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COMPLETE SPECIFICATION

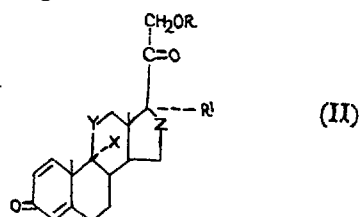
New Steroids of the $\Delta^{1,4}$ -Pregnadiene Series and the preparation of $\Delta^{1,4}$ -Pregnadienes

We, AMERICAN CYANAMID COMPANY, a corporation organized under the laws of the State of Maine, United States of America, of 30, Rockefeller Plaza, New York, State of New York, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to new steroids of the $\Delta^{1,4}$ -pregnadiene series and to a process for their production.

Recently a number of steroids of the pregnene and pregnadiene series, such as hydrocortisone and 1-dehydro-hydrocortisone, have become important therapeutic agents and useful as intermediates for the preparation of other therapeutically useful steroids. The new $\Delta^{1,4}$ -pregnadienes of the present invention are useful as anti-inflammatory agents in the treatment of arthritis, asthma, burns, bursitis, and the like, and also in the treatment of skin disorders and collagen diseases. The compounds are used in combination with fillers, excipients, etc., in tablets, powders, pills etc. They can also be used parenterally in a solution or in a suspension.

In accordance with the invention there are provided new steroids of the $\Delta^{1,4}$ -pregnadiene series having the general formula



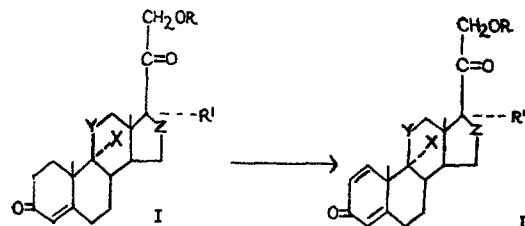
in which X is a hydrogen or halogen atom, Y is a methylene, hydroxymethylene, or carbonyl group, Z is a hydroxymethylene group, or an

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alkanoyloxymethylene group in which the alkanoyl portion contains up to 6 carbon atoms, R^1 is a hydrogen atom or a hydroxyl group, and R is a hydrogen atom or an alkanoyl group containing up to 6 carbon atoms.

The invention also provides a process for producing new steroids having the general formula II which comprises subjecting the corresponding Δ^1 -pregnene to the fermentative enzymatic action of fungi microorganisms of the genus *Nocardia* or to the fermentative enzymatic action of the microorganism *Corynebacterium simplex*.

The process of the present invention can be illustrated by the following equation, in which general Formula I represents the Δ^1 -pregnene and general formula II represents the resulting novel $\Delta^{1,4}$ -pregnadiene.



in which X is a hydrogen or halogen atom, Y is a methylene, hydroxymethylene, or carbonyl group, Z is a hydroxymethylene group or an alkanoyloxymethylene group in which the alkanoyl portion contains up to 6 carbon atoms, R^1 is a hydrogen atom or a hydroxyl group and R is a hydrogen atom or an alkanoyl group containing up to 6 carbon atoms. In certain preferred compounds X is a fluorine atom, Y is CHOH , Z is CHOR^{11} , R^1 is a hydroxyl group, R and R^{11} each being a hydrogen atom or an alkanoyl group containing up to 6 carbon atoms.

In carrying out the process of the present invention, a fungus of the genus *Nocardia*,

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CAP. Y /

(Bergey, Manual, 6th Edition), such as *corallina*, ATCC 999 and ATCC 4273, (Lederle Culture No. 13); *asteroides*, ATCC 3308; *keratolyticus*, ATCC 12484; *transvalensis*, ATCC 12485; *erythropolis*, ATCC 4277; *convoluta*, ATCC 4275; *gardneri*, ATCC 9604; *leishmanii*, ATCC 6855; *opaca*, ATCC 4276; *blackwellii*, ATCC 6846; *caviae*, ATCC 6848; *comituli*, ATCC 6864; *globerula*, ATCC 9356; *polychromogenes*, ATCC 3409; *sylvodifera*, ATCC 7372; *farcinica*, ATCC 3318; *sylvodifera*, ATCC 4919; or other specie of *Nocardia*, or *Corynebacterium simplex* is cultivated aerobically in a suitable nutrient medium with a Δ^4 -steroid of the pregnene series. During the growth of the organism under favourable conditions, two hydrogen atoms are eliminated from steroid ring A, and a double bond is thereby obtained in the 1,2-position. The exact mechanism of this dehydrogenation is obscure, but is it the result of enzymes produced by the organism in the process of the growth. A suitable nutrient medium contains an assimilable source of carbon, nitrogen, and mineral materials. Sources of carbon include sugars, such as glucose, sucrose, maltose, dextrose, xylose, and galactose, also alcohols, such as glycerol or mannitol; corn starch; organic acids, such as citric acid, malic acid, and acetic acid; and various natural products containing carbohydrates, such as corn steep liquor, soybean meal; cotton seed meal, and many other available materials which have been used heretofore as a source of carbon in fermentation processes. Usually a variety of the above can be employed in the medium with good results. Suitable sources of nitrogen include some of the above-named materials, such as corn steep liquor, soybean meal, and cotton seed meal and various other substances, such as beef extract, casein, yeast, enzymatically digested proteins, and degradation products, including peptones, amino acids, and many other available proteinaceous materials which have been found to be suitable in supporting the like, may be used in the medium as a growth of fungi. Inorganic sources of nitrogen, including urea, ammonium salts, nitrates, and source of assimilable nitrogen to provide a favourable growth medium for the organism.

