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STERIODS

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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 *Penicillium*.

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 *Penicillium*, e. g., *Penicillium janthinellum*, 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 *Penicillium*. 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 *Penicillium*. Another object of the invention is to provide a process of oxygenating 3-keto steroids by means of *Penicillia*. Still another object of the invention is the provision of a process of modifying steroid structure by means of *Peni-*

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cillia. Other and more particularly objects of the invention will become apparent hereinafter.

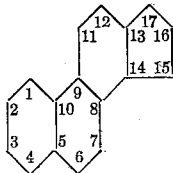
It has now been found that eleven desoxy steroids, which contain the cyclopentanopolyhydrophenanthrene nucleus, especially the 10,13 - dimethylcyclopentanopolyhydrophenanthrenes, can be readily converted in high yields to corresponding oxygenated steroids by subjecting the steroid compound to the action of a species of fungus of the genus *Penicillium*. 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 *Penicillium*. 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 thereinto. 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 pro-

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cedure 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 unoxygenated or oxygenatable position, such as, for example, an unoxygenated eleven position; illustratively, a methylene group, as in an eleven 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,-oxidopregnane-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-allopregnane-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-oxidopregnane-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) - dioxidopregnane-20-one, 3 β - hydroxy-5(6),16(17)-dioxido-21-bromo-

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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-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; ergosterol, stigmaterol, stigmastanol, and 3-acyl, e. g. acetyl, esters of the foregoing; ergostenone, stigmastanone, stigmastanone, cholestenone, cholic acid, desoxycholic acid, lithocholic acid, cholanic acid, norcholanic acid, bisnorcholanic 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 β -methyltestosterone, 17 α -methyl-17a-hydroxy-D-homo - 4 - androstene-3,17-dione, and their 4,5-dihydro, ring A saturated analogs, D-homo-androstene-3 α - or 3 β -hydroxyandrostene-17a-one), and D-homo-epidehydroandrostene (3 β -hydroxy-D-homo-4-androstene-16a-one). All of these are amenable to fermentation with *Penicillium* 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 *Penicillium*. The genus *Penicillium* belongs to the family Aspergillaceae of the order Aspergillales (Plectascales). The classification and definition of *Penicillium* as herein employed is that of Raper, K. B., and Thom, C., "A Manual of the *Penicillia*," Williams and Wilkins Company, Baltimore, 1949. Among species of the genus *Penicillium* useful in the fermentation of steroids may be mentioned those of the group or subgenus: *Monoverticillata*, *Asymmetrica-divaricata*, *Asymmetrica-velutina*, *Asymmetrica-lanata*, *Asymmetrica-funiculosa*, *Asymmetrica-fasci-*

culata, Biverticillata-symmetrica, and Polyverticillata, for example: *Penicillium adametzi*, *brevi-compactum*, *camemberti*, *canescens*, *cap-sulatum*, *charlesii*, *citrinum*, *claviforme*, *com-mune*, *cyclopium*, *decumbens*, *digitatum*, *duclauxi*, *expansum*, *frequentans*, *funiculosum*, *fuscum*, *gladioli*, *granulatum*, *herquei*, *implicatum*, *itali-cum*, *janthinellum*, *lavendulum*, *levitum*, *lilaci-num*, *luteum*, *nigricans*, *notatum*, *novae-zeelandi-ae*, *ochraceum*, *oxalicum*, *pallidum*, *purpuro-genum*, *purpurogenum* var. *rubrisclerotium*, *raistrickii*, *roqueforti*, *roseo-purpureum*, *ruglo-sum*, *terrestre*, *thomii*, *urticae*, and *viridicatum*.

Culture of the fungi, for the purpose and prac-tice 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 ma-terial, such as copra, chestnuts, or lupine seeds may be used. These can be extracted with al-cohol, ether or other organic solvents, to remove objectionable contaminants and growth inhibitors prior to fermentation. The carriers may option-ally contain added growth factors and nutrients and may be used in layers or trays with or with-out 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 adapt-ed 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 condi-tions.

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 pro-teins. 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 as-similable 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 pro-teins, soybean meal, lactalbumin, casein, egg al-bumin, peptones, polypeptides or amino acids, urea, ammonium salts, ammonia trapped on base exchange resins or on zeolites, ammonium chlo-ride, sodium nitrate, potassium nitrate, mor-pholine. Whey, distillers solubles, corn steep liquor, or yeast extract have been useful.

