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Preparation of steroidal hormones with an emphasis on transformations of phytosterols and cholesterol - a review

Yosef Al Jasem[1], Mubashar Khan[2], Ahmed Taha[3] and Thies Thiemann[4,] *

[1]Department of Chemical and [2]Petroleum Engineering, Faculty of Engineering, [3]Central University Library and [4]Department of Chemistry, Faculty of Science, United Arab Emirates University, Al Ain, Abu Dhabi, United Arab Emirates

Abstract: Today, there is a big market for steroidal hormones as well as for their derivatives. This review traces the development in steroidal production from the first milligram-scale isolation of the products to their semi-synthesis from sapogenins, their total synthesis and finally their microbial enzymatic preparation from phytosterols.

Keywords: steroids, hormones, cholesterol, phytosterols, C-17 side chain cleavage, biotransformation, enzymes.

Contents

Introduction - Hormonal steroids

Steroids form an integral part of the mammalian endocrinal system. Some of the better known and important steroids are estradiol (**1**, E2), testosterone (**2**), progesterone (**3**, P4), aldosterone (**4**) and cortisol (**5**) (Figure 1). As the major estrogen, estradiol (**1**, E2) serves as a growth hormone for tissues of reproductive organs, being co-responsible for the development of secondary sex characteristics in females, and has an important effect of maintaining bone structure. Progesterone (**3**, P4) as the major progestogen is involved in the female menstrual cycle. It supports pregnancies and is involved in embryogenesis. Testosterone (**2**) as the major androgen is co-responsible for the development of secondary sex characteristics in males and helps regulate sperm production. Also, it has anabolic properties. Aldosterone (**4**) as a mineralocorticoid helps regulate the blood pressure and influences the salt and water

**Corresponding author: Thies Thiemann*
E-mail address: thies@uaeu.ac.ae, thiesthiemann@yahoo.de
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balance in the body. It supports Na^+ and water retention, but lowers the K^+ plasma levels. Lastly, cortisol (**5**) as a glucocorticoid regulates blood sugar and aids fat, carbohydrate and protein metabolism.

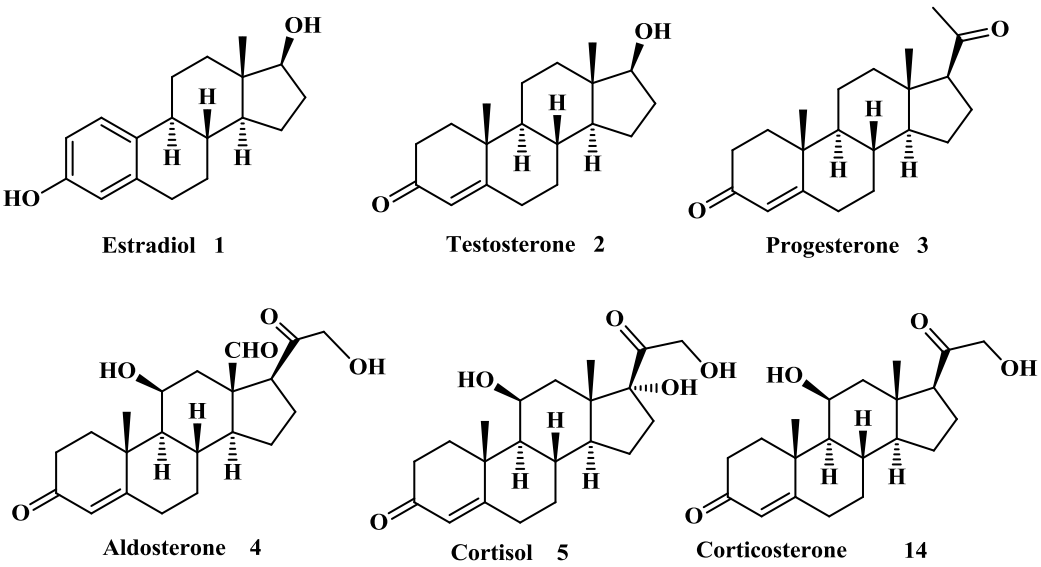
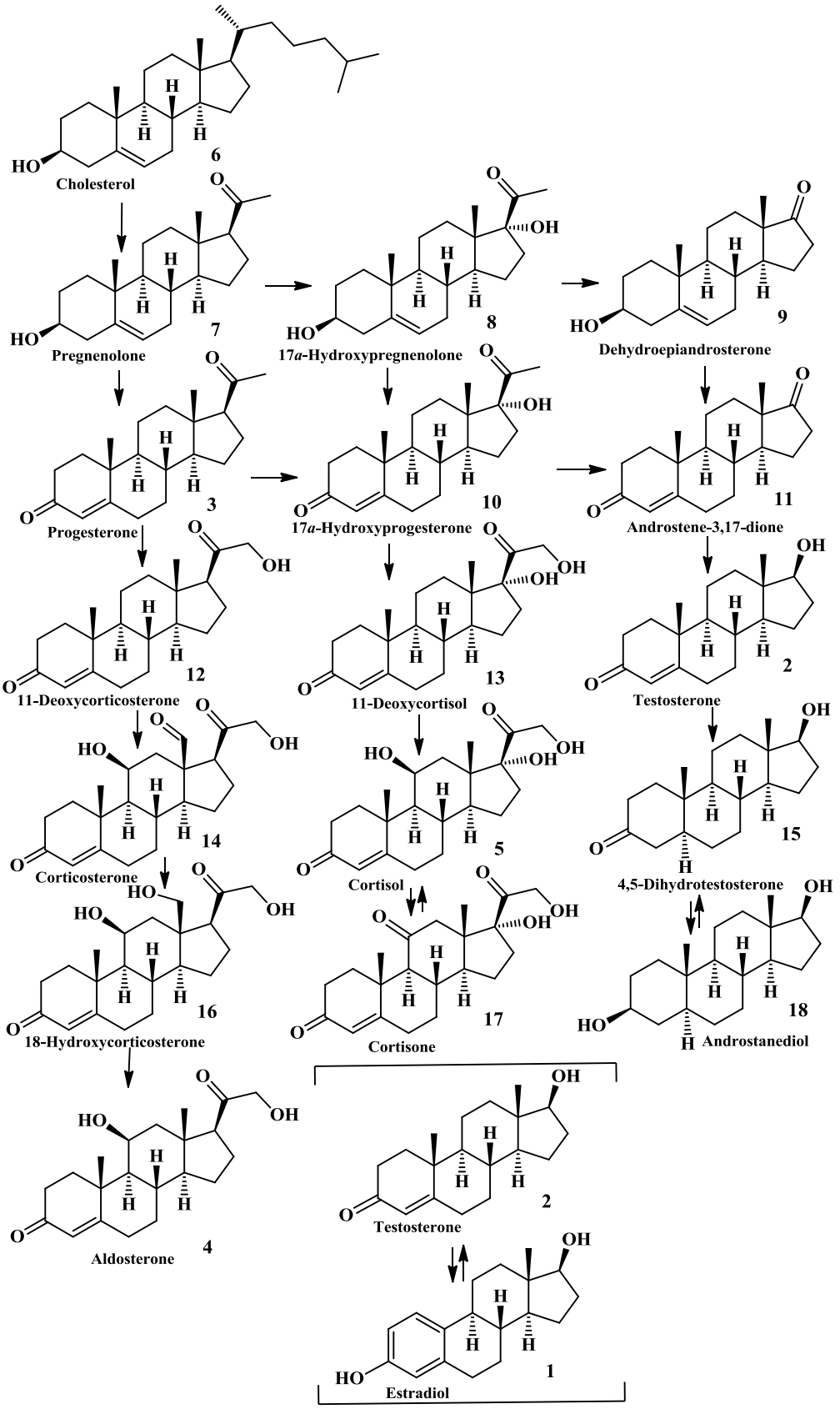


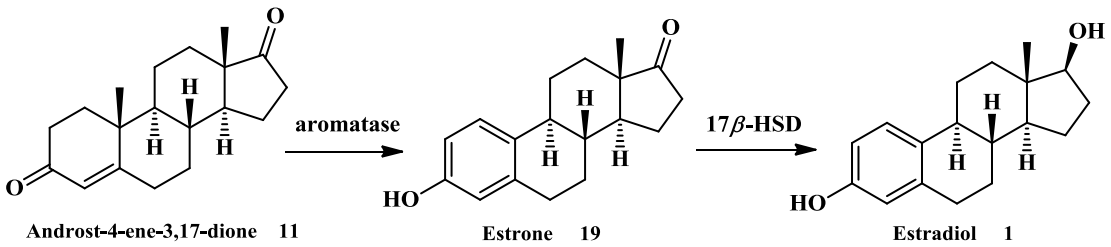
Figure 1. Important steroidal hormones

As an example of the production of hormonal steroids in humans, let us look at the simplified schematic of the biogenesis of estradiol. In the human body, estradiol (**1**, E2) is produced from cholesterol as virtual all human hormonal steroids are (Scheme 1). A pivotal intermediate in the synthesis is androstene-3,17-dione (**11**), some of which is reduced to testosterone (**2**) by the 17β-hydroxysteroid dehydrogenase (17β-HSD) (Scheme 1). An aromatase (estrogen synthase) converts androstenedione (**11**) to estrone (**19**, E1), where the 19-methyl group of androstenedione (**11**) is eliminated oxidatively (Scheme 2). 17β-HSD can convert estrone (**19**) to estradiol (**1**) (Scheme 2).

A second pathway takes us from testosterone (**2**) directly to estradiol (**1**), a conversion mediated by aromatase (Scheme 1). Androstenedione (**11**), which is also a very important intermediate in the synthetic production of steroidal hormones from phytosterols (plant derived steroids) and cholesterol, is derived from pregnenolone (**7**). Pregnenolone (**7**) can be converted to either progesterone (**3**) using both 3β-hydroxysteroid dehydrogenase (3β-HSD) and delta 4-5-isomerase. These conversions lead into the delta-4-pathway, where progesterone (**3**) transforms to 17α-hydroxyprogesterone (**10**) via 17α-hydroxylation, and **10** converts to androstenedione (**11**) by 17,20-lyation. In this regard, the human cytochrome enzyme P45scc (CYP17A1) has both 17α-hydroxylase and 17, 20-lyase activities (Scheme 1).

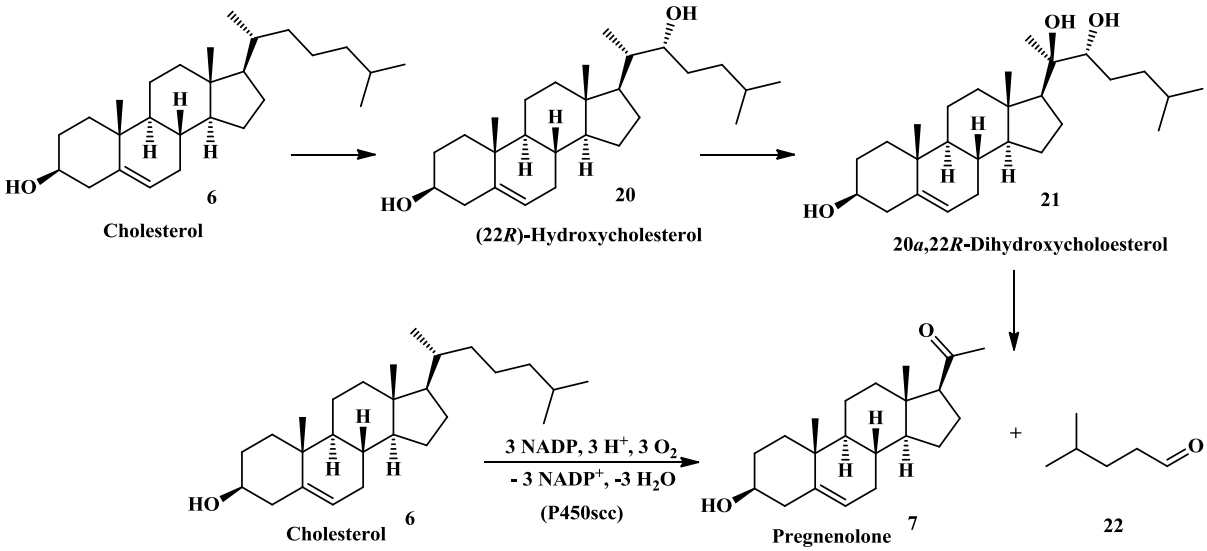


Scheme 1. Schematic representation of the biogenesis cascades of steroidal hormones



Scheme 2. *In vivo* conversion of androst-4-ene-3,17-dione (AD, **11**) to estradiol (**1**)

This leaves to be answered how pregnenolone (**7**) is produced from cholesterol (**6**). To understand this C-17 side chain cleavage from **6** to **7** is also of fundamental importance for the synthetic (eg., the industrial) production of steroidal hormones from phytosterols and cholesterol. In the human body, the conversion of cholesterol to pregnenolone (**7**) is catalysed by the cholesterol side-chain cleavage enzyme P45scc. The reaction proceeds in three steps. First, a hydroxylation step of cholesterol (**6**) yields 22*R*-hydroxycholesterol (**20**). A second hydroxylation step gives 20α,22*R*-dihydroxycholesterol (**21**) (Scheme 3).



Scheme 3. *In vivo* conversion of cholesterol (**6**) to pregnenolone (**7**)

Both of these steps are monooxygenase steps involving electron transfer from NADPH to enzyme P450scc via two electron transfer proteins - adrenodoxin and adrenodoxin reductase. A final step cleaves the 1,2-diol in **21** to furnish pregnenolone (**7**) with isocaproic acid as an isolable side product in humans[1]. *In vitro*, isocaproaldehyde (**22**) (see Scheme 3) and other products such as isohexanol have been found[2]. This may in part indicate follow-up reactions of the isocaproaldehyde (4-methylpentanal, **22**), *ie.*,through oxidation or reduction.

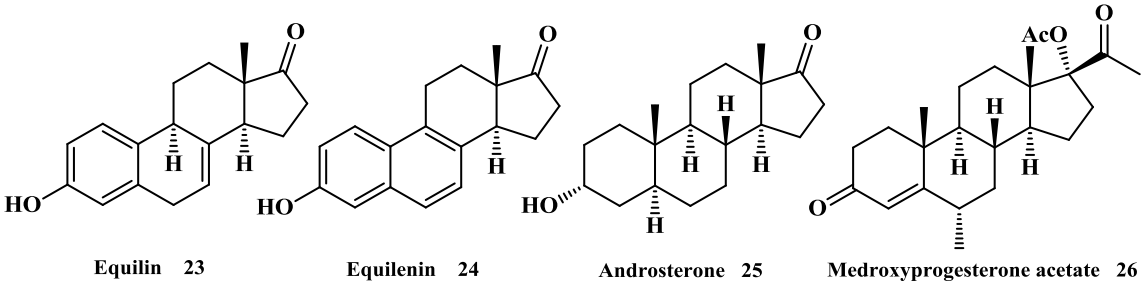


Figure 2. Structure of equilin (**23**), equilenin (**24**), androsterone (**25**), medroxyprogesterone (**26**)

Hormonal steroids - first isolation

Androsterone (**25**) was first isolated from the urine of policemen[3]. Quickly, however, it became evident that natural isolation of hormonal steroids would not satisfy the demand, as, for instance, in 1931, 15.000 L of urine yielded only 15 mg of pure androsterone (**25**)[3]. Similarly, 4 tons of sows' ovaries were needed to isolate 12 mg of estradiol (**1**)[4,5], while extraction from 625 kg of sows' ovaries produced 20 mg of progesterone (**3**)[6]. Also, estrone was isolated from pregnant female human urine as well as from mare's urine. 100 kg of bull's testicles yielded 10 mg of testosterone (**2**) and 100 kg of adrenal glands from 20000 cows yielded 75 mg of cortisone and 55 mg of hydrocortisone. Aldosterone (**4**) was also isolated from adrenal glands[7]. While these isolations were of fundamental importance for the general understanding of the structure and function of steroidal hormones, it did not contribute much to a method of production, at a scale that these compounds would be needed in future years. In this respect, however, it has to be noted that the estrogens of Premarin®, a hormone replacement drug containing mainly the sulfates of estrone, equilin (**23**), and equilenin (**24**) have been produced from the urine of pregnant horses from the early 1940s until today. The same is true for Prempro®, which includes additionally the synthetic medroxyprogesterone acetate (**26**). In the 1930s, efforts were devoted to the wet-chemical transformation of cholesterol into steroidal hormones. Today, natural steroids and their synthetic derivatives find use as contraceptives, hormone replacement agents, muscle enhancers, and anti-inflammatory agents in rheumatic arthritis. By 1975, the total revenue from steroid sales is estimated to have been $ 3 billion[8a]. By 2011, the revenues from topical corticosteroids alone account for $ 900 million[8b].