The mineral requirements of fermentation are usually supplied in the crude materials which are often used as sources of carbon and nitrogen or occur in water that is used in the process. However, it is usually advisable to supplement the minerals normally present with added amounts to obtain a maximum growth of *Nocardia*. Cations and anions which may be desirable in added amounts include sodium, potassium, calcium, magnesium, phosphate, sulphate, chloride, cobalt, manganese, and various others. The use of trace elements, such as boron, copper, cobalt, molybdenum, and chromium, is often desirable.

Growth of the *Nocardia* fungus or of the

Corynebacterium simplex takes place under aerobic conditions, and aeration in flasks, for example, can be achieved by agitation on a reciprocating or rotary shaker or in bottles or tanks by forcing sterile air through the fermentation mixture. It is desirable that the sterile air be forced through the medium in an amount of from $\frac{1}{4}$ to 2 volumes of air per volume of medium per minute. Agitation in the bottles or fermenter tanks is provided by a mechanical impeller. The *Nocardia* fungus will grow at temperatures between 5° and 45°C., but it is preferable to carry out the process using the same at a temperature of from 25° to 37°C. *Corynebacterium simplex* will grow at temperatures between 10°C. and 45°C. but is preferably used at a temperature of from 25°C. to 38°C.

To prepare inocula, 1.0 ml. of washed vegetative cell suspension of a fungus of the genus *Nocardia* or *Corynebacterium simplex* is used to inoculate 100 ml. of sterile Trypticase soy broth in a 500 ml. Erlenmeyer flask. The Trypticase soy broth contains 3% of tryptic digest of soya proteins as a dehydrated powder (Baltimore Bacteriological Laboratories), glycerine — 5%, beef extract (Armour) — 0.3%, which mixture is sterilized for 15 minutes at a temperature of 120°C. (15 pounds steam pressure). After sterilization, the pH is in the range of 7.4—7.7. The inoculated flask is incubated at 37°C. on a shaker for about 4—8 hours. Such inocula may be used to inoculate larger batches of sterile medium in bottles, and such bottle cultures, after fermentation, may be used to inoculate large batches of medium in fermenter tanks. In place of the Trypticase soy broth described above, other media can be used, as shown in the examples hereinafter.

The Δ^4 -steroids of the pregnene series which can be used in the process of the present invention include:

Δ^4 - pregnene - 11 β ,16 α ,17 α ,21 - tetrol - 3,20-dione, Δ^4 - pregnene - 9 α - fluoro - 11 β ,16 α ,17 α ,21 - tetrol - 3,20 - dione and Δ^4 - pregnene - 16 α ,17 α ,21 - triol - 3,11,20 - trione and esters thereof. When using the above steroid substrates in the fermentation, the products formed are the free steroids. These steroids are generally added to the fermentation in solution or in finely-divided form. A preferred method is to dissolve the steroid in ethanol or other water-miscible solvents and add it to the fermentation medium at the desired stage in the process. Although the steroid may precipitate from solution when so added, it is dispersed throughout the medium as a fine suspension and becomes readily available to the organism for oxidation. The amount of steroid added to the fermentation may vary considerably, but it is generally on the order of 1/10 to one gram per litre of medium.

During the fermentation process, it may be desirable to add antifoaming agents, such as silicones and glyceride oils. These compounds

silicones/



unicula



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are added from time to time and in the amounts needed.

In the process of the present invention, the 10ml. batches of inoculated medium in 100 ml. shaker tubes are usually incubated for a period of about 16 to 40 hours at a temperature of about 32°C. At this point, 2 mgm. of sterile substrate (Δ^4 -pregnene steroid) dissolved in 0.2 ml. of ethanol is added to each tube and the fermentation continued at about 32°C. The fermentation is allowed to proceed for a period of time long enough to achieve maximum conversion of the Δ^4 -pregnene to the $\Delta^{1,4}$ -pregnadiene. This period of time may vary from 1 to 72 hours or longer.

At the conclusion of the fermentation process, the desired $\Delta^{1,4}$ -steroid of the pregnadiene series is recovered from the fermentation medium by the following procedure, which describes in particular a 10 ml. fermentation. This is a general procedure and is operative for fermentations of various sizes.

The contents of a fermentation tube are extracted four times with four volumes of methylene chloride. The four extracts are pooled and the resulting solution is then washed once with 2% aqueous sodium bicarbonate saturated with sodium chloride and then washed twice with saturated sodium chloride solution. The washed methylene chloride solution can be dried over anhydrous magnesium sulfate and filtered. The filtrate is concentrated on the steam bath at atmospheric pressure to a small volume, and the concentrate is transferred to a 10 ml. volumetric flask and brought up to volume with methylene chloride. This solution is used for characterization of steroid content as described hereinafter.

In large-scale fermentations, the crude product or products may be recovered from the fermentation beer by simple solvent extraction, using a suitable water-immiscible solvent, such as chlorinated lower hydrocarbons, alcohols, esters and ketones. Further purification and separation of steroid products from extracts may be accomplished by methods well understood by those skilled in the art. Separation and purification of steroid mixture often require the use of chromatography.

The process employed to identify the steroids present in the extracted fermentation beer previously described is by paper strip chromatography. The solvent system used is water-methanol-benzene prepared by shaking approximately 50% water-50% methanol with benzene in a separating funnel and then allowing the two layers to separate. A portion of the lower layer is placed in an open dish on the floor of a large glass cylinder. The upper layer is the solvent phase and is used to fill the trough-shaped well within the cylinder. A standard steroid solution is prepared by dissolving 10 mgm. each of the following steroids in 10 ml. of methylene chloride:

Δ^4 - pregnene - 11 β ,16 α ,17 α ,21 - tetrol-3,20-dione

Δ^4 - pregnene - 11 β ,16 α ,17 α ,21 - tetrol-3,20-dione 16,21-diacetate

Δ^4 - pregnene - 9 α - fluoro - 11 β , 16 α ,17 α , 21-tetrol-3,20-dione

Δ^4 - pregnene - 9 α - fluoro - 11 β , 16 α ,17 α , 21-tetrol-3,20-dione 16,21-diacetate

(Other steroids can be used in the standard solution when appropriate.)

