LABELED STEROIDS OF POTENTIAL BIOLOGICAL INTEREST: SYNTHESIS AND PROPERTIES OF ^{18}O-LABELED 17 α-HYDROPEROXYPROGESTERONE.

Pierre Falardeau and Liat Tan.
Department of Biochemistry, Faculty of Medicine,
University of Sherbrooke, Sherbrooke, Quebec, Canada.
Received on December 20th 1973.

SUMMARY

^{18}Oxygen labeled 17α-hydroperoxyprogesterone has been synthesized via a 3-step reaction sequence in an all-glass, high vacuum transfer system. The hydroperoxidation reaction was found to proceed smoothly only, when an adequate oxygen pressure in the reaction vessel is maintained. Comparisons are made between the mass spectral fragmentations of the ordinary ^{16}O, and the isotopically labeled ^{18}O-17α-hydroperoxyprogesterones. Their NMR-spectra display a splitting of the 18-, and 21-methyl signals, probably due to the coexistence of two finite conformations of the acetyl side-chain.

INTRODUCTION

The synthesis of 17α-hydroperoxyprogesterone, incorporating the natural, ^{16}O-oxygen isotope, has been originally described by BARTON and his group already more than a decade ago (1). The stereoselective, peroxidative attack on the α-keto hydrogen atom at C-17, was initially carried out at room temperature in a basic medium with a solution of tert. potassium butoxide in anhydrous tert. butanol. Later, the rather low yields obtained in this reaction were improved by VAN RHEENEN and

VISSER (2), who introduced the use of a binary solvent system composed of a mixture of tetrahydrofurane and tert. butanol, permitting them to carry out the oxygenation reaction at below minus 20°C; in this way preventing the formation of undesirable side-products.

Recently, we have emitted the hypothesis that 17α-hydroperoxyprogesterone may be implicated as one of the first intermediates formed in the biosynthesis of the steroid hormones of the adrenal cortex, and which involves an intramolecular, peroxide rearrangement reaction, linking all the known oxygenated positions in corticosteroids (3). To gather evidence for the existence of such a biogenetic relationship, for instance between positions 17α and 21 in the pregnene molecule, the acquisition of 17α-, ^{18}O-labeled, hydroperoxyprogesterone became imperative. Because the synthesis of this ^{18}O-labeled, pregnene-17α-hydroperoxide has necessitated the exploration of a new set of parameters, and also because our synthesis differs in some crucial details from the procedures described earlier by BARTON and VAN RHEENEN and VISSER, we believe that a full description of our synthetic approach would be in order.

EXPERIMENTAL

Materials and methods.

Progesterone was purchased from Organon, Oss, The Netherlands. It gave a single peak in gas chromatography and was therefore used without further purification. Triethylorthoformate was from BDH Chemicals, Toronto, Ontario. All solvents for column chromatography and recrystallizations were distilled prior to use. Anhydrous tetrahydrofurane and tert. butanol were obtained by distillation over resp. sodium and calcium hydride. Melting points were determined on a Kofler hot-stage with microscopic magnification; they were not corrected.

Gas chromatography.

Gas chromatographic analyses were carried out on a Hewlett-Packard 7610A instrument. The 180 x 0.2(i.d.) cm glass column was packed with a dual layer, consisting of 3% OV-1 and 3% OV-210 coated on 100-120 mesh Gaschrom Q, used in combination as described previously (4). The peaks were eluted isothermally at 220°C using nitrogen as a carrier gas at a flow rate of 55 ml/min.

Liquid chromatography.

Two, 60 x 1.5(i.d.) cm columns were screwed together and used in series. They were packed with aluminium oxide, which was previously desactivated with 6% of water. The bands were eluted first with hexane, then with benzene, and finally with a mixture of benzene and increasing amounts of ethylacetate. Fractions of ca. 20 ml were collected and analyzed by gas chromatography. The fractions containing the desired compound were then pooled, the organic solvent removed by evaporation under vacuum, the residue weighed and recrystallized from the appropriate solvent system.

Mass spectrometry.

