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PROCESS FOR THE OXYGENATION OF STEROIDS WITH NIGROSPORA

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This invention relates to a novel method for the introduction of oxygen into a steroid molecule, and is particularly concerned with the process of oxygenating steroids by subjecting them to the oxygenating activity of fungi of the genus Nigrospora.

Various processes have previously been described for the bio-oxygenation of steroids by the use of organisms entirely different from those employed in the process of the present invention. The process of transforming steroids by means of the oxygenating activity of fungi of the genus Nigrospora affords certain advantages and improvements over the bio-oxygenation processes of the prior art. One of the important and novel results of the process of the present invention is that it provides means for the introduction of an oxygen atom into position 15 of the steroid molecule. Steroid compounds oxygenated at the 15 position have useful physiological properties, but have not heretofore been conveniently or economically available from known synthetic or biochemical processes. The process of the present invention provides an excellent route to oxygenated steroids, including 15-oxygenated steroids, and constitutes an important advance over the prior art.

The organisms employed in the process of the present invention are oxygenating strains of fungi of the genus Nigrospora, which genus belongs to the order Moniliales of the class Fungi Imperfecti. Comprehended within the genus Nigrospora are various strains which have sometimes, especially in the earlier literature, been assigned different names. The classification of E. W. Mason, Transactions of the British Mycological Society, 12, 152–165 (1927) proposes the acceptance of three species of this genus, *N. oryzae, N. sphaerica* and *N. sacchari.* In this classification strains included within *N. oryzae* are *Monotospora oryzae, Memnonium palmicolum, Trichosporium palmicolum, Acremoniella occulta, Basisporium gallarum, Glenospora elasticate,* and possibly *Sporotrichum maydis, Trichosporium maydis,* and *Mycogone nigra.* Strains included within *N. sphaerica* are *Trichosporium sphaericum, Hadrotrichum arundinaceum, Epicoccum levisporum, Epicoccum hyalopes, Coniosporium gecevi, Coniosporium extremorum,* and possibly *Monotospora nigra. N. sacchari* is used synonymously with the earlier designation *Glenospora sacchari.* Other strains of organisms of the genus Nigrospora have appeared under the names *N. panici, N. javanica* and possibly *Coniosporium micans.*

Cultures of organisms of the genus Nigrospora, suitable for carrying out the process of this invention, can be obtained from public culture collections. Alternatively, organisms of this fungal genus can be isolated from natural sources by standard procedures and techniques well known to microbiologists. Nigrospora fungi in nature are found growing on such diverse vegetative sources as rice, corn, wheat, bananas, tomatoes and sugar cane.

In carrying out the process of the present invention the selected strain of organism is cultured on a medium favorable to its propagation. Moist cereal grits, illustratively hominy grits, are suitable media for the propagation of Nigrospora in the laboratory. However, for the process of the present invention, synthetic or partially synthetic liquid media are generally preferred, as such liquid media afford certain advantages. Incorporation of available carbon, nitrogen, minerals and accessory nutrient factors is facilitated by the use of a liquid medium. Use of a liquid culture medium affords additional advantages in permitting efficint aeration, intimate contact of the steroid substrate with the oxygenating enzymes of the fungus and conveniences in the isolation of the final product. Such a culture medium can incorporate a carbohydrate as a source of available carbon, and proteins, hydrolysis products of proteins, or inorganic nitrates or ammonium salts as a source of available nitrogen. Additional nutrient factors can be supplied by the incorporation of corn steep liquor into the medium. Mineral constituents and other accessory growth factors recognized in the prior art as being necessary or desirable for the growth of fungi are usually naturally present in the culture media described hereinafter. When synthetic media are employed which are deficient in minerals and other accessory growth factors, such constituents are added as required.

The sterilized culture medium is inoculated with the selected species of Nigrospora fungus and growth of the fungus is allowed to proceed under aerobic conditions at a suitable temperature. Temperatures within the range of 20–30° C. have been found desirable for the propagation of Nigrospora fungi although appreciable growth also takes place outside of these limits. When culture media are used which are primarily solid and particulate, such as moist cereal grits, satisfactory aeration is achieved by such means as growing the fungus in relatively flat layers or trays or by growing it in a rotating drum. Aeration of liquid culture media is conveniently accomplished by agitation such as is achieved by stirring or by use of a shaking platform, by blowing air through the culture medium, or by a combination of these means.