At least one standard steroid solution is chromatographed simultaneously each time an unknown solution is tested. Exactly 0.025 ml. of the standard steroid test solution is applied to the paper strip at the starting line, four inches from the upper end of the strip, which is folded over the edge of the trough and immersed in the solvent phase within. The strip is then developed for 2-4 hours at about 37°C. Similarly, 0.1 ml. of the unknown solution is applied to another strip, which is then folded into the same trough and developed simultaneously with the steroid standard strip. The trough permits development of many strips simultaneously. After proper development of the paper strips, they are removed from the apparatus and dried with air at about 37°C. After drying, the strips are sprayed with an alkaline solution of Blue Tetrazolium, which develops color at the spots where the steroids are present. Color-developed strips are lined up with at least one "standard" strip and compared. The different steroids can then be identified by their positions on the strips.

The desired $\Delta^{1,4}$ -steroids will be more polar than their corresponding Δ^4 -steroid. It should be understood, moreover, that the desired $\Delta^{1,4}$ -steroids, once they have been isolated and characterized, may themselves be used in a standard steroid solution for process improvement.

The specific examples which follow illustrate in detail the dehydrogenation of the Δ^4 -steroids to produce the corresponding $\Delta^{1,4}$ -steroids.

EXAMPLE 1

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A potato dextrose agar test tube slant of *Nocardia gardneri* (ATCC 9604) was washed with 5 ml. of sterile saline solution, and 1 ml. lots of the resulting vegetative cell suspension were used to inoculate 10 ml. lots of sterile medium containing 1% "Cerelease" (Registered Trade Mark), 0.1% yeast extract, 0.25% sodium chloride, 0.4% beef extract and 0.4% Peptone, adjusted to pH 7, each lot in a 100 ml. shaker tube. The tubes were shaken on a reciprocating shaker at 32°C. for 40 hours at which time 2 mgm. of Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione dissolved in 0.2 ml. of 70% aqueous ethanol, was added to each tube. The tubes were harvested 6, 24 and 72 additional hours of incubation after addition of substrate.

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The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were heated on a steam cone for 10—15 minutes, and then they were concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone and appropriate volumes of the resulting solutions were chromatographed as described hereinbefore. The chromatograms showed that the substrate had been converted into $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 2

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A potato dextrose agar test tube slant of *Nocardia leishmanii* (ATCC 6855) was washed with 5 ml. of sterile saline solution, and 1 ml. lots of the resulting vegetative cell suspension were used to inoculate 10 ml. lots of sterile medium as described in Example 1, adjusted to pH 7, each lot in a 100 ml. shaker tube. The tubes were shaken on a reciprocating shaker at 32°C. for 40 hours, at which time 2 mgm. of substrate, Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, dissolved in 0.2 ml. of 70% aqueous ethanol, was added to each tube. The tubes were harvested 6, 24 and 72 additional hours of incubation after addition of substrate.

The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were heated on a steam cone for 10—15 minutes and then concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone, and appropriate volumes of the resulting solutions were chromatographed as described hereinbefore. The chromatograms showed that the substrate had been converted into $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 3

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A potato dextrose agar test tube slant of *Nocardia caviae* (ATCC 6848) was washed with 5 ml. of sterile saline solution, and 1 ml. lots of the resulting vegetative cell suspension were used to inoculate 10 ml. lots of sterile medium as described in Example 1, adjusted to pH 7, each lot in a 100 ml. shaker tube. The tubes were shaken on a reciprocating shaker at 32°C. for 40 hours, at which time 2 mgm. of substrate, Δ^4 -pregnene-11 β ,16 α ,17 α ,21-

tetrol-3,20-dione, dissolved in 0.2 ml. of 70% aqueous ethanol, was added to each tube. The tubes were harvested 6, 24 and 72 additional hours of incubation after addition of substrate.

The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were heated on a steam cone for 10—15 minutes and then concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone, and appropriate volumes of the resulting solutions were chromatographed as described hereinbefore. The chromatograms showed that the substrate had been converted into $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 4

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A potato dextrose agar test tube slant of *Nocardia convoluta* (ATCC 4275) was washed with 7 ml. of sterile saline solution, and 1 ml. of the resulting vegetative cell suspension was used to inoculate 10 ml. of sterile medium as described in Example 1, adjusted to pH 7 prior to autoclaving (pH after autoclaving was 6.75) in a 100 ml. shaker tube. The tube was shaken on a reciprocating shaker for 16 hours at 32°C. One ml. of the resulting growth were used to inoculate five 10 ml. lots of sterile medium as described in Example 1, in 100 ml. shaker tubes. These tubes were incubated on the reciprocating shaker for 16 hours at 32°C. at which time 2 mgm. of substrate, Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, in 0.2 ml. of 70% aqueous ethanol was added to each tube. The tubes were harvested after subsequent periods of incubation of $\frac{1}{2}$, 2, 7, 24 and 72 hours.

