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Stereoselective Microbial Hydroxylation of Progestin, Norethisterone by Using *Aspergillus niger* and *Penicillium citrinum*

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Abstract – Microbial transformation of a potent progestin, norethisterone (17*β*-hydroxy-19-nor-17*α*-pregn-4-en-20-yn-3-one) (**1**) was carried out by using two filamentous fungi *Aspergillus niger* and *Penicillium citrinum.* Biotransformation of **1** with *A. niger* yielded a hydroxylated transformed product 10*β*,17*β*-diydroxy-19-nor-17*α*-pregn-4-en-20-yn-3-one (**2**) whereas 11*β*,17*β*-diydroxy-19-nor-17*α*-pregn-4-en-20-yn-3-one (**3**) was obtained through microbial transformation of **1** by *P. citrinum*. It is the first report of their production from **1** by using *A. niger* and *P. citrinum* with complete ^{1}H- and ^{13}C-NMR assignment. The structures of both metabolites were characterized by various spectroscopic techniques and reported data.
Keywords – Microbial transformation, Progestin, Norethisterone, *Aspergillus niger*, *Penicillium citrinum*

Introduction

Microbial transformation for the stereoselective modification of bioactive steroidal compounds has attracted great interest in last few decades. The main advantages of microbial transformation over traditional chemical synthesis are environmental friendly, mild conditions and highly stereoselective reactions at inaccessible sites.[1] Filamentous fungi have cytochrome P450 enzymes, which are responsible for stereoselective hydroxylation.[2] Microbial hydroxylation is widely used in organic chemistry to get more potent metabolites because it is observed that about 80% transformed products are more potent than their parent compounds.[3] It is also observed that highly oxygenated steroidal compounds possesses antibiotic, antioxidant and anti-cancer activities so biotransformation process helps to get more potent drugs to treat diseases.[3]

Progestins are synthetic progestogens, which have similar biological function to natural hormone progesterone. They are commonly used in hormonal birth control and menopausal hormone therapy. Norethisterone (NET) (**1**) is a potent progestin, which was developed in 1952 by Djerassi but it was launched in 1973 as the first so-called minipill.[4] About 50 million women are taking norethisterone (**1**) or its precursors as oral contraceptive pills to prevent unwanted pregnancies in a number of formulations because it is the safest among the female contraceptives.[5-7] It is also used to postpone menstruation and to treat endometriosis.

In the current study, we are reporting the fermentation of antifertility agent, NET (**1**) with *A. niger* and *P. citrinum* in liquid phase medium to get structurally diverse forms of **1** because both these fungus were not tested for the biotransformation of **1** so for. Fermentation of **1** with *A. niger* and *P. citrinum* yielded mono hydroxylated products **2** and **3**, respectively (Fig. 1). Both transformed products were reported here for the first time from these fungal strains with complete ^{1}H- and ^{13}C-NMR assignment.

Experimental

General experimental procedures – Norethisterone (**1**) was gifted by a local pharmaceutical company. The melting point was determined on a ST15 OSA, UK

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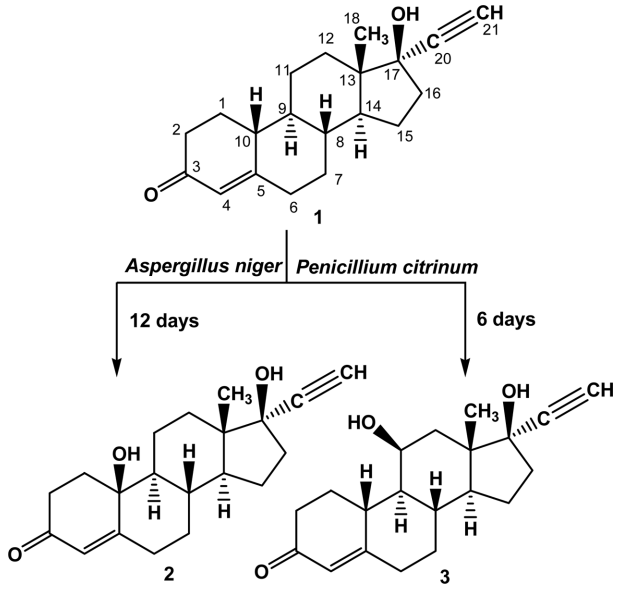