The exact time and manner of introducing the steroidal substrate to the orygenating activity of the Nigrospora fungus is a relatively non-critical factor and these operating details can be selected from within fairly wide limits. For example, the steroid can be added to the culture medium prior to sterilization, at the time of inoculation of the medium with the fungus, or after the fungus has been allowed to grow for a period of time, conveniently up to 48 hours. The steroid to be oxygenated can be added to the culture medium as a solid, a suspension, or a solution. A desirably fine state of dispersion of the steroid is achieved by adding it to the fermentation medium as a solution in a small amount of ethanol. Oxygenation of the steroid can be achieved by subjecting it to the action of the entire culture medium, or alternatively, with the use of procedures known to the prior art, by subjecting it to the action of the separated oxygenating enzymes, free of nutritional factors which are needed for the growth of the fungus.

Satisfactory yields of oxygenated product are obtained by allowing a contact time of, typically, 24 or 48 hours, although substantial conversion begins almost immediately after the addition of the steroid to the fermentation mixture. The minimum period of time required for a satisfactory degree of conversion of the steroid depends on various factors such as the stage of growth of the fungus at the time of the addition of the steroid, the temperature of fermentaiton and the rate of aeration. In general, extensive growth of the fungus before the addition of the steroid substrate tends to lessen the contact time required for satisfactory oxygenation of the steroid. A period of fungal growth of 48 hours, followed by a steroid contact

time of 24 hours, has been found to give generally good results when the process of the present invention is carried out as described in the examples given hereinafter.

At the conclusion of the fermentation period, the resulting steroid or mixture of steroids is recovered from the fermentation mixture. Depending on the contact time and other operating factors, the recovered steroidal product may contain unreacted starting material as well as oxygenated steroid. The crude steroidal product can be recovered by extracting the fermentation mixture with a water-immiscible solvent. Water-immiscible ethers, esters and chlorinated hydrocarbons are especially suitable for this purpose. It is convenient to separate the mycelium from the fermentation liquor by filtration prior to extraction with the organic solvent, as emulsions are minimized and the mycelium and liquid phase are more easily processed when they are treated separately. The mycelium and filtrate are both extracted with a suitable solvent and the combined extract, which can be washed with dilute aqueous sodium bicarbonate solution and with water, is concentrated to dryness, affording a crude steroidal product which usually contains other components such as nitrogenous materials. Purification by chromatographic fractionation or by recrystallization affords the desired oxygenation products.

Steroidal compounds which are oxygenated in the 15 position are physiologically active and are useful in chemotherapeutics. They have hormonal properties and are useful in the treatment of states of hormonal deficiencies. The compounds described herein are valuble hypotensive agents; for example, both the 15-hydroxyprogesterone and the dihydroxyprogesterone resulting, as described hereinafter, from the fermentation of progesterone with Nigrospora produce a fall in blood pressure following their administration. Since Nigrospora fungi are capable of oxygenation at position 11 as well as at position 15, as disclosed hereinafter, the process of the present invention is also useful for the production of intermediates of value in the synthesis of adrenocortical hormones.

This invention will appear more fully from the examples which follow. These examples are set forth by way of illustration only and it will be understood that the invention is not to be construed as limited in spirit or in scope by the details contained therein. It will be apparent to those skilled in the art that many modifications in materials and methods can be made without departing from the scope of this invention. In these examples, temperatures are given in degrees centigrade (°C.). Except where otherwise indicated, quantities of materials are given as parts by weight.

