PREPARATION OF SOME C_{19} STEROID-PROTEIN CONJUGATES

R. S. Rosenfeld, Burton Rosenberg, Jacob Kream and Leon Hellman

Institute for Steroid Research and the Department of Oncology, Montefiore Hospital and Medical Center, New York, N. Y. 10467

Received: 1/11/73

ABSTRACT

17-Carboxymethoximino derivatives of DHA (1), androsterone and etiocholanolone, as well as the 7-carboxymethoximino derivatives of 3β-hydroxy-androst-5-ene-7,17-dione and 7-keto-androst-5-ene-3β,17β-diol have been prepared and conjugated to BSA for use in producing antisera to the corresponding C_{19} steroids.

The analysis of steroid hormones in blood has been greatly facilitated by the use of competitive protein-binding techniques (2). However, 17-ketosteroids have not been measured in this way because they bind poorly to the proteins commonly used in these assays. Thus, unconjugated dehydroisoandrosterone (DHA), androsterone, and etiocholanolone have been

measured by double-isotope derivative (3) and gas-liquid chromatographic methods (4). DHA in plasma has been assayed by competitive protein-binding but only after conversion to androst-5-ene-3β,17β-diol which then binds to the appropriate protein (5). The recent application of radioimmunoassay techniques to the problem has resulted in procedures with more specificity and sensitivity in blood hormone analysis (6) which can, by preparing the appropriate antibodies, be used for the assay of circulating 17-ketosteroids. In this connection, Nieschlag, Loriaux and Lipsett have published a radioligand assay for plasma DHA and its sulfate (7). It is the purpose of this communication to report the synthesis of some antigens of DHA, androstenediol, androsterone and etiocholanolone which are potentially useful for the analysis of circulating C_{19} steroids; a preliminary report has appeared (8).

The 17-carboxymethoximino derivatives of DHA, androsterone and etiocholanolone as well as androsterone hemisuccinate were prepared by procedures identical to those described by Erlanger et al for the synthesis of the corresponding derivatives of testosterone (9); their physical constants appear in Table I of the experimental section.

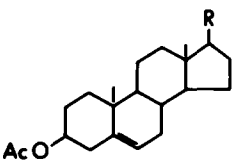
In order to obtain a DHA antigen which would elicit specific antibody, we prepared the 7-carboxymethoximino derivative (V) which has both the A and D rings available as antigenic determinants. DHA-7α-^{3}H, 4-^{14}C was converted to the 17-ethylene ketal (10) and the reaction mixture was acetylated. DHA-3-acetate-17-ethylene ketal (Ia) was separated from DHA-acetate by chromatography on alumina. Oxidation of Ia with chromic oxide-pyridine

complex in methylene chloride (11) afforded a mixture which consisted of about 22% starting material and 78% of the 7-keto derivative IIa. Efforts to separate the two compounds were unsuccessful; consequently the mixture was reacted with carboxymethoxylamine hemihydrochloride and after removal of the neutral fraction, the acidic portion afforded 7-carboxymethoximino-androst-5-en-3β-ol-17-one-17-ethylene ketal (IV) in good yield. Compound IV contained ^{14}C but no ^{3}H, as would be expected if oxidation at C-7 had taken place. Under the reaction conditions, the 3-acetoxy group was hydrolyzed. Removal of the protecting group at 17 was achieved by treatment with hydrochloric acid in ethanol to give 7-carboxymethoximino-androst-5-en-3β-ol-17-one (V).

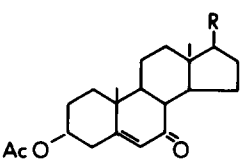
Androst-5-en-3β,17β-diol occurs in significant quantities in the circulation (5) and, because of its cross reactivity with antiserum generated against DHA-17-BSA, may be measured by radioimmunoassay techniques using the same antiserum after the diol is separated from DHA (12). Since an antiserum against androstenediol-7-BSA should possess greater specificity for the diol, the hapten VI was prepared from androst-5-en-3β,17β-diol diacetate (Ib) by a reaction sequence similar to that used for making the 17-keto analog V. Oxidation of Ib with the chromium trioxide-pyridine complex (11) gave the 7-keto derivative IIb in about 63% yield, the remainder being starting material. Reaction of the oxidation product with carboxymethoxylamine hemihydrochloride gave a mixture which was separated into a neutral fraction (Ib) and an acid fraction; the latter appeared to consist of VI and an

acetylated derivative of VI. After saponification at room temperature, 7-carboxymethoximino-androst-5-en-3,17-diol (VI) was obtained in good yield.