Fig. 1. Biotransformation of norethisterone (**1**) with *A. niger* and *P. citrinum*.

apparatus. Column chromatography (CC) was carried on silica gel column (60 - 270 mesh). Purity of the samples was checked by TLC on preparative plates (pre-coated silica gel GF-254; 20 × 20 cm, 0.25 mm thick, Merck) and were detected under the UV light (254 and 366 nm). The LREI-MS were recorded on Finnigan MAT 311 mass spectrometer with a MASPEC data system while Jeol JMS 600 and HX 110 mass spectrometers with the data system DA 5000 were used to perform HREI-MS. IR spectroscopy was carried out on a Jasco A-302 spectrophotometer. The ^{1}H-NMR spectra were recorded in $CDCl_3$ on Bruker Avance-400 NMR spectrometer with TMS as an internal standard using the UNIX operating system at 400 MHz. The ^{13}C-NMR spectra were recorded in $CDCl_3$ on same instrument at 100 MHz.

Fungal cultures – Fungal cultures were obtained from the American Type Culture Collection (A.T.C.C). *Aspergillus niger* (ATCC 9142) and *Penicillium citrinum* (ATCC 9849) were grown on Sabouraud dextrose agar (SDA) slant and stored at 4 ºC.

Media preparation – Liquid phase culture media (3 L each) for *A. niger* (ATCC 9142) and *P. citrinum* (ATCC 9849) were prepared by dissolving glucose (30 g), peptone (15 g), yeast extract (15 g), KH_2PO_4 (15 g), NaCl (15 g) and glycerol (15 mL) into distilled water (3 L). The fermentation media of each fungus was distributed equally among 40 conical flasks of 250 mL (approximately 75 mL in each flask). The mouths of all flasks were plugged with cotton swab and autoclaved at 121 ºC for 1 hour.

Seed flasks preparation and inoculation of the culture – The spores of *A. niger* and *P. citrinum* were inoculated from stored fungal culture (SDA slant) into corresponding five seed flasks, containing freshly prepared autoclaved media and placed on rotary shaker (90 rpm) for incubation at 28 ± 2 ºC for three days.

When well-developed growth was seen in seed flasks, the spores were transferred into 33 Erlenmeyer flasks of each fungus and again placed on shaker (90 rpm) at 28 ± 2 ºC for few days. One negative control flask (contained media and fungus) for each fungus was also made to check the fungal metabolites in media.

Fermentation of norethisterone (1) – After three days of inoculation, suitable growth of fungus was seen. Norethisterone (**1**) (300 mg) was dissolved in acetone (16.5 mL) and equally distributed among 33 Erlenmeyer flasks (approximately 9 g/0.5mL in each flask). The fermentation of **1** in the culture of *A. niger* and *P. citrinum* was continued for 12 and 6 days, respectively. One positive control flask (contained media and compound) was also made to check the stability of **1** in respective media.

Filtration and extraction procedure – On completion of fermentation, the biomass of fungus was filtered and washed with CH_2Cl_2. The filtrate was extracted with three times volume of CH_2Cl_2. The extract was dried over anhydrous sodium sulphate and evaporated to dryness, which afforded a brown gummy crude extract. The negative and positive control flasks for each fungus were also extracted in a same manner after 12 days.

TLC analysis – The transformation of **1** by *A. niger* and *P. citrinum* were checked by a comparative TLC of crude extract of both fungus with substrate (**1**), positive and negative control flask extracts on pre-coated silica gel plates using acetone:pet.ether (20:80) system. A more polar metabolite was seen on each TLC, which was absent in positive and negative control crude extracts showing the transformation of **1**.

Purification of metabolites – The brown gums (0.36 g and 0.38 g), which were obtained from *A. niger* and *P. citrinum*, respectively were subjected to column chromatography with increasing polarity of ethyl acetate into pet. ether. Elution with ethyl acetate:pet.ether (15.0:85.0) yielded **2** from the crude extract of *A. niger* while **3** was obtained with ethyl acetate:pet.ether (15.5:84.5) from the crude extract of *P. citrinum* (Table 3).

Timescale study – Timescale study was also conducted during the fermentation of **1** with both fungi. For this study, one Erlenmeyer flask of each fungus was harvested, extracted and analyzed by TLC in order to check the degree of transformation of substrate **1** after

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

Carbon No.	1	2	3
1	α 1.52 (m), β 2.23 (m)	α 1.74 (m), β 2.48 (m)	α 1.59 (m), β 2.29 (m)
2	α 2.24 (m), β 2.37 (m)	α 1.98 (m), β 2.30 (m)	α 2.30 (m), β 2.38 (m)
3	-	-	-
4	5.80 (s)	5.87 (br s)	5.84 (br s)
5	-	-	-
6	α 2.44 (ddd, *J* =14.4, 3.2 and 3.2), β 2.23 (m)	α 2.46 (ddd, *J* =14.3, 3.2 and 3.2), β 2.24 (m)	α 2.43 (ddd, *J* =14.5, 3.4 and 3.4), β 2.25 (m)
7	α 1.04 (m), β 1.80 (m)	α 0.92 (m), β 1.74 (m)	α 1.07 (m), β 1.91 (m)
8	β 1.35 (m)	β 1.38 (m)	β 1.42 (m)
9	α 0.86 (m)	α 1.18 (m)	α 1.26 (m)
10	β 2.06 (ddd, *J* =8.4, 8.4 and *J* = 4.6)	-	β 2.02 (ddd, *J* = 8.2, 8.2 and 3.5)
11	α 1.89 (m), β 1.22 (m)	α 1.90 (m), β 1.24 (m)	α 4.27 (ddd, *J* =3.0, 3.0 and 3.0)
12	α 1.60 (m), β 1.69 (m)	α 1.59 (m), β 1.70 (m)	α 2.01 (m), β 1.94 (m)
13	-	-	-
14	α 1.50 (m)	α 1.49 (m)	α 1.46 (m)
15	α 1.68 (m), β 1.32 (m)	α 1.69 (m), β 1.38 (m)	α 1.70 (m), β 1.41 (m)
16	α 2.26 (m), β 1.97 (m)	α 2.19 (m), β 2.04 (m)	α 2.28 (m), β 1.99 (m)
17	-	-	-
18	0.88 (s)	0.92 (s)	0.95 (s)
20	-	-	-
21	2.54 (s)	2.62 (s)	2.56 (s)

[a]) Assignments based on COSY and HMQC.

Table 2. ^{13}C NMR data of norethisterone (**1**) and its metabolites **2** and **3** in $CDCl_3$. (δ in ppm, 100 MHz)[a,b]

Carbon No.	1	2	3
1	26.5 (CH_2)	33.2 (CH_2)	26.0 (CH_2)
2	36.4 (CH_2)	33.6 (CH_2)	35.8 (CH_2)
3	199.3 (C)	198.3 (C)	199.9 (C)
4	124.5 (CH)	125.5 (CH)	124.7 (CH)
5	166.5 (C)	167.3 (C)	167.8 (C)
6	35.4 (CH_2)	35.7 (CH_2)	35.1 (CH_2)
7	30.6 (CH_2)	31.5 (CH_2)	30.7 (CH_2)
8	40.9 (CH)	37.2 (CH)	35.4 (CH)
9	49.0 (CH)	56.1 (CH)	54.2 (CH)
10	42.5 (CH)	71.2 (C)	37.5 (CH)
11	26.1 (CH_2)	21.4 (CH_2)	66.6 (CH)
12	32.3 (CH_2)	35.2 (CH_2)	40.7 (CH_2)
13	46.8 (C)	47.6 (C)	42.4 (C)
14	49.1 (CH)	50.1 (CH)	52.2 (CH)
15	22.8 (CH_2)	23.7 (CH_2)	22.4 (CH_2)
16	38.7 (CH_2)	39.5 (CH_2)	37.8 (CH_2)
17	79.6 (C)	80.2 (C)	79.9 (C)
18	12.6 (CH_3)	13.3 (CH_3)	15.2 (CH_3)
20	87.2 (C)	88.1 (C)	87.8 (C)
21	74.0 (CH)	75.4 (CH)	74.5 (CH)

[a]) Multiplicities were determined by DEPT experiments.
[b]) Assignment based on HMQC and HMBC.

every 2 days of fermentation. The % degree of transformation of NET (**1**) into its transformed products **2**, **3** and unknown was determined after the purification of compounds (Fig. 3 and 6).