EXAMPLE 1

A nutrient medium is prepared, having a composition of 20 grams of a commercial enzymatic digest of lactalbumin, 50 grams of dextrose and 3 grams of corn steep liquor, diluted to 1 liter with tap water and adjusted to a pH of about 4.5 with concentrated hydrochloric acid. A total of 8 liters of this medium is dispensed in equal portions of 400 milliliters into 20 Erlenmeyer flasks having a capacity of 2 liters each. The flasks are plugged with non-absorbent cotton, and are sterilized by heating them in an autoclave for 5 minutes at about 120° C. The flasks are cooled and the contents of each are inoculated with a culture of *Nigrospora oryzae*, American Type Culture Collection No. 8667. They are then mounted on a shaking platform which operates in a manner such that every point on the platform describes a circle of about 2¼ inches in diameter at a rate of approximately 225 revolutions per minute. While these specific operating conditions are recommended, it has been found that within fairly wide limits the rate of aeration of the culture media is not a critical factor, and satisfactory results are obtained with other rates of agitation or by other means of aeration, such as by the introduction of sterilized air or oxygen through an inlet tube. Growth of the organism is allowed to proceed for about 48 hours. To each of the flasks there is then added a solution of 100 milligrams of progesterone in 3 milliliters of ethanol. Incubation is continued on the shaking platform at approximately 25° C. for 26 hours after the addition of the steroid substrate. After this period of fermentative oxygenation of the steroid, the contents of the 20 flasks are combined and filtered in order to separate the mycelium from the liquid phase. The mycelium is washed with 2.8 liters of methylene chloride, and this wash liquor is added to fresh methylene chloride which is used to extract the aqueous filtrate. The aqueous filtrate is extracted with a total of 11.2 liters of methylene chloride in 2 portions. The combined methylene chloride extracts are concentrated to dryness under reduced pressure, affording about 4.2 grams of a non-crystalline residue. This material is dissolved in a 10 volume percent solution of ethyl acetate in benzene and fractionated on a chromatography column prepared from 420 grams of silica. The column is developed with 1 liter portions of solvents. Results of a representative fractionation are given in Table I and in the text which follows. It will be obvious to anyone possessing ordinary skill in chromatographic separations that the absolute positions of elution from the column are subject to variations in different runs, and that residues from successive eluates are combined for purification on the basis of evidence of substantial homogeneity, such as is afforded by a showing that successive residues have identical or similar melting points.

Table I

Fractions	Solvent, Volume Percent Ethyl Acetate in Benzene	Eluate Solids, Milligrams
1-6	10	593
7-10	15	53
11-16	20	877
17-20	25	113
21-22	30	53
23-24	30	117
25	35	52
26	35	41
27	40	33
28-32	40	426
33	40	52
34	40	57
35-38	50	300
39-40	50	126
41-44	60	219
45-46	70	94
47-48	80	175
49-50	100% Ethyl Acetate	329

The crystalline residues from fractions 23 through 25, washed with ether, melt in the range of 120–166° C. Purification by recrystallization from a mixture of acetone and cyclohexane, then from dilute acetone, and finally again from a mixture of acetone and cyclohexane affords a compound melting at about 187–188° C. and exhibiting a specific rotation of about +176.5° in chloroform solution. The crystalline residues from fractions 28 through 32, washed with ether, melt in the range of 215–229° C. They are combined and recrystallized from acetone and then from dilute methanol to yield 15α-hydroxy-4-pregnene-3,20-dione (15α-hydroxyprogesterone) melting at 228–230° C. This preparation exhibits a specific rotation of +226° in chloroform solution and an ultraviolet absorption maximum at 241 millimicrons with a molecular extinction coefficient of 18,200. The crystalline residues from fractions 34 through 38, washed with ether, melt in the range of 150–164° C. and consist chiefly of a diketopiperazine. The crystalline residues from fractions 49 and 50, washed with ether, melt in the range of 180–190° C. They are combined and recrystallized twice from a mixture of acetone and cyclohexane to yield 11α,15β-dihydroxy-4-pregnene-3,20-dione (11α,15β-dihydroxyprogesterone) melting at 202–203° C. This compound has a specific rotation of +134° in chloroform solution and an ultraviolet absorption maximum at 240 millimicrons with a molecular extinction coefficient of 17,100. Its ultraviolet absorption spectrum does not

change appreciably with time in aqueous potassium hydroxide.

