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Fusarium lini Potential for the Biotransformation of Norandrostenedione and Evaluation of Urease and Chymotrypsin Properties of the Transformed Products

Simeon Pierre Chegaing Fodouop[1,2*], Alex Doris Kengni Mboussaah[2,3], Didiane Yemele Mefokou[1], Alain Bertrand Fowa[4], Mahwish Siddiqui[2,5], Gabriel T. Kamsu[4], Donatien Gatsing[4], Muhammad Iqbal Choudhary[2,5]

[1]Research Unit of Metabolic and Infectious Diseases, Department of Biomedical Sciences, Faculty of Science, University of Ngaoundéré, Ngaoundéré, Cameroon
[2]H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan
[3]Research Unit of Applied Chemistry, Department of Chemistry, Faculty of Science, University of Dschang, Dschang, Cameroon
[4]Research Unit of Microbiology and Antimicrobial Substances, Department of Biochemistry, Faculty of Science, University of Dschang, Dschang, Cameroon
[5]International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan
Email: *saafodouop@yahoo.fr

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Abstract

Several androgenic steroids have been biotransformed by fungi into metabolites with numerous biological properties. Incubation of norandrostenedione (**1**) with *Fusarium lini* NRRL 2204 was carried out for the first time, yielding two new metabolites, 3,7*β*-dihydroxy-19-norandrost-1,3,5-trien-17-one (**3**) and 6*α*,10*β*,17*β*-trihydroxy-19-nor-4-androsten-3-one (**4**), along with three known compounds, 3-hydroxy-19-norandrost-1,3,5-trien-17-one (**2**), 10*β*,17*β*-dihydroxy-19-nor-4-androsten-3-one (**5**) and 10*β*-hydroxy-19-nor-4-androsten-3,17-dione (**6**). Their structures were elucidated by extensive spectroscopic analyses, including 1D-, 2D-NMR, and HR-MS experiments. Substrate **1** and its derivatives **2**-**6** were evaluated *in vitro* for their urease and chymotrypsin inhibitory properties. Compounds **2** and **3** were found to have strong urease activity with IC_{50} = 23.7 ± 0.17 and 10.2 ± 0.28 µm, respectively, as compared to the standard drug thiourea (IC_{50} = 21.6 ± 0.12 µm). Compounds **4**, **5** and **6** showed good chymotrypsin activity with IC_{50} values of 6.4 ± 0.19, 15.6 ± 0.46 and 18.4 ± 0.65 µm, respectively, as compared to standard chymostatin with IC_{50} = 5.7 ± 0.14 µm. These transformed metabolites may form the basis for the future development of new drugs against ulcer, inflammation, bacterial and viral diseases.

Keywords

Chymotrypsin Inhibition, Norandrostenedione, *Fusarium lini*, Urease Inhibition

1. Introduction

Increased microbial resistance to pharmaceuticals has prompted researchers to move towards the use of naturally occurring antimicrobial substances. Despite the effectiveness of natural substances in the treatment of diseases, the limited amount of natural resources forces researchers to seek alternative sources to obtain new active compounds. Several studies have shown that bioactive molecules can be biotransformed into other compounds with new biological properties [1] [2] [3]. Biotransformations are organic reactions using biological catalysts. They are used in many different conditions such as free enzymes or whole cells. Biocatalysts can be used for regio- and stereo-selective reactions, or to introduce chirality in a way that would be very difficult, if not impossible, with classical chemical syntheses [4]. A number of studies have shown that several androgenic steroids have been biotransformed by fungi into metabolites with numerous biological properties [5] [6] [7]. Because of their stereochemistry (presence of many asymmetric centres and inert hydrocarbon skeletons), the hydroxylation, oxidation and epoxidation of steroids are difficult tasks for organic chemists. However, these reactions are readily possible by appropriate biotransformations [5].

Norandrostenedione is an anabolic steroid taken as dietary supplements by athletes who want to build up muscle mass and aggression. 19-norandrostenedione refers to two steroid isomers that were once marketed as dietary supplements and mainly used by bodybuilders. They can also be taken by people who are HIV-positive and others with muscle atrophy caused by illness. When taken either orally or by injection, norandrostenedione is metabolised in the body to 19-norandrosterone [8].

Ureolytic activity plays a key role in the pathogenesis of bacteria such as *Clostridium perfringens*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Salmonella* spp., *Staphylococcus saprophyticus*, *Ureoplasma urealyticum*, *Yersinia enterocolitica* [9] [10], and fungi such as *Cryptococcus neoformans* and *Coccidioides posadasii* [11]. Ammonia produced during the hydrolysis of urea by ureases is toxic for human cells and may help the disease to spread into a systemic infection. Bacterial ureases are thus involved in the pathogenesis of many diseases. Urease is also an immunogenic protein and is recognized by antibodies present in human sera. The presence of such antibodies is connected with progress of several long-lasting diseases, like rheumatoid arthritis, atherosclerosis or urinary tract infections. Taking into consideration that the human genome does not contain urease-encoding genes [11], the inhibition of urease activity can be considered an effective means of treating certain microbial infections. On the other hand, proteolytic enzymes such as chymotrypsin have been used to facilitate tissue repair since ancient times. It provides better resolution of inflammatory symptoms and promotes faster recovery of acute tissue injury [12] [13].