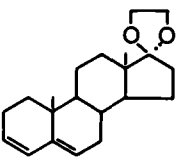
The steroid carboxymethoximes and androsterone hemisuccinate were covalently linked to bovine serum albumin by the mixed anhydride technique (9). These conjugates are being injected into animals for the preparation of antibodies and the results and applications to measurements of steroids in blood will be the subject of a future communication.



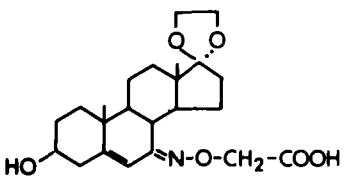
I a,b



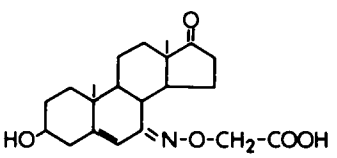
II a,b



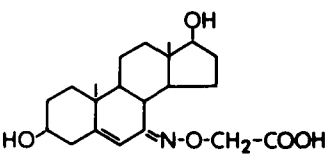
III



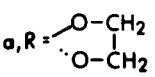
IV



V



VI

a, R = 

b, R = O Ac

EXPERIMENTAL

Melting points were taken on a micro hot stage and are corrected. Optical rotations were measured in absolute ethanol. Infrared spectra were determined in potassium bromide dispersions.

Dehydroisoandrosterone-3-acetate-17-ethylene ketal (Ia). Tracer amounts of DHA-7α-^{3}H and DHA-4-^{14}C (New England Nuclear Corp., Boston, Mass.) were mixed with 10 g of DHA so that the specific activity of the material was 70 cpm per mg ^{3}H and 235 cpm per mg ^{14}C. The material was dissolved in 550 ml of benzene to which 9 ml of ethylene glycol was added and the mixture was refluxed for one hour; water was removed via a distillate collector adapter. Following the introduction of 660 mg of p-toluenesulfonic acid, refluxing was continued for 21 hours in the same apparatus, drawing off the water during the first few hours (10). The cooled solution was diluted with ether containing a few drops of pyridine and was washed with 2.5% sodium hydroxide and water. The ether was removed in vacuo to yield an oil (10.7 g) which crystallized on standing. GLC analysis showed 2 peaks, corresponding to about 35% of the starting material and 65% of DHA-17-ethylene ketal.

The entire product was acetylated by remaining overnight at 37° in 10 ml of pyridine and 5 ml acetic anhydride. After the addition of ice, the mixture was extracted with ether and the solution was washed with sodium hydroxide and water to afford, after removing the ether, a crystalline mass (11 g) which, by GLC showed a mixture of DHA-3-acetate and DHA-3-acetate-17-ketal (Ia) in the same proportion as above.

3 Grams of the acetate mixture was chromatographed on 200 g of neutral alumina (Woelm, Waters Associates, Framingham, Mass. 01701) made to grade II (Brockmann). The column was developed with petroleum ether-benzene mixtures; 1.81 g of DHA-3-acetate-17-ethylene ketal (Ia) was obtained in the 1:1 petroleum ether-benzene eluates and 1.05 g of DHA-3-acetate was recovered in the benzene fraction. Ia was crystallized from benzene-isooctane, m.p., 140-141°, and showed an infrared spectrum identical with that of an authentic sample. The GLC showed a single peak with an RRT identical to the later peak in the mixture before chromatography.

3β-Acetoxy-androst-5-en-7,17-dione-17-ethylene ketal (IIa). DHA-3-acetate-17-ethylene ketal (Ia) was oxidized with the CrO_3-(pyridine)$_2$ complex (11). The reagent was prepared as described by stirring dry chromium trioxide with pyridine at -15° for 5 hours. The red slurry was quickly filtered through a course glass funnel and the precipitate was washed with dry petroleum ether and stored in a desiccator over phosphorus pentoxide at reduced pressure

(water pump). 1.12 g of Ia was dissolved in 60 ml of methylene chloride in a round bottom flask equipped with a nitrogen inlet, drying tube, and stirrer. While mixing at room temperature under a nitrogen atmosphere, a slurry of 7 g of CrO_3-(pyridine)$_2$ in 15 ml of methylene chloride was introduced and stirring was continued for 19 hours. A further addition of 2.5 g of the oxidizing agent in 10 methylene chloride was made and the reaction was permitted to run for 6 additional hours. The methylene chloride solution was diluted with ether and the tarry residue in the flask and on the stirrer was washed with ether. The combined ether solutions were washed 6 times with saturated sodium bicarbonate and most of the color passed into the aqueous alkali. After 3 water washes, the ether was removed to afford 990 mg of oil which crystallized on cooling. GLC analysis revealed two products (Table II), corresponding to the starting substance (Ia, 22%) and the oxidized material (IIa, 78%). Ultraviolet spectra, maxima at λ = 234 mμ (ethanol), E = 10000, corresponding to 80% of Δ^5-7-ketone (E_{max} = 12300) (13).