EXAMPLE 2

A solution of 1 part of 11α,15β-dihydroxy-4-pregnene-3,20-dione in a mixture of 40 parts of pyridine and 40 parts of acetic anhydride is allowed to stand at room temperature for about 16 hours. The reaction mixture is diluted with water to several times its original volume and extracted with methylene chloride. The organic phase is washed with dilute hydrochloric acid and with water, and is then dried over sodium carbonate, filtered and concentrated to dryness. The residue in benzene solution is poured on a chromatography column prepared from 60 parts of silica. The column is eluted with mixtures of ethyl acetate and benzene containing increasingly higher percentages of ethyl acetate. Elution with a 30 volume percent solution of ethyl acetate in benzene affords 11α,15β-diacetoxy-4-pregnene-3,20-dione melting at 179–181° C. This compound has a specific rotation of +136° in chloroform solution and an ultraviolet absorption maximum at 239 millimicrons with a molecular extinction coefficient of 17,200.

EXAMPLE 3

A solution prepared from 1 part of 11α,15β-dihydroxy-4-pregnene-3,20-dione, 5 parts of pyridine and 5 parts of acetic anhydride is allowed to stand at room temperature for 30 minutes. The reaction mixture is diluted to several times its original volume with ice water, and the precipitated product is collected on a filter and washed. Recrystallization from a mixture of acetone and cyclohexane yields 11α - acetoxy - 15β - hydroxy-4-pregnene-3,20-dione melting at 233–235° C. This compound has a specific rotation of +111.6° in chloroform solution and an ultraviolet absorption maximum at 239.5 millimicrons with a molecular extinction coefficient of 17,000.

EXAMPLE 4

A solution of 1 part of 11α,15β-dihydroxy-4-pregnene-3,20-dione in 40 parts of pyridine is stirred with a mixture of 2 parts of chromium trioxide in 80 parts of pyridine and allowed to stand at room temperature for about 16 hours. The reaction mixture is poured into several times its volume of water and extracted with 3 portions of methylene chloride. The methylene chloride phase is washed with water, dried over sodium sulfate, filtered and concentrated to dryness. The residue in benzene solution is poured on a chromatography column prepared with 60 parts of silica, and the column is eluted with mixtures of benzene and ethyl acetate containing gradually increasing amounts of ethyl acetate. Elution with a 30 volume percent solution of ethyl acetate in benzene, followed by concentration of the eluates and recrystallization of the residues from a mixture of acetone and cyclohexane and finally from aqueous acetone, yields 4-pregnene-3,11,15,20-tetrone (11,15 - dioxoprogesterone) melting at 218.5–221.5° C. This compound has an ultraviolet absorption maximum at 238.5 millimicrons with a molecular extinction coefficient of 17,700. The infrared absorption spectrum includes a prominent band at 5.75 microns indicative of a 5-membered ring ketone. Other principal infrared absorption maxima appear at 5.89, 5.97, 6.19, and 7.36 microns.

EXAMPLE 5

A solution of 5 parts of 11α,15β-dihydroxy-4-pregnene-3,20-dione in 300 parts of glacial acetic acid is mixed with a solution of 2 parts of chromium trioxide in 100 parts of water and allowed to stand at room temperature for 1 hour. The mixture is diluted with several times its volume of water and extracted with methylene chloride. The organic phase is concentrated to dryness, and the residue in benzene solution is poured on a chromatography column prepared from 300 parts of silica. The column is eluted with mixtures of benzene and ethyl acetate containing gradually increasing proportions of ethyl acetate. Elution with a 30 volume percent solution of ethyl acetate in benzene affords 4-pregnene-3,11,15,20-tetrone, identical with the product of Example 4. Elution with a 40 volume percent solution of ethyl acetate in benzene, followed by concentration of the eluates and recrystallization of the residues from a mixture of acetone and cyclohexane yields 11α-hydoxy-4-pregnene-3,15,20-trione having a melting point of 187–189° C., and an ultraviolet absorption maximum at 240 millimicrons with a molecular extinction coefficient of 17,700. Prominent infrared absorption maxima appear at 2.94, 5.76, 5.92, 6.04, 6.24, and 7.36 microns. Elution with a 70 volume percent solution of ethyl acetate in benzene removes a small amount of unreacted 11α,15β-dihydroxy-4-pregnene-3,20-dione from the column.