The crystalline samples were dried under vacuum over phosphorous pentoxide and then introduced via the direct insertion lock into the ion source of a Hitachi RMU-6E mass spectrometer. The temperature of the solids inlet probe was gradually raised from 80° to 140°C until sufficient evaporation occurred. The source temperature was held at 250°C, the ionizing energy at 70 eV.

NMR spectrometry.

NMR spectra were recorded on a Varian A-60 instrument at room temperature. The samples were dissolved in deuterated dimethylsulfoxide and tetramethylsilane was added as an internal standard.

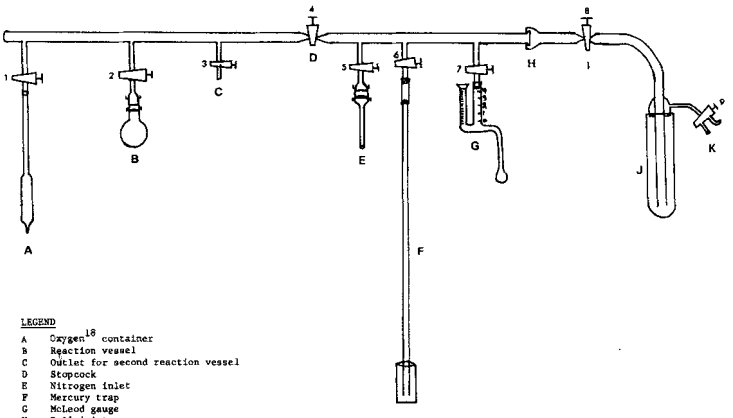
3-Ethoxy-pregna-3,5-dien-20-one.

To a solution of 10 g of progesterone in 250 ml of anhydrous benzene were added 10 ml of triethylorthoformate and 200 mg of p-toluenesulfonic acid. The mixture was boiled gently for two hours, whereby the initially dark brown coloured solution gradually turned into yellow-orange. A second portion of 200 mg of the acid was then added. The enolation reaction, which was monitored by gas chromatography at regular time intervals, never went completely one way, but achieved a certain equilibrium with a conversion ration of 60-70%. After boiling for a total of 4 hours, the reaction mixture was cooled to room temperature, then washed resp. with sodiumhydrogencarbonate and water, dried over sodium sulfate and evaporated under vacuum. The siropous residue (10.6 g) was then subjected to column chromatography using 175 g of alumina as the stationary phase, as described above to yield finally 6.2 g of a white solid. Recrystallization from methanol/water gave 5.7 g of colourless crystalls; m.p. 100-101°C; IR(KBr): 1706, 1655, 1630 and 1180 cm^{-1}. This compound gave rise to a single peak in gas chromatography with t_R 0.44 at 220°C (calculated relative to progesterone).

3-Ethoxy-17α-^{18}O-hydroperoxy-pregna-3,5-dien-20-one.

The synthesis was carried out in the high vacuum transfer system shown in Fig.1. The system was checked previously for leakage by standing overnight under a vacuum of 10^{-2} mm Hg. Using a reaction vessel of 50 ml volume, 1 g of 3-ethoxy-pregna-3,5-dien-20-one was dissolved in an anhydrous mixture of 12 ml of tetrahydrofurane and 4 ml of tert. butanol. The solution was magnetically stirred and cooled to -10°C. Under nitrogen atmosphere was then added 810 mg of tert. potassiumbutoxide. The reaction vessel B was then immersed in liquid nitrogen, and the entire system was degassed until a vacuum of better than 0.1 mm Hg was attained. Stopcock D was then closed, and after breaking the glass seal 100 ml of ^{18}oxygen (90% purity, Miles Laboratories, Illinois) were transferred by opening stopcock 1, and warming of the container A. After an equilibration period

