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ENZYMATIC OXYGENATIVE PRODUCTION OF 11- OR 19-POSITION HYDROXYLATED STEROIDS

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The present invention relates to a process for the oxygenation of steroids at the 11- or 19-position. The 11-position-oxygenated steroids include a number of important compounds, such as 17-hydroxycorticosterone (4-pregnene-11β,17α,21-triol-3,20-dione) and 17-hydroxy-11-dehydrocorticosterone (4-pregnene-17α,21-diol-3,11,20-trione), which are useful medicinally. Other 11-position-oxygenated steroids, although not employed as medicines as they are, are useful intermediates in the synthesis of such useful compounds as mentioned precedently. And, 19-position-oxygenated steroids such as 17,19-dihydroxydesoxycorticosterone (4-pregnene-17α,19,21-triol-3,20-dione), 19-hydroxydesoxycorticosterone (4-pregnene-19,21-diol-3,20-dione), 4-androsten-19-ol-3,17-dione and 19-hydroxyprogesterone (4-pregnen-19-ol-3,20-dione) can be converted into the corresponding 19-norsteroids through several steps of reactions. These 19-norsteroids are useful intermediates for preparing 19-norsteroids such as 19-norprogesterone (corpus luteum hormone), 19-nor-4-androstene-3,17-dione (an intermediate for known medicines, and the course leading to such medicines will be stated later).

Accordingly, much work has hitherto been done in connection with the oxygenation of steroids at the 11- or 19-position. Such work has included biochemical oxygenation with the aid of enzymes produced by microorganisms. As to the oxygenation of steroids at the 11-position, most work of this category has involved the use of fungi belonging to the order Mucorales (class Phycomyces), order Moniliales (class *Fungi imperfecti*) or order Sphaeropsidales (class *Fungi imperfecti*). On the other hand, the oxygenation of steroids at the 19-position has never been accomplished with the aid of enzymes of microorganisms.

The present invention is based on the discovery that oxygen can be introduced into the 11- or 19-position of the steroids by utilizing the enzyme produced by fungi belonging to genus Corticium (order Agaricales, class Basidiomycetes) which does not come under any order hereinbefore mentioned as the source of the enzyme capable of oxygenating the 11-position of the steroids, and that the yield is very good. It is the present inventors that have succeeded for the first time in oxygenating steroids at the 19-position with the aid of the enzymes of the microorganisms.

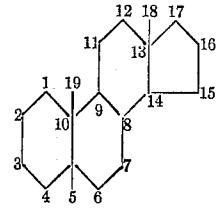
Corticium, in the present specification, is the name of a genus belonging to the family Thelephoraceae, order Agaricales and class Basidiomycetes, the classification being in accordance with that in "A Dictionary of the Fungi" by G. C. Ainsworth and G. R. Bisby, published by the Commonwealth Mycological Institute, Kew, Surrey, England, in 1954.

Among the species which can be used in the method of the present invention are involved *Corticium sasakii* (Shirai) Matsumoto, *Corticium microsclerotia* (Matz) Weber, and *Corticium praticola* Kotila, for example. The classification of fungi is, however, very complicated and is sometimes entangled. For instance, the above-mentioned *Corticium sasakii* (Shirai) Matsumoto is often named *Hypochnus sasakii* Shirai, *Corticium vagum* Br. et Cav., *Rhizoctonia solani* Kuhn, or *Pellicularia filamentosa* (Pat.) Rogers. According to D. P. Rogers (Farlowia vol. 1 (1943), pp. 95–118) and B. Exner (Mycologia vol. 45 (1953), pp. 698–719) the above exemplified three species (*Corticium sasakii, Corticium microsclerotia* and *Corticium praticola*) are involved in the genus Pellicularia. The generic name Pellicularia, however, has been rarely used by European taxonomists as seen from the list of the genera in the above-cited "A Dictionary of the Fungi A1954)." The list indicates that "Pellicularia" is one of "synonym or uncertain names" on page 412 of the dictionary. In this specification, therefore, the genus Corticium usable in the present invention includes the genus Pellicularia segregated from the genus Corticium in the Rogers' report supra. At any rate, the fungi having the ability of introducing oxygen into the 11-position or the 19-position of steroids and belonging to the genus Corticium according to the classification by G. C. Ainsworth and G. R. Bisby are all involved in what is called genus Corticium in this specification, even if they may come under other generic names in other nomenclatures.

Strains belonging to Corticium, which are employed in the process of this invention, are all available in known culture collections, such as Centraalbureau voor Schimmelcultures, Holland, or Institute for Fermentation, Osaka, Japan, or Northern Regional Research Laboratories, Peoria, Illinois, or American Type Culture Collection, Washington, D.C. Or, their wild cultures can easily be obtained from cultivated plant diseases such as banded sclerotial disease and web blight on rice, cane, beans, figs, etc.

According to the process of this invention, oxygen can be introduced into the 11-position or 19-position of steroids by the action of the enzyme contained in a fungus belonging to genus Corticium. The oxygenation may be effected by bringing the steroids in contact with a strain of a fungus belonging to the genus Corticium or with the enzyme produced by such a microorganism.

The steroids to be oxygenated in this invention are, for example, progesterone (4-pregnene-3,20-dione), 17α,21-dihydroxyprogesterone (4-pregnene-17α,21-diol-3,20-dione), 21-hydroxyprogesterone (4-pregnen-21-ol-3,20-dione), 1-dehydroprogesterone (1,4-pregnadiene-3,20-dione), 4-androstene-3,17-dione, or other cyclopentanopolyhydrophenanthrene derivatives having one or more of such substituents as oxo, hydroxyl, halogen and alkyl radicals at one or more positions of the sixteen positions of the dimethylcyclopentanopolyhydrophenanthrene nucleus except its 11- or 19-position as well as 10- and 13-positions. Moreover one or more double bonds in the nucleus or in the side chain may not obstruct the method of this invention. The position numbers of the dimethylcyclopentanopolyhydrophenanthrene nucleus are:



The enzyme to be employed in this invention may be a crude enzyme, but if desired, it can be used in comparatively pure state. The purification of the enzyme is conducted by any method conventional in the enzymological field.

The enzyme generally exists in the mycelium of the

fungi to be used. Therefore, the following two processes are most conveniently employed for the purpose of this invention: (1) a strain of the fungi is incubated in a medium containing suitable nutrients as well as a steroid to be oxygenated, or (2) a strain of the fungi is incubated in a nutrient medium, and the resulting mycelium is separated from the broth and then brought in contact with a steroid to be oxygenated under proper conditions.

In the above two processes the incubation of the microorganism is generally conducted in an aqueous nutrient medium as in the incubation of ordinary microorganisms. And, as carbon source there are used starch, cane sugar, lactose, dextrin, glycerol and maltose, and as nitrogen source various organic or inorganic nitrogen-containing substances such as soybean meal, meat extract, peptone, casein, yeast, cornsteep liquor, nitrates, urea and ammonium salts, for example. A small quantity in inorganic salts or trace elements may be added, if necessary.

When the oxygenation is effected by the process (1) described above, it is desirable to conduct a pre-incubation of the strain for a suitable period and then to continue the incubation in the presence of the steroid to be oxygenated. The time for the pre-incubation varies with conditions such as the kinds of the strain and the medium, though in general two to three days are most profitable. Suitable temperature and pH for the pre-incubation are, in most cases, about 20 to 30° C. and 4 to 8, respectively. These, however, may be varied with conditions. In any event, it is most important to conduct the pre-incubation so as to bring the enzyme to its most active state.

The broth of the pre-incubation is then mixed with a suspension or a solution of the steroid to be oxygenated and the incubation is continued for one to three more days, for example. In most cases, the solvent of the suspension or the solution is an aqueous solvent, but sometimes an organic solvent such as acetone, methanol, ethanol, ethylene glycol or propylene glycol may be used. A dispersion supplement such as a surface active agent may be added together with the steroid to make the oxygenation proceed smoothly, if desired.

When the oxygenation is effected by the process (2) supra, the mycelium in which the enzyme has been sufficiently accumulated is separated from the medium, and then added to a suitable solvent such as Ringer's solution, together with the steroid to be oxygenated. In this case, too, the steroid may be added in the form of a suspension or a solution in a proper solvent such as acetone, methanol, ethanol, ethylene glycol, propylene glycol.

In this way the steroid is oxygenated at the 11-position or the 19-position and which of the two positions is oxygenated depends upon the kind of microorganisms or the substrate used or upon other conditions. Or, the product oxygenated at the 11-position and that oxygenated at the 19-position can simultaneously be obtained under certain conditions. In the case of the oxygenation at the 11-position, the steric configuration of the introduced hydroxyl group is, in most cases, β-configuration, but in some cases it takes α-configuration, in other cases a mixture of two products having 11β- and 11α-configurations respectively is simultaneously formed. For example, when 17-hydroxy-desoxycorticosterone (4-pregnene-17α,21-diol-3,20-dione) is oxygenated with the aid of enzyme of *Corticium vagum*, 11β-hydroxyl compound, 11α-hydroxyl compound and 19-hydroxyl compound are simultaneously obtained. And, it is found that 19-hydroxylation dominates when *Corticium microsclerotia* is employed as the source of the enzyme. It is therefore preferable that the oxygenation is effected with the aid of the enzyme of *Corticium microsclerotia* when 19-hydroxylation is chiefly desired. But even when the enzyme of *Corticium microsclerotia* is employed, 11-hydroxylation may be the main reaction according to the kind of the substrate or other conditions. For example, hydrocortisone (4-pregnene-11β,17α-21-triol-3,20-dione) is the main product when the enzyme of *Corticium microsclerotia* is acted on 17α - hydroxydesoxycorticosterone (4 - pregnene - 17α,21-diol-3,20-dione).

The product is isolated from the reaction mixture by utilizing differences between the product and impurities in such properties as solubility, adsorbability, distribution coefficient between two solvents, etc. These processes can also be applied for the separation of two or more oxygenated steroids in the reaction mixture.

The most convenient process for isolation of one or more of the products is to extract the reaction mixture with a solvent which can readily dissolve the product or products. Chlorinated hydrocarbons such as chloroform, methylene chloride, and acetic acid esters such as ethyl acetate, butyl acetate can be used for the purpose, for instance.

In general, the oxygenated steroids thus obtained are not pure, and sometimes a mixture of two or more products. They may be purified by conventional methods such as recrystallization, chromatography and countercurrent distribution. As the solvent for recrystallization there are used alcohols, acetone, water, chlorinated hydrocarbons, and their mixtures, and as the support for chromatography there may be employed alumina, magnesium silicate and activated carbon. Other general methods for purifying steroids are all applicable for the purification of the products of the present invention.

Utility of the products of this invention was outlined in the opening of this specification. And the utility of 19-hydroxylated steroids is not clear in the literature, and the utility is stated below. 19-hydroxylated steroids can be converted into 19-norsteroids through 10-carboxy-19-norsteroids. More concretely, oxidation with e.g. chromium trioxide of 19-hydroxysteroids gives 10-carboxy-19-norsteroids, which, when heated together with a mineral acid or alkali hydroxide, give 19-norsteroids. In general, 19-norsteroids show medicinal effect superior to that of the corresponding steroids, which is so far clarified in certain reports. Δ^4-3-oxo-19-hydroxysteroids, which are included in the products of this invention, can be converted into $\Delta^{1,4}$-3-oxo-19-hydroxysteroids through dehydrogenation with e.g. selenium dioxide, and then further into $\Delta^{1,3,5(10)}$-3-hydroxy-19-norsteroids by the action of e.g. mineral acid. $\Delta^{1,3,5(10)}$-3-hydroxy-19-norsteroids thus produced are useful female sex hormones, e.g. estratriene.

The following examples set forth presently preferred illustrative embodiments of the present invention. In these examples, temperatures are uncorrected, analytical values are given in percent, IR-spectrum and UV-spectrum mean infrared absorption spectrum and ultraviolet absorption spectrum, respectively, and the relationship between part by weight and part by volume is the same as that between gram and millilitre. And, the names of culture collections abbreviated as IFO, NRRL and ATCC mean Institute for Fermentation, Osaka, Northern Regional Research Laboratories, Peoria, Illinois and American Type Culture Collection, Washington, D. C., respectively.

Example 1

To a mixture of 30 parts by weight of cane sugar, 0.5 part by weight of potassium chloride, 0.5 part by weight of potassium dihydrogen phosphate, 40 parts by volume of malt extract and 250 parts by volume of 20% potato juice, tap water is added until 1000 parts by volume of a medium is obtained. This medium is inoculated with a strain of *Corticium vagum*, and the strain incubated aerobically for 70 to 80 hours with shaking. Then a solution of 0.2 part by weight of 17-hydroxydesoxycorticosterone (4-pregnene-17α,21-diol-3,20-dione) in 20 parts by volume of methanol is added and the incubation is continued for additional 48 hours under the same conditions. The broth separated from the mycelium by filtration is extracted with three 300 parts by volume portions of methylene chloride. The mycelium is extracted with

hot acetone three times, and then the acetone is distilled off. The residue is dissolved in methylene chloride and the solution is combined with the aforementioned methylene chloride extract. After washing with water, the combined solution is concentrated under reduced pressure in an atmosphere of nitrogen to leave 0.25 part by weight of a yellow residue. The residue is purified by adsorption chromatography on a column of magnesium silicate, and the product is recrystallized from acetone to give 0.075 part by weight of 4-pregnene-11β,17α,21-triol-3,20-dione, M.P. 205–207° C.

The *Corticium vagum* used in this example and in Example 3 was received from the National Institute of Agricultural Science, Tokyo, Japan under the name of *Corticium vagum* Br. et Cav., but the strain resembles Corticium sasakii (Shirai) Matsumoto in microbiological properties. The strain was deposited at IFO under the accession number IFO–6192.

Example 2

In the same medium as in Example 1, a strain of *Corticium sasakii* is aerobically incubated for 70 to 80 hours with shaking. The mycelium separated from the culture broth is washed with two 500 parts by volume portions of Ringer's solution, and then immediately suspended in 1000 parts by volume of Ringer's solution, a solution of 0.2 part by weight of 17-hydroxydesoxycorticosterone (4-pregnene-17α,21-diol-3,20-dione) in 20 parts by volume of ethanol being added simultaneously. The mixture is then kept at 28° C. for 16 hours with agitation. Both the mycelium and the Ringer's solution are extracted three times with 500 parts by volume each of ethyl acetate. The ethyl acetate layer is washed once with one-fifth its volume of 1% aqueous sodium bicarbonate solution and twice with water, successively. The ethyl acetate layer is concentrated under reduced pressure in an atmosphere of nitrogen to leave 0.096 part by weight of a crude product. The crude product is recrystallized from chloroform to give 0.054 part by weight of hydrocortisone (4-pregnene-11β,17α,21-triol-3,20-dione).

The *Corticium sasakii* used in this example and Examples 4 and 5 was deposited at IFO, ATCC and NRRL under the accession numbers IFO–5254, ATCC–13269 and NRRL–2705, respectively.

Example 3

To a mixture of 600 parts by weight of cane sugar, 10 parts by weight of potassium chloride, 10 parts by weight of magnesium sulfate, 20 parts by weight of potassium dihydrogen phosphate and 5000 parts by volume of malt extract, tap water is added until 20,000 parts by volume of a medium is obtained. The medium is then inoculated with a strain of *Corticium vagum*, and the strain incubated aerobically for 70 hours in a tank. A solution of 15 parts by weight of 17-hydroxydesoxycorticosterone (4-pregnene-17α,21-diol-3,20-dione) in 700 parts by volume of ethanol is then added, and the incubation is continued aerobically for further 24 hours. The broth containing mycelium is extracted with 30,000 parts by volume of ethyl acetate, and the ethyl acetate solution is concentrated to 500 parts by volume. After washing with 1% aqueous sodium bicarbonate solution, the ethyl acetate solution is further concentrated under reduced pressure to leave a yellow crystalline residue (16 parts by weight). The residue is washed with petroleum ether to give 10.5 parts by weight of a crude product. This crude product is a mixture of oxygenated steroids containing a small quantity of unchanged 17-hydroxydesoxycorticosterone.

The mixture is washed with chloroform, whereupon 3.5 parts by weight of crystals are obtained, contaminants such as hydrocortisone going into the mother liquor. The crystals are recrystallized from methanol to give 1.2 parts by weight of "Crystal A" melting at about 200° C. The methanol is distilled off from the mother liquor from the recrystallization, and the residue is recrystallized from chloroform to give 2.0 parts by weight of "Crystal B" melting at 190–198° C.

The "Crystal A" is recrystallized from methanol several times. The product has the properties: melting point; 233–236° C., $[\alpha]_D^{27}=+143°$ (ethanol), $[\alpha]_D^{27}=+128°$ (dioxane), UV-spectrum: $\lambda_{max}=243.5$ mμ (ε, 15,500), empirical formula; $C_{21}H_{30}O_5$. And the "Crystal A" shows the following qualitative reactions: Bluetetrazolium reaction (α-Ketol) is positive, NaOH-fluorescence is blue-green and color-reaction with concentrated sulfuric acid is red.

Analysis.—Calcd. for $C_{21}H_{30}O_5$: C, 69.58, H, 8.34. Found: C, 69.89, H, 8.11.

The diacetate of this product melts at 231–232° C. The analytical values are in accord with those of dihydroxydesoxycorticosterone. And the afore-indicated natures of this compound are all in accord with those of 17,19-dihydroxydesoxycorticosterone whose natures have already been reported in Helvetica Chimica Acta vol. 39 (1956), p. 2062 by R. Neher et al. From the foregoing, "Crystal A" is identified as 17,19-dihydroxydesoxycorticosterone (4-pregnene-17α,19,21-triol-3,20-dione).

The "Crystal B" is recrystallized several times from methanol and the product has the properties: melting point; 212–216° C., $[\alpha]_D^{20}=+120°$ (ethanol), UV-spectrum: $\lambda_{max}=242$ mμ (ε, 14,400), empirical formula; $C_{21}H_{30}O_5$.

Analysis.—Calcd. for $C_{21}H_{30}O_5$: C, 69.58, H, 7.68. Found: C, 69.36, H, 8.27.

The diacetate of "Crystal B" melts at 201–203° C., and it shows $[\alpha]_D^{20}=+120°$ (chloroform), UV-spectrum: $\lambda_{max}=240$ mμ (ε, 15,000).

Analysis.—Calcd. for $C_{24}H_{34}O_7$: C, 67.24, H, 7.68. Found: C, 67.09, H, 7.48.

These properties are substantially in accord with those of epihydrocortisone. Constants of epihydrocortisone are: M.P. 209–212° C., $[\alpha]_D=+117°$ (ethanol), UV-spectrum: λ_{max} 242 mμ (ε, 14,700); and those of epihydrocortisone diacetate are: M.P. 202–203° C., $\lambda_{max}=240$ mμ (ε, 14,900). The IR-spectrum of "Crystal B" is in accord with that of epihydrocortisone, and moreover no depression of melting point is observed when the diacetate of "Crystal B" is melted with an authentic specimen of the diacetate of epihydrocortisone. It is thus clear that "Crystal B" is nothing but epihydrocortisone (4-pregnene-11α,17α,21-triol-3,20-dione).

Example 4

A strain of *Corticium sasakii* is aerobically pre-incubated for 72 hours in 1000 parts by volume of the same medium as that used in Example 1. A solution of 0.2 part by weight of desoxycorticosterone (4-pregnene-21-ol-3,20-dione) in 20 parts by volume of methanol is then added and incubation is further continued aerobically for 48 hours with shaking. At the end of this time, the broth is treated in the same manner as in Example 1 to give about 0.28 part by weight of a yellow residue. This residue is subjected to chromatography on a column of alumina, and the fraction showing the same behavior as corticosterone on a paper chromatogram is collected. The so-obtained product is recrystallized from acetone-hexane to give 0.06 part by weight of crystals, M.P. 178–180° C., $[\alpha]_D^{25}=+216°$ (ethanol).

Analysis.—Calcd. for $C_{21}H_{30}O_4$: C, 72.80, H, 8.73. Found: C, 72.76, H, 8.55.

These properties are in good agreement with those of corticosterone as given in the literature. M.P. 178–180° C., $[\alpha]_D=+210.5°$ (ethanol). Moreover, the IR-spectrum of the product of this example is also in good accord with that of an authentic specimen of corticosterone. It is thus clarified that the product is corticosterone (4-pregnen-11β,21-diol-3,20-dione).

Example 5

A strain of *Corticium sasakii* is pre-incubated aerobically for 72 hours in 1000 parts by volume of the medium

described in Example 1, and a solution of 0.2 part by weight of 1-dehydro-17-hydroxydesoxycorticosterone (1,4-pregnadiene-17α,21-diol-3,20-dione) in 20 parts by volume of methanol is added, and the incubation is continued aerobically for further 48 hours with shaking. After the incubation, the broth is processed as described in Example 1 to give 0.35 part by weight of a residue. As its paper chromatograms (using propylene glycol-toluene solvents) shows, the said residue is a mixture containing three oxygenated steroids in addition to 1-dehydrohydrocortisone (1,4 - pregnadiene-11β,17α,21-triol-3,20-dione). The residue is subjected to chromatography on a column of magnesium silicate and the fraction containing 1-dehydrohydrocortisone is collected. The crude substance thus obtained melts at 223–226° C. The yield is 0.075 part by weight. Recrystallization of the crude substance from acetone gives 0.04 part by weight of 1-dehydrohydrocortisone, M.P. 232–236° C. (decomposition),

$$[\alpha]_D = +100°$$

(dioxane). Both UV- and IR-spectra of the product are in good accord with those of an authentic sample of 1-dehydrohydrocortisone (1,4 - pregnadiene-11β,17α,21-triol-3,20-dione).

Example 6

To a mixture consisting of 30 parts by weight of corn starch, 0.5 part by weight of magnesium sulfate, 1 part by weight of potassium dihydrogen phosphate and 250 parts by volume of 20% potato juice, tap water is added to make 1000 parts by volume of a medium. The medium so-prepared is then sterillized under a steam pressure of 15 pounds per square inch for 15 minutes. To the medium, 100 parts by volume of a culture of a strain of *Corticium microsclerotia* is added and aerobically incubated for 48 hours at 28° C. To the broth thus obtained, there is added a solution of 0.1 part by weight of 17 - hydroxy - desoxycorticosterone (4-pregnene-17α,21-diol-3,20-dione) in 20 parts by volume of ethanol, and the incubation is aerobically continued further for 48 hours. At the end of this incubation period, steroids are extracted from the broth by shaking with three 500 parts by volume portions of ethyl acetate. The ethyl acetate solution is washed with 200 parts by volume of 1% aqueous sodium hydroxide solution and two 200 parts by volume portions of distilled water, successively. The ethyl acetate solution is then concentrated under reduced pressure in an atmosphere of nitrogen to give 0.11 part by weight of an oily residue. The residue is subjected to chromatography on a column of magnesium silicate and the portion corresponding to hydrocortisone (4-pregnene-11β,17α,21-triol-3,20-dione) (as indicated by paper chromatogram test) is collected. Recrystallization of the product from acetone gives 0.015 part by weight of hydrocortisone (4 - pregnene - 11β,17α,21-triol-3,20-dione), M.P 205–207° C., $[\alpha]_D^{25} = +162°$ (ethanol).

The strain of *Corticium microsclerotia* used in this example and Examples 8, 9, 10, 11 and 12 was deposited at IFO and NRRL under the accession number IFO–6298 and NRRL–2727, respectively.

Example 7

A medium as described in Example 1 is sterilized with steam under a pressure of 15 pounds per square inch for 15 minutes. To the medium, 100 parts by volume of a culture of a strain of *Corticium praticola* is added and aerobically incubated for 48 hours at 28° C. A solution of 0.1 part by weight of 17-hydroxydesoxycorticosterone (4-pregnene-17α,21-diol-3,20-dione) in 20 parts by volume of ethanol is then added, and the incubation is aerobically continued for 48 more hours under the aforeindicated conditions. The broth is then processed as in Example 6 to give an oily residue. From this residue, 0.0032 part by weight of hydrocortisone (4-pregnene-11β,17α,21-triol-3,20-dione) is obtained by treating in the manner described in Example 6.

The strain of *Corticium praticola* used in this example was deposited at IFO and NRRL under the accession number IFO–6252, NRRL–2724, respectively.

Example 8

To a mixture of 900 parts by weight of soluble starch, 60 parts by weight of ammonium nitrate, 30 parts by weight of dibasic potassium phosphate, 15 parts by weight of ferrous chloride, 15 parts by weight of potassium chloride, 15 parts by weight of magnesium sulfate and 300 parts by volume of vitamin solution, tap water is added to make 30,000 parts by volume of a medium. One liter of the above used vitamin solution contains 0.2 milligram of biotin, 40 milligrams of calcium pantothenate, 0.2 milligram of pteroylglutamic acid, 200 milligrams of inositol, 40 milligrams of niacinamide, 20 milligrams of paraaminobenzoic acid, 40 milligrams of pyridoxine hydrochloride, 20 milligrams of riboflavin mononucleotide and 40 milligrams of thiamine hydrochloride. To the medium is added about one percent portion of a culture of a strain of *Corticium microsclerotia*, and is incubated at 28° C. for 48 hours under agitation and aeration. Then a solution of 20 parts by weight of 4-pregnene-17α,21-diol-3,20-dione 21-acetate in 600 parts by volume of propylene glycol is added. Aerobic incubation is further continued for 12 hours with agitation. The broth so-obtained is treated in the same manner as in Example 3 to give a crude steroid. Paper chromatrographic quantitative analysis of the crude steroid shows that it contains about 65% relative to the starting steroid of 4-pregnene-17α,19,21-triol-3,20-dione and about 7 to 8% relative to the starting steroid of 4-pregnene-11β,17α,21-triol-3,20-dione. Recrystallization of the crude product gives 8.4 parts by weight of 4-pregnene-17α,19,21-triol-3,20-dione which occurs as crystals showing M.P. 235–236° C., $[\alpha]_D^{19} = +121°$ (ethanol). The yield is 42%.

In the mother liquor from the recrystallization is observed the existence of a large amount of 4-pregnene-17α,19,21-triol-3,20-dione and a little amount of 4-pregnene-11β,17α,21-triol-3,20-dione.

Example 9

In accordance with the manner described in Example 8, 30,000 parts by volume of the same medium as in Example 8 is prepared, and a strain of *Corticium microsclerotia* is inoculated into the medium and incubated at 28° C. for 48 hours under aeration and agitation. Then a solution of 15 parts by weight of desoxycorticosterone (4-pregnen-21-ol-3,20-dione) in 300 parts by volume of ethanol is added to the culture, and incubation is continued aerobically for further 8 hours. The culture broth so-obtained is extracted with ethyl acetate, and the ethyl acetate is evaporated. The extract is subjected to adsorption chromatography on a column of alumina, and from the fraction eluted with benzene:ethanol (9:1) is obtained a crude product. Recrystallization of the crude product from methanol-acetone-ether gives 4-pregnene-19,21-diol-3,20-dione, M.P. 153–156° C. Acetylation of this product by the conventional method gives the diacetate thereof (4-pregnene-19,21-diacetoxy-3,20-dione) which occurs as crystals having M.P. 122–124° C., $[\alpha]_D^{18} = +215°$ (chloroform).

The above two products do not show depression of melting point when they are melted with respective authentic specimens. And, IR-spectra of these are in good agreement with those of the authentic specimens.

Example 10

After the incubation of *Corticium microsclerotia* as in Example 8, a solution of 12 parts by weight of 4-androstene-3,17-dione in 200 parts by volume of ethanol is added, and the aerobic incubation is continued further for 8 hours with stirring. The so-obtained broth is extracted with ethyl acetate, and the ethyl acetate solution is concentrated. The residue is subjected to adsorption chro-

matography on a column of alumina, and the column is eluted with benzene. From the eluate there can be collected a crude product showing M.P. 156° C. Recrystallization of the product from acetone-ether gives 4-androsten-19-ol-3,17-dione which occurs as crystals, M.P. 165° C., $[\alpha]_D^{20}=+180°$ (chloroform). IR-spectrum of the product is in good accord with that of an authentic specimen.

Example 11

In a similar manner to the method described in Example 8, a strain of *Corticium microsclerotia* is incubated aerobically. Then a solution of 20 parts by weight of progesterone (4-pregnene-3,20-dione) in 800 parts by volume of propylene glycol is added to the culture. Further aerobic incubation is continued for 12 hours with stirring. The isolation of the resulting steroid is conducted as in Example 10 to obtain 4-pregnen-19-ol-3,20-dione which occurs as crystals showing M.P. 170–171° C., $[\alpha]_D=+175°$ (ethanol)

$\lambda_{Max.}^{Ethanol}$ 242 mμ

The product can be converted into its acetate through a conventional means, and the acetate shows M.P. 125–126° C., $[\alpha]_D=+215°$

$\lambda_{Max.}^{Ethanol}$ 239 mμ

Example 12

To a mixture of 600 parts by weight of cane sugar, 10 parts by weight of potassium chloride, 10 parts by weight of magnesium sulfate, 20 parts by weight of dibasic potassium phosphate and 5000 parts by volume of malt extract, water is added to make 20,000 parts by volume of a medium. The medium is inoculated with a strain of *Corticium microsclerotia,* and incubated for 48 hours with agitation and aeration. Then a solution of 15 parts by weight of 4-pregnene-17α,21-diol-3,20-dione in 700 parts by volume of propylene glycol is added to the culture, and aerobic incubation is continued for further 16 hours. The broth is extracted with 30,000 parts by volume of ethyl acetate, and the extract is concentrated to 500 parts by volume. The concentrate is washed with 1% aqueous solution of sodium bicarbonate. The ethyl acetate is distilled off under reduced pressure to obtain 16 parts by weight of a yellow crystalline residue. The residue is washed with petroleum ether to leave 10.5 parts by weight of crude steroid which is contaminated with a small quantity of the unchanged starting steroid.

After washing with chloroform, the crude steroid is recrystallized from methanol to give 3.0 parts by weight of crystals which melt at about 200° C. Further recrystallization from methanol gives a crystalline product, M.P. 235–236° C.

Analysis.—Calcd. for $C_{21}H_{30}O_5$: C, 69.58, H, 8.34. Found: C, 69.46, H, 8.35.

$[\alpha]_D=+123°$ (ethanol), UV-spectrum:

$\lambda_{Max.}^{EtOH}$ 242.5 mμ (ε, 15,500)

The product was identified as 4-pregnene-17α,19,21-triol-3,20-dione, which was obtained in Example 3.

The diacetate of the product obtained through a conventional manner melts at 231–232° C., and no depression of melting point was observed when this diacetate is melted together with the diacetate of 4-pregnene-17α,19,21-triol-3,20-dione obtained in Example 3.

This application is a continuation-in-part of our copending application Serial No. 696,960, filed November 18, 1957 (abandoned since the filing of the present application).

Having thus disclosed the invention, what is claimed is:

1. A process for the production of a member selected from the group consisting of 11β-position hydroxylated steroids, 11α-position hydroxylated steroids and 19-position hydroxylated steroids, which comprises the step of subjecting a steroid which is unsubstituted at the 11- and 19-positions to the action of oxygenating enzymes produced from a fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into a position selected from the group consisting of the 11- and 19-positions.

2. A process for the production of a member selected from the group consisting of 11β-position hydroxylated steroids, 11α-position hydroxylated steroids and 19-position hydroxylated steroids, which comprises the steps of subjecting steroid which is unsubstituted at the 11- and 19-positions to the action of oxygenating enzymes produced from a fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into a position selected from the group consisting of the 11- and 19-positions, and then recovering the so-produced hydroxylated steroid from the reaction mixture.

3. A process for the production of steroid selected from the group consisting of 11β-position hydroxylated steroids, 11α-position hydroxylated steroids and 19-position hydroxylated steroids, which comprises the steps of bringing a steroid which is not hydroxylated at the 11- and 19-positions into contact with the growing mycelium of a fungus of the genus Corticium (order Agaricales, class Basidiomycetes) under aerobic conditions, whereby an OH-group is introduced into a position selected from the group consisting of the 11- and 19-positions and then recovering the so-produced hydroxylated steroid from the reaction mixture.

4. A process for the production of a steroid selected from the group consisting of 11β-position hydroxylated steroids, 11α-position hydroxylated steroids and 19-position hydroxylated steroids, which comprises the steps of bringing a steroid which is not hydroxylated at the 11- and 19-positions into aerobic contact with the growing mycelium of a fungus of the genus Corticium (order Agaricales, class Basidiomycetes) whereby an OH-group is introduced into a position selected from the group consisting of the 11- and 19-positions, and then recovering the so-produced hydroxylated steroid from the reaction mixture.

5. A process for the production of an 11β-hydroxylated steroid, which comprises the steps of subjecting a steroid which is unsubstituted at the 11-position to the action of oxygenating enzymes produced from a fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into the 11β-position, and then recovering the so-produced 11β-position hydroxylated steroid from the reaction mixture.

6. A process for the production of an 11β-position oxygenated steroid, which comprises the steps of bringing a steroid which is not hydroxylated at the 11-position into contact with the growing mycelium of a fungus of the genus Corticium (order Agaricales, class Basidiomycetes) under aerobic conditions, whereby an OH-group is introduced into the 11β-position, and then recovering the so-produced 11β-position hydroxylated steroid from the reaction mixture.

7. A process for the production of a 19-position oxygenated steroid, which comprises the steps of subjecting a steroid which is unsubstituted at the 19-position to the action of oxygenating enzymes produced from a fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into the 19-position, and then recovering the so-produced 19-position hydroxylated steroid from the reaction mixture.

8. A process for the production of a 19-position oxygenated steroid, which comprises the steps of bringing a steroid which is not oxygenated at the 19-position into contact with the growing mycelium of a fungus of the genus Corticium (order Agarcales, class Basidiomycetes) under aerobic conditions, whereby an OH-group is introduced to the 19-position, and then recovering the so-produced 19-position hydroxylated steroid.

9. The process of claim 1, wherein the fungus of the genus Corticium is a strain of *Corticium sasakii.*

10. The process of claim 1, wherein the fungus of the

genus Corticium is a strain of *Corticium microsclerotia.*

11. The process of claim 1, wherein the fungus of the genus Corticium is a strain of *Corticium praticola.*

12. The process of claim 2, wherein the fungus of the genus Corticium is a strain of *Corticium sasakii.*

13. The process of claim 2, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

14. The process of claim 2, wherein the fungus of the genus Corticium is a strain of *Corticium praticola.*

15. The process of claim 3, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

16. The process of claim 3, wherein the fungus of the genus Corticium is a strain of *Corticium praticola.*

17. The process of claim 3, wherein the fungus of the genus Corticium is a strain of *Corticium sasakii.*

18. The process of claim 4, wherein the fungus is the genus Corticium is a strain of *Corticium sasakii.*

19. The process of claim 4, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

20. The process of claim 4, wherein the fungus of the genus Corticium is a strain of *Corticium praticola.*

21. The process of claim 5, wherein the fungus of the genus Corticium is a strain of *Corticium sasakii.*

22. The process of claim 5, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

23. The process of claim 5, wherein the fungus of the genus Corticium is a strain of *Corticium praticola.*

24. The process of claim 6, wherein the fungus of the genus Corticium is a strain of *Corticium sasakii.*

25. The process of claim 6, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

26. The process of claim 6, wherein the fungus of the genus Corticium is a strain of *Corticium praticola.*

27. The process of claim 7, wherein the fungus of the genus Corticium is a strain of *Corticium sasakii.*

28. The process of claim 7, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

29. The process of claim 7, wherein the fungus of the genus Corticium is a strain of *Corticium praticola.*

30. The process of claim 8, wherein the fungus of the genus Corticium is a strain of *Corticium sasakii.*

31. The process of claim 8, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

32. The process of claim 8, wherein the fungus of the genus Corticium is a strain of *Corticium praticola.*

33. The process of claim 1, wherein the starting steroid is a member selected from the group consisting of 4-pregnen-21-ol-3,20-dione, 4-pregnene-17α,21-diol-3,20-dione, 1,4-pregnadiene-17α,21-diol-3,20-dione, 4-pregnene-3,20-dione and 4-androstene-3,17-dione.

34. The process of claim 2, wherein the starting steroid is a member selected from the group consisting of 4-pregnen-21-ol-3,20-dione, 4-pregnene-17α,21-diol-3,20- dione, 1,4-pregnadiene-17α,21-diol-3,20-dione, 4-pregnene-3,20-dione and 4-androstene-3,17-dione.

35. The process of claim 3, wherein the starting steroid is a member selected from the group consisting of 4-pregnen-21-ol-3,20-dione, 4-pregnene-17α,21-diol-3,20- dione, 1,4-pregnadiene-17α,21-diol-3,20-dione, 4-pregnene-3,20-dione and 4-androstene-3,17-dione.

36. The process of claim 4, wherein the starting steroid is a member selected from the group consisting of 4-pregnen-21-ol-3,20-dione, 4-pregnene 17α,21-diol-3,20- dione, 1,4-pregnadiene-17α,21-diol-3,20-dione, 4-pregnene-3,20-dione and 4-androstene-3,17-dione.

37. The process of claim 5, wherein the starting steroid is a member selected from the group consisting of 4-pregnen-21-ol-3,20-dione, 4-pregnene-17α,21-diol-3,20-dione, 1,4-pregnadiene-17α,21-diol-3,20-dione, 4-pregnene-3,20-dione and 4-androstene-3,17-dione.

38. The process of claim 6, wherein the starting steroid is a member selected from the group consisting of 4-pregnen-21-ol-3,20-dione, 4 pregnene-17α,21-diol-3,20-dione, 1,4-pregnadiene-17α,21-diol-3.20-dione, 4-pregnene-3,20-dione and 4-androstene-3,17-dione.

39. The process of claim 7, wherein the starting steroid is a member selected from the group consisting of 4-pregnen-21-ol-3,20-dione, 4 pregnene-17α,21-diol-3,20-dione, 1,4-pregnadiene-17α,21-diol-3,20-dione, 4-pregnene-3,20-dione and 4-androstene-3,17-dione.

40. The process of claim 8, wherein the starting steroid is a member selected from the group consisting of 4-pregnen-21-ol-3,20-dione, 4 pregnene-17α,21-diol-3,20-dione, 1,4-pregnadiene-17α, 21- diol-3,20-dione, 4-pregnene-3,20-dione and 4-androstene-3,17-dione.

41. A process for the production of 4-pregnene-11β, 17α,21-triol-3,20-dione, which comprises the steps of subjecting 4-pregnene-17α,21-diol-3,20-dione to the action of oxygenating enzymes produced from a fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into the 11β-position, and then recovering the so-produced hydroxylated steroid from the reaction mixture.

42. The process of claim 41, wherein the fungus of the genus Corticium is a strain of *Corticium sasakii.*

43. A process for the production of both 4-pregnene-17α,19,21-triol-3,20-dione and 4-pregnene-11α,17α,21-triol-3,20-dione, which comprises the steps of bringing 4-pregnene-17α,21-diol 3,20-dione into aerobic contact with the growing mycelium of a fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby OH-groups are introduced into 11α- and 19-positions, and then recovering the so-produced hydroxylated steroids from the reaction mixture separately.

44. The process of claim 43, wherein the fungus of the genus Corticium is a strain of *Corticium vagum.*

45. A process for the production of 4-pregnene-17α, 19,21-triol-3,20-dione, which comprises the steps of bringing 4-pregnene-17α,21-diol-3,20-dione into aerobic contact with the growing mycelium of the fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into the 19-position, and then recovering the so-produced 19-position hydroxylated steroid from the reaction mixture.

46. The process of claim 45, wherein the fungus of the genus Corticium is a strain of *Corticium vagum.*

47. The process of claim 45, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

48. A process for the production of 4-pregnene-19,21-diol-3,20-dione, which comprises the steps of bringing 4-pregnen-21-ol-3,20-dione into aerobic contact with the growing mycelium of the fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into the 19-position, and then recovering the so-produced 19-position hydroxylated steroid from the reaction mixture.

49. The process of claim 48, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

50. A process for the production of 4-androstene-19-ol-3,17-dione, which comprises the steps of bringing 4-androstene-3,17-dione into aerobic contact with the growing mycelium of the fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into the 19-position, and then recovering the so-produced 19-position hydroxylated steroid from the reaction mixture.

51. The process of claim 50, wherein the fungus of the genus Corticium is a strain of *Corticium microsclerotia.*

52. A process for the production of 4-pregnen-19-ol-3,20-dione, which comprises the steps of bringing 4-pregnene-3,20-dione into aerobic contact with the growing mycelium of the fungus of the genus Corticium (order Agaricales, class Basidiomycetes), whereby an OH-group is introduced into the 19-position, and then recovering the so-produced 19-position hydroxylated steroid from the reaction mixture.

53. The process of claim 52, wherein the fungus of the genus Corticium is *Corticium microsclerotia.*

No references cited.