[34] Synthesis of Affinity Labels for Steroid-Receptor Proteins

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A. Introduction

The general considerations underlying active-site-directed irreversible enzyme inhibition[1] (affinity labeling of enzymes[2]) have been applied to the study of steroid-binding proteins,[3] restricting attention to cytoplasmic androgen[4,5] and progestagen[6,7] receptors.

There are two requisites for the affinity labeling of a steroid receptor: The steroid must bind reversibly at the active site of the receptor, and the steroid must possess a functional group capable of forming a covalent bond with an amino acid residue either within (endo[1]) or adjacent to (exo[1]) the binding site.

The first requisite seems relatively easy to fulfill. The progesterone receptor of chick oviduct forms a highly stable ($k_d \simeq 10^{-10}$) complex with progesterone,[6,7] and competition studies show that other steroids form complexes somewhat less stable but with $k_d \leq 10^{-8}$.[8] Thus, in the absence of progesterone, the progesterone-binding protein should strongly bind other steroids.

The second requisite is more difficult. The amino acids in the polypeptide chain at the binding site are not known. Cysteine may be one since it has been shown for both a rat prostate 5α-dihydrotestosterone receptor[5] and the chick oviduct progesterone receptor[6] that sulfhydryl groups are involved either in binding of the steroid to the receptor or in maintenance of the active structure of the protein. We do assume that one or both of the oxygen functions of 5α-dihydrotestosterone (C-3 and C-17) and

[1] B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibition." Wiley, New York, 1967.
[2] L. Wofsy, H. Metzger, and S. J. Singer, *Biochemistry* **1**, 1031 (1962).
[3] U. Westphal, "Steroid-Protein Interaction." Springer-Verlag, Berlin and New York, 1971.
[4] S. Fang and S. Liao, *J. Biol. Chem.* **246**, 16 (1971).
[5] W. I. P. Mainwaring. *J. Endocrinol.* **45**, 531 (1969).
[6] M. R. Sherman, P. L. Corvol, and B. W. O'Malley, *J. Biol. Chem.* **245**, 6085 (1970).
[7] W. T. Schrader and B. W. O'Malley, *J. Biol. Chem.* **247**, 51 (1972).
[8] H. E. Smith, R. G. Smith, D. O. Toft, J. R. Neergaard, E. P. Burrows, and B. W. O'Malley, *J. Biol. Chem.*, in press.

of progesterone (C-3 and C-20) are involved in binding. A reactive substituent at a position near one of these oxygen functions would probably be closer to the polypeptide chain in the steroid–receptor complex than a more remotely positioned substituent and consequently would be more likely to undergo reaction.

Four general classes of chemical reactions that have been used in attempts at affinity labeling apply here: alkylation reactions, photochemical insertion reactions, disulfide bond formation, and mercaptide bond formation. An amino acid with a nucleophilic group such as cysteine, serine, threonine, tyrosine, tryptophan, histidine, lysine, arginine, and methionine may participate in an alkylation reaction with a steroid appropriately substituted with a leaving group such as a halogen atom.[9] Insertion reactions of carbenes generated by the photolysis of diazo steroids can take place with any amino acid.[2] Disulfide bond formation may occur between a sulfur-containing steroid derivative and cysteine.[10] Mercaptide bond formation involves the formation of a mercury–sulfur bond between a mercurated steroid and cysteine.[11,12]

Steroid reagents for these reactions may be divided into general classes according to the functional group present for the formation of a covalent bond with the receptor. A portion of the work in these laboratories has involved the preparation and use of halo and haloacetoxy derivatives of progesterone for alkylation reactions, but this discussion will be limited to the preparation and utility of diazo, sulfur-containing, and mercurated steroids for affinity labeling of androgen and progestagen receptor proteins.

In the procedures outlined below, we give details for the formation and purification of compounds of each of these types. Sufficient spectral data are included so that the identity and purity of subsequent preparations can be evaluated.