The oxidation was performed 3 more times with no improvement in the proportion of the 7-keto-derivative (IIa). Two recrystallizations from methanol and a third from benzene-isooctane were unsuccessful in separating the mixture. Chromatography on alumina failed to yield the product; while Ia was obtained in pure form, the major portion of IIa lost the elements of acetic acid to yield the corresponding $\Delta^{3,5}$-7 ketone (III), λ = 277 mμ (E = 20200) (12). GLC data are also in accord with this structure (Table II).

7-Carboxymethoximino-androst-5-en-3β-ol-17-one-17-ethylene ketal (IV). A mixture (990 mg) of Ia and IIa (20:80) was dissolved in 35 ml of ethanol. To this was added the solution prepared by mixing 675 mg of carboxymethoxylamine hemihydrochloride in 2.8 ml of 2N sodium hydroxide and the solution was refluxed for 3 hours. Water (70 ml) was added to the solution followed by 2N alkali to pH 10 and the alkaline solution was extracted with ether to afford, after the usual procedure, 295 mg of neutral fraction which, by GLC, was a mixture of DHA-17-ketal, Ia and unreacted IIa. It was not examined further.

The aqueous solution was acidified to pH 1 with 1 ml of concentrated hydrochloric acid. A precipitate formed which was extracted 3 times with ethyl acetate. The ethyl acetate solution was washed with water and dried over sodium sulfate. Removal of the solvent in vacuo yielded 776 mg of crystalline material, UV maxima at 238 mμ ($\overline{E = 11000}$). GLC (Table II) of the derivative prepared by treatment with Sil-Prep (Applied Science Laboratories, State College, Pa.) showed only one peak consistent with structure IV. Recrystallized from ethyl acetate: m.p., 204° (dec); $[\alpha]_D^{26}$, = - 192.1° (ethanol); infrared spectrum, acetate absent, bands at 1170 and 1310 cm^{-1} (17-ethylene ketal); specific activity, 177 cpm per mg ^{14}C, no ^3H. Analysis: calculated for $C_{23}H_{33}O_6N$ (MW, 419); C, 66.0, H, 7.89, N, 3.34. Found: C, 65.8, H, 7.87, N, 3.33.

7-Carboxymethoximino-androst-5-en-3β-ol-17-one (V). 800 Milligrams of IV was dissolved in 50 ml ethanol containing 2 ml concentrated hydrochloric acid. On standing overnight at room temperature, the solution was diluted with water and extracted with ethyl acetate to afford, after the usual procedures, 715 mg of an oil which resisted all attempts at crystallization. GLC showed a single peak (Table II) consistent with V. For characterization, a portion was chromatographed on a 20 x 20 cm silica gel plate in benzene:dioxane:acetic acid (75:20:2) (14). Extraction of the major band yielded a colorless oil which on drying in vacuo afforded a noncrystalline solid: UV maxima at 239 mμ (E = 12900) in ethanol; RRT, 4.71; specific activity, 170 cm per mg ^{14}C. The infrared spectrum was in accord with structure V; broad OH at 3460 cm^{-1} (3-hydroxyl and the carboxyl group); ketone band at 1740 cm^{-1} (17-ketone); shoulder at 1760 cm^{-1} (carboxyl carbonyl). Analysis: calculated for $C_{21}H_{29}O_5N$ (MW, 375); C, 67.2, H, 7.73, N, 3.73. Found; C, 66.9, H, 7.95, N, 3.74.

7-Keto-androst-6-en-3β,17β-diol-3,17-diacetate (IIb). 6.52 Grams of Ib, SA, 298 cpm per mg was reacted with the chromium trioxide-pyridine reagent as described above. After washing the ether extract, 6.34 g of crystalline product was obtained which, by GLC (Table II) showed 2 peaks corresponding to 63% IIb and 37% of starting material; the UV maximum at 235 mμ indicated a 70% yield of the Δ^5-7-ketone.

