03
9	NA[b]
10	NA[b]
11	NA[b]
12	653.0 ± 0.02
13	<70.0
14	<70.0
15	340.0 ± 0.02
16	NA[b]
Deoxynojirimycin[c]	425.6 ± 8.14
Acarbose[c]	780.0 ± 0.28

Metabolites **17**–**20** were not tested due to insufficient quantities.
[a] S.E.M. is the standard error of the mean.
[b] The inactive compounds.
[c] The standard inhibitors of the α-glucosidase.

These metabolites also showed significant inhibitory activity against α-glucosidase enzymes (Table 5). The metabolites **4**, **6**, **7**, **8**, **12**, **13**, **14** and **15**, which contains unsaturation in ring A and hydroxylations at C-6, C-10 and C-15 positions were found to be potent inhibitors of α-glucosidase enzyme, while metabolites **10** and **11** containing unsaturation in ring B and hydroxylations at C-11 and C-15 in metabolites **9** and **10** showed no inhibitory activities against α-glucosidase enzyme. This concluded that both unsaturation in ring A and hydroxylations in ring A and hydroxylations in ring B enhanced the inhibition of α-glucosidase enzyme.

Table 6
Tyrosinase inhibitory activities of tibolone (**1**) and its analogs **4**–**16**, as compared to the reference inhibitors.

Compounds	$IC_{50} \pm$ S.E.M.[a] (in μM)
1	NA[b]
4	7.45 ± 0.21
5	50.86 ± 0.36
6	8.19 ± 0.54
7	NA[b]
8	119.44 ± 0.20
9	5.12 ± 0.67
10	7.10 ± 0.13
11	NA[b]
12	36.14 ± 0.21
13	NA[b]
14	NA[b]
16	25.15 ± 0.24
Kojic acid (KA)[c]	16.67 ± 0.51
L-Mimosine (LM)[c]	3.68 ± 0.022

Metabolites **15** and **17**–**20** were not tested due to insufficient quantities.
[a] S.E.M. is the standard error of the mean.
[b] The inactive compounds.
[c] The standard inhibitors of the tyrosinase.

4. Conclusions

In conclusion, the transformation of tibolone (**1**) by fungus yielded thirteen metabolites **4**–**16**. While incubation of hydroxytibolones (**2** and **3**) with *C. elegans* yielded four polar metabolites **17**–**20**. The main hydroxylations occurred in rings B and D, especially at C-6 and C-15 positions. The metabolites **4**, **5**, **9**, **10**, **12**, **13**, **14**, **15**, and **16** were identified as the main metabolites of the fermentations. Hydroxylations at C-6, C-10 and C-15 positions in metabolites **4**, **8**, **12**, **13**, **14**, and **15** showed pronounced inhibitory activity against α-glucosidase enzyme, while α,β-unsaturated system (C-4/C-5) in metabolite **6** and C-1/C-2, C-4/C-5 in metabolite **7** also showed a good inhibitory activity (Table 5). The analogs of tibolone (**1**) also exhibited a mild to potent inhibition of the enzyme tyrosinase, except compounds **7**, **11**, **13**, and **14**. Compound **9** was the most potent inhibitor of the tyrosinase (Table 6).