The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were heated on a steam cone for 10—15 minutes and then concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone, and appropriate volumes of the resulting solutions were chromatographed. The chromatograms showed that the substrate had been converted into $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 5

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A yeast extract agar test tube slant of *Nocardia corallina* (ATCC 999) was washed



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with 7 ml. of sterile saline solution. The resulting suspension was added to 100 ml. of Trypticase soy broth medium without glycerol in a 500 ml. Erlenmeyer flask. The mixture was incubated with shaking at 37°C. for 8 hours. One ml. of this inoculum was used to inoculate each of 15 Erlenmeyer flasks, each flask containing 100 ml. of sterile medium as described in Example 1. The flasks were incubated with shaking at 32°C. for 40 hours. Twenty mg. of Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione dissolved in 2 ml. of ethanol was added to each flask. The flasks were then incubated with shaking at 32°C. for 8½ hours. The contents of the flasks were pooled, giving a mash of pH 7.3. The pooled beer after harvest was extracted in the usual manner, giving 352 mg. of crude crystals. Crystallization from acetone-petroleum ether gave 145 mg., melting point 216–218°C. Recrystallization from acetone-petroleum ether raised the melting point to 229–231°C. The infrared spectrum was identical to that of an authentic sample of $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 6

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A potato dextrose agar test tube slant of *Nocardia globerula* (ATCC 9356) was washed with 5 ml. of sterile saline solution, and 1 ml. lots of the resulting vegetative cell suspension was used to inoculate 10 ml. lots of sterile medium as described in Example 1, adjusted to pH 7, each lot in a 100 ml. shaker tube. The tubes were shaken on a reciprocating shaker at 32°C. for 40 hours, at which time 2 mgm. of substrate, Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, dissolved in 0.2 ml. of 70% aqueous ethanol, was added to each tube. The tubes were harvested 6, 24 and 72 additional hours of incubation after addition of substrate.

The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were heated on a steam cone for 10–15 minutes and then concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone, and appropriate volumes of the resulting solutions were chromatographed. The chromatograms showed that the substrate had been converted into $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 7

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A yeast extract agar slant of the culture *Nocardia corallina* (ATCC 999) was washed

with 7 ml. of sterile saline solution and the resulting suspension was used to inoculate 100 ml. of sterile medium as described in Example 1 in a 500 ml. Erlenmeyer flask. The mixture was incubated with shaking for 7½ hours at 37°C. One ml. portions of this culture were used to inoculate 10 ml. lots of the sterile medium in 100 ml. tubes. The tubes were incubated at 32°C. for 40 hours, at which time 2 mg of Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate, dissolved in 0.2 ml. of ethanol, was added to each tube. The tubes were then incubated for 24 hours. The contents of one tube (10 ml.) were extracted four times with 40 ml. each time of methylene chloride. The extracts were pooled and concentrated to a dry residue under reduced pressure. The residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. This mixture was heated on a steam cone for 10–15 minutes and then concentrated to a dry residue under vacuum. The residue was dissolved in 2 ml. of acetone, and an appropriate volume of the resulting solution was chromatographed. The chromatogram showed that the substrate had been converted into $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 8

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A fermentation experiment such as described in Example 5 was carried out using *Nocardia sp.* (ATCC 12483) instead of *Nocardia corallina*. The product when tested on a paper strip chromatogram gave $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione which was identical with the product of Example 5.

EXAMPLE 9

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione

A potato dextrose agar test tube slant of *Nocardia polychromogenes* (ATCC 3409) was washed with 5 ml. of sterile saline solution, and 1 ml. lots of the resulting vegetative cell suspension was used to inoculate 10 ml. lots of sterile medium as described in Example 1, adjusted to pH 7, each lot in a 100 ml. shaker tube. The tubes were shaken on a reciprocating shaker at 32°C. for 40 hours, at which time 2 mgm. of substrate, Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, dissolved in 0.2 ml. of 70% aqueous ethanol, was added to each tube. The tubes were harvested 6, 24 and 72 additional hours of incubation after addition of substrate.

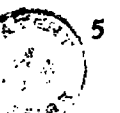
The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were





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heated on a steam cone for 10—15 minutes and then concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone, and appropriate volumes of the resulting solutions were chromatographed. The chromatograms showed that the substrate had been converted into $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 10

Preparation of $\Delta^{1,4}$ -Pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

A yeast extract agar slant of *Nocardia corallina* (ATCC 999) was washed with 7 ml. of sterile saline solution, and the resulting suspension was used to inoculate 100 ml. of sterile medium as described in Example 1 in a 500 ml. Erlenmeyer flask. The mixture was incubated with shaking at 37°C. for 7½ hours. One ml. portions of this culture were then used to inoculate 10 ml. lots of the sterile medium as described in Example 1 in 100 ml. fermentation tubes. The inoculated tubes were incubated for 40 hours at 32°C. At this time 2 mg. of Δ^1 -pregnene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione dissolved in 0.2 ml. of ethanol was added to each tube. The tubes were incubated for 72 hours. After this the contents of one tube were extracted four times with 40 ml. each time of methylene chloride. The extracts were pooled and concentrated to a dry residue under vacuum. The residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. This mixture was heated on a steam cone for 10—15 minutes and then concentrated to a dry residue under vacuum. The residue was dissolved in 2 ml. of acetone, and an appropriate volume of the resulting solution was chromatographed. The chromatography gave substantial amounts of $\Delta^{1,4}$ -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate. Thus it was shown that $\Delta^{1,4}$ -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione has formed in sizeable quantities during the fermentation.

EXAMPLE 11

Preparation of $\Delta^{1,4}$ -Pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione/16,21-diacetate.