For nuclear magnetic resonance spectra (NMR), chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane as an internal standard and were determined as solutions in deuteriochloroform. Abbreviations used for these spectra are: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; and m, multiplet. Infrared spectra (IR) were determined as potassium bromide disks. Ultraviolet spectra (UV) were determined as solutions in absolute ethanol. Silica

[9] C.-C. Chin and J. C. Warren, *Biochemistry* **11**, 2720 (1972).
[10] I. Field, *in* "Organic Chemistry of Sulfur" (S. Oae, ed.). Plenum, New York, in press.
[11] P. D. Boyer, *J. Amer. Chem. Soc.* **76**, 4331 (1954).
[12] C.-C. Chin and J. C. Warren, *J. Biol. Chem.* **243**, 5056 (1968).

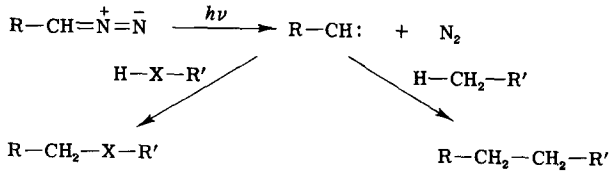

FIG. 1

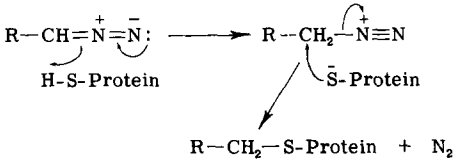

FIG. 2

gel was used for all thin-layer chromatograms (TLC). Magnesium sulfate was the drying agent for organic solutions.

B. Diazo Steroids

Diazo steroids are of potentially great utility for photochemical insertion reactions at a receptor active site. On irradiation, a carbene is generated which then can insert into a carbon–hydrogen bond or react with other groups such as an hydroxyl, an amino, or a thiol group (Fig. 1).[13] Diazo compounds may also alkylate a protein thiol group (Fig. 2) as shown by the reaction of azaserine with the reactive sulfhydryl group of 2-formamido-*N*-ribosylacetamide 5′-phosphate amidotransferase.[14]

Two types of diazo steroids are discussed here: α-diazo keto steroids, and ethyl diazomalonate and diazoacetate esters of hydroxy steroids. 2-Diazo-3-keto steroids are obtained by oximination of the 3-keto steroid with 2-octyl nitrite (*n*-butyl nitrite is unsatisfactory)[15] followed by treatment of the α-oximino ketone with chloramine in aqueous tetrahydrofuran[16] (THF) (Fig. 3). 21-Diazoprogesterone constitutes a special case as it is conveniently prepared from sodium 4-androsten-3-one-17β-carboxylate in two steps[17] (Fig. 4).

[13] W. Kirmse, "Carbene Chemistry," 1st ed. Academic Press, New York, 1964.
[14] T. C. French, I. B. Dawid, and J. M. Buchanan, *J. Biol. Chem.* **238**, 2186 (1963).
[15] H. E. Smith and A. A. Hicks, *J. Org. Chem.* **36**, 3659 (1971).
[16] M. P. Cava and B. R. Vogt, *J. Org. Chem.* **30**, 3775 (1965).
[17] A. L. Wilds and C. H. Shunk, *J. Amer. Chem. Soc.* **70**, 2427 (1948).