In continuity of our work developing new bioactive substances, we reported in our previous biotransformation investigation of norandrostenedione by *Cunninghamella blakesleeana* ATCC 8688A, the hemisynthesis and structure elucidation of four steroids, including one new, 6α,10β-dihydroxy-19-nor-4-androsten-3-one, along with three know compounds: 10β-hydroxy-19-nor-4-androsten-3,17-dione, 6β,10β,17β-trihydroxy-19-nor-4-androsten-3-one and 10β,17β- dihydroxy-19-nor-4-androsten-3-one [14]. The present study is aimed to follow up the biotransformation of norandrostenedione by *Fusarium lini* and to evaluate the urease and chymotrypsin inhibition properties of the transformed products.

2. Results and Discussion

Six metabolites **2**-**6**, including two new (**3** and **4**) and three known ones (**2**, **5** and **6**) were isolated from the fermentation of norandrostenedione (**1**) ($C_{18}H_{24}O_2$, *m/z* = 272.1), with *Fusarium lini* NRRL 2204 (**Figure 1**). This transformation has not been previously reported in the literature. The chemical structures of three known steroids **2**, **5** and **6**, which are here obtained for the first time by biotransformation from *Fusarium lini*, were identified by comparison of their physical and spectral data with previously reported data as 3-hydroxy-19-norandrost-1,3,5-trien-17-one (**2**) [15], 10β,17β-dihydroxy-19-nor-4-androsten-3-one (**5**) [5] [14] [16] and 10β-hydroxy-19-nor-4-androsten-3,17-dione (**6**) [5] [14]. Compounds **5** and **6** were previously synthesized by the biotransformation of **1** with *Cunninghamella blakesleeana* ATCC 8688A [14], while compound **2** was reported from biotransformation of estradiol valerate by *Fusarium lini* NRRL 2204 [15].

Compound **3** was obtained as a white solid, m.p. = 152˚C - 155˚C, soluble in $CDCl_3$. HR-EI-MS showed the quasi-molecular ion peak $[M]^+$ at *m/z* = 286.1563, indicating the formula $C_{18}H_{22}O_3$ (calcd. *m/z* = 286.1567), consistent with eight degrees of unsaturation, which is consistent with dehydrogenation (aromatization) of the A-ring, followed by hydroxylation of compound **1**. Absorption bands in the IR spectrum at 3405, 1720 and 1657 cm^{-1} indicated the presence of hydroxyl (OH), carbonyl (C=O) and aromatic groups (C=C), respectively. The ultraviolet spectrum showed absorption maxima at λ_{max} 230 and 243 nm which indicate the presence of a benzene ring in the molecule. In the ^1H NMR spectrum (**Table 1**), three aromatic protons resonate as an ABX system at δ_H = 7.18 (1H, d,

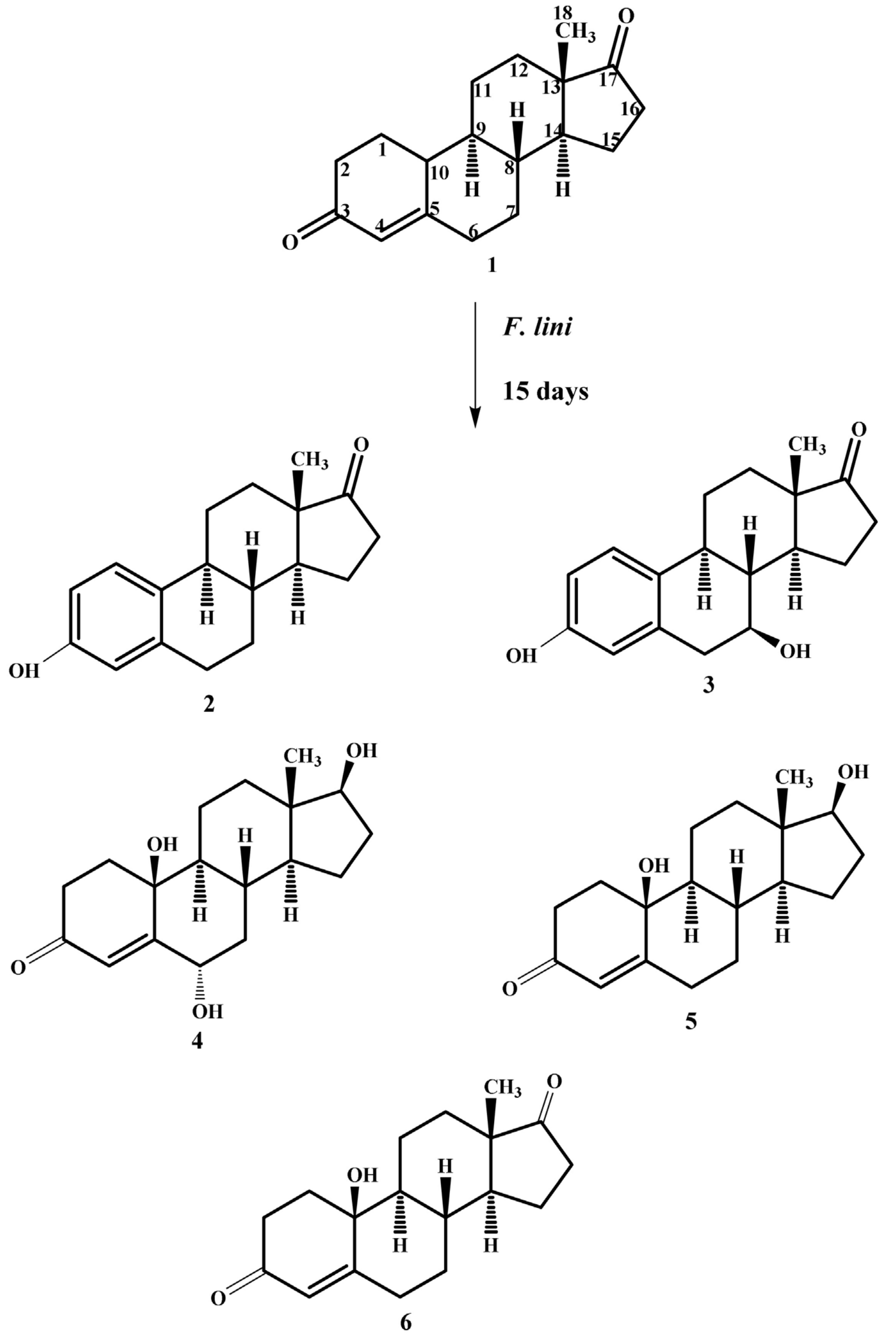


Figure 1. Biotransformation of norandrostenedione (**1**) with *Fusarium lini*.

J = 8.8 Hz), 6.65 (1H, dd, *J* = 2.5 - 8.8 Hz) and 6.56 ppm (1H, d, *J* = 2.5 Hz), which could be assigned, to the protons H-1, H-2 and H-4 of the mono-oxygenated A-ring of **3** [16]. The oxygen was located at C-3 using the HMBC ^2J and ^3J correlations of H-1 at δ_H = 7.18 ppm with C-4 (δ_C = 116.1 ppm); C-5 (δ_C = 134.3 ppm) and C-3 (δ_C = 153.7 ppm) and those of H-2 at δ_H = 6.65 ppm with C-4 (δ_C = 116.1 ppm); C-3 (δ_C = 153.7 ppm) and C-10 (δ_C = 131.1 ppm). Thus the A-ring of **1** is totally dehydrogenated, *i.e.* a benzene ring. The ^{1}H and ^{13}C NMR spectra showed three additional downfield methine signal at δ_H =

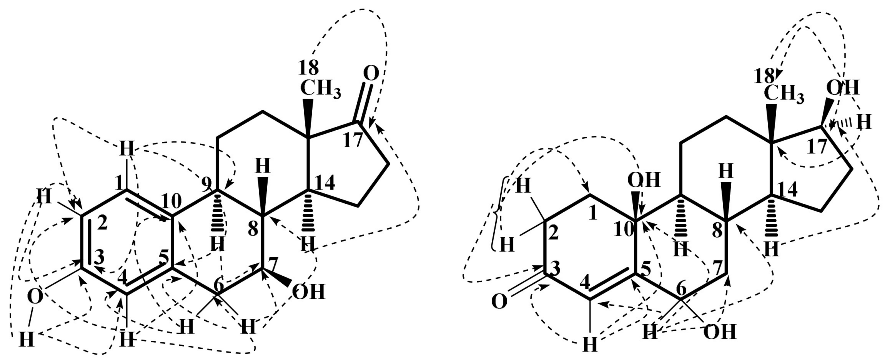
Table 1. ^{1}H and ^{13}CNMR data (coupling constants *J* in Hz) of compounds **1**, **3** and **4** (chemical shifts *δ* in ppm) respectively.