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References

[1] Choudhary MI, Azizuddin, Jali S, Musharraf SG, Atta-ur-Rahman. Chem Biodiv 2008;5:324–31.
[2] Choudhary MI, Nasir M, Khan SN, Atif M, Ali RA, Khalil SM, et al. Naturforschung 2007;62b:593–9.
[3] Choudhary MI, Yousuf S, Samreen, Shah SAA, Ahmed S, Atta-ur-Rahman. Chem Pharm Bull 2006;54:927–30.
[4] Choudhary MI, Atif M, Nawaz SA, Fatmi MQ, Atta-Ur-Rahman. Nat Prod Res 2006;20:1074–81.
[5] Choudhary MI, Siddiqui ZA, Musharraf SG, Nawaz SA, Atta-Ur-Rahman. Nat Prod Res 2005;19:311–7.
[6] Choudhary MI, Shah SAA, Sami A, Ajaz A, Shaheen F, Atta-ur-Rahman. Chem Biodiv 2005;2:516–24.
[7] Choudhary MI, Batool I, Shah SAA, Nawaz SA, Atta-ur-Rahman. Chem Pharm Bull 2005;53:1455–9.
[8] Choudhary MI, Sultan S, Jalil S, Anjum S, Rahman AA, Fun HK, et al. Chem Biodiv 2005;2:392–400.
[9] Choudhary MI, Sultan S, Khan MTH, Atta-ur-Rahman. Steroids 2005;70:798–802.
[10] Da-Fang Z, Lu S, Lei L, Hai-Hua H. Acta Pharmacol 2003;24:442–7.
[11] Moody JD, Zhang D, Heinze TM, Cerniglia CE. Appl Environ Microbiol 2000;66:3646–9.
[12] Abourashed EA, Clark AM, Hufford CD. Curr Med Chem 1999;6:359–74.
[13] Chatterjee P, Kauzi SA, Pezzuto JM, Hamann MT. Appl Environ Microbiol 2000;66:3850–5.
[14] Kent UM, Jushchyshyn MI, Hollenberg PF. Curr Drug Metab 2001;2:215–43.
[15] Hu SH, Tian XF, Sun YH, Han GD. Steroids 1996;61:407–10.
[16] Hu SH, Tian XF, Han GD. Steroids 1998;63:88–92.
[17] Choudhary MI, Musharraf SG, Ali RA, Atif M, Atta-ur-Rahman Z. Naturforschung 2004;59b:323–8.
[18] Ederveen AJ, Kloosterboer HJ. J Bone Miner Res 1999;14:1963–70.
[19] Kloosterboer HJ. J Steroid Biochem Mol Biol 2001;76:231–8.
[20] Vos RME, Krebbers SFM, Verhoever CHJ, Delbressine LPC. Drug Metab Dispos 2002;30:106–12.
[21] Steckelbroeck S, Jin Y, Oyesanmi B, Kloosterboer HJ, Penning TM. Mol Pharmacol 2004;66:1702–11.
[22] Helenius J, Kloosterboer HJ. Maturitas 2004;48:30–40.
[23] Yazigi R, Sahid S, Contreras L, Rodriguez T. Gynecol Oncol 2004;93:568–70.
[24] Morin-Papunen LC, Vauhkonen I, Ruokonen A, Tapanainen JS, Raudaskoski T. Eur J Endocrinol 2004;150:705–14.
[25] Choudhary MI, Siddiqui ZA, Nawaz SA. Prod Res 2009;23:507–13.
[26] Choudhary MI, Shah SAA, Atta-ur-Rahman. Nat Prod Res 2008;22:1289–96.
[27] Choudhary MI, Batool I, Shah SAA, Khan SN, Atta-Ur-Rahman. Nat Prod Res 2008;22:489–94.
[28] Choudhary MI, Musharraf SG, Khan MTH, Abdelrahman D, Parvaz M, Shaheen F, et al. Helv Chim Acta 2003;86:3450–60.

[29] Choudhary MI, Shah SAA, Musharraf SG, Shaheen F, Atta-ur-Rahman. Nat Prod Res 2003;17:215–20.
[30] Choudhary MI, Musharraf SG, Shaheen F, Atta-ur-Rahman. Nat Prod Lett 2002;16:377–82.
[31] Choudhary MI, Azizuddin, Atta-ur-Rahman. Nat Prod Lett 2002;16:101–6.
[32] Atta-ur-Rahman, Choudhary MI, Asif F, Farooq A, Yaqoob M. Nat Prod Lett 2000;14:217–24.
[33] Atta-ur-Rahman, Choudhary MI, Asif F, Farooq A, Yaqoob M, Dar A. Phytochemistry 1998;49:2341–2.
[34] Atta-ur-Rahman, Yaqoob M, Farooq A, Anjum S, Asif F, Choudhary MI. J Nat Prod 1998;61:1340–2.
[35] Atta-ur-Rahman, Choudhary MI, Shaheen F, Ashraf M, Jahan S. J Nat Prod 1998;61:428–31.
[36] Atta-ur-Rahman, Farooq A, Anjum S, Choudhary MI. Curr Org Chem 1999;3:309–12.
[37] Atta-ur-Rahman, Farooq A, Choudhary MI. J. Nat Prod 1997;60:1038–40.
[38] Crespo CJ, Smit E, Snelling A, Sempos CT, Andersen RE. Diab Care 2002;25:1675–80.
[39] Egarter C, Huber J, Haidbauer R, Pusch H, Fischl F, Putz M. Maturitas 1996;23:55–62.
[40] Castelo-Branco C, Vicente JJ, Figueras F, Sanjuan A, deOsaba MJM, Casals E, et al. Maturitas 2000;32:161–8.
[41] Kamel HK, Perry HM, Morley JE. J Am Geriatr Soc 2001;49:179–87.
[42] Iqbal MM. South Med J 2000;93:2–18.
[43] Lindsay R, Cosman F, Lobo RA, Walsh BW, Harris ST, Reagan JE, et al. J Clin Endocrinol Metab 1999;84:3076–81.
[44] Chiba S. Biosci Biotechnol Biochem 1997;61:1233–9.
[45] Shiino M, Watanabe Y, Umezawa K. Bioorg Med Chem 2001;9:1233–40.
[46] Hanson JR, Nasir H, Parvez A. Phytochemistry 1996;42:411–5.
[47] Lee P, Kitamura Y, Kaneko K, Shiro M, Xu GJ, Chen YP. Chem Pharm Bull 1988;36:4316–29.
[48] Hearing VJ. Methods Enzymol 1987;142:154–65.
[49] Ali MS, Jahangir M, Shazad-ul-Hussan S, Choudhary MI. Phytochemistry 2002;60:295–9.