EXAMPLE 6

By the procedure of Example 1, progesterone is fermented with a 48 hour growth of *Nigrospora sp.*, ATCC 12773. The oxygenation products are recovered by extraction with methylene chloride and chromatographic fractionation on silica gel.

EXAMPLE 7

By the procedure of Example 1, progesterone is fermented with a 48 hour growth of *Nigrospora sphaerica*, ATCC 12772. The oxygenation products are obtained by extraction with methylene chloride and chromatographic fractionation on silica gel.

EXAMPLE 8

By the procedure of Example 1 a 48 hour growth of *Nigrospora oryzae* ATCC 12771, is prepared. To each of the 20 culture flasks there is then added a solution of 100 milligrams of 17α-hydroxy-4-pregnene-3,20-dione (17-hydroxyprogesterone) in 5 milliliters of ethanol. Incubation is continued on the shaking platform at approximately 25° C. for 72 hours after the addition of the steroid substrate. After this period of fermentative oxygenation of the steroid, the contents of the 20 flasks are combined and filtered in order to separate the mycelium from the liquid phase. The mycelium and the filtrate are extracted with methylene chloride, and the combined methylene chloride extracts are concentrated to dryness under reduced pressure, affording about 2.7 grams of a non-crystalline residue. This residue is dissolved in a 5 volume percent solution of ethyl acetate in benzene and fractionated on a chromatography column prepared from 200 grams of silica. The column is developed with 500 milliliter portions of solvents. Results of a representative fractionation are given in Table II and in the text which follows.

Table II

Fractions	Solvent, Volume Percent Ethyl Acetate in Benzene	Eluate Solids, Milligrams
1-2	5	467
3-6	10	184
7-10	15	34
11-14	20	75
15-16	30	36
17-21	40	77
22	40	48
23-24	50	212
25	50	81
26-28	50	239
29-32	60	214
33-34	70	63
35-36	80	56
37-38	90	74
39-42	100% Ethyl Acetate	89

The crystalline residues from fractions 22 through 24, washed with ether, melt in the range of 200–215° C. Purification by recrystallization from mixtures of acetone and cyclohexane yields 15β,17α-dihydroxy-4-pregnene-

3,20-dione (15β,17-dihydroxyprogesterone) having a melting point of 213–215° C. and a specific rotation of +73° in chloroform solution. This compound has an ultraviolet absorption maximum at 240 millimicrons with a molecular extinction coefficient of 16,500. The crystalline residues from fractions 26 through 28, washed with ether, melt in the range of 150–155° C. and consist chiefly of a diketopiperazine.

EXAMPLE 9

A solution of 5 parts of 15β,17α-dihydroxy-4-pregnene-3,20-dione in 300 parts of pyridine is added to a suspension of 5 parts of chromium trioxide in 400 parts of pyridine and allowed to stand at room temperature for 24 hours. The reaction mixture is diluted with several times its volume of water and extracted with 3 portions of a mixture of equal parts of benzene and ether. The organic phase is separated, dried over sodium sulfate, filtered, and concentrated under reduced pressure. Purification of the residue by recrystallization from a mixture of acetone and cyclohexane and finally from dilute acetone yields 17β-hydroxy-4-pregnene-3,15,20-trione melting at 218–220° C. and having a specific rotation of +133° in chloroform solution. This compound has an ultraviolet absorption maximum at 238.5 millimicrons with a molecular extinction coefficient of 17,500.