Norethisterone (1) – Colorless crystalline solid. m.p. 201 - 203 °C; $[\alpha]_D^{25}$: -30.6° (*c* = 0.1, $CHCl_3$); IR ($CHCI_3$) ν_{max}: 3400, 3300, 2130, 1650, 1590 cm^{-1}; LREI-MS *m/z*: 298 $[M]^+$; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data listed in Tables 1 and 2, respectively.

10β-Hydroxy norethisterone (2) – White to off white crystalline solid. m.p. 263 - 265 °C; $[\alpha]_D^{25}$: = -13.4° (*c* = 0.1, $CHCl_3$); IR ($CHCI_3$) ν_{max}: 3410, 3310, 2135, 1665, 1575 cm^{-1}. LREI-MS *m/z*: 314 $[M]^+$. ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data listed in Tables 1 and 2, respectively.

11β-Hydroxy norethisterone (3) – Colorless crystalline solid. m.p. 223 - 224 °C; $[\alpha]_D^{25}$: -5.6° (*c* = 0.1, $CHCl_3$); IR ($CHCI_3$) ν_{max}: 3415, 3308, 2140, 1660, 1580 cm^{-1}; LREI-MS *m/z*: 314 $[M]^+$; ^{1}H ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data listed in Tables 1 and 2, respectively.

Result and Discussion

Small scale screening experiments with *A. niger* and *P.*

citrinum were carried out, which showed that both fungi have ability to transform NET (**1**) into more polar derivatives, thus large scale experiments with these fungi were performed. Fermentation of **1** with these fungi yielded two metabolites, **2** and **3**.

The LREI-MS of **2** displayed M^+ at *m/z* 314, which was 16 units greater than **1**, showing the hydroxylation of **1**. The molecular formula of **2** was deduced from the HREI-MS as $C_{20}H_{26}O_3$, corresponding to the M^+ at *m/z* 314.2318 (calcd 314.2306). The IR spectrum ($CHCl_3$) of **2** was almost similar to **1**, indicating that there was no change in the functional groups of **1**. The ^{1}H-NMR spectrum ($CDCl_3$, 400 MHz, Table 1) of **2** did not show any downfield carbinolic proton signal, indicating the presence of tertiary -OH group at C-8, -9, -10 or -14 but the absence of downfield signal at δ_H 2.06 (ddd, J = 8.4, 8.4 and 3.6 Hz, H-10) in **2** as compared to **1**, suggesting hydroxylation at C-10. The remaining ^{1}H-NMR spectrum of **2** was almost similar to **1** as it showed two characteristic singlets for a methyl at δ_H 0.92 (s, H3-18) and an acetylenic proton at δ_H 2.62 (s, H-21) as well as a broad singlet of olefinic methine at δ_H 5.87 (br s, H-4). The spectrum showed lots of multiplets for remaining protons. The ^{13}C-NMR spectra ($CDCl_3$, 100 MHz, Table 2) of **2** showed the resonances for all twenty carbon atoms with the appearance of a new quaternary carbon signal at δ_C 71.2 (C-10) and disappearance of C-10 methine carbon atom as compared to **1**, which clearly indicated the presence of hydroxyl group. The spectrum showed a downfield signal for methine at δ_C 56.1 (C-9), which was appeared at δ_C 49.0 (C-9) in **1**, indicating the presence of -OH group at C-10 position. Similarly the methylene at δ_C 26.5 (C-1) of **1** was also shifted downfield at δ_C 33.2 in **2**, indicating hydroxylation at C-10. The rest of the spectrum closely resembled with **1**. In the HMBC spectrum of **2**, the C-4 and C-9 methine protons at δ_H 5.87 and δ_H α 1.18, respectively showed HMBC correlations with C-10 (δ_C 71.2). The HMBC spectrum also showed J^2 and J^3 correlations of C-1 and C-11 methylene protons (δ_H α 1.74 and δ_H β 2.48) and (δ_H α 1.90 and δ_H β 1.24), respectively with C-10 (δ_C 71.2) showing hydroxylation at C-10 position (Fig. 2). According to the rule of hydroxylation in steroids, the newly hydroxyl group has the same configuration as hydrogen atom had so it was suggested that the -OH group has β configuration.[8] A comparative study of the spectral data with the reported data indicated that it was the known metabolite, 10β-hydroxy norethisterone (**2**) previously obtained from the fungal transformation of **1** by using *Fusarium lateritium*, *Rhizopus arrhizus*, *Sclerotiorum sclerotiorum*, *Absidia orchidis*, *Curvularia lunata*, *Cladosporium herbarum*, *Cephalosporium asperum*[9] *Acremonium kiliense*[10] and *Rhizopus nigricans*[8] with low yield and along with another product but we are reporting first time **2** from **1** by using a new microbial source, *A. niger* with high yield (24.6%). The enzymatic system of *A. niger* is well known for 10β-hydroxylation of 19-nor steroidal compounds.[11,12] We also investigated the 10β-hydroxylating ability of *A. niger* by the hydroxylation of **1** at C-10 position. Furthermore the time scale study was also conducted, which showed that *A. niger* slowly hydroxylated **1** even after 12 days of fermentation some quantity of **1** was also present, which was separated during column chromatography using ethyl acetate:pet.ether (5.0:95.0) system (Fig. 3).