As mineral constituents the media or menstru-m may contain, naturally present or added, available aluminum, calcium, chromium, cobalt, copper, gallium, iron, magnesium, molybdenum, potassium, scandium, uranium, vanadium, and boron. Sulfur may be provided by sulfates, alkyl sulfonates, sulfoxylates, sulfamates, sulfinate, free sulfur, hyposulfite, persulfate, thiosulfate, methionine, cystine, cysteine, 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 ovovitell-

lin. Boron, iodine and selenium 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 fungus.

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 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, pep-tones or amino acids; and mineral constituents, illustratively phosphates and magnesium sulfate; and other art recognized, desirable or adventi-tious, 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 4 to 6 is preferred for the growth of *Penicillium*. Low pH values in-hibit bacterial contamination and facilitate sterilization. For example, at a pH of 2 to 3, ef-fective sterilization of media may be accom-plished by heating the media for thirty minutes at 100 degrees centigrade, whereas, at a pH of 4 to 4.5, thermal sterilization may require super-atmospheric pressure. Alternatively or con-comitantly, bacterial contamination may be re-tarded by the presence of antiseptic or anti-biotic agents such as benzoates, sulfites, pencil-lin or circulin.

Inoculation of the fungal growth-supporting medium with the selected fungus of the genus *Penicillium* may be accomplished in any suit-able manner. *Penicillia* grow over a wide range of temperatures from about eight degrees centi-grade to about 35 degrees centigrade and prefer-ably at or about room temperature or between about fifteen degrees centigrade and 30 degrees centigrade.

The developmental period of fungal growth re-quired before the steroid to be fermented is ex-posed to the fungus does not appear to be critical. For example, the steroid may be added either before thermal or other sterilization of the me-dium, at the time of inoculating the medium with the selected *Penicillium* species, or at some time, as 24 to 48 hours, later. The steroid to be fer-mented may be added at any suitable concentra-tion although for practical reasons steroid sub-strate 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, de-pending upon the particular steroid, may be used with some inhibition of mycelial development. Either a purified steroid, a crude material con-taining 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 ad-dition 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. The *Penicillium* fermentation processes as illustratively disclosed in United States Patents 2,458,495 and 2,538,721 or aerobic processes using other penicillia such as *P. cycloptum*, *P. nigricans*, *P. notatum* and *P. roqueforti*, with the addition of steroids as above described, are useful in the simultaneous oxygenation of steroids and the production of *Penicillium* 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

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, 0.95 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 was adjusted to a pH of 7.0 with hydrochloric acid and sodium hydroxide. Twelve liters of this medium was sterilized by heat. The pH after sterilization was 6.35. This sterile medium was maintained at room temperature and inoculated with spores of *Penicillium adametzi*, ATCC 10497; the aeration was adjusted to one liter per minute and the agitation to 200 revolutions per minute. After 66 hours of growth, the pH was 3.75 and three grams of progesterone dissolved in 160 milliliters of acetone was added. After 47 hours of bio-conversion time the fermentation was stopped. The final pH was 3.4.

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 portions 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, weighed 1.6388 grams. This residue was dissolved in 100 milliliters of benzene and chromatographed over 50 grams of alumina (hydrochloric acid washed, water washed, and dried at 120 degrees centigrade) using 100-milliliter portions of developing solvent as indicated in Table I. Paper chromatography analysis using a toluene-propylene glycol system demonstrated production of a steroid component having a mobility similar to but different from 14 α -hydroxyprogesterone. Fractions 13 through 16 were combined, dissolved in methylene chloride, filtered, and evaporated to a syrup of three milliliters. To this syrup, thirty milliliters of ether was added with stirring to effect immediate precipitation of small white crystals weighing 0.425 gram, melting at 200 to 208 degrees centigrade, and having a melting point upon recrystallization of 208 to 209 degrees centigrade, an $[\alpha]_D^{23}$ of plus 43 degrees (1.00 in chloroform), and an analysis of C₂₅H₃₄O₄. Infrared spectrum showed this compound to contain a 3-keto- Δ 4 grouping, 20-keto, an additional carbonyl group and probably an ether group. This compound has estrogenic activity.

Chromatographic analysis Table I

Fraction	Solvent	Eluate Solids, Milligrams
1	benzene	91.5
2	do	46.6
3	benzene-ether 20:1	22.3
4	do	39.6
5	benzene-ether 10:1	7.1
6	do	5.5
7	benzene-ether 1:1	14.0
8	do	33.0
9	ether	27.0
10	do	19.0
11	ether-chloroform 20:1	64.0
12	do	153.0
13	ether-chloroform 10:1	123.0
14	do	133.0
15	ether-chloroform 1:1	142.0
16	do	75.0
17	do	26
18	do	20
19	chloroform	80
20	do	76
21	do	41
22	do	25
23	chloroform-acetone 20:1	41
24	acetone	88
25	methanol	11
26	do	
27	do	

EXAMPLE 2

Otherwise in the same manner as Example 1, the inoculant was *Penicillium raistrickii*, ATCC 10490.