EXAMPLE 10

A total of 8.8 liters of the nutrient medium described in Example 1 is dispensed in equal portions of 400 milliliters into 22 Erlenmeyer flasks having a capacity of 2 liters each. The contents of the flasks are sterilized, cooled and inoculated with a culture of *Nigrospora oryzae*, ATCC 12771. By the procedure described in Example 1, a 24 hour growth of this organism is obtained. To each of the flasks there is then added a solution of 100 milligrams of 21-acetoxy-4-pregnene-3,20-dione (desoxycorticosterone acetate) in 5 milliliters of ethanol and incubation is continued on the shaking platform at approximately 25° C. for 96 hours after the addition of the steroid substrate. The contents of the 22 flasks are combined and filtered in order to separate the mycelium from the liquid phase, and both are extracted with methylene chloride. The methylene chloride extracts are concentrated to dryness under reduced pressure, affording about 4.1 grams of a non-crystalline residue. This material is dissolved in a 15 volume percent solution of ethyl acetate in benzene and fractionated on a chromatography column prepared from 350 grams of silica. The column is developed with 1 liter portions of solvents. Results of a representative fractionation are given in Table III and in the text which follows.

Table III

Fractions	Solvent, Volume Percent Ethyl Acetate in Benzene	Eluate Solids, Milligrams
1–2	15	813
3–8	20	449
9–12	25	74
13–16	30	86
17–20	40	120
21–24	45	119
25–30	50	381
31–34	60	435
35–38	65	583
39–42	65	201
43–44	70	40
45–46	90	41

Unconverted desoxycorticosterone acetate is recovered from the column in the fractions containing 20 volume percent of ethyl acetate in benzene. Crystalline fractions obtained by elution of the column with from 40 to 50 volume percent solutions of ethyl acetate in benzene consist, in part, of a diketopiperazine. The crystalline residues from fractions 31 through 38, washed with ether, melt in the range of 170–210° C. Purification by recrystallization from acetone affords a monohydroxy substitution product of 21-hydroxy-4-pregnene-3,20-dione melting at 210–213° C. and having a specific rotation of +214° in chloroform solution. This compound has an ultraviolet absorption maximum at 241.5 millimicrons with a molecular extinction coefficient of 17,100. It is 15α,21-dihydroxy-4-pregnene-3,20-dione, derived from desoxycorticosterone acetate by hydrolysis of the ester grouping and monohydroxylation at position 15.

EXAMPLE 11

A culture of *Nigrospora sp.*, ATCC 12774, is grown for 66 hours in 20 flasks, according to the procedure of Example 1. To each of the flasks there is then added a solution of 100 milligrams of progesterone in 3 milliliters of ethanol and incubation is continued on the shaking platform at approximately 25° C. for 22 hours after the addition of the steroid substrate. The contents of the 20 flasks are then combined and filtered in order to separate the mycelium from the liquid phase. The mycelium and the filtrate are extracted with methylene chloride and the combined methylene chloride extracts are concentrated to dryness under reduced pressure, affording about 4.2 grams of a non-crystalline residue. This material is dissolved in a 5 volume percent solution of ethyl acetate in benzene and fractionated on a chromatography column prepared from 400 grams of silica. The column is developed with 1 liter portions of solvents. Results of a representative fractionation are given in Table IV and in the text which follows.

Table IV

Fractions	Solvent, Volume Percent Ethyl Acetate in Benzene	Eluate Solids, Milligrams
1–6	10	790
7–10	20	107
11–14	30	114
15–18	35	149
19–23	40	151
24–28	40	393
29–31	50	185
32	50	42
33	60	65
34–36	60	245
37–38	60	129
39–40	70	118
41–42	80	95
43–44	90	90
45–48	100% Ethyl Acetate	294

The crystalline residues from fractions 24 through 31, washed with ether, melt in the range of 185–208° C. Combination of these residues and purification by recrystallization from acetone and from dilute methanol yield 15α-hydroxy-4-pregnene-3,20-dione, identical with the product of Example 1. The crystalline residues from fractions 34 through 36 consist principally of a diketopiperazine.

EXAMPLE 12

A solution of 54 parts of 15α-hydroxy-4-pregnene-3,20-dione in 3000 parts of pyridine is added to 58 parts of chromium trioxide in 4000 parts of pyridine and the mixture is allowed to stand at room temperature for 18 hours. The mixture is diluted with several times its volume of water and extracted with 3 portions of a solvent mixture containing equal parts of benzene and ether. The combined organic extract is washed with water and concentrated to dryness in a vacuum. Recrystallization of the residue from dilute methanol and then from a mixture of acetone and petroleum ether affords 4-pregnene-3,15,20-trione melting at 154–155° C. and having a specific rotation of +218.6° in chloroform solution. The infrared absorption spectrum shows a prominent band at about 5.76 microns, indicative of a 5-membered ring ketone.