Hormonal steroids from steroidal sapogenins of *Dioscoreae*

One such natural source is diosgenin (**27**), a steroidal sapogenin, first isolated by Tsukamoto from the yam *Dioscorea tokoro* (Makino)[9], but which can also be found in the tubers of other species of the *Dioscoreae* (*Dioscorea villosa, D. mexicana* and *D. composita*). Diosgenin (**27**) is also a constituent of many other plants such as *Trillium erectum*[10], *Solanum lyratum*[11], *Tribulus terrestis*[12] and *Paris polyphylla*[13]. The hydrogenated form of diosgenin, tigogenin (**28**), can be found in *Chlorogalum pomeridianum* bulbs[14], *Tribulus terrestis*[15], and *Solanum paniculatum*[16]. Hecogenin (**29**) as a third such sapogenin was extracted from the sisal plant *Agave sisalana*[17]. In the so-called Marker synthesis, diosgenin (**27**) could be transformed to progesterone (**3**)[18,19] (Scheme 4), which was used in the first combined oral contraceptive pills[20]. While Dioscorea plants grow in various countries such as India, South Africa and China[21], the large-scale production of steroidal hormones by semi-synthesis from diosgenin (**27**) and related sapogenins left a mark on the Mexican chemical industry[8] and led to the founding of Syntex in Mexico[22,23]. Due to protection and limited availability of *Dioscorea* plants, however, the price for diosgenin (**27**) increased dramatically over time, so that other sources needed to be found, especially in regions outside of China.

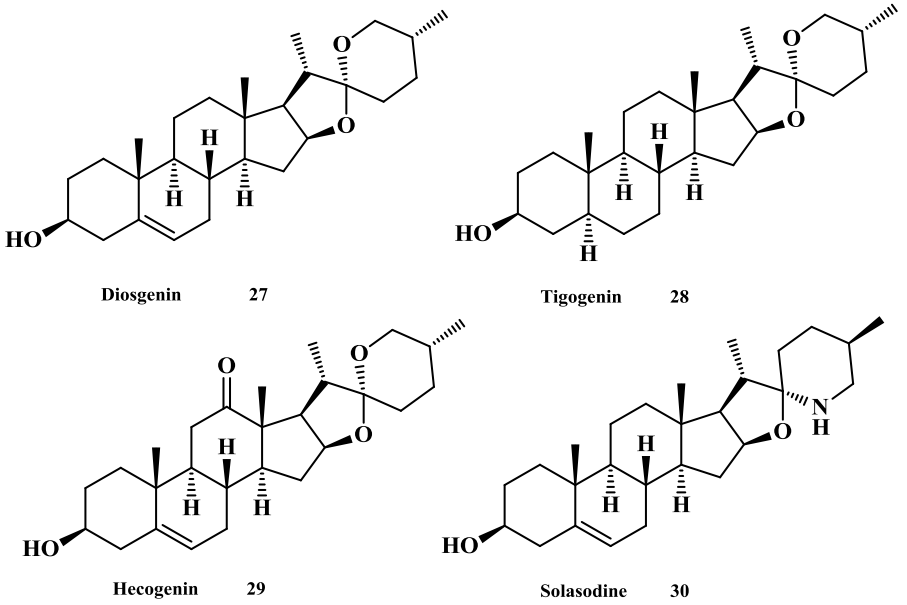
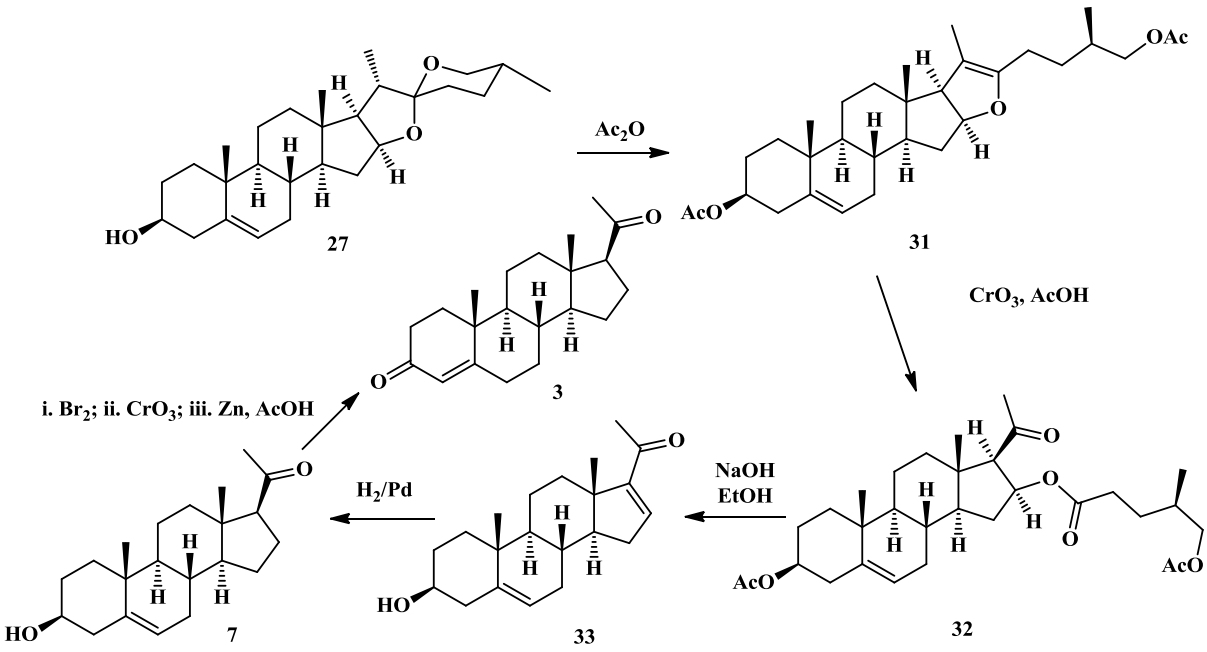
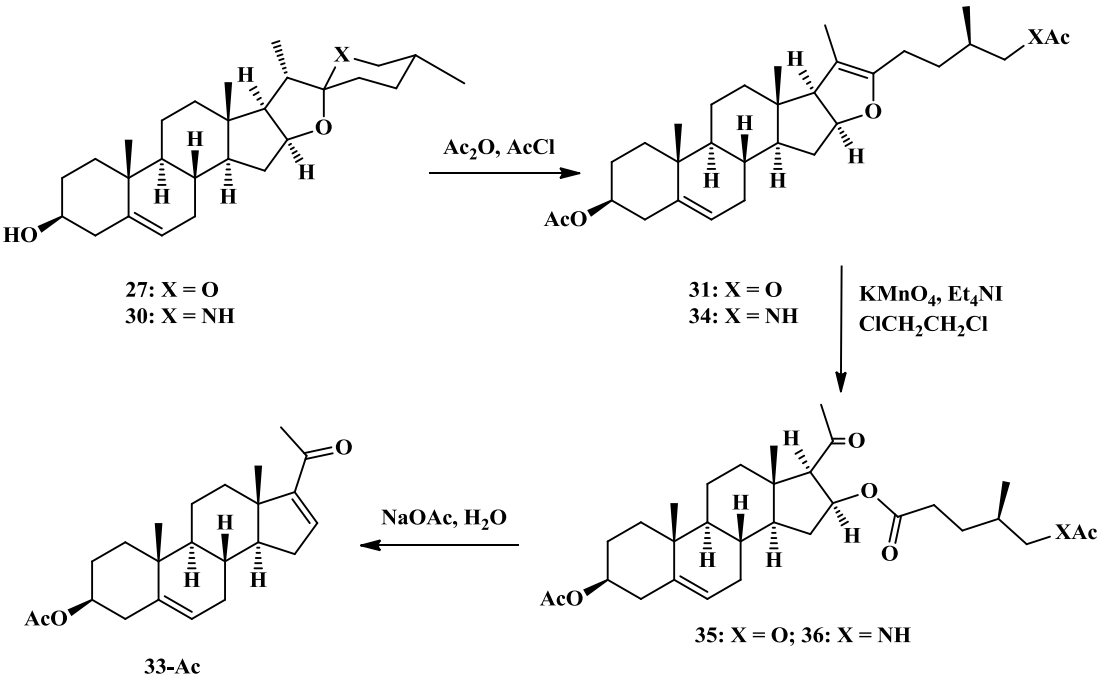


Figure 3. Steroidal sapogenins



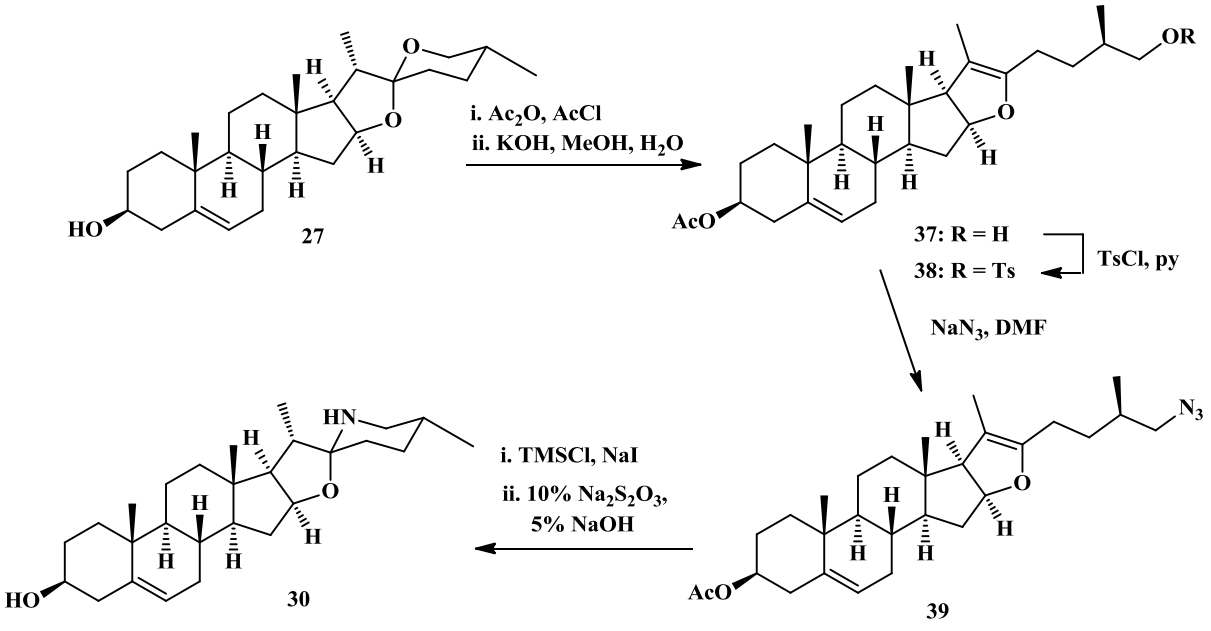
Scheme 4. Marker's degradation of diosgenin (**27**) to pregnenolone (7) and progesterone (**3**)

Thereafter, processes developed use solasodine (**30**) as a starting material[24] (eg, Scheme 5). Solasodine has been found in a number of plants of the genus *Solanum*, where steroidal manufacturers have tried to cultivate such plants as *Solanum auriculatum* for the purpose of harvesting solasodine (**30**).



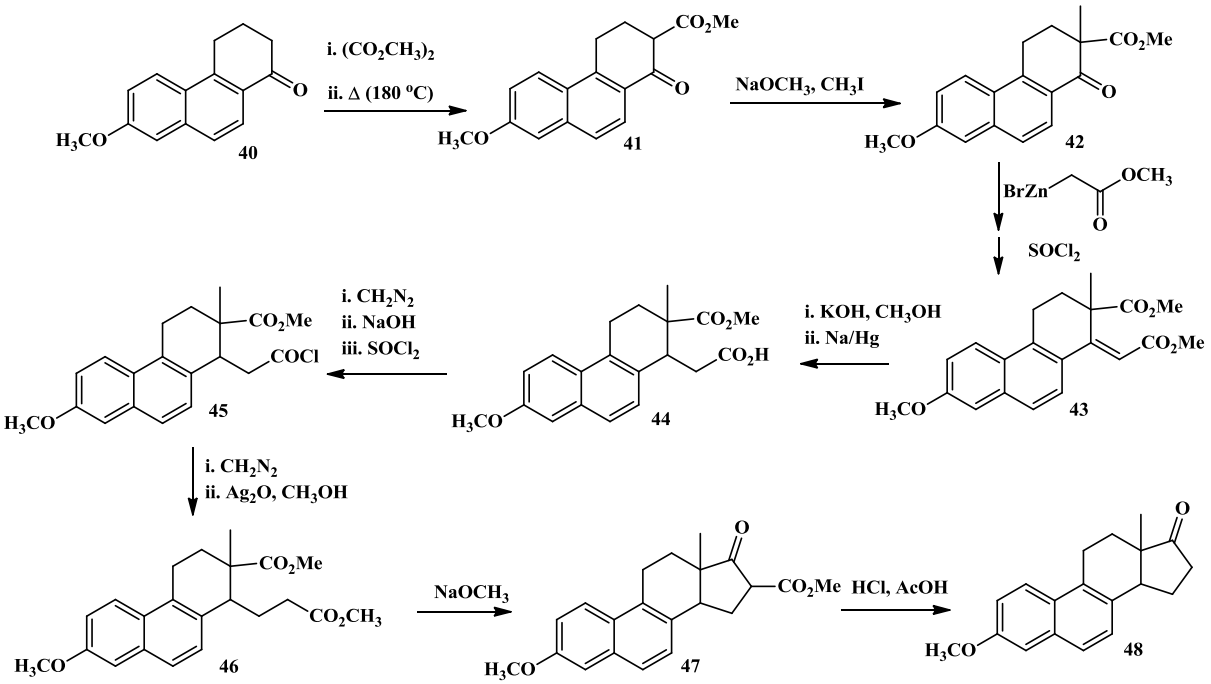
Scheme 5. High-yielding process converting diosgenin (**27**) and solasodine (**30**) into dehydropregnenolone acetate (**33-Ac)**

However, solasodine (**30**) has been found to have anti-tumour activities, where a plant preparation (SBP002, Coramsine®), containing solasonine and solamargine (both solasodine glycoalkaloids), is currently in phase-II clinical trial for treating skin cancers[25]. Thus, in China, where diosgenin (**27**) is more readily available, a synthetic process has been developed to transform diosgenin (**27**) into solasodine (**30**)[26] (Scheme 6). At the same time, numerous Chinese patents underline the continuing importance of diosgenin as a source of steroidal hormones. Nevertheless, the worldwide pharmaceutical industry with a global market for steroidal drugs of US$ 4-8 billion had to find an alternative source for the production of hormonal steroids.



