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STEROIDS

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1

The present invention relates to a novel method for the introduction of oxygen into a steroid molecule. This application is a continuation-in-part of our prior-filed applications Serial No. 297,242, filed July 5, 1952, Serial No. 272,944, filed February 23, 1952, and issued as Patent 2,602,769, and Serial No. 180,496, filed August 19, 1950, and now abandoned, and relates more especially to an aerobic fermentation process wherein fermentation and oxygenation of steroids may be accomplished by means of fungi of the genus *Aspergillus*.

It is already known to introduce oxygen into a steroid molecule, particularly into the eleven position, for the production of intermediates useful in the preparation of therapeutic compounds, or for the production of the drugs themselves, which intermediates or drugs have an oxygenated structure, particularly an eleven oxygenated structure, among which may, for example, be mentioned corticosterone, 11-dehydrocorticosterone (compound E, cortisone), and 17-hydroxycorticosterone (compound F). Such result has been accomplished, heretofore, only through highly involved organic synthesis necessitating a considerable number of steps. More recently, the introduction of oxygen into a steroid molecule by means of Mucorales fungi has been described by Murray and Peterson, United States Patent 2,602,769, issued July 8, 1952. It has additionally been found that fungi of the genus *Aspergilli*, e. g., *Aspergillus niger*, are useful in the oxygenation of steroids, including eleven desoxy steroids, although not necessarily producing the same result as the fungi of the Mucorales order.

It is an object of the present invention to provide a novel method for the introduction of oxygen into a steroid molecule. Another object of the invention is the provision of such method whereby an eleven desoxy steroid (the term "eleven desoxy steroid" is employed throughout to indicate a steroid which contains no oxygen in the eleven position) is converted to an eleven oxygenated steroid by the action of a species of fungus of the genus *Aspergillus*. Another object of the invention is the provision of a process for the introduction of oxygen into at least the eleven position of an eleven desoxy steroid through the action of fungus of the genus *Aspergillus*. Another object of the invention is to provide a process of oxygenating 3-keto steroids by means of *Aspergilli*. Still another object of the invention is the provision of a process of modifying steroid structure by means of *Aspergilli*. Other and more particular objects of the invention will become apparent hereinafter.

It has now been found that eleven desoxy steroids, which contain the cyclopentanopolyhydrophenanthrene nucleus, especially the

2

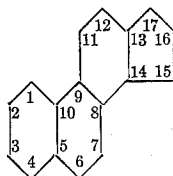
10,13 - dimethylcyclopentanopolyhydrophenanthrenes, can be readily converted in high yields to corresponding oxygenated steroids by subjecting the steroid compound to the action of a species of fungus of the genus *Aspergillus*. By the method of the present invention, an efficient, economical, and commercially satisfactory method of introducing oxygen into the eleven position of an eleven desoxy steroid molecule is provided. Accordingly, a novel and simple approach to the production of eleven oxygenated steroid drugs is afforded, which is, as previously stated, of great importance to the chemical, pharmaceutical and medical professions, and of especial value in the treatment of physiological abnormalities known to be beneficially affected only by such eleven oxygenated drugs.

The method of the present invention, in its broader aspects, consists in fermenting a steroid or an eleven desoxy steroid by means of a species of fungus of the genus *Aspergillus*. Another way of expressing one of the results of the process of the present invention is to say that the steroid is "oxygenated" since an oxygen atom is introduced thereto. Other positions as well as the eleven position of the steroid molecule may undergo transformation due to the action of the fungus, but such transformations are not to be regarded as undesirable, since the introduction of oxygen into other portions of the steroid molecule may result in valuable therapeutic products or intermediates, for example those containing a hydroxy group at the 17 position. In case such additional groups are not considered desirable, methods are available for the removal of such groups with facility. An important advantage of the present invention is the oxygenation of eleven desoxy steroids in the eleven position. Hydroxy groups which are themselves capable of oxidation to keto groups, when present in the molecule of a steroid to be oxygenated, may, if considered necessary, as where exceedingly high yields of eleven oxygenated hydroxysteroid product are sought to be produced, be protected from attack of various types, including attack by the oxidizing fungi, by conversion, as for example by esterification, etherification, halogenation, or the like, to a group which is reconvertible to a hydroxy group. However, such procedure is not a prerequisite to the introduction of oxygen, especially eleven oxygen, into a hydroxysteroid by the method of the present invention.

The steroids operative in the method of the present invention are not limited as to type or number of substituents, and for operativeness in the process need only contain a nuclear unoxxygenated or oxygenatable position, such as, for example, an unoxxygenated eleven position; illustratively, a methylene group, as in an eleven

3

desoxy steroid. Such compounds contain the nucleus:



which may in addition contain substituents or combinations of substituents about the nucleus, as in the 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16, and 17 positions, especially 10,13-dimethyl groups, 3,7, or 12 keto, hydroxy, or acyloxy groups; 17-side chains of which the progesterone and corticosterone (Ketol) side chains deserve special mention; a 17 keto group; a 17 hydroxy group, and the like; as well as double bonds in the 4, 5, 6, 7, 8, 9 (11), 11(12), 16(17) and other positions, or combinations of positions, about the nucleus; or double bonds saturated by addition thereto of halogen or hydrogen halide; adducts of dienophiles such as maleic acid, maleic anhydride, or maleic acid esters with steroids having a conjugated double bond system, as at 5,7; and other substituents and combinations of substituents, double bonds and so forth too numerous for special mention, a great many of which are known in the steroid art. The presence or absence of unsaturation at the 9(11) or 11(12) positions of the nucleus is not a critical factor in the method of the present invention, for, while it is preferred to apply the process to a steroid having an eleven methylene group, i. e., a steroid having two hydrogen atoms at carbon atom eleven or no unsaturation in the 9(11) or 11(12) positions, for reasons of economy and to obviate unnecessary transformations of saturated to unsaturated compounds, the fermentation may be applied with equal facility to either the saturated or unsaturated compounds.