EXAMPLE 13

In the same manner as Example 1, testosterone is fermented with a culture of *Nigrospora oryzae*, ATCC 8667, and the resulting steroidal product is recovered by extraction with methylene chloride and chromatographic fractionation on silica gel.

EXAMPLE 14

In the same manner as Example 1, 4-androstene-3,17-dione is fermented with a culture of *Nigrospora oryzae*, ATCC 8667, and the resulting steroidal product is recovered by extraction with methylene chloride and chromatographic fractionation on silica gel.

EXAMPLE 15

In the same manner as Example 1, 17α,21-dihydroxy-4-pregnene-3,20-dione is fermented with a culture of *Nigrospora oryzae*, ATCC 8667, and the resulting steroidal product is recovered by extraction with methylene chloride and chromatographic fractionation on silica gel.

EXAMPLE 16

In the same manner as Example 1, 21-hydroxy-4-pregnene-3,20-dione (desoxycorticosterone) is fermented with a culture of *Nigrospora oryzae*, ATCC 8667, and the resulting steroidal product is recovered by extraction with methylene chloride and chromatographic fractionation on silica gel.

EXAMPLE 17

In the same manner as Example 1, 16,17-epoxy-4-pregnene-3,20-dione is fermented with a culture of *Nigrospora oryzae*, ATCC 8667, and the resulting steroidal product is recovered by extraction with methylene chloride and chromatographic fractionation on silica gel.

EXAMPLE 18

A stainless steel fermentation tank having a capacity of about 40 liters is charged with a solution of 1000 grams of dextrose, 200 grams of a commercial enzymatic digest of lactalbumin, 90 milliliters of corn steep liquor and 15 milliliters of concentrated hydrochloric acid in 25 liters of tap water. Five grams of an antifoaming agent, suitably of a silicone-type, is added and the contents of the vessel are sterilized by the introduction of live steam under pressure to a final temperature of 110° C. and a final volume of about 30 liters. The contents of the fermentor are cooled and inoculated with a culture of *Nigrospora oryzae*, ATCC 8667. The contents of the fermentor are kept agitated by a stirrer operating at about 200 revolutions per minute. A stream of air which has been sterilized by filtration through a glass wool filter is introduced through an inlet tube into the fermentor at a rate of from 15 to 22 liters of air per minute. This rate of aeration is measured by means of a rotameter placed in the sterile portion of the air line. Growth of the organism is allowed to continue for 48 hours at 25° C., during which time the pH of the fermentation medium drops from about 4.4 to 3.7. During the 48 hour period of growth of the organism, two additional portions of 5 grams each of an antiforming agent are added. A solution of 7.5 grams of progesterone in 500 milliliters of ethanol is added and fermentation in the presence of the steroid substrate is continued for an additional 24 hours at 25° C., with the same rates of stirring and aeration. The contents of the fermentor are stirred for one hour with 18 liters of methylene chloride. The mycelium is separated by filtration and washed with about twice its volume of methylene chloride. The aqueous broth collected as a filtrate in the separation of the mycelium is stirred for an additional hour with a fresh portion of methylene chloride. The combined methylene chloride extracts, amounting to a total volume of about 40 liters, are concentrated under reduced pressure to a volume of about 650 milliliters. This concentrate is washed with dilute sodium bicarbonate solution and with water and is then concentrated to dryness. The non-crystalline residue is dissolved in a 10 volume percent solution of ethyl acetate in benzene and fractionated on a silica gel chromatography column by elution of the column with mixtures of ethyl acetate and benzene containing gradually increasing percentages of ethyl acetate. In this manner there are obtained purified 15α-hydroxy-4-pregnene-3,20-dione and 11β,15α-dihydroxy-4-pregnene-3,20-dione, identical with the products of Example 1.