Seven ml. of sterile saline solution was used to wash a yeast extract agar test tube slant of *Nocardia corallina* (ATCC 999) and the resulting suspension was used to inoculate 100 ml. of sterile medium as described in Example 1 in a 500 ml. Erlenmeyer flask. The mixture was incubated for 8 hours at 37°C., and 1 ml. lots of this growth were used to inoculate 100 ml. lots of sterile medium in thirty-two 500 ml. flasks. These flasks were incubated at 32°C. for 40 hours, after which time 25 mg. of Δ^1 -pregnene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate dissolved in 2.5 ml. of

ethanol was added to each flask. The flasks were incubated for 53 hours. The contents of the flasks were pooled, and an equal volume of acetone was added. The solution was filtered through diatomaceous earth and the acetone was evaporated under reduced pressure. The aqueous phase was saturated with salt and extracted five times with 500 ml. portions of *n*-butanol. The butanol extract was evaporated under reduced pressure to dryness. The residue was slurried with approximately 1 litre of boiling acetone to remove the organic residue from the inorganic salts and then filtered through diatomaceous earth. The acetone was evaporated and the residue was dissolved in pyridine and acetylated with acetic anhydride (room temperature overnight). Methanol was added and the solution was evaporated under reduced pressure to dryness (weight 1.17 g.). Partition chromatography (solvent system by volume: 3 parts ethyl acetate, 2 parts petroleum ether (90—100°C.), 3 parts methanol, 2 parts water) afforded 0.50 g. of the crude desired product, $\Delta^{1,4}$ -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol 16,21-diacetate. Crystallization of the residue from acetone-petroleum ether gave 341 mg., melting point 153°—233°C. with previous softening. The wide melting point range is presumably a result of solvation.

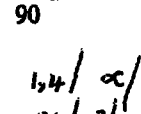
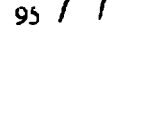
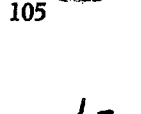
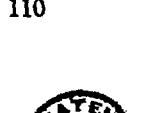
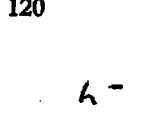
EXAMPLE 12

Preparation of $\Delta^{1,4}$ -Pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

A yeast extract agar test tube slant of *Nocardia sp.* (ATCC 12483) was washed with 7 ml. of sterile saline solution, and the resulting vegetative cell suspension was used to inoculate 100 ml. of sterile Trypticase soy broth (described hereinbefore) in a 500 ml. Erlenmeyer flask. The flask was incubated for seven hours at 37°C. This inoculum was then used to inoculate sterile Trypticase soy broth medium in 100 ml. shaker tubes. One ml. of inoculum was used to inoculate each 10 ml. of a medium containing 2% molasses, 1% beef extract and 1% glucose in each 100 ml. shaker tube. The tubes were incubated at 32°C. for forty hours. At this time 2 mg. of Δ^1 -pregnene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione dissolved in 0.2 ml. of ethanol was added to each tube. The incubation was then continued for an additional 24 hours.

The 10 ml. of mash from one of the above tubes was extracted four times with 40 ml. each time of methylene chloride. The extracts were pooled and evaporated to dryness under vacuum. The residue was taken up in 2 ml. of acetone, and an appropriate sample was analyzed by the paper strip techniques described hereinbefore. The paper strip chromatogram indicated the presence of $\Delta^{1,4}$ -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

65





EXAMPLE 13

Preparation of Δ^4 -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

1,4 /
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A potato dextrose agar test tube slant of *Nocardia asteroides* (ATCC 3308) was washed with 5 ml. of sterile saline solution, and 1 ml. lots of the resulting vegetative cell suspension were used to inoculate 10 ml. lots of sterile medium as described in Example 1, adjusted to pH 7, each lot in a 100 ml. shaker tube. The tubes were shaken on a reciprocating shaker at 32°C. for 40 hours, at which time 2 mgm. of substrate, Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, dissolved in 0.2 ml. of 70% aqueous ethanol, was added to each tube. The tubes were harvested 6, 24 and 72 additional hours of incubation after addition of substrate.

The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were heated on a steam cone for 10—15 minutes and then concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone, and appropriate volumes of the resulting solutions were chromatographed. The chromatograms showed that the substrate had been converted into Δ^4 -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 14

Preparation of Δ^4 -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

1,4 /
h-
h
40
45
50
55
60

A potato dextrose agar test tube slant of *Nocardia cuniculi* (ATCC 6864) was washed with 7 ml. of sterile saline solution and 1 ml. of the resulting vegetative cell suspension was used to inoculate 10 ml. of sterile medium as described in Example 1, adjusted to pH 7 prior to autoclaving (pH after autoclaving was 6.75) in a 100 ml. shaker tube. The tube was shaken on a reciprocating shaker for 16 hours at 32°C. One ml. lots of the resulting growth were used to inoculate five 10 ml. lots of sterile medium as described in Example 1 in 100 ml. shaker tubes. These tubes were incubated on the reciprocating shaker for 16 hours at 32°C., at which time 2 mgm. of substrate, Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, in 0.2 ml. of 70% aqueous ethanol was added to each tube. The tubes were harvested after subsequent periods of incubation of $\frac{1}{2}$, 2, 7, 24 and 72 hours.