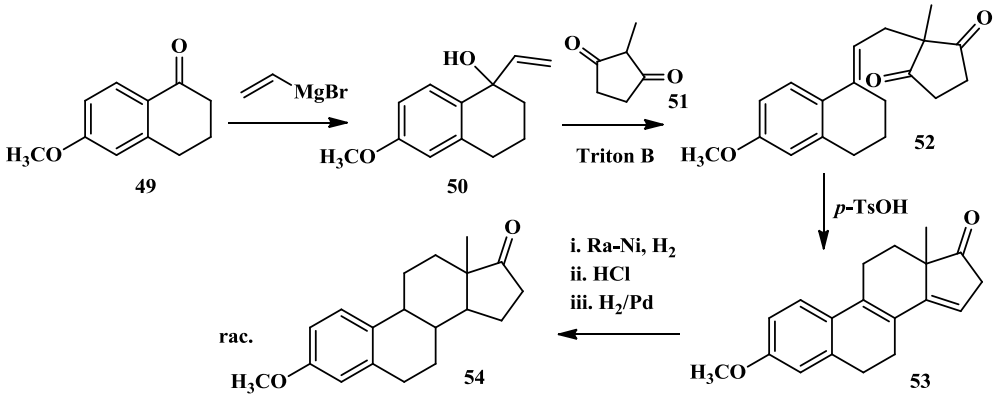
Scheme 6. Solasodine (**30**) from diosgenin (**27**)

Hormonal steroids by total synthesis



Scheme 7. Bachmann's total synthesis of *O*-methylequilenin (**48**)

Early, a synthetic route for equilenin (**24**), a steroid found in horses, was devised by Bachmann[27]. Thus, in part, an alternative access to steroidal hormones presented itself with *de novo* synthesis of steroids such as estradiol and its derivatives. A number of total syntheses[28] of research groups from pharmaceutical companies and academic institutions were disseminated in the late 1950s – early 1960s, namely by Velluz of Roussel-Uclaf, Smith of Wyeth Laboratories (and Manchester University), Rufer and Schroeder of the German Schering AG, and Torgov of the USSR Academy of Sciences. Here, only two of the main synthetic strategies can be shown. The first is the Torgov synthesis (Scheme 8).



Scheme 8. Torgov total synthesis of *rac*-estrone

Torgov's synthesis[29] was exploited by Jenapharm, where by the 1980s up to 5 tons of steroids were produced per year, after the process had been improved through various modifications as documented by over 100 patents[30]. Initially, the 3-*O*-methylestone was separated into its antipodes by diastereomeric of D-acetyl tartaric acid methyl ester[31] and also as diastereomeric cinchonine salts derived from racemic hemiphthalates[32]. Bucourt et al.

from Roussel-Uclaf let the secosteroid dione**52**react with L-tartramic acid and separated the two adducts **55a** and **55b** by their different solubility in methanol in the presence of trace amounts of acetic acid (Figure 4)[33].

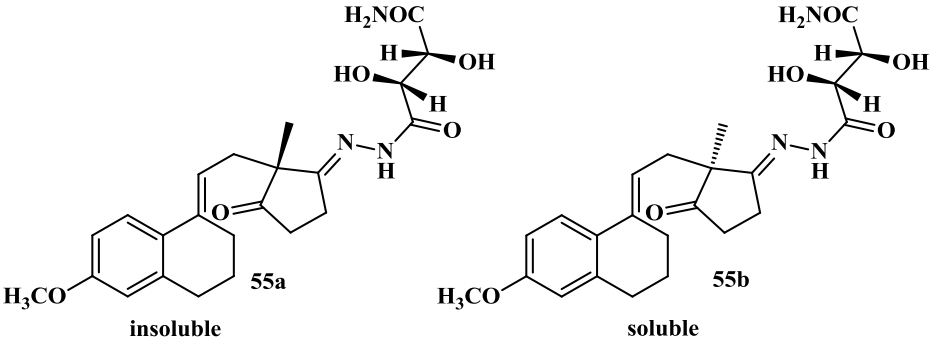
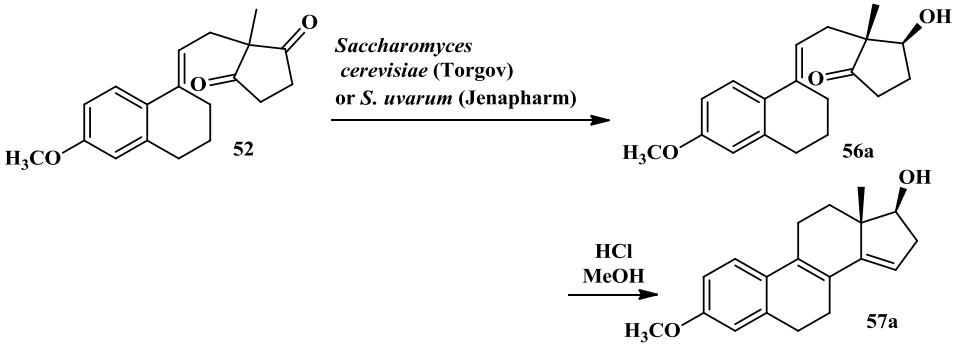


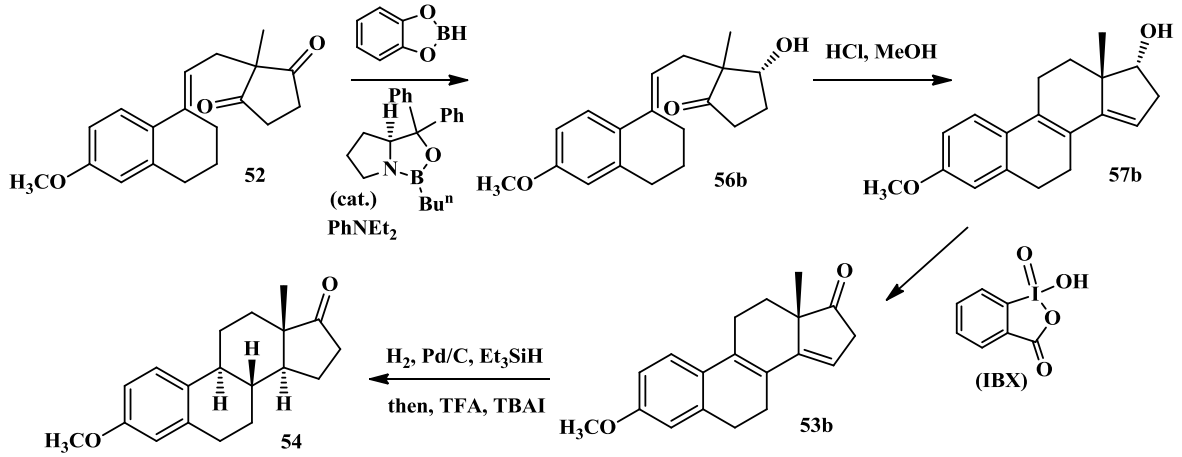
Figure 4. Separation of the antipodes of secosteroid **52**by reaction with tartramic acid

Also, an enantioselective reduction of the secosteroid **52** was developed by Torgov et al., and by the Schering steroidal chemists, which gave enantiopure 3-*O*-methylestrone (Scheme 9)[34].



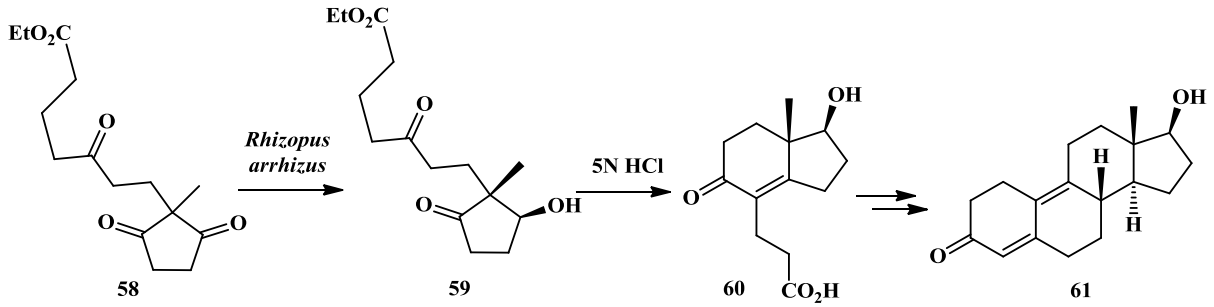
Scheme 9. Towards enantiopure estrone/estradiol by enantioselective reduction of secosteroid**52**

In 2007, Corey *et al.* published a further enantioselective variation of the Torgov estrone synthesis using the enantiotopo-selective Itsuno-Corey reduction of secosteroid **52** (Scheme 10)[35].



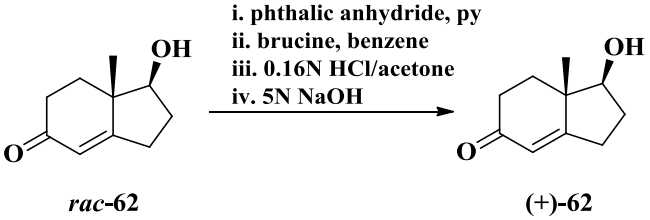
Scheme 10. Corey's enantioselective variant of the Torgov synthesis

Many syntheses of hormonal steroids employed the approach of preparing a suitable enantiomeric enriched 1-hydroxylated tetrahydroindan-5-one system of type **60**. For the most part, this involved the desymmetrization of an 2-alkylcyclopenta-1,3-dione derivative.

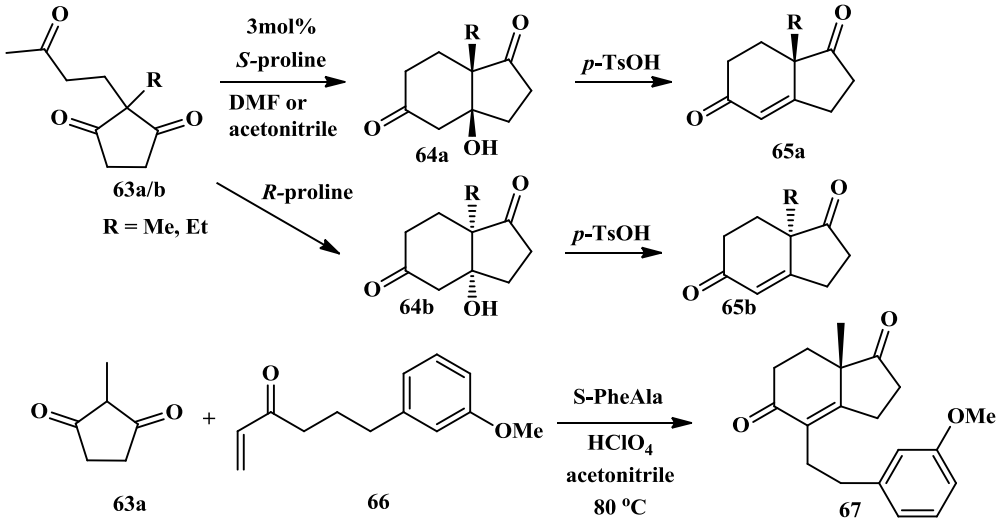


Scheme 11. Desymmetrization of an 2-alkylcyclopenta-1,3-dione derivative by enzymatic reduction

This could be carried out by an enzymatic reduction at the stage of the 2-alkylcyclopenta-1,3-dione as shown in Bellet's synthesis of **61** (Scheme 11)[36]. In other cases, tetrahydroindan-5-on-1-ols were resolved into antipodes by reacting first an auxiliary group to the molecule. Thus, *rac*-**62**, obtained by $NaBH_4$ reduction of **65**, was reacted with phthalic anhydride and the resulting ester-acid was resolved using brucine(Scheme 12)[37]. In further instances, the symmetric cyclopenta-1,3-diones were reacted with molecules which had already a controlled stereocenter introduced into their structure.

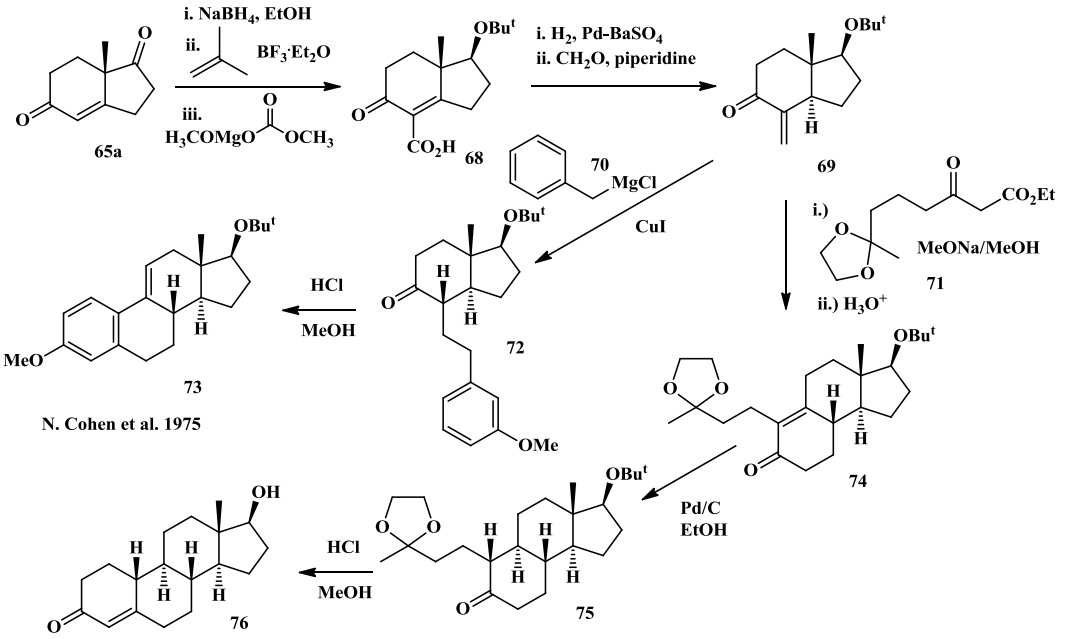


Scheme 12. Separation of optical antipodes by brucinium phthalates



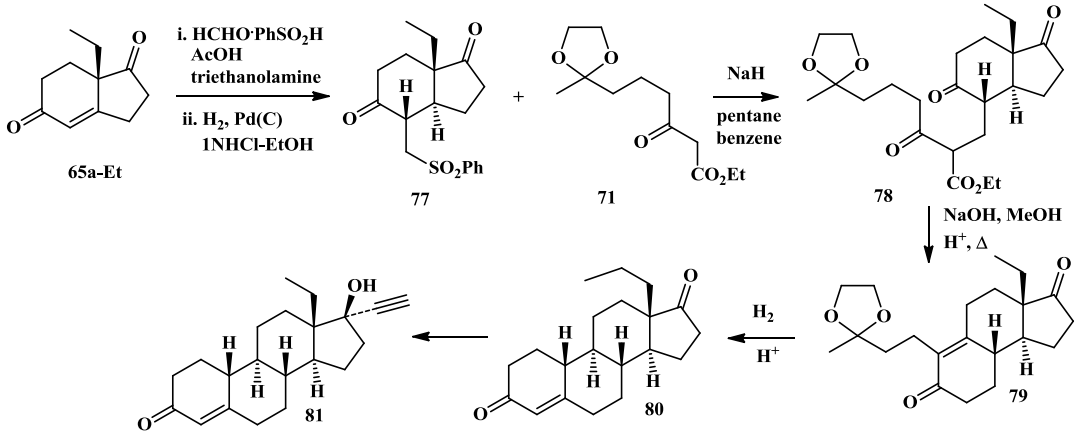
Scheme 13. Enantioselective synthesis of Hajos-Parrish diketone **65** and other tetrahydroindandiones with chiral amino acids as organocatalyst

A big step forward was the possibility of an early organic catalytic reaction using proline in the intramolecular Aldol reaction to produce the Hajos-Parrish diketone **65** in an enantioselective manner (Scheme 13)[38]. The chiral organocatalyst used was proline, where *R*- and *S*-proline led to the respective enantiomers of the Aldol reaction products. Not only 2-butan-3-onyl-2-methylcyclopenta-1,3-dione but also other 2-butan-3-onyl-2-alkyl / arylcyclopenta-1,3-diones could be used as starting materials in the reaction. Moreover, the butanonyl residue of the starting material could be elaborated further. Also, the Michael addition with the 2-alkylcyclopenta-1,3-dione as Michael acceptor and the subsequent intramolecular Aldol reaction could be run in one-pot, as shown in Scheme 12 in the transformation of **63a** and **66** to **67**, where S-phenylalanine was used as the organocatalyst. This text-book reaction was used as a key-step in the synthesis of (+)-3-*O*-methylestrone **54**[39] and of a number of optically active 19-norsteroids such as 19-nortestosterone **76**[40] (Scheme 14).



Scheme 14. Use of chiral Hajos-Parrish diketone as a building block in the syntheses to *O*-protected estra-1,3,5(10),9(11)-tetraen-3,17β-diol (**73**) and to nortestosterone **76**

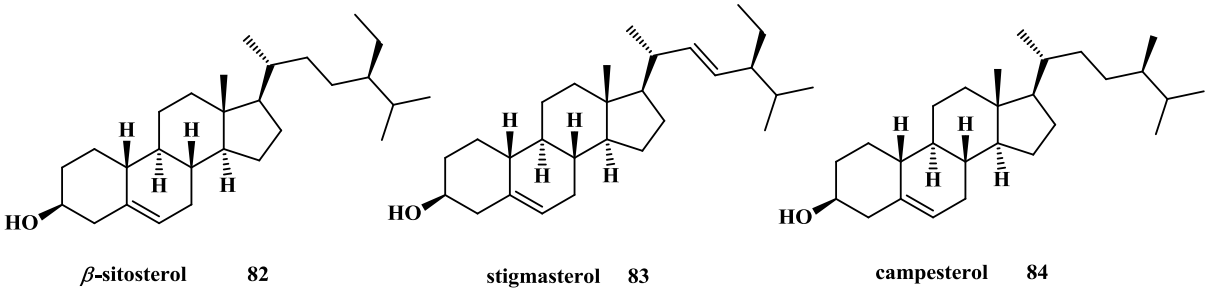
Sauer et al. also used diketone **65a-Et** as their starting material for norgestrel (**81**)[41], used in hormonal contraceptives.



Scheme 15. Synthesis of norgestrel (**81**) from Hajos-Parrish-type diketone **65a-Et**

Chemical cleavage of the C17-side chain in cholesterol - early routes to steroidal hormones from cholesterol

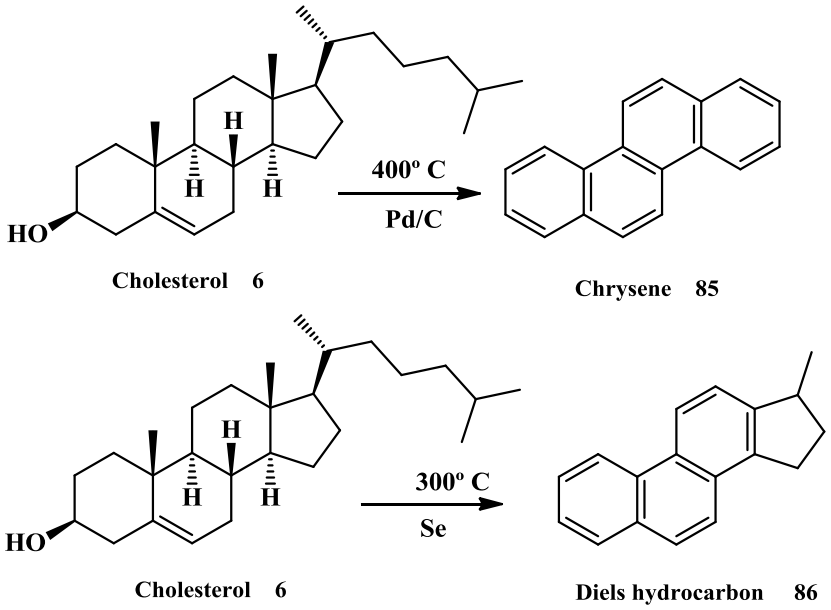
At the same time as some of the earliest total syntheses towards steroids, efforts were devoted to finding ways to prepare hormonal steroids from cholesterol (**6**) and plant phytosterols.Among these sterols,*β*-sitosterol (**82**), stigmasterol (**83**) and campesterol (**84**) (Figure 5) are abundant in soy bean oil, rape seed and paper pulp industrial waste. Lastly, for the most part the human body provides itself also with hormonal steroids through the enzymatic degradation of the C17-side chain of cholesterol (**6**).



β-sitosterol 82 stigmasterol 83 campesterol 84

Figure 5. Structures of *β*-sitosterol (**82**), stigmasterol (**83**), and campesterol (**84**)

At first, efforts were undertaken to chemically cleave the C17-side chain in cholesterol (**6**) at high reaction temperatures. Initially, these reactions were carried out to elucidate the structure of cholesterol. Thus, Diels subjected cholesterol (**6**) to a reaction over palladized charcoal at 400^{o}C to yield chrysene (**85**)[42,43], albeit in poor yield, at 500^{o}C a mixture of chrysene (**85**) and naphthalene[44,45].



Cholesterol 6 Chrysene 85

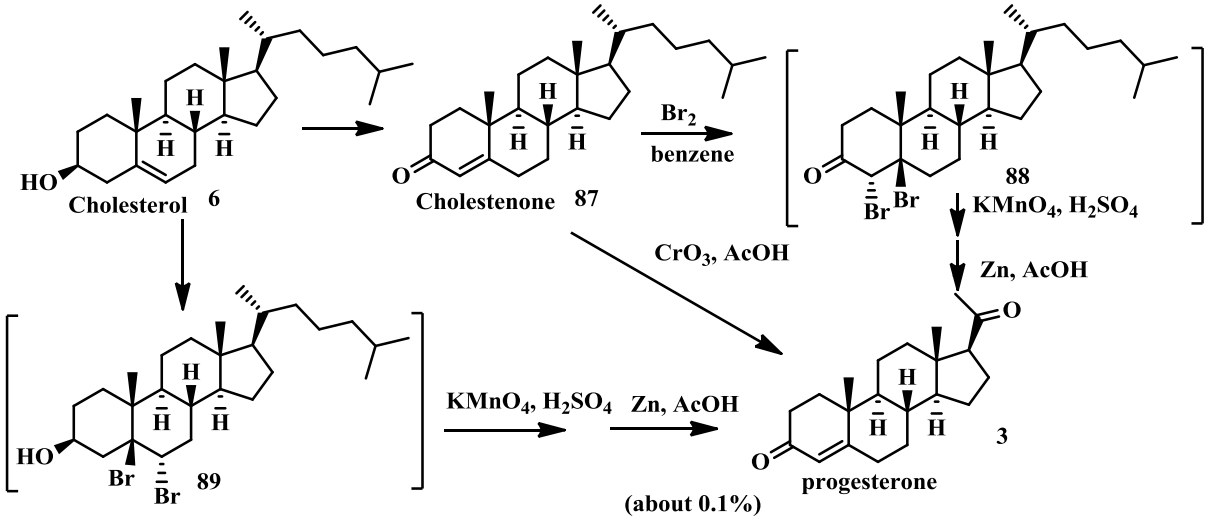
Cholesterol 6 Diels hydrocarbon 86

Scheme 16. C-17-side chain cleavage in cholesterol (**6**) under pyrolysis conditions

Later, also experiments on carbon alone and on a mixture of sulfur and carbon were shown to transform cholesterol (**6**) to chrysene (**85**)[46]. Nevertheless, these reactions gave mixtures of products, often in poor yield, where sulfur is known to insert into cyclic carbon structures. An analogous reaction of cholesterol over selenium, albeit at 300^{o}C, was more successful to yield cyclopentanophenanthrene **86**, subsequently named Diels hydrocarbon. This derivative could be prepared independently starting from 9,10-dihydrophenanthrene[47].

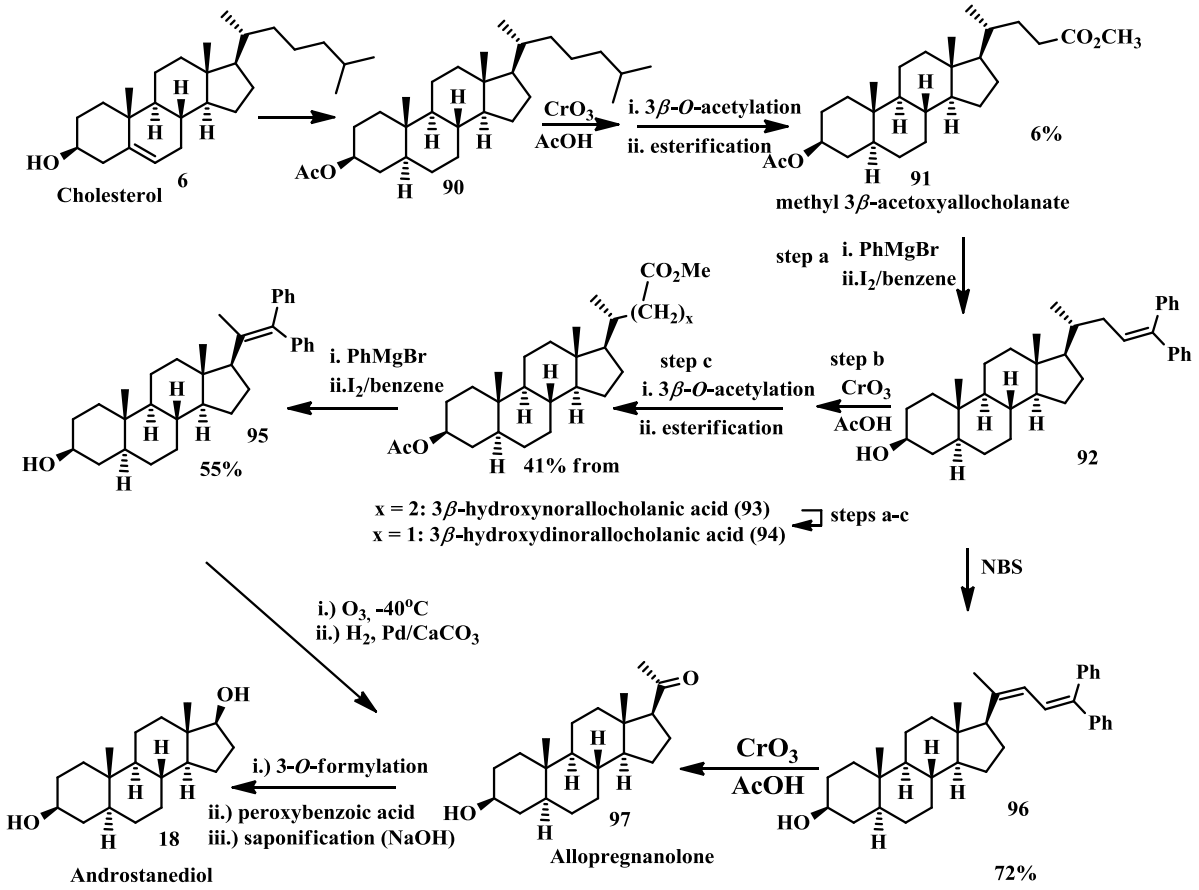
The formation of the polycyclic aromatic compounds was believed to be the basis of the cancerogenocity of the pyrolysis oils of cholesterol (**6**) and caused concern, when reviewing the fate of food sterols, when subjected to heating in cooking processes.

Early endeavors of chemically converting cholesterol (**6**) to hormonal steroids such as progesterone have produced the target compounds in poor yield. Thus, the oxidation of cholestenone dibromide **88** with potassium permanganate in a mixture of sulfuric acid and benzene has been reported to give progesterone in 1% yield[48]. An oxidation of the cholesteryl dibromide is also possible, where cholesterol is brominated. Thereafter, without purification.the cholesteryl dibromide **89** is oxidized with potassium permanganate in sulfuric acid. After crude separation of MnO_2 formed, the mixture of oxidation products is debrominated with zinc in glacial acetic acid to give progesterone in 0.2% yield[49]. The oxidation of cholestenone (**87**) with chromic acid in acetic acid to progesterone (**3**) in unspecified yield has also been communicated[50] (Scheme 17).

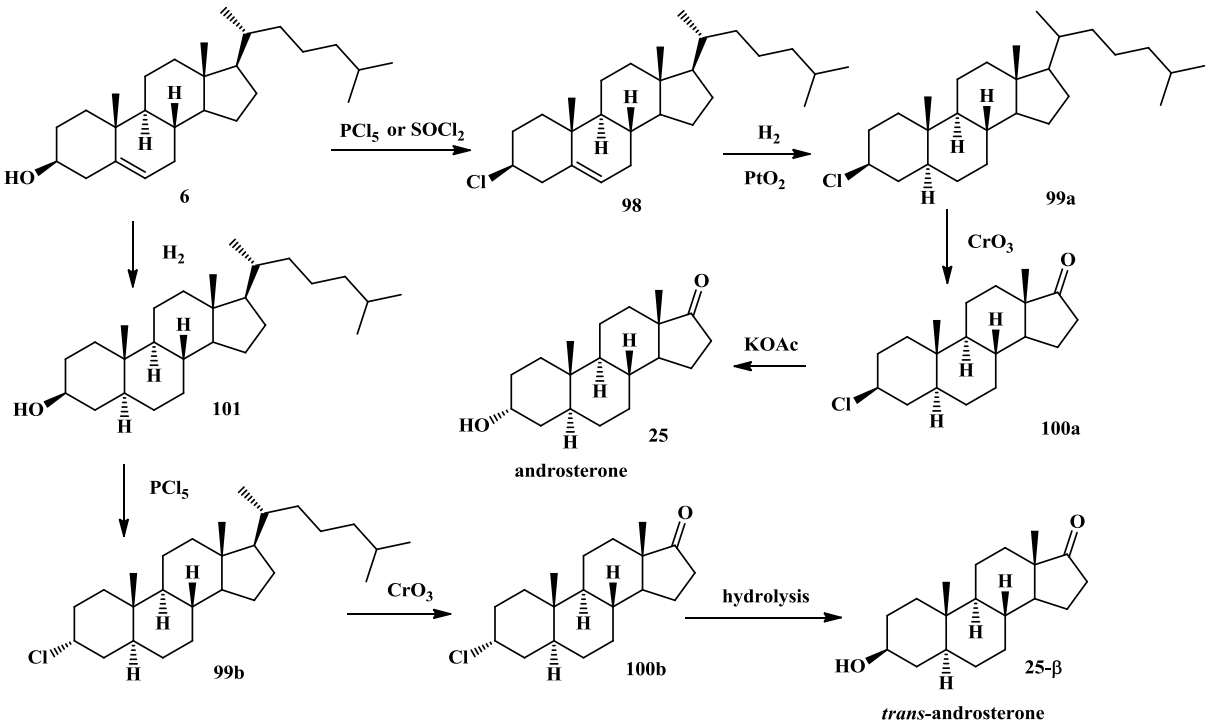


Scheme 17. Early chemical conversions of cholesterol (**6**) to progesterone (**3**)

Cholesterol (**6**) could be converted to 3β-hydroxyallocholanic acid (**91-OH**) by oxidation of the intermediate dihydrocholesterol acetate**90**[51]. Subsequently, **90** was transformed to 3β-hydroxynorallocholanic acid by Barbier-Wieland degradation, ie., by reacting 3β-hydroxyallocholanate **91**with phenylmagnesium bromide, dehydrating the resulting diphenylcarbinol with iodine in benzene and oxidizing the olefin, thus prepared, with chromic acid[52]. Three further cleavages to 3β-hydroxynorallocholanic acid (**93**), subsequently to 3β-hydroxybisnorallocholanic acid (**94**) and then to 3β-allopregnanol-20-one (**97**), respectively, were affected along the same lines, with the oxidative cleavage of the final olefin **95** carried out with ozone in ethyl acetate at -40^{o}C with a reductive work-up by hydrogenation of the ozonide on Pd/$CaCO_3$[52]. Diphenyl-3-β-acetoxyallocholene **92** could be reacted with *N*-bromosuccinimide (NBS) to diene **96**, which could be cleaved with chromic acid to yield 3β-allopregnanol-20-one (**97**), directly. It must be noted that the procedure was not meant as a synthetic process from cholesterol (**6**) to hormonal steroids, but rather as a clarification of the biogenesis of cholesterol through degradation of a ^{14}C labelled cholesterol, prepared enzymatically by incubating rat liver slices with 1-^{14}C-acetate and 2-^{14}C-acetate[52].



Scheme 18. Degradation of cholesterol (**6**) to allopregnanolone (**97**) and androstanediol (**18**) through repeated Barbier-Wieland degradation



Scheme 19. Marker's andRuzicka's syntheses of androsterone (**25**) and 3-epiandrosterone (**25-β**) from cholesterol (**6**)

Tetrahydroergosterol was acetylated and subsequently the C17-side chain was cleaved with chromic acid[53]. No yield is given, but it is expected to be low for this type of reaction. Similarly α-cholestanyl chloride (**99a**) was treated with CrO_3 in acetic acid to give chloroandrosterone (**100a**) in 2.5% yield[54] (Scheme 19). A similar sequence was carried out by Ruzicka, with a change in the sequence chlorination - hydrogenation of Marker to hydrogenation - chlorination of the starting material cholesterol (**6**)[51] (Scheme 19).

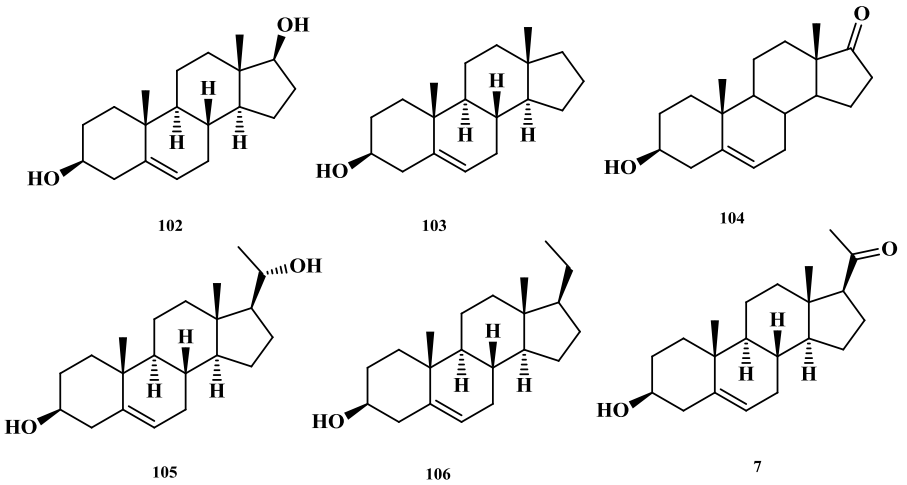
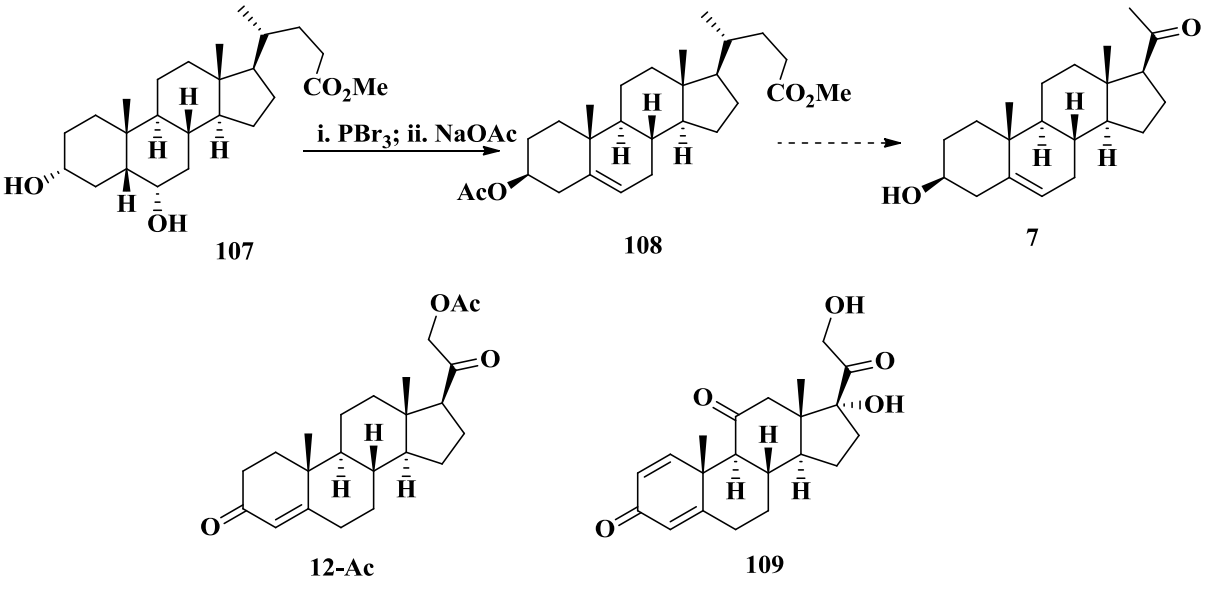


Figure 6. Identified reaction products from the autooxidation of cholesterol in air

It is interesting to note, however, that autooxidation of cholesterol (**6**) in air can also lead to steroids of the androstane and the pregnane series, namely androst-5-ene-3β,17β-diol (**102**), androst-5-en-3-β-ol (**103**), 3β-hydroxyandrost-5-en-17-one (**104**), pregn-5-ene-3β,20α-diol (**105**), pregn-5-en-3β-ol (**106**), and to 3β-hydroxypregn-5-en-20-one (**7**)[55] (Figure 6). It has been suggested that the oxidation proceeds via the 20α- and 25-hydroperoxyderivatives, which can be detected when ultrapure cholesterol is reacted with air at elevated temperatures[55].

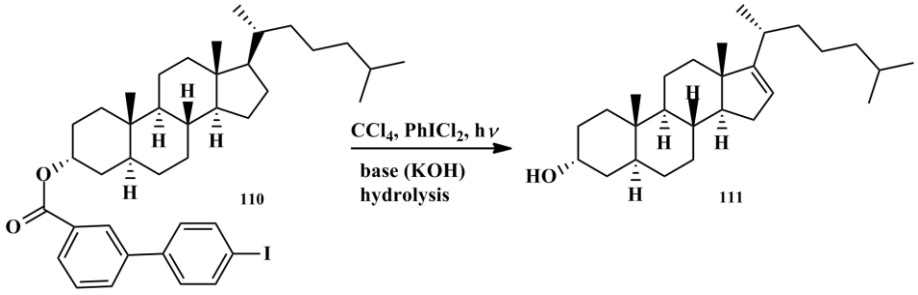
For a time, Jenapharm also used hyodeoxycholic acid (**107**), derived from hog bile, as anindustrial starting material for pregnenolone (**7**)[30,56] (Scheme 20).



Scheme 20. Hyodeoxycholic acid (**107**) from hog bile in the synthesis of steroidal hormones

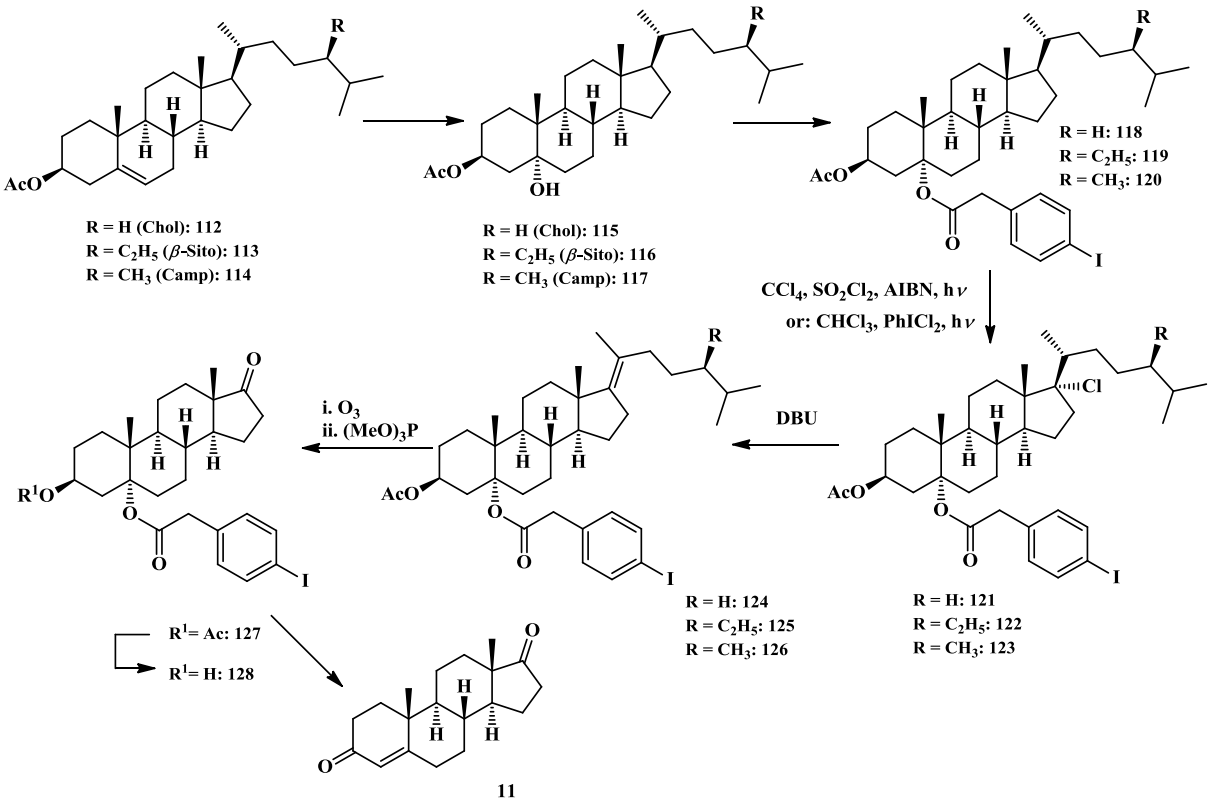
In the mid-1950s, Jenapharm was already producing kilogram quantities of progesterone (**3**) and deoxycorticosterone acetate (**12-Ac**), utilizing this process. This was the starting point of a whole production cascade leading also to testosterone (**2**), cortisone (**17**), cortisol (**5**) and prednisone (**109**), a synthetic corticosteroid drug. One of the key reactions was to differentiate the 3α- and 5α-hydroxy groups in hyodeoxycholic acid (**107**). This could be done by reacting **107** with phosphorus tribromide in the absence of pyridine followed in a second step by a nucleophilic substitution of an acetate for the bromo function at C-3 in the intermediate to yield methyl 3β-acetoxy cholanate (**108**).

In 1970, Breslow and Baldwin developed site-specific, template-directed functionalisation reactions in ring D of steroids[57-59], where 3α-cholestanol and analogous derivatives were esterified with 4-benzoylphenyl containing alkanoic acids of differing chain lengths, and the resulting esters were photolysed. Photolyis would lead to the substituted benzophenone triplet, which would abstract a hydrogen atom from the steroidal framework nearby. It was found that depending on the chain lengths of the attached ester, either macrolide formation would dominate in the case of shorter chain lengths or dehydrogenation would be favored. The site of the initial hydrogen abstraction would also differ, with abstraction occurring at C12 and C14 with shorter chain length of the ester and at C14 and C17 with longer chain esters. When the photolysis was carried out in the presence of chlorinated solvents, the radical pair thus prepared was found to abstract a chloro atom from the solvent to form chlorinated steroids. This fact was utilized by Breslow's group with further, published examples of site-specific, template-directed halogenations of steroidal frameworks. Within this context, they also achieved halogenation at C17[60,61].

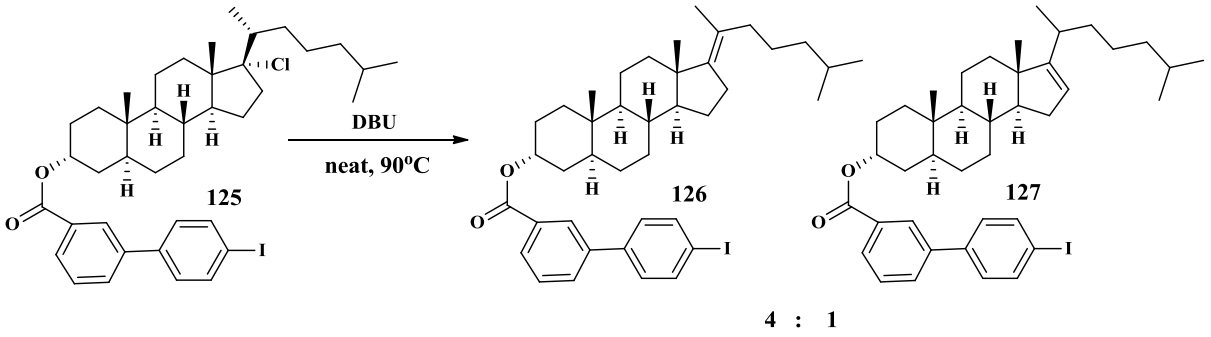


Scheme 21. Photochemical chlorination of a 3α-cholestanol derived ester **110** with subsequent dehydrohalogenation to 3α-cholest-16-enol (**111**)[61]

This allowed for the introduction of a double at C17-C20 via dehydrohalogenation. In a number of cases the creation of an unsaturation at C16-C17 competed effectively (Scheme 20), so that in these instances an isomerisation step had to be added to obtain the C17-C20 olefins in good yield. These could be subjected to oxidative cleavage to produce the C17-keto steroids, eg., by ozonolysis. Nevertheless, the sequence of reactions was used effectively to prepare androst-4-en-3,17-dione (**11**) from cholesterol, β-sitosterol and from campesterol (Scheme 22). Best suited base for the dehalogenation reaction with the highest ratio of 17(20)-ene over 16(17)ene formed was found to be 1,5-diazabicyclo[5.4.0]undec-5-ene (DBU)[62] (Scheme 22). The reaction was run solventless, where the addition of solvent was found to increase the proportion of 16(17)ene[63] (Scheme 23). Also, it was found that an oxo function or acetoxy function in C11 of the steroidal intermediate would direct the dehydrohalogenation involving the C17α-chloro substituent to provide predominantly the 17(20)-enes[64]. This strategy would be useful for the preparation of corticosteroid derivatives[64].



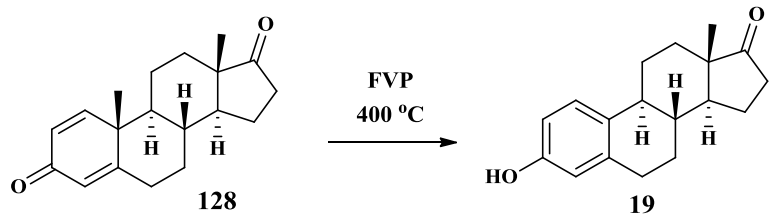
Scheme 22. Use of 1,5-diazabicyclo[5.4.0]undecene (DBU) to furnish steroidal 17(20)-enes after radical template directed chlorination of C17[62]



Scheme 23. 4:1 selectivity of steroidal 17(20)-enes vs. 16(17)-enes when using DBU[63]

Enzymatic cleavage of the C17-side chain in cholesterol, sitosterol, stigmasterol, campesterol and analogs

All the methods of preparation mentioned above were not adequate to address the size of the market for steroidal hormones, which in 2001 was for androsta-1,4-dien-3,17-dione (ADD) and androst-4-ene-3,17-dione (AD) derived compounds over 1000 tons per year[65]. Thus, new resources for steroids needed to be found. Phytosterols were deemed to be such a resource. Already in 1970, Wiechert noted that such phytosterols as β-sitosterol are regaining importance as raw materials for steroidal hormones due to the fact that enzymatic degradations of the C-17 side chain of phytosterols became better understood[66].



Scheme 24. Transformation of androsta-1,4-dien-3,17-dione (**128**) to estrone (**19**) by flash vacuum pyrolysis

The enzymatic cleavage of phytosterols such as sitosterol builds on the accumulation of androsta-1,4-dien-3,17-dione (**128**), which can be transformed to other hormonal steroids such as to estrone (**19**), eg., by flash vacuum pyrolysis. Soybean Oil Deodorizer Destillate (SODD), which is a waste product in the soybean oil production, has been forwarded as a source especially rich inphytosterols, campesterol (**83**), β-sitosterol (**81**), stigmasterol (**82**) and brassicasterol (**129**)[67-70].

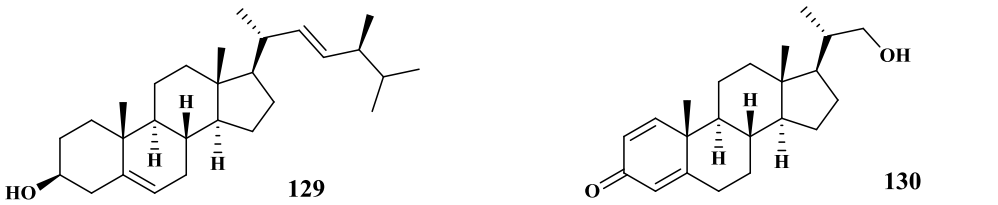
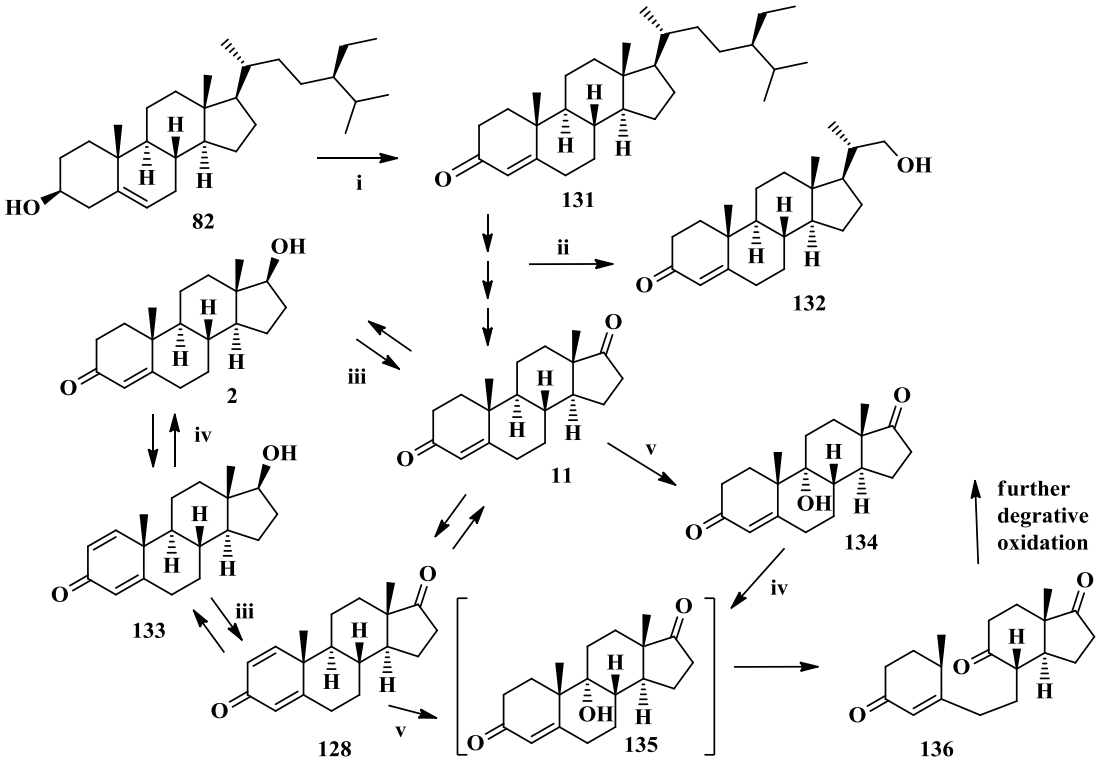
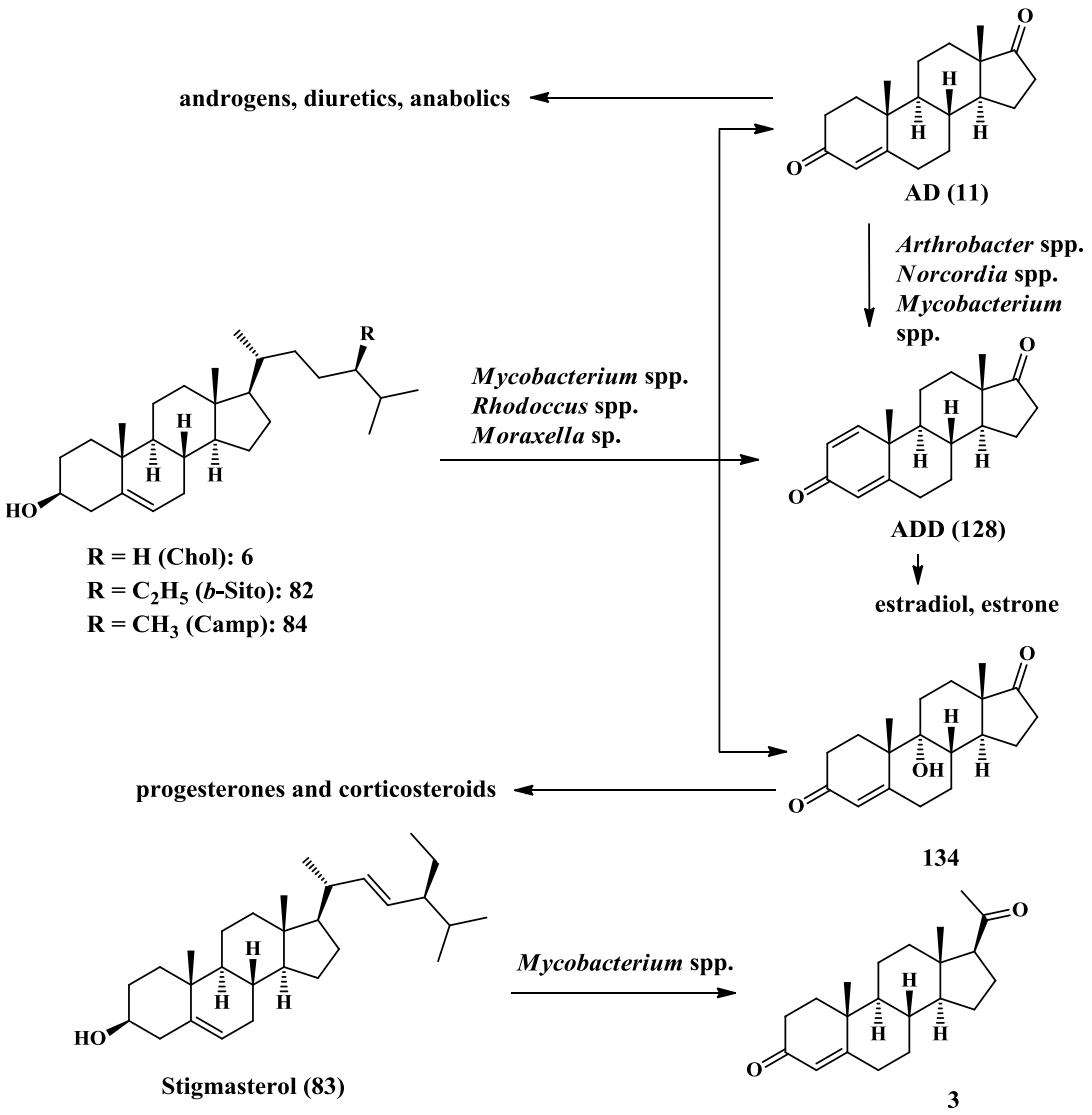


Figure 7. Structures of brassicasterol (**129**) and 20α-hydroxymethylpregna-1,4-dien-3-one (**130**)



Scheme 25. Pathway of the conversion of β-sitosterol with *M.* spp. (adopted from ref. 73)

One of the earliest viable enzymatic transformations of C17-side chain containing steroids to androsta-1,4-dien-3,17-dione (**128**) was the conversion of cholesterol with *Mycobacteriumphlei* in the presence of nickel sulfate[71]. The steroid degradation with *Mycobacterium* strains is a complicated, multienzyme process, incorporating the often unwanted action of 1,2-dihydrogenases and 9α-hydroxylase[72]. In the case of the action of *Mycobacterium* spp. on β-sitosterol (**82**), 9α-hydroxylation of accumulated AD (**11**) and subsequent 1,2-dehydrogenation leads to the instable 9α-hydroxyandrosta-1,4-diene-3,17-dione (**135**), which undergoes A-ring aromatization with concurrent cleavage of ring B, leading to the 9(10)-secosteroid **136**, which is then metabolized further[73] (Scheme 25). Early on, it was noted that the presence of chelating agents such as *o*-phenanthroline or α,α'-bipyridyl inhibits the enzymatic ring cleavage, providing the opportunity to transform for instance cholesterol (**6**) into androst-1,4-diene-3,17-dione (**128**) in high yield. Other inhibitors of enzymatic ring cleavage were found to be hydroxyquinoline, β-naphthol, copper sulfate and nickel sulfate. Thus, cholesterol (**6**) was incubated with *Arthrobacter simplex* (IAM 1660) in the presence of 0.8 mM bipyridyl to yield androst-1,4-diene-3,17-dione (**128**) in 14.9% yield after 44h at 30^{o}C[74]. At some point, however, it was tried to develop strains of bacteria, in which the synthesis of 9α-hydroxylase and 1,2-dihydrogenases is inhibited. This was successful with a number of *Mycobacterium* strains, and patents followed. Kraychy et al. described the fermentation of steroids of the cholestane and stigmastane series with *Mycobacterium* sp.NRRL-B-3683, providing a mixture of AD (**11**), ADD (**128**), and 20α-hydroxymethylpregna-1,4-dien-3-one (**130**)[75]. Weber and Kennecke of Schering A.G. patented a microbial degradation of ergosterol to AD (**11**) and ADD (**128**) with a mycobacterium, with an example given, where ergosterol was transformed into ADD in 30% yield with 67% of ergosterol unreacted[76]. In the patent, it was expressly stated that the process was to be 9α-hydroxylation inhibitor free. *M. fortuitum*, NRRL B-8153, obtained through mutation of *M. fortuitum*, ATC 6842, a non-selective degrader of steroids, by addition of nitrosoguanidin, was found to be a selective degrader.Herein, stigmasterol (**83**), cholesterol (**6**), campesterol (**84**), and sitosterol (**82**) were transformed into a mixture of AD (**11**) and ADD (**128**)[77]. Other patents included the use of *M. vaccae* for preparing AD from steroids of the cholestane and stigmastane series[78], the use of a *M. fortuitum mutant* (B-11359) for preparing AD (**11**) from steroids sitosterol (**82**), cholesterol (**6**), stigmasterol (**83**), campesterol (**84**) or a combination thereof (all examples given experimentally in the patent) with the advantage of obtaining very little ADD (**128**) as by-product. A comprehensive overview of microbial reactions leading to C17-cleavage in cholesterol and phytosterols can be found in Figure 8 and Table 1. Today, more than 1000 tons of steroids are produced annually from phytosterols by microbial transformation, alone. Some of the production streams have been schematized below.



Scheme 26. Major pathways of microbial production of hormonal steroids from phytosterols and cholesterol (**6**); adopted from ref. 79

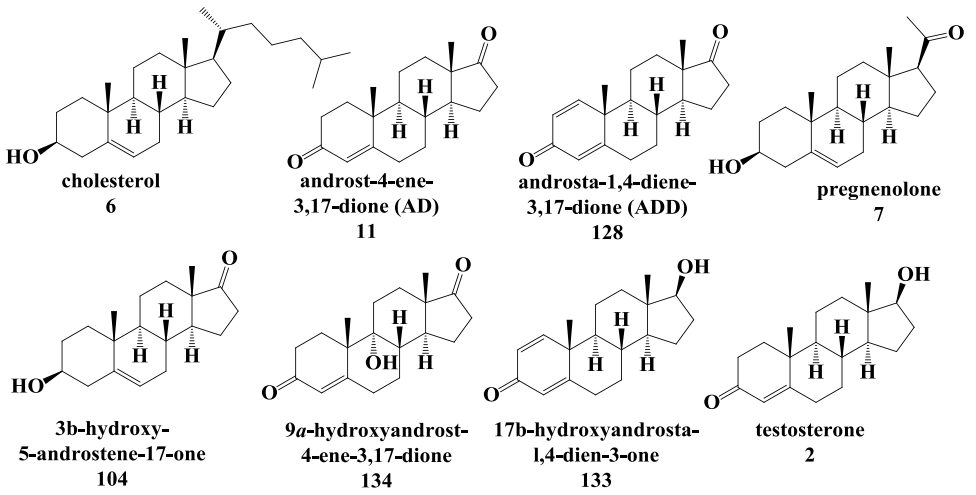


Figure 8.

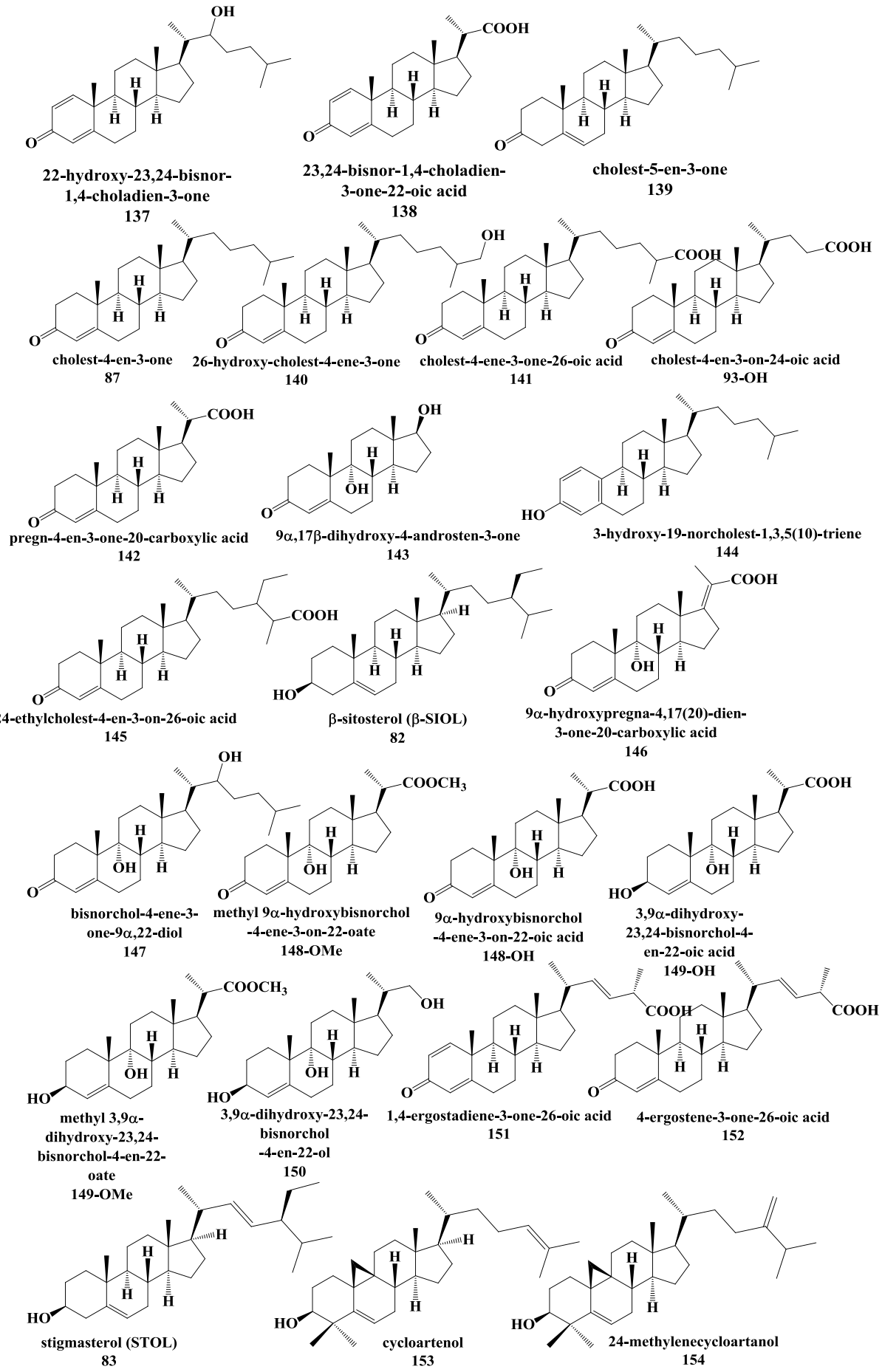


Figure 8 (Continued)

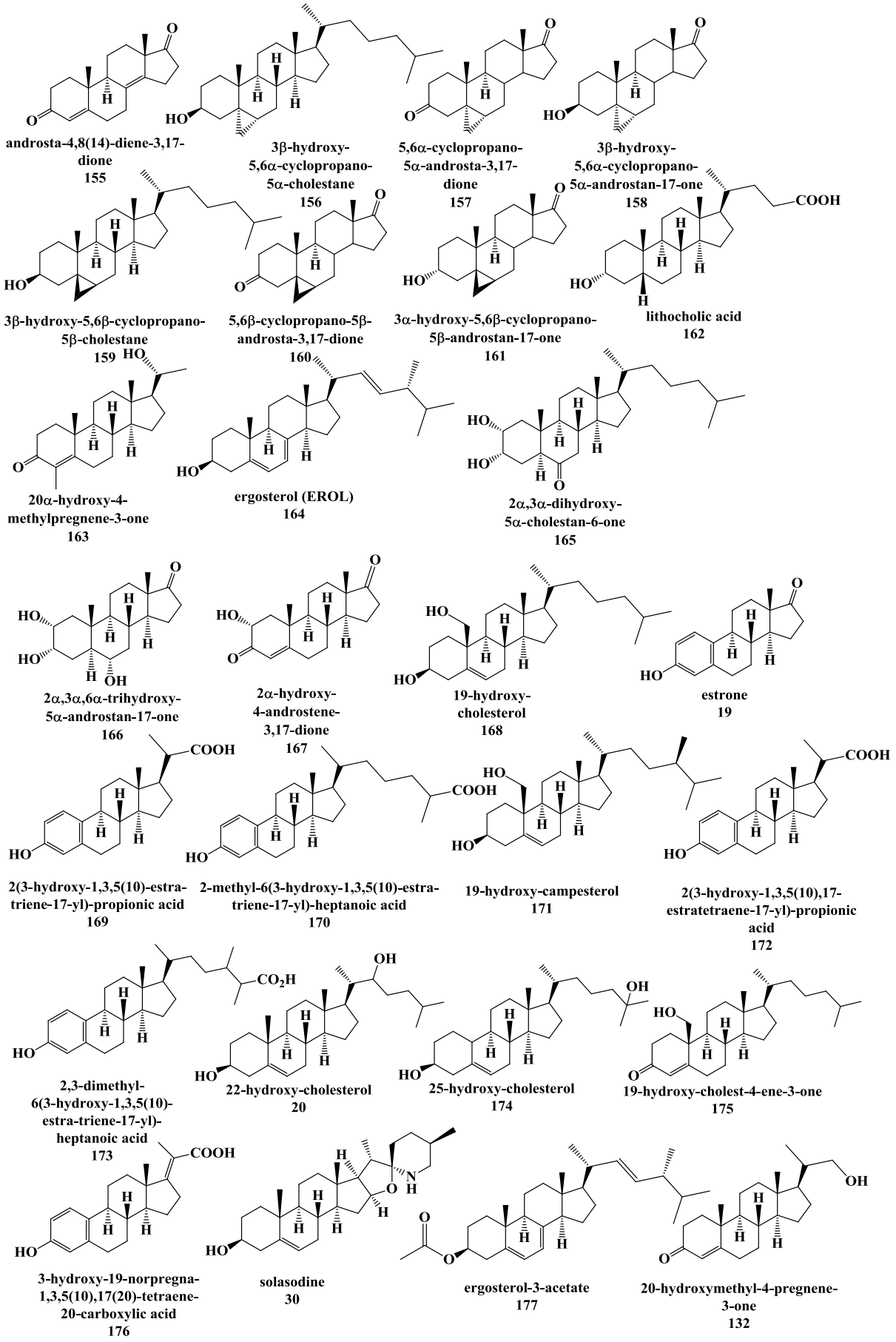
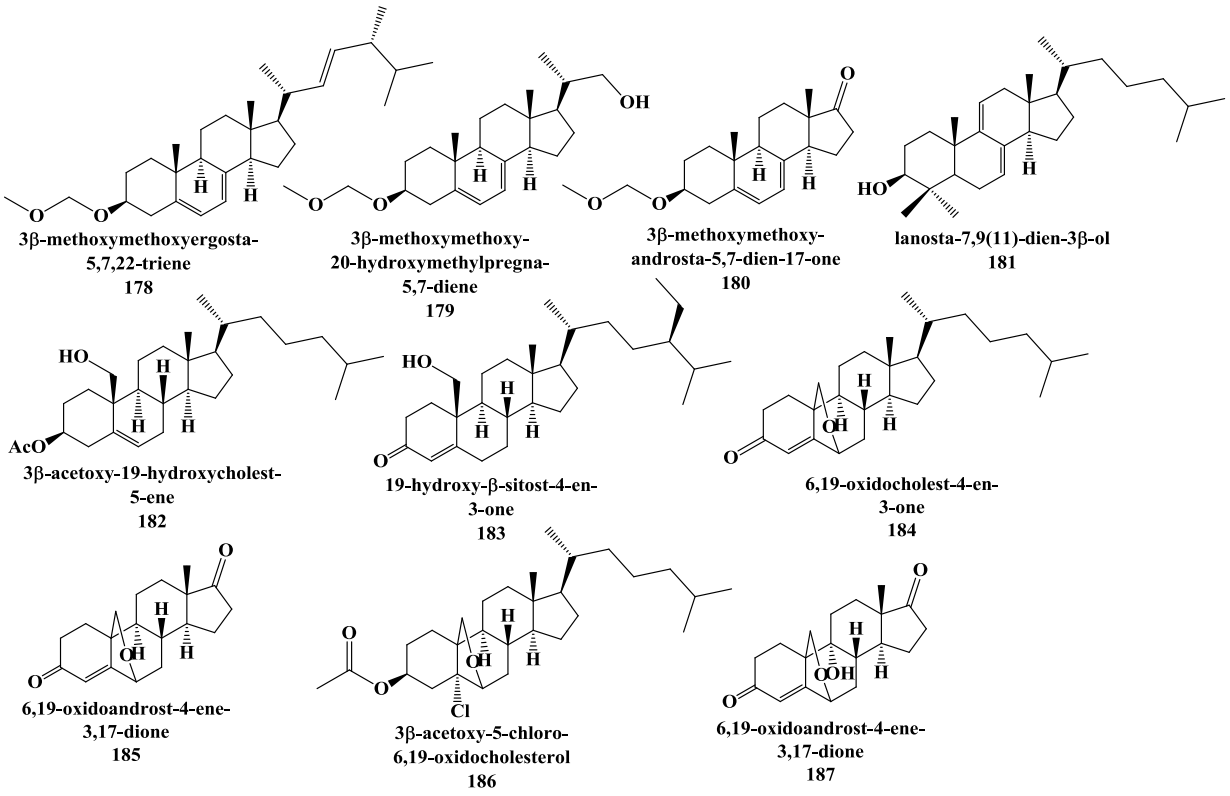


Figure 8. (Continued)



Acknowledgement: Thies Thiemann thanks the Emirates Foundation for grant 21S023.

Table 1: Microbial transformation of cholesterol and phytosterols with C17-side chain cleavage

Substrate*	**Microorganism****	**Product (Yield %)**	**Reference**
CHOL (**6**)	*Arthrobacter simplex*	AD (**11**)	Malaviya 2008[80] Mathur 1992[81]
CHOL (**6**)	*Arthrobacter simplex* and *M.* sp. NRRL B-3683	ADD (**128**)	Lee 1993[82] Wilbrink 2011[83]
CHOL (**6**)	*Nocardia* sp.soil isolate	AD (**11**), ADD (**128**)	Whitmarsh 1964[84]
CHOL (**6**)	*Brevibacterium lipolyticum*	AD (**11**), ADD (**128**)	Malaviya 2008[80] Mahato 1984[85]
	M. smegmatis	AD (**11**), ADD (**128**)	
	M. parafortuitum MC1-0801	AD (**11**), ADD (**128**)	
CHOL (**6**)	Bovine Adrenal Cortex Mitochondria	pregnenolone (**7**)	Kraaipoel 1978[86]
CHOL (**6**)	Moraxella bacteria	3β- hydroxy-androst-5-en-17-one (**104**)	Niven 2001[87] Bhattacharyya 1984[88]
CHOL (**6**)	*M.fortuitum* NRRL B-8153	3β- hydroxy-5-androsten-17-one (**104**)	Srivastava 1994[89] Wilbrink 2011[83]
CHOL (**6**)	*M.parafortuitum*	9α- hydroxy-4-androstene-3,17-dione (**134**)	Donova 2007[90] Voishvillo 1992[91]
CHOL (**6**)	*M. phlei*	AD (**11**)	Stadtman 1954[92] Wilbrink 2011[83]
CHOL (**6**)	*M.smegmatis* PTCC 1307	AD (**11**), ADD (**128**)	Naghibi 2002[93]
CHOL (**6**)	*M.* sp.	AD (**11**), ADD (**128**)	Smith 1993[94] Wilbrink 2011[83]
		17β- hydroxyandrosta-l,4-dien-3-one (**133**)	
CHOL (**6**)	*M.* sp.	testosterone (**2**)	Liu 1994[95] Wilbrink 2011[83]
CHOL (**6**)	*M.* sp.	AD (**11**), ADD (**128**)	Malaviya 2008[80] Smith 1993[94]
CHOL (**6**)	*M.* sp. CCM 3528	ADD (**128**) (51%)	Mickova 1985[96]
CHOL (**6**)	*M.* sp. CCM 3529	AD (**11**) (1.4%)	
		ADD (**128**) (66.8%)	
		22- hydroxy-23,24-bisnor-1,4-choladien-3-one (**137**) (15.4%)	
		17β- hydroxyandrosta-l,4-dien-3-one (**133**) (2.8%)	
CHOL (**6**)	*M.* sp. NRRL B 3805	AD (**11**)	Malaviya 2008[80] Shukla 1992[97] Lee 1992[98]

Substrate*	**Microorganism****	**Product (Yield %)**	**Reference**
CHOL (**6**)	*M.* sp. NRRL B-3805	testosterone(**2**) (51%)	Fernandes 2003[79] Liu 1997[99]
CHOL (**6**)	*M.* sp. NRRL B 3683	ADD (**128**)	Malaviya 2008[80] Shukla 1992[97]
CHOL (**6**)	*M.tuberculosis*	AD (**11**), ADD (**128**)	Nesbitt 2010[100]
CHOL (**6**)	*M.vaccae*	9α- hydroxy-4-androstene-3,17-dione (**134**)	Donova 2007[101] Mahato 1985[85]
CHOL (**6**)	*R. corallina*	ADD (**128**)	Shi 1992[102] Wilbrink 2011[83]
CHOL (**6**)	*R. equi*	AD (**11**), ADD (**128**)	Ahmed 1993[103] Ahmed 1993[104] Wilbrink 2011[83]
CHOL (**6**)	*R. equi*	ADD (**128**)	Malaviya 2008[80] Ahmad 1992[105]
CHOL (**6**)	*R. rhodochrous* DSM43269 mutant RG32	ADD (**128**)	Wilbrink 2011[83] Wilbrink 2011[106]
		23,24-bisnor-1,4-choladien-3-on-22-oic acid (**138**)	
CHOL (**6**)	*Pseudomonas* sp.	ADD (**128**)	Owen 1983[107]
		cholest-5-en-3-one (**139**)	
		cholest-4-en-3-one (**87**)	
		26- hydroxy-cholest-4-en-3-one (**140**)	
		cholest-4-en-3-on-26-oic acid (**141**)	
		cholest-4-en-3-on-24-oic acid (**93-OH**)	
		pregn-4-en-3-one-20-carboxylic acid (**142**)	
		pregna-1,4-dien-3-one-20-carboxylic acid (**138**)	
3-hydroxy-19-norcholest-1,3,5(10)-triene (**144**)	*Nocardia restrictus*	estrone (**19**)	Niven 2001[87] Afonso 1966[108]
24-ethylcholest-4-en-3-on-26-oic acid (**145**)	*M.* sp. NRRL 3805	AD (**11**)	Fujimoto 1982[109]
SIOL	*Arthrobacter simplex*	AD (**11**), ADD (**128**)	Malaviya 2008[80] Mathur 1992[81]
β-SIOL (**82**)	*Arthrobacteroxydans*	cholest-4-en-3-on-24-oic acid (**93-OH**)	Dutta 1992[110] Wilbrink 2011[106]
		27-nor-4-cholestene-3,24-dione	
β-SIOL (**82**)	*Arthrobacter simplex*	AD (**11**), ADD (**128**)	Mathur 1992[81] Wilbrink 2011[83]
β-SIOL (**82**)	*M.*	AD (**11**)	Kurakov 1993[111] Wilbrink 2011[83]

Substrate*	**Microorganism****	**Product (Yield %)**	**Reference**
SIOL	*M.flavum*	ADD (**128**)	Malaviya 2008[80]
	M.fortuitum	AD (**11**), ADD (**128**)	
	M. sp. NRRL B 3683	ADD (**128**)	
	M. vaccae	ADD (**128**)	
SIOL	*M. fortuitum*	9*α*- hydroxy-pregna-4,17(20)-dien-3-one-20-carboxylic acid (**146**)	Donova 2007[101] Wovcha 1978[112] FR 2505360, 1982[113]
β-SIOL (**81**)	*M. fortuitum*	9α- hydroxy-4-androstene-3,17-dione (**134**)	Birke 1992[114] Wilbrink 2011[83]
SIOL	*M. fortuitum*	9*α*,17*β*-dihydroxy-4-androsten-3-one (**143)**	Donova 2007[101] JP Pat. 7 959 395, 1979[115]
		bisnorchol-4-en-3-one-9*α*,22-diol (**147**)	
		methyl 9*α*-hydroxy-bisnorchol-4-en-3-on-22-olate (**148-OMe**)	
		9*α*-hydroxy-bisnorchol-4-en-3-on-22-oic acid (**148-OH**)	
SIOL	*M.parafortuitum*	9*α*,17*β*-dihydroxy-4-androsten-3-one (**143)**	Donova 2007[101] JP Pat. 7 959 395, 1979[115]
		3,9*α*-dihydroxy -23,24-bisnorchol-4-en-22-oic acid (**149-OH**)	
		Methyl 3,9*α*-dihydroxy-23,24-bisnorchol-4-en-22-olate (**149-OMe**)	
		3,9*α*,22-trihydroxy -23,24-bisnorchol-4-ene (**150**)	
β-SIOL (**82**)	*R.equi* k-3	3,9*α*-dihydroxy -23,24-bisnorchol-4-en-22-oic acid (**149-OH**)	Murohisa 1993[116] Wilbrink 2011[83]
		23,24-bisnor-1,4-choladien-3-on-22-oic acid (**138**)	
		1,4-ergostadien-3-one-26-oic acid (**151**)	
		4-ergosten-3-one-26-oic acid (**152**)	
		AD (**11**)	
		ADD (**128**)	
STOL (**83**)	*Arthrobacter simplex*	ADD (**128**)	Oda 1994[117] Wilbrink 2011[83]
STOL (**83**)	*M. fortuitum*	9*α*- hydroxy-4-androstene-3,17-dione (**134**)	Atrat 1992[118] Wilbrink 2011[83]
STOL (**83**)	*M. fortuitum*	9*α*- hydroxy-4-androstene-3,17-dione (**134**)	Seidel 1992[119] Wilbrink 2011[83]
STOL (**83**)	*M.* NRRL B- 3805	AD (**11**)	Lee, 1990[120] Wilbrink 2011[83]
STOL (**83**)	*M.* sp.	AD (**11**), ADD (**128**)	Zhang 1992[121] Wilbrink 2011[83]
STOL (**83**)	*M.vaccae*	ADD (**128**)	Gottschaldt 1993[122] Reiche 1992[123] Wilbrink 2011[83]

Substrate*	**Microorganism****	**Product (Yield %)**	**Reference**
cycloartenol (**153**)	*M.* sp. (NRRL B-3805)	4,8(14)-androstadiene-3,17-dione (**155**) (34%)	Yan 2000[124]
24-methylenecycloartanol (**154**)	*M.* sp. (NRRL B-3805)	4,8(14)-androstadiene-3,17-dione (**155**) (35%)	Yan 2000[124]
3*β*-hydroxy-5,6*α*-cyclopropano-5*α*-cholestane (**156**)	*M.* sp. (NRRL B-3805)	5,6*α*-cyclopropano-5*α*-androstane-3,17-dione (**157**) (43%)	Yan 2000[124]
		3*β*-hydroxy-5,6*α*-cyclopropano-5*α*-androstan-17-one (**158**) (3.6%)	
		AD (**11**) (6%)	
3*β*-hydroxy-5,6*β*-cyclopropano-5*β*-cholestane (**159**)	*M.* sp. (NRRL B-3805)	5,6*β*-cyclopropano-5*β*-androsta-3,17-dione (**41**) (38%)	Yan 2000[124]
		3*α*-hydroxy-5,6*β*-cyclopropano-5*β*-androsta-17-one (**42**) (15%)	
		AD (**11**) (4%)	
lithocholic acid (**162**)	*M.* sp.	20*α*-hydroxy-4-methylpregnen-3-one (**163**)	Wang 1994[125] Wilbrink 2011[83]
CHOL (**6**), SIOL (**82**), STOL (**83**), EROL (**164**)	*M.* sp. NRRL B 3805 &*M.* sp. NRRL B 3683	AD (**11**), ADD (**128**)	Malaviya 2008[80] Sripalakit 2006[126]
CHOL (**6**), PHOL, EROL (**164**)	*M.* sp. Ac-1815D	AD (**11**)	Ivashina 2012[127]
2*α*,3*α*-dihydroxy-5*α*-cholestan-6-one (**165**)	*M. vaccae*	2*α*,3*α*,6*α*-trihydroxy-5*α*-androstan-17-one (**166**)	Vorbrodt 1991[128] Wilbrink 2011[83]
		2*α*-hydroxy-4-androstene-3,17-dione (**167**)	
19-hydroxy-CHOL (**168**)	*R.* mutant k-3	estrone (**19**)	Murohisa 1993[129] Wilbrink 2011[83]
		2(3-hydroxy-1,3,5(10)-estra-triene-17-yl)-propionic acid (**169**)	
		2-methyl-6(3-hydroxy-1,3,5(10)-estratrien-17-yl)-heptanoic acid (**170**)	
		2(3-hydroxy-1,3,5(10),17-estratetraen-17-yl)-propionic acid (**172**)	
19-hydroxy-campesterol (**171**)	*R.* mutant k-3	2(3-hydroxy-1,3,5(10),17-estratetraen-17-yl)-propionic acid (**172**)	Murohisa 1993[129] Wilbrink 2011[83]
		2,3-dimethyl-6(3-hydroxy-1,3,5(10)-estratrien-17-yl)-heptanoic acid (**173**)	
22-hydroxy-CHOL (**20**)	bovine adrenal enzyme	pregnenolone (**7**)	Chaudhuri 1962[130]
25-hydroxy-CHOL (**174**)	cytochrome *P*-450scc in isolated mitochondria	pregnenolone (**7**)	Tuckey 1989[131]
19-hydroxy-cholest-4-en-3-one (**175**)	*Nocardia restrictus* (ATCC 14887)	3-hydroxy-19-norpregna-1,3,5(10)-triene-20-carboxylic acid (**172**)	Sih 1967[132]
		3-hydroxy-19-norpregna-1,3,5(10),17(20)-tetraene-20-carboxylic acid (**176**)	
		acidic products, besides estrone (**19**)	
β-SIOL (**82**)	*M.* sp.	9*α*- hydroxy-4-androstene-3,17-dione (**134**)	Borman 1992[133] Wilbrink 2011[83]
α-SIOL	*M.* sp. NRRL B-3805	AD (**11**) (25%)	Fernandes 2003[79] US Pat. 5,516,649
	M. sp. NRRL B-3683	ADD (**128**) (20%)	
β-SIOL (**82**)	*M.* NRRL B-3683	ADD (**128**)	Roy 1992[134]

Substrate*	**Microorganism****	**Product (Yield %)**	**Reference**
β-SIOL (**82**)	*M.* sp. NRRL B-3805	AD (**11**) (90%)	Fernandes 2003[79] Rumijowska 1997[135]
SIOL	*M.* sp. NRRL 3805	AD (**11**)	Fujimoto 1982[109]
β-SIOL (**82**)	*M.* sp. NRRL B 3683	ADD (**128**)	Malaviya 2008[80]
	M.vaccae	ADD (**128**)	
β-SIOL (**82**)	*M.* sp. VKM Ac-1815D	AD (**11**) (70% - 75%)	Egorova 2002[72]
β-SIOL (**82**)	*M.* sp. VKM Ac-1815D ET1	AD (**11**) (72%)	Fernandes 2003[79] Egorova 2002[72]
β-SIOL (**82**)	*M.* sp. VKM Ac-1815D	AD (**11**), ADD (**128**)	Malaviya 2008[80] Egorova 2002[72] Donova 2005[136] Donova 2005[137] Donova 2005[138]
β-SIOL (**82**)	*M.* sp. VKM Ac-1816D	testosterone (**2**) (50% - 55%)	Egorova 2009[73]
β-SIOL (**82**)	*M.vaccae*	AD (**11**)	Spassov 1993[139] Wilbrink 2011[83]
β-SIOL (**82**)	*Nocardia* sp. M 29	AD (**11**), ADD (**128**)	Martin[140] Wilbrink 2011[83]
β-SIOL (**82**)	*Pseudomonas* sp. NCIB 10590	ADD (**128**)	Malaviya 2008[80] Owen 1985[141]
β-SIOL (**82**)	*R. rhodochrous* DSM43269 mutant RG32	ADD (**128**)	Reiche 1992[123]
		23,24-bisnor-1,4-choladien-3-on-22-oic acid (**138**)	Wilbrink 2011[83]
Mixture of SIOL, CMOL (**84**)& STOL (**83**)	*M.fortuitum*	9*α*- hydroxy-4-androstene-3,17-dione (**134**)	Donova 2007[90] East (German) Pat. 298278, 1992[142]
Solasodine (**30**)	*M.* sp. NRRL B 3805	AD (**11**)	Malaviya 2008[80] Shukla 1992[97]
Mixture of *β*-SIOL, CMOL & STOL	*M.*sp. NRRL B-3683	ADD (**128**)	Wang 2006[143]
PHOL	*M.* MB 3683	AD (**11**) (80%-90%)	Fernandes 2003[79] Kutney 1999[144]
PHOL (SIOL, CMOL (**84**), STOL (**83**), Brassicasterol (**129**))	*M.neoaurum*ZJUVN-08	AD (**11**)	Zhang 2013[145]
EROL (**164**)	*M.* sp. NRRL B-3805	AD (**11**) (35%)	Fernandes 2003[79]
	M. sp. NRRL B-3805	ADD (**128**) (30%)	Ambrus 1995[146]
EROL (**164**)	*M.* sp. VKM Ac-1815D	AD (**11**) (45%)	Dovbnya 2010[147]
		ADD (**128**) (30%)	
		20-hydroxymethyl-4-pregnen-3-one (3%) (**132**)	

Substrate*	**Microorganism****	**Product (Yield %)**	**Reference**
EROL-3-acetate (**177**)	*M.* sp. VKM Ac-1815D	AD (**11**) (20%) ADD (**128**) (15.5%) 20-hydroxymethyl-4-pregnen-3-one (0.5%) (**132**)	Dovbnya 2010[147]
3*β*-methoxymethoxyergosta-5,7,22-triene (**178**)	*M.* sp. VKM Ac-1815D	3*β*-methoxymethoxy-20-hydroxymethylpregna-5,7-diene (**179**) 3*β*-methoxymethoxy-androsta-5,7-dien-17-one (**180**)	Dovbnya 2010[147]
lanosta-7,9(11)-diene-3*β*-ol (**181**)	*M.* sp. NRRL B-3805	androsta-4,8(14)-diene-3,17-dione (30%) (**155**)	Fernandes 2003[79] Wang 1995[148]
3*β*-acetoxy-19-hydroxycholest-5-ene (**182**)	*Moraxella* sp.	estrone (**19**) (15%)	Fernandes 2003[79] Madyastha 1994[149]
19-hydroxy-cholest-4-en-3-one (**175**)	*Nocardia restrictus* CSD-10 (isolated from soil)	estrone (**19**) (8%) estrone (**19**) (30%)	Sih 1965[150]
19-hydroxy-*β*-sitost-4-en-3-one (**183**)	CSD-10 (isolated from soil)	estrone (**19**) (10%)	Sih 1965[150]
3*β*-acetoxy-19-hydroxy-cholest-5-ene (**182)**	CSD-10 (isolated from soil)	estrone (**19**) (72%)	Sih 1965[151]
6,19-oxidocholest-4-en-3-one (**184**)	CSD-10 (isolated from soil)	6,19-oxidoandrost-4-ene-3,17-dione (**185**) (57%) 6,19-oxido-9*α*-hydroxyandrost-4-ene-3,17-dione (**187**)	Sih 1965[151]
3*β*-acetoxy-5-chloro-6,19-oxidocholesterol (**186**)	CSD-10 (isolated from soil)	6,19-oxidoandrost-4-ene-3,17-dione (**185**) (36%)	Sih 1965[151]

* CHOL: Cholesterol; SIOL: Sitosterol; STOL: Stigmasterol; CMOL: Campesterol; PHOL: Phytosterol; EROL: Ergosterol.
** *M.*: *Mycobacterium* ; *R.*: *Rhodococcus*

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