What is claimed is:

1. A process for the manufacture of a member of the group consisting of steroids oxygenated at position 11 and steroids oxygenated at position 15 which comprises: subjecting a steroid compound having a carbon skeleton of fewer than 22 carbon atoms and a methylene group in at least one of positions 11 and 15 to the oxygenating activity of a fungus of the genus Nigrospora, and isolating the resulting fermented steroid.

2. A process for the manufacture of a steroid oxygenated at position 15 which comprises: subjecting a steroid compound having a carbon skeleton of fewer than 22 carbon atoms and a methylene group in position 15 to the oxygenating activity of a fungus of the genus Nigrospora, and isolating the resulting fermented steroid.

3. A process for the manufacture of a steroid oxygenated at position 15 which comprises: subjecting a steroid compound containing the pregnane carbon skeleton and a methylene group in position 15 to the oxygenating activity of a fungus of the genus Nigrospora, and isolating the resulting fermented steroid.

4. A process which comprises: growing a fungus of the genus Nigrospora in an aqueous nutrient medium, dispersing progesterone in said medium, maintaining said dispersion under aerobic agitated conditions, and isolating a 15-hydroxy-4-pregnene-3,20-dione.

5. A process which comprises: growing a fungus of the genus Nigrospora in an aqueous nutrient medium, dispersing 17α-hydroxy-4-pregnene-3,20-dione in said medium, maintaining said dispersion under aerobic agitated conditions, and isolating a 15,17α-dihydroxy-4-pregnene-3,20-dione.

6. A process which comprises: growing a fungus of the genus Nigrospora in an aqueous nutrient medium, dispersing a member of the class consisting of 21-hydroxy-4-pregnene-3,20-dione and 21-acetoxy-4-pregnene-3,20-dione in said medium, maintaining said dispersion under aerobic agitated conditions, and isolating a 15,21-dihydroxy-4-pregnene-3,20-dione.

7. A process for the manufacture of a steroid oxygenated at position 15 which comprises: aerobically contacting a steroid compound having a carbon skeleton of fewer than 22 carbon atoms and a methylene group in position 15 with a living culture of a fungus of the genus Nigrospora, and isolating the resulting fermented steroid.

8. A process for the manufacture of a steroid oxygenated at position 15 which comprises: aerobically contacting a steroid compound having a carbon skeleton of fewer than 22 carbon atoms and a methylene group in position 15 with a living culture of *Nigrospora oryzae* and isolating the resulting fermented steroid.

9. A process which comprises: growing an organism of the species *Nigrospora oryzae* in an aqueous nutrient medium, dispersing progesterone in said medium, maintaining said dispersion under aerobic agitated conditions, and isolating a member of the class consisting of a 15-hydroxy-4-pregnene-3,20-dione and an 11,15-dihydroxy-4-pregnene-3,20-dione.

References Cited in the file of this patent

Meister et al.: Jour. Am. Chem. Soc., 76, Aug. 5, 1954, pages 4050–4051.

Disclaimer

2,823,170.—*Robert D. Muir*, Deerfield, and *Raymond M. Dodson*, Park Ridge, Ill. PROCESS FOR THE OXYGENATION OF STEROIDS WITH NIGROSPORA. Patent dated Feb. 11, 1958. Disclaimer filed Oct. 21, 1965, by the assignee *G. D. Searle & Co.*

Hereby enters this disclaimer to claims 1 and 9 of said patent.

[*Official Gazette April 5, 1966*]

UNITED STATES PATENT OFFICE

Certificate of Correction

Patent No. 2,823,170 February 11, 1958

Robert D. Muir et al.

It is hereby certified that error appears in the printed specification of the above numbered patent requiring correction and that the said Letters Patent should read as corrected below.

Column 2, line 10, for "efficint" read —efficient—; column 6, line 9, for "-hydoxy-" read — -hydroxy- —; column 7, line 21, for "17β-" read —17α- —; column 10, line 10, for "11β,15α-" read —11α,15β- —.

Signed and sealed this 29th day of April 1958.

[SEAL]

Attest:
KARL H. AXLINE,
Attesting Officer,

ROBERT C. WATSON,
Commissioner of Patents.