[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF UTAH]

A Synthesis of Progesterone Labeled in Position 4[1]

BY G. I. FUJIMOTO AND JULIANNE PRAGER

RECEIVED FEBRUARY 16, 1953

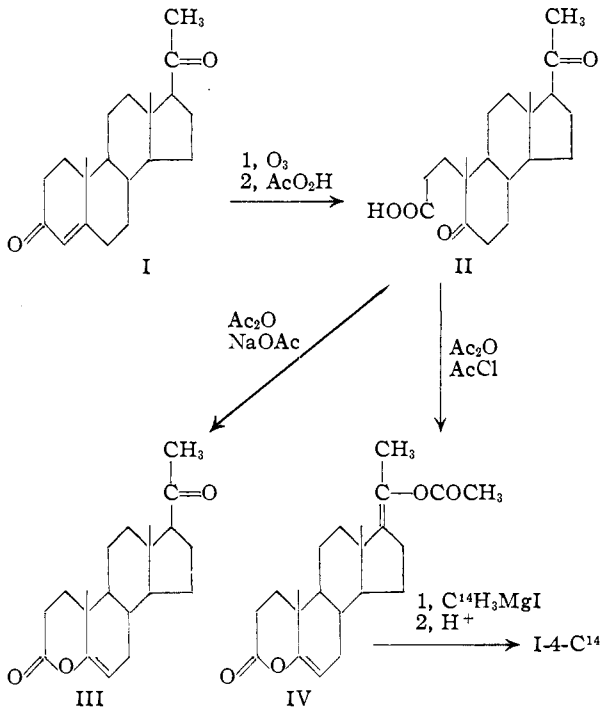
A method of introducing carbon 14 in ring A of progesterone while keeping the side chain intact is described. The Grignard reaction with the enol lactone in ring A has been applied to progesterone by protecting the C-20 ketone by acetylation. Greatly improved yields and an easily purified product are obtained.

At present, one of the fundamental problems of endocrinology is the mechanism of cellular action of the steroid hormones. The synthesis and use of side chain labeled progesterone has afforded some valuable information on the distribution and fate of the injected steroid but little information was obtained on its mode of action.[2] Furthermore, these studies indicated that the side chain of approximately 5 to 10% of the progesterone administered was metabolized to CO_2. The functional significance of this oxidation could further be elucidated by use of the biologically more stable ring-labeled molecule.

It is generally accepted that the steroid hormones function in extremely minute concentrations. Consequently, for physiological investigation, it is desirable that the specific activity of the steroid studied be at a maximum. The specific activities of the preparations used in the above studies were in no case greater than 2,700 disintegrations per minute per microgram of progesterone-21-C^{14}. We are reporting the synthesis of progesterone labeled in position 4 of the A ring and having a specific activity of approximately 38,000 disintegrations per minute per microgram.

Previous methods for labeling of steroids in the nucleus have involved the preparation and use of isotopically labeled phenyl acetate or bromoacetic ester. Cholestenone and testosterone labeled in ring A have been described by Turner.[3] The Reformatsky method was extended to the synthesis of ring-A-labeled Δ^4-3-ketoetiocholenic acid ester which was then converted by several steps to ring-labeled progesterone and desoxycorticosterone.[4] Because of the many reactions and the low yields, this procedure is undesirable as a preparative method. Also, in any synthesis involving many steps, there is, of necessity, the technical difficulty of satisfactorily separating the product from radioactive intermediates at each step.

Recently, we reported a procedure for the synthesis of cholestenone-4-C^{14} and testosterone-4-C^{14}, by which the labeled carbon is introduced in the final stage by means of the Grignard reaction with the corresponding enol lactone.[5] Our first attempts to synthesize radioprogesterone were directed at reproducing this reaction leaving the C-20 ketone intact. Rupture of the A ring of progesterone (I) with loss of a carbon was effected by ozonization to form 5,20-diketo-3,5-seco-A-norpregnan-3-oic acid (II). This has been reported in 28% yield by Reichstein and Fuchs[6] but by decomposing the ozonide with peracetic acid instead of zinc we were able to improve the yield of the diketo acid II to 80%. Then the enol lactone in the A ring was formed by treatment with sodium acetate–acetic anhydride. Infrared spectra and analyses confirmed the formation of the enol lactone III and the presence of the intact C-20 carbonyl group.



However, when the enol lactone III was treated with one equivalent of methylmagnesium iodide, and then with alkali, we obtained a mixture of products which could not be readily separated and which contained little if any progesterone. The mixture showed an absorption maximum at 240 mμ. Since progesterone could not be isolated there was probably present a product of the Grignard reagent with both the C-20 carbonyl and the enol lactone.

Thus, it appeared necessary to protect the C-20 carbonyl group and this was done successfully by making the enol–acetyl derivative at this position.

