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A REVIEW ON BIOTRANSFORMATION OF A SYNTHETIC PROGESTIN, NORETHISTERONE

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ABSTRACT

A biotransformation is a key tool for the structural modification of natural and synthetic complex compounds. It is employed to synthesize those compounds, which are arduous to get from chemical synthesis. During the last few decades' research has been focused on the modification of biologically potent compounds. Biotransformation is one of the effective ways in this regard. The current article covers the biotransformation of norethisterone (**1**) by using various bacteria and fungi as well as metabolism in man and animals i.e. rat, rabbit, monkey and dog, which brought mono-, di-, and tri-hydroxylation, oxidation, reduction, epoxidation and deethynylation of **1**. The review covers 26 metabolites (**2** to **27**) of **1**, obtained during 1906 to 2018.

Keywords: *Biotransformation, Norethisterone, Progestin, Bacteria, Fungi, Human, Metabolites*

INTRODUCTION

Steroids are widely distributed in living organisms of plants and animal kingdoms. The basic skeleton of steroids contains 17 carbon atoms, which are arranged in the form of four rings, is called perhydrocyclopentanophenanthrene. They are also used as a raw material for the preparation of important biologically active compounds of pregnane, androstane and estrane series, which possess different hormonal activities (Bhatti-andKhera, 2012).

The biological system of plants, animals, fungi, bacteria and algae is rich of biological catalysts (enzymes), which catalyze chemical reactions in living organisms with higher stereo-, regio- and chemo-selectivity without forming undesirable side products. It also avoids tedious addition and removal of protecting groups as compared to traditional chemical synthesis. Nowadays the enzymatic system of aliving organism is routinely used for hydroxylation, oxidation, dehydrogenation, epoxidation, double bond formation, reduction, hydrolysis, acetylation and isomerization (Mahato and Mukherjee, 1984; Mahato and Banerjee, 1985; Mahato and Majumdar, 1993).

Norethisterone (NET) (17β-Hydroxy-19-nor-17α-pregna-4-en-20-yn-3-one) (**1**) (Fig. 1) is a potent synthetic sex steroidal hormone with very weak androgenic and estrogenic properties. It was synthesized and patented in 1951 by Syntex Company, Mexico City. About 50 million women are taking NET (**1**) or its precursors including norethisterone acetate, lynestrenol, norethynodrel and ethynodiol diacetate as oral contraceptive pills for birth control in several formulations (Hümpel, 1982; Schoonen *et al*., 2000; Perusquía *et al*., 2003). It is an antifertility agent, used to delay in menstruation and the treatment of endometriosis. It is also used to prevent unwanted pregnancies. Fertility control synthetic progestin **1** is considered to be the safest among the female contraceptives (Taniguchi *et al.*, 2017).

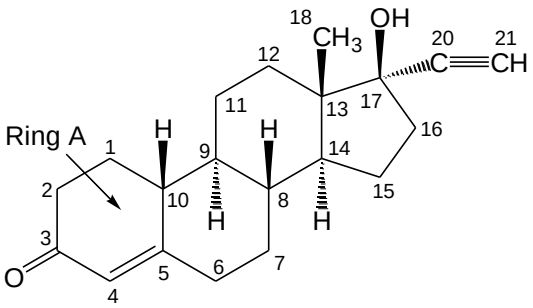


Fig. 1: Norethisterone (**1**)

