# SYNTHESIS OF 9α-HYDROXYSTEROIDS BY A <u>RHODOCOCCUS</u> <u>SP.</u>

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Received December 20, 1988
Revised June 13, 1989

ABSTRACT

9α-Hydroxylation of $\Delta^5$-3β-hydroxysteroids (of androstane, pregnane, 24-nor- and 21,24-bisnorcholane groups) was carried out by a <u>Rhodococcus sp.</u>, isolated from a petroleum-containing soil sample. A large number of the investigated steroids was transformed into 9α-hydroxy-$\Delta^4$-3-ketones in satisfactory yields (50-90%) at high initial concentrations of the substrates (0.5-5.0 g/L). The influence of some structural features of the steroid molecule on the progress and effectiveness of the microbial transformation was also shown.

INTRODUCTION

9α-Hydroxysteroids can be used as key intermediates in the synthesis of 9α-halo-11β-hydroxysteroids, which as a rule have a high specific physiological activity (1).

Despite the obvious practical significance of the 9α-hydroxysteroids, the available information about cultures capable of achieving a selective 9α-hydroxyla-

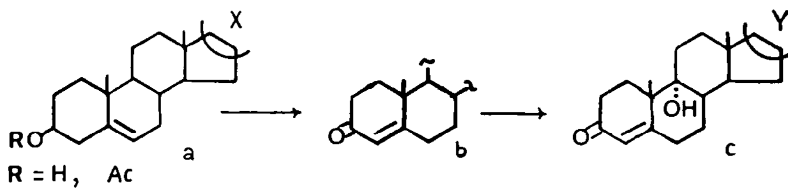
tion is rather limited (2). The microorganisms producing 9α-hydroxysteroids are either fungi or mutant strains of Schizomycetes in which the enzyme system responsible for the destruction of the steroid molecule is inhibited. Apart from 9α-hydroxylation, however, the fungi also hydroxylate the 6β, 7α, 12α, 14α, or the 15β positions. We have summarized here data about the culture Nocardia conicruria (3), which hydroxylates selectively a number of $\Delta^4$-3-oxosteroids at a concentration of 0.6 g/L, and the culture Corynespora cassicola, which converts 1.5 g/L androstenedione into 9α-hydroxyandrostenedione in yields above 70% (4). A Japanese patent (5) offers brief information about the capability of Rhodococcus equi to introduce a 9α-hydroxy group into pregnane $\Delta^4$-3-oxosteroids at high concentrations. However, no mention is made of the substrate specificity of the strain.

Using the culture Rhodococcus sp., we transformed the $\Delta^5$-3β-acetoxysteroids 1a-22a and/or some of their derivatives: the respective 3β-alcohols and $\Delta^4$-3-ketones (Scheme 1). The results are given in Tables 1, 2, and 3.

## EXPERIMENTAL

### General

Analytical thin-layer chromatography was carried out on Kieselgel $60F_{254}$ plates (Merck, FRG) using a solvent system ether-heptane: No.1 (1:2) for compounds 1c-3c, 15c-17c; No.2 (1:1) for compounds 4c-6c, 18c; No.3 (2:1)




1. X = y = (OAc)

2. X = y =

3. X = y = (OH)

4. X = (AcO, OH), y = (OH, OH)

5. X = ($CH_2OAc$, OH), y = ($CH_2OH$, OH)

6. X = (AcO, OAc), y = (HO, OAc)

7. X = (AcO), y = (HO)

8. X = y = (AcO, OH)

9. X = (AcO, AcO, OH), y = (OH, AcO, OH)

10. X = (AcO, $CH_2OAc$, OH), y = (AcO, $CH_2OH$, OH)

11. X = y =

12. X = y =

13. X = y =

14. X = y =

15. X = y =

16. X = y =

17. X = y =

18. X = y =

19. X = y =

20. X = y =

21. X = (OAc, OH), y = (OH, OH)

22. X = y = (H, $OCH_2CH_3$)

for compounds 11c, 12c, 14c, 19c, 20c, and 22c; and No.4: ether-heptane-methanol (5:5:0.1) for compound 21c. The developed chromatograms were examined under 254 nm UV light or by spraying with a solution of $Ce(SO_4)_2$, followed by warming on a hot plate.