Position	**1**[a]		**3**[b]		**4**[a]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.53 m; 2.28 m	33.6	7.18, d, *J* = 8.5	126.8	1.83 m; 2.24 m	33.9
2	2.29 m; 2.36 m	36.4	6.65, dd, *J* = 2.5 - 8.5	113.5	2.58 m; 2.33 m	33.5
3	–	199.6	–	153.7	–	200.1
4	5.82 s	124.8	6.56, d, *J* = 2.5	116.1	4.83 s	126.1
5	–	165.6	–	134.3	–	158.6
6	2.08 m; 2.53 m	35.7	2.88, d, *J* = 17.5; 3.14, dd, *J* = 3.5 - 17.5	38.7	4.46 bs.r	73.3
7	1.13 m; 1.96 m	29.8	4.23, bsr	65.2	2.08, overlap; 1.26 m	38.6
8	1.50 m	39.8	1.64, m	41.9	2.06, overlap	29.2
9	0.93 m	49.5	2.65, m	35.7	1.02 m	53.2
10	–	42.4	–	131.1	–	70.9
11	1.32 m; 1.94 m	25.9	1.58 m; 2.10 m	25.8	1.74 m 2[H]	19.5
12	1.30 m; 1.83 m	31.3	1.52 m; 1.92 m	31.4	1.16 m; 1.88 m	36.2
13	–	47.3	–	50.9	–	43.1
14	1.34 m	50.6	1.84 m	46.1	1.04, overlap	49.9
15	1.54 m; 1.98 m	21.4	1.61 m; 2.11 m	21.1	1.38 m; 1.61 m	23.2
16	2.26 m; 2.45 m	35.2	2.18 m; 2.46 m	35.7	1.46 m; 2.07 m	30.4
17	–	220.3	–	220.4	3.64 t	81.6
18	0.94 s	10.5	0.88 s	13.9	0.82 s	10.9

[a]100 MHz. [b]150 MHz.

4.23 (brs) and δ_C = 65.2 ppm, indicating the presence of a second hydroxyl group in the transformed metabolite (**3**). The position of this hydroxyl group has been deduced on the basis of COSY and HMBC correlations. The HMBC correlations of H-6 (δ_H = 2.88 ppm, d, *J* = 17.5 Hz) with C-7 (δ_C = 65.2 ppm); C-8 (δ_C = 41.9 ppm) and C-5 (δ_C = 134.3 ppm) supported the presence of a hydroxyl group at C-7 (**Figure 2**). This position of the hydroxyl group was also further confirmed by COSY correlations of H-6 (δ_H = 3.05 ppm, dd, *J* = 3.5 - 17.5 Hz) with H-7 (δ_H = 4.17 ppm, brs). The stereochemistry of the transformed metabolite **3** was deduced on the basis of NOESY correlations. H-7 (δ_H = 4.23 ppm, brs) showed NOESY correlations with H-14 (δ_H = 1.84 ppm, m). As H-14 is *α*-oriented in substrate **1**, therefore its NOESY correlation with H-14 suggested *α*-hydroxymethylene at C-7 and the hydroxylation at C-7 was deduced to be *β*-oriented (**Figure 3**). Finally, metabolite **2** was identified as 3,7*β*-dihydroxy-19-norandrost-1,3,5-trien-17-one.

Metabolite **4** was obtained as a yellow solid, m.p. = 253˚C - 258˚C, soluble in $CDCl_3$ The HRMS ((–)-FAB) spectrum of **4** showed the quasi-molecular ion peak $[M–H]^-$ at *m/z* = 305.1719 indicating the formula $C_{18}H_{26}O_4$ (calcd. *m/z* = 305.1747), consistent with six degrees of unsaturation. This mass indicated that



3 4

Figure 2. KeyHMBC correlations in compounds **3** and **4**.

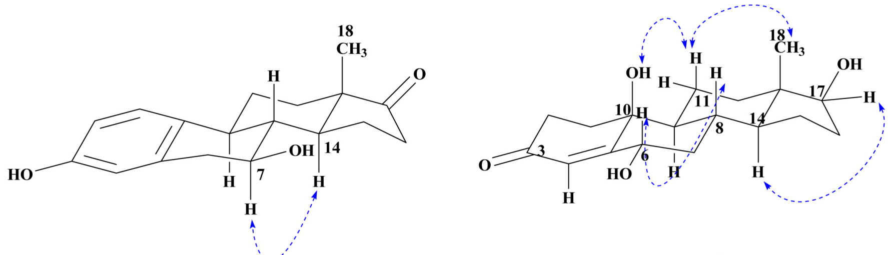


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Figure 3. KeyNOESY correlations in compounds **3** and **4**.

compound **4** contains three additional OH groups as compared to compound **1** (*m/z* = 272.1). The IR bands at 3418 and 1657 cm^{-1} indicated the presence of -OH and ketone functionalities, respectively. Compound **4** showed characteristic fluorescence in the UV spectrum at 229 and 243 nm, which indicates the presence of a conjugated ketone. The ^1H NMR spectrum displayed signals of two new methine protons at δ_H = 3.64 ppm (t, *J* = 8.1 Hz) and δ_H = 3.64 ppm (brs) (**Table 1**). The presence of a new quaternary (δ_C = 70.9 ppm) and two methine carbons (δ_C = 73.3, 81.6 ppm) in the ^{13}C NMR spectrum further supported the trihydroxylation of the substrate **1** (**Table 1**). The position of these hydroxyl groups was deduced using the HMBC spectrum. Protons H-2 (δ_H = 2.32, 2.58 ppm) and H-4 (δ_H = 5.83 ppm) showed HMBC correlations with C-10 (δ_C = 70.9 ppm), which indicated a hydroxylation at C-10. H-4 (δ_H = 5.83 ppm) and H-7 (δ_H = 2.05 ppm) showed HMBC correlations with the new C-6 atom (δ_C = 70.8 ppm) which indicated a second hydroxylation at C-6. H-18 (δ_H = 0.82 ppm) and H-14 (δ_H = 1.04 ppm) showed HMBC correlations with C-17 (δ_C = 81.6 ppm), hence the third hydroxyl group occurs at C-17 (**Figure 2**). NOESY correlations of H-11 (δ_H = 1.74 ppm) and H-18 (δ_H = 0.92 ppm) with OH (δ_H = 5.04 ppm) at C-10 were used to support the stereochemistry of the hydroxyl group at C-10 asβ. H-8(-β) at δ_H = 2.06 ppm showed NOESY correlations with H-6 (δ_H = 4.46 ppm). Therefore, α-OH was placed at C-6. In addition, the NOESY correlation between H-14 (δ_H = 1.04 ppm) with H-17 (δ_H = 3.64 ppm) allowed us to assign a β-orientation of OH at C-17 (**Figure 3**). Thus the metabolite **4** was identified as 6α,10β,17β-trihydroxy-19-nor-4-androsten-3-one.

The transformed compounds and substrate **1** were screened for their potential against enzymes involved in ulcer and viral diseases by various *in vitro* biological assays (Table 2).

When the activities of compounds **1**-**6** were screened against urease, compound **3** showed remarkable urease inhibition with an IC_{50} value of 10.2 ± 0.28 µm as compared to the standard thiourea with IC_{50} = 21.6 ± 0.12 µm. This clearly indicates that compound **3** is more potent than thiourea. In addition, compound **2** also showed excellent urease inhibition activity with an IC_{50} value of 23.7 ± 0.17 µM.

With regard to chymotrypsin inhibitory activity, compound **4** exhibits excellent protease inhibitor potential against chymotrypsin with an IC_{50} value of 6.4 ± 0.19 µm as compared to standard chymostatin with IC_{50} = 5.7 ± 0.14 µm. In addition, compounds **5** and **6** also show promising activity with IC_{50} value of 15.6 ± 0.46 and 18.4 ± 0.65 µM, respectively. It shows that these compounds **4**-**6** can be potential serine inhibitors. NS3 serine protease of HCV and HIV protease are key enzymes that catalyse replication of virus and are the target for anti-HCV and anti-HIV drugs [17], therefore compound **4** can be effective as anti-viral compounds and can be further used for anti-viral studies in future but compounds **5** and **6** required incomparably higher concentration than standard chymostatin to inhibit the enzyme with less potency.

3. Materials and Methods

3.1. General Experimental Conditions

Norandrostenedione (**1**) ($C_{18}H_{24}O_2$) was obtained from Sigma-Aldrich (cat no. 74640, USA). Media constituents including Sabouraud dextrose agar were acquired from Sigma-Aldrich (cat no. 146392, Germany). The quality of the substrate purity (**1**) was checked by thin layer chromatography (TLC, silica-coated (PF254)). Silica gel (70 - 230 mesh) column chromatography was performed for

Table 2. IC_{50} values of pure compounds **1**-**6** in enzyme inhibition.

Compounds	IC_{50} ± MSE (values in *MM*)	
	Urease inhibition	Chymotrypsin inhibition
1	81.2 ± 0.28^a	00 ± 0.00^a
2	23.7 ± 0.17^b	75.3 ± 0.26^b
3	10.2 ± 0.28^c	61.2 ± 0.24^c
4	78.5 ± 0.27^d	6.4 ± 0.19^d
5	76.3 ± 0.46^e	15.6 ± 0.46^e
6	00 ± 0.00^f	18.4 ± 0.65^f
Thiourea	21.6 ± 0.12^g	–
Chymostatin	–	5.7 ± 0.14^g

[a,b,c,e,f,g]On the same column, values with different letters are differ significantly ($p > 0.05$) to the standard the control. MSE: Mean standard error.

fractionation of crude extract. Fractions were finally purified through recycling with preparative RP-HPLC (LC-908, Japan), equipped with JAIGEL-ODS-L-80 (L = 250 mm, I.D. = T20 mm). HR-FAB-MS and HR-IE-MS were recorded on Jeol JMS-600H mass spectrometer. 1D-, and 2D-NMR of compounds **1**-**6** were performed in deuterated chloroform ($CDCl_3$) on Bruker Avance spectrometers AMX (400 and 100 MHz) with tetramethylsilane as an internal standard. A Büchi M-560 apparatus (model M-560) was used for determining the melting points. Optical rotations of isolated compounds were measured on a JASCO P-2000 polarimeter. UV absorbance (λ in nm) was done on a Shimadzu UV 240 spectrophotometer (Shimadzu Corporation, Tokyo, Japan; model: Evolution-300). Infrared (IR) spectra (in cm^{-1}) were recorded with an FT-IR-8900 spectrophotometer.