Fig. 1 HIGH-VACUUM TRANSFER LINE



LEGEND

A	Oxygen^{18} container
B	Reaction vessel
C	Outlet for second reaction vessel
D	Stopcock
E	Nitrogen inlet
F	Mercury trap
G	McLeod gauge
H	Ball joint
I	Main stopcock
J	Liquid nitrogen trap
K	High vacuum pump inlet
1-9	Stopcocks

of ca. 10 minutes, stopcock 2 was closed, the liquid nitrogen bath was removed from under B and replaced by an ice-salt bath, maintaining the temperature of the reaction flask at about -10°C. After 90 minutes of stirring, the steps of cooling B with liquid nitrogen, warming A, and equilibration were repeated. The reaction was then allowed to proceed for another 90 minutes at -10°C. The yellowish reaction mixture was then poured into icecold, saturated sodium chloride solution and brought to pH 7. The biphasic mixture thus formed was then extracted exhaustively with ethylacetate and ether. The organic extracts were pooled, washed with water, dried over sodium sulfate and evaporated to dryness under vacuum, whereby the waterbath temperature was maintained at below 45°C. A white residue of 640 mg was obtained, which was recrystallized twice from dioxane/water to yield colourless crystalls of m.p. 136-138°C (the literature reports m.p. 135-137°C). IR(KBr): 3360 (very broad), 1690, 1650, 1628, 1175, 1045, and 850 cm^{-1}.

17α-^{18}O-Hydroperoxy-4-pregnene-3,20-dione.

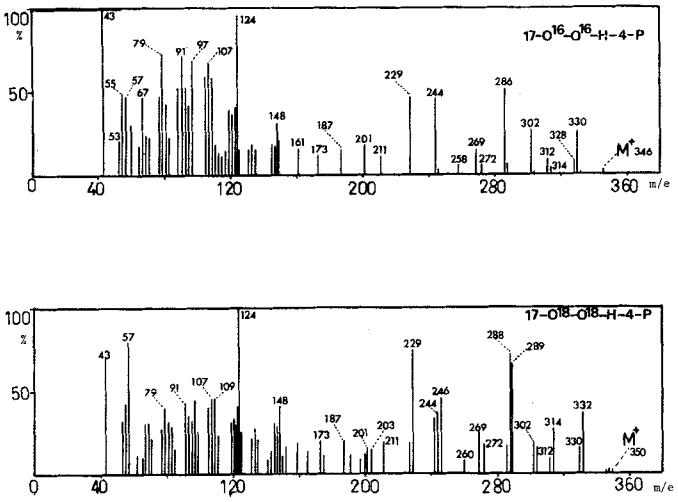
3-Ethoxy-17α-^{18}O-hydroperoxypregna-3,5-dien-20-one (500 mg) was dissolved in 25 ml of warm methanol. After addition of 25 ml of glacial acetic acid, the solution was rapidly cooled to room temperature with stirring. After 120 minutes, the solution was concentrated under vacuum and then poured into 100 ml of icecold water. The thus obtained white precipitate was filtered off and recrystallized from dioxane/water. The first crop gave 420 mg of colourless needles, m.p.: 183-185°C. IR(KBr): 3190, 1710, 1660, 1640 (sh.), 1610, 1120, and 872 cm^{-1}. Anal. Calcld. for $C_{21}H_{30}{}^{16}O_2{}^{18}O_2$: C, 72.00; H, 8.57. Found: C, 72.64; H, 8.67. Gas chromatographic analysis gave rise to the characteristic pyrolysis pattern which we have described earlier (5).

RESULTS AND DISCUSSION

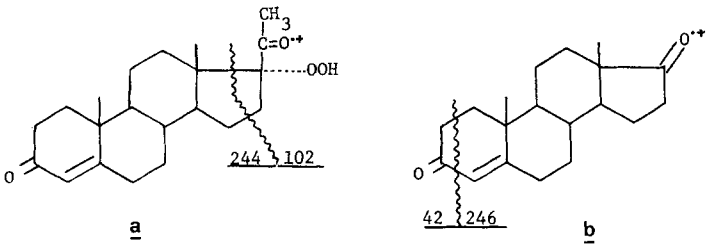
The succesful synthesis of ^{18}O-labeled 17α-hydroperoxyprogesterone depends upon a number of factors, such as: pressure, temperature, use of the right solvents, concentration of the reactants, complete dryness of the system, and the reaction time. We found that the best results were obtained when the reaction was not allowed to proceed beyond 3 hours. At the same time the temperature should be kept at lower than -10°C, as otherwise a host of other side-products is formed. To minimize any contamination with ordinary ^{16}oxygen from the air, a high vacuum had to be achieved. On the other hand, an adequate oxygen pressure must be present for the reaction to proceed. With only a limited amount of the very expensive ^{18}O-gas at our disposal, this incompatability has created problems. An attempt to dilute the ^{18}O-gas with nitrogen in order to achieve a positive overall pressure had to be abandoned, because under these conditions the hydroperoxidation reaction became excessively slow, giving rise instead to other undesirable products. This finding corroborates the previous report that α-ketohydroperoxides are unstable when kept for longer periods under basic conditions (1). We finally succeeded in overcoming these difficulties by keeping the volume of the reaction flask to a minimum, and by transferring as much as possible of the ^{18}O-gas into it. In the closed system which we were forced to use, the pressure of ^{18}O-gas diminishes in the course of the reaction to attain a certain limit, beyond which the reaction becomes too slow. Therefore, the yield of 57,9% which we have obtained was not quantitative. In fact, VAN RHEENEN and VISSER (2), who were using an open system with a large excess of oxygen, have obtained much better yields of the natural, ^{16}O-labeled compound.

Fig.2 shows the mass spectra of the naturally abundant, and the ^{18}O-labeled 17α-hydroperoxyprogesterones. At 70 eV, by slowly heating the

Fig. 2



solid injection probe, the molecular ions of resp. 346 and 350 were still discernable. Below m/e 201, there are no significant quantitative differences. In both spectra, the m/e 124 peak due to the ring-A fragment formed by scission of the C-9/C-10 and the C-6/C-7 bonds, is predominant. At the higher mass ranges, the differences between the spectra of the two compounds are more striking.



Usually, 20-ketopregnenes give rise to a m/e 244 peak (fragment a) due to release of a C_4 fragment by scission of the bonds between

C-13/C-17, and C-15/C-16 (6,7). This type of scission can hardly explain, however, the great enhancement of a m/e 246 ion in the spectrum of the ^{18}O-labeled compound. The 2 additional mass units most certainly originate from the ^{18}O-label, which means that the 246 ion must in some way still have one ^{18}O-labeled atom attached to it. We explain this by expulsion from the parent molecule of the acetyl side-chain and an OH-group to give first 4-androstene-3,17-dione, followed by loss of ketene from ring-A, to yield fragment b.

Based upon a calculation of the intensity ratios of the 346 and 350 molecular ion peaks in both spectra, the ^{18}O-labeled 17α-hydroperoxyprogesterone which we have synthesized has a relative ^{18}O-isotope content of 64.5%.

Our previous studies have indicated that 17α-hydroperoxyprogesterone is not stable under gas chromatographic conditions and decomposes into a number of lower molecular weight steroids (5,8). This apparently happens also when the substance is subjected to the heated environment of the mass spectrometer's ion source. The various compounds, which can be identified in this manner are shown in the following table (Table 1):

Table 1.

Steroid assigned	MS-ions from ^{16}O-compound	MS-ions from ^{18}O-compound
4,16-Androstadien-3-one	269*	269*
4-Androsten-3-one	272	272
4-Androstene-3,17-dione	286	288
16-Dehydroprogesterone	312	312
Progesterone	314	314
16,17α-Epoxyprogesterone	328	330
17α-Hydroxyprogesterone	330	332

*minus 1-H.

Of these decompositions, perhaps the most interesting is the one which is characterized by the loss of an oxygen molecule without the simultaneous introduction of a C-16 double bond, thus leading to the formation of progesterone. The mechanism of this deoxygenation reaction can best be described by a McLafferty type rearrangement, proceeding through the formation of a 6-membered cyclic transition state with participation of the 20-ketone, and the hydroperoxyl groups (5). Indeed, the IR-spectra of the hydroperoxides, both in the solid state as well as in dilute solution, show strong hydrogen-bonded OH-absorption bands.

To our knowledge, no data have been published thus far dealing with the NMR-spectra of 17α-hydroperoxyprogesterone and -pregnenolone. Since we were interested in the conformation of the 20-ketopregnene side-chain when a geminal OOH-group is present, we have compared the spectra of various 17α-oxygenated and non-substituted pregnenes (Table 2).