The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were heated on a steam cone

for 10—15 minutes and then concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone, and appropriate volumes of the resulting solutions were chromatographed. The chromatograms showed that the substrate had been converted into Δ^4 -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 15

Preparation of Δ^4 -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

1,4 /
h-
75
80
85
90
95
100
105
110
115
120
125

A potato dextrose agar test tube slant of *Nocardia sylvodifera* (ATCC 4919) was washed with 5 ml. of sterile saline solution, and 1 ml. lots of the resulting vegetative cell suspension were used to inoculate 10 ml. lots of sterile medium as described in Example 1 adjusted to pH 7, each lot in a 100 ml. shaker tube. The tubes were shaken on a reciprocating shaker at 32°C. for 40 hours, at which time 2 mgm. of substrate, Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, dissolved in 0.2 ml. of 70% aqueous ethanol, was added to each tube. The tubes were harvested 6, 24 and 72 additional hours of incubation after addition of substrate. The fermented mash was each extracted four times with 40 ml. each time of methylene chloride. The extracts of each mash were pooled and concentrated to a dry residue under reduced pressure. Each residue was taken up in 2 ml. of pyridine, and 0.5 ml. of acetic anhydride was added. These mixtures were heated on a steam cone for 10—15 minutes, and then concentrated to dry residues under vacuum. Each residue was dissolved in 2 ml. of acetone, and appropriate volumes of the resulting solutions were chromatographed. The chromatograms showed that the substrate had been converted into Δ^4 -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

EXAMPLE 16

Preparation of Δ^4 -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

1,4 /
h-
105
110
115
120
125

A yeast extract agar slant of *Nocardia coralina* (ATCC 999) was washed with sterile saline. The resulting vegetative cell suspension was inoculated into 500 ml. Erlenmeyer flask containing 100 ml. of sterile Medium # 13. This flask was placed on a reciprocating shaker at 37°C. for 7 $\frac{1}{2}$ hours. One ml. of the resulting growth was inoculated into each of five 100 ml. shaker tubes containing 10 ml. of sterile medium as described in Example 1. The tubes were incubated on a reciprocating shaker at 32°C. for 40 hours, at which time 2 mg. of Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione-16,21-diacetate dissolved in 0.2 ml. of ethanol was added to each tube. The tubes were harvested 2, 7 $\frac{1}{2}$, 24, 48, and 72 hours subsequently. Paper strip assays on the extracted fermentation beers showed that a substantial quantity of starting material had been transformed to Δ^4 -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.



1,4/ h-

EXAMPLE 17

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

A Trypticase soy agar test tube slant was washed with 5 ml. of sterile water, and the resulting cell suspension of *Corynebacterium simplex* was used to inoculate 100 ml. of sterile Trypticase soy broth medium in a 500 ml. Erlenmeyer flask. This mixture was incubated with shaking at 37°C. for 8 hours. Twenty-five 500 ml. Erlenmeyer flasks, each containing 100 ml. of sterile Trypticase soy broth medium without glycerol, were each inoculated with 1 ml. of the 8 hour old inoculum. These flasks were incubated at 32°C. for 40 hours. At this time, 40 mg. of Δ^4 -pregnene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione dissolved in 4 ml. of ethanol was added to each flask, and the fermentation was continued for 8 hours at 32°C. The contents of all 25 flasks were pooled, giving a mash of pH 8.1.

The pooled beer after harvest was extracted once with 3 litres of methylene chloride and three times with 2-liter portions of methylene chloride. The combined extract was washed

Anal. Calc'd for $C_{27}H_{46}O$ (376.44):
Found: C, 66.80; H, 7.62.

The physical and chemical properties of the compound were those for $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.



5

10



15

h,

20



25

1,4/ 55
h-

EXAMPLE 18

Preparation of $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

The pooled beer from a fermentation as described in Example 17 (above) after harvest was extracted with seven 2-liter portions of methylene chloride, and the combined extracts evaporated to dryness under reduced pressure. This gave 1.585 g. of a crude semisolid, which was chromatographed. The frac-

Anal. Calc'd for $C_{27}H_{46}O$ (376.44):
Found: C, 66.82; H, 7.27.

The product was identical with a sample of $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione obtained as in Example 17.

EXAMPLE 19

 $\Delta^{1,4}$ -Pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate

A mixture of 100 mg. $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione in 10 ml. of pyridine containing 2ml. of acetic anhydride was allowed to stand overnight at room temperature after which the solvent was evaporated to dryness under reduced pressure. The solid residue was crystallized from ethyl acetate-petroleum ether (90°—100°C.), giving 105 mg. (86%) of $\Delta^{1,4}$ -pregnadiene-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate, melting point 147°—150°C. Recrystallization from the same solvent pair raised the melting point

once with saturated saline and evaporated to dryness under reduced pressure. This gave 509 mg. of oily residue, which was dissolved in 1.5 ml. of the stationary phase from the system by volume, 3 parts ethyl acetate: 2 parts petroleum ether (90—100°C.): 3 parts methanol: 2 parts water, and mixed with 3 g. of diatomaceous earth. This impregnated diatomaceous earth was then packed on top of a 1.5 x 35 cm/glass column containing 25 g. of diatomaceous earth impregnated with 12.5 ml. of the stationary phase from the above system. The desired compound was then eluted with the mobile phase from the above system, giving 207 mg. of crude solid. This was crystallized from acetone-petroleum ether (60—70°C.) to give 56 mg., melting point 195°—200°C. (Köfler block). Recrystallization from the same solvent pair raised the melting point to 202°—205°C. (block); melting point, 229°—231°C. (capillary method). Ultraviolet spectrum: $\lambda_{\text{Max.}}^{\text{EtOH}}$ 241 m μ (ϵ 14,500).

Infrared spectrum: $\nu_{\text{Max.}}^{\text{KBr}}$ 3412, 1718, 1667, 1622, 1612 (shoulder), 1098, 1072 cm.⁻¹

C, 67.00; H, 7.50

tions containing the desired compound were combined and evaporated to dryness to give 601 mg. of crystalline residue. Crystallization from acetone-petroleum ether gave 278 mg., melting point 229°—231°C. Recrystallization from the same solvent pair raised the melting point to 231°—232°C. $[\alpha]_D^{24}$ + 77°C.

(methanol). Ultraviolet spectrum: $\lambda_{\text{Max.}}^{\text{EtOH}}$ 241—242 m μ (ϵ 14,800). Infrared spectrum: $\nu_{\text{Max.}}^{\text{KBr}}$ 3436, 1715, 1664, 1621, 1603, 1129, 1063 cm.⁻¹.

