United States Patent [19]

Becher et al.

[11] **3,853,707**

[45] **Dec. 10, 1974**

[54] **METHOD FOR PRODUCING HEXANOR-5,9-SECO-PREGNAN-5-OIC ACID**

[75] Inventors: **Elisabeth Becher**, Basel; **Arno Johannes Schocher**, Benken; **Erich Widmer**, Arlesheim, all of Switzerland

[73] Assignee: **Hoffmann-LaRoche Inc.**, Nutley, N.J.

[22] Filed: **Aug. 16, 1973**

[21] Appl. No.: **389,041**

[30] **Foreign Application Priority Data**

Aug. 31, 1972 Switzerland 12838/72

[52] **U.S. Cl.** ..... **195/51 R**, 260/514 R

[51] **Int. Cl.** **C12d 1/02**

[58] **Field of Search** ..... 195/51 R, 51 G

[56] **References Cited**

UNITED STATES PATENTS

3,379,621	4/1968	Casas-Campillo	195/51 G
3,616,228	10/1971	Schubert et al.	195/51 R

Primary Examiner—Alvin E. Tanenholtz

Attorney, Agent, or Firm—Samuel L. Welt; Jon S. Saxe; W. H. Epstein

[57] **ABSTRACT**

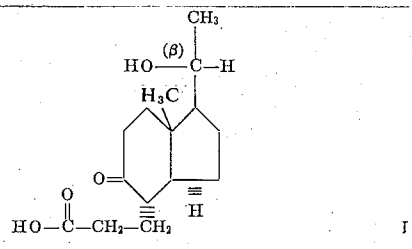
The present invention relates to a microbiological method for producing 20beta-hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid, an intermediate for known steroids, from 3beta, 20beta-dihydroxypregn-5-ene or 2beta, 20-dihydroxy-pregn-5-ene.

4 Claims, No Drawings

METHOD FOR PRODUCING HEXANOR-5,9-SECO-PREGNAN-5-OIC ACID

SUMMARY OF THE INVENTION

In accordance with this invention, a process is provided for the preparation of 20beta-hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid which has the formula:



I

The process of this invention is carried out by fermenting 3beta,20beta-dihydroxypregn-5-ene or 20beta-hydroxy-pregn-4-en-3-one with Mycobacterium phlei ATCC 19249.

The compound of formula I is utilized as an intermediate in the preparation of known steroids by procedures described in Belgian Pat. No. 741,826.

DETAILED DESCRIPTION OF THE INVENTION

In the pictorial representation of the compound of formula I, the symbol ≡ indicates a substituent which is in the alpha-orientation, i.e., below the plane of the molecule and the symbol (β) indicates a substituent which is above the plane of the molecule.

By the term "Mycobacterium phlei ATCC 19249" it is intended to cover all microorganisms of the genus Mycobacterium phlei which produce the compound of formula I and which cannot be definitely differentiated from the strain deposited with the American Type Culture Collection, Rockville, Maryland under ATCC No. 19249 and its subcultures including mutants and variants.

According to the process provided by the present invention, the hexanor-5,9-seco-pregnan-5-oic acid of formula I is manufactured by fermenting 3beta,20beta-dihydroxypregn-5-ene or 20beta-hydroxypregn-4-en-3-one with Mycobacterium phlei ATCC 19249 adapted to aliphatic hydrocarbons.

The adaption of Mycobacterium phlei ATCC 19249 to aliphatic hydrocarbons can be carried out in a manner known per se by inoculating the microorganism from culture substrates which contain glucose on to those substrates in which the glucose is replaced in increasing quantity by aliphatic hydrocarbons, especially C_{14}–C_{18} alkanes.

The term "mutants" as used herein can be spontaneous mutants or mutants produced in a physical or chemical manner. Mutants can be produced by irradiation (e.g., with ultraviolet light or gamma rays) or by treatment with mutating agents (e.g., N-methyl-nitro-nitrosoguanidine or bromouracil). Spontaneous mutants are preferably used in the present process. The selection of the mutants can be carried out by spreading single colonies on agar plates which contain, as the carbon source, the steroid starting material to be fermented. Single colonies obtained from such agar substrates are finally spread on agar plates which contain,

as the carbon source, the hexanor-5,9-seco-pregnan-5-oic acid of formula I. Those cultures which show no growth on such media are used for the present fermentation.

