3-Hydroxy-13-methyl-9,11,12,14,15,16-hexahydro-6H-cyclopenta(a)phenanthren-17-one
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1. André Girard

The discovery of equilin in 1932 goes back to André Girard and his co-workers. André Girard was a French chemist who lived from 1901-1968 and was employed by Goupe Roussel in the Laboratoires des Proxystases, which mainly produced insulin and vitamin B12 from horse organs. In 1927 Girard succeeded in producing a fat-soluble bismuth solution, which was known as Bivatol and was used for syphilis, the following year Sterogyl, a drug based on vitamin D. Finally, in 1932, he succeeded in isolating hormones from an aqueous solution using the Girard-reagents and presented them in London in 1932 at an International Conference of the Classification of Sex Hormones.

2. Steroid hormones

Equilin is an estrogen found in the urine of pregnant mares and has a typical steran basic structure with 4 annealed carbon cycles. For analytical purposes, equilin has a specific light adsorption of 280 nm wave length. Cycle A has an aromatic system and a hydroxyl group at the C-3 atom. It is unique in its C-C double bond at the C-7 and C-8 atoms, which has no conjugation with the cyclic ring A. Equilin contains stereogenic centers at the C-9, C-13 and C-14 atoms, which are uniformly configured S. With its secondary carbon-cycle system, equilin contains the same chemical properties as other steroid hormones. It is not soluble in water but the hydroxyl and keto group make hydrophilic interactions possible. Its hydrophobic properties therefore makes it possible for equilin to diffuse through the outer cell membrane and infiltrate the cytoplasm of cells to take effect inside the cell.

As a part of the steroid hormone group, equilin has a common effect on cells targeted by hormones, which are distributed throughout the organism through its cardiovascular system. equilin has to bind to lipoproteins in order to ensure its solubility in the aqueous solution and to allows targeted transport into the target cells. When this target cell is reached, equilin diffuses through the cell membrane and then binds to receptors within the nucleus. This receptor-ligand influences certain DNA sequences and thus acts as a transcription factor that changes gene expression.

Compared to the human estrone, equilin differs only by means of the C-C double bond between the C-7 and C-8 atom. Nevertheless, estrone has a far higher estrogenic activity than equilin in the human body.

2-B. Kleine, W. Rossmanith; Hormone und Hormonsystem- Lehrbuch der Endokrinologie 3. Auflage, Springer Spektrum
3-J.M. Berg, J.L. Tymoczko, L. Stryer; Biochemie 7. Auflage, Springer Spektrum
3. Extraction and Synthesis

The collection of equilin can be divided in three alternate approaches:

- the extraction of equilin from its natural source, the urine of a pregnant mare
- a synthesis by biothechnological methods
- a chemical synthesis

Biosynthesis:

The most common method of gaining equilin is by extracting it from mares’ urine, in which the concentration of equilin varies throughout a horse’s pregnancy. Its biosynthesis takes place in the mares’ placenta; the process, however, is not fully understood. It seems that the biosynthesis is independent from cholesterol, however its precursors or intermediates as well as the stereo chemical development are not known. In the horse’s urine, equilin as well as equilenin is present in the form of hormone-esters, where a hormone is bound to sulphuric acid. The ester bond can be broken enzymatically by using sulfatases or chemically by using hot mineral acids, which is a simple ester hydrolysis. The sulphate influences the hormones effect on the body. Sulphate containing estrone shows about 1/7 of the activity compared to estrone when consumed by injection and it shows two times the activity when used orally.

Extraction:

The process of extraction is divided in four steps:

- Fluid-fluid extraction
- Processing
- Exchange of the solvent
- Fluid-fluid extraction

Firstly, the hormone-esters are extracted from the urine with butanol. The butanol containing the hormone-ester, acids and free phenols is washed with sodium hydroxide. This process removes the free phenols and acids from the butanol without washing out the hormone-esters. After that, the butanol is vaporized and the remains are soluted by a chloroform- and ethanol solution. From that solution, the hormone-esters can be extracted by water. The product of this process is not a pure hormone but a mixture of hormones. To gain a pure product they need to be separated, for instance by their melting points, which are:

- Equilin: ca. 240°C
- Equilenin: ca. 259°C
- Estrone: ca. 261-264°C

5-Louis Fieser und Mary Fieser, Steroide, S.523
6-Rudolf Abderhalden, Lehrbuch der Physiologie, Die Hormone, S.17
7-Rudolf Abderhalden, Lehrbuch der Physiologie, Die Hormone, S.17
**Biotechnological Synthesis:**

Over 20 microorganisms have been screened of their ability of synthesising equilin out of 19-hydroxy-androsta-4,7-diene-3,17-dione. The best results were achieved by Nocardia rubra, which achieved a transformation yield of 15%. However, the yield has been optimised by usage of mutants. These mutants were created by exposing N. rubra to radiation for up to 30 min. By optimising the conditions given by substrate and medium transformation rates of 40% have been recorded.

The substrate should have following properties to ensure a synthesis. The 19-position or the 19- and the 3-position should be hydroxylated. Substrates having hydroxylgroups at both positions will be transformed slower than substrates with only one hydroxylgroup at the 19-position.

If one of the named positions is acetylated, the velocity and yield of the process are also determined by the rate of hydrolyses.

If a stable group as a methyl ether is attached to 19- or 3-position no conversion has been recorded.¹⁰

**Chemical Synthesis:**

The chemical synthesis of equilin has been a challenge for a long time, it was rated as a key requirement for an extended clinical usage of equilin and to gain new derivates for further biological examination. Its unusual properties, such as its non-conjugated double bond in the B-ring, lead to an isomerisation in the presence of acids and to the aromatisation of equilenin in the presence of palladium and hydrogen. This dehydration contradicts the usual hydrating effect of palladium and hydrogen on double bonds.

To access a chemical synthesis, there had to be found conditions not affecting the non-conjugated double bond. This had been achieved in 1958 and was published in the Journal of the American Chemical Society. In the described synthesis, equilin was synthesized from 19-nortestosterone acetate.

There are multiple intermediate stages as a dehydrobromination (II – III), the intermediate is brought to its enol diacetate form (IV) by the procedure of Velluz and his co-workers. After that, the enol diacetate is reduced with sodium borohydride to \( \Delta^{5,7}-19\)norandrostadiene-3\(\beta\),17\(\beta\)-diol (V).

Oppenauer oxidation of V provides (VI). In the last step, Substrate VI is transformed by a microbiological dehydrogenation, Corynebacterium simplex led to over 60% yield in this step¹¹

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⁹ [https://de.wikipedia.org/wiki/Nocardia](16.06.2017)
¹¹ [http://pubs.acs.org/doi/pdf/10.1021/ja01543a072](16.06.2017)
4. Premarin©

Equilin is one of the most frequently used hormones in hormone replacement therapy for postmenopausal women in form of the drug Premarin© (one of many possible drugs), which was first sold by Wyeth Ayerst in 1942 and today marketed by Pfizer. The name Premarin© derives from the origin of the basic substance, Pregnant Mare's urine.

In the middle of the sixties, estrogen preparations were most popular mainly because they were used as cure for osteoporosis, dementia and other diseases. Premarin© enjoyed an outstanding popularity due to its effectiveness and became the third most sold drug worldwide in the 1970s. At the same time, a study found out that women who had not had their uterus removed medically should be given a gestagen therapy in addition to Premarin© to prevent endometrial cancer because hormone replacement therapies were suspected of increasing the risk of cancer. In the following years further studies emerged which gradually revealed side effects of the estrogen preparations and therefore diminished the reputation of hormone preparations. In 1991 the Women's Health Initiative (WHI) was founded, which explicitly deals with the topic of women in the post-menopause and carries out studies until today. One of these studies in 2002/2003 found out that hormone replacement therapies in combination of a gestagen therapy increased the risk of heart attacks, strokes and blood clots as well as the risk of getting breast cancer, which lead to a drastic downfall of Premarin© sales. At the same time (2013), sales of Premarin© were about US $ 1.07 billion, making it the 10th most frequently sold drug from Pfizer as low-dose Premarin© preparations sales rose.

Premarin© itself is a drug used in hormone replacement therapy to fight the symptoms of menopause. This is a reduced production of the body's hormones in the ovaries of a woman and leads to a drop in estrogen, which causes deficiency symptoms. On the other hand the production of male hormones is reduced by about 10-15 years after woman menopause, causing a disequilibrium of female and male hormones during menopause, which can lead to discomfort, sweating, heart disease, sleep disorders, vaginal infections as well as an increased risk of cardiovascular disease and osteoporosis. Equilin (sodium-equilinsulfate), which is one component of Premarin©, is intended to restore the equilibrium and reduce these symptoms and risks. Nevertheless the effect of equilin cannot be equated with the effects of Premarin© as Premarin© consists of a mixture of estrogens.

Due to this mixture of estrogens, the effects of the individual components were investigated and discovered the following.

5. Effects inside the human body

Naturally produced estrogen in the body can be hydroxylated at the 2- or 4-position catalyzed by cytochrom P450 (CYP) isoenzym. This enzyme, which is mainly found in the liver, serves as a monooxygenase and leads to derivatives called catechol estrogens. The hydroxylation preferably takes place at the 2-position of endogenous estrogens. At the end of the eighties it was found that the resulting 4-hydroxyestrogen has carcinogenic effects. One possible explanation of equilin's carcinogenic properties is a different decomposition compared to the human estrogen metabolism. In contrast to endogenous hormones equilin is mainly hydroxylated at its 4-position in a reaction catalysed by CYP. It is thought that the properties of equilin are due to the fact that the C-C double bond of the unsaturated B-ring causes a different reaction at the cytochrom P450 isoenzym deriving a 4-hydroxyequilin as the resulting catechol estrogen and therefore metabolises differently to the 2-position hydrolysed endogenous estrogen. 4-hydroxyequilin is autoxidised to an o-quinone, the 4-hydroxyequilin-o-quinone, which then isomerises and forms a quinone-methide, that reacts to 4-hydroxyequilenin. It is assumed that the return to aromaticity is the driving force of this reaction. 4-hydroxyequilenin in turn oxidises voluntarily to a 4-hydroxyequilin-o-quinone, without the addition of a metal ion or an enzyme as a catalyst. The resulting quinone is more stable compared to the 4-hydroxy-o-quinone formed from endogenous hormones due to the π-conjugation of the two aromatic systems of ring A and B. In comparison, 2-hydroxyestrogens do not oxidize to o-quinones, regardless of whether they are endogenous or exogenous. Many important biological effects of equilin are attributed to 4-hydroxy-o-quinone, including toxic effects on human detoxifying enzymes and DNA-damages. This metabolite interacts with the deoxynucleosides of the DNA and forms a bulky DNA, while interaction with thymidine is not possible due to the lack of an exocyclic amino group. In addition, 4-hydroxyequilenin-o-quinone can lead to oxidation of the DNA bases as well as apurinic sites containing neither a purine nor a pyrimidine. Both, 4-hydroxyequilin-o-quinone and 4-hydroxyequilenin-o-quinone can also consume reduction equivalents such as NAD(P)H, causing further damage.
Besides this negative effect the oxidation to 4-hydroxyestrone results into a positive effect regarding the inhibition of lipid peroxidation. It can act as an antioxidant. The oxidation of the low density lipoproteins (LDL), which carry cholesterin to cells, can lead to calcification of the vascular inner walls and thus to cardiovascular diseases (arteriosclerosis etc.). For arteriosclerosis, glucose metabolism decomposes less glucose, which can lead to hyperinsulinaemia. Conjugated estrogens can counteract this development (Wilcox et al.). Equilin sulfate, for example, increases the glucose degradation thus counteracts the development to hyperinsulinaemia.19,20,21,22,23

23-Fagen Zhang et all, The Major Metabolite of Equilin, 4-Hydroxyequilin, Autoxidizes to an o-Quinone Which Isomerizes to the Potent Cytotoxin 4-Hydroxyequilenin-o-quinone, 1998