7-Carboxymethoximino-androst-5-en-3β,17β-diol (VI). A portion of the above mixture, 950 mg, was refluxed for 3 hours in 35 ml ethanol containing 495 mg carboxymethoxylamine hemihydrochloride and 2.05 ml 2N sodium hydroxide. After reducing the volume to about 15 ml in a rotovap, the residue was diluted with water and made alkaline to pH 11 by the addition of 2N sodium hydroxide. Extraction with ether yielded 380 mg of crystalline neutral fraction whose infrared spectrum was identical with that of the diol-diacetate Ib. The aqueous phase was acidified with concentrated hydrochloric acid, then extracted with ethyl acetate which was washed 3 times with water and concentrated to afford 615 mg of acid fraction, UV maximum at 235 mμ. That the product was a mixture of VI and an acetoxy derivative of VI was shown by GLC of the TMS derivative (2 peaks, RRT: 2.66 and 5.05) and by the NMR spectrum which displayed a signal, δ = 2 ppm, characteristic of an acetoxy methyl group.

The final product VI was obtained by dissolving 196 mg of the acid fraction in 50 ml of 2N sodium hydroxide in 80% methanol and storing overnight at room temperature. After acidification and extraction with ether, the usual processing gave 160 mg of crystalline 7-carboxymethoximino-androst-5-en-3β, 17β-diol (VI), SA 301 cpm per mg (calculated on basis of starting material Ib, 296 cpm per mg), RRT of TMS derivative, 2.66. The acetoxy methyl signal, δ = 2 ppm, was absent in the NMR spectrum. After two recrystallizations from ethanol, the substance decomposed at 227-229°; $[\alpha]_D^{27.5}$, − 192.7° (ethanol).

Analysis: Calculated for $C_{21}H_{31}O_5N$ (MW, 377.2); C, 66.8; H, 8.27; N, 3.71. Found: C, 66.8; H, 8.35; N, 3.59.

17-Carboxymethoxyimino derivatives of DHA, androsterone and etiocholanolone. These substances were prepared as described for IV according to procedures of Erlanger and co-workers (9). All of the derivatives were crystalline and analytical data are reported in Table I.

TABLE I

Analyses and Physical Constants of the 17-Carboxymethoximino Derivatives of 17-Ketosteroids

17-Carboxymethoximino derivative of:	m.p.	$[\alpha]_D^{24}$ (ethanol)	Elemental Analysis* C	H	N
DHA	264-266 (dec)	-37.9	70.18	8.66	3.85
Androsterone	173-175	+38.7	69.47	9.31	3.79
Etiocholanolone		+45.7	69.50	9.28	3.75

* The analyses are in good agreement with the theoretical values.

Androsterone Hemisuccinate. Androsterone was refluxed with succinic anhydride in pyridine for 4 hours according to Erlanger et al (9). Isolation of the product yielded crystals, m.p. 185-186° (15), $[\alpha]_D^{24}$ = + 59.3 (ethanol). C and H analyses were in good agreement with calculated values.

Conjugation of steroid derivatives to BSA. The substances were conjugated by the mixed anhydride technique (9). For 7-carboxymethoximino-androst-5-en-3β-ol-17-one-^{14}C (V): 772 mg, specific activity, 170 cpm per mg, was dissolved in 18 ml dry dioxane to which 0.50 ml of tri-n-butylamine was added and the solution was cooled to 10°. After the addition of 0.264 ml of isobutylchloro-carbonate, the mixture was cooled to below 10°, at about the freezing point of the solution. Twenty minutes later this was added, in one portion, to a cold, stirred solution of 2.76 g BSA in 120 ml of dioxane:water (1:1) containing 2.52 ml of N sodium hydroxide. After 1 hour, a further 1.2 ml of alkali was added and stirring was continued in the cold for 3 more hours. The solution was dialyzed against cold running deionized water overnight and the turbid protein solution

was brought to pH 4.5 with N hydrochloric acid; a precipitate formed. After storing for 5 days in the refrigerator, the suspension was centrifuged at 5° and the precipitate was suspended in 100 ml cold distilled water. Saturated sodium bicarbonate solution was added until the suspension largely dissolved and the material was dialyzed as before. Lyophilization of the turbid solution afforded a fluffy powder; specific activity, 14.3 cpm per mg. Assuming a molecular weight of 69000 for BSA, this amounted to 16.5 moles of V per mole of protein.