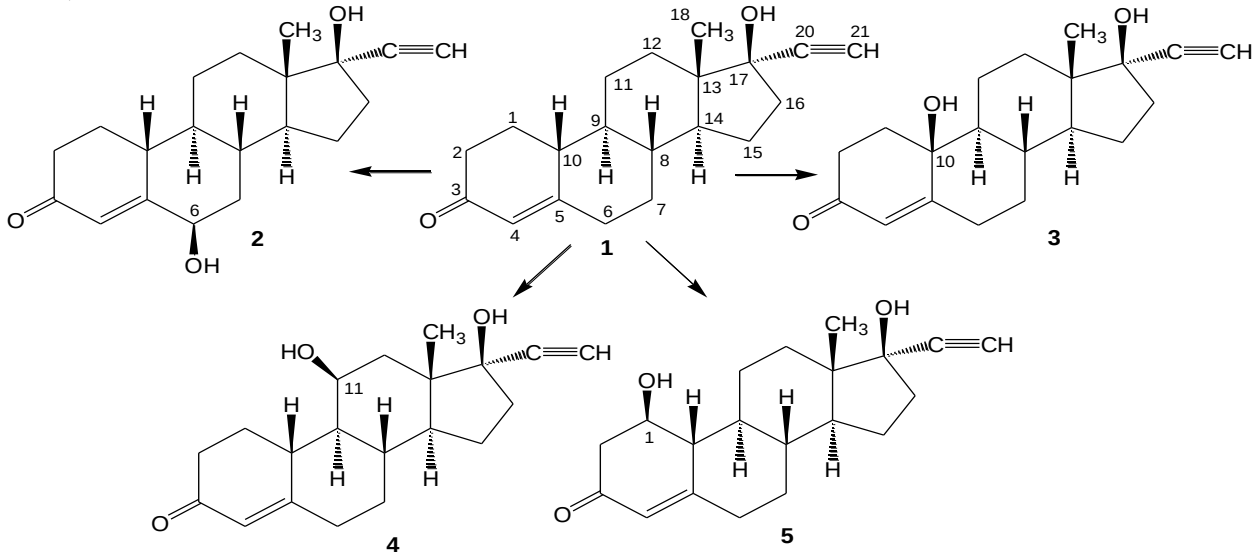
BIOTRANSFORMATION OF NORETHISTERONE (1): For the extension of our work on review article for biotransformation studies of bioactive drugs (Azizuddin and Iqbal, 2016; Azizuddin, 2017), the current work was carried out. This review article presents mainly the structures

of 26 metabolites (**2** to **27**) of **1** (Schemes 1a, 1b, 2, 3a, 3b and 4; Table 1), which were obtained *in vitro* and *in vivo* from a variety of biological media having specific enzymes during 1906-2018.

Biotransformation of norethisterone (1) by using fungi: Fungal reactions are unique in their importance and diversity. A fungal culture is rich with specific enzymes, which offer access to inaccessible sites not only in natural but also in synthetic steroidal compounds. Fermentation of **1** with various fungi yielded ten metabolites in which **2**-**8** were mono-hydroxylated at C-1β, C-6β, C-10β, C-11β, C-15β, C-1α and C-15α positions while **9** and **10** were di-hydroxylated at C-6β, C-10β and C-10β, C-11β positions, respectively whereas **11** was found to be aromatic metabolite, which was formed by the $\Delta^{1,2}$ dehydrogenation of ring-A (Schemes1 and 2).

Ambrus *et al.*, (1972) reported the first microbial hydroxylation of **1** by using seven fungi; *Fusarium lateritium, Rhizopus arrhizus, Sclerotinia sclerotiorum, Absidiaorchidis, Curvularialunata, Cladosporiumherbarum* and *Cephalosporium asperum*, which afforded two mono-hydroxylated metabolites, 6β,17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**2**) and 10β, 17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**3**).

Greenspan *et al.*, (1974) incubated **1** into secondary stage culture of *Botryodi plodiamolorum*, which transformed **1** into 11β, 17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**4**) and 1β, 17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**5**) as major and minor, respectively after 3 to 5 days of incubation.



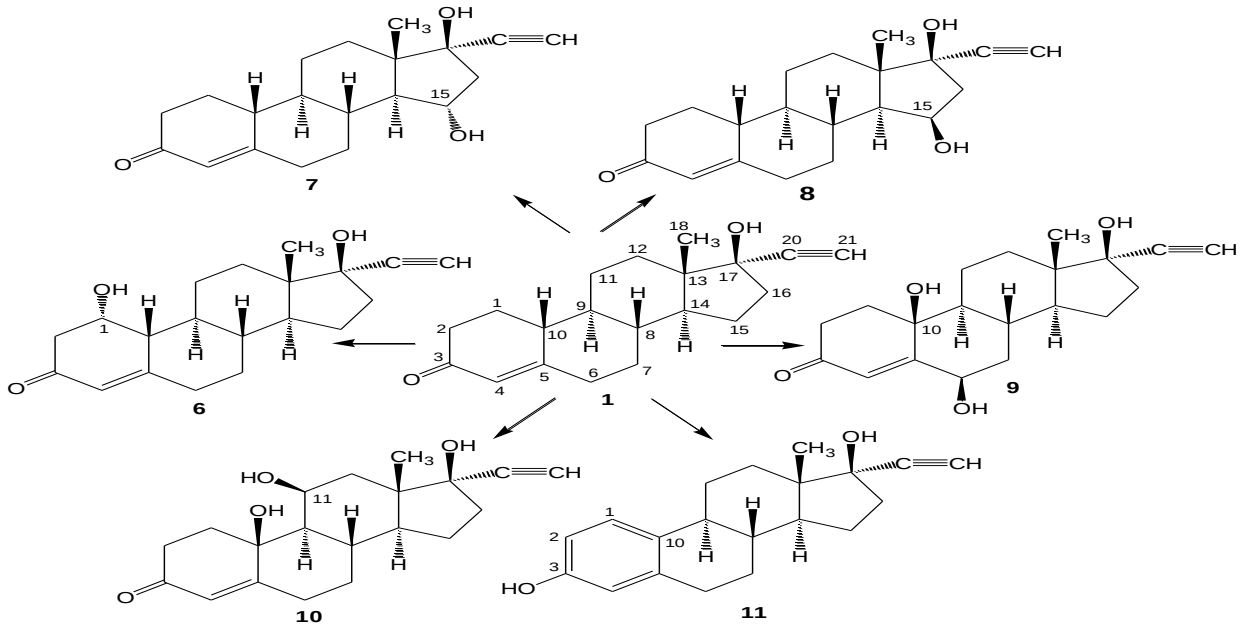
Scheme 1: Biotransformation of norethisterone (**1**) by using fungi.