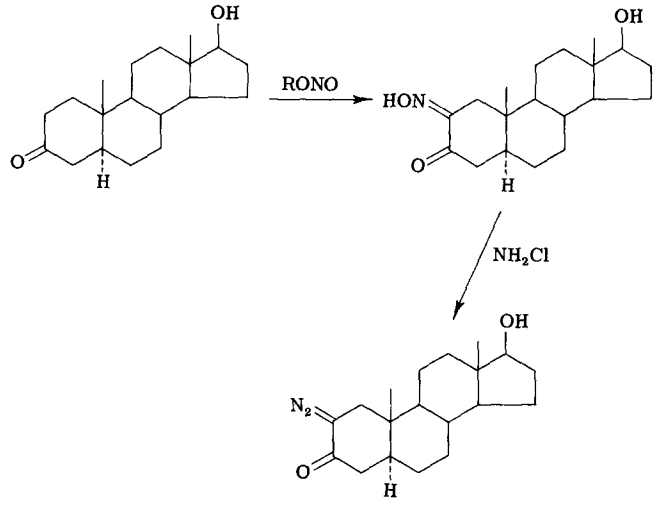

FIG. 3

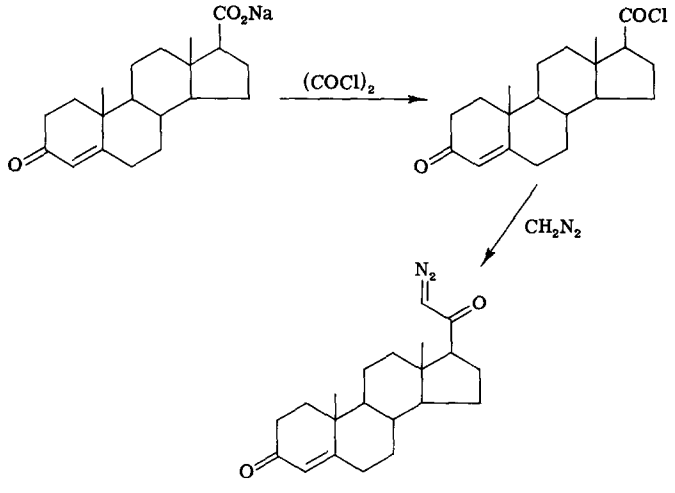

FIG. 4

Initially, ethyl diazomalonate esters of steroidal alcohols, rather than the respective diazoacetates, were the compounds of choice because of their simplicity of synthesis. Reaction of ethyl diazomalonyl chloride, prepared by reaction of phosgene with ethyl diazoacetate,[18] with an ap-

[18] H. Staudinger, J. Becker, and H. Hirzel, *Ber. Deut. Chem. Ges.* **49**, 1978 (1916); R. J. Vaughan and F. H. Westheimer, *Anal. Biochem.* **29**, 305 (1969).

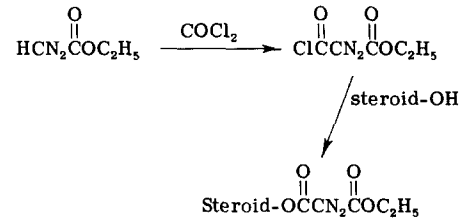

FIG. 5

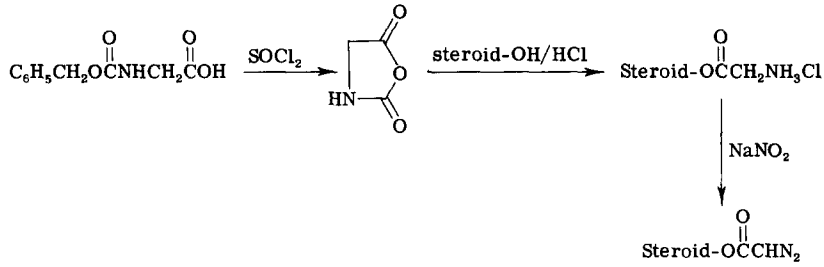

FIG. 6

propriate steroidal alcohol gives the ethyl diazomalonate ester in good yield (Fig. 5).

For better success in receptor protein-labeling experiments, however, the less readily prepared and less sterically hindered diazoacetates may be required. The latter are prepared as shown in Fig. 6. Treatment of *N*-carbobenzoxyglycine with thionyl chloride yields *N*-carboxyglycine anhydride,[19,20] which, on heating with the appropriate steroidal alcohol in ethyl acetate-containing dry hydrogen chloride, gives the steroid glycinate hydrochloride. Treatment of the latter with aqueous sodium nitrite gives the desired diazoacetate (Fig. 6).