C, 67.00; H, 7.50.

to 161°—163°C.. Infrared spectrum: $\nu_{\text{Max.}}^{\text{KBr}}$ 3458, 1758, 1668, 1632, 1612 (shoulder), 1234, 1060 cm.⁻¹. Ultraviolet spectrum: $\lambda_{\text{Max.}}^{\text{EtOH}}$ 242 m μ (ϵ 14,200).

EXAMPLE 20

Preparation of $\Delta^{1,4}$ -Pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol 16,21-diacetate

A test tube agar slant of the culture was washed with 5 ml. of sterile saline solution, and the resulting spore suspension of *Corynebacterium simplex* was added to 100 ml. of sterile Trypticase soy broth medium in a 500 ml. Erlenmeyer flask. The mixture was incubated at 32°C. for 8 hours. One ml. of this culture was used to inoculate each of 10 flasks, each flask containing 100 ml. of sterile



35

40

45

65



75



100

105

110



80

h-

85

h, solution/

90

h-

95





5 flask
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25
30
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Trypticase soy broth medium. The 10 flasks were incubated with shaking at 32°C. for 16 hours. Twenty mg. of Δ^4 -pregnene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate dissolved in 2 ml. of ethanol was added to each flask, and the flasks were pooled. The pooled solution was extracted several times with a large volume of methylene chloride, washed with saturated saline, and evaporated under reduced pressure. The residue was dissolved in methanol, treated with activated charcoal, filtered through diatomaceous earth, and re-evaporated to afford 277 mg. of oil and acetylated overnight. Paper strip chromatography showed approximately equal amounts of substrate and a more polar product ($\Delta^{1,4}$ -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate) together with very small amounts of two less polar products. Partition chromatography of 0.25 g. of the residue (diatomaceous earth column; system: 2 parts ethyl acetate, 3 parts petroleum ether (90°—100°C.), 3 parts methanol, and 2 parts water) separated the less polar products and the substrate. The desired most polar product remained on the column and was eluted with 500 ml. of methanol. The residue (90 mg.) from the evaporated methanol was repartitioned on diatomaceous earth (system: 3 parts ethyl acetate, 2 parts petroleum ether (90°—100°C.), 3 parts methanol, and 2 parts water), and the cut containing the desired product (determined by ultraviolet absorption spectrum) was evaporated under reduced pressure to afford 18 mg. of solid. Crystallization from acetone-petroleum ether gave 13 mg. of colorless

needles of $\Delta^{1,4}$ -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate; melting point (Köfler block) ca 150°—240°C. with apparent loss of solvent at 150°C. Recrystallization from acetone-petroleum ether did not alter the melting point. Ultraviolet spectrum:

λ abs. alc. max. 239 m μ (ϵ 15,200); spectrum in

sulfuric acid: λ H_2SO_4 max. 261, 308, 387 m μ .

The bis-2,4-dinitrophenylhydrazone of the above compound was prepared and its ultraviolet absorption spectrum determined:

CHCl_3 max. 258, 300 (inflection), 312 (infl.) and 400 m μ .

EXAMPLE 21

$\Delta^{1,4}$ -Pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

A solution of 100 mg. of $\Delta^{1,4}$ -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate was dissolved in 10 ml. of methanol and cooled to 0°C. After flushing with nitrogen, a solution of 35 mg. of potassium hydroxide in 2 ml. of methanol was added to the steroid solution. After standing at room temperature for 1 hour, the solution was neutralized and the glacial acetic acid evaporated under nitrogen atmosphere to leave a white solid. Water was added, and after cooling the product was filtered and washed with water to afford 52 mg. of $\Delta^{1,4}$ -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, melting point 246°—249°C. Three crystallizations from acetone-petroleum ether gave 29 mg. of the tetrol, melting point 260°—262.5°C.

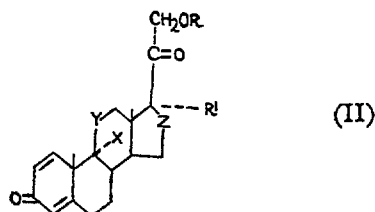
Anal. Calc'd for $\text{C}_{21}\text{H}_{27}\text{O}_5\text{F}$: C, 63.94; H, 6.90; F, 4.82.

Found: C, 64.19; H, 7.17; F, 4.90.

Patent Specification No. 789,363 describes and claims a process for the nuclear (1,2)dehydrogenation of a 3-keto- Δ^4 -steroid compound having from 18 to 21 atoms in the carbon skeleton, which process comprises contacting the steroid with the dehydrogenating activity of an organism of the genus *Nocardia*, but it does not disclose the novel products herein claimed or their production by the use of the microorganism *Corynebacterium simplex*.

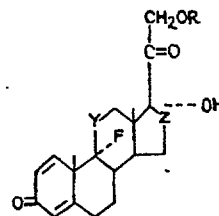
WHAT WE CLAIM IS:—

1. New steroids of the $\Delta^{1,4}$ -pregnadiene series having the general formula



in which X is a hydrogen or halogen atom, Y is a methylene, hydroxymethylene, or carbonyl group, Z is a hydroxymethylene group, or an alkanoyloxymethylene group in which the alkanoyl portion contains up to 6 carbon atoms, R¹ is a hydrogen atom or a hydroxyl group and R is a hydrogen atom or an alkanoyl group containing up to 6 carbon atoms.

2. New steroids of the $\Delta^{1,4}$ -pregnadiene series having the general formula



in which Y is CHOH, Z is CHOR¹¹,R and R¹¹ being hydrogen or an alkanoyl radical containing up to 6 carbon atoms.



5 flask
10
15
20
25
30
35
Trypticase soy broth medium. The 10 flasks were incubated with shaking at 32°C. for 16 hours. Twenty mg. of Δ^1 -pregnene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate dissolved in 2 ml. of ethanol was added to each flask, and the flasks were pooled. The pooled solution was extracted several times with a large volume of methylene chloride, washed with saturated saline, and evaporated under reduced pressure. The residue was dissolved in methanol, treated with activated charcoal, filtered through diatomaceous earth, and re-evaporated to afford 277 mg. of oil and acetylated overnight. Paper strip chromatography showed approximately equal amounts of substrate and a more polar product (Δ^1 -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate) together with very small amounts of two less polar products. Partition chromatography of 0.25 g. of the residue (diatomaceous earth column; system: 2 parts ethyl acetate, 3 parts petroleum ether (90°—100°C.), 3 parts methanol, and 2 parts water) separated the less polar products and the substrate. The desired most polar product remained on the column and was eluted with 500 ml. of methanol. The residue (90 mg.) from the evaporated methanol was repartitioned on diatomaceous earth (system: 3 parts ethyl acetate, 2 parts petroleum ether (90°—100°C.), 3 parts methanol, and 2 parts water), and the cut containing the desired product (determined by ultraviolet absorption spectrum) was evaporated under reduced pressure to afford 18 mg. of solid. Crystallization from acetone-petroleum ether gave 13 mg. of colorless

needles of Δ^1 -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate; melting point (Köfler block) ca 150°—240°C. with apparent loss of solvent at 150°C. Recrystallization from acetone-petroleum ether did not alter the melting point. Ultraviolet spectrum:

λ abs. alc. max. 239 m μ (ϵ 15,200); spectrum in

sulfuric acid: λ H_2SO_4 max. 261, 308, 387 m μ .

The bis-2,4-dinitrophenylhydrazone of the above compound was prepared and its ultraviolet absorption spectrum determined:

$CHCl_3$ max. 258, 300 (inflection), 312 (infl.) and 400 m μ .

EXAMPLE 21

Δ^1 -Pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione.

A solution of 100 mg. of Δ^1 -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione 16,21-diacetate was dissolved in 10 ml. of methanol and cooled to 0°C. After flushing with nitrogen, a solution of 35 mg. of potassium hydroxide in 2 ml. of methanol was added to the steroid solution. After standing at room temperature for 1 hour, the solution was neutralized and the glacial acetic acid evaporated under nitrogen atmosphere to leave a white solid. Water was added, and after cooling the product was filtered and washed with water to afford 52 mg. of Δ^1 -pregnadiene-9 α -fluoro-11 β ,16 α ,17 α ,21-tetrol-3,20-dione, melting point 246°—249°C. Three crystallizations from acetone-petroleum ether gave 29 mg. of the tetrol, melting point 260°—262.5°C.

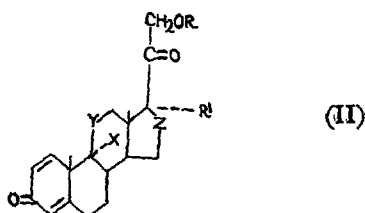
Anal. Calc'd for $C_{27}H_{42}O_6F$: C, 63.94; H, 6.90; F, 4.82.

Found: C, 64.19; H, 7.17; F, 4.90.

Patent Specification No. 789,363 describes and claims a process for the nuclear (1,2)-dehydrogenation of a 3-keto- Δ^1 -steroid compound having from 18 to 21 atoms in the carbon skeleton, which process comprises contacting the steroid with the dehydrogenating activity of an organism of the genus *Nocardia*, but it does not disclose the novel products herein claimed or their production by the use of the microorganism *Corynebacterium simplex*.

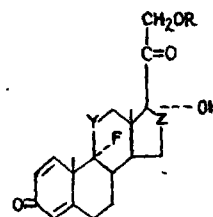
WHAT WE CLAIM IS:—

1. New steroids of the Δ^1 -pregnadiene series having the general formula



in which X is a hydrogen or halogen atom, Y is a methylene, hydroxymethylene, or carbonyl group, Z is a hydroxymethylene group, or an alkanoyloxymethylene group in which the alkanoyl portion contains up to 6 carbon atoms, R¹ is a hydrogen atom or a hydroxyl group and R is a hydrogen atom or an alkanoyl group containing up to 6 carbon atoms.

2. New steroids of the Δ^1 -pregnadiene series having the general formula

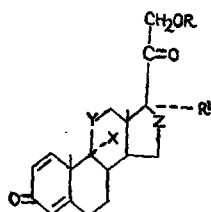


in which Y is CHOH, Z is CHOR¹¹, R and R¹¹ being hydrogen or an alkanoyl radical containing up to 6 carbon atoms.





5. A process for producing new steroids of the Δ^4 -pregnadiene series having the general formula:



in which X is a hydrogen or halogen atom, Y is a methylene, hydroxymethylene, or carbonyl group, Z is a hydroxymethylene group, or an alkanoyloxymethylene group in which the alkanoyl portion contains up to 6 carbon atoms, R¹ is a hydrogen atom or a hydroxyl group, and R is a hydrogen atom or an alkanoyl group containing up to 6 carbon atoms, said process comprising subjecting the corresponding Δ⁴-pregnene to the fermentative enzymatic action of fungi microorganisms of the genus

10. Steroids of the $\Delta^{1,4}$ -pregnadiene series whenever produced by the process according to any of claims 5 to 9.

STEVENS, LANGNER, PARRY &
ROLLINSON,
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Agents for the Applicants.

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