Ambrus and co-workers (1975a) tested more than 300 fungal strains for the biotransformation of **1** and observed that only limited fungi were responsible for the bioconversion of **1** into five mono- and two di-hydroxylated products; **2**, **3**, 1α,17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**6**), 15α, 17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**7**), 15β, 17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**8**), 6β, 10β-17β-trihydroxy-19-nor-17α-pregna-4-en -20-yn-3-one (**9**) and 10β, 11β, 17β-trihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**10**). It was observed that **6** was more superior contraceptive than **1** (Ambrus *et al*., 1974).

Ambrus *et al.*, (1975b) isolated two fungi, *Acremonium strictum* and *Acremonium kiliense* from soil and used them for the microbial hydroxylation of **1**. The culture of *Acremonium strictum* converted **1** into **6** after 24 hours of incubation while the culture of *Acremonium kiliense* transformed **1** into greater amount of **6** and smaller amount of **3** after 16 hours incubation.

Žakelj-Mavrič *et al.*, (1986) incubated **1** with *Rhizopus nigricans*, which yielded again two mono-hydoxylated transformed products, **2** and **3**.

Choudhary *et al.*, (2004) added **1** in the 24 hours old stage-II culture of fungus *Cephalosporium aphidicola* and allowed fermentation for 8 days, which brought the biotransformation of **1** into a single less polar aromatic metabolite 3, 17β-dihydroxy-19-nor-17α-pregna-1, 3, 5(10)-triene-20-yn (**11**) through $\Delta^{1,2}$ dehydrogenation of ring-A. Metabolite **11** is a popular estrogen and also used as oral contraceptive.

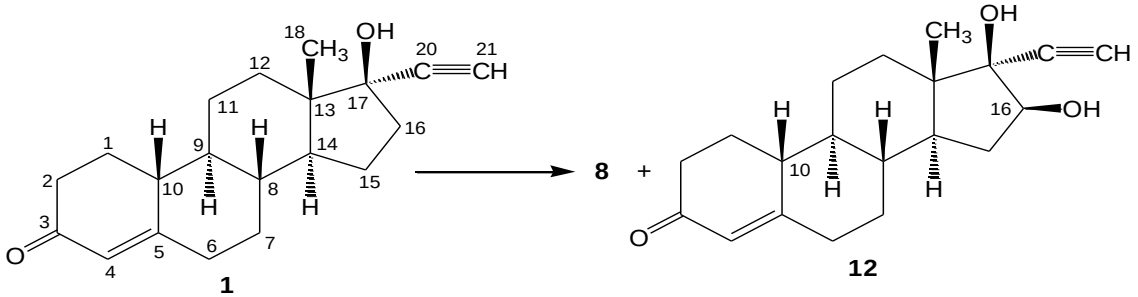


Scheme 2: Biotransformation of norethisterone (**1**) by using fungi.

Biotransformation of norethisterone (1) by using mutant bacteria: The prokaryotic micro-organism, bacteria has Cytochrome P450 BM3 enzyme, which has the highest catalytic activity ever seen for a monoxygenase. The natural substrates of Cytochrome P450 BM3 are long chain fatty acids but now a day several engineered mutants are used to convert bioactive compounds. Fermentation of **1** with mutant bacteria yielded two mon-ohydroxylated metabolites (**8** and **12**) at C-15β and C-16β positions, respectively (Scheme 3).

Rea *et al.*, (2012) brought the A82W mutation of Cytochrome P450 of bacteria, *Bacillus megaterium* in two templates M11 and M01, and get two P450 BM3 mutants M11 A82W and M01 A82W. They transformed **1** into **8** and 16β, 17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**12**) by M11, M11 A82W, M01 and M01 A82W. They also compared the % transformation of **1** between M11 and M11 A82W and between M01 and M01 A82W. The result on comparison showed that both mutants M11 A82W and M01 A82W increase selectivity for 16β-hydroxylation (42 to 88% and 58 to 77%, respectively) as compared to non-mutants.

Reinen and co-workers (2015) also presented the regio and stereoselective hydroxylation of **1** by the resting whole cells culture of CYP-BM3 mutants (MT80 and MT102) of *Escherichia coli*, which afforded **8** and **12**, respectively.



Scheme 3: Biotransformation of norethisterone (**1**) by using mutant bacteria.

Biotransformation of norethisterone (1) in amimals: The biotransformation, pharmacokinetics, absorption, distribution etc. of all drugs are study in animals before given to human. The biotransformation of potent synthetic progestin **1** in different animals such as beagle, rabbit, rat, African green monkey and grey hound bitch were reported by different researchers. They obtained thirteen transformed products in which only two compounds (**20a** and **20b**) retained the 4-ene moiety of **1** while in all other transformed product (**13–19**, **21-23b**) 4-ene moiety were reduced (Schemes 4 and 5).

Cook *et al.*, (1974) incubated **1** with a supernatant fraction of beagle liver and isolated two novel transformed products. One of the

metabolites was formed by the epoxidation of 4-ene moiety and identified as 17β-hydroxy-19-nor-4β, 5β-epoxy-17α-pregna-20-yn-3-one (**13**) while the other was oxidized product having the additional ketonic group as compared to **1** and characterized as 17β-hydroxy-19-nor-5α, 17α-pregna-20-yn-3, 6-dione (**14**). Both metabolites were less uterotropic than **1** whereas**14** was found to be less antifertility agent than **1** and **13**.

Khan and Fotherby (1979) dissected white female rabbits of New Zealand and separated hepatic tissues. The liver tissues were incubated with **1** and studied the metabolism. They observed the reduction of ring-A, which led to tetrahydro derivative of **1** and identified as 17α-ethynyl-5β-estran-3β, 17β-diol (**15**).

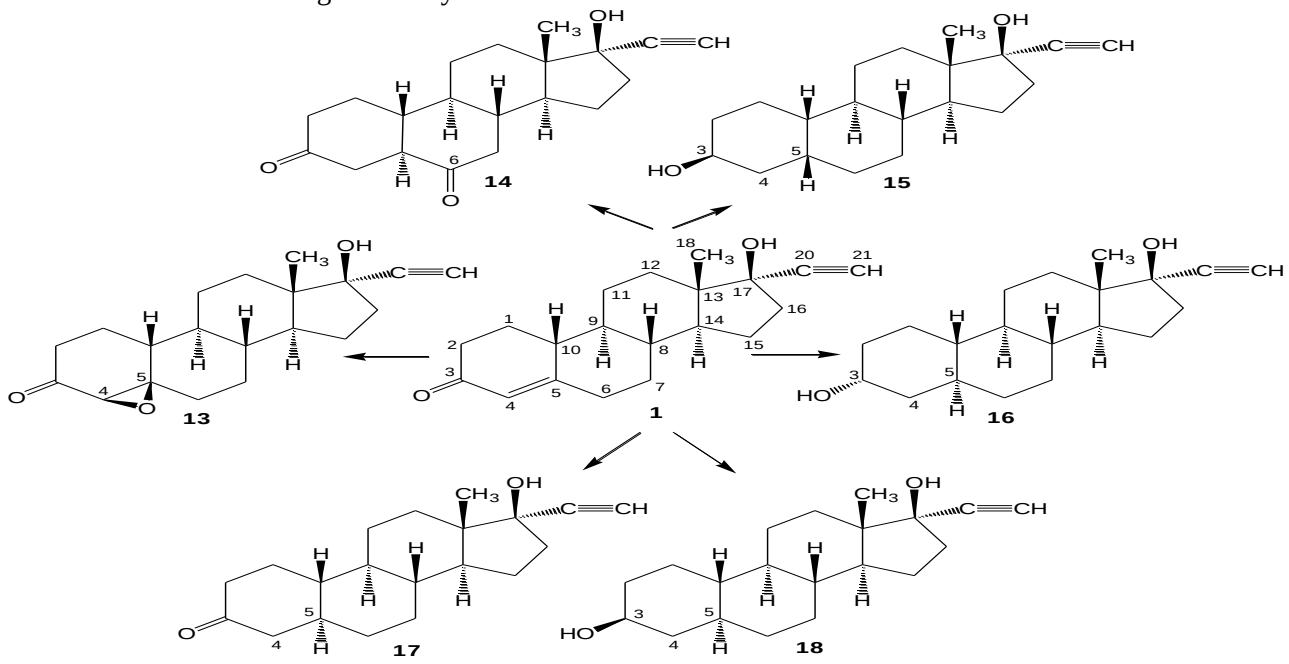
When seven cases of hepatic tumours in women were reported by those who take oral contraceptives; Peter and co-workers (1981) decided to study the metabolism of **1**. They incubated **1** in rat liver microsomes *in vitro* and obtained **13**. These researchers could not found the mutagenicity of **13** in the Ames-test and rejected the idea of a direct carcinogenic effect of **13**.

Mendoza *et al.,* (1993) obtained the anterior pituitaries from 3-month-old female Wistar rats at random stages of the estrous cycle. The ethanolic-aqueous solution of **1** was added to the cells and obtained the ring-A tetrahydro reduced metabolite 17α-ethynyl-5α-estran-3α, 17β-diol (**16**) and C-5α reduced metabolite 17β-hydroxy-19-nor-5α-17α-pregna-20-yn-3-one (**17**). Metabolite **16** binds the estrogen receptor while **17** binds the androgen receptor, showing both estrogen and androgen like effects on gonadotropin secretion, respectively.

Blom and co-workers (2001) investigated whether the metabolism of **1** can take place in organs, which are target to hormone replacement therapy (HRT). Mature female proestrus Wistar rats were sacrificed and the uterus, vagina and aorta were separated for the tissue fragment incubations. Two major metabolites of **1** in all three tissues were identified as **16** and **17** whereas ring-A reduced metabolite 17α-ethynyl-5α-estran-3β, 17β-diol (**18**) was obtained more in the vagina and less in the uterus.

Pasapera *et al.,* (2002) explored the biotransformation of **1** into ring-A reduced metabolite **16** and its isomer **18** in African green monkey breast cancer T-47D cells and kidney CV-1 cells.

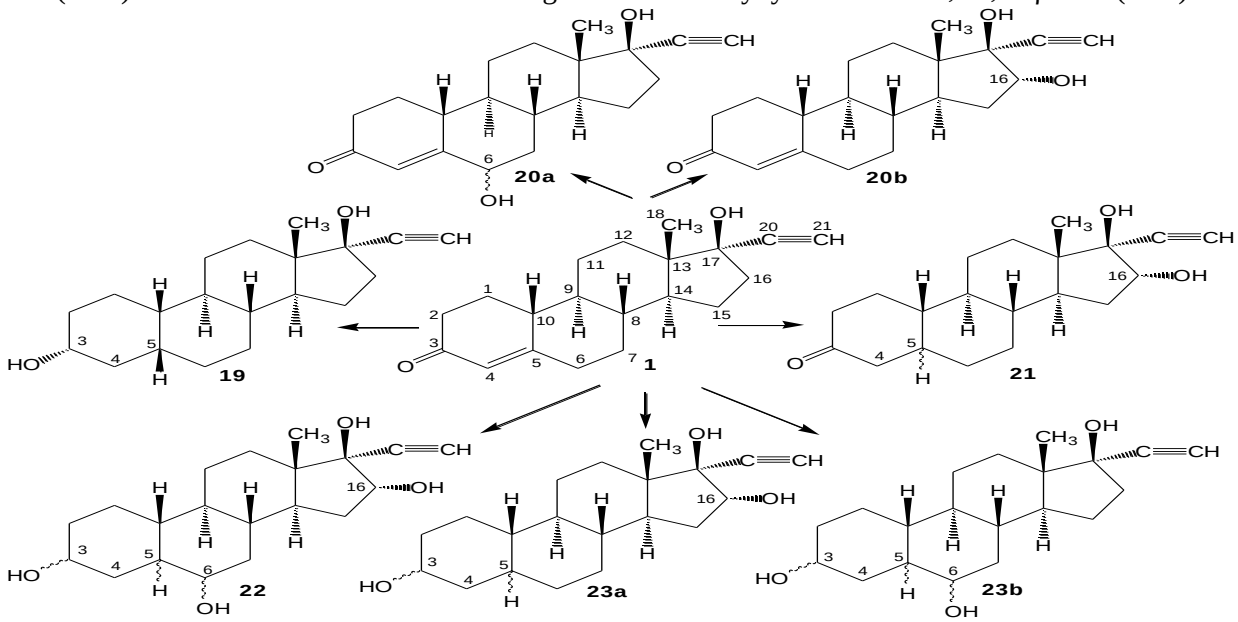
Lemus and co-workers (2009) incubated radiolabeled **1** with female rate and observed that osteoblastic cells of female neonatal Wistar rats efficiently biotransformed **1** into three ring-A tetrahydro reduced products **16**, **17** and **18**. They also concluded that first **1** is reduced into **17** then it is further reduced into **16** and **18**.



Scheme 4: Biotransformation of norethisterone (**1**) in animals.

Biddle and co-workers (2013) first time studied the metabolism of **1** in greyhound bitch. A dose of 5mg/day of **1** was given orally to animals for eight days. The urine samples were collected after 2, 4, 8, 24, 36, 48 and 72 hours and 4, 5, 6, 7 and 8 days, and kept at -20 ºC. The urinary metabolites were identified by using mass spectrometry and proposed metabolic pathway. The major

metabolic pathway was the reduction of 4-ene-3-one group of ring-A into **18** and 17α-ethynyl-5β-estran-3α, 17β-diol (**19**). One of the metabolite was hydroxylated but the position of –OH was not sure so it was assigned as either 6z, 17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**20 a**) or 16α, 17β-dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**20 b**). One reduced metabolite was assigned as 16α, 17β-dihydroxy-19-nor-5z-17α-pregna-20-yn-3-one (**21**). Other metabolites showed the reduction of the 4-ene-3-one moiety of **1** along with hydroxylation of C-6 and C-16 in which one metabolite was assigned as 17α-ethynyl-5z-estran-3z, 6z, 16α, 17β-tetrol (**22**) while the other was either 17α-ethynyl-5z-estran-3z, 16α, 17β-triol (**23 a**) or 17α-ethynyl-5z-estran-3z, 6z, 17β-triol (**23 b**).



Scheme 5: Biotransformation of norethisterone (**1**) in animals.

Biotransformation of norethisterone (1) in man: Incubation of **1** in different body parts of human yielded eleven transformed products in which four compounds (**24-27**) were new while two compounds (**5** and **11**) were similar to fungal metabolites while the rest (**15-19)** were similar to animal metabolites (Scheme 6).

Gerhards *et al.*, (1971) investigated the metabolism of **1** in man and found metabolite **19** in the glucuronosidic urinary fraction of man.

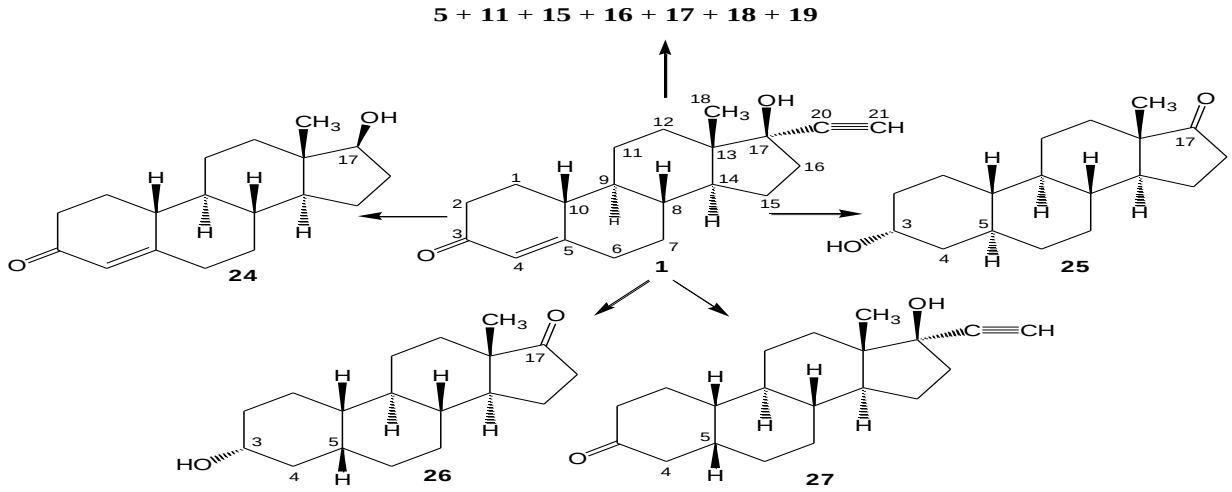
Stillwell and co-workers (1972) administrated 10 mg of **1** orally in man for two days and collected the urine samples during the 24 hours period, diluted to a volume of 2L and stored at low temperature (-15 ºC). The steroidal drug metabolites were found in a fraction of urine (27% ethyl acetate in benzene), which was then further separated into ketone and non-ketonic fractions. The non-ketonic fraction was subjected to mass spectrometry and gas chromatography. The metabolites of **1** were characterized as **16** and **19**.

Honjo (1975) incubated **1** with human placental microsomes, which was supplemented with NADPH-generating system, transformed **1** into **5** and **11**.

Masse and co-workers (1989) studied various anabolic steroids and their metabolites in human urine. After administration of 10 mg of **1** to female, they proposed de-ethynylation of **1** yielded a metabolic intermediate 17β-hydroxy-19-norandrost-4-ene-3-one (**24**) through which **1** is transformed into 5α-estran-3α-ol-17-one (**25**) and 5β-estran-3α-ol-17-one (**26**).

Ye and co-workers (1995) presented the gas chromatography-mass spectrometric method for the separation of four 19-androsterone metabolites of **1** from samples of human urine after administration of **1**. The main metabolites were identified as **16** and **19** while two other metabolites **25** and **26** were also present.

Pommier *et al.*, (1995) described capillary gas chromatography-mass-selective analysis for the simultaneous determination of **1** and its metabolites in human plasma after transdermal application of norethisterone acetate (NETA) (Precursor of norethisterone). After enzymatic hydrolysis of NETA, the major compound in blood was **1** before hepatic metabolism. Plasma concentrations of eight post-menopausal women contained two dihydro derivatives of **1**, which were found to be **17** and 17β-hydroxy-19-nor-5β-17α-pregna-20-yn-3-one (**27**) while four tetrahydro derivatives of **1** were also identified as **15**, **16**, **18** and **19**.



Scheme 6: Biotransformation of norethisterone (**1**) in man.

CONCLUSION

Biotransformation of organic compounds has been studied for centuries, which brought the modification of compounds with highly regio-, stereo- and chemo-selectivity through a green approach. The current article shows the structure diversity of norethisterone. This review article will be helpful for those researchers who work on green reactions (environmental friendly). It will not only beneficial for further modification in norethisterone but also for structure-activity relationship among its transformed products.

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Table 1: Norethisterone (**1**) and its reported biotransform edproducts **2-27**.

S. No.	NET (1) and its biotransformed products	Biological system	Reference
1	17β-Hydroxy-19-nor-17α-pregna-4-en-20-yn-3-one) (**1**)	-	-
2	6β,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**2**)	*Fusarium lateritium, Rhizopus arrhizus, Sclerotinia sclerotiorum, Absidia orchidis, Curvulari alunata, Cladosporium herbarum, Cephalosporium asperum, Rhizopus nigricans*	Ambrus *et al*., (1972); Žakelj-Mavrič *et al*., (1986)
3	10β,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**3**)	*Fusarium lateritium, Rhizopus arrhizus, Sclerotinia sclerotiorum, Absidia orchidis, Curvulari alunata, Cladosporium herbarum, Cephalosporium asperum, Acremonium kiliense, Rhizopus nigricans*	Ambrus *et al*., (1972); Ambrus *et al*., (1975b); Žakelj-Mavrič *et al*., (1986)
4	11β,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**4**)	*Botryodiplo diamolorum*	Greenspan *et al*., (1974)
5	1β,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**5**)	*Botryodiplo diamolorum*, human placental microsomes	Greenspan *et al*., ,(1974); Honjo (1975)
6	1α,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**6**)	Unknown fungus, *Acremonium kiliense*, *Acremonium strictm*	Ambrus *et al*., (1975a); Ambrus *et al*., (1975b)
7	15α,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**7**)	Unknown fungus	Ambrus *et al*., (1975a)
8	15β,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**8**)	Unknown fungus, mutant *Bacillus magaterium* M11 A82W and M01 A82W, mutants *Escherichia coli* MT80 and MT102	Ambrus *et al*., (1975a); Rea *et al*., (2012); Reinen *et al*., (2019)
9	6β,10β-17β-Trihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**9**)	Unknown fungus	Ambrus *et al*., (1975a)
10	10β,11β,17β-Trihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**10**)	Unknown fungus	Ambrus *et al*., (1975a)
11	3,17β-Dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-20-yn (**11**)	*Cephalosporium aphidicola*, human placental microsomes	Choudhary *et al*., (2004); Honjo (1975)
12	16β,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**12**)	Mutant *Bacillus magaterium* M11 A82W and M01 A82W, mutants *Escherichia coli* MT80 and MT102	Rea *et al*., (2012); Reinen *et al*., (2019)
13	17β-Hydroxy-19-nor-4β,5β-epoxy-17α-pregna-20-yn-3-one (**13**)	Beagle liver, rat liver microsomes	Cook *et al*., (1974); Peter *et al*., (1981)
14	17β-Hydroxy-19-nor-5α-17α-pregna-20-yn-3,6-di-one (**14**)	Beagle liver	Cook *et al*., (1974)
15	17α-Ethynyl-5β-estran-3β, 17β-diol (**15**).	Hepatic tissues of rabbits, human plasma	Khan and Fotherby (1979); Pommier *et al*., (1995)
16	17α-Ethynyl-5α-estran-3α,17β-diol (**16**)	Anterior pituitaries, uterus, vagina and aorta tissues of Wistar rat; osteobalstic cells of female neonatal Wistar rat; African green monkey breast cancer T-47D cells and kidney CV-1 cells; human; human plasma	Blom *et al*., (2001); Lemus *et al*., (2009); Mendoza *et al*., (1993); Pasapera *et al*., (2002); Pommier *et al*., (1995); Stillwell *et al*., (1972); Ye *et al*., (1995)
17	17β-Hydroxy-19-nor-5α-17α-pregna-20-yn-3-one (**17**)	Anterior pituitaries, uterus, vagina and aorta tissues of Wistar rat; osteobalstic cells of female neonatal Wistar rat; human plasma	Blom *et al*., (2001); Lemus *et al*., (2009); Mendoza *et al*., (1993); Pommier *et al*., (1995)

18	17α-Ethynyl-5α-estran-3β,17β-diol (**18**)	Uterus and vagina tissues of Wistar rat; African green monkey breast cancer T-47D cells and kidney CV-1 cells; osteobalstic cells of female neonatal Wistar rat; Greyhound bitch; human plasma	Biddle *et al*., (2013); Blom *et al*., (2001); Lemus *et al*., (2009); Pasapera *et al*., (2002); Pommier *et al*., (1995)
19	17α-Ethynyl-5β-estran-3α,17β-diol (**19**)	Greyhound bitch, human, human plasma	Biddle *et al*., (2013); Gerhards *et al*., (1971); Pommier *et al*., (1995); Stillwell *et al*., (1972); Ye *et al*., (1995)
20	6z,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**20 a**)	Greyhound bitch	Biddle *et al*., (2013)
21	16α,17β-Dihydroxy-19-nor-17α-pregna-4-en-20-yn-3-one (**20 b**)	Greyhound bitch	Biddle *et al*., (2013)
22	16α,17β-Dihydroxy-19-nor-5z-17α-pregna-20-yn-3-one (**21**)	Greyhound bitch	Biddle *et al*., (2013)
23	17α-Ethynyl-5z-estran-3z,6z,16α,17β-tetrol (**22**)	Greyhound bitch	Biddle *et al*., (2013)
24	17α-Ethynyl-5z-estran-3z,16α,17β-triol (**23 a**)	Greyhound bitch	Biddle *et al*., (2013)
25	17α-Ethynyl-5z-estran-3z,6z,17β-triol (**23 b**)	Greyhound bitch	Biddle *et al*., (2013)
26	17β-Hydroxy-19-norandrost-4-ene-3-one (**24**)	Human	Masse *et al*., (1989)
27	5α-Estran-3α-ol-17-one (**25**)	Human	Masse *et al*., (1989); Ye *et al*., (1995)
28	5β-Estran-3α-ol-17-one (**26**)	Human	Masse *et al*., (1989); Ye *et al*., (1995)
29	17β-Hydroxy-19-nor-5β-17α-pregna-20-yn-3-one (**27**)	Human plasma	Pommier *et al*., (1995)