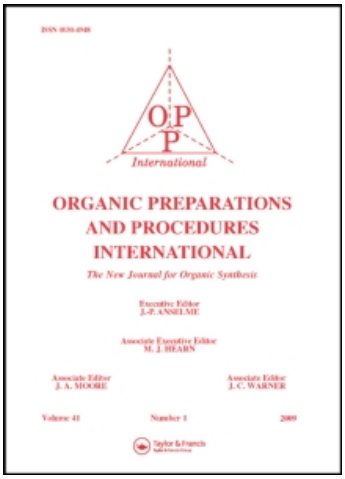

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CHEMICAL AND BIOCHEMICAL HYDROXYLATIONS OF STEROIDS. A REVIEW

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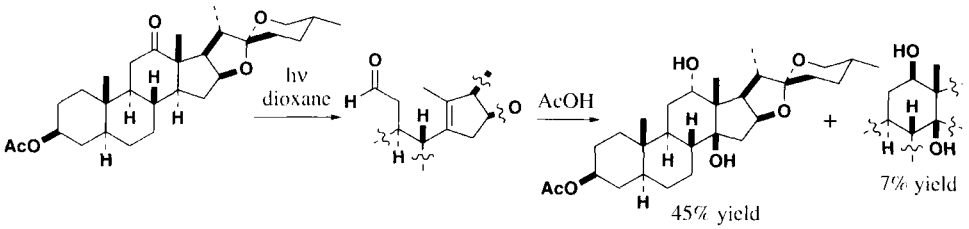
INTRODUCTION

Nature's ability to catalyze the monooxygenation of unactivated C-H bonds in steroids employing enzymatic systems (cytochromes P-450) has long been recognized. In contrast, chemists' attempts to mimic nature by replacing a hydrogen atom attached to an unactivated carbon of a steroid with a hydroxyl group while maintaining the integrity of the carbon atom, constitute a formidable challenge. Despite the fact that the use of covalently attached templates to catalyze the remote functionalization of steroids was introduced by Breslow over 20 years ago,[1] the direct remote hydroxylation of steroids with high predictability and specificity has yet to be accomplished and thus, constitutes an area of increasing interest. In spite of the very large number of results, abstract coverage has been extended only to publications offering acceptable yields to the end of 1999.

I. CHEMICAL HYDROXYLATION OF STEROIDS

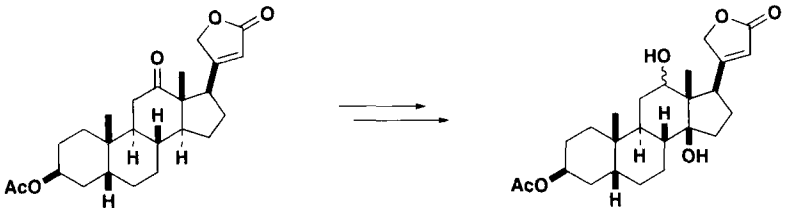
1. Hydroxylation by Photochemical Process and/or Iodine Derivatives

Welzel has reported the photochemical rearrangement of hecogenin acetate into the intermediate unsaturated aldehyde followed by a Prins reaction which leads efficiently to 14β-hydroxy steroids.[2]



(1)

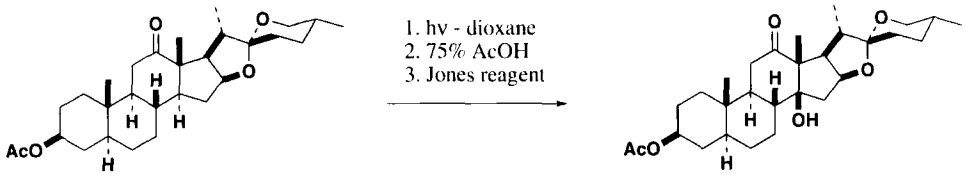
The syntheses of digoxigenin and digitoxygenin employing the same strategy have been achieved.[3]



12α-OH, 44% yield
12β-OH, 28% yield

(2)

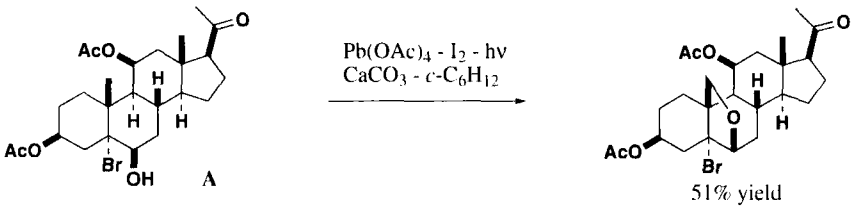
Cephalostatin is among the most powerful anticancer agents ever tested by the National Cancer Institute. The first total synthesis of this molecule is very recent and involves a photolysis/Prins protocol on hecogenin acetate to install the Δ^{14} functionality.[4]



94% yield

(3)

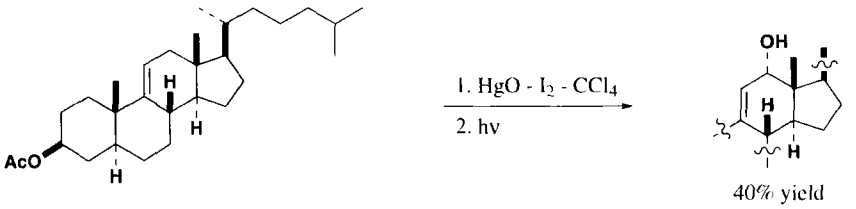
Application of the "hypoiodite reaction" [$Pb(OAc)_4$, I_2, hv] converted the bromohydrin **A** into 6,19-epoxypregnane derivative which constitutes a key intermediate in the synthesis of 19-hydroxycorticosterone.[5]



51% yield

(4)

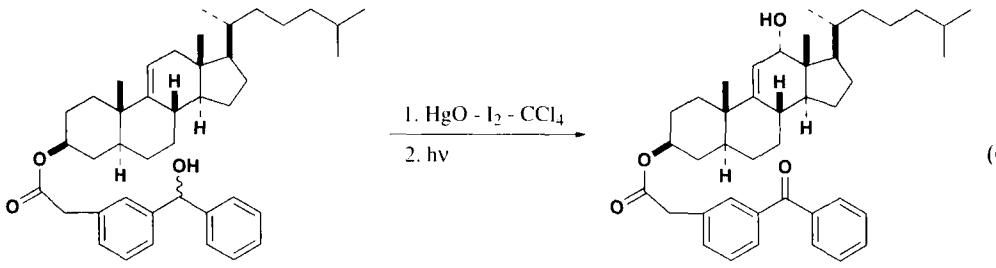
The same reaction has been applied by Suginome to cholestane derivatives with the involvement of mercury oxide instead of lead tetraacetate. In this case, a selective C-12α hydroxylation was observed.



40% yield

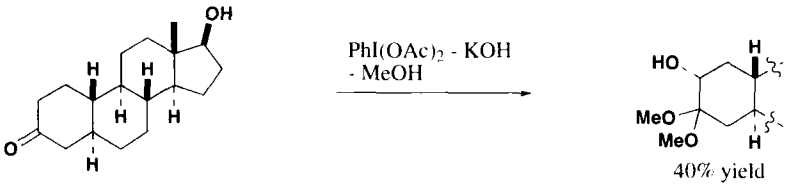
(5)

A long range intramolecular hydrogen abstraction by alkoxy radicals generated from the esters of 3-(hydroxyphenylmethyl)phenyl acetate gave also 12α-allylic alcohol.[6]



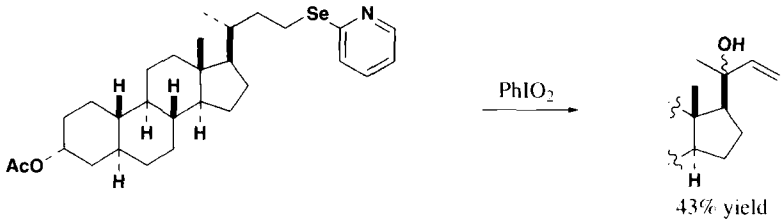
36% yield (6)

Moriarty has demonstrated the synthetic usefulness of hypervalent iodines such as diacetoxyphenyliodine, iodosylbenzene and *o*-iodosylbenzoic acid for the α-hydroxylation of enolizable ketones.[7] More recently, Daum has reported that hypervalent iodine in MeOH and excess base when reacted with a 19-nor-5α-androstan-3-one gave mainly the 2α-hydroxy-3,3-dimethoxy derivative.[8]



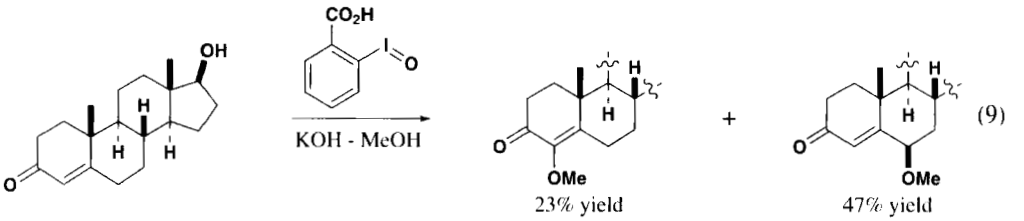
40% yield (7)

During his work on elimination of alkylaryl selenides, Crich showed that treatment of 2-selenopyridine with iodoxybenzene at reflux in toluene provided allylic alcohol instead of the desired olefin. Seemingly, the former arises from the allylic oxidation of the olefin.[9]



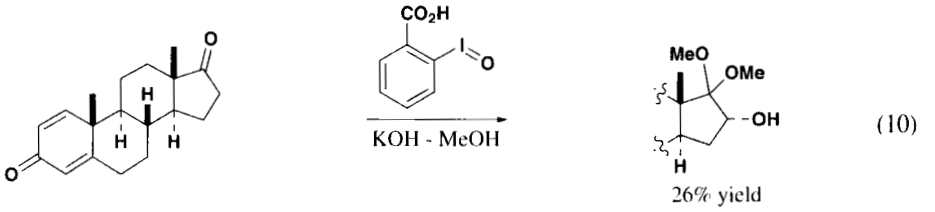
43% yield (8)

In 1988, Numazawa reported on the *o*-iodosobenzoic acid-MeOH-KOH reaction applied to enolizable steroidal α,β-unsaturated ketones, as the 3-oxo-4-androsten-derivatives. This reaction represents a striking way of functionalizing the C-4 and C-6 positions without affecting the C-17 carbonyl function. The 4-methoxysteroid is an obvious precursor for the synthesis of 4-hydroxy-4-androstene-3,17-dione, a potent inhibitor of estrogen synthetase.



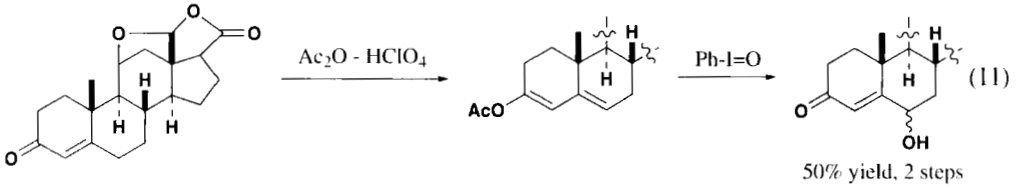
(9)

The reaction was then applied to a series of 17-oxo steroids. In the case of the two first compounds having a 5-en-3β-ol system or 5α-3β-ol system, the hypervalent iodine oxidation provided the 16α-hydroxy-17,17-dimethylacetal in moderate yield. By the same way, other 17-oxo steroids having a 1,4-dien-3-one system or phenolic ring A were similarly converted into the corresponding 16α-hydroxy acetals. After hydrolysis, these acetals afford 16α-hydroxy-17-one steroids which are major metabolites of C-18 and C-19 steroids.[10]



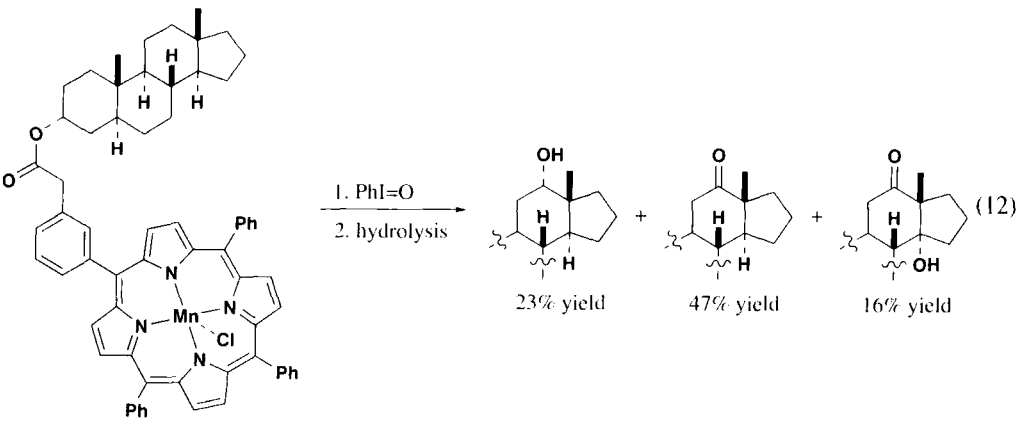
(10)

Some metabolites of aldosterone are physiologically important in the regulation and / or expression of this hormone's action. Among them, the 6β-hydroxy aldosterone γ-lactone has been prepared by Kirk and Morris employing iodosobenzene as oxidant.[11]



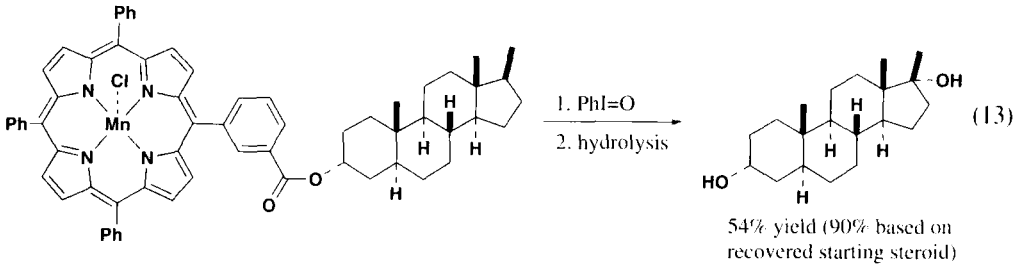
(11)

Grieco has shown that hydroxylation of unactivated carbons in steroids was possible when a synthetic metalloporphyrin is attached to the steroid backbone. Iodosylbenzene constitutes the source of oxygen. By manipulation of the length of the tether linking the steroid to the template, the intermediate oxometalloporphinate can be directed to abstract a hydrogen atom at either the C(12), C(14) or C(17) position, thereby leading to hydroxyl incorporation at these sites. For instance, 5α-androstan-3α-ol gave rise in these conditions to a mixture of corresponding androstan-3α-ol-12-one, androstan-3α,12α-diol and androstan-3α,14α-diol-12-one in good yields.



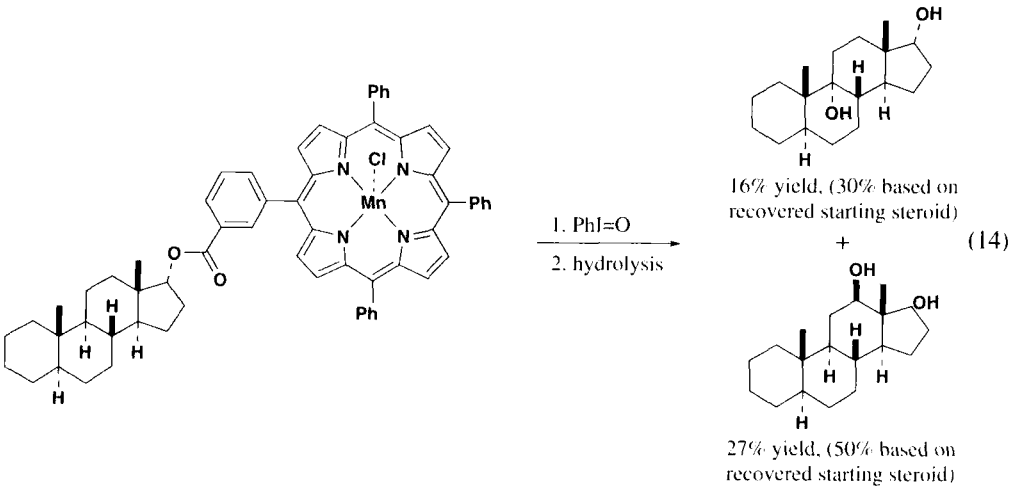
(12)

On the other hand, attachment of the same rigid metalloporphyrin fragment to 17β-substituted-5α-androstan-3α-ol led to hydroxylation at C(17).[12]



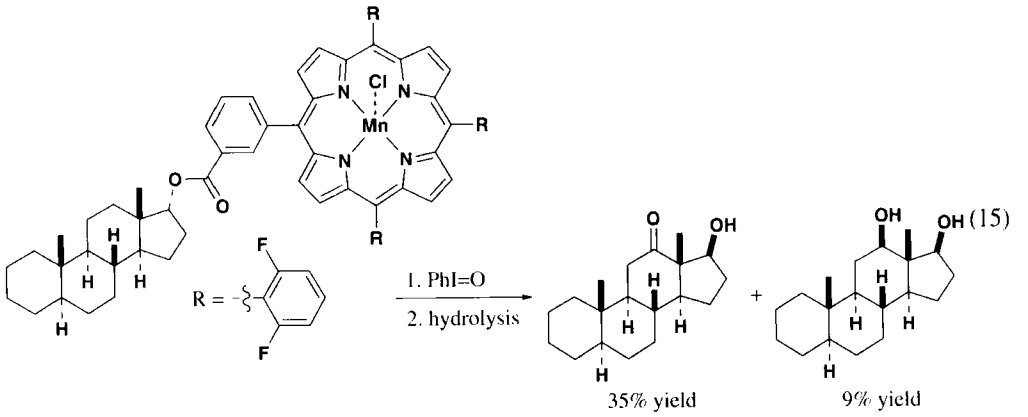
(13)

When the same synthetic manganese(III)porphyrin was attached to steroidal substrates at C(17), the reaction gave rise to hydrogen atom abstraction at C(9) or C(12), thereby leading to hydroxyl incorporation at these sites.



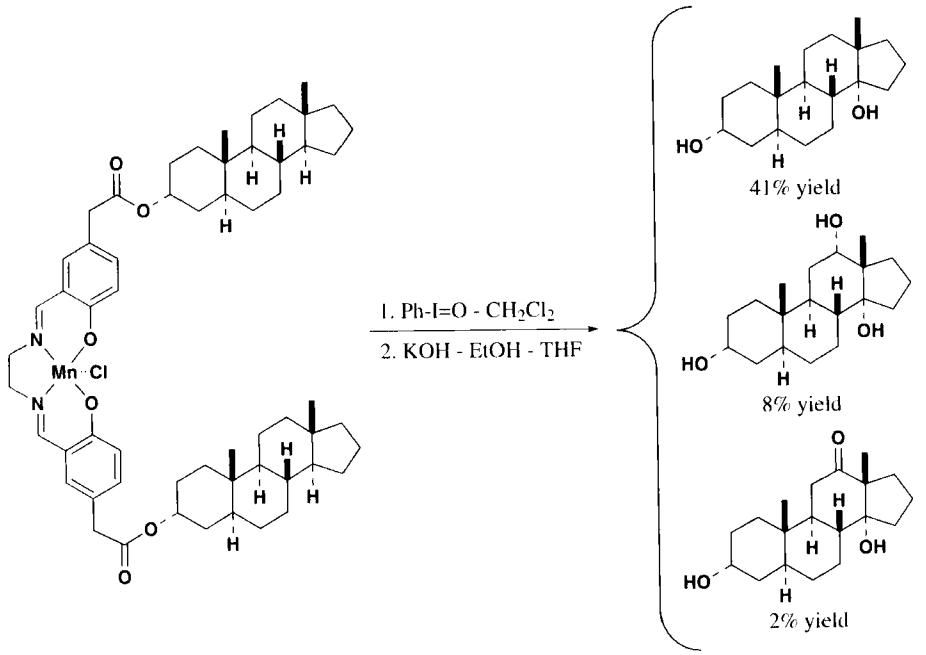
(14)

The major factor limiting the yields of these hydroxylations is the ease with which tetraphenyl-porphyrin is oxidatively degraded. In order to limit this problem, several substituted tetraphenylporphyrins bearing electron-withdrawing substituents on the phenyl rings have been designed. For instance, incorporation of fluorine atoms in the 2,6-positions of the C(10), C(15), and C(20) phenyl groups of the same metalloporphyrin that above dramatically increased the isolated yield of 12-oxo steroid from 10 to 35%.[13]



(15)

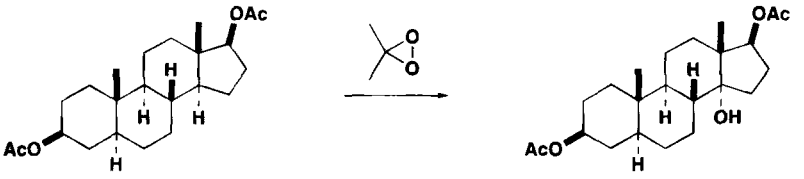
Unexpectedly, manganese (III) salen(*N*,*N*'-bis-(salicylideneamino)ethane) complexes attached at C(3) to steroid substrates mediate the C(14)-hydroxylation in presence of three equivalents of iodosylbenzene.[14]



(16)

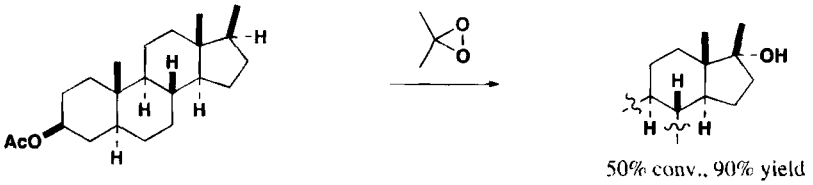
2. Hydroxylation with Dioxiranes and Oxaziridines

Dioxiranes appear to be very effective for the selective hydroxylation of steroidal carbons. These reagents which can be isolated or prepared *in situ*, present a high reactivity combined with a neutral pH.[15] For instance, 5α-androstan-3β-ol acetate was selectively oxyfunctionalized at C-14 to give the corresponding 14α-hydroxy derivative. With regards to synthetic implications of the C-14 hydroxy functionalization at steroids, new access to the ecdysonic hormones having the C-14α-OH could result. The preferential attack of dimethyldioxirane is explained by the less hindered steric environment as shown by molecular models.[16]



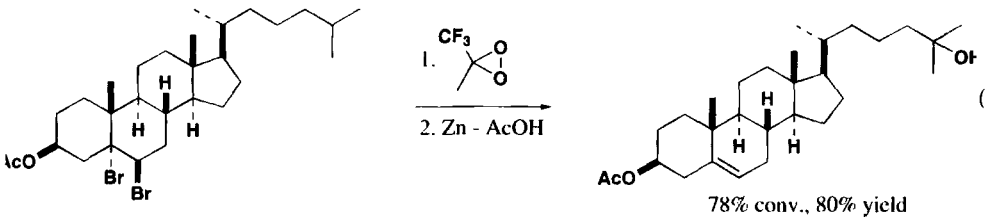
50% conv., 85% yield (17)

In the case of 17β-methyl-5α-androstan-3β-ol acetate, a selective and unexpected hydroxylation at C-17 was observed. It seems that finer steric controls regulate the reactivity of the C-17-H bond.



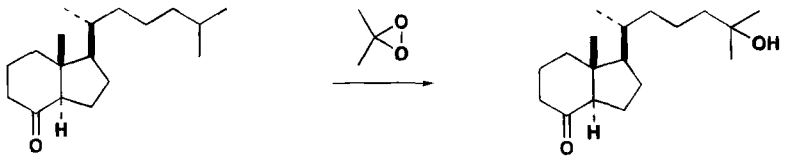
50% conv., 90% yield (18)

Some saturated cholestane derivatives can be selectively C-25 hydroxylated under the same conditions as before. Transformations are much faster, without change in selectivity, with the more reactive methyl(trifluoromethyl)dioxirane. The origin of the selectivity might be traced to rather stringent steric and stereoelectronic demands. The acetoxydibromocholestane could be cleanly (yield 93%) converted into 25-hydroxycholesteryl acetate.[17]



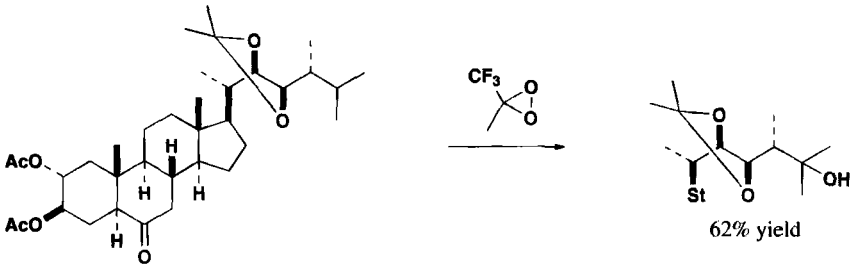
78% conv., 80% yield (19)

Interestingly, the Windaus-Grundmann ketone can be selectively hydroxylated giving rise to an important synthon.



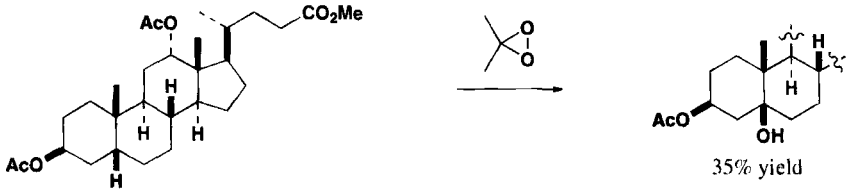
80% conv., 86% yield (20)

Similarly, the following precursor of brassinolide afforded by treatment with methyl(trifluoromethyl)dioxirane, the desired 25-hydroxy derivative in 62% yield.[18]



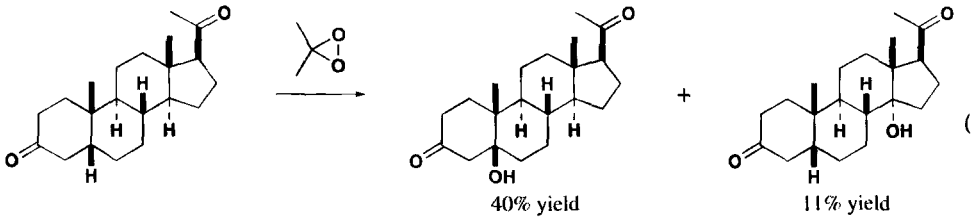
62% yield (21)

Several 5β-hydroxy cardioactive steroids occur in nature, but there have been only a few attempts to prepare 5β-hydroxy cardenolides or bufadienolides. This may be due to the fact that the introduction of a 5β-hydroxy function generally comprises at least four steps. In 1993, Heerden reported a single step procedure for the introduction of a 5β-hydroxy function into 5β-steroids.



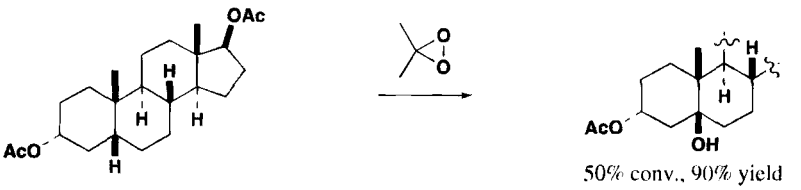
35% yield (22)

The difference in reactivity of *trans*- and *cis*-fused rings towards oxidation with dioxirane can probably be attributed to greater steric hindrance in the case of the *trans* compounds and indicates that reactions with dioxirane are influenced by steric effects.[19]



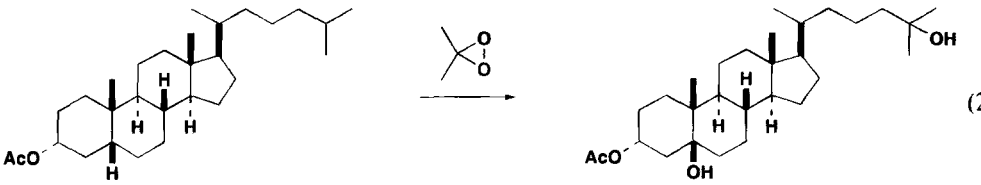
40% yield + 11% yield (23)

Using a similar strategy, various coprostane steroids have also been C-5 oxyfunctionalized. This methodology constitutes an improved access to progestogen and androgen hormones.



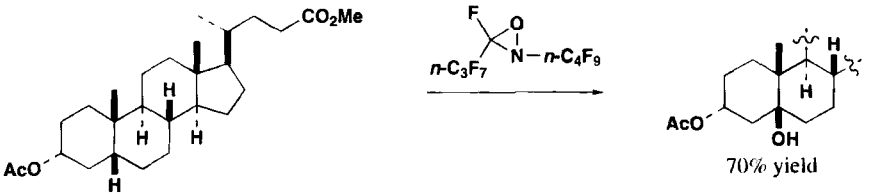
50% conv., 90% yield (24)

With 5β-cholestan-3α-ol acetate, double oxyfunctionalization occurred at C(5) and C(25)(yield not given).[20]



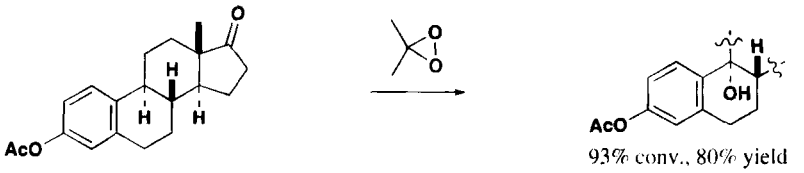
(25)

In 1994, Resnati reported the 5β oxyfunctionalization of other 5β-steroids performed in the presence of perfluorodialkyloxaziridines in good yields.[21] These reagents are easily prepared from commercially available perfluorotrialkylamines.[22]



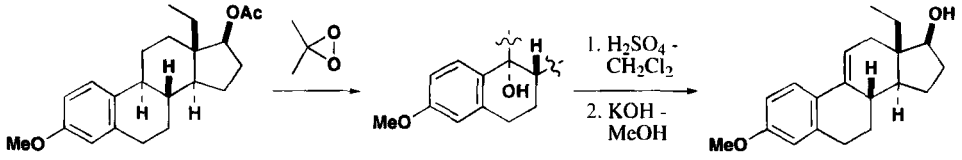
70% yield (26)

Curci has also applied the dioxirane methodology to estrone acetate. In this case, a selective C-9 functionalization was observed.[23]



93% conv., 80% yield (27)

The 9α-hydroxyestrone acetate constitutes a key intermediate in the synthesis of corticosteroids and androsteroids. A few years later, Schwarz described a partial synthesis of the steroid hormone desogestrel involving a dimethyldioxirane approach. Indeed, the 18α-homosteroid could be selectively converted to its corresponding 9α-hydroxy derivative as depicted below.[24]

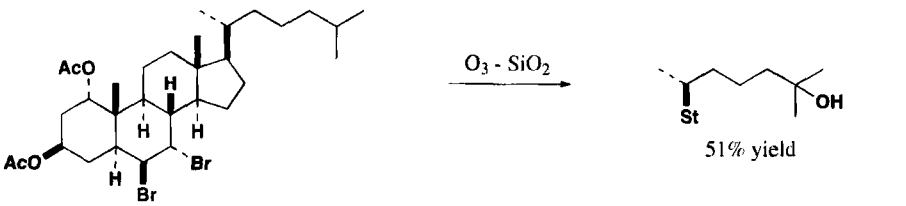


(28)

Finally, the dioxirane approach has very recently proved to be the method of choice for the stereoselective oxyfunctionalization of steroids.

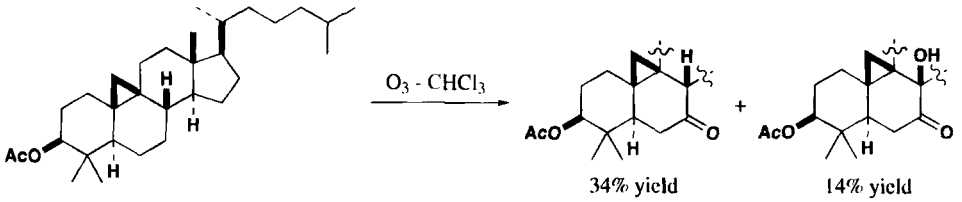
3. Hydroxylation with Ozone

A convenient synthesis of 1α,25-dihydroxyvitamin D_3, the natural calcium regulating hormone, has been elaborated by Mazur. This latter is based on a regioselective C25-hydroxylation of 1α,3β-diacetoxy-6β,7α-dibromocholestane by means of ozone absorbed on silica gel.[25]



(29)

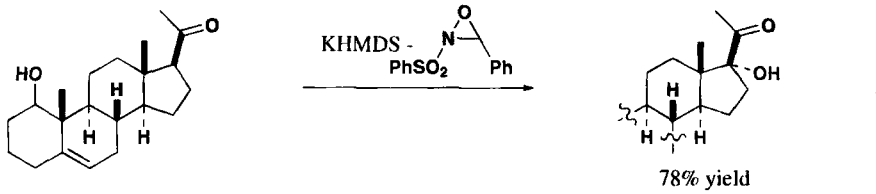
However, treatment of cycloartanyl acetate with oxygen containing ozone gave rise to the major 7-oxo corresponding derivative along with 7-oxo-8β-hydroxy steroid.[26]



(30)

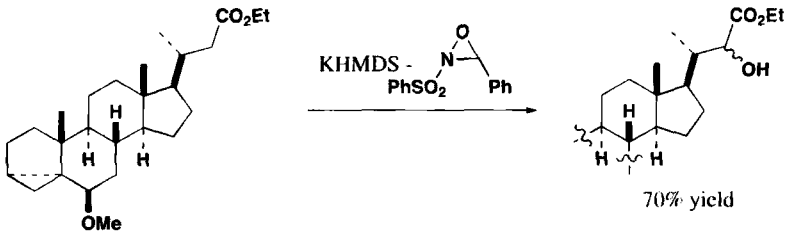
4. Oxidation at Activated Carbon

Davis reported that enolate oxidation using 2-(phenylsulfonyl)-3-phenyloxaziridine affords α-hydroxy carbonyl compounds in high yield.[27]



(31)

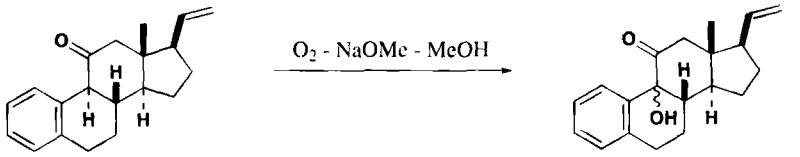
Hydroxylation of the potassium enolate generated from the following *i*-steroid with Davis'oxaziridine reagent yielded the corresponding hydroxylated ester in good yield. This selective C-22 hydroxylation method has considerable potential in the synthesis of structurally diverse side chains of physiologically active steroids.[28]



(32)

70% yield

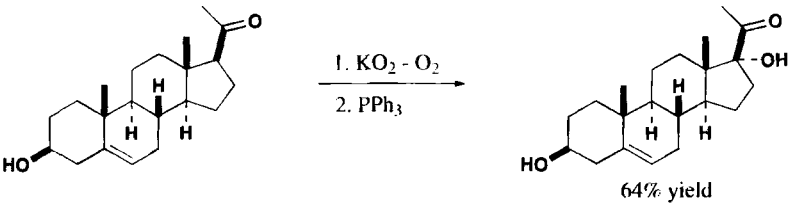
With the aim of epimerizing the 9-position of estrone derivative, it has been observed the unexpected formation of corresponding 9-hydroxy steroid resulting from an oxidation of the enolate by the adventitious dissolved oxygen.[29]



(33)

90% yield

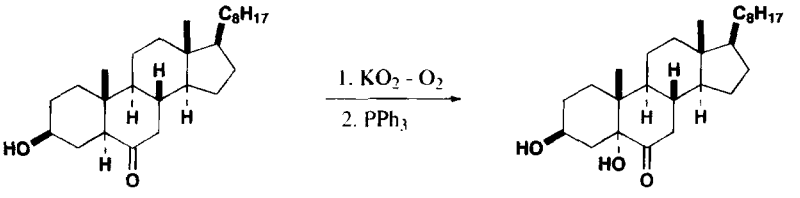
In 1988, Freire described an efficient two step procedure to transform pregnan-20-ones into their corresponding α-ketols. The intermediate hydroperoxides obtained by reaction with KO_2 and oxygen under pressure are later reduced with PPh_3 to give the corresponding α-ketols.



(34)

64% yield

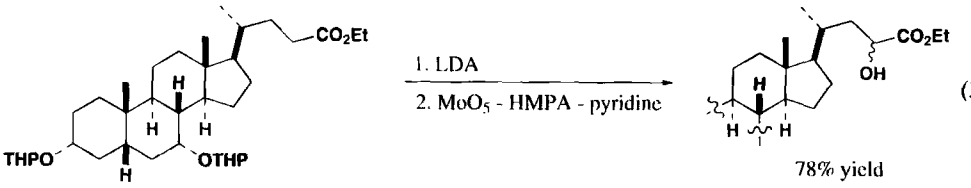
Extension of this procedure to 3β-hydroxy-5α-cholestan-6-one gave rise to the 5-hydroxy derivative.[30]



(35)

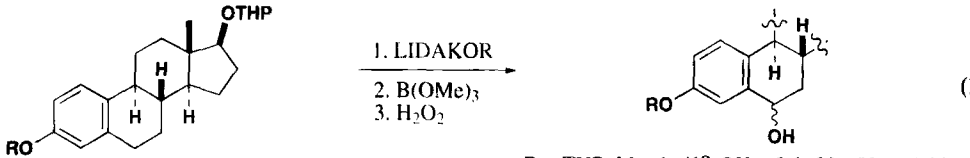
66% yield

The oxidoperoxymolybdenum/hexamethylphosphoric triamide/pyridine/complex (MoOPH)[31] was able to hydroxylate at C-23 the lithium enolate of completely protected chemodeoxycholic acid.[32]



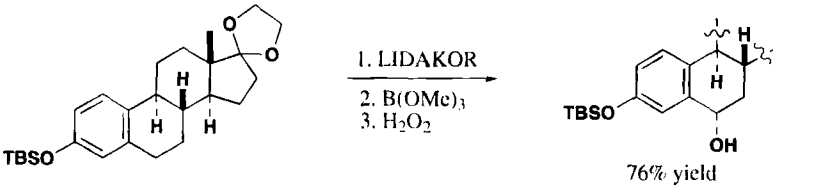
78% yield (36)

The oxidation of organometallic derivatives obtained by hydrogen-metal exchange is a general method for hydrocarbon functionalization, whose main limitation is the problem of achieving metalation at a specific position within a complex molecule. In 1995, Napolitano reported a C-6 selective metalation of estradiols using superbases. The metalated intermediates can be oxidized by quenching with trimethylborate followed by treatment with hydrogen peroxide. This method allows the easy introduction of a 6-hydroxy group into the estradiol framework.[33]



R = THP, Me; 6α/6β-OH = 9:1; 88 - 90% yield (37)

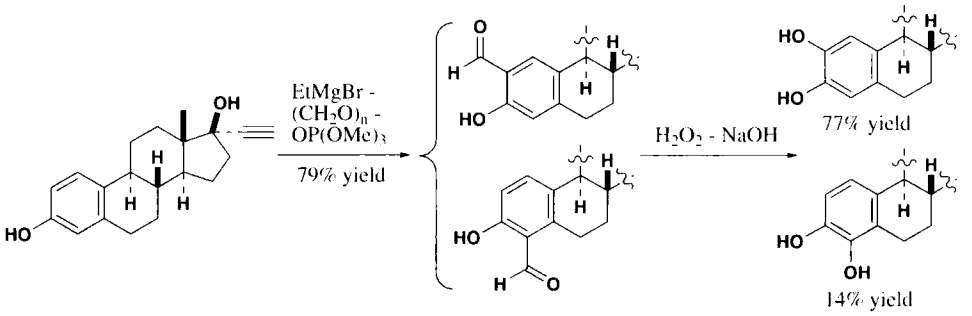
In order to elaborate Δ^6-estrogens, which represent useful intermediates in dye-sensitized photo-oxygenation studies and in the synthesis of [6,7-$^3H_2$]- and [7-^{127}I]-labelled estrogens used in metabolic studies, radioimaging and radioimmuno assay, Rao and Wang have applied the same methodology, followed by a dehydration.[34]



76% yield (38)

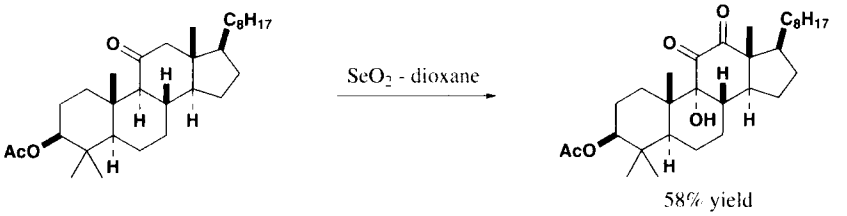
Synthetic methods for catechol estrogens cannot be generally applied to the hydroxylation of 17α-ethynylestradiol. On the other hand, the reaction of ethyl magnesium bromide and 17α-ethynylestradiol with formaldehyde in the presence of triethylphosphate or hexamethylphosphoramide gave the 2-formyl-17α-ethynylestradiol in high yield, along with 4-formyl derivative. Treatment of

these latter with an alkaline solution of hydrogen peroxide (Dakin reaction) afforded the desired corresponding catechols.[35]



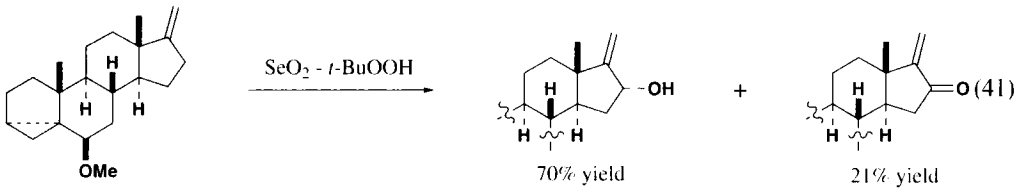
(39)

The use of selenium dioxide in the case of oxidation of acetoxy-lanostanone has led to the corresponding C-9,12 deoxidized compound.[36]



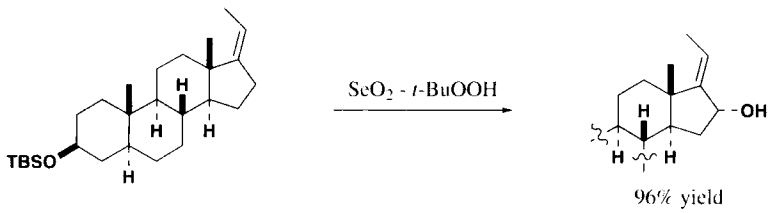
(40)

In the synthesis of cholesterol from dehydroepiandrosterone, Trost has shown that allylic oxidation of $\Delta^{17(20)}$ steroid to 16α-hydroxysteroid using the procedure developed by Sharpless proved to be superior to the classical selenium dioxide procedure.[37]



(41)

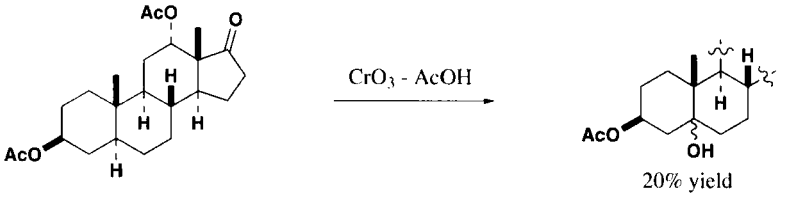
Gros has explored several strategies for the synthesis of steroidal 20,16-δ-carbolactones from androstanes. These results should provide access to a variety of functionalized sapogenins and steroidal alkaloids. The synthetic pathway providing the desired β-oriented δ-lactone involves an allylic hydroxylation with TBHP in the presence of catalytic amounts of SeO_2.[38]



(42)

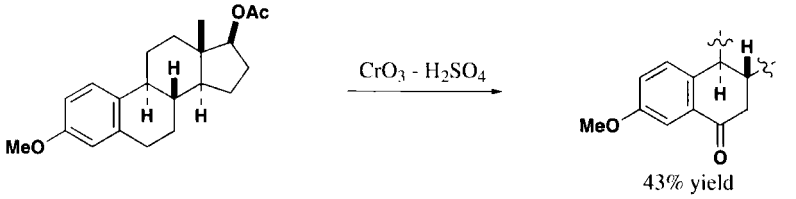
5. Hydroxylation with Chromic Anhydride

Regioselective hydroxylations of steroids can be achieved using chromium trioxide. For instance, in 1964, Nambara has described the oxidation of 3β,12α-dihydroxy-5α-androstan-17-one diacetate into the corresponding 5-hydroxy derivative.[39]



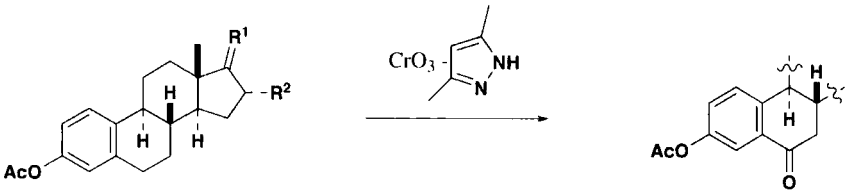
20% yield (43)

In the same year, 6-oxo-derivatives of estrogens were prepared with 8N chromic acid in acetone solution.[40]



43% yield (44)

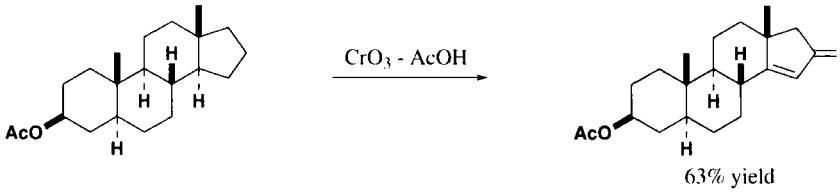
In order to prepare these steroids in improved yields, an oxidation procedure involving the use of 3,5-dimethylpyrazole-chromium trioxide complex at low temperature has been developed.[41]



(45)

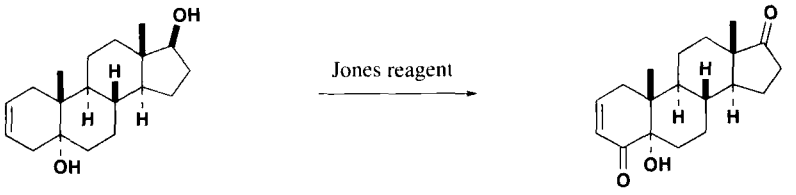
R^1 = O, R^2 = H, 36% yield
R^1 = β-OAc, α-H, R^2 = H, 37% yield
R^1 = β-OAc, α-H, R^2 = OAc, 37% yield

Steroidal estrogens with an oxygen function at the 6-position are of interest in estrogen metabolism as well as in the preparation of steroid "haptens" capable of generating monospecific anti-serum suitable for radio immuno assay of estrogens. On the other hand, 5α-androstane and 3β-acetoxy-5α-androstane were converted to their corresponding 14-en-16-oxo-derivatives by Schäfert.[42]



63% yield (46)

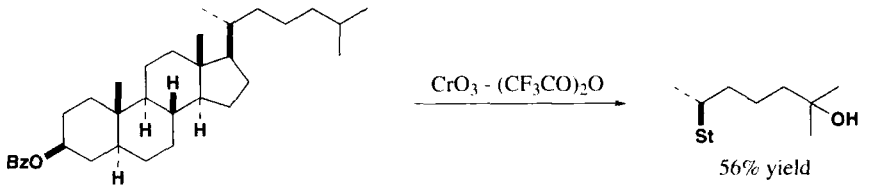
5α,17β-Dihydroxyandrost-2-ene treated with 8N chromium trioxide led efficiently to 5α-hydroxyandrost-2-ene-4,17-dione. Hence, this reaction affords an easy route to relatively inaccessible 4-oxo-5α-hydroxy-steroids.[43]



(47)

76.5% yield

A simple and direct synthesis of C-25 hydroxysteroids by the use of chromyl trifluoroacetate has been reported.[44]

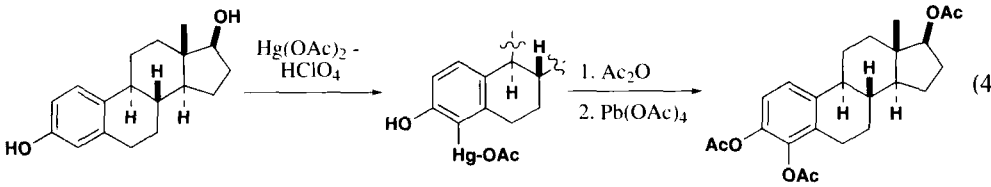


(48)

56% yield

6. A-ring Hydroxylation with Chromic and Mercury Derivatives

The hydroxylation of the aromatic ring in estrogens is recognized as the most significant path in the oxidative metabolism of those compounds in man. The synthesis of 2- and 4-substituted estrogens can be accomplished by classical electrophilic substitutions, but these procedures generally lead to equimolar amounts of the two *ortho* isomers.[45] It has been reported that treatment of estradiol with $Hg(OAc)_2$ in acetic acid afforded the 4-acetoxymercurioestradiol.[46] On the basis of these results, Kirk has described in 1982, a simple method for introducing a hydroxy group into estrogens specifically at C-4. This procedure involves lead tetra-acetate in a metal-metal exchange reaction.[47]



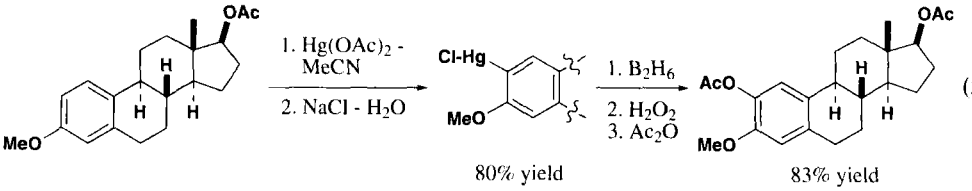
(49)

90% yield

48% yield

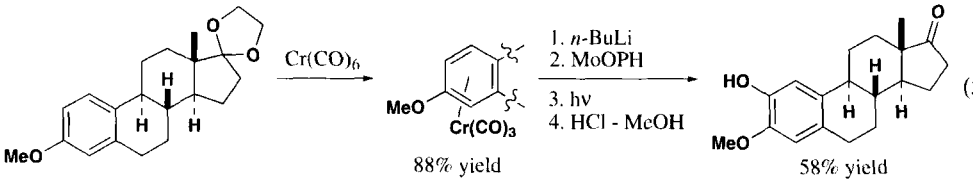
Unlike previous work, Santaniello has obtained the sole 2-chloromercurio derivative by treatment of estradiol derivative with $Hg(OAc)_2$ in dry acetonitrile.[48] It seems that the regioselectivity of the mercuration depends upon whether the 3-methyl ether or the free phenol is present on the aromatic A-ring of the steroid. Unfortunately, the metal-metal exchange of the steroid with $Pb(OAc)_2$ showed no positive result, in contrast with the successful reaction reported above. Nevertheless, treat-

ment of the chloromercurio compound with a solution of diborane followed by oxidation with H_2O_2 and then acetylation afforded the 2-hydroxyestrogen acetate.[49]



(50)

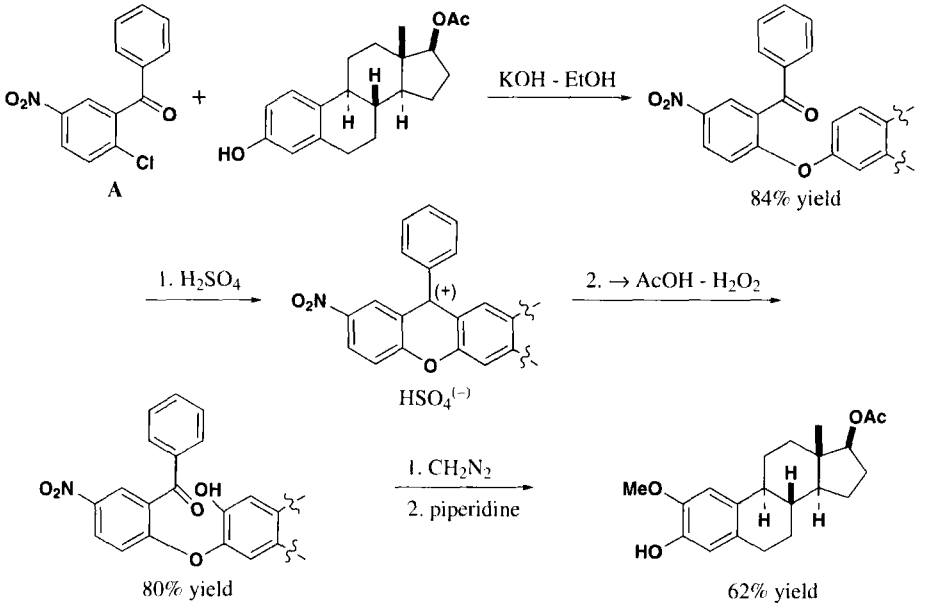
In 1987, Marples reported a very satisfactory 2-hydroxylation of 3-methoxyestra-1,3,5(10)-trienes achieved by deprotonation at C-2 of the (η^6-arene)$Cr(CO)_2$ complexes with BuLi followed by reaction with MoOPH (MoO_5-pyridine-HMPA) and then decomplexation.[50]



(51)

7. A-ring Hydroxylation *via* Xanthylium Salts

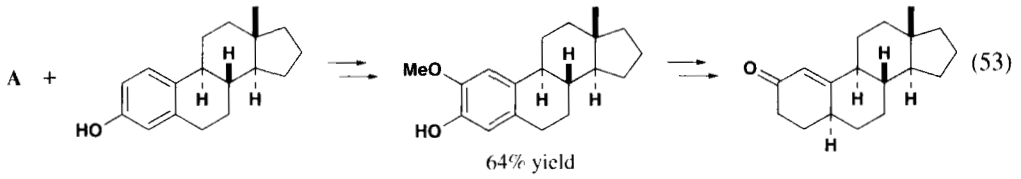
2-Hydroxylation is the major metabolic pathway of estradiol in man, which makes the preparation of compounds containing this feature of considerable interest. In 1958, Fishman reported the efficient synthesis of 2-methoxyestrogens from estradiol. The Scheme involves reaction of phenol with 2-chloro-5-nitrobenzophenone **A**, cyclization of the resultant diarylether to the xanthylium salt and oxidation of the latter with hydrogen peroxide to give the *o*-hydroxylated derivative.[51]



(52)

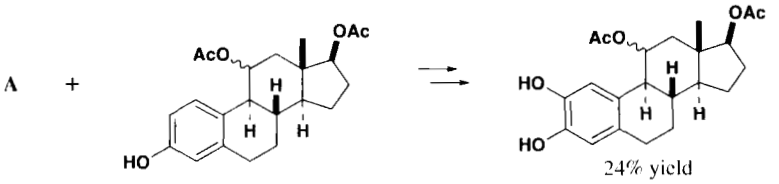
The cyclization with sulfuric acid appeared to be exclusively with C-2 and no C-4 substituted products were isolated. Molecular models indicate that the formation of the xanthylium ion at C-4 would result in some steric interference between the unsubstituted benzene ring and the hydrogens at C-6. An even more likely explanation for the non cyclization at C-4 is that the xanthylium ion at that position would be forced out of planarity and thus lose some of its stabilizing resonance forms.

Using the same procedure, 19-nor 5α 1(10)androsten-2-one was prepared from estrone.[52]



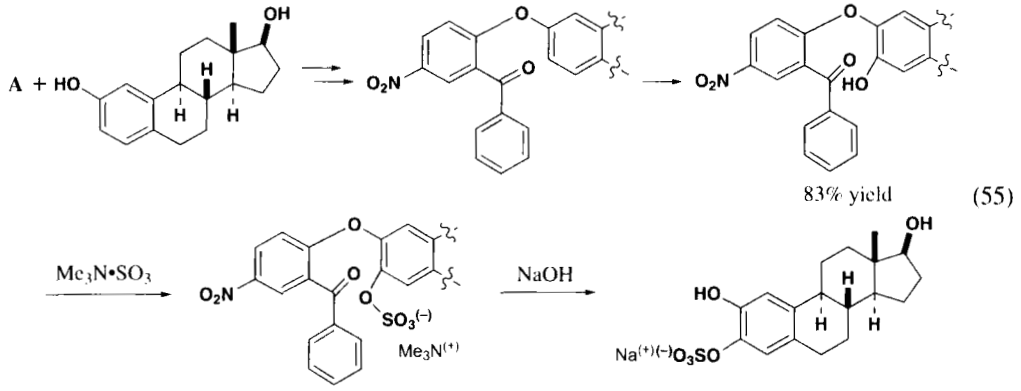
(53)

11-Oxygenated estrogen catechols were also synthesized by Fishman.[53]



(54)

2-Hydroxyestrogens are among the most significant derivatives of the female hormones. In order to achieve their synthesis, the Fishman's method has been applied to the 3-hydroxylation of estrogens and the preparation of isomeric aryl monosulfates of estrogen catechols.[54]



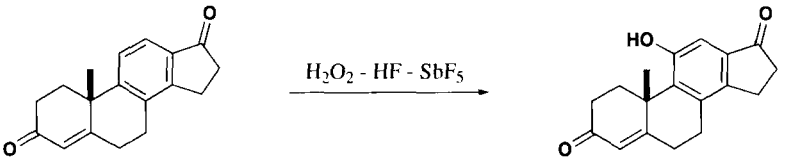
(55)

No evidence for the alternative cyclization to C-1 was obtained. This is presumably due to the steric interference of the C-1 xanthylium ion and the resultant loss of resonance stabilization.

8. Hydroxylation in Superacids

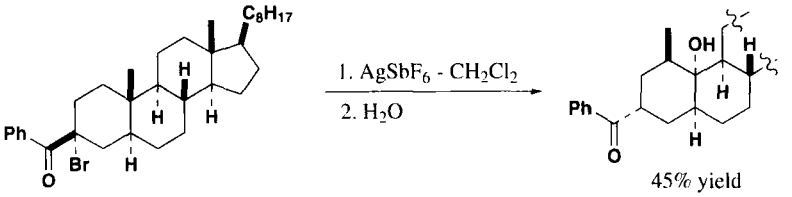
Electrophilic hydroxylation using hydrogen peroxide in superacids can be performed on

aromatics with various functional groups. This reaction has been successfully applied to many polyfunctional substrates such as steroids. For instance, treatment of ketone exhibiting an aromatic C-ring with HF-SbF_5 in presence of H_2O_2 led to selective hydroxylation at C-11.[55]



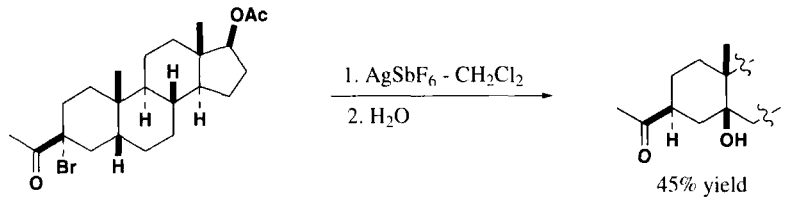
55% yield (56)

The same authors encountered lower selectivities in the case of hydroxylation of estrone derivatives, since a mixture of C-10, C-1 and C-(2+4) hydroxylated steroids were formed.[56] Under nonnucleophilic conditions, the $AgSbF_6$ dehalogenation of steroidal α-bromo ketones derived from cholestan-3-one and (5α)- and (5β)-17-hydroxyandrostan-3-one leads, after hydrolysis, to hydroxylation of the A-ring. In compounds with A/B *trans* ring fusion, hydroxylation takes place at the 10α-position leading to 5α, A/B *cis* steroids.



45% yield (57)

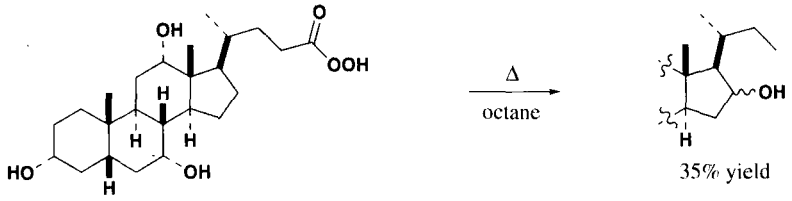
On the other hand, in compounds with A/B *cis* ring fusion, hydroxylation takes place at the 5β-position.[57]



45% yield (58)

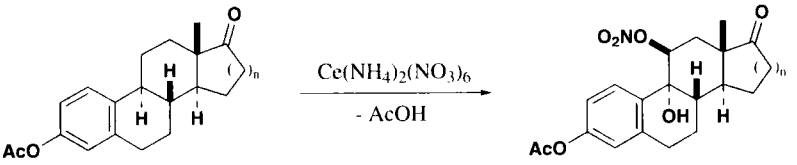
9. Other Hydroxylation Methods

The thermal decarboxylation of peroxycholanic acid led, by a radical chain reaction to the epimeric 16-hydroxy norcholane. This regioselective D-ring functionalization is the result of an intramolecular homolytic 1,5-hydrogen shift.[58]

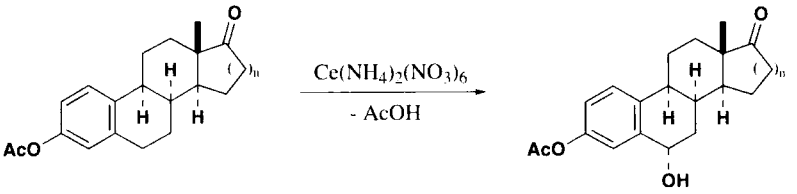


35% yield (59)

For biosynthetic studies, Ivanenko and co-workers have prepared several estrone derivatives by oxidation with ceric ammonium nitrate (CAN). It seems that the regioselectivity of the reaction is determined by the stereochemistry of the joining of rings B/C of the estrogens. Thus, the oxidation of 8-*iso*estrogens with CAN proceeds with preferable attack of the C-6 atom and leads to introduction of one hydroxyl group, in contrast to the oxidation of estrogens with the natural 8β-configuration, for which attack of Ce^{4+} on the tertiary C-9 atom leads to the 9α,11β-dihydroxylated compounds.[59]

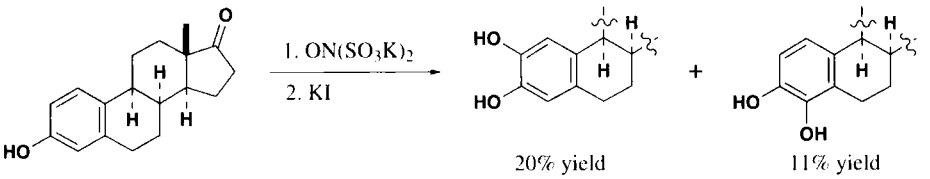


(60)



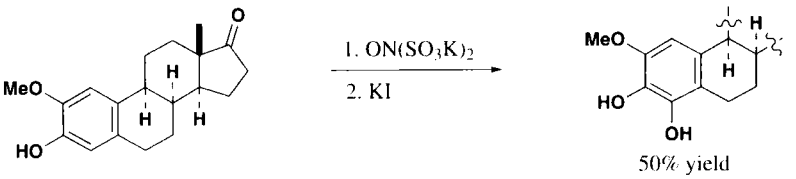
(61)

Potassium nitrosodisulfonate or Fremy's radical is used to selectively oxidize phenols to the corresponding quinones.[60] Afterwards, the latter compounds may be submitted to a reduction with KI, leading to the corresponding catechols. This simple one-step reaction has been applied by Knuppen to estrone and estradiol in order to prepare corresponding catechol estrogens.[61]

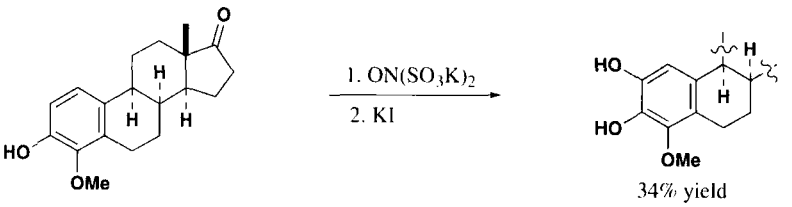


(62)

A few years later, the same authors revealed that the presence of solid KH_2PO_4 in the reaction mixture led to a marked increase in yields. By this way, 2-methoxy-4-hydroxyestrone and 2-hydroxy-4-methoxyestrone could be obtained in acceptable yields.[62]



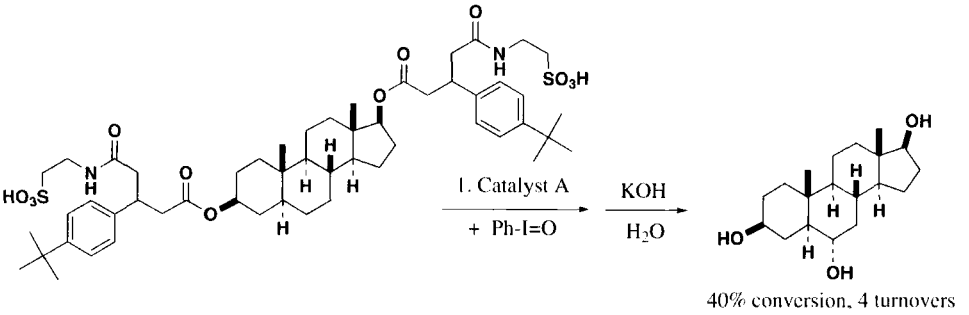
(63)

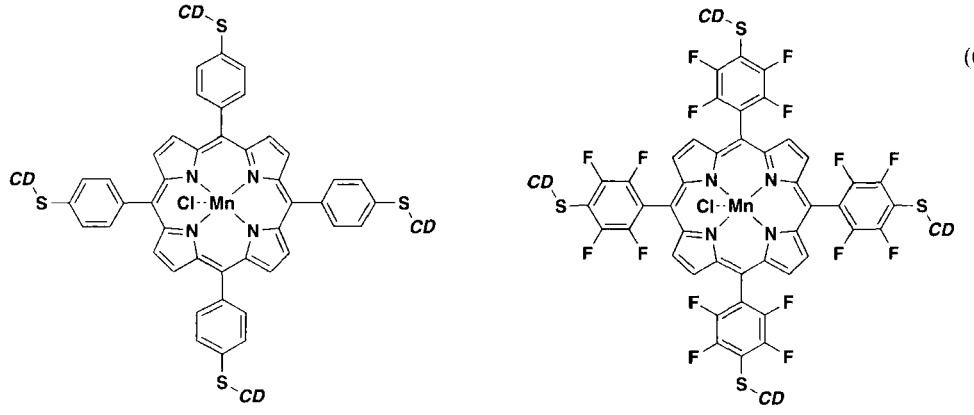


(64)

10. Catalytic Hydroxylations by Metal Porphyrins

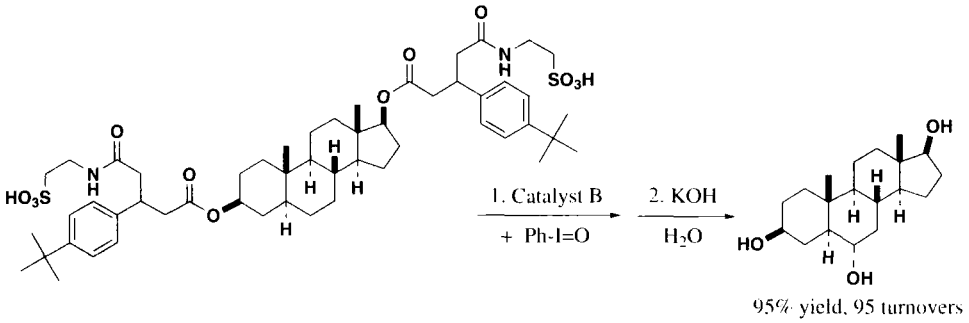
In Nature, the relevant enzymatic reactions also involve oxidation by metalloporphyrins, but in this case, a catalytic process and turnover are observed. The very interesting Gif systems (iron cluster-metallic zinc-pyridine-aqueous acetic acid) have been applied by Barton to a series of steroids. Almost without exception it gave the 20-ketone as major product, but yields are generally low.[63] In the aim to propose an artificial cytochrome P450 enzyme, Breslow has recently described a system[64] that indeed binds a substrate, performs the C(6) regioselective hydroxylation catalyzed by a metalloporphyrin (*catalyst A*) attached to cyclodextrins (***CD***), and then releases the product to perform true catalysis. However, the hydroxylation proceeded with only 3-5 turnovers before the catalyst was oxidatively destroyed.[65]





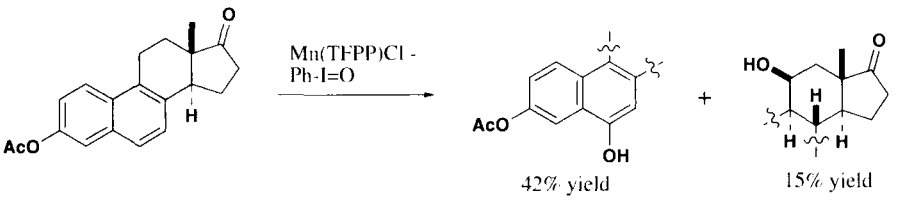
(65)

In 1998, Breslow synthesized a new metalloporphyrin (*catalyst B*, see page 22) carrying a β-cyclodextrin group on tetrafluorophenyl rings at the 4-*meso* positions of the porphyrin. This stabilized fluorocatalyst performed the selective hydroxylation of an androstanediol derivative with complete positional selectivity and 95 turnovers.[66]



(66)

More recently, the same authors reported that the aromatic steroid equilenin acetate can be hydroxylated at C-6 with good catalytic turnover by iodosobenzene, catalyzed by chloro[5,10,15,20-tetrakis(pentafluorophenyl)porphyrinato]manganese(III) [Mn(TFPP)Cl].

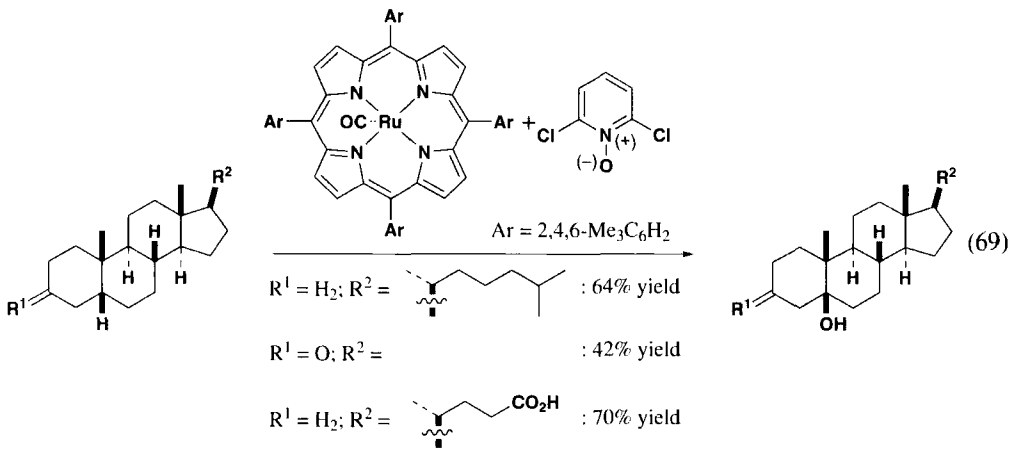


(67)

This reaction could have been generalized to a very specific 11β-amidation by using *N*-tosyliminophenyliodinane instead of iodosobenzene.[67]

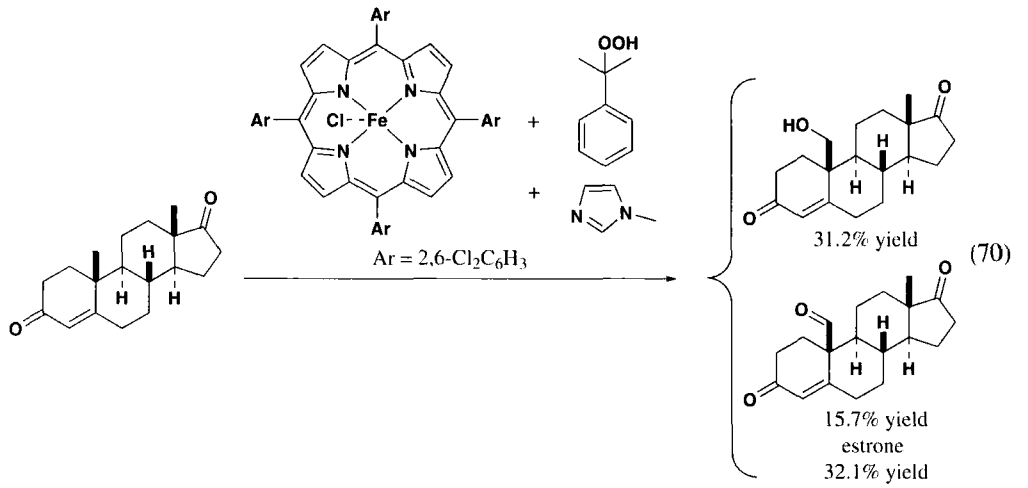
(68)

Recently, Nagano investigated the oxidation of 5β-cholestanes with 2,6-dichloropyridine *N*-oxide catalyzed by Ru(TMP)CO in the presence of HBr. Thus, he could observe a C-5 regioselective hydroxylation with retention of the configuration, since the corresponding 5α-derivatives led in moderate yields to the 5α-hydroxy compounds.[68]



(69)

The oxidation of 4-androsten-3,17-dione with cumene hydroperoxide, catalyzed by chloro-iron (III)-5,10,15,20-tetraarylporphinate/*N*-methylimidazole systems has been reported by Chauhan. An oxidation at C-19 was observed along with aromatization of the A-ring.[69]



(70)

II. BIOCHEMICAL HYDROXYLATION OF STEROIDS

1. Introduction

The importance of microbial biotechnology in the production of steroidal drugs and hormones was realized for the first time in 1952 when Murray and Peterson patented the process of 11α-hydroxylation of progesterone by a *Rhizopus species*.[70] Since then, microbial reactions for the transformation of steroids have proliferated, and specific microbial transformations have been incorporated into numerous partial syntheses of new steroids for evaluation as drugs and hormones.[71,72]

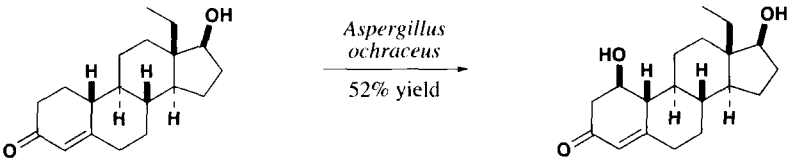
The enzymes responsible for this reaction were slow to yield their secrets, but their essential nature is now understood. Their characterization as cytochrome P-450 dependent monooxygenases

preceded investigation of enzyme-substrate interactions.[73]

2. Monohydroxylations

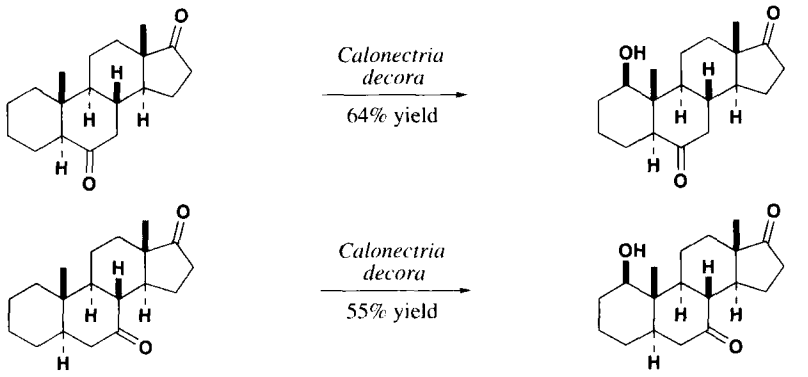
a. *C-1 Hydroxylation*

The microbiological attack of *Aspergillus ochraceus* on racemic 13β-ethyl-17β-hydroxygon-4-en-3-one resulted primarily in C-1 monohydroxylation of both the two antipodes.[74]



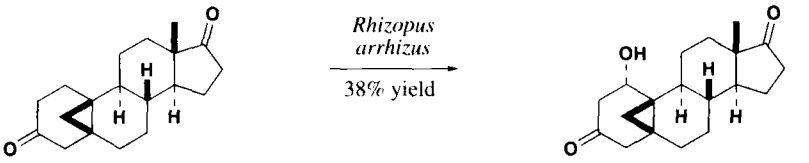
(71)

When cultures of the fungus *Calonectria decora* are employed, it has been observed that mono-oxygenated steroids were twice hydroxylated, whereas only one hydroxyl group was introduced into the diketones or the keto-alcohols. Thus, 1β-hydroxy-compounds could be readily obtained by hydroxylation of dioxygenated 5α-androstanes with cultures of the fungus *Calonectria decora*.[75,76]



(72)

Holland has prepared a series of steroids containing a cyclopropyl ring adjacent to the position at oxidation and submitted them to enzymatic oxidation using fungus *Rhizopus arrhizus*. Thus, in the case of 5β,19-cycloandrostane-3,17-dione, a selective C-1α oxidation was observed without rearrangement of the cyclopropyl unit.[77]

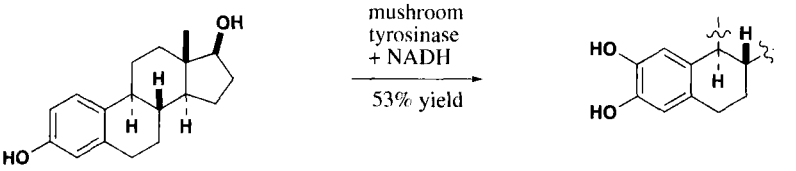


(73)

b. *C-2 Hydroxylation*

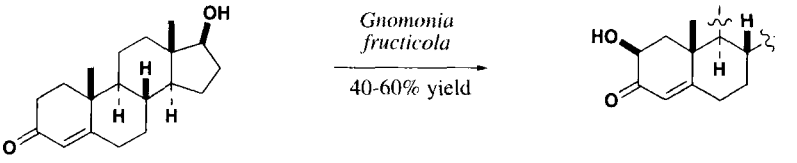
Hydroxylation at C-2 is a significant metabolic fate of estrogens both *in vivo* and *in vitro*

and in addition, 2-hydroxylated estrogens have been shown to influence the enzymatic methylation of catecholamines. Using mushroom tyrosinase in the presence of NADH constitutes a simple method to prepare these derivatives.[78]



(74)

In 1989, Holland has reported the hydroxylation of testosterone and related steroids by *Gnomonia fructicola* which led to 2β-hydroxy derivatives.[79]



(75)

c. *C-3 Hydroxylation*

As with *Calonectria decora*, E. R. H. Jones and coll. have shown that di-oxygenated substrates were preferentially monohydroxylated when *Rhizopus nigricans* incubations were employed.[76,80]

(76)

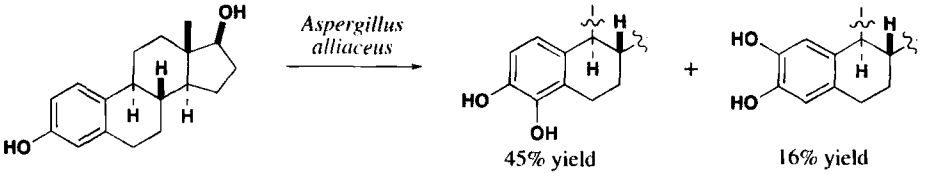
Table 1.

5α-Androstane	Product	Yield (%)
5α-androstan-7,17-dione	5α-androstan-3α-ol-7,17-dione	37
5α-androstan-17β-ol-7-one	5α-androstan-3α,17β-diol-7-one	52
5α-androstan-17β-ol-11-one	5α-androstan-3α,17β-diol-11-one	47
5α-androstan-7α-ol-17-one	5α-androstan-3β,7α-diol-17-one	77
5α-androstan-11,16-dione	5α-androstan-3α-ol-11,16-dione and 5α-androstan-3β-ol-11,16-dione	56

d. *C-4 Hydroxylation*

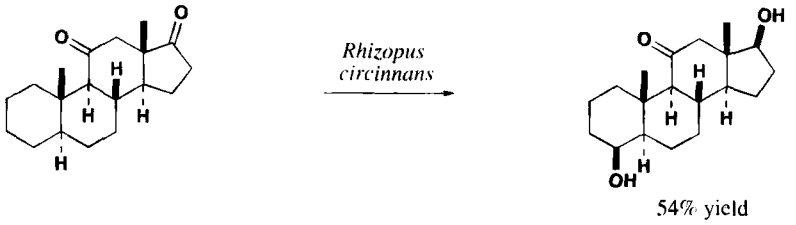
Catechol estrogens are implicated in a wide variety of biological actions. Microbial transformations by the fungus *Aspergillus alliaceus* have been applied for the production of 4- and 2-hydrox-

yestradiols starting from 17β-estradiol. Unfortunately, the same conditions applied to estrone did not provide catechol estrogens for unclear reasons.[81]



(77)

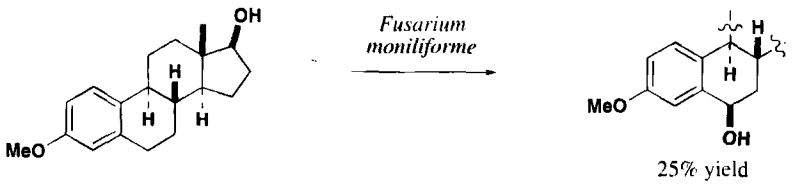
Rhizopus circinnans presents a greater ability compared with the other *Rhizopus* species to introduce a 4-hydroxy group as shown by the conversion of 5α-androstan-11,17-dione into 5α-androstan-4α,17β-diol-11-one.[80b]



(78)

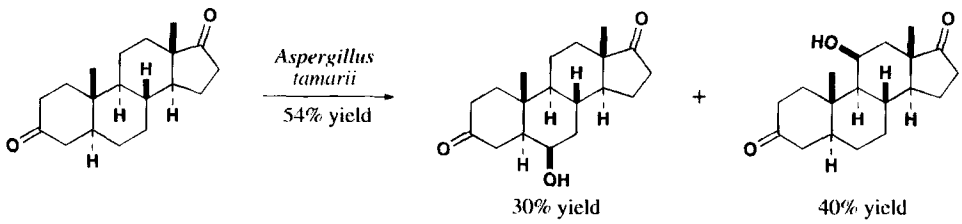
e. *C-6 Hydroxylation*

Incubation of estradiol 3-methylether with *Fusarium moniliforme* led to the major 6β-hydroxy derivative.[82]



(79)

It has been proven that *Aspergillus tamarii* was able to provide the same selectivity in similar yields.[83]



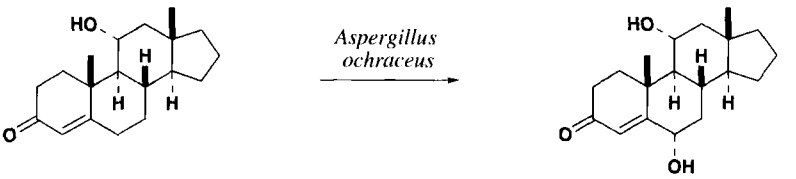
(80)

Microbiological hydroxylation of androstanes by the fungus *Calonectria decora* has been intensively studied by Meakins and co-workers. A 6α-hydroxylation was observed with dioxygenated androstanes.[75,84]

Table 2.

5α-Androstane	Product	Yield (%)
5α-androstan-11,17-dione	5α-androstan-6α-ol-11,17-dione	32
5α-androstan-17β-ol-2-one	5α-androstan-6α,17β-diol-2-one	52
5α-androst-2-en-11,17-dione	5α-androst-2-en-6α-ol-11,17-dione	54

With *Aspergillus ochraceus*, good selectivities have also been observed by the same author.[85]



76% yield (81)

The fungus *Cephalosporium aphidicola* has been shown to hydroxylate testosterone and some relatives at the C-6β position in contrast to the results observed with progesterone which gave majoritary in presence of this organism the 11α-hydroxylated compound.[86]

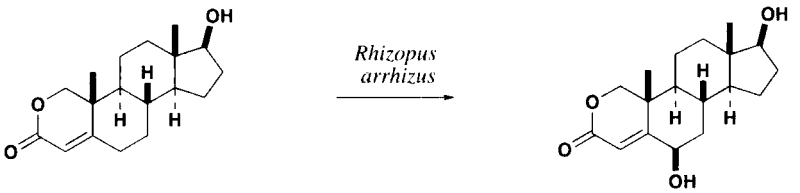
Table 3.

Steroid	Product	Yield (%)
Testosterone	6β-hydroxytestosterone	47
19-nortestosterone	6β-hydroxy-19-nortestosterone	47
1-dehydrotestosterone	6β-hydroxy-1-dehydrotestosterone	48
1α-methyltestosterone	6β-hydroxy-1α-methyltestosterone	51

In 1991, Hamada reported the same selectivity for the hydroxylation of testosterone by a green cell suspension culture of *Marchantia polymorpha* (6β-hydroxytestosterone, 18% yield).[87]

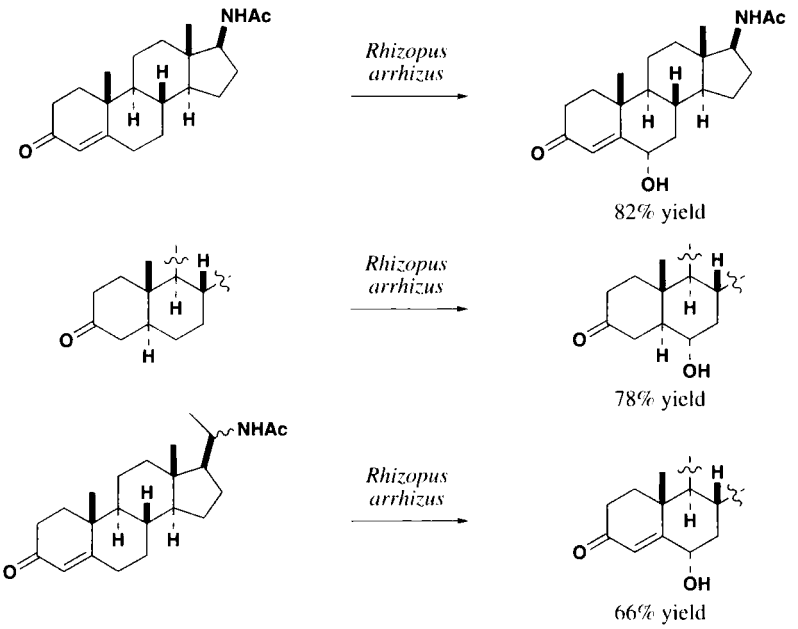
The potential pharmacological properties of hydroxylated derivatives of heterosteroids remains largely unexplored as microbial transformations of ring heteroatom-substituted steroids have not been systematically studied. In order to examine the influence of the presence of an oxygen heteroatom in the substrate on the outcome of the hydroxylation, Holland has prepared 2-oxatestosterone and then submitted it to biotransformation. The use of *Aspergillus ochraceus* led to selective

11α-hydroxylation (cf 11α-hydroxylation) whereas *Rhizopus arrhizus* produced only the 6β-hydroxy derivative.[88]



59% yield (82)

In microbial hydroxylations of other, non-steroid substrates, one of the more efficient substituent groups for the direction of microbial hydroxylation to specific sites within the substrate is known to be the amide functionality. In view of this observation, Holland studied the microbial hydroxylation of a range of aminosteroids. Using *Rhizopus arrhizus* allowed to obtain good yields in C-6-hydroxylated derivatives.[89]



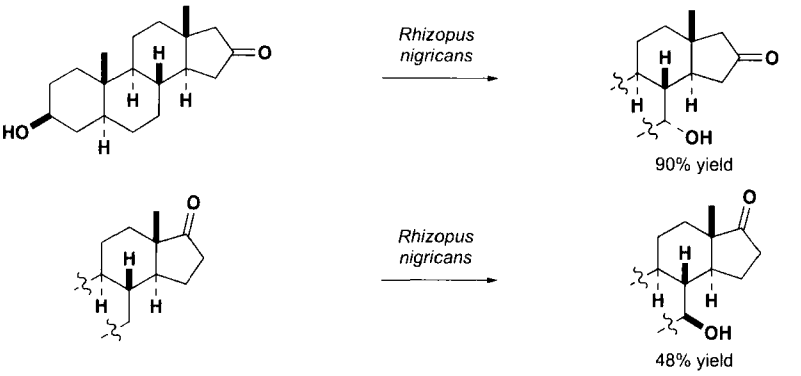
82% yield

78% yield (83)

66% yield

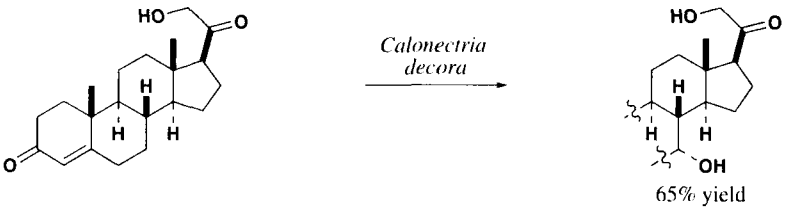
f. *C-7 Hydroxylation*

Rhizopus nigricans sometimes introduces an hydroxyl group in 7β-position of steroids according to the position of oxygen functions in the substrate.[80a] The previous yields could be increased when *Rhizopus arrhizus* was employed instead of *Rhizopus nigricans*.[80b]



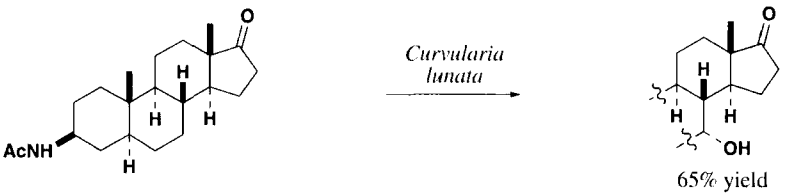
(84)

In order to study the possible anti-aldosterone activity of desoxycorticosterone, Kieslich has developed a synthesis involving a microbial 7α-hydroxylation with *Calonectria decora*.[90]



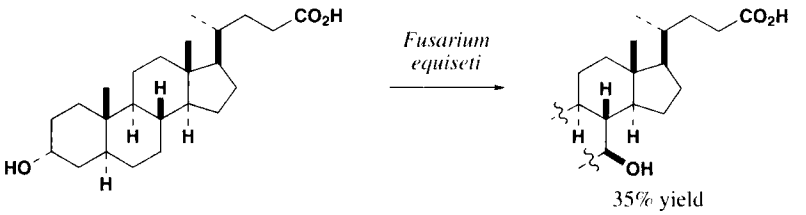
(85)

In 1981, Crabb reported that 3α-hydroxy-5α-androstan-17-one underwent predominant 7β-mono-oxygenation by treatment with the fungus *Cunninghamella elegans*.[91]



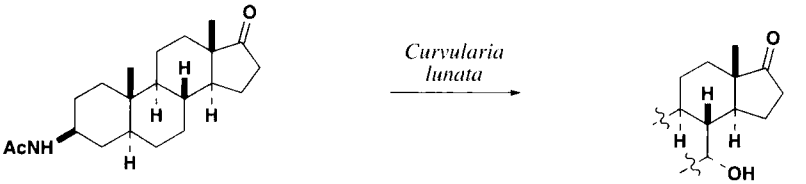
(86)

Ursodeoxycholic acid is chemotherapeutically effective as a cholagogue and is known to solubilize cholesterol in gallstones. The chemical synthesis is a seven-step procedure and offers fairly low yields. Nevertheless, *Fusarium equiseti*, a soil fungus, has been used by Sawada to prepare efficiently this acid in a one-step reaction.[92]



35% yield (87)

Because many aminosteroids exhibit a wide range of physiological activities, Holland studied their microbial transformations. The fungus *Curvularia lunata* is well known to generate 11β-hydroxylation of steroids. Unexpectedly, this author observed the formation of the 7α-hydroxy derivative as the sole product from 3β-acetylamino-5α-androstan-17-one.[89]



65% yield (88)

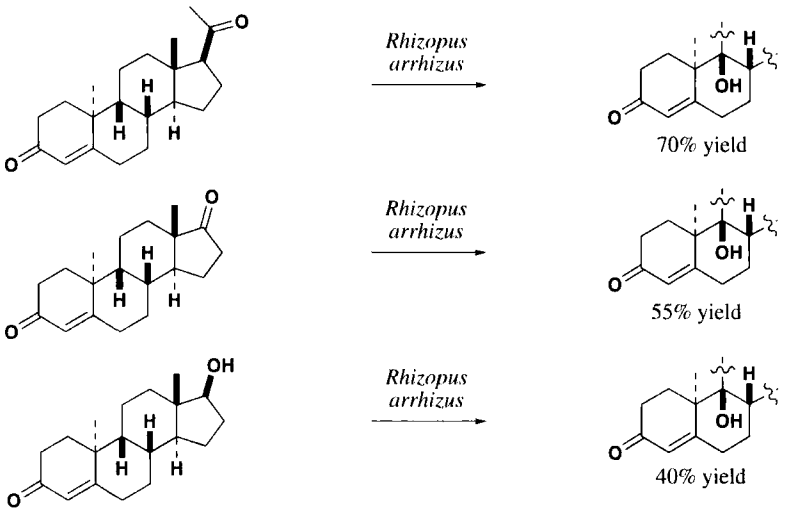
g. *C-8 Hydroxylation*

Nearly all positions of the steroid skeleton can be hydroxylated by different strains of fungi and bacteria. However, hydroxylation at C-8 from the β-face of the steroid is extremely hindered by the steric shielding effect of the β-oriented groups at C-10 and C-13. The exclusive formation of 8β,17α-dihydroxyprogesterone by bioconversion of 17α-hydroxyprogesterone with *Corynespora melonis* constitutes the sole example of C-8-hydroxylation of steroids.[93]

8% yield (89)

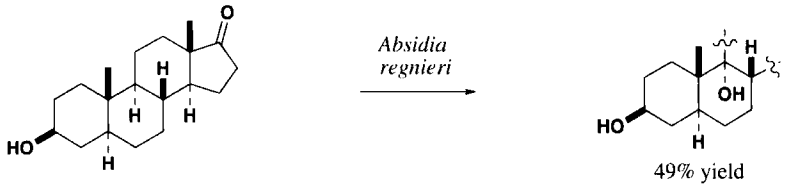
h. *C-9 Hydroxylation*

Rhizopus arrhizus is well known to hydroxylate normal (9α,10β) steroids in 11α or 6β position. In contrast, when a (9β,10α) steroid is involved, the hydroxylation takes place in the 9β-position as it is shown below for 9β,10α-progesterone, 9β,10α-androst-4-en-3,17-dione and 9β,10α-testosterone.[94]



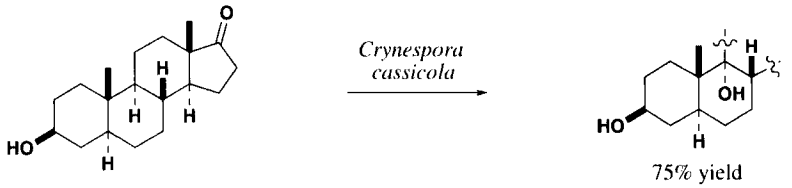
(90)

On the other hand, Meakins has reported a 9α-hydroxylation of 3β-hydroxy-5α-androstan-17-one by *Absidia regnieri* in the presence of cobalt (II) sulfate which is known to inhibit hydroxylation at tertiary positions.[95]



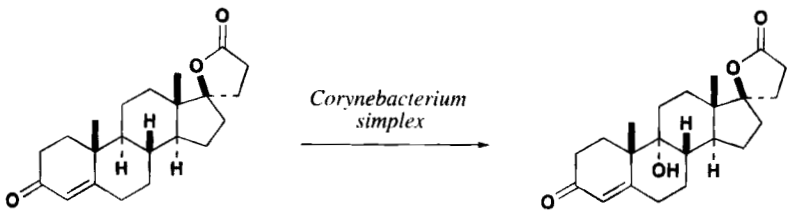
(91)

9α-Halocorticoids have a great pharmacological importance and are usually synthesized from $\Delta^{9,11}$ steroids. These latter arise generally from 9α-hydroxy derivatives. In the aim to reproduce the degradation of sitosterol, Kieslich has used the fungal strain *Crynespora cassicola*:[90]



(92)

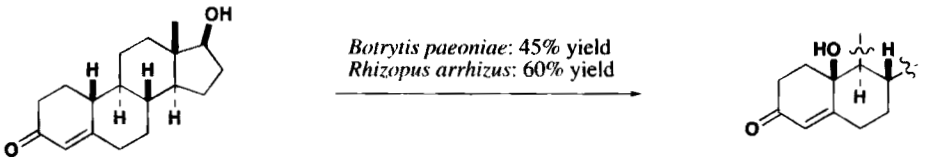
In order to prepare various 9α,11-epoxy-steroids which are potent aldosterone antagonists, Kalvoda has reported the 9α-hydroxylation of a carbolactone by *Corynebacterium simplex*.[96]



42% yield (93)

i. *C-10 Hydroxylation*

Generally, the 10β-position seems to be a favored place of attack with microbiological hydroxylations in the 19-nor-series.[97] For instance, fermentation of 19-nortestosterone with *Botrytis paeoniae* or *Rhizopus arrhizus* yielded the corresponding 10β-hydroxylated derivative.[98]



(94)

j. *C-11 Hydroxylation*

Peterson and Murray were the first to report the 11-hydroxylation of a steroid (progesterone) with *Rhizopus arrhizus*.[99] Further studies revealed that *Rhizopus nigricans* was more effective (85-95%).[100]

85-95% yield (95)

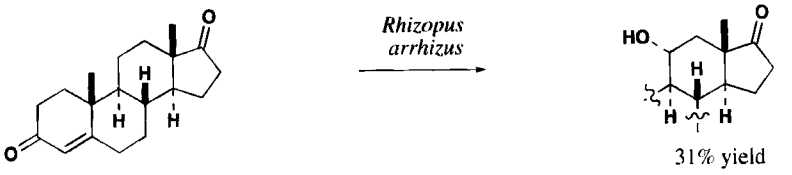
Since the conversion of progesterone to corticosteroids necessitated introduction of an oxygen function at the C-11 position, this reaction became the first commercialized microbial process in the steroid field. 11α- along with 16α-microbial hydroxylations are the most commercially important biohydroxylations since they lead to corticosteroids. These reactions are now exclusively achieved in the steroid industry by bioconversions.

To the best of our knowledge, C-11-hydroxylations of steroids have been carried out with six different fungi: *Rhizopus arrhizus*, *Rhizopus nigricans*, *Aspergillus ochraceus*, *Aspergillus*

tamarii, *Calonectria decora* and *Curvularia lunata*. The many results will be classified by range of micro-organisms.

Biotransformations by *Rhizopus arrhizus*:

In the continuation of their work, Peterson and Murray have submitted several androgens to the action of *Rhizopus arrhizus* such as 4-androstene-3,17-dione.[101]

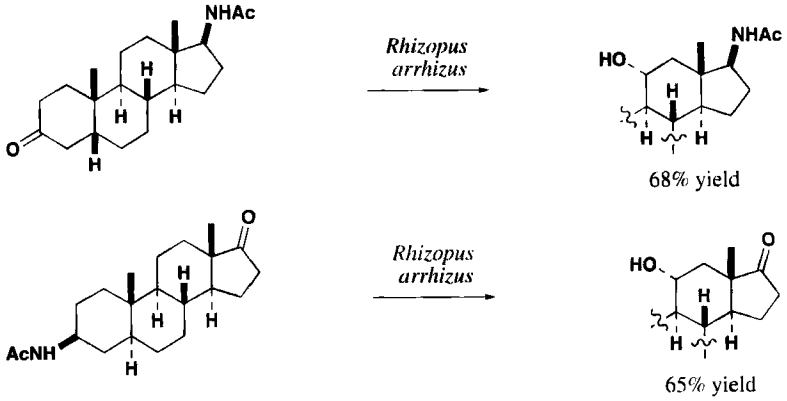


31% yield (96)

In 1973, Meakins and Jones studied the hydroxylation of several dioxygenated 5α-androstanes by *Rhizopus arrhizus*.[80b]

72% yield (97)

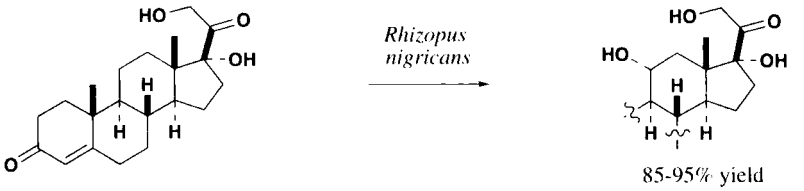
More recently, Holland has examined the influence of the presence of an amide functionality on the steroidal skeleton, on the hydroxylation process. Thus, these authors observed stereoselective C-11α-hydroxylation in the two following examples.[89]



(98)

Biotransformations by *Rhizopus nigricans*

In 1953, Peterson and Murray reported an efficient route to a precursor of cortisone involving a fermentation of dihydroxyprogesterone by *Rhizopus nigricans*.[102]



85-95% yield (99)

The sapogenin tigogenin has been chemically converted to the following epoxide in order to prepare 11α-hydroxy-5α-derivatives, which are potential cortical hormones. The corresponding 11α-hydroxy steroid by treatment with *Rhizopus nigricans*.[103]

60% yield (100)

In 1962, Djerassi described the efficient C-11α-hydroxylation of 19-norprogesterone with *Rhizopus nigricans*.[104]

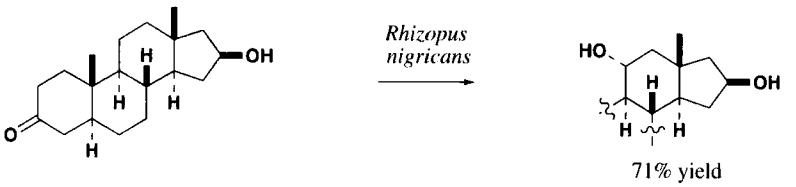
70% yield (101)

One year later, Murray reported another highly stereoselective C-11α-hydroxylation using the same fungus.[105]

82% yield (102)

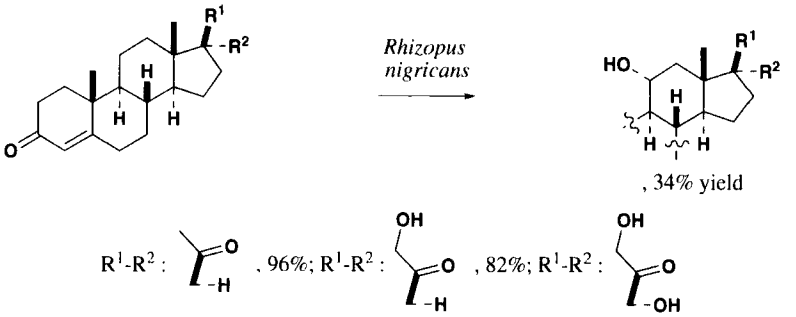
Meakins and Jones have shown that varying the positions and oxidation levels of the oxygen functions borne by 5α-androstanes led to hydroxylation at different positions. C-11α-hydroxylation was majoritary observed in the case of incubation of 16β-hydroxy-3-oxo-5α-androstane with

Rhizopus nigricans.[76,80a]



(103)

Belic revealed that the polarity of the side chain at C-17 of progesterone was an important factor determining the yield of C-11α-hydroxylation by *Rhizopus nigricans*.[106]



(104)

Biotransformations by *Aspergillus ochraceus*

Aspergillus ochraceus, fungal mycelium, is well-known as an 11α-hydroxylating agent with many substrates, it has been invariably effected 11α-hydroxylation. Different authors have prepared 11α-hydroxylated derivatives of 19-nortestosterone by fermentation with *Aspergillus ochraceus*.[74,98a]

(105)

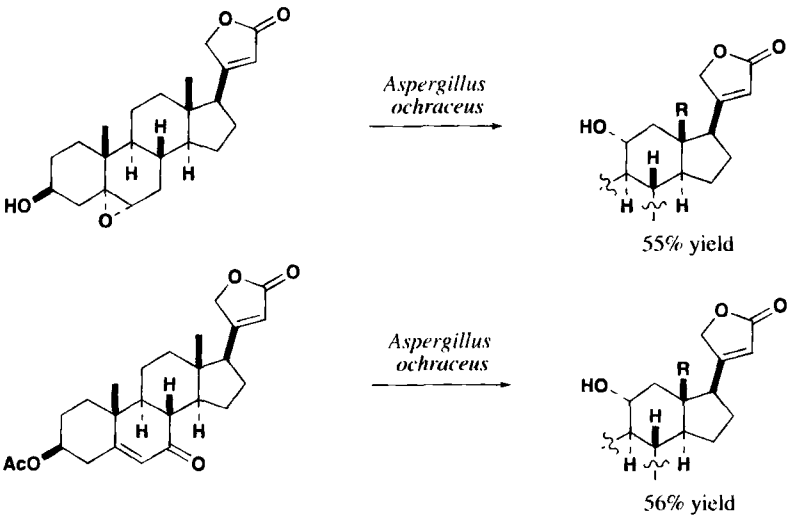
Various functionalized 5α-androstanes and 5α-estranes have been employed by Meakins and Jones in order to study the enzyme systems involved in the 11α-hydroxylations with *Aspergillus*

ochraceus. Even fluorinated compounds led cleanly to the expected 11α-hydroxylated derivatives as it can be observed in the following table.[85,107]

Table 4.

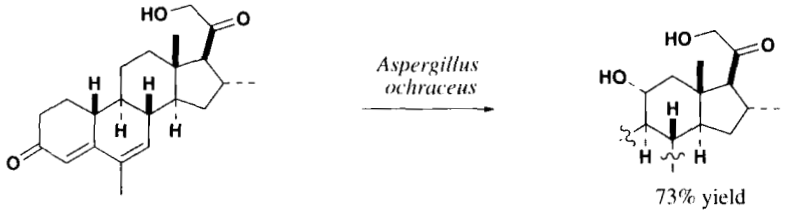
Steroid	Product	Yield (%)
androstan-2,16-dione	androstan-11α-ol-2,16-dione	61
androstan-2,17-dione	androstan-11α-ol-2,17-dione	57
androstan-3,6-dione	androstan-11α-ol-3,6-dione	60
androstan-6β-ol-3-one	androstan-6β,11α-diol-3-one	76
4-androsten-6β-ol-3-one	4-androsten-6β,11α-diol-3-one	61
androstan-3,7-dione	androstan-11α-ol-3,7-dione	74
androstan-3,16-dione	androstan-11α-ol-3,16-dione	55
androstan-3β-ol-16-one	androstan-3β,11α-diol-16-one	71
androstan-17β-ol-3-one	androstan-17β,11α-diol-3-one	79
4-androsten-17β-ol-3-one	4-androsten-17β,11α-diol-3-one	73
androstan-3α-ol-17-one	androstan-3α,11α-diol-17-one	81
5-androsten-3β-ol-17-one	5-androsten-3β,11α-diol-17-one	87
3α-fluoroandrostan-17-one	3α-fluoroandrostan-11α-ol-17-one	66
6β-fluoroandrostan-17-one	6β-fluoroandrostan-11α-ol-17-one	54

In 1976, Kreiser reported the 11α-hydroxylation of various cardenolides by treatment with *Aspergillus ochraceus*.[108]



(106)

An another example of 11α-hydroxylation was described by Krieslich with the pregnadien-3,20-dione depicted below.[109]



73% yield (107)

A mutant of *Aspergillus ochraceus* has been developed by Somal and Chopra, which converts progesterone to 11α-hydroxyprogesterone in high yields. Thus, this result proposes the optimization of parameters for the 11α-hydroxylation of *Aspergillus ochraceus*.[110] Very recently, Holland has carried out *Aspergillus ochraceus* fermentations of various acetylaminosteroids and obtained excellent yields in 11α-hydroxy-derivatives.[89]

R = Me, 85% yield
Δ^4, R = H, 75% yield
Δ^4, R = Me, 86% yield (108)

85% yield

Since the potential pharmacological properties of hydroxylated derivatives of heterosteroids remains largely unexplored, Holland has studied the 11α-hydroxylation of 2-oxatestosterone by *Aspergillus ochraceus*.[88]

82% yield (109)

Biotransformations by *Aspergillus tamarii*

Aspergillus tamarii has also been employed for the 11-hydroxylation of dioxygenated androstanes by Brannon. Nevertheless, the yields were generally lower than those observed with other micro-organisms.[83]

Biotransformations by *Calonectria decora*

In microbial conversion of steroids by *Calonectria decora*, the 11α-position is sometimes hydroxylated, especially when a hydroxyl group is present at the C-6 position.[84b,111]

(110)

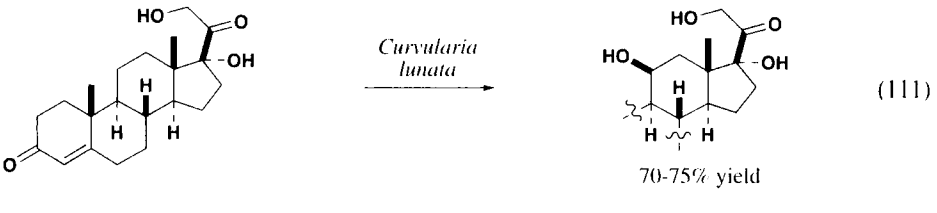
Biotransformations by *Curvularia lunata*

Unlike the microbial 11α-hydroxylation of progesterone, which can be performed in almost quantitative yields, the introduction of the hydroxyl group into position 11β was until 1992 a less directed process and was often accompanied by undesirable by-products formation. However, the 11β-hydroxylation of steroids by *Curvularia lunata* is one of the classic steroid biotransformations and exhibits a wide tolerance for variation in substrate structure.

Table 5.

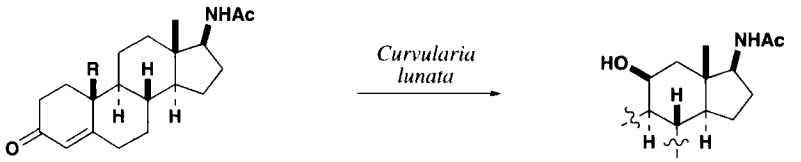
Steroid	Product	Yield (%)	Ref.
19-norprogesterone	11β-hydroxy-19-norprogesterone	21	104
4-pregnen-17β-ol-3,20-dione	11β-hydroxy derivative	32.5	112
21-acetoxy-5α,6-epoxypregnan-3β-ol-20-one	11β-hydroxy derivative	55	113
21-acetoxy-5α,6-epoxy-16α-methylpregnan-3β-ol-20-one	11β-hydroxy derivative	55	113
6,16α-dimethyl-4,6-pregnadien-21-ol-3,20-dione	11β-hydroxy derivative	16	109

In 1992, Russian workers studied the 11β-hydroxylation of 11-desoxycortisol by the mycelium of *Curvularia lunata* in the presence of β-cyclodextrin. The yield of expected hydrocortisone was 70-75%.[71e]



(111)

Recently, Holland reported the very efficient 11β-hydroxylation of acetylaminosteroids by this micro-organism.[89]

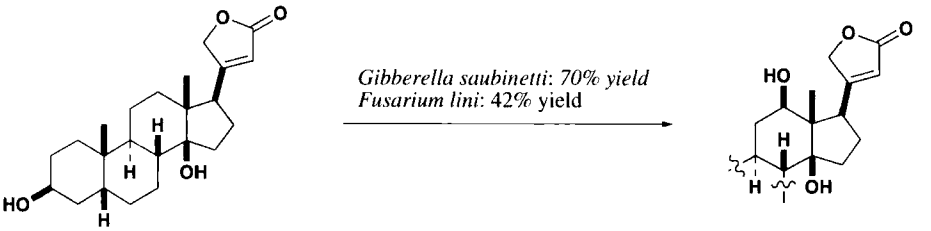


(112)

R = H, 78% yield
R = Me, 84% yield

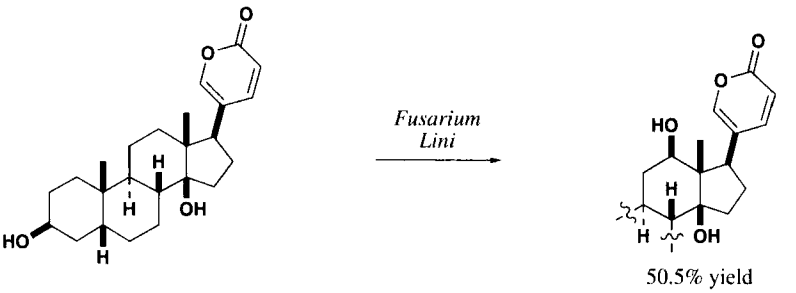
k. *C-12 Hydroxylation*

The 12β position of steroidal aglycones appears to be particularly susceptible to hydroxylation, since several authors have observed the microbial conversion of digitoxigenin to digoxigenin.[114] The cardiac aglycones are unique among naturally occurring steroids in possessing a 14β-hydroxyl and a *cis* C/D ring junction. The hydroxyl and the lactone ring can be seen in models to lie in a position to hinder access to the D-ring. It may be that steric hindrance accounts for the observations of Tamm and Gubler that *Fusarium lini*, which normally hydroxylates the 15α position, does not attack this position on the cardenolides, but reacts the 12β position.[115]



(113)

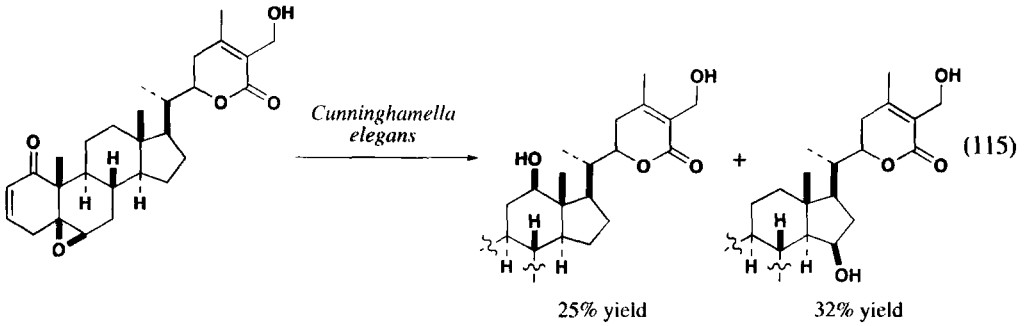
A similar result was observed with bufalin.[116]



(114)

50.5% yield

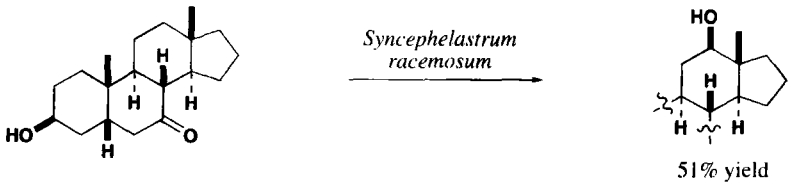
The withanolides are a group of growth inhibitory steroidal lactones of interest as potential antitumor agents. The antitumor lactone withaferin A has been converted to its 12β-hydroxy derivative by microbial transformation with *Cunninghamella elegans*.[117]



(115)

In 1980, Hayakawa reported the 12β-hydroxylation of lithocholic acid (40% yield) using the fungus *Helicostylum piriforme*.[118]

According to Meakins and Jones, in general the fungus *Syncephelastrum racemosum* gives complex mixtures of oxygenated androstanes. The one notable exception is the efficient 12α-hydroxylation of 3β-hydroxy-5α-androstan-7-one with *Syncephelastrum racemosum*.[95]



(116)

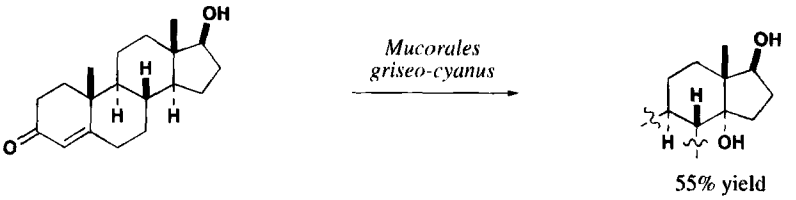
In order to prepare 12α-halo-hydrocortisone which exhibits greatly reduced systemic side-effects, Kieslich has synthesized 11-deoxy-12α-hydroxyhydrocortisone with the fungus *Cercospora kaki*.[90]

(117)

On the other hand, 17α-acetoxy-progesterone such as progesterone was selectively hydroxylated in position 12β whereas 17α-hydroxy-progesterone led to 15α-hydroxylated compound.[119]

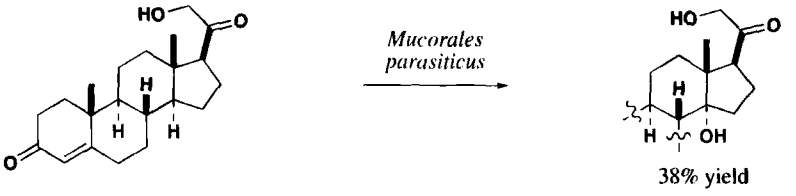
1. *C-14 Hydroxylation*

In 1958, Murray and Peterson reported a study concerning the 14α-hydroxylation of several steroids by fungi of the order *Mucorales*. The best result was obtained with testosterone and *Mucorales griseo-cyanus*.[120]



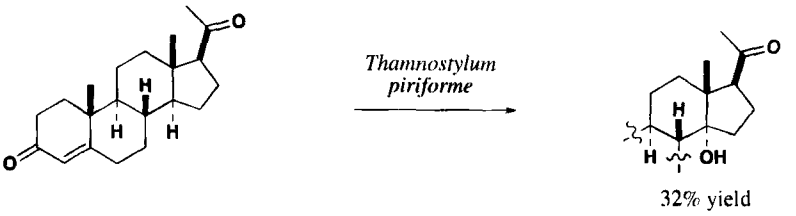
55% yield (118)

Mucor parasiticus has been used by Kieslich to hydroxylate desoxycorticosterone in position 14α.[90]



38% yield (119)

A screening of ten other micro-organisms adequate for the production of several 14α-hydroxylated steroids has been done by Azerad and Hu in 1995. *Thamnostylum piriforme* appeared to be the preferred micro-organism for the selective preparation in moderate yields of 14α-steroids.[121]



32% yield (120)

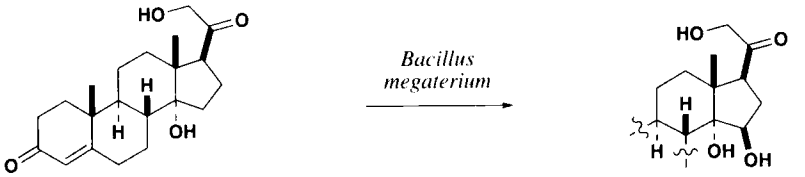
m. *C-15 Hydroxylation*

In 1972-1973, Meakins and Jones reported some hydroxylations of 5α-androstanes in position 15α in moderate yields by the use of *Calonectria decora*.[75,76]

36% yield (121)

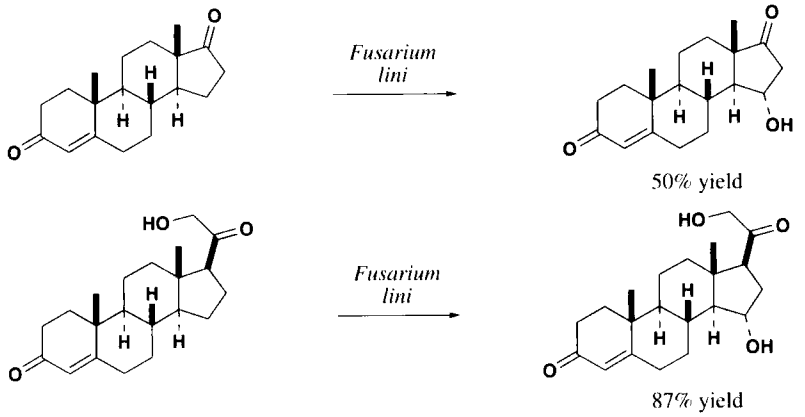
In 1980, Kieslich showed that it was possible to hydroxylate 14α-desoxycorticosterone either in 15α position or in 15β position according to the nature of the micro-organism. Indeed, the

use of *Bacillus megaterium* provided the 15β-derivative whereas the involvement of *Calonectria decora* led to the 15α-steroid in similar and moderate yields.[90]



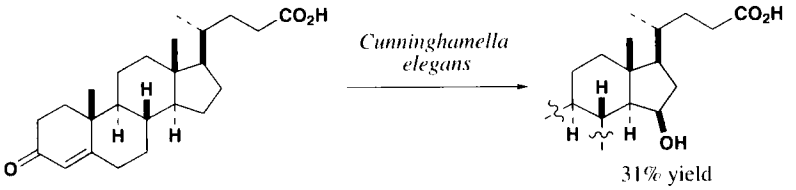
27% yield (122)

This result constitutes an example of a sequential introduction of two hydroxy groups in two stages in the preparation of 14,15-dihydroxydesoxycorticosterone compounds, since the 14α-desoxy-corticosterone is firstly prepared by treatment of desoxycorticosterone with *Mucor parasiticus* (cf. position 14). *Fusarium lini* has also been employed sometimes to generate 15α-hydroxy-steroids such as 15α-hydroxyprogesterone.[90,122]



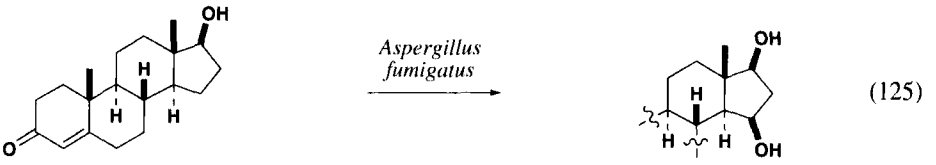
(123)

In the course of their studies on the biotransformation of bile acids by means of fungi, Nihira and co-workers have described the 15β-hydroxylation of lithocholic acid by *Cunninghamella elegans* which was the first bile acid derivative containing 15β-hydroxy moiety.[123]



(124)

Whereas treatment of progesterone with *Aspergillus fumigatus* led to various hydroxylated compounds, the incubation of testosterone with this micro-organism unexpectedly provided 15β-hydroxytestosterone as the only major biotransformed product.[124]



59% yield (125)

It may be argued that in the case of progesterone, the bigger substituent at C-17 produces conformational change in the molecule which is responsible for the generation of metabolites having functions at other sites.

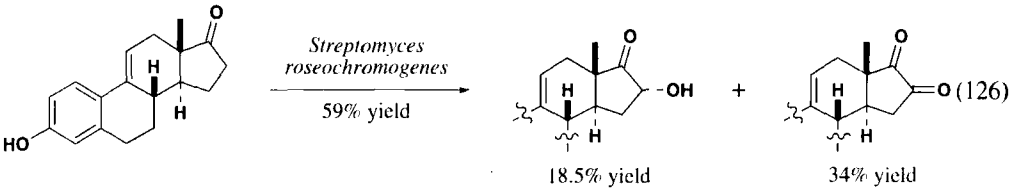
n. *C-16 Hydroxylation*

16-Hydroxylations of steroids along with hydroxylations at C-11 are the most commercially important microbial processes since they lead to adrenal cortex hormones and their analogues. Jones and Meakins have extensively studied the fermentation of various dioxygenated androstanes by *Rhizopus nigricans.*[76,80a]

Table 6.

Steroid	Product	Yield (%)
androstan-3β-ol-6-one	androstan-3β,16α-diol-6-one	43
androstan-7α-ol-3-one	androstan-7α,16β-diol-3-one	33
androstan-3β,6β-diol	androstan-3β,6 β,16β-triol	38
4-androsten-6β-ol-3-one	4-androsten-6β,16β-diol-3-one	61
androstan-7β-ol-3-one	androstan-7β,16β-diol-3-one	52
androstan-11α-ol-3-one	androstan-11α,16β-diol-3-one	45
androstan-6β-ol-3-one	androstan-6β,16β-diol-3-one	39
androstan-3β-ol-6-one	androstan-3β,16β-diol-6-one	43

Estratrienes and estratetraenes have been microbiologically oxidized in position 16 by *Streptomyces roseochromogenes.* The best yield is obtained in the case of 3-hydroxyestra-1,3,5(10),9(11)-tetraen-17-one.[125]



18.5% yield 34% yield

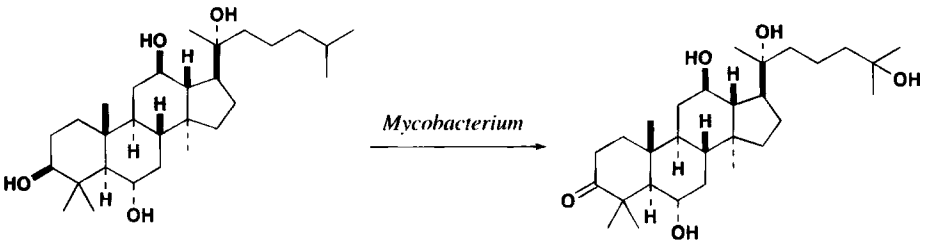
o. *C-19 Hydroxylation*

To the best of our knowledge, the sole example of hydroxylation at C-19 presenting an acceptable yield has been described by Meakins and Jones. It seems that the presence on the substrate of three oxygenated functions in positions C-1, C-6 and C-17 involve the formation of the C-19 hydroxylated compound as the main product of the incubation with *Calonectria decora*.[84]

56% yield (127)

p. *C-25 Hydroxylation*

The saponins protopanatriol derivatives possess the dammarane skeleton which is similar to lanostane except for the replacement of Me-13 by Me-8. Lee investigated whether transformation of these compounds by *Mycobacterium sp.* would follow a similar oxidative degradation pathway and afford C-19 steroids. Unexpectedly, transformation of the mixture of the two epimers of (20*R*)-dihydroprotopanaxatriol by *Mycobacterium sp.* produced the corresponding 3-oxo-compound along with the 3-oxo-25-hydroxylated derivative. Thus, formation of the 25-hydroxylated product showed an inconsistency with the expected degradation pathway.[126]



21% yield (128)

3. Dihydroxylations

Dihydroxylations of steroids can also be performed with fairly non-polar substrates in one fermentation step.

a. *C-1,C-6 Dihydroxylation*

In their course to know the factors which determine the specific positions at which steroids are hydroxylated by *Calonectria decora*, Meakins and Jones have shown that the presence of a 17-

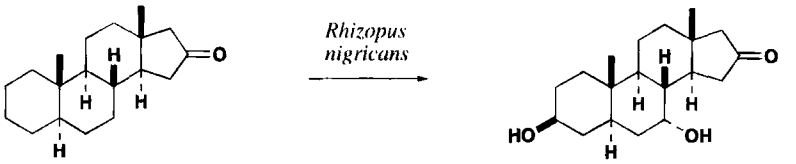
keto or -hydroxy group led to substitution at positions 1 and 6. Their significant results emphasized the fact that mono-oxygenated substrates are dihydroxylated. The two hydroxyl groups are introduced on carbon atoms ca 4Å apart from the directing group.[75,76,84,107b,c,127]

Table 7.

Steroid	Product	Yield (%)
androstan-17-one	androstan-1β,6α-diol-17-one	47
2-androsten-17-one	2-androsten-1β,6α-diol-17-one	52
3α-methoxyandrostan-17-one	3α-methoxyandrostan-1β,6α-diol-17-one	60
3-methyleneandrostan-17α-ol	3-methyleneandrostan-1β,6α,17α-triol	82
3-fluoroandrostan-17-one	3-fluoroandrostan-1β,6α-diol-17-one	41
3-chloroandrostan-17-one	3-chloroandrostan-1β,6α-diol-17-one	70
3-bromoandrostan-17-one	3-bromoandrostan-1β,6α-diol-17-one	65

b. *C-3,C-7 Dihydroxylation*

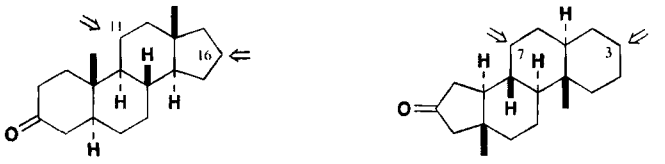
Rhizopus nigricans is well known to dihydroxylate 5α-androstanes exhibiting one oxygenated function.[80a]



(129)

50% yield

The position of the carbonyl group influences the direction of the hydroxylation process. 16- and 17-ketones lead to 3,7-dihydroxylation whereas 11,16-dihydroxylation is observed in the case of 3-ketones. The comparison of these two processes suggests that the reversal effect is operating.



(130)

Reversal effect

c. *C-6,C-11 Dihydroxylation*

Aspergillus ochraceus is well known as an efficient 11α-hydroxylator of steroids, and the 6β-position appears to be an additional, or occasionally an alternative, site for hydroxylation. Several steroids have been efficiently converted by Meakins and Jones to their 6β,11α-dihydroxylated derivatives using this micro-organism.[107b,85]

Table 8.

Steroid	Product	Yield (%)
4-androsten-3-one	4-androsten-6β,11α-diol-3-one	75
19-nor-4-androsten-3-one	19-nor-4-androsten-6β,11α-diol-3-one	53
androstan-3-one	androstan-6β,11α-diol-3-one	84

Calonectria decora was also able to dihydroxylate 3α-fluoro-5α-androstan-16-one in both positions 6α and 11α.

56% yield

(131)

D-homoprogesterone could be converted to 6β,11α-dihydroxylated derivative by microbial treatment with *Rhizopus arrhizus*.[128]

51.5% yield

(132)

More recently, the fungus, *Cephalosporium aphidicola*, has been shown to hydroxylate progesterone predominantly at the 6β- and 11α-positions. The sequence involves hydroxylation at 11α and then 6β-positions.[129]

50% yield

(133)

d. *C-7,C-11 Dihydroxylation*

In 1964, Hayakawa reported the hydroxylation of the steroidal alkaloid tomatidine by the fungus *Helicostylum piriforme* yielding 7α,11α-dihydroxytomatidine.[130]

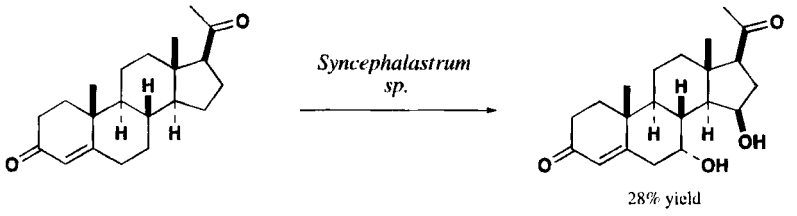
(134)

On the other hand, hydroxylation by *Aspergillus ochraceus* of 3-fluoro-17-keto-5α-androstanes has led with both 3α-fluoro and 3β-fluoro substrates to the 7β,11α-dihydroxylated steroids.[107b]

(135)

e. *C-7,C-15 Dihydroxylation*

In order to prepare adrenocortical hormones, Tsuda and co-workers have studied the microbiological hydroxylation of progesterone. Thus, they observed the formation in a moderate yield of 7α,15β-dihydroxyprogesterone by using *Syncephalastrum sp.*[131]

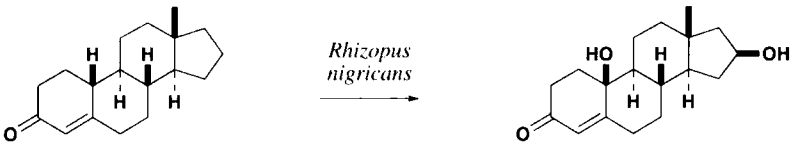


(136)

f. *C-10,C-16 Dihydroxylation*

An interesting feature of fungus *Rhizopus nigricans* is its propensity for attack at the 16-position. While the behavior of 5α-estran-3-one is similar to that of 5α-androstan-3-one (dihydroxylations

in positions 11 and 16 were observed in this case), the hydroxylation of 19-nor-4-estren-3-one leads to a 10β,16β-dihydroxy-product. It is reasonable to suppose that the site responsible for 11-hydroxylation of 10-methyl-substrates is suitably disposed for 10-hydroxylation of 19-norestrane derivatives.[132]



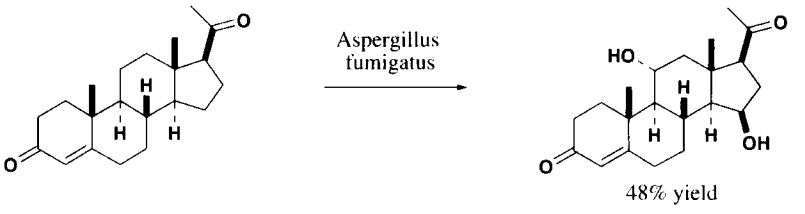
(137)

g. *C-11,C-15 Dihydroxylation*

D-Homoprogesterone has been submitted to various fungi by Wiechert and co-workers. They observed an interesting dihydroxylation in positions 11α and 15α in the case of using *Glomerella cingulata*.[128]

(138)

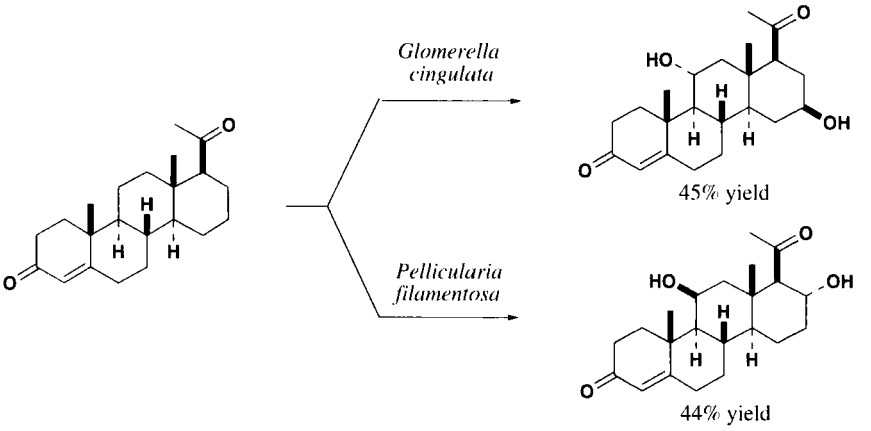
Aspergillus fumigatus has led after three hours of incubation with progesterone to 11α- and 15β-hydroxyprogesterones as major products along with trace amounts of various minor products, one of which was 11α,15β-dihydroxy-progesterone. Smith showed that after 72 hours of incubation, the dihydroxyprogesterones were the sole metabolites. Thus, 11α,15β-dihydroxyprogesterone could be obtained in 48% yield.[133]



(139)

h. *C-11,C-16 and C-11,C-17 Dihydroxylations*

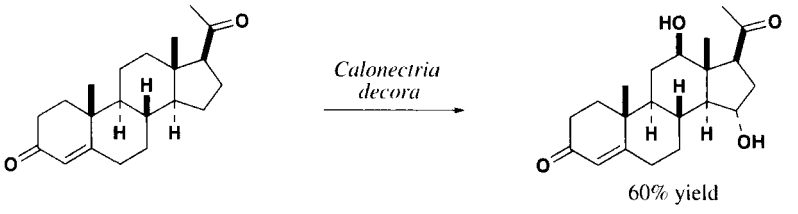
D-Homoprogesterone may be transformed into 11α,16β-dihydroxy- and 11β,17α-dihydroxy derivatives by treatment with *Glomerella cingulata* and *Pellicularia filamentosa*, respectively.[90]



(140)

i. *C-12,C-15 Dihydroxylation*

In 1958, Schubert reported the efficient dihydroxylation of progesterone by *Calonectria decora* in positions 12β and 15α.[90,134]



(141)

In the literature on steroid hydroxylation, *Calonectria decora* is indeed recorded as a 12β,15α-dihydroxylator. Meakins and Jones' results have confirmed this, particularly for 3-oxygenated substrates, but that by varying the location of a single oxygen group in the steroid, hydroxylation can be effected in other positions. Dihydroxylation is only observed with relatively lipophilic substrates.[76,127]

Table 9.

Steroid	Product	Yield (%)
androstan-3-one	androstan-12β,15α-diol-3-one	52
4-androsten-3-one	4-androsten-12β,15α-diol-3-one	55
androstan-4-one	androstan-12β,15α-diol-4-one	34

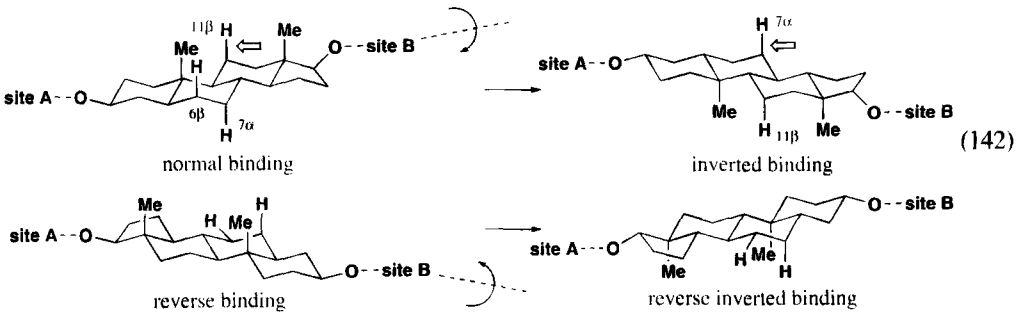
III. CONCLUSION

To explain the observation that hydroxylation by one micro-organism can occur in two or three positions, Brannon and co-workers proposed the following explanation: "in view of the stereose-

lectivity of microbial transformations it is probable that the steroid substrate is at some time bound to the surface of enzyme causing the transformation, and that the points of binding having a fixed spatial relationship to the site of hydroxylase activity on the enzyme. In most steroidal substrates, the most likely sites for binding the steroid to the enzyme surface are the oxygen functions at C-3 and C-17 (or C-20)".[83] The steroid substrate may bind to the surface of the enzyme according to one of the four (or more) possible orientations, in each orientation a different hydrogen is nearest to the site of hydroxylase activity.[135] This theory is confirmed by the results observed in the case of dihydroxylation. The main results are summarized below.

Table 10.

site of hydroxylation in the normal binding mode	Corresponding sites of hydroxylation in the other binding modes
1β	6α in inverted binding
3β	7α in reverse binding
11α	6β in inverted binding
11α	7α in inverted binding
11α	7β in inverted binding
11α	15α and 15β in inverted binding
12β	15α in inverted binding
15β	7α in reverse inverted binding
16β	10β in reverse binding



(142)

It is apparent that more than forty years after the discovery and development of the process, significant progress in the area of microbial steroid hydroxylations continues to be made. Although new biocatalysts and hydroxylation products do not appear as frequently as before, the examination of new substrate groups continues to reinforce the value of microbial hydroxylation for the preparation of novel products, and continued research, particularly in such areas as methodological development and the use of artificial enzymes, holds the promise of exciting new applications for the future. Considerable progress has been made in understanding steroid hydroxylation reactions. However, the exact mechanism of the hydroxylation step is not yet fully understood.

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