Preparative separation was done using the flash technique as reported by Still and co-workers (6). Column packing was Silica gel 40/100 μm (Lachema, Czechoslovakia). The same solvent system as the one described above was used for elution. All metabolites were recrystallized from methanol or acetone.

Steroids 1a-14a (R=Ac), 20a,b, and 22b were synthesized according to the previously described methods (8-11). Dehydroepiandrosterone acetate, 15a, androstenedione, 15b, pregnenolone, 16a, $\Delta^{16}$-pregnenolone and its acetate, 17a, 16α,17α-epoxypregnenolone, 18a, 16β-methyl-16α,17α-epoxypregnenolone, 19a, menadione were purchased from Sigma Chemical Co. (USA), cortexolone and its 21-acetate, 21b, were supplied by Akrikhin (USSR). The media chemicals were Koch-Light Ltd. (UK) commercial products.

Melting points were determined on a Köfler apparatus and were uncorrected. IR spectra were recorded on a Carl-Zeiss IR-20 spectrometer, and UV spectra on a Unicam SP-700. $^1$H-NMR spectra were taken in deuteriochloroform at 250 MHz on a Brüker WM-250 spectrometer and chemical shifts were given in ppm (δ) relative to tetramethylsilane as an internal standard. Low resolution mass spectra were obtained on JEOL JMS D-300 spectrometer with chemical ionization (isobutane). All isolated 9α-hydroxy-$\Delta^4$-3-ketones absorb UV light at 240 nm, ε~10000. The physical constants obtained are listed in Table 4.

Transformation products 1b, 6b, 9b, 10b, 15c, 16c, and 21c proved to be identical in all respects with the known material (3,4,7,11).

Description of a Rhodococcus sp.

Gram-positive, slightly acid-fast, occurs as short, sometimes branched rod forms (0.6-0.7 μm to 8-10 μm) fragmented into coccoids upon prolonged incubation. Mycelium is not formed. Non-spore forming. Forms rough and lightly mucoid pinkish pigmented colonies on agar media. Pigmentation of colonies enhanced by light exposure. Growth occurs at 18-37 but not at 45C. Resistant to 5% ethanol and 5% NaCl. Utilizes glucose, sucrose, fructose, glycerol, n-propanol, acetate, citrate, succinate, malate, and pyruvate as sole carbon sources in the presence of ammonium nitrogen. Does not utilize benzamide. Mycolic acids are composed of 36-48 carbon atoms.

The coryneform was identified in The All Union Collection of Microorganisms, Moscow, USSR.

Organism and culture conditions

*Rhodococcus sp.* used in this work was maintained and stored at 4 C on agar slants (corn-steep liquor 10 g, glucose 10 g, agar 20 g, distilled water 1 L, pH 7.2-7.4) Periodic transfer (every 1-2 months) preserved the culture.

Two types of fermentation media were used in this study. Medium 1 was the same as above with the agar excluded, and medium 2 consisted of (per liter of tap water) corn-steep liquor 8 g, $Na_2HPO_4.12H_2O$ 4.5 g, $KH_2PO_4$ 1 g, pH 7.3-7.5.

Shaken cultures for biotransformation experiments (220 strokes/min, 28 C) were generated by a three-stage fermentation procedure.

Stage I culture was initiated by suspending the surface growth from a 7-day-old slant in 50 mL of medium 1 in a 250 mL Erlenmeyer flask, and was incubated for two days; 5mL of stage I culture were then withdrawn to inoculate 50 mL of medium 1 for a II stage culture. The stage III culture was grown in a 250 mL Erlenmeyer flask or in a 750 mL conical flask containing 50 and 100 mL of medium 2 respectively, and was inoculated with 10 vol% of the 24-h stage II culture.