Representative steroids which may be fermented by the method of the invention include, for example, progesterone, 9(11) or 11(12)-dehydroprogesterone, 7,9(11)-bisdehydroprogesterone, 17-hydroxyprogesterone, 17 α -progesterone, testosterone, pregnenolones, 3-hydroxy-5-pregnene-20-one, pregnenolone, 3 β -hydroxy-5,16-pregnadiene-20-one, acyloxy-pregnenolones such as pregnenolone acetate, 3-hydroxy-5,6-oxido-pregnane-20-one (α - or β -oxido), 3-hydroxy-5-chloropregnane-20-one, 5,6-oxido-pregnane-3,20-dione (α - or β -oxido), 4-bromo and 4-chloropregnane-3,20-dione, 5-chloropregnane-3,20-dione, 3-ketopregnane-20-ol, 3-keto-allo-pregnane-20-ol, 3 β -hydroxy-16,17-oxido-21-acetoxy-5-pregnene-20-one, 3 β -hydroxy-16,17-oxido-5-pregnene-20-one, 3 β -hydroxy-5,6,21-tribromo-16,17-oxido-pregnane-20-one, 3 β -hydroxy-16-bromo-17-hydroxy-5-pregnene-20-one, 3 β -hydroxy-16-chloro-17-hydroxy-5-pregnene-20-one, 3 β -hydroxy-5(6),16(17)-dioxido-pregnane-20-one, 3 β -hydroxy-5(6),16(17)-dioxido-21-bromo-pregnane-20-one, 3 β -hydroxy-5(6),16(17)-dioxido-21-acetoxypregnane-20-one, 3 β -hydroxy-5(6),16(17)-dioxido-21-hydroxypregnane-20-one, 11-desoxycorticosterone, delta-9(11) or 11(12)-desoxycorticosterone, 11-desoxy-17-hydroxycorticosterone and acyloxy derivatives, such as the acetoxy derivative, thereof, 21-hydroxypregnenolone and 21-acyl, e. g. acetyl, esters thereof, 17,21-dihydroxypregnenolone and 17,21-diacyloxy derivatives thereof, e. g. the diacetoxy derivative, androstenedione, androstan-

4

17-ol, 9(11) or 11(12) dehydroandrostenedione, 3-hydroxy-9(11) or 11(12)-pregnen-20-ones, 3,21-dihydroxy-9(11) or 11(12)-pregnen-20-ones, 3,17,21-trihydroxy-9(11) or 11(12)-pregnen-20-ones, 4-androsten-3-ol-17-one and 3-acyl, e. g. acetyl, esters thereof, 5-androsten-3-ol-17-one and 3-acyl, e. g. acetyl, esters thereof; ergosterol, stigmaterol, stigmastanol, and 3-acyl, e. g. acetyl, esters of the foregoing; ergostenone, stigmastene, stigmastamone, chloestene, cholic acid, desoxycholic acid, lithocholic acid, cholanolic acid, norcholanolic acid, bisnorcholanolic acid, cholenic acid, norcholenic acid, bisnorcholenic acid, and 3-hydroxy-, 3-keto-, 3,7-dihydroxy-, 3,7-diketo-, 3,7,12-trihydroxy-, 3,7,12-triketo-, 9(11) or 11(12)-unsaturated, ester, thiolester, and further derivatives of the foregoing acids, and the like. Suitably a steroid having up to and including 22 carbon atoms in the carbon to carbon skeleton or a steroid having a two carbon atom side chain at the 17 position and an eleven methylene group may be used. The 10-nor-methyl, the 13-nor-methyl, and the 10,13-bisnor-methyl forms of each of the above steroids, in which either one or both of the 18 and 19 position angular methyl groups are replaced by hydrogen, are included within the purview of those steroids which may be fermented by the method of this invention. In the event that the 11-position is already oxygenated or substituted, the dominant product may be oxygenated additionally in another position. The 16-dehydro form of each of the above steroids is likewise included. Within the purview of this invention is the fermentation of D-homosteroids otherwise known as perhydrochrysenes, for example, D-homo-4-androstene-3,17a-dione, D-homo-testosterone, D-homo-17 α -methyltestosterone, D-homo-17 $\alpha\beta$ -methyltestosterone, 17 α -methyl-17 α -hydroxy-D-homo-4-androstene-3,17-dione, and their 4,5-dihydro, ring A saturated analogs, D-homo-androstene-3,17-dione, and D-homo-epidehydroandrostene-3,17-dione, and D-homo-epidehydroandrostene-3,17-dione-16a-one. All of these are amenable to fermentation with *Aspergillus* fungi in accordance with the presented examples.

In the process of the present invention, the operational conditions and reaction procedure and details may be those of parent application Serial No. 180,496, filed August 19, 1950, utilizing the action of a species of fungus of the genus *Aspergillus*. The genus *Aspergillus* belongs to the family Aspergillaceae of the order Aspergillales (Plectascales). The classification and definition of *Aspergillus* as herein employed is that of Thom, C., and Church, M. G., "The Aspergilli," Williams and Wilkins Company, Baltimore, 1926, and includes certain species previously grouped under *Eurotium*. Species of the genus *Aspergillus* useful in the fermentation of steroids include the *Aspergillus clavatus* group, *A. clavatus*, *A. clavellus*, *A. fusco-cinereus*, *A. giganteus*, *A. pseudo-clavatus*; *A. glaucus* group, *A. amstelodami*, *A. chevalieri*, *A. disjunctus*, *A. echinulatus*, *A. fontoyonti*, *A. herbariorum* series major, *A. herbariorum* series minor, *A. medius*, *A. mutabilis*, *A. repandus*, *A. repens*, *A. ruber*, *A. scheelei*, *A. sejunctus*, *A. umbrinosus*; intermediate species related to *A. glaucus*, *A. conicus*, *A. gracilis*, *A. penicilloides*, *A. varians*; *A. fumigatus* group, *A. fischeri*, *A. fumigatus*; *A. nidulans* group, *A. nidulans*; *A. versicolor* group, *A. sydowii*, *A. versicolor*; *A. terreus* and *A. ustus* group, *A.*

gratioti, *A. insuetus*, *A. terreus*, *A. ustus*; *A. flavipes* group, *A. flavipes*, *A. candidus* group, *A. alliaceus*, *A. candidus*, *A. cinnamomeus* obtained by Schiemann as a mutant from *A. niger*, *A. okazakii*; *A. niger* group, *A. atropurpureus*, *A. carbonarius*, *A. fumaricus*, *A. japonicus*, *A. luteoniger*, *A. nanus*, *A. niger*, *A. phoenicis*, *A. pulchellus*, *A. pulverulentus*, *A. schiemanni*, *A. violaceo-fuscus*; *A. wentii*; *A. ochraceus* group, *A. citrisporus*, *A. ochraceus*, *A. ostianus*, *A. quercinus*, *A. sulphureus*, *A. terricola*, *A. terricola* var. *americana*; *A. tamarii* group, *A. erythrocephalus*, *A. penicillopsis*, *A. tamarii*; *A. flavus-oryzae* group, *A. flavus*, *A. oryzae*, *A. parasiticus*; *A. niveo-glaucus*, *A. awomori*, *A. aereus*, *A. gymosarce* and *A. luteo-virescens*. Of the listed species, *Aspergillus niger* is especially preferred for the practice of the present invention.

Culture of the fungi, for the purpose and practice of the present invention, is in or on a medium favorable to the development of the fungi. Solid media may be utilized, but the preferred media are those which permit quantity growth under aerobic conditions. Moist solid particulate media such as bran, cereal grains, cereal grits, wood chips, shavings, sawdust, cornhusks, fibrous material such as copra, chestnuts, or lupine seeds may be used. These can be extracted with alcohol, ether or other organic solvents, to remove objectionable contaminants and growth inhibitors prior to fermentation. The carriers may optionally contain added growth factors and nutrients and may be used in layers or trays with or without auxiliary aeration, in towers as in the vinegar process or under conditions of agitation as for example by tumbling in a rotating drum. Liquid media, illustratively brewer's wort, are well adapted to use under aerobic layer or more especially aerobic submerged fermentation conditions. Suitably the media should contain sources of available carbon, nitrogen and minerals although of course there can be significant growth and development under less than optimum conditions.

Available carbon may be from carbohydrates, starches, gelatinized starches, dextrin, sugars, molasses as of cane, beet and sorghum, glucose, fructose, mannose, galactose, maltose, sucrose, lactose, pentoses, amino acids, peptones or proteins. Carbon dioxide, glycerol, alcohols, acetic acid, sodium acetate, citric acid, sodium citrate, lower fatty acids, higher fatty acids, or fats are illustrative of other materials which provide assimilable carbon for the energy requirements of the fungi. Mixtures of various carbon sources are sometimes advantageous.

Nitrogen in assimilable form may be provided by soluble or insoluble vegetable or animal proteins, soybean meal, lactalbumin, casein, egg albumin, peptones, polypeptides or amino acids, urea, ammonium salts, ammonia trapped on base exchange resins or on zeolites, ammonium chloride, sodium nitrate, potassium nitrate, morpholine. Whey, distillers solubles, corn steep liquor, or yeast extract have been useful.

As mineral constituents the media or menstrium may contain, naturally present or added, available calcium, cobalt, copper, gallium, iron, magnesium, molybdenum, potassium, scandium, uranium, vanadium, and boron. Sulfur may be provided by sulfates, alkyl sulfonates, sulfoxylates, sulfamates, sulfonates, free sulfur, hypodisulfite, persulfate, thiosulfate, methionine,

cystine, cystein, thiamin or biotin. Phosphorus, preferably pentavalent, suitably in a concentration at or about 0.001 to 0.07 molar and particularly at or about 0.015 to 0.02, may be present as ortho-, meta-, or pyrophosphates, salts or esters, phytin, phytic acid, phytates, glycerophosphate, sodium nucleinate, casein or ovovitellin. Boron and iodine in traces may be advantageous. Desirably boron, in the form of boric acid or sodium borate, borax, may be present or added especially after germination and early growth of the *Aspergilli*.

Other accessory growth factors, vitamins, auxins and growth stimulants may be provided as needed or desired.

While solid or liquid media may be utilized, liquid media is preferred as it favors mycelial growth.

Suspending or mycelial carriers such as filter earths, filter aids, finely divided cellulose, wood chips, bentonite, calcium carbonate, magnesium carbonate, charcoal, activated carbon or other suspendable solid matter, methyl cellulose, carboxymethyl cellulose or alginates may be added to facilitate fermentation, aeration and filtration.

The selected species of fungus of the genus *Aspergillus* is grown either in light or darkness on a medium containing available carbon, illustratively carbohydrates such as sugars or starches; assimilable nitrogen, illustratively soluble or insoluble proteins, peptones or amino acids; and mineral constituents, illustratively phosphates and magnesium sulfate; and other art recognized, desirable or adventitious, additions. The medium may desirably have a pH before inoculation of between about 2.8 and 8.8 although a higher or lower pH may be used. A pH of about 3 to 7 is preferred for the growth of *Aspergilli*. Low pH values inhibit bacterial contamination and facilitate sterilization. For example, at a pH of 2 to 3, effective sterilization of media may be accomplished by heating the media for thirty minutes at 100 degrees centigrade, whereas, at a pH of 4 to 4.5, thermal sterilization may require superatmospheric pressure. Alternatively or concomitantly, bacterial contamination may be retarded by the presence of antiseptic or antibiotic agents such as benzoates, sulfites, penicillin or circulin.

Inoculation of the fungal growth-supporting medium with the selected fungus of the genus *Aspergillus* may be accomplished in any suitable manner. *Aspergilli* grow over a wide range of temperatures from about eight degrees centigrade to about 45 degrees centigrade and preferably at or about room temperature or between about twenty degrees centigrade and 33 degrees centigrade.

The developmental period of fungal growth required before the steroid to be fermented is exposed to the fungus does not appear to be critical. For example, the steroid may be added either before thermal or other sterilization of the medium, at the time of inoculating the medium with the selected *Aspergillus* species, or at some time, as 24 to 48 hours, later. The steroid to be fermented may be added at any suitable concentration although for practical reasons steroid substrate at a concentration of about or up to about 0.6 gram per liter or even 0.8 gram per liter of medium is satisfactory and two grams per liter is operative although higher concentrations, depending upon the particular steroid, may be used with some inhibition of mycelial de-

velopment. Either a purified steroid, a crude material containing steroid, or a steroid material comprised of or consisting predominantly or essentially of steroid, for example, a mixture of steroid and fat or solvent, may be used as substrate. The addition of steroid substrate to be fermented may be accomplished in any suitable manner especially so as to promote a large surface of contact of the steroid substrate with the oxygenating activity of the fungus, such as by dispersing the steroid substrate, either alone, with a dispersing agent, or in solution in a water-miscible organic solvent, by mixing or homogenizing the steroid substrate with the fungal medium to form a suspension or dispersion of steroid. Either submerged or surface culture procedures may be used with facility, although submerged culture is preferred. Alternatively, steroid fermenting enzymes of a growth of the fungus may be separated from the fungus or medium, admixed with the steroid or a solution or dispersion thereof, and the mixture subjected to aerobic conditions to accomplish fermentation of the steroid.

The temperature during the period of fermentation of the steroid may be the same as that found suitable for fungal growth. It need be maintained only within such range as supports life, active growth, or the enzyme activity of the fungus.

While any form of aerobic incubation is satisfactory for the growth of the selected fungus or fermentation of the steroid substrate, the efficiency of steroid fermentation is related to aeration. Therefore, aeration is usually controlled, as by agitation and/or blowing air through the fermentation medium. Aeration may be effected by surface culture or under submerged fermentation conditions. Aerobic conditions include not only the use of air to introduce oxygen, but also other sources or mixtures containing oxygen in free or liberatable form. In using air as the aerating medium, a desirable rate of aeration is about four to twenty millimoles of oxygen per hour per liter as determined by the method of Cooper, Fernstrom and Miller, Ind. Eng. Chem., 36, 504 (1944). In the accompanying working examples, aeration concomitant with agitation and stirring corresponds to four, eight, or twenty millimoles of oxygen per hour per liter at stirring speeds of 176, 250, or 360 revolutions per minute, respectively. Under some conditions it is desirable to utilize different rates of aeration during the fungus growing or developing stage as contrasted with the steroid fermentation stage. Aeration is suitably modified by using superatmospheric or subatmospheric pressures, for example thirty pounds per square inch or ten pounds per square inch absolute. Oxygen uptake may be facilitated by the presence of various catalysts such as ascorbic acid, glutamic acid, citric acid, lactic acid, tyrosine, or tryptophane.

The time required for the fermentation of steroids varies somewhat with the procedure. When the steroid substrate is present at the time of inoculation of the medium, periods of from eight to 72 hours may be used. However, when the steroid is added to the fungus, after substantial aerobic growth of the fungal organism, for example after 16 to 24 hours at optimum temperature, the conversion of steroid substrate begins immediately and high yields are obtained in from one to 72 hours, 24 hours being generally satisfactory. The steroids may be fermented in

a simultaneous or sequential heterofermentative procedure resulting in other useful products, which are recoverable according to procedures known in the art, including enzymes and acids, for example amylase, invertase, lipase, maltase, protease, proteolytic enzymes, rennet, urease, citric acid, fumaric acid, gluconic acid, itaconic acid, kojic acid and oxalic acid. These fermentation products may be separated from the fermentation beer at the same time, before or after the fermentation is complete with respect to the steroid fermentation products.

After completion of the steroid fermentation, the resulting fermented steroid is recovered from the fermentation reaction mixture. An especially advantageous manner of recovering the fermented steroid involves extracting the fermentation reaction mixture, including the fermentation liquor and mycelia with a water-immiscible organic solvent for steroids, for example, methylene chloride, ethylene chloride, trichloroethylene, ether, amyl acetate, and the like. The fermentation liquor and mycelia may be separated and then separately extracted with suitable solvents. The mycelia may be extracted with either water-miscible or water-immiscible solvents, acetone being effective. The fermentation liquor, freed of mycelia, may be extracted with water-immiscible solvents. The extracts can be combined, either before or after washing with an alkaline solution, illustratively sodium bicarbonate, suitably dried, as for example over anhydrous sodium sulfate, and the purified fermented steroid obtained by recrystallization from organic solvents or by chromatography.

The following examples are illustrative of the process of the present invention and are not to be construed as limiting.