2-Oximino-5α-androstan-17β-ol-3-one. This compound was prepared previously[15] in 12% yield from 5α-androstan-17β-ol-3-one and 2-octyl nitrite. It is advised that a procedure strictly analogous to that described below for the preparation of 17α-methyl-2-oximino-5α-androstan-17β-ol-3-one be used. 2-Oximino-5α-androstan-17β-ol-3-one has m.p. 265–268° dec; TLC R_f 0.3 (9:1 benzene–methanol); $[\alpha]^{26}D$ +67° (*c* 0.243, absolute ethanol); IR 1620 (C=N), 1720 (C=O), 3150 (OH), and 3480 cm^{-1} (OH); UV_{max} 243 (ε 7300) and 340 nm (33) (shoulder).

2-Diazo-5α-androstan-17β-ol-3-one. To a stirred, ice-cooled mixture of 2-oximino-5α-androstan-17β-ol-3-one (302 mg, 0.951 mmole) in 10%

[19] M. Bergmann and L. Zervas, *Ber. Deut. Chem. Ges.* **65**, 1192 (1932).
[20] Y. Go and H. Tani, *Bull. Chem. Soc. Jap.* **14**, 510 (1939).

aqueous sodium hydroxide (3 ml), water (1.5 ml), and tetrahydrofuran (15 ml) was added, in one portion, concentrated ammonium hydroxide (3 ml), and then followed by 5.25% aqueous sodium hypochlorite (Clorox) (7.5 ml) dropwise during 10 minutes. The mixture was stirred 45 minutes at 0°, then allowed to come to room temperature and stirred 2 hours longer. The tetrahydrofuran layer was separated and extracted with half-saturated sodium chloride until the aqueous layer was neutral (3 portions). After drying, removal of the tetrahydrofuran gave a yellow, crystalline residue (253 mg, 84%) of 2-diazo-5α-androstan-17β-ol-3-one, homogeneous on TLC, R_f 0.5 (9:1 benzene–methanol): $[\alpha]^{25}D + 37°$ (*c* 1.04, chloroform); NMR δ 0.76 (s, 3, C-18 H), 0.95 (s, 3, C-19 H), and 3.68 ppm (t, 1, $J = 8$ Hz, C-17 H); IR 1620 (C=O), 2100 (C=N=N), and 3460 cm^{-1} (OH); UV_{max} 262 (ε 4700) (shoulder) and 291 nm (8400). It had no definite melting point but gradually decomposed with gas evolution when heated above 170°. An analytical sample was recrystallized from chloroform–acetone.[21]

17α-Methyl-2-oximino-5α-androstan-17β-ol-3-one. Under nitrogen, potassium (about 100 mg) was dissolved in *tert*-butyl alcohol (15 ml), and to the stirred solution was added in one portion 17α-methyl-5α-androstan-17β-ol-3-one (304 mg, 1.00 mmole) followed by a solution of 2-octyl nitrite[22] (162 mg, 1.02 mmoles) in *tert*-butyl alcohol (10 ml) dropwise during 10 minutes. The mixture was stirred for 5 hours at room temperature, then diluted with water (125 ml) and extracted with methylene chloride (50 ml). The aqueous layer was separated, acidified with 5% hydrochloric acid, and extracted with methylene chloride. The methylene chloride layer was washed with water, dried, and evaporated to a white, crystalline residue (86 mg, 26%), homogeneous on TLC, R_f 0.3 (9:1 benzene–methanol): $[\alpha]^{25}D + 84°$ (*c* 2.05, pyridine); IR 1605 (C=N), 1710 (C=O), 3180 (OH), and 3440 cm^{-1} (OH); UV_{max} 228 (ε 5100) (shoulder), 243 (6700), and 330 nm (60) (shoulder). An analytical sample was recrystallized from methanol and had m.p. 256–260° dec.[21]

