# SYNTHESIS AND CHARACTERISTICS OF ALLYLIC 4-PREGNENE-3,20-DIOLS FOUND IN GONADAL AND BREAST TISSUES

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**ABSTRACT**

Recently several allylic steroids have been found in gonadal and breast tissues. In order to establish their presence and identity in tissues and determine the possible biological properties, a method for the synthesis of 4-pregnene-3α,20α-diol, 4-pregnene-3α,20β-diol, 4-pregnene-3β,20α-diol, and 4-pregnene-3β,20β-diol was developed using 4-pregnene-3,20-diome (progesterone) as substrate and freshly-prepared aluminum isopropoxide in isopropyl alcohol as reducing agent. The yields were about 19%, 30%, 13%, and 38% for the 3α,20α-, 3α,20β-, 3β,20α-, and 3β,20β-diols, respectively. The structures and stereochemistry of these diols were established using proton and carbon NMR spectroscopy and infrared and mass spectrometry.

## INTRODUCTION

In previous studies it was shown that, in culture, Sertoli cells from young rats produce a number of steroids from progesterone. Ten of these steroids, constituting the majority of the metabolites formed, have been systematically identified (1). The major metabolite was shown to be 20α-hydroxy-4-pregnen-3-one (2), while a novel labile allylic metabolite was identified as 3α-hydroxy-4-pregnen-20-one (3α-DHP) (3). Recently, 3α-DHP was also identified as a metabolite of progesterone produced by theca cells from adult domestic chicken (4), and human breast and brain tissues (5). In order to test for possible biological activity of 3α-DHP a convenient chemical synthesis of this steroid was developed (6), and it has now been shown that 3α-DHP is the first known steroid to selectively inhibit follicle stimulating hormone (FSH) *in vivo* without a similar effect on luteinizing hormone (7,8).

On the basis of preliminary evidence it appeared that the remaining

unidentified progesterone metabolites of Sertoli cells consisted of one or more isomeric 4-pregnene-3,20-diols. Also in vitro conversion of progesterone or 20α-hydroxy-4-pregnen-3-one to allylic 4-pregnene-3,20-diols has been reported for embryonic rat fibroblasts (9), uterine tissue of non-pregnant rats (10), and rat (11) and mouse (12) mammary gland tissue. In addition, we have recently obtained evidence that human breast tissue may contain or produce one or more of these allylic 4-pregnene-3,20-diols (5). In order to help establish the presence and identity of these steroids in gonadal and breast tissue and also for use in ongoing biological work it was necessary to have the four allylic steroidal diols in reasonable amounts. Here we report a convenient synthesis of 4-pregnene-3α,20α-diol **2**, 4-pregnene-3α,20β-diol **3**, 4-pregnene-3β,20α-diol **4**, and 4-pregnene-3β,20β-diol **5**, starting with progesterone **1** and a readily available reduction mixture. The structures and stereochemistry of the compounds reported here were established using proton and carbon NMR spectroscopy and infrared and mass spectrometry.

## MATERIALS AND METHODS

Melting points were determined in capillary tubes. Infrared spectra were recorded on a Beckman Acculab 4 instrument with $CHCl_3$ solutions. $^1H$ NMR spectra were recorded on a Varian XL-200 spectrometer with $CDCl_3$ solutions containing tetramethylsilane; only relevant peaks from the $^1H$ NMR spectra are reported (Table 1). The $^{13}C$ NMR spectra were obtained using $CDCl_3$ solutions with an XL-300 (75.4 MHz) instrument. Comparison of the fully decoupled spectra with those obtained with either the APT (13) or DEPT (14) sequences served to identify the methyl, methylene, methine, and quaternary signals. The $^{13}C$ data are collected in Table 2. Exact masses were determined on an MAT 311A mass spectrometer. Capillary GC retention times and ion fragmentation patterns were determined on a Hewlett-Packard 5790A GC/5970A MS. Camag DF-5 silica gel was used for thin- and thick-layer chromatography. Preparative plates (20 x 20 cm) contained 20 g of silica gel each. For the high performance liquid chromatography (HPLC) a Beckman Model 332 gradient liquid chromatograph with Altex Model 420 microprocessor and Model 155 variable wavelength detector was interfaced with a Hewlett-Packard 5840A terminal; a Whatman Partisil 10 (Magnum 9) ODS-3 preparative HPLC column was used for the

purifications. Commonly, a liquid phase consisting of 70% methanol and 30% water was employed. A wavelength of 240 nm was used for detection of 3-keto-4-ene steroids and 206 nm for steroids with 3-hydroxy-4-ene substitution.

The following commercially available chemicals were used: progesterone (Aldrich Chemical Co.), digitonin (Sigma), aluminum metal (Fisher, 20 mesh and finer), mercuric chloride (B.D.H.). Isopropyl alcohol (Fisher, ACS grade) was distilled from sodium.

Aluminum Isopropoxide Preparation

The method of Vogel (15) was used. A mixture of 2.5 g of aluminum metal, 50 mg of mercuric chloride, and 25 mL of dry isopropyl alcohol was heated gently. External heating was discontinued when the exothermic reaction had set in. After the reaction had subsided, the mixture was refluxed for 24 h. After distilling the unreacted isopropyl alcohol at atmospheric pressure, the residual viscous mass was distilled under reduced pressure, giving aluminum isopropoxide as a colorless viscous liquid with b.p. of 145-148 °C/3.0 Torr. The material solidified on standing at room temperature.

Reaction of Progesterone with Aluminum Isopropoxide - Isopropyl Alcohol

A solution of 1.0 g progesterone (3.2 mmol), 30 mL dry isopropyl alcohol, and 2.6 g (12.8 mmol) of freshly prepared aluminum isopropoxide was refluxed until acetone was no longer detected in the distillate. At the end of the reaction, most of the isopropyl alcohol had been distilled off. The residue was taken up in excess ether and extracted with 10% NaOH. The organic layer was washed with water, dried, and concentrated, resulting in 980 mg of an oily solid. Analysis of this mixture by HPLC (206 nm, $CH_3OH:H_2O$, 7:3, 1 mL/min) showed 4 peaks with retention times at 9.7, 11.9, 13.3, and 16.5 min. Ratios of the areas under these peaks were determined by comparison with purified diols (see below) and were found to be 19:30:13:38 for the 3α,20α:3α,20β:3β,20α:3β,20β diols, respectively.

Purification and Identification of 4-Pregnene-3,20-diols

To a solution of the above material in 50 mL ethanol was added a warm solution of 1.0 g digitonin in ethanol (25 mL) and water (15 mL), and the mixture was allowed to stand overnight at 5 °C. The precipitated digitonide was filtered, washed with ether and dried, giving 1.2 g of colorless solid. The filtrate was concentrated to a colorless solid; it was thoroughly washed with ether and then concentrated, resulting in an oily solid. The oily solid was chromatographed on eight preparative plates developed twice in chloroform:ether (90:10) and once in chloroform:ether (75:25). The band at $R_f$ 0.32 yielded 270 mg of colorless solid which the proton NMR indicated to be a mixture of 3α,20α- and 3α,20β-diols. The band at $R_f$ 0.49 yielded 280 mg of colorless solid which the proton NMR indicated to be a mixture of 3β,20α- and 3β,20β-diols.

A mixture of the digitonide, 25 mL pyridine, and 60 mL anhydrous ether was allowed to stand at room temperature for 30 min. The gelati-

nous mass was filtered, washed with ether and chloroform, and the filtrate was concentrated. This material was chromatographed on two preparative plates developed in chloroform:ether (90:10) and chloroform: ether (75:25). From the band at $R_f$ 0.5, 145 mg of crystalline solid was obtained. Proton NMR indicated this substance to be a mixture of 3β,20α- and 3β,20β-diols.

The above mixtures of diols were further purified using preparative HPLC (methanol:water, 7:3; flow rate, 3.7 mL/min; 210 nm). The peak with retention time of 24 min gave 80 mg of colorless granules of 4-pregnene-3β,20α-diol **4**. The peak with retention time of 30 min gave 288 mg of colorless plates of 4-pregnene-3β,20β-diol 5. The peak with retention time of 39 min gave 48 mg of 4-pregnene-3α,20α-diol 2 as colorless needles. The peak with retention time of 48 min gave 110 mg of 4-pregnene-3α,20β-diol 3 as colorless granules. The structures of the 4-pregnene-3,20-diols are represented in Figure 1.

## Capillary GC/MS Retention Times and Mass Spectra

To establish capillary GC retention times and fragmentation spectra a Hewlett Packard 5790A GC/5970A MS was employed with a 12.5 m cross-linked methyl silicone capillary column. The conditions were as follows: splitless mode, 0.7 kg/cm$^2$ helium, 205 °C injection temperature, column temperature 150 °C (initial) to 210 °C at 15 °C/min, and scan speed of 690 amu/sec. Steroids were dissolved in methanol (1 mg/mL), and 1 μL aliquots were injected. Under these conditions the retention times were 18.38 min for 4-pregnene-3α,20β-diol 3, 18.90 min for 4-pregnene-3β,20β-diol 5, 19.42 min for 4-pregnene-3α,20α-diol 2, 19.96 min for 4-pregnene-3β,20α-diol 4, and 24.51 min for 4-pregnene-3,20-dione 1. The GC conditions resulted in dehydration of 30-60% of the allylic diols; the dehydration fragments had short relative retention times (12.7-13.7 min).

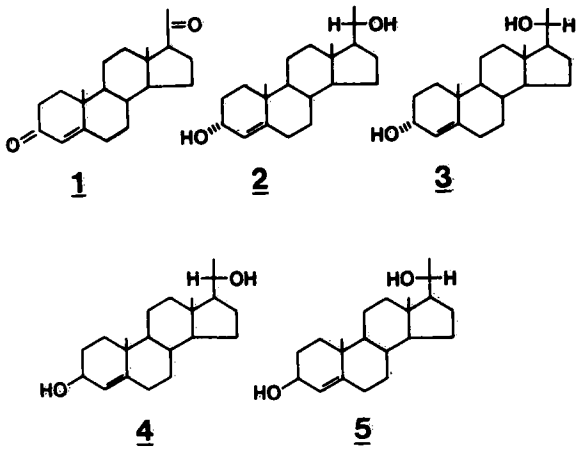


**FIGURE 1**. Progesterone and the 4-pregnene-3,20-diols.
(1) 4-pregnene-3,20-dione,
(2) 4-pregnene-3α,20α-diol, (3) 4-pregnene-3α,20β-diol,
(4) 4-pregnene-3β,20α-diol, (5) 4-pregnene-3β,20β-diol.

**TABLE 1.** Physical constants and spectral characteristics of the diols

| Diol[a,b] | m.p.[c] °C | % Diol in Mixture | δ (ppm)[d] | | | | | | Mass Spec. | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 18-$CH_3$ | 19-$CH_3$ | 21-$CH_3$[e] | 3-H | 4-H | 20-H[f] | m/e[g] | |
| 3α,20α **2** | 198-202 | 19 | 0.69 | 0.98 | 1.22 | 4.07 | 5.44 | 3.70 | 318.2563 | 25 |
| 3α,20β **3** | 159-163 | 30 | 0.73 | 0.94 | 1.09 | 4.03 | 5.40 | 3.67 | 318.2557 | 25 |
| 3β,20α **4** | 190-195 | 13 | 0.66 | 1.03 | 1.20 | 4.13 | 5.26 | 3.70 | 318.2558 | 24, 26 |
| 3β,20β **5** | 177-182 | 38 | 0.74 | 1.03 | 1.10 | 4.12 | 5.25 | 3.71 | 318.2563 | 24,26,27 |

[a] Numbers refer to compounds in Figure 1.

[b] The four diols showed hydroxyl absorption in IR at 3800 $cm^{-1}$.

[c] TLC of melted solid from all 4 diols show more than one spot, indicating possible decomposition (dehydration ?) during melting.

[d] The signal for 4-H in 4-pregnen-3α-ol appears as doublet of doublets with J=1.70;5.0 Hz and for 4-pregnen-3β-ol appears as doublet of triplets with J=1.5;1.7 Hz; 3-H resonance of both appears as a broad multiplet.

[e] Doublet with coupling constant 6.15 Hz.

[f] With $J_{20\alpha\text{-H},\ 17\alpha\text{-H}} = 10$ Hz and $J_{20\beta\text{-H},17\alpha\text{-H}} = 8$ Hz.

[g] Calculated value for $C_{21}H_{34}O_2$ is 318.2558.

TABLE 2. $^{13}C$ Shieldings[a] of diols 2-5

| Carbon | 4-Pregnene-3,20-diols | | | |
|---|---|---|---|---|
| No. | 2 | 3 | 4 | 5 |
| 1 | 31.3 | 31.7 | 35.2 | 35.5 |
| 2 | 27.5 | 27.9 | 29.5 | 29.6 |
| 3 | 63.8 | 64.2 | 67.9 | 68.0 |
| 4 | 120.2 | 120.7 | 123.2 | 123.3 |
| 5 | 149.6 | 150.2 | 147.3 | 147.5 |
| 6 | 32.4 | 32.8 | 33.0 | 33.2 |
| 7 | 32.0 | 32.4 | 32.1 | 32.3 |
| 8 | 35.1 | 35.7 | 35.5 | 35.9 |
| 9 | 53.6 | 54.1 | 54.3 | 54.5 |
| 10 | 37.2 | 37.6 | 37.3 | 37.5 |
| 11 | 20.9 | 21.4 | 20.7 | 21.0 |
| 12 | 38.5 | 40.0 | 38.8 | 40.0 |
| 13 | 41.4 | 42.4 | 41.7 | 42.5 |
| 14 | 55.5 | 55.6 | 55.9 | 55.7 |
| 15 | 23.8 | 24.5 | 24.1 | 24.6 |
| 16 | 25.3 | 25.6 | 25.6 | 25.7 |
| 17 | 58.0 | 58.5 | 58.3 | 58.5 |
| 18 | 12.2 | 12.5 | 12.6 | 12.6 |
| 19 | 17.7 | 18.1 | 18.9 | 19.0 |
| 20 | 69.9 | 70.5 | 70.2 | 70.6 |
| 21 | 23.1 | 23.7 | 23.5 | 23.8 |