The present process can be carried out by any conventional procedure of aerobic fermentation. Especially suitable culture substrates are liquid media which contain a source of assimilable nitrogen and a source of assimilable carbon as well as inorganic salts. Any conventional source of assimilable nitrogen and assimilable carbon can be utilized. As the source of assimilable nitrogen there can be used animal, vegetable, microbial and inorganic nitrogen compounds such as meat extracts, peptone, cornsteep, yeast extract, glycine and sodium nitrate. As the source of assimilable carbon there can be used sugars such as glucose and, especially, the steroid starting material to be fermented. The nutrient medium may, if desired, also contain trace elements such as iron, sulfur and phosphorus as well as growth factors (e.g., vitamins such as biotin or pyridoxine) or auxins (e.g., indolylacetic acid). In order to protect against infections, the medium can be sterilized and/or can be provided with substances such as benzoates, antibiotics, etc., which inhibit the growth of foreign organisms. The fermentation is preferably carried out in a neutral or weakly acidic pH-range (e.g., at pH 6–7). The fermentation can be carried out at a temperature of between 18°C. and 40°C. It is preferably carried out at about 28°C.

The steroid used as the starting material is preferably added to the fermentation culture in solution. Any conventional inert organic solvent can be used as the solvent medium in accordance with this invention. Among the preferred solvents are included dimethyl sulfoxide, dimethylformamide, ethanol or acetone. It has proved to be advantageous to add the steroid gradually to the fermentation solution and to wait for the complete decomposition of the previously added steroid before each new addition.

At the end of the fermentation, the hexanor-5,9-seco-pregnan-5-oic acid aforesaid can be isolated from the culture solution by conventional procedures. Among these procedures are included extraction, countercurrent extraction and chromatography.

The 20beta-hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid is a useful intermediate in the synthesis of steroids into which it can be converted according to known methods (see Belgian Pat. No. 741,826).

The following examples illustrate the process provided by the present invention.

EXAMPLE 1

Production of the fermentation culture:

Mycobacterium phlei ATCC 19249, adapted to C_{14}–C_{18}n-alkane, was incoluated from sloping agar cultures on to a culture medium of the following composition:

Difco Bacto Nutrient Broth*	23 g.
C_{14}–C_{18}n-alkanes	10 ml.
Trace element solution	1 ml.
Distilled water	1000 ml.

*Difco Manual, 9th edition.

Trace element solution: 11 g. $ZnSO_4.7H_2O$; 6 g. $MnSO_4.4H_2O$; 1 g. $FeSO_4.7H_2O$; 0.3 g. $CoSO_4.7H_2O$; 0.04 g. $CuSO_4.5H_2O$; 0.06 g. H_3BO_3; 0.01 g. KI; 5 g.

ethylenediamine-tetraacetic acid in 1000 ml. of distilled water.

The cultures were cultivated in 500 ml. Erlenmeyer flasks each containing 100 ml. of nutrient solution for 48 hours at 28°C. on a rotary shaking machine (160 r.p.m.). With the thus-obtained cultures there were incolulated 7 liter small fermenters containing a nutrient medium of the following composition:

3β,20β-dihydroxypregn-5-ene	5	g.
NH_4NO_3	2	g.
KH_2PO_4	1	g.
K_2HPO_4	1	g.
Na $2HPO_4.2\ H_2O$	1	g.
$MgSO_4.7\ H_2O$	0.2	g.
KCl	0.2	g.
$FeSO_4.7\ H_2O$	0.001	g.
Tap Water, pH 7.0	1000	ml.

The cultures were incubated for 15 days at 28°C. with aeration and stirring. From the thus-obtained cultures, there were taken plate-cultures on agar plates which contained only 3β,20β-dihydroxypregn-5-ene as the carbon source. The plate-cultures were repeated several times, by which means the steroid content in the agar plates was increased. Finally, further plate-cultures were used in which the agar contained only 20-β-hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid as the carbon source. Those cultures which showed no growth on these plates were used for the fermentation. In order to store the cultures, a suspension in sterile 10 percent skim milk was prepared from the biomass and was filled into 2 ml. ampoules and stored under liquid nitrogen.

Example 2

100 ml. of a nutrient solution containing:		
Glucose (separately sterilized)	1	%
NH_4NO_3	0.2	%
KH_2PO_4	0.1	%
K_2HPO_4	0.2	%
$NaHPO_4.7\ H_2O$	0.1	%
$MgSO_4.7\ H_2O$	0.02	%
KCl	0.02	%
$FeSO_4.7\ H_2O$	0.0001	%

in tap water, pH 7.0 (adjusted with sodium hydroxide), was inoculated with the contents of 2 ampoules prepared according to Example 1. The culture was incubated for 48 hours at 28°C. with shaking. This preculture was used to inoculate 8 liters of nutrient medium in a small fermenter following which incubation was carried out for 2 days at 28°C. with stirring and aeration (640 r.p.m., 2 liters air/minute), the pH value being adjusted to pH 7.0 by the addition of 28 percent sodium hydroxide. The contents of this fermenter were used as the inoculant for 180 liter fermenter containing the same nutrient solution. After the inoculation, incubation was carried out at 32°C., 1,100 r.p.m. and an aeration rate of 100–130 liters/minute. After 27 hours, the glucose content of the nutrient medium had fallen to 0.2 percent by weight. At this point, 180 g. of 3 β,20β-dihydroxypregn-5-ene dissolved in 3600 ml. of dimethyl sulfoxide were added. The reaction of the steroid was followed by thin layer chromatography carried out on samples. The pH was held at between 6.5 and 7.0 by the addition of sodium hydroxide.

After 63 hours, the steroid used as the starting material had completely reacted, whereupon another 180 g. of 3 β,20β-dihydroxypregn-5-ene in dimethyl sulfoxide were added to the fermentation solution. After complete reaction of the steroid (90 hours), a third addition of 180 g. of steroid was carried out. After 155 hours, all of the steroid had reacted.

The fermentation mass was centrifuged, rinsed with 20 liters of water and adjusted to pH 2.0 with concentrated aqueous hydrochloric acid. The solution was extracted twice with the same volume and once with half the volume of methylene chloride. The extracts were concentrated in a rotary evaporator and worked up by countercurrent extraction. The yield of 20β-hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid amounted to 82 percent. 10 percent of 9,20-dioxo-1,2,3,4,10,19-hexanor-5-seco-pregnan-5-oic acid was also isolated.

EXAMPLE 3

20β-Hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid is converted to 20 beta-hydroxy-deA-pregn-9-en-5-one as follows:

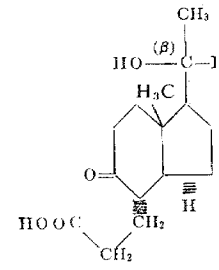
A solution of 13.4 g of 20beta-hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid and 4 g of sodium acetate in 250 ml of acetic anhydride is boiled at reflux under nitrogen for 4 hours. The acetic anhydride is removed in vacuum, the residue twice evaporated with toluene and finally taken up in 750 ml of methylene chloride. The methylene chloride solution is washed five times with water, dried over Na_2SO_4 and evaporated in vacuum. The resulting 7-(1R-acetoxyethyl)-6a-methyl-3(2H)-oxo-1,6,6a,7,8,9,9a,9b-octahydro-cyclopenta[f][1]benzopyran is directly employed in the next step.

A solution of 8.8 g of 7-(1R-acetoxyethyl)-6a-methyl-3(2H)-oxo-1,6,6a,7,8,9,9a,9b-octahydro-cyclopenta[f][1]benzopyran in 250 ml of absolute ether is slowly treated with 60 ml of a 0.5 N ethereal solution of ethyl magnesium bromide at −15°. After 24 hours, the reaction mixture is poured onto ice-cold saturated ammonium chloride solution. The ethereal solution is washed with water (1 ×), ice-cold 0.5 N caustic potash (2 ×) and again with water (2 ×). The organic phase is dried over sodium sulfate, the solvent is removed in vacuum and the residue is filtered through a column of 25 g of silica gel.

1.1 g of the thus obtained crude product are stirred with a solution of 3 g of potassium hydroxide in 100 ml of methanol and 10 ml of water for 15 hours at room temperature. The reaction mixture is poured onto ice-water and extracted with ether. The ethereal extract is washed neutral with water, dried and evaporated. Chromatography of the residue on silica gel yields pure 20beta-hydroxy-deA-pregn-9-en-5-one.

We claim:

1. A process for the manufacture of 20beta-hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid of the formula:



which process comprises fermenting 3beta,20beta-dihydroxypregn-5-ene or 20beta-hydroxy-pregn-4-en-3-one with Mycobacterium phlei ATCC 19249 adapted to aliphatic hydrocarbons in a nutrient medium containing an assimilable nitrogen and an assimilable carbon source.

2. A process according to claim **1**, wherein there are used spontaneous mutants which show no growth on a nutrient medium containing 20beta-hydroxy-9-oxo-1,2,3,4,10,19-hexanor-5,9-seco-pregnan-5-oic acid as the carbon source.

3. The process of claim **1** wherein the fermentation is carried out aerobically.

4. The process of claim **1** wherein the fermentation is carried out at a pH of from 6 to 7.

* * * * *