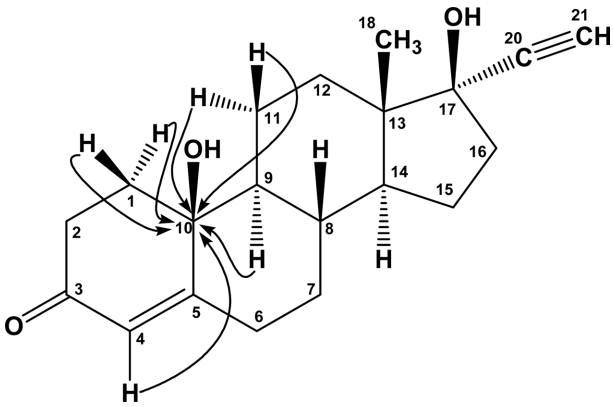


Fig. 2. Key HMBC (→) correlations of **2**.

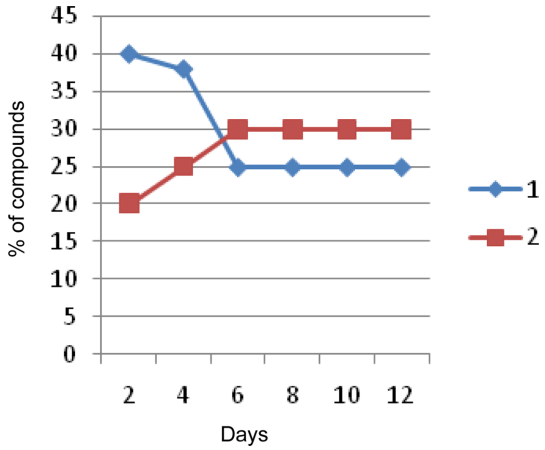


Fig. 3. Degree of transformation of norethisterone (**1**) into **2** by using *A. niger*.

The LREI-MS and HREI-MS of **3** were almost similar to **2**, showing same molecular mass and formula. The IR spectrum of **3** was also similar to **1** and **2**. The ^{1}H-NMR spectrum ($CDCl_3$, 400 MHz, Table 1) of **3** showed

Table 3. % Yield of metabolites of norethisterone (**1**) by fermentation with *Aspergillus niger* and *Penicillium citrinum*.

Names of Fungus	Fermentation time period (days)	% Yield of 2	% Yield of 3
Aspergillus niger	12	24.6	-
Penicillium citrinum	6	-	45.4

additional downfield shifted carbinolic proton signal at δ_H 4.27 (ddd, $J = 3.0$, 3.0 and 3.0, H-11) as compare to **1**, indicating the presence of secondary -OH group in **3** at C-1, -7, -11 or -15 but the absence of methylene proton signal at δ_H 1.89 and 1.22 (m, H-11) in **3** as compare to **1**, suggesting hydroxylation at C-11. The downfield shifting of C-9 methine δ_H 1.26 (m) and C-12 methylene protons δ_H 1.94 (m) and 2.01 (m) was also specifying hydroxylation at C-11. The remaining ^{1}H-NMR spectrum of **3** showed resemblance to **1**. The ^{13}C-NMR spectra ($CDCl_3$, 100 MHz, Table 2) of **3** revealed the transformation of methylene carbon of **1** into a deshielded methine at δ_C 66.6 in **3**. The spectrum showed downfield signals for methine at δ_C 54.2 (C-9) and methylene at δ_C 40.7 (C-12), which was appeared at δ_C 49.0 and δ_C 32.3, respectively in **1**, indicating the presence of -OH group at C-11 position. In the HMBC spectrum of **3**, the C-12 methylene protons (δ_H α 2.01 and δ_H β 1.94) showed correlations with C-11 (δ_C 66.6) while the C-11 methine proton (δ_H 4.27) showed HMBC correlations with C-12 (δ_C 40.7) and C-9 (δ_C 54.2). Similarly the C-9 methine proton (δ_H 1.26) showed correlation with C-11 (δ_C 66.6). The HMBC spectrum also showed correlations between H-11 (δ_H 4.27)/C-13 (δ_C 46.4) and H-11 (δ_H 4.27)/C-8 (δ_C 35.4), these correlations permitted us to conclude that hydroxylation occurred at C-11 position. In the COSY-45° spectrum of **3**, the C-11 methine proton (δ_H 4.27) showed vicinal coupling with the C-9 methine proton (δ_H 1.26) and C-12 methylene protons (δ_H α 2.01 and β 1.84) further indicating the hydroxylation at C-11 (Fig. 4). The relative stereochemistry of hydroxyl group (-OH) at C-11 was assigned as β from the NOESY interaction of the geminal H-11 (δ_H 4.27) with α-oriented H-9 (δ_H 1.26) (Fig. 5). A comparative study of the spectral data with the reported data indicated that it was the known metabolite, 11β-hydroxy norethisterone (**3**) previously obtained from the biotransformation of **1** by using fungus *Botryodiplodia malorum* with low yield and incomplete NMR spectroscopic data[13] but here we are reporting first time the bioconversion of **1** into **3** by using a new fungal source, *P. citrinum* with high yield (45.4%). Additionally we investigated the timescale study for the rate of transformation of **1** which showed that the rate of hydroxylation of **1** by *P. citrinum* was very fast. Metabolite **3** was appeared in a significant quantity after two days of fermentation. The

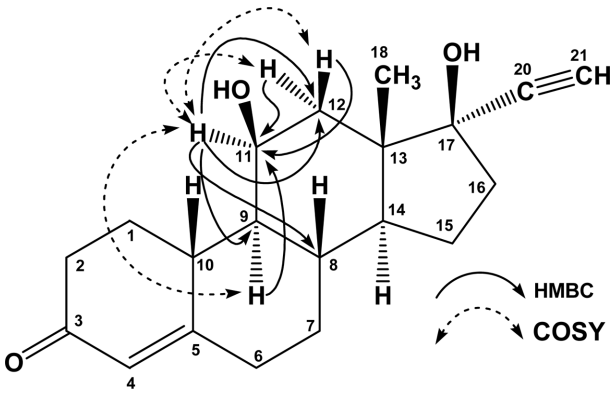


Fig. 4. Key HMBC (→) and COESY (⋯→) correlations of **3**.

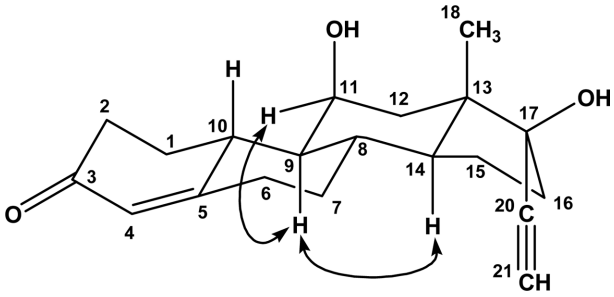


Fig. 5. Key NOESY (↔) correlations of **3**.

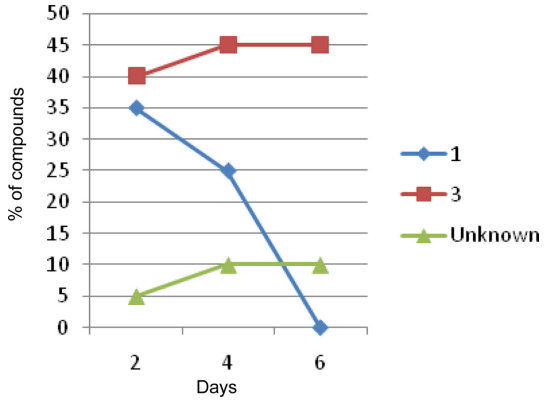
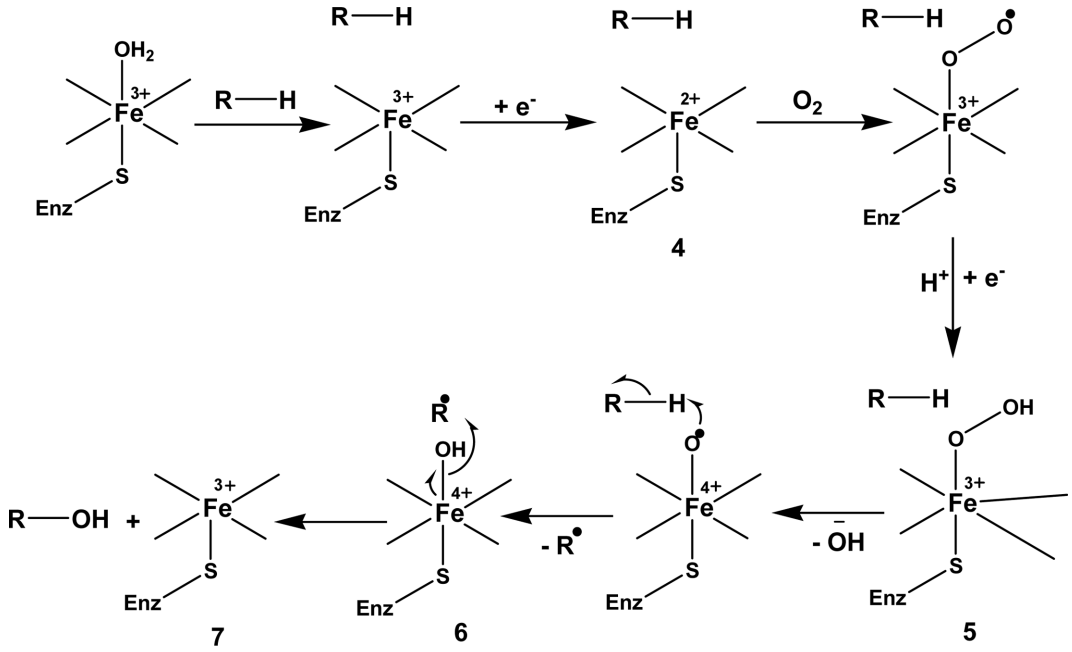


Fig. 6. Degree of transformation of norethisterone (**1**) into **3** by using *P. citrinum*.

concentration of **1** was decreasing day by day. After 6 days of fermentation, the substrate was completely transformed into **3** (Fig. 6) along with one another transformed product, which could not be characterized due to its insufficient amount for spectroscopic techniques.



Scheme 1. Hydroxylation of R-H by Cytochrome P450.

Microorganisms have monooxygenase system, which employ cytochrome P450 and also need an electron source, which is usually NADPH.[14] When steroidal compounds are incubated with fungal culture the monooxygenase enzymes brought the hydroxylation of steroids with high regio- and stereoselective by transferring one oxygen atom to the steroids which was difficult to achieved by traditional chemical synthesis. The series of events involves for the reduction of the Fe^{3+} in the cytochrome P450 heme complex to Fe^{2+} (**4**), which gain an oxygen, electron and proton to form an Fe^{3+}–OOH species (**5**), which loses a hydroxyl anion to form an oxygen radical which abstract hydrogen atom from steroid to generate a carbon radical and Fe^{4+} hydroxy species (**6**). The carbon radical of steroid then accepts a hydroxyl radical from **6** to form the hydroxylated biotransformed product and Fe^{3+} (**7**) (Scheme 1). Since these hydroxylations involve the replacement of hydrogen by a hydroxyl group so the stereochemistry remains same.

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