[a] In ppm from internal TMS for $CDCl_3$ solutions.

## RESULTS AND DISCUSSION

Steroid ketone reductions are frequently carried out with sodium borohydride. In our experience, reaction of progesterone with sodium borohydride afforded products with reduced double bonds as well as the desired alcohols as shown by mass spectral analysis. Use of lithium aluminum hydride did not reduce the double bond of progesterone, but the yield of 3α-hydroxy-4-ene steroids (according to proton NMR spectral analysis) was only 8-10%. In the hope of improving the yield of 3α-hydroxy steroids we employed aluminum isopropoxide in isopropyl alcohol which had been reported (16) to reduce 4-cholesten-3-one to a 1:1 mix-

ture of 3α-hydroxy-4-cholestene and 3β-hydroxy-4-cholestene without affecting the double bond (17).

Carrying out the reaction of progesterone with freshly prepared aluminum isopropoxide in isopropyl alcohol gave equilibrium mixtures of four diols as reported for other compounds (18-20). High performance liquid chromatography of the crude reaction mixture was compared with HPLC of purified isomeric diols and indicated that the 3α,20α-, 3α,20β-, 3β,20α-, and 3β,20β-diols were present in the ratio of 19:30:13:38, respectively.

Following digitonin treatment, the diols were purified using preparative TLC and HPLC. Infrared spectra of these purified products showed the absence of carbonyl and the presence of hydroxyl groups. Assignment of the stereochemistry of the 3-hydroxyl group in each of the four diols was based on our previous findings (4, 21). For 3α-hydroxy-4-ene steroids the olefinic 4-proton gives rise to a doublet of doublets with J=1.7,5.0 Hz, while, for the 3β-hydroxy-4-ene isomers, the olefinic proton absorption is a doublet of triplets, J=1.5,1.7 Hz. These different patterns reflect the changes in coupling interactions with the 3- and 6- protons.

Assignments of the stereochemistry for the 20-hydroxyl group followed from the data of Lee and Wolff (22), which indicated that the 21-methyl protons in the 20α-epimers absorb downfield from those in the 20β-epimers and that the 18-methyl protons in the 20β-epimers appear at lower field than those in the 20α-epimers. These trends have been employed by others (23) for stereochemical assignments of 20-hydroxylated steroids and are attributed to the effect of the 20-hydroxyl group on the preferred conformation of the 17β-side chain in the two epimers in each of which the separation between the 20-hydroxyl and the

18-methyl will differ. It may be noted that the vicinal coupling between the 20- and 17α-protons is ~10 Hz in the 20β-hydroxyl epimers and ~8 Hz for their 20α-hydroxyl counterparts. For each pair of 20-hydroxyl epimers the major product has 20β-substitution, which is consistent with the favored 20R(20β) configuration for alkoxide reduction, in complete agreement with the NMR results (see Table 1). The carbon-13 shielding data for the four diols are collected in Table 2.

Initially it was thought that aluminum isopropoxide-isopropyl alcohol, under the reaction conditions used, might cause epimerization of the 17β-acetyl side chain before reduction of the 20-oxo group. However, spectral and HPLC comparisons of the product mixture obtained from lithium aluminum hydride and aluminum isopropoxide-isopropyl alcohol reactions showed that the diols formed in the two reactions were the same, thus ruling out possible epimerization at C-17 before reduction. The mass spectra of the isomeric pregnenediols at the conditions employed are shown in Figure 2. Characteristic peaks are the ions m/e 318, 300, 285, 267, 229, 215, 203, 174, 159, 145, 135, 121, 119, 105, 91, 79, 67, and 55. Table 3 shows the three highest intensity ions for the 4 isomers. The m/e ratios of 229/203 for the 3α- and the 3β-isomers are approximately 1.0 and 0.3, respectively; the m/e ratios of 148/145 and 105/93 for the 3β,20α-diol are different from those of the other 3 isomers (Table 3).

In our previous paper (6) we reported the isolation of a diol from KS-Selectride reduction of progesterone, and it was tentatively assigned the structure 4-pregnene-3α,20β-diol 3. With all four purified diols on hand we have now been able to make a definitive assignment of the stereochemistry for this diol. Comparison of spectra and HPLC retention

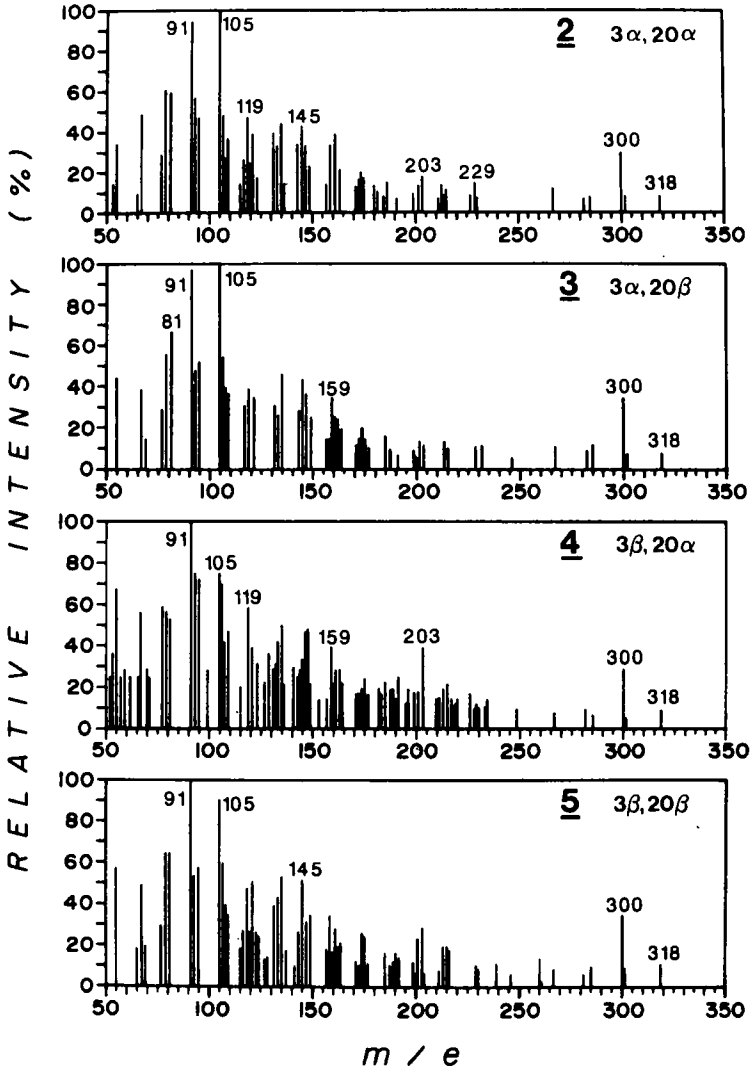


**FIGURE 2.** Mass spectra of (2) 4-pregnene-3α,20α-diol, (3) 4-pregnene-3α,20β-diol, (4) 4-pregnene-3β,20α-diol, and (5) 4-pregnene-3β,20β-diol.

**TABLE 3.** GC/MS Analysis of 4-pregnene-3,20-diols

| Steroid[a] | Retention Time (min) | Highest Intensity Ions (m/e) | | | m/e Ratios | | |
|---|---|---|---|---|---|---|---|
| | | 1st | 2nd | 3rd | 229/203 | 148/145 | 105/93 |
| 3α,20α | 19.42 | 105 | 91 | 79 | 0.90 | 0.53 | 1.75 |
| 3α,20β | 18.38 | 105 | 91 | 81 | 1.00 | 0.56 | 2.08 |
| 3β,20α | 19.96 | 91 | 93 | 105 | 0.32 | 1.45 | 1.00 |
| 3β,20β | 18.90 | 91 | 105 | 79 | 0.35 | 0.66 | 1.69 |
| PROGESTERONE | 24.51 | 124 | 91 | 229 | 11.00 | - | 1.17 |

The analyses were carried out as described in Materials and Methods, and the ratios are calculated from the fragmentation patterns (Fig. 2).
[a] Abbreviations refer to the respective 4-pregnene-3 , 20-diols.

times confirmed that the progesterone/KS-Selectride diol is 4-pregnene-3α,20β-diol **3**. In addition, with the help of these synthesized steroids, it has now been possible to detect allylic 4-pregnene-3,20-diols and to identify differences between tumorous and non-tumorous human breast tissues with respect to these steroids and to study their *in vitro* effects on cell lines (5).

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## NOTES AND REFERENCES

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