(1) This work was supported in part by a Grant-in-Aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, by the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and by Armour and Company.

(2) B. Riegel, W. L. Hartop, Jr., and G. W. Kittinger, *Endocrinology*, [5] **47**, 311 (1950); M. C. Barry, M. L. Eidinoff, K. Dobriner and T. F. Gallagher, *ibid.*, [6] **50**, 587 (1952); H. J. Grady, W. H. Elliott, E. A. Doisy, Jr., B. C. Bocklage and E. A. Doisy, *J. Biol. Chem.*, **195**, 755 (1952).

(3) R. B. Turner, THIS JOURNAL, **72**, 579 (1950).

(4) R. D. H. Heard and P. Ziegler, *ibid.*, **72**, 4328 (1950).

(5) G. I. Fujimoto, THIS JOURNAL, **73**, 1856 (1951); see also R. D. H. Heard and P. Ziegler, *ibid.*, **73**, 4036 (1951).

(6) T. Reichstein and H. G. Fuchs, *Helv. Chim. Acta*, **23**, 682 (1940).

The diketo acid II was both lactonized and acetylated in acetic anhydride–acetyl chloride solution. By analyses and infrared study, the products were shown to be a mixture of the Δ^{17}-enol-20-acetates IV. This mixture most likely contained both the α- and β-forms for, when the reaction was reproduced on pregnenolone acetate, the α- and β-forms of the Δ^{17}-enol-20-acetates described by Fieser and Huang-Minlon[7] were obtained.

The mixture of the 20-isomers IV was treated with methylmagnesium iodide and without further purification the Grignard product was hydrolyzed and cyclized in acetic and hydrochloric acid solution. Final purification of the progesterone was accomplished by partition chromatography according to the method developed by Butt, *et al.*[8] The 46% yield compares closely with the yields we have obtained for the synthesis of cholestenone and testosterone[5] indicating that for the most part the reaction had proceeded in the desired direction.

Hydrolysis of the side chain enol acetate might be expected to give rise to a mixture of progesterone and isoprogesterone. However, as in the case of the hydrolysis of the Δ^{17}-enol-20-acetates from pregnenolone acetate,[7] only the 17-normal product was obtained.

Final identification was made by melting point, mixed melting point and infrared analysis. Hooker–Forbes microbioassay in the mouse demonstrated that the enol lactone III was 1/1000 as active as progesterone and the final product contained the same activity as authentic progesterone.[9]

When unlabeled methyl iodide was used the final yield on the basis of methyl iodide was 46%. In the radioactive synthesis from methyl iodide-C^{14}, a yield of 25% was obtained. It is probable that this lower yield was due to the decomposition of the radiomethyl iodide which had been in storage for almost a year.

Experimental

Melting points were taken on a Kofler micro hot-stage and have been corrected. Infrared spectra were taken on a Perkin–Elmer double beam recording spectrophotometer model 21. The microanalyses were carried out by Dr. A. Elek.[10]

5,20-Diketo-3,5-seco-A-norpregnan-3-oic Acid (II).—A solution of 1.6 g. of progesterone in 75 ml. of 5:4 ethyl acetate–glacial acetic acid was ozonized by passing through it 3 mole equivalents of ozone at a rate of 150 ml./minute. In order to decompose the ozonide 2 ml. of 30% hydrogen peroxide was added and the solution was placed in the refrigerator overnight. The solvents were then removed *in vacuo* at room temperature. The residual oil was dissolved in ether and extracted with dilute sodium carbonate. Some of the starting material was recovered from the ether layer. The sodium carbonate extract was acidified with dilute sulfuric acid and 1.35 g. (79%) of the diketo acid II was obtained; m.p. 170–175°. On recrystallization from acetone, the melting point was raised to 174–178° (reported[6] m.p. 173–175°).

Anal. Calcd.: C, 71.82; H, 9.04. Found: C, 71.81; H, 8.92.

The disemicarbazone of the diketo acid II was prepared by the customary method and recrystallized from methanol; m.p. 268° with decomposition.

Anal. Calcd.: C, 58.90; H, 8.09; N, 18.74. Found: C, 58.84; H, 8.18; N, 18.29.

5-Hydroxy-20-keto-3,5-seco-A-norpregn-5-en-3-oic-3,5-lactone (III).—A mixture of 2.3 g. of diketo acid II, 1.5 g. of anhydrous sodium acetate and 100 cc. of acetic anhydride was heated for 48 hours in an atmosphere of nitrogen in an oil-bath (80–95°). The acetic anhydride was removed *in vacuo* and the residue carefully extracted with acetone. The crude enol lactone *III* obtained upon evaporation of the acetone solution was recrystallized from acetone, yielding 1.5 g. (69%) of white crystalline material, m.p. 153–156°. The analytical sample was recrystallized several times from acetone, m.p. 154–156°. The infrared spectral bands at 5.67, 5.85 (C-20 carbonyl), 5.92, 7.40, 7.95, 8.25, 8.37, 8.64, 8.81 and 9.01 μ were characteristic of steroid enol lactones.

Anal. Calcd.: C, 75.91; H, 8.92. Found: C, 75.61; H, 8.58.

Mixture of C-20 Isomers of 5-Hydroxy-20-acetoxy-3,5-norpregna-5,17-dien-3-oic-3,5-lactone (IV).—A solution of 2.5 g. of diketo acid II in 10 ml. of acetic anhydride and 10 ml. of acetyl chloride was refluxed gently for 48 hours in a dry atmosphere. The solvents were removed *in vacuo* and the brown crystalline residue was recrystallized from acetone. The yield of colorless enol lactone enol acetate (IV, a mixture of the position 20 isomers) was 1.2 g. (45%), m.p. 172–182°. An analytical sample was prepared by recrystallizing several times from acetone; m.p. 181–185°. The absorption bands in the infrared were at 5.67, 5.91, 7.30, 7.95, 8.12, 8.31, 8.62, 8.83 and 9.00 μ.

Anal. Calcd.: C, 73.71; H, 8.44. Found: C, 73.65; H, 8.50.

Progesterone from IV.—A flask containing 90 mg. of magnesium and 10 ml. of dry ether was cooled in liquid nitrogen and 351 mg. of methyl iodide *in vacuo* was added through a break seal joint. After the addition, the solution was warmed to room temperature and kept at 30° for two hours. An atmosphere of nitrogen was introduced and the Grignard solution was added dropwise to a stirred solution of 951 mg. of enol lactone enol acetate (IV) in 100 ml. of dry ether.

The first addition caused a white, curd-like precipitate to form; this precipitate soon filled the solution. The mixture was stirred continuously for 30 minutes after addition was complete and allowed to stand overnight. Then, the ether solution was poured into 30 ml. of cold ammonium chloride solution and swirled frequently for 20 minutes. The aqueous layer was thoroughly extracted with ether and the combined ether extracts were washed with water. The ether was carefully removed by distillation. To the residue was added 16 ml. of glacial acetic acid and 1.5 ml. of concd. hydrochloric acid. After 36 hours at room temperature in an atmosphere of nitrogen, the acids were removed *in vacuo* without heating, leaving an orange-colored oil. This oil was dissolved in ether, washed with dilute sodium carbonate and then with water. The ether was distilled and the residue, a light yellow, partially crystalline material, was dried thoroughly. This material was divided into 10 portions; each was chromatographed on Hyflo Supercel using 70% aqueous methanol (equilibrated with hexane), as the stationary phase, and hexane (equilibrated with 70% methanol) as the moving phase, as described by Butt, *et al.*[8] The column, 20 by 2.8 cm., was packed with 24 g. of the Supercel wet with 16 cc. of stationary phase. The material to be chromatographed was placed on the column in 0.8 ml. of stationary phase. This was eluted with the moving phase and collected in 10-ml. fractions. Upon evaporation of the solvent, the 4th to 8th fractions crystallized. After recrystallization from acetone–hexane, the melting point was 120–122°. There was no depression on admixture with an authentic sample of progesterone. The total yield was 380 mg., 46% based on the methyl iodide used.

When the above reaction was carried out using 1.0 mmole of methyl iodide carbon-14[11] (1.2×10^{10} disintegrations/minute) and 360 mg. of enol lactone enol acetate (IV), there was obtained 80 mg. (25% based on the radiomethyl

(7) L. F. Fieser and Huang-Minlon, THIS JOURNAL, **71**, 1840 (1949).

(8) W. R. Butt, Peggy Morris, C. J. O. R. Morris and D. C. Williams, *Biochem. J.*, **49**, 434 (1951).

(9) The enol lactone III was assayed by Dr. M. X. Zarrow and the radioprogesterone by Drs. H. A. Salhanick and A. G. Olsen.

(10) Elek Micro Analytical Laboratories, 4763 W. Adams Blvd., Los Angeles 16, Calif.

(11) J. D. Cox, H. S. Turner and R. J. Warne, *J. Chem. Soc.*, 3167 (1950).

iodide) of progesterone-4-C^{14} (38×10^6 disintegrations/minute/mg.). The infrared spectra of progesterone-4-C^{14} were identical to those of an authentic sample of progesterone. The major absorption bands occurred at 5.86 (C-20 carbonyl), 5.98 (C-3 carbonyl), 6.17 and 7.36 μ.

SALT LAKE CITY, UTAH

[CONTRIBUTION FROM THE LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID COMPANY]

Synthesis of Degradation Products of Aureomycin. IV[1]

BY J. H. BOOTHE, S. KUSHNER, J. PETISI AND J. H. WILLIAMS

RECEIVED FEBRUARY 18, 1953

One of the degradation products of Aureomycin, 4-chloro-3-hydroxy-7-methoxy-3-methylphthalide has been synthesized by chlorination of 3-hydroxy-7-methoxy-3-methylphthalide. The normal and pseudo esters of these compounds have been prepared and characterized.

During the degradation of Aureomycin,[2] a compound was isolated which was postulated to be 4-chloro-3-hydroxy-7-methoxy-3-methylphthalide (III). This postulation was based partially on the observation that the compound formed two different methyl esters depending on the method of esterification. This fact indicated a normal and a pseudo ester formation which is characteristic for *o*-acyl- or *o*-aroylbenzoic acids[3] or similar compounds in the aliphatic series.[4] It has been postulated that this type of compound exists as an equilibrium mixture of the keto acid and the phthalide type structure. In this paper these acids are named as phthalides rather than as carboxylic acids.

3-Hydroxy-7-methoxy-3-methylphthalide (II) was prepared by hydrolysis of 2-cyano-3-methoxyacetophenone (I)[5] in dilute sodium hydroxide. Hydrolysis of this cyano compound did not go well in acid solution although small yields could be obtained by hydrolysis in boiling 6 *N* hydrochloric acid. Ammonia determinations were run on the hydrolysis mixture to follow the course of the reaction and it was found that after 30 minutes about 35% of the theoretical amount of ammonia was liberated. This figure only increased to about 40% after four hours. The alkaline hydrolysis however gave good yields and a minimum of side reactions.

This acid, 3-hydroxy-7-methoxy-3-methylphthalide (II), on treatment with diazomethane gave a low melting normal ester (IV). The pseudo ester (V) was prepared by acid–methanol esterification or by treating the acid chloride with methanol and pyridine. The normal ester proved to be quite unstable in acid solution and rearranged to the pseudo ester at room temperature in dilute hydrochloric acid. It was also found that some batches of 3-hydroxy-7-methoxy-3-methylphthalide yielded only the pseudo ester on treatment with diazomethane. This unexpected reaction was probably catalyzed by small amounts of mineral acids because after thorough washing of these batches with water, they would then yield the normal ester.

Kuhn and Dury[6] have reported the preparation of "6-methoxy-2-acetylbenzoic acid methyl ester" from the degradation of Terramycin and by a synthetic route different from the one presented here. In each case the esterification was done on a crude mixture with diazomethane. The authors assumed their product was a normal ester as their name for it indicates. However, their reported m.p. is identical with that found by us for the pseudo ester (V). This anomaly might be explained by the presence of small amounts of acidic impurities in their product before esterification as noted in the above paragraph, or perhaps the normal ester is isomerized by distillation.

Chlorination of 3-hydroxy-7-methoxy-3-methylphthalide (II) with chlorine in acetic acid yielded 4-chloro-3-hydroxy-7-methoxy-3-methylphthalide (III). This compound was identical in all respects with the product from Aureomycin degradation as were the various derivatives described below. A normal (VII) and a pseudo ester (VI) were prepared in the same manner as described above for the unchlorinated compound. The normal ester also rearranged to the pseudo ester on treatment with dilute hydrochloric acid.

The position of the entering chlorine atom in these compounds is proved by the fact that they are identical with the degradation product of Aureomycin, since the position of the chlorine in Aureomycin has already been proved.[5]

The ultraviolet absorption data of these acids, normal esters and pseudo esters in methanol are shown in Table I. An inspection of these data shows that in the unchlorinated compounds the acid and the pseudo ester absorb similarly probably

TABLE I

ULTRAVIOLET ABSORPTION DATA

Compound	Maxima mμ	ε	mμ	ε
Acid (II)	232	6940	300	4140
Normal ester (IV)	245	6010	312	3160
Pseudo ester (V)	237	6190	300	5090
Chlorinated acid (III)	235	8610	307	3760
Normal ester (VII)	230	8620	302	3165
	End absorption			
Pseudo ester (VI)	237	9080	312	5240

(1) Portions of this work were presented in a preliminary communication: S. Kushner, *et al.*, THIS JOURNAL, **74**, 3709 (1952).

(2) B. L. Hutchings, *et al.*, *ibid.*, **74**, 3710 (1952).

(3) M. S. Newman, *et al.*, (a) *ibid.*, **63**, 1537 (1941); (b) *ibid.*, **66**, 731 (1944); (c) *ibid.*, **73**, 4625 (1952).

(4) R. E. Lutz and A. W. Winne, *ibid.*, **56**, 445 (1934).

(5) S. Kushner, *et al.*, *ibid.*, **75**, 1097 (1953).

(6) R. Kuhn and K. Dury, *Ber.*, **84**, 848 (1951).