EXAMPLE 3

Otherwise in the same manner as Example 1, the medium was adjusted to pH 4.2 before sterilization and the inoculant was *Penicillium canescens*, ATCC 10419.

EXAMPLE 4

Otherwise in the same manner as Example 1, the medium was adjusted to pH 7 before sterilization, the inoculant was *Penicillium urticae*, ATCC 10120, and the agitation was 160 revolutions per minute. After 66 hours of growth, three grams of progesterone dissolved in 160 milliliters of acetone was added and fermentation was continued for 47 hours at which time the pH was 4.2.

Extraction as in Example 1 produced 4.196

grams of crude extract solids. This residue was dissolved in 300 milliliters of benzene and chromatographed over 150 grams of alumina (hydrochloric acid washed, water washed, and dried at 120 degrees centigrade for four hours) using 300-milliliter portions of developing solvent as indicated in Table II.

Fractions 20 and 21 were combined and crystallized from three milliliters of methylene chloride by the addition of forty milliliters of ether to produce fine white crystals which were filtered, and washed with ether to give 745.7 milligrams of crystals melting at 205 to 218 degrees centigrade. Recrystallization from 25 milliliters of ethyl acetate gave 632 milligrams of crystals of melting point 219 to 228 degrees centigrade. Recrystallization twice more from methylene chloride-ether (1:10) gave 517.5 milligrams of crystalline product having a melting point of 234 to 235 degrees centigrade, and progestational activity. According to infrared spectra, this is a new monohydroxyprogesterone.

Fractions 5 through 10 were combined and crystallized from five milliliters of methylene chloride by the addition of fifty milliliters of ether. After refrigerating for two hours, the crystals were filtered and washed with ether. Recrystallization from fifteen milliliters of ethyl acetate followed by recrystallization from seven milliliters of ethyl acetate gave 0.259 milligram of allopregnanedione having a melting point of 185 to 198 degrees centigrade.

Chromatographic analysis Table II

Fraction	Solvent	Eluate Solids, Milligrams
1	benzene	98
2	do	35
3	benzene-ether 20:1	24
4	do	54
5	benzene-ether 10:1	124
6	do	177
7	benzene-ether 1:1	206
8	do	245
9	ether	191
10	do	142
11	ether-chloroform 20:1	68
12	do	64
13	ether-chloroform 10:1	13
14	do	12
15	ether-chloroform 1:1	17
16	do	71
17	do	104
18	do	93
19	chloroform	42
20	do	632
21	do	301
22	do	84
23	chloroform-acetone 20:1	42
24	acetone	51
25	methanol	151
26	do	13
27	do	17

EXAMPLE 5

Otherwise in the same manner as Example 1, the medium was adjusted to pH 4.5 before sterilization, the inoculant was *Penicillium luteum*, ATCC 10465, and the agitation was 250 revolutions per minute. After a culture development time of 96 hours, progesterone was added for a bioconversion time of 92 hours and the resulting fermented steroid was recovered.

EXAMPLE 6

Otherwise in the same manner as Example 1, the medium was adjusted to pH 4.0 before sterilization, and inoculated with *Penicillium expansum*, ATCC 7861. Progesterone was added with no previous culture time. After a bioconversion time of 48 hours, the resulting fermented steroid was extracted and recovered.

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EXAMPLE 7

Otherwise in the same manner as Example 1, the medium was adjusted to pH 6.65 before sterilization, inoculated with *Penicillium notatum*, ATCC 9479, and agitated at 250 revolutions per minute. Progesterone was added with no previous culture time. After a bioconversion time of 48 hours, the resulting fermented steroid was extracted and recovered.

EXAMPLE 8

In the same manner as Example 1, the starting steroid was 17 α -hydroxyprogesterone and the resulting fermented steroid was recovered.

EXAMPLE 9

In the same manner as Example 1, the starting steroid was 17 α ,21-dihydroxyprogesterone and the resulting fermented steroid was recovered.

EXAMPLE 10

In the same manner as Example 1, the starting steroid was pregnane-3,20-dione and the resulting fermented steroid was recovered.

EXAMPLE 11

In the same manner as Example 1, the starting steroid was 21-hydroxy-4-pregnene-3,20-dione and the resulting fermented steroid was recovered.

EXAMPLE 12

In the same manner as Example 1, the starting steroid was 16-dehydropregesterone and the resulting fermented steroid was recovered.

EXAMPLE 13

To twelve liters of a medium having a composition of fifty grams of Cerelose 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, inoculated with *Penicillium urticae*, ATCC 10120, and cultured for 48 hours with agitation of 176 revolutions per minute, three grams of progesterone dissolved in a minimum of acetone was added. Following a bioconversion time of 48 hours, the resulting fermented steroids were extracted and recovered as in Example 1.

EXAMPLE 14

Otherwise in the same manner as Example 13, the medium was adjusted to an initial pH of 3.5, the inoculant was *Penicillium lilacinum*, ATCC 10114, the agitation was 250 revolutions per minute, and the resulting fermented steroids were recovered.

EXAMPLE 15

Otherwise in the same manner as Example 13, the medium was adjusted before sterilization to a pH of 3.5, was inoculated with *Penicillium brevi-compactum*, ATCC 9056, and agitated at 176 revolutions per minute. With no previous culture time, progesterone was added. After a bioconversion time of 48 hours, the resulting fermented steroids were recovered.

EXAMPLE 16

Otherwise in the same manner as Example 15, the inoculant was *Penicillium citrinum*, ATCC 10105.

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EXAMPLE 17

To twelve liters of medium having a composition of five grams of soybean meal, twenty grams of Cerelose 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 4.0, sterilized, inoculated with *Penicillium thomii*, ATCC 10506, 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 176 revolutions per minute, the resulting fermented steroids were extracted and recovered as in Example 1, with aliquots being subjected to paper chromatography.

EXAMPLE 18

In the same manner as Example 17, the medium adjusted to an initial pH of 6.55, inoculated with *Penicillium brevi-compactum*, ATCC 4056, and agitated at 250 revolutions per minute produced fermented steroids which were recovered.

EXAMPLE 19

Otherwise in the same manner as Example 17, the medium was inoculated with *Penicillium lilacinum*, ATCC 10114, and agitated at 176 revolutions per minute. With no previous culture time, progesterone was added. After a bioconversion time of 48 hours, the resulting fermented steroids were recovered.

EXAMPLE 20

In the same manner as Example 17, the medium was adjusted to an initial pH of 4.1, inoculated with *Penicillium novae zeelandiae*, ATCC 10473, and agitated at 176 revolutions per minute. With no previous culture time, progesterone was added. After a bioconversion time of 48 hours, the resulting fermented steroids were recovered.

EXAMPLE 21

In the same manner as Example 17, the medium was inoculated with *Penicillium urticae*, ATCC 10120, and agitated at 250 revolutions per minute. Progesterone was added with no previous culture time. After a bioconversion time of 48 hours, the resulting fermented steroids were recovered.

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 4.6, sterilized, and cultured for 48 hours with *Penicillium nigricans*, ATCC 10115, 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.

EXAMPLE 23

Otherwise in the same manner as Example 22, the medium was adjusted before sterilization to a pH of 4.35, inoculated with *Penicillium oxalicum*, ATCC 10476, and agitated at 176 revolutions per minute. Progesterone was added with

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no previous culture time. After a bioconversion time of 48 hours, the resulting fermented steroids were extracted and recovered as in Example 1.

EXAMPLE 24

A medium was prepared from five milliliters of corn steep liquor, twenty grams of Edamine commercial lactalbumin digest and fifty milligrams of Cerelese commercial dextrose per liter of tap water and adjusted to a pH of between about 5.5 and 5.9. To four liters of this medium containing a 24-hour growth, at room temperature with aeration, of *Penicillium janthinellum*, ATCC 10455, was added one gram of progesterone in fifty milliliters of acetone. After a bioconversion time of 48 hours at room temperature with aeration, the fermented steroid was extracted and recovered as in Example 1.

EXAMPLE 25

To twelve liters of medium having a composition of twenty grams of corn steep liquor, twenty grams of dextrose, 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 4.7, sterilized, and cultured with *Penicillium janthinellum*, ATCC 10455, with agitation at 176 revolutions per minute, there was added three grams of progesterone dissolved in a minimum of acetone. Following a bioconversion time of 48 hours, the resulting fermented steroids were extracted and recovered as in Example 1.

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 a *Penicillium* 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 a *Penicillium* under submerged aerobic conditions in a fermentation medium containing assimilable non-steroidal carbon and a steroid having an eleven methylene group, and recovering the resulting oxygenated steroid.

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

4. A process of oxygenating a steroid which comprises: aerobically subjecting a steroid containing an eleven methylene group to the oxygenating activity of a growth of *Penicillium* and isolating the resulting oxygenated steroid.

5. A process of fermenting a steroid which comprises: growing a *Penicillium* 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

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group, and recovering the resulting fermented steroid.

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

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

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

10. A process of oxygenating a steroid which comprises: growing a *Penicillium* under aerobic agitated conditions in a nutrient fermentation medium containing carbohydrate and a steroid and extracting the resulting oxygenated steroid.

11. A process which comprises: growing *Penicillium chrysogenum* under aerobic agitated conditions in a nutrient fermentation medium containing carbohydrate and steroid and extracting the resulting fermented steroid.

12. A process which comprises: dispersing a steroid substrate, consisting essentially of a steroid, having an eleven methylene group and up to and including 22 carbon atoms in the carbon to carbon skeleton, in a fermentation medium and subjecting such dispersed steroid to the action of viable *Penicillium* under aerobic agitated conditions and separating the resulting fermented steroid.

13. A process comprising growing a species of fungus of the genus *Penicillium* under aerobic agitated conditions in a nutrient fermentation medium containing a steroid substrate, consisting essentially of a steroid having an eleven methylene group and up to and including 22 carbon atoms in the carbon to carbon skeleton, and recovering the resulting oxygenated steroid.

14. A process comprising growing a species of fungus of the genus *Penicillium* under aerobic conditions in a nutrient fermentation medium containing progesterone.

15. A process comprising dispersing progesterone in a nutrient fermentation medium, and growing *Penicillium chrysogenum* in said medium under aerobic conditions.

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

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

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

19. A process comprising growing a species of fungus of the genus *Penicillium* 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.

20. A process which comprises: dispersing a steroid substrate, consisting essentially of a steroid having an eleven methylene group and up to and including 22 carbon atoms in the carbon to carbon skeleton, 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 *Penicillium adametzi*, *Penicillium brevi-compactum*, *Penicillium citrinum*, *Penicillium nigricans*, *Penicillium oxalicum*, and *Penicillium raistricki*.

21. A process of oxygenating a steroid which comprises: growing a *Penicillium* under aerobic submerged agitated 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.

22. A process which comprises: dispersing a 3-keto steroid having an eleven methylene group in an aqueous fermentation medium containing assimilable nitrogen, phosphate and carbohydrate, and therein growing under aerobic agitated conditions a species of fungus of the genus *Penicillium*.

23. A process which comprises: growing a species of fungus selected from the group consisting of *Penicillium adametzi*, *Penicillium brevi-compactum*, *Penicillium citrinum*, *Penicillium nigricans*, *Penicillium oxalicum*, and *Penicillium janthinellum* under aerobic agitated 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 up to and including 22 carbon atoms in the carbon to carbon skeleton.

24. A process which comprises: growing a species of fungus selected from the group consisting of *Penicillium adametzi*, *Penicillium brevi-compactum*, *Penicillium citrinum*, *Penicillium nigricans*, *Penicillium oxalicum*, and *Penicillium janthinellum* under aerobic agitated conditions in a fermentation medium containing assimilable nitrogen, phosphate, carbohydrate, and progesterone.

25. A process which comprises: growing a species of fungus selected from the group consisting of *Penicillium adametzi*, *Penicillium brevi-compactum*, *Penicillium citrinum*, *Penicillium nigricans*, *Penicillium oxalicum*, and *Penicillium janthinellum* under aerobic agitated conditions in a fermentation medium containing assimilable

nitrogen, phosphate, carbohydrate, and 17 α -hydroxyprogesterone.

26. A process which comprises: growing a species of fungus selected from the group consisting of *Penicillium adametzi*, *Penicillium brevi-compactum*, *Penicillium citrinum*, *Penicillium nigricans*, *Penicillium oxalicum*, and *Penicillium janthinellum* under aerobic agitated conditions in a fermentation medium containing assimilable nitrogen, phosphate, carbohydrate, and 17 α ,21-dihydroxyprogesterone.

27. A process which comprises: growing a species of fungus selected from the group consisting of *Penicillium adametzi*, *Penicillium brevi-compactum*, *Penicillium citrinum*, *Penicillium nigricans*, *Penicillium oxalicum*, and *Penicillium janthinellum* under aerobic agitated conditions in a fermentation medium containing assimilable nitrogen, phosphate, carbohydrate, and pregnane-3,20-dione.

28. A process which comprises: growing a species of fungus selected from the group consisting of *Penicillium adametzi*, *Penicillium brevi-compactum*, *Penicillium citrinum*, *Penicillium nigricans*, *Penicillium oxalicum*, and *Penicillium janthinellum* 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.

29. 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 *Penicillium* genus, and exposing a steroid to the oxygenating activity of enzymes produced by the said culture.

30. 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 *Penicillium*.

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