Steroids were added to a 6-h stage III culture as a 2.5% solution in dimethylformamide to a final medium concentration of 0.5-1.0 g/L. or as a powder with grain size of 5-10 μm and concentration 3-5 g/L. Incubation was continued for 15 h (c = 0.5-1.0 g/L) or 40 h (c = 3-5 g/L) after steroid addition.

The entire culture was extracted three times with equal volumes of chloroform. The extracts were dried ($MgSO_4$) and evaporated with heptane (2 X 10 mL).

## RESULTS AND DISCUSSION

The structure and in particular the position of the newly introduced hydroxyl group at C(9) of the 9α-hydroxysteroids, was determined by the usual analytical methods (Table 4).

For all 9α-hydroxy-$\Delta^4$-3-ketones, it was found that

the signal of the 19-methyl group in the $^{1}$H-NMR spectrum appears at lower field, $\delta$ = 1.31-1.38 ppm, as compared to the corresponding $\Delta^4$-3-ketones, $\delta$ = 1.18-1.25 ppm.

The absolute configuration of the 9α-hydroxysteroid 2c was confirmed by X-ray analysis (12).

The unequivocally determined structure of compound 2c, the identityof spectral data reported for 9α-hydroxysteroids 15c, 16c, and 21c (3,4), and the data obtained from the IR and MS spectral analysis, led us to the assumption that the hydroxylation of the investigated substrates by a Rhodococcus sp. takes place at the C(9) position.

Steroids of 24-nor- and 21,24-bisnorcholane group

A microbial hydroxylation of a series of $\Delta^5$-3β-acetoxy compounds 1a-14a was performed at a concentration of 0.5 g/L in the course of our investigations on the synthesis and biological activity of a new class of modified polyfunctional steroids with an additional ring E (13). It was found that four versions of the microbial process are possible depending on the substituents in ring E and on its structure (Table 1):

a) $\Delta^5$-3β-Acetates or their $\Delta^4$-3-ketoderivatives are oxidized to 9α-hydroxy-$\Delta^4$-3-ketones (compounds 2a, 3a, 4b, 5b, 7b, 12a-14a).

b) The process stops at the stage of $\Delta^4$-3-ketone formation (compounds 1a, 6a, 9a, 10a).

c) The starting steroids remain unchanged, or are hydrolyzed (compounds 4a, 5a, 7a, 8a, 8b).

d) Transformation proceeds with formation of only $\Delta^{1,4}$-3-ketone (compound 11a).

The hydrolysis of some sterically accessible primary and secondary acetoxy groups in ring E takes place simultaneously with the changes in the A/B part of the steroid molecule (compounds 4c, 5c, 7c, 6b, 9b, 10b).

The $\Delta^5$-3β-acetoxy steroids 2a, 3a, and 12a-14a were transformed to 9α-hydroxy-$\Delta^4$-3-ketones with a yield of 50-90%. The acetate 14a was transformed in a lower yield (40%) due to the presence of unmetabolized substrate (30%).

We assumed that 9α-hydroxylation of the acetates 1a and 4a-10a fails to take place because of the very low rate of transformation at the initial stages, i.e. hydrolysis and oxydation of the $\Delta^5$-3β-acetoxy group. With this in mind, we made use of the corresponding $\Delta^4$-3-ketones: 1b and 4b-10b as starting products. Indeed, we obtained the 9α-hydroxy derivatives 4c, 5c, and 7c with a yield of 40-60%. The remaining $\Delta^4$-3-ketones 1b, 6b, and 8b-10b were recovered quantitatively from the cultu-

TABLE 1. Metabolites obtained from 24-nor- and 21,24-bisnorcholanic steroids 1a-14a

| No. | Subst. at C(3) | Metabolites (%)* | | | Starting product (%) |
|---|---|---|---|---|---|
| | | 9α-hydroxy-$\Delta^4$-3-ketone | $\Delta^4$-3-ketone | $\Delta^{1,4}$-3-ketone | |
| 1a | $CH_3COO$- | - | 60 | - | 0 |
| 2a | $CH_3COO$- | 90 | - | - | 0 |
| 3a | $CH_3COO$- | 80 | - | - | 0 |
| 4a | $CH_3COO$- | - | - | - | 95 (22α-OH) |
| 4b | O= | 40 | - | - | 0 |
| 5a | $CH_3COO$- | - | - | - | 95 (23-OH) |
| 5b | O= | 60 | - | - | 0 |
| 6a | $CH_3COO$- | - | 60 (20β-OH) | - | 0 |
| 7a | $CH_3COO$- | - | - | - | 95 (20β-OH) |
| 7b | O= | 50 | - | - | 0 |
| 8a | $CH_3COO$- | - | - | - | 95 |
| 8b | O= | - | - | - | 95 |
| 9a | $CH_3COO$- | - | 50 (22α-OH) | - | 0 |
| 10a | $CH_3COO$- | - | 60 (23-OH) | - | 0 |
| 11a | $CH_3COO$- | - | - | 80** | 0 |
| 12a | $CH_3COO$- | 50 | - | - | 0 |
| 13a | $CH_3COO$- | 50 | - | - | 0 |
| 14a | $CH_3COO$- | 40 | - | - | 30 |

*The sign "-" means that no metabolite is formed, or that it does not accumulate in the culture medium.
**The physical constants are stated in (14).

re medium.

The results clearly indicate that the possibility of obtaining 9α-hydroxy steroids and the efficiency of the process very much depends on the structure and substituents of ring E. At this stage, however, we are unable to offer any plausible explanation of this pronounced substrate specificity of the investigated microorganism.

Transformation of androstanes

The 9α-hydroxyandrostenedione, 15c, was obtained in high yield from both dehydroepiandrosterone, 15a, and androstenedione, 15b, at a concentration of 1-3 g/L (Table 2).

The effectiveness (40%) of the transformation of androstenedione at a concentration of 5 g/L was very unsatisfactory: 40% of the starting substrate remained unchanged in the culture medium. Extending the fermentation period resulted in the destruction of the 9α-hydroxyandrostenedione.

It should be noted that all known microbial methods for obtaining 9α-hydroxyandrostenedione employ cultures, which convert 0.5-1.5 g/L androstenedione for 50-60 h (2,3).

Transformation of pregnanes

A considerable number of steroids with pregnane

TABLE 2. Synthesis of 9α-hydroxyandrostenedione, 15c

| No. | Subst. at C(3) | C g/L | 9α-hydroxy-androstenedione (%) | Starting product (%) |
|---|---|---|---|---|
| 15a | $CH_3COO$- | 1 | 65 | 0 |
| 15a | $CH_3COO$- | 3 | 70 | 0 |
| 15b | O= | 1 | 70 | 0 |
| 15b | O= | 3 | 70 | 0 |
| 15b | O= | 5 | 40 | 40 |

skeleton, e.g. 16a-21a, 20b-22b, were transformed into 9α-hydroxy-$\Delta^4$-3-keto derivatives by a *Rhodococcus sp.* at concentrations of 1-5 g/L (Table 3).

TABLE 3. Synthesis of 9α-hydroxypregnanes 16c-22c

| No. | Subst. at C(3) | C g/L | 9α-hydroxy-$\Delta^4$-3-ketones (%) | Starting product (%) |
|---|---|---|---|---|
| 16a | HO- | 1 | 60 | 0 |
| 17a | $CH_3COO$- | 3 | 40 | 0 |
| 17a | HO- | 3 | 60 | 0 |
| 18a | HO- | 3 | 70* | 0 |
| 19a | HO- | 1 | 80 | 0 |
| 20a | $CH_3COO$- | 1 | 0** | 0 |
| 20b | O= | 1 | 70 | 0 |
| 21a | HO- | 5 | traces | 0 |
| 21b | O= | 5 | 50 | 30 (21-OH) |
| 22b | O= | 1 | 50 | 40 |

*16α,17-Epoxy-20β-hydroxypregn-4-en-3-one (15%) was isolated as a side product; the physical constants are stated in (15).
**$\Delta^4$-3-Ketoderivative, 20b, yield 75%, was obtained.

In the case of compound 20b, the activity of the strain is only revealed in the presence of a $\Delta^4$-3-oxo group in the molecule of the substrate, as was shown earlier for some steroids belonging to the group of 24-nor- and 21,24-bisnorcholane.

The 3β-acetoxy groups of the steroids 16a and 17a were hydrolyzed very slowly which prevented the accumulation of 9α-hydroxy derivatives. The transformation of 17a was more effective when the corresponding alcohol was used; the yield of the metabolite 17c increased from 40% to 60% at equal concentrations of the starting substrate (3 g/L). In the case of the steroid 16a, the 9α-hydroxy derivative, 16c, was obtained only from the 3β-alcohol.

A comparison of the results in Table 3 allows the assumption that the protected side chain of the pregnane steroids (especially the dihydroxyacetone one) and the presence of substituents in ring D improve the stability of the 9α-hydroxy-$\Delta^4$-3-ketones towards a subsequent degradation (compounds 19a, 20b, 22b).

At a concentration of 5 g/L, the transformation of cortexolone 21-acetate, 21a, proceeded with almost complete destruction of the substrate (only traces of 9α-hydroxy derivative were found). However, when the mena-

TABLE 4. Physical constants of 9α-hydroxysteroids

| No. | m.p. (°C) | MS (m/z) | IR ($cm^{-1}$, $CHCl_3$) | $^1$H-NMR (δ, ppm) |
|---|---|---|---|---|
| 2c | 242-244 | $358 (M)^+$<br>340 | 1620<br>1665<br>1695 | 0.93s(3H,18-$CH_3$), 1.34s(3H,19-$CH_3$), 1.79s (OH), 3.68m(1H,16-H), 4.20m(1H,23-H), 4.32m (1H,23-H), 5.88br.s(1H,4-H). |
| 3c | 216-218 | $374 (M)^+$<br>356<br>348 | 1620<br>1670<br>1700 | 1.00s(3H,18-$CH_3$), 1.34s(3H,19-$CH_3$), 3.28s (OH), 3.75m(1H,16-H), 3.98m,4.25m(2H,23-2H), 5.87br.s(1H,4-H). |
| 4c | oil | $390 (M)^+$<br>302<br>278 | 1618<br>1670<br>1755 | 0.92s(3H,18-$CH_3$), 1.35s(3H,19-$CH_3$), 3.93m (2H,23-2H), 4.23m(1H,22-H), 4.43m(1H,16-H), 5.87br.s(1H,4-H). |
| 5c | oil | $390 (M)^+$<br>328<br>302 | 1620<br>1670<br>1755 | 1.03s(3H,18-$CH_3$), 1.35s(3H,19-$CH_3$), 3.90m (2H,23-2H), 4.27m(1H,22-H), 4.65m(1H,16-H), 5.88br.s(1H,4-H). |
| 7c | 238-240 | $360 (M)^+$<br>342<br>324 | 1618<br>1665 | 1.14s(3H,18-$CH_3$), 1.34s(3H,19-$CH_3$), 3.34td (J=2 and 8,1H,20-H), 4.24m(2H,16-H,23-H), 4.00m(1H,23-H), 5.88br.s(1H,4-H). |
| 12c | 238-241 | $370 (M)^+$ | 1616<br>1667<br>1716 | 0.89s(3H,18-$CH_3$), 1.34s(3H,19-$CH_3$), 2.06s (3H,21-$CH_3$), 4.56m(1H,16-H), 5.78br.s(1H,22-H), 5.90br.s(1H,4-H). |

(continued)

| | | | | |
|---|---|---|---|---|
| 13c | 208-210 | 370(M)$^+$<br>326<br>308 | 1618<br>1680<br>1732 | 0.97s(3H,18-$CH_3$), 1.34s(3H,19-$CH_3$), 1.84s (3H,21-$CH_3$), 2.42s(OH), 5.06br.s(1H,16-H), 5.89s(1H,4-H). |
| 14c | oil | 368(M)$^+$<br>350 | 1533<br>1618<br>1665<br>1710 | 1.05s(3H,18-$CH_3$), 1.38s(3H,19-$CH_3$), 2.15s (3H,21-$CH_3$), 5.88br.s(1H,22-H), 5.92br.s(1H, 4-H). |
| 17c | 140<br>(decomp.) | 328(M)$^+$<br>310 | 1604<br>1665 | 0.96s(3H,18-$CH_3$), 1.35s(3H,19-$CH_3$), 2.27s (3H,21-$CH_3$), 5.87br.s(1H,4-H), 6.73br.s(1H, 16-H). |
| 18c | 229-230 | 344(M)$^+$<br>326<br>266 | 1620<br>1667<br>1703 | 1.08s(3H,18-$CH_3$), 1.31s(3H,19-$CH_3$), 2.02s (3H,21-$CH_3$), 2.40s(OH), 3.70br.s(1H,16-H), 5.84br.s(1H,4-H). |
| 19c | 208-210 | 358(M)$^+$<br>340<br>280 | 1617<br>1667<br>1703 | 1.08s(3H,18-$CH_3$), 1.32s(3H,19-$CH_3$), 1.46s (3H,16-$CH_3$), 2.22s(3H,21-$CH_3$), 5.86br.s(1H, 4-H). |
| 20c | 244-246 | 402(M)$^+$<br>384 | 1620<br>1666<br>1708 | 0.65s(3H,18-$CH_3$), 1.17s(3H,$CH_3$-C-$CH_3$), 1.32s (3H,19-$CH_3$), 1.47s(3H,$CH_3$-C-$CH_3$), 2.24s(3H, 21-$CH_3$), 2.42s(OH), 5.04d(J=5,1H,16-H), 5.92br.s(1H,4-H). |
| 22c | 180-182 | 418(M)$^+$<br>372<br>354<br>328 | 1618<br>1665<br>1727 | 0.72s(3H,18-$CH_3$), 1.21t(3H,-$OCH_2CH_3$), 1.33s (3H,19-$CH_3$), 3.60m(2H,-$OCH_2CH_3$), 3.97d(J=5, 1H,21-H), 4.28d(J=5,1H,21-H), 5.68s(1H,$HCO_3$-) 5.88br.s(1H,4-H). |

dione, which inhibited the reduction of the 20-carbonyl group, we succeeded in obtaining 9α-hydroxy-cortexolone in a yield of 50% (and 30% cortexolone). Marshek and coworkers (3) reported also a low yield of 9α-hydroxy derivatives from 17,21-dihydroxysteroids.

The assumption that Rhodococcus sp. is capable of reducing the 20-carbonyl group of pregnane steroids was confirmed by the transformation of 16α,17α-epoxypregnenolone, 18a. As a side product, 15% 16α,17-epoxy-20β-hydroxypregn-4-en-3one was isolated. Its physical characteristics coincide with the literature data for the 20β-isomer (15).

APPENDIX

Dehydroepiandrosterone; 3β-(acetyloxy)-androst-5-en-17-one
Androstenedione; androst-4-ene-3,17-dione
9α-Hydroxyandrostenedione; 9-hydroxyandrost-4-ene-3,17-dione
Pregnenolone; 3β-hydroxypregn-5-en-20-one
$\Delta^{16}$-Pregnenolone; 3β-hydroxypregna-5,16-dien-20-one
16α,17α-Epoxypregnenolone; 16α,17-epoxy-3β-hydroxypregn-5-en-20-one
16β-Methyl-16α,17α-epoxypregnenolone; 16α,17-epoxy-3β-hydroxy-16-methylpregn-5-en-20-one
Cortexolone; 17,21-dihydroxypregn-4-ene-3,20-dione
Menadione; 2-methyl-1,4-naphthoquinone

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