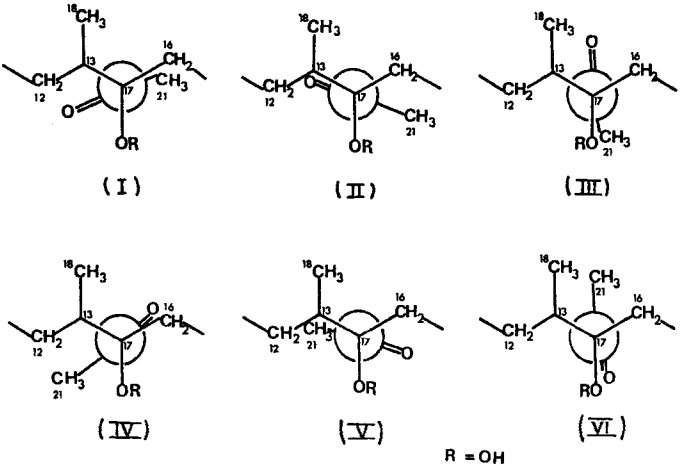
Table 2. PROTON MAGNETIC RESONANCE DATA (DMSO-^{2}H).

COMPOUND	18-Me (ppm)	19-Me (ppm)	21-Me (ppm)
PREGNENOLONE	0.908	1.316	2.436
17α-HYDROXYPREGNENOLONE	0.870	1.313	2.466
17α-HYDROPEROXYPREGNENOLONE	0.990 ; 0.933	1.330	2.583 ; 2.506
PROGESTERONE	0.941	1.516	2.433
17α-HYDROXYPROGESTERONE	0.908	1.516	2.466
17α-HYDROPEROXYPROGESTERONE	0.950 ; 0.916	1.516	2.553 ; 2.473

For solubility reasons, the spectra were determined in d-dimethylsulfoxide solution, while tetramethylsilane was used as an internal reference. The 19-methyl protons are too far removed to be influenced by a 17α-substituent. However, the 18-, and 21-methyl protons are shifted downfield in 17α-hydroperoxyprogesterone and -pregnenolone. Moreover, their

signals are split into a pseudo-doublett with an intensity ratio of ca. 2:1. In Fig.3, we have drawn the possible conformations, which the

Fig.3 NEWMAN PROJECTIONS VIEWED ALONG THE C-17 TO C-20 AXIS



progesterone side-chain may assume. The most stable conformation would be IV, in which the 20-carbonyl dipole is pointed towards the C-16 methylene moiety and the C-20,21 bonds are oriented more or less anti-parallel with the C-16,17 bonds. This conformation has recently been established for the decanoate esters of 17α-hydroxyprogesterone by X-ray analysis (9). Apart from this preferred conformation, due to the restricted rotation of the side-chain, a second one may exist to some extent, i.e. conformation VI. In this conformation, the hydroperoxyl and ketone groups assume the most suitable position for the formation of a hydrogen-bond and a 6-membered cyclic transition state. The finite existence of these 2 conformations may account for the observed splittings of the 18-, and 21-methyl signals in the NMR-spectra.

ACKNOWLEDGEMENTS

We wish to thank the Medical Research Council of Canada and the Banting Research Foundation for financial support of this work.

REFERENCES

1. Bailey, E.J., Barton, D.H.R., Elks, J., and Templeton, J.F., J.CHEM.SOC., 1578 (1962).
2. Van Rheenen, V. and Visser, V., CHEM.ABSTR., 72, 79324b (1970).
3. Tan, L. and Falardeau, P., STEROIDOLOGIA, 2, 65 (1971).
4. Tan, L., Clemence, M., and Gass, J., J.CHROMATOG., 53, 209 (1970); see also, Dougan, J. and Tan, L., IBID., 86, 107 (1973).
5. Tan, L., Wang, H.M., and Falardeau, P., CAN.J.BIOCHEM., 50, 706 (1972).
6. Peterson, L., ANAL.CHEM., 34, 1781 (1962).
7. Takemoto, C., Nakano, H., Sato, H., and Tamaoki, B., BIOCHIM.BIOPHYS. ACTA, 152, 749 (1968).
8. Wang, H.M. and Tan, L., PROC.CAN.FED.BIOL.SOC., 15, 37 (1972).
9. Watson, W.H., Go, K.T., and Purdy, R.H., ACTA CRYST., B29, 199 (1973).