Example 1.—Biooxygenation of 11-desoxycorticosterone acetate to 11 α ,21-dihydroxy-4-pregnene-3,20-dione (11-epicorticosterone) by Aspergillus

A medium having a composition of twenty grams of Edammine enzymatic digest of lactalbumin, three grams of corn steep liquor and fifty grams of technical dextrose diluted to one liter with tap water was adjusted to a pH of 6.4 with hydrochloric acid and sodium hydroxide. Twelve liters of this medium was sterilized in a five-gallon bottle equipped with stirrer and sparger. The pH after sterilization was 5.75. This sterile medium was inoculated with spores of *Aspergillus nidulans* (ATCC 10074); the aeration was adjusted to one liter per minute and the agitation to 200 revolutions per minute. Forty-eight hours later three grams of 11-desoxycorticosterone acetate, dissolved in 100 milliliters of acetone, was added and washed in with thirty milliliters of acetone. The pH was now 4.9. After 24 hours of bioconversion time the fermentation was stopped. The final pH was 4.8.

The mycelium was separated from the whole beer by squeezing through gauze. The separated mycelium was extracted twice, each time with a volume of acetone approximately equal to the volume of the mycelium and again extracted twice, each time with a volume of methylene chloride approximately equal to the volume of the mycelium. The acetone and methylene chloride extracts including solvent were added to the beer filtrate. The mixed extracts and beer filtrate were then extracted four times, each time with three liters of methylene chloride. The combined methylene chloride extract was washed twice, each time with one-tenth by volume por-

tions of a two percent aqueous solution of sodium bicarbonate and then twice with one-tenth by volume portions of water. The methylene chloride extract was dried with anhydrous sodium sulfate and then concentrated to a small volume on a steam bath.

The concentrated extract, freed of solvent, was a dark brown gummy mass weighing 7.039 grams. This residue was dissolved in 430 milliliters of ethylene dichloride and chromatographed over 240 grams of Florisil using 430-milliliter portions of developing solvent as indicated in Table I. Paper chromatography analysis using a toluene-propylene glycol system is included in Table I and is based upon the application of 160-microgram aliquots of eluate solids. Fraction 24, showing a sharp peak of 329.8 milligrams, was dissolved in excess methylene chloride, filtered to remove insoluble material and evaporated to a gum. This was dissolved with warming in one milliliter of ethyl acetate and then cooled whereupon crystallization occurred. The crude crystals melted at 140 to 162 degrees centigrade. Fraction 21 similarly treated yielded crystals melting at 151 to 158 degrees centigrade. Infrared absorption spectroscopy confirmed this compound as 11-epi-corticosterone. Fractions 20 through 24, weighing 1.097 grams, were combined in methylene chloride, decolorized by agitation with 0.3 gram Magnesol magnesium silicate, filtered and evaporated to dryness. The residue was dissolved in three milliliters of boiling ethyl acetate. On cooling to room temperature, crystallization was initiated and refrigeration for two hours completed crystallization. It was then filtered and washed three times with one-milliliter portions of cold ethyl acetate and then with ether. The crystals melted at 153 to 158 degrees centigrade and had an $[\alpha]_D^{23}$ of plus 166 degrees (0.764 in chloroform); yield 15.5 percent.

CHROMATOGRAPHIC ANALYSIS TABLE I

Fraction	Solvent	Eluate Solids, Milligrams	Desoxy-corticosterone, γ	11 α ,21-Dihydroxy-4-pregnene-3,20-dione, γ
1.....	ethylene dichloride (3 vols.)	633.6	-----	-----
2.....	ethylene dichloride (1 vol.)	63.1	-----	-----
3.....	ethylene dichloride-acetone 25:1.	32.4	-----	-----
4.....	do	180.8	-----	-----
5.....	ethylene dichloride-acetone 15:1.	120.1	-----	-----
6.....	do	252	-----	-----
7.....	ethylene dichloride-acetone 12:1.	297.2	-----	-----
8.....	do	311.9	70	-----
9.....	ethylene dichloride-acetone 10:1.	266.7	-----	-----
10.....	do	264.9	-----	-----
11.....	ethylene dichloride-acetone 8:1.	165.1	-----	-----
12.....	do	175	-----	-----
13.....	do	128	-----	-----
14.....	do	95.6	-----	-----
15.....	ethylene dichloride-acetone 5:1.	70.4	-----	-----
16.....	do	81.9	-----	-----
17.....	do	61.0	-----	-----
18.....	do	53	-----	-----
19.....	ethylene dichloride-acetone 2:1.	42.3	-----	-----
20.....	do	180	1	90
21.....	do	273	-----	80
22.....	do	179.5	trace	80
23.....	acetone	135	-----	-----
24.....	do	329.8	-----	70
25.....	do	72.5	-----	-----
26.....	do	39.9	-----	-----
Total.....		4,504.7	-----	-----

Example 2

In the same manner as Example 1, using As-

pergillus niger, ATCC 10577, and 17 α -hydroxyprogesterone as the starting steroid produced 11 α ,17 α -dihydroxyprogesterone.

Example 3

In the same manner as Example 1, using *Aspergillus niger*, ATCC 10577, and 17 α ,21-dihydroxyprogesterone as the starting steroid produced 11 α ,17 α ,21-trihydroxyprogesterone.

Example 4

In the same manner as Example 1, using *Aspergillus niger*, ATCC 10577, and pregnane-3,20-dione as the starting steroid produced 11 α -hydroxypregnane-3,20-dione.

Example 5

In the same manner as Example 1, using *Aspergillus niger*, ATCC 10577, and 21-hydroxy-4-pregnene-3,20-dione as the starting steroid produced 11 α ,21-dihydroxy-4-pregnene-3,20-dione.

Example 6

In the same manner as Example 1, using *Aspergillus niger*, ATCC 10577, and 16-dehydroprogesterone as the starting steroid produced 11 α -hydroxy-17 α -progesterone.

Example 7

In the same manner as Example 1, except as otherwise stated, in a medium adjusted to a pH of 6.35, and inoculated with *Aspergillus niger*, ATCC 10577, progesterone was added. With no previous culture time, after a bioconversion time of 48 hours with agitation at 360 revolutions per minute (aeration equivalent to twenty millimoles of oxygen per liter per hour), the resulting 11 α -hydroxyprogesterone was recovered.

Example 8

In the same manner as Example 1, except as otherwise stated, the medium was adjusted to a pH of 5.3, inoculated with *Aspergillus wentii*, ATCC 10583, and cultured for 48 hours. Then twelve grams of progesterone dissolved in a minimum of acetone was added to the culture and, after fermentation for 24 hours with agitation at 250 revolutions per minute, the resulting 11 α -hydroxyprogesterone was recovered.

Example 9

In the same manner as Example 1, except as otherwise stated, the medium was adjusted to a pH of 6.0, inoculated with *Aspergillus fischeri*, ATCC 1020, and cultured for 48 hours. Then three grams of desoxycorticosterone acetate dissolved in a minimum of acetone was added to the culture and, after fermentation for 24 hours with agitation at 250 revolutions per minute, the resulting epicorticosterone was recovered.

Example 10

In the same manner as Example 9, except as otherwise stated, the inoculant was *Aspergillus clavatus*, ATCC 9598, and the resulting fermented steroids were recovered.

Example 11

In the same manner as Example 9, except as otherwise stated, the inoculant was *Aspergillus nidulans*, ATCC 10074, and the resulting fermented steroids were recovered.

Example 12

In the same manner as Example 9, except as otherwise stated, the medium was adjusted to a

11

pH of 5.9, the inoculant was *Aspergillus ustus*, ATCC 10032, and the resulting fermented steroids, including a good yield of epicorticoosterone, were recovered.

Example 13

To twelve liters of medium having a composition of five grams of soybean meal, twenty grams of Cerelese commercial dextrose, five grams of Pabst debittered brewers' yeast extract, five grams of sodium chloride, and five grams of monobasic potassium phosphate diluted to one liter with tap water and adjusted to a pH of 6.6, sterilized, inoculated with *Aspergillus niger*, ATCC 10577, and cultured for 48 hours, three grams of progesterone dissolved in a minimum of acetone was added. Following a bioconversion time of 48 hours with agitation at 250 revolutions per minute, the resulting fermented steroids were extracted and recovered as in Example 1, with aliquots being subjected to paper chromatography.

Example 14

In the same manner as Example 13, except as otherwise stated, fermentation with *Aspergillus itaconicus*, ATCC 10021, similarly produced 11 α -hydroxyprogesterone.

Example 15

In the same manner as Example 13, except as otherwise stated, the medium was adjusted to a pH of 3.7 and inoculated with *Aspergillus niger*, ATCC 6275, to produce 11 α -hydroxyprogesterone.

Example 16

In the same manner as Example 13, except as otherwise stated, the medium was adjusted to a pH of 6.5 and inoculated with *Aspergillus clavatus*, ATCC 10058. Immediately thereafter, progesterone was dispersed in the culture. After a bioconversion time of 48 hours with agitation at 176 revolutions per minute, the resulting fermented steroid, including 11 α -hydroxyprogesterone, was recovered.

Example 17

In the same manner as Example 13, except as otherwise stated, the medium was adjusted to a pH of 3.7 and inoculated with *Aspergillus ustus*. Immediately thereafter, progesterone was dispersed in the culture. After a bioconversion time of 48 hours with agitation at 250 revolutions per minute, the resulting fermented steroid, including 11 α -hydroxyprogesterone, was recovered.

Example 18

To twelve liters of medium having a composition of fifty grams of Cerelese commercial dextrose, thirty grams of sucrose, two grams of ammonium nitrate, one gram of monobasic potassium phosphate, 0.5 gram of magnesium sulfate heptahydrate, 0.01 gram of ferrous sulfate, 0.2 gram of zinc sulfate, 0.1 gram of manganese sulfate, and two grams of yeast extract diluted to one liter with tap water, adjusted to a pH of 6.65, sterilized, and cultured for 48 hours with *Aspergillus wentii*, ATCC 10583, there was added three grams of progesterone dissolved in a minimum of acetone. Following a bioconversion time of 48 hours with agitation at 250 revolutions per minute, the resulting fermented steroids were extracted and recovered as in Example 1 to yield 11 α -hydroxyprogesterone.

12

Example 19

In the same manner as Example 18, except as otherwise stated, inoculation of the medium with *Aspergillus ochraceus*, ATCC 1009, was immediately followed by the addition of progesterone. After a bioconversion time of 48 hours, the resulting fermented steroid, including 11 α -hydroxyprogesterone, was recovered.

Example 20

In the same manner as Example 18, except as otherwise stated, to a 48-hour culture of *Aspergillus flavus*, ATCC 9170, in the medium which was first adjusted to a pH of 4.5 before sterilization, progesterone was added and fermented for 48 hours with agitation of 360 revolutions per minute, and then the resulting fermented steroid was recovered by extraction and chromatography.

Example 21

Otherwise in the same manner as in Example 18, to a 48-hour culture of *Aspergillus flavus*, ATCC 9170, in the medium which was first adjusted to a pH of 4.5 before sterilization, progesterone was added and fermented for 48 hours with an agitation of 360 revolutions per minute, and then the resulting fermented steroid was recovered by extraction and chromatography.

Example 22

To twelve liters of medium having a composition of twenty grams of corn steep liquor, twenty grams of dextrin, one gram of monobasic potassium phosphate, two grams of sodium nitrate, 0.5 gram of magnesium sulfate, 0.2 gram of potassium chloride, 0.01 gram of ferrous sulfate, and two grams of sodium acetate diluted to one liter with tap water, adjusted to a pH of 3.90, sterilized, and cultured for 48 hours with *Aspergillus flavus*, ATCC 9170, there was added three grams of progesterone dissolved in a minimum of acetone. Following a bioconversion time of 48 hours with agitation at 250 revolutions per minute, the resulting fermented steroids were extracted and recovered as in Example 1 to yield 11 α -hydroxyprogesterone.

Example 23

Twelve liters of the same medium as in Example 22 was inoculated with *Aspergillus ustus*, ATCC 10032, and immediately followed by the addition of three grams of progesterone in a minimum of acetone. After a bioconversion time of 48 hours with agitation at 176 revolutions per minute, the resulting fermented steroids were extracted and recovered as in Example 1 to yield 11 α -hydroxyprogesterone.

Example 24

To twelve liters of Raulin's medium having a composition of fifty grams of dextrose, three grams of tartaric acid, three grams of ammonium nitrate, 0.4 gram of dibasic ammonium phosphate, 0.5 gram of potassium carbonate, 0.2 gram of ammonium sulfate, 0.05 gram of zinc sulfate, 0.05 gram of ferrous sulfate, one gram of sodium acetate, and 0.3 gram of magnesium carbonate diluted to one liter with distilled water, adjusted to a pH of 3.95, sterilized, and cultured for 48 hours with *Aspergillus niger*, ATCC 10549, there was added three grams of progesterone dissolved in a minimum of acetone. Following a bioconversion time of 48 hours with agitation at 250 revolutions per minute, the resulting fermented steroids were extracted and recovered as in Example 1 to yield 11 α -hydroxyprogesterone.

13

Example 25

Twelve liters of the same medium as in Example 24 was inoculated with *Aspergillus nidulans*, ATCC 10074, and immediately followed by the addition of three grams of progesterone in a minimum of acetone. After a bioconversion time of 48 hours with agitation at 176 revolutions per minute, the resulting fermented steroids were extracted and recovered as in Example 1 to yield 11 α -hydroxyprogesterone.

Example 26

In the same manner as Example 24, using medium adjusted to a pH of 4.5 before sterilization, inoculation with *Aspergillus luchuensis*, ATCC 10061, and fermentation with agitation at 250 revolutions per minute produced 11 α -hydroxyprogesterone.

It is to be understood that the invention is not to be limited to the exact details of operation or exact organisms and compounds shown and described, as obvious modifications and equivalents will be apparent to one skilled in the art, and the invention is therefore to be limited only by the scope of the appended claims.

We claim:

1. A process for the introduction of oxygen into a steroid which comprises: growing an *Aspergillus* under aerobic conditions in the presence of a fermentation medium containing assimilable non-steroidal carbon and a steroid having an eleven methylene group and recovering the resulting oxygenated steroid.

2. A process of fermenting a steroid which comprises: growing an *Aspergillus* under submerged aerobic conditions in a fermentation medium containing assimilable non-steroidal carbon and a steroid having an eleven methylene group.

3. A process of fermenting a steroid which comprises: growing an *Aspergillus* under submerged aerobic conditions in a fermentation medium containing carbohydrate and a steroid having an eleven methylene group.

4. A process of fermenting a steroid which comprises: growing an *Aspergillus* under aerobic conditions in a fermentation medium containing carbohydrate and a steroid having an eleven methylene group and up to and including 22 carbon atoms in the carbon to carbon skeleton.

5. A process of fermenting a steroid which comprises: growing an *Aspergillus* under aerobic submerged agitated conditions in a fermentation medium containing assimilable non-steroidal carbon and a steroid substrate, consisting essentially of steroid having an eleven methylene group, and recovering the resulting fermented steroid.

6. A process of oxygenating steroid which comprises: dispersing a steroid substrate, consisting essentially of a 3-keto steroid, in an aqueous nutrient medium, and growing an *Aspergillus* in said medium under aerobic conditions.

7. A process of fermenting a steroid which comprises: dispersing a steroid substrate, consisting essentially of a 3-keto steroid, in an aqueous nutrient medium, growing an *Aspergillus* in said medium under aerobic conditions, and isolating the resulting fermented steroid.

8. A process of fermenting a steroid which comprises: aerobically contacting growing *Aspergillus* with a steroid substrate, consisting essentially of a 3-keto steroid having up to and including 22 carbon atoms in the carbon to carbon skeleton, and recovering the resulting fermented steroid.

14

9. A process of oxygenating a steroid which comprises: growing an *Aspergillus* under aerobic agitated conditions in a nutrient fermentation medium containing assimilable non-steroid carbon and a steroid having an eleven methylene group, and isolating the resulting oxygenated steroid.

10. A process comprising dispersing progesterone in a nutrient fermentation medium, and growing a species of fungus of the genus *Aspergillus* in said medium under aerobic conditions.

11. The process of claim 10 wherein the fungus is *Aspergillus niger*.

12. A process comprising growing a species of fungus of the genus *Aspergillus* under aerobic conditions in a nutrient fermentation medium containing 17 α -hydroxyprogesterone.

13. The process of claim 12 wherein the fungus is *Aspergillus niger*.

14. A process comprising growing a species of fungus of the genus *aspergillus* under aerobic conditions in a nutrient fermentation medium containing 17 α ,21-dihydroxyprogesterone.

15. The process of claim 12 wherein the fungus is *Aspergillus niger*.

16. A process comprising growing a species of fungus of the genus *Aspergillus* under aerobic conditions in a nutrient fermentation medium containing pregnane-3,20-dione.

17. The process of claim 16 wherein the fungus is *Aspergillus niger*.

18. A process comprising growing a species of fungus of the genus *Aspergillus* under aerobic conditions in a nutrient fermentation medium containing a steroid selected from the group consisting of 21-hydroxy-4-pregnene-3,20-dione and 16-dehydroprogesterone.

19. The process of claim 18 wherein the fungus is *Aspergillus niger*.

20. A process which comprises: dispersing a steroid substrate, consisting essentially of a 3-keto steroid, in an aqueous fermentation medium and aerobically subjecting such dispersed 3-keto steroid to the action of viable *Aspergillus niger*.

21. A process which comprises: dispersing a steroid substrate, consisting essentially of a 3-keto steroid, in an aqueous nutrient medium and growing *Aspergillus niger* in said dispersion under aerobic agitated conditions.

22. A process which comprises: dispersing a 3-keto steroid substrate in an aqueous fermentation medium and aerating and agitating said dispersion in the presence of a species of fungus selected from the group consisting of *Aspergillus clavatus*, *Aspergillus fischeri*, *Aspergillus flavus*, *Aspergillus itaconicus*, *Aspergillus luchuensis*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ustus*, and *Aspergillus wentii*.

23. A process of oxygenating a steroid which comprises: growing an *Aspergillus* under aerobic submerged conditions in a fermentation medium containing assimilable nitrogen, phosphate, carbohydrate, and a steroid substrate, consisting essentially of a steroid having an eleven methylene group, and recovering the resulting oxygenated steroid.

24. A process which comprises: dispersing a 3-keto steroid having an 11-methylene group in an aqueous fermentation medium containing assimilable nitrogen, phosphate and carbohydrate, and therein growing under aerobic agitated conditions a species of fungus selected from

the group consisting of *Aspergillus clavatus*, *Aspergillus fischeri*, *Aspergillus flavus*, *Aspergillus itaconicus*, *Aspergillus luchuensis*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ustus*, and *Aspergillus wentii*.

25. A process which comprises: dispersing progesterone in an aqueous fermentation medium containing assimilable nitrogen, phosphate and carbohydrate, and therein growing under aerobic agitated conditions a species of fungus selected from the group consisting of *Aspergillus clavatus*, *Aspergillus fischeri*, *Aspergillus flavus*, *Aspergillus itaconicus*, *Aspergillus luchuensis*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ustus*, and *Aspergillus wentii*.

26. A process which comprises: growing a species of fungus selected from the group consisting of *Aspergillus clavatus*, *Aspergillus fischeri*, *Aspergillus flavus*, *Aspergillus itaconicus*, *Aspergillus luchuensis*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ustus*, and *Aspergillus wentii* under aerobic agitated conditions in a fermentation medium containing assimilable nitrogen, phosphate, carbohydrate, and 17 α -hydroxyprogesterone.

27. A process which comprises: growing a species of fungus selected from the group consisting of *Aspergillus clavatus*, *Aspergillus fischeri*, *Aspergillus flavus*, *Aspergillus itaconicus*, *Aspergillus luchuensis*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ustus*, and *Aspergillus wentii* under aerobic agitated conditions in a fermentation medium containing assimilable nitrogen, phosphate, carbohydrate, and 17 α ,21-dihydroxyprogesterone.

28. A process which comprises: growing a species of fungus selected from the group consisting of *Aspergillus clavatus*, *Aspergillus fischeri*, *Aspergillus flavus*, *Aspergillus itaconicus*, *Aspergillus luchuensis*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ustus*, and *Aspergillus wentii* under aerobic agitated conditions in a fermentation medium containing assimilable nitrogen, phosphate, carbohydrate, and pregnane-3,20-dione.

29. A process which comprises: growing a species of fungus selected from the group consisting of *Aspergillus clavatus*, *Aspergillus fischeri*, *Aspergillus flavus*, *Aspergillus itaconicus*, *Aspergillus luchuensis*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus ustus*, and *Aspergillus wentii* under aerobic agitated conditions in a fermentation medium containing assimilable nitrogen, phosphate, carbohydrate and a steroid selected from the group consisting of 21-hydroxy-4-pregnene-3,20-dione and 16-dehydroprogesterone.

30. A process for the production of an oxygenated steroid comprising the steps of aerobically growing a culture of an oxygenating strain of an organism of the *Aspergillus* genus, and exposing a steroid to the oxygenating activity of enzymes produced by the said culture.

31. A process for the production of an eleven oxygenated steroid comprising the aerobic fermentation of a nutrient-containing substrate, containing a steroid having an eleven methylene group, by means of a submerged growth of an organism of the genus *Aspergillus*.

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References Cited in the file of this patent

UNITED STATES PATENTS

Number	Name	Date
1,893,819	Currie et al.	Jan. 10, 1933
2,006,086	May et al.	June 25, 1933
2,236,574	Koester et al.	Apr. 1, 1941

OTHER REFERENCES

- Chem. Abstracts, vol. 7, page 1739 (1913).
Chem. Abstracts, vol 30, page 4534 (1936).
Turfitt, J. Bact., vol. 54, (1947) page 562.
Kamli et al., Nature, vol. 162, October 16, 1948, page 619.
Peterson et al., J. Am. Soc., vol. 74, pages 1871-2, April 5, 1952.
Colingsworth et al., J. Am. Chem. Soc., vol. 74, pages 2381-82, May 5, 1952.