TABLE II

Relative Retention Times of Some C_{19} Steroid Derivatives

SUBSTANCE	RRT*
Cholestane	1.00
Dehydroisoandrosterone	1.07
Dehydroisoandrosterone-3-acetate	1.32
Dehydroisoandrosterone-3-acetate-17-ethylene ketal (Ia)	2.00
Androst-5-en-3β,17β-diol-diacetate (Ib)	1.55
3β-acetoxy-androst-5-en-7,17-dione-17-ethylene ketal (IIa)	4.65
7-keto-androst-5-en-3β,17β-diol-3,17-diacetate (IIb)	3.45
3β-hydroxy-androst-5-en-7,17-dione-17-ethylene ketal	4.25
Androsta-3,5-diene-7,17-dione-17-ethyleneketal (III)	2.16
Androsta-3,5-diene-7,17-dione	1.30
7-carboxymethoximino-androst-5-en-3β-ol-17-one-17-ethylene ketal (IV)	6.44+
7-carboxymethoximino-androst-5-en-3β-ol-17-one (V)	4.72+
7-carboxymethoximino-androst-5-en-3β,17β-diol (VI)	2.66+

* RRT, relative retention time to cholestane (5.4 minutes). Gas-liquid chromatography on 3% OV-22 on 80/100 supelcoport (Supelco, Bellefonte, Pa.), T, 275°.

+ Chromatographed as trimethylsilyl derivatives.

ACKNOWLEDGMENTS

We are grateful to Miss Inge Paul and Mrs. Julia S. C. Liang for their invaluable assistance. This work has been supported by Grants from the National Heart and Lung Institute (HL 14734), The National Cancer Institute (CA-07304), and the General Clinical Research Centers Branch (RR-53) of the National Institutes of Health.

REFERENCES

1. Abbreviations: Androstenediol, androst-5-en-3β,17β-diol; DHA, dehydroisoandrosterone, 3β-hydroxy-androst-5-en-17-one; androsterone, 3α-hydroxy-5α-androstan-17-one; etiocholanolone, 3α-hydroxy-5β-androstan-17-one; BSA, bovine serum albumin; GLC, gas-liquid chromatography; RRT, relative retention time.

2. Murphy, B. P., Engelberg, W., and Pattee, C. J., J. Clin. Endocrinol. Metab. 23, 293 (1963); Murphy, B. P., J. Clin. Endocrinol. Metab. 27, 973 (1967); Horton, R., Kato, T., and Sherins, R., Steroids 10, 245 (1967); Mayes, D., and Nugent, C. A., J. Clin. Endocrinol. Metab. 28, 1169 (1968); Mayes, D., and Nugent, C.A., Steroids 15, 389 (1970).

3. Kirschner, M. A., Lipsett, M. B., and Collins, D. R., J. Clin. Invest. 44, 657 (1965); Rivarola, M. A., Saez, J. M., Meyer, W. J., Kenny, F. M., and Migeon, C. J., J. Clin. Endocrinol. Metab. 27, 371 (1967); Gandy, H. M., and Peterson, R. E., J. Clin. Endocrinol. Metab. 28, 949 (1968); George, J. M., Wolff, S. M., Diller, E., and Bartter, F. C., J. Clin. Invest. 48, 558 (1969); Rosenfeld, R. S., Hellman, L., Roffwarg, H., Weitzman, E. D., Fukushima, D. K., and Gallagher, T.F., J. Clin. Endocrinol. Metab. 33, 87 (1971).

4. Goldfein, A., Jones, J., Yannone, M. E., and White, B., Gas Chromatography of Steroids in Biological Fluids, Editor, M. B. Lipsett, Plenum Press, New York, 1965, p. 35; Klokke, A. H., and Thijssen, J. H., Clin. Chem. Acta 20, 168 (1968).

5. Rosenfield, R. L., Steroids 17, 689 (1971).

6. Abraham, G. E., and Grover, P. K., Principles of Competitive Protein-Binding Assays, Editors, W. D. Odell and W. H. Daughaday, J. B. Lippincott Co., Philadelphia, 1971, p. 140 and references cited therein.

7. Nieschlag, E., Loriaux, D. L., and Lipsett, M. B., Steroids 19, 669 (1972).

8. Kream, J., Rosenfeld, R. S., Rosenberg, B. J., and Hellman, L., Clinical Chem. 18, 713 (1972).

9. Erlanger, B. F., Borek, F., Beiser, S. M., and Lieberman, S., J. Biol. Chem. 228, 713 (1957); ibid, 234, 1090 (1959).

10. Fieser, L. F., J. Am. Chem. Soc. 76, 1945 (1954).

REFERENCES (continued)

11. Dauben, W. G., Lorber, M., and Fullerton, D. S., J. Org. Chem. 34, 3587 (1969).

12. Loriaux, D. L., and Lipsett, M. B., Steroids 19, 681 (1972).

13. Dorfman, L., Chem. Revs. 53, 47 (1953).

14. Lisboa, B. P., Methods in Enzymology, XV, Editors, S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1969, p. 3.

15. Ruzicka, L., Goldberg, M. W., and Meyer, J., Helv. Chim. Acta. 18, 994 (1935).