3.2. Fermentation of Norandrostenedione (1) with *Fusarium lini* and Isolation of Metabolites

3.2.1. Microbial Culture and Media Preparation

Fungus culture was obtained from the international culture collection centre Northern Regional Research Laboratory (NRRL). The fungus used was *Fusarium lini* NRRL 2204 which was grown on Sabouraud dextrose agar (SDA) and preserved at $T = 4˚C$. The ingredients used for the preparation of the culture medium (1 L) for the microorganism *Fusarium lini* was as follows: 5.0 g peptone, 5.0 g NaCl, 10.0 g glucose, 5.0 g potassium dihydrogen phosphate, and 10.0 mL glycerol in 1 L of distilled water [5] [7] [14].

3.2.2. Fermentation, Extraction and Purification of Norandrostenedione (1) with *Fusarium lini*

The biotransformation experiment was performed in two steps, on a small and a large scale. Based on small-scale screening results, 20 L of culture medium for the growth of *F. Lini* was prepared by using the above mentioned media ingredients in 18 L of distilled water, and transferred to 10 conical flasks of 5 L (each flask containing 2 L of medium). They were cotton-plugged, autoclaved at $T = 121˚C$, cooled to room temperature and inoculated with seed flasks of *F. lini* under aseptic conditions on a shaker (121 rpm) at 20˚C ± 2˚C until a good biomass (4 days) was obtained. After mature growth of *F. lini*, 2.5 g of norandrostenedione (**1**) ($C_{18}H_{24}O_2$) was dissolved in 80 mL of acetone, and transferred to the flasks (8 mL in each flask). The flasks were placed on a shaker for 15 days at 26˚C ± 2˚C. After 15 days of incubation, the reaction was stopped by adding 1 L of ethyl acetate to each flask. All culture flasks were combined and filtered to remove mycelia. The aqueous filtrate was extracted thrice with EtOAc and after drying with sodium sulfate, concentrated under reduced pressure with a rotavapor. This operation yielded 4.5 g yellow gummy crude which was subjected to silica-gel (0.032 - 0.063 mesh) column chromatography (CC) with a mobile phase of hexanes-acetone (100:0; 95:5; 90:10; 85:15; 80:20; 75:25; 70:30; 65:35; 60:40; 55:45 and 50:50).

Thin layer chromatography (TLC) was used to determine the profile of different fractions. Three fractions (F-1 to F-3) were obtained from column chromatography. Compounds were further purified on reverse phase recycling HPLC. The fraction F-3 was re-chromatographed on silica gel and yielded compounds **4** (RT = 16 min, 18.7 mg), **5** (RT = 20 min, 33.5 mg) and **6** (RT = 24 min, 41.5 mg), on elution with methanol and water (MeOH-H_2O 90:10). Compounds **2** (RT = 21 min, 18.5 mg) and **3** (RT = 27 min, 24.5 mg), were purified from fraction **3** through purification on reverse phase HPLC eluted with (MeOH-H_2O 80:20).

Compound (3): White solid, m.p. = 152˚C - 155˚C. $[\alpha]_D^{25}$ = +3380.4 (*c* = 0.003, MeOH). UV (MeOH, nm): λ_{max} ($\log\varepsilon_{max}$) = 230 (1.41), 243. IR (KBr, cm^{-1}): ν = 3405 (-OH stretching), 1720 (C=O stretching), 1657 (C=C). HRMS ((+)-EI): *m/z* = 286.1563 (calcd. 286.1567 for $C_{18}H_{22}O_3$, $[M]^+$). ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz): **Table 1**.

Compound (4): Yellow solid, m.p. = 253˚C - 258˚C; $[\alpha]_{25}^D$ = +3380.4 (c 0.003, MeOH). UV λ_{max} (MeOH, nm): λ_{max} ($\log\varepsilon_{max}$) = 243 (2.40), 229. IR ($CHCl_3$, cm^{-1}): ν_{max} = 3418 (-OH stretching), 1657 (C=O stretching). HRMS ((−)-FAB): *m/z*: 305.1719 $[M-H]^-$ (calcd. for $C_{18}H_{26}O_4^+$ 306.1747); EI-MS ((+)-EI): *m/z* (relative intensity, %) = 306 (100) $[M]^+$, 259 (10), 138 (18.8), 133 (16), 91 (14.4). ^1H NMR ($CDCl_3$, 400 MHz): **Table 1**; ^{13}C NMR ($CDCl_3$, 100 MHz): **Table 1**.

3-Hydroxy-19-norandrost-1,3,5-trien-17-one (2): Yellow solid; m.p. = 183˚C - 189˚C; $[\alpha]_{25}^D$ = −338.4 (c 0.03, MeOH); UV λ_{max} (MeOH $\log\varepsilon$2.38) nm: 230 and 281 [5]. IR ν_{max} ($CHCl_3$, cm^{-1}): 3278 (-OH stretching); 1709 and 1618 (C=O stretching). ^1H NMR ($CDCl_3$, 500 MHz): δ_H 7.14 (1H, d, *J* = 8.5 Hz, H-1); 6.62 (1H, dd, *J* = 2.5 - 8.5 Hz, H-2); 6.56 (1H, d, *J* = 2.5 Hz, H-4); 2.28 (2H, d, *J* = 17.5 Hz, H-6); 1.43 (1H, m, H-7α); 1.99 (1H, m, H-7β); 1.57 (1H, m, H-8); 2.23 (1H, m, H-9); 5.04 (10-OH, *bsr*); 1.55 (1H, m, H-11α); 2.53 (1H, m, H-11β); 1.47 (1H, m, H-12α); 1.94 (1H, m, H-12β); 1.51 (1H, m, H-14); 1.64 (1H, m, H-15α); 2.04 (1H, m, H-15β); 2.13 (1H, m, H-16α); 2.48 (1H, m, H-16β); 0.91 (3H, *s*). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 126.5 (C-1); 112.8 (C-2); 153.4 (C-3); 115.2 (C-4); 138.1 (C-5); 29.4 (C-6); 26.8 (C-7); 38.3 (C-8); 43.9 (C-9); 132.1 (C-10); 26.3 (C-11); 31.5 (C-12); 48.9 (C-13); 50.4 (C-14); 21.5 (C-15); 35.8 (C-16); 221.2 (C-17); 13.8 (C-18). HREI-MS *m/z*: 270.1617 $[M]^+$ (calcd. for $C_{18}H_{22}O_2^+$ 270.1620).

10β,17β-dihydroxy-19-nor-4-androsten-3-one (5): White solid; m.p. = 214˚C; $[\alpha]_{25}^D$ +3380.4 (c 0.003, MeOH); UV λ_{max} (MeOH $\log\varepsilon$3.32) nm: 230, and 260. IR ν_{max} (KBr, cm^{-1}): 3395 (-OH stretching); 1661 (C=O stretching).^1H NMR ($CDCl_3$, 400 MHz): δ_H 1.98 (1H, m, H-1α); 2.19 (1H, m, H-1β); 2.35 (1H, m, H-2α); 2.51 (1H, m, H-2β); 5.77 (1H, *s*, H-4); 2.24 (1H, m, H-6α); 2.65 (1H, m, H-6β); 1.33 (1H, m, H-7α); 1.89 (1H, overlap, H-7β); 1.95 (1H, overlap, H-8); 1.12 (1H, *bsr*, H-9); 1.74 (2H, m, H-11); 1.11 (1H, m, H-12α); 1.98 (1H, m, H-12β); 1.32 (1H, overlap, H-14); 1.58 (1H, m, H-15α); 1.97 (1H, m, H-15β);

2.10 (1H, m, H-16*α*); 2.50 (1H, m, H-16*β*); 3.64 (1H, *t*, H-17); 0.92 (3H, *s*); 5.04 (1H, m, 10-OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 33.8 (C-1); 33.6 (C-2); 198.9 (C-3); 124.9 (C-4); 164.1 (C-5); 31.9 (C-6); 31.3 (C-7); 35.3 (C-8); 52.6 (C-9); 70.4 (C-10); 19.9 (C-11); 36.1 (C-12); 43.1 (C-13); 50.1 (C-14); 23.3 (C-15); 30.5 (C-16); 81.6 (C-17); 10.8 (C-18). HREI-MS *m/z*: 290.1 $[M]^+$ (calcd. for $C_{18}H_{26}O_3^+$ 290.1).

10*β*-hydroxy-19-nor-4-androsten-3,17-dione (6): White solid; m.p. = 205˚C - 210˚C, (203˚C - 208˚C) [15]; $[\alpha]_{25}^{D}$ = −3380.4 (c 0.003, MeOH). IR ν_{max} (KBr, cm^{-1}): 3402 (-OH stretching); 1720 and 1665 (C=O stretching). ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 1.98 (1H, m, H-1*α*); 2.18 (1H, m, H-1*β*); 2.31 (1H, m, H-2*α*); 2.54 (1H, m, H-2*β*); 5.77 (1H, *s*, H-4); 2.21 (1H, m, H-6*α*); 2.68 (1H, m, H-6*β*); 1.31 (1H, m, H-7*α*); 1.81 (1H, overlap, H-7*β*); 1.91 (1H, overlap, H-8); 1.17 (1H, *bsr*, H-9); 1.70 (2H, m, H-11); 1.13 (1H, m, H-12*α*); 2.01 (1H, m, H-12*β*); 1.35 (1H, overlap, H-14); 1.57 (1H, m, H-15*α*); 1.96 (1H, m, H-15*β*); 2.12 (1H, m, H-16*α*); 2.54 (1H, m, H-16*β*); 3.66 (1H, *t*, H-17); 0.92 (3H, *s*); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 33.7 (C-1); 33.5 (C-2); 198.7 (C-3); 124.9 (C-4); 163.7(C-5); 31.7 (C-6); 31.1 (C-7); 34.8 (C-8); 52.5 (C-9); 70.3 (C-10); 19.6 (C-11); 30.5 (C-12); 47.5 (C-13); 50.4 (C-14); 21.7 (C-15); 35.7 (C-16); 220.3 (C-17); 13.6 (C-18) HREI-MS *m/z*: 288.1734 $[M]^+$ (calcd. for $C_{18}H_{24}O_3^+$ 288.1725).

3.3. Urease Inhibition Assay

Reaction mixtures comprising 25 µL of enzyme (Jack bean Urease) solution and 55 µL of buffers containing 100 mm urea were incubated with 5 µL of test compounds (1 µm concentration) at $T = 30˚C$ for 15 min in 96-well plates. Urease activity was determined by measuring ammonia production using the indophenol method as described by Khan [18]. Briefly, 45 µL of phenol reagent (1% *w*/*v* phenol and 0.005% *w*/*v* sodium nitroprusside) and 70 µL of alkali reagent (0.5% *w*/*v* NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 µL. The results (change in absorbance per min) were processed by using SoftMaxPro software (Molecular Device, USA). Assays were performed at pH = 8.2 (0.01 m $K_2HPO_4 \cdot 3H_2O$, 1 mM EDTA and 0.01 m LiCl). Percentage inhibition was calculated from the formula $100-(OD_{testwell}/OD_{control})\times 100\%$. where OD stands for optical density. Thiourea was used as the standard inhibitor of urease.

3.4. *In Vitro α*-Chymotrypsin Assay

The *α*-chymotrypsin inhibitory activity was determined using a previously described method [19]. Briefly, *α*-chymotrypsin (9 units per mL in 50 mm Tris-HCl buffer, pH = 7.6; Sigma Chemical Co. USA) was pre-incubated with the test compounds at a concentration varying from 5 to 500 µm for 20 min at

T = 25˚C. 100 µL of substrate solution (*N*-succinyl-phenylalanine-*p*-nitroanilide, 0.01 - 0.06 mm in 50 mm Tris-HCl buffer, pH = 7.6) was added to start the enzyme reaction. The absorbance of released *p*-nitroaniline was continuously monitored at 410 nm until a considerable colour change was achieved. The final DMSO concentration in the reaction mixture was 7%. Best inhibition was found at concentration 3.02×10^{-5} g/100µL. The percentage (%) inhibition was calculated as $(E - S)/E \times 100\%$ where *E* is the activity of the enzyme without test compound, and *S* is the activity of the enzyme in the presence of the test compound. The concentrations of test compounds that inhibited the hydrolysis of substrate up to 50% (IC_{50}) were determined by monitoring the effect of various concentrations of these compounds in the assays. The IC_{50} values were then calculated using the EZ-Fit Enzyme Kinetics program (Version PerrellaScientific Inc., Amherst, USA).

3.5. Statistical Analysis

The data are presented as the mean ± one standard deviation (SD). One-way ANOVA followed by Dennett's test, as specified, was performed using GraphPad Prism, version 7.00 (GraphPad Software, San Diego, California, USA). One-way ANOVA was used to compare the control and test groups. Values $p \leq 0.05$ were considered to be statistically significant.

4. Conclusion

In conclusion, microbial transformation of the androgenic steroid norandrostenedione (**1**) with *Fusarium lini* yielded two new metabolites **3** and **4**, along with three known metabolites **2**, **5** and **6**. Biocatalytic transformation of **1** with *Fusarium lini* provided an efficient route for the mono-hydroxylation(10-*β*) in metabolite **6,** dihydroxylation (10-*β* and 17-*β*) in metabolite **5**, trihydroxylation (6-*α*, 10-*β* and 17-*β*) in metabolite **4** and aromatisation of A-ring in metabolite **2** and **3**. We can conclude on the basis of literature and previous studies that, *Fusarium lini* possess enzymes such as monooxygenase, dioxygenase and trioxygenase systems, which catalyze the *α*- and *β*-hydroxylation, and also dehydrogenases which catalyse the dehydrogenation at various positions of the steroidal skeleton. In addition, compounds **2** and **3** show potent growth inhibition against drug-resistant strains of urease. Compounds **4**, **5** and **6** show good chymotrypsin activity. This study indicates that special attention should be paid to the biotransformation process as it can contribute to the development of new bioactive compounds.

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Competing Interests

The authors declare that they have no conflict of interests.

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