2-Diazo-17α-methyl-5α-androstan-17β-ol-3-one. The procedure outlined above for the preparation of 2-diazo-5α-androstan-17β-ol-3-one was used with 17α-methyl-2-oximino-5α-androstan-17β-ol-3-one (175 mg, 0.525 mmole), 10% sodium hydroxide (2 ml), water (1 ml), concentrated ammonium hydroxide (2 ml), and Clorox (5 ml). The mixture was stirred

[21] This previously unreported compound, on combustion analysis for carbon, hydrogen, and nitrogen, showed elemental composition in agreement with the assigned structure.

[22] M. Pezold and R. L. Shriner, *J. Amer. Chem. Soc.* **54**, 4707 (1932). Prepared by a modification of the procedure described for *n*-butyl nitrite [W. A. Noyes, *Org. Syn. Collect. Vol.* **2**, 108 (1943)], and distilled, b.p. 54–55° (10 mm).

45 minutes at 0°, then allowed to come to room temperature and stirred 5 hours longer. Work-up as before afforded a yellow semicrystalline solid (63 mg) which on one recrystallization from methanol gave pure crystalline 2-diazo-17α-methyl-5α-androstan-17β-ol-3-one (42 mg, 24%): TLC R_f 0.5 (9:1 benzene–methanol; $[\alpha]^{25}D$ +47° (*c* 1.1, absolute ethanol); NMR δ 0.88 (s, 3, C-18 H), 0.95 (s, 3, C-19 H), and 1.22 ppm (s, 3, C-17 CH_3); IR 1610 (C=O), 2100 (C=N=N), and 3420 cm^{-1} (OH); UV_{max} 262 (ϵ 4700) (shoulder) and 291 nm (8600). Its behavior on melting was similar to that of 2-diazo-5α-androstan-17β-ol-3-one.[21]

21-Diazoprogesterone. A solution of 17β-carboxy-4-androsten-3-one (1.00 g; 3.16 mmoles) in 0.1 *N* sodium hydroxide (38 ml) was lyophilized, and the resulting pale yellow powder was dried under reduced pressure at 100° for 8 hours. A stirred solution of this salt in dry benzene (10 ml) was cooled to 0° and pyridine (3 drops) was added followed by excess oxalyl chloride (4 ml) dropwise. The mixture was allowed to warm slowly to 15°, and when no further evolution of gas occurred, the solvent was evaporated under reduced pressure. A solution of the residual acid chloride in benzene (20 ml) was filtered, diluted with ether (20 ml), and added dropwise to a stirred ethereal solution of excess diazomethane kept at −15°. The mixture was kept at −15° for 30 minutes and at 0° for another 30 minutes, and the solvents were evaporated under reduced pressure. Trituration of the residue with ether followed by filtration yielded crude crystalline 21-diazoprogesterone (459 mg, 43%). It was dissolved in hot acetone, and the solution was filtered and cooled to give pure 21-diazoprogesterone: m.p. 174–176° (lit[17] 177–178° dec); TLC R_f 0.7 (9:1 benzene–methanol); NMR δ 0.95, 1.21 (2 s, 6, C-18 and C-19 H), 5.22 (s, 1, C-21 H), and 5.76 ppm (s, 1, C-4 H); IR 1635 (C-20 C=O), 1660 (C-3 C=O), 2180 (C=N=N), and 3160 cm^{-1} (C-21 C-H stretch); UV_{max} 244 (ϵ 23,000) and 273 nm (7800) (shoulder).

Ethyl Diazomalonyl Chloride. Phosgene was bubbled in benzene (43.5 ml) until the total volume of the solution was 50 ml. This solution was cooled in an ice bath, and ethyl diazoacetate (22.1 g, 0.194 mole) in benzene (65 ml) was added dropwise with stirring. The mixture was allowed to come to room temperature and then was stored for 6 hours. Most of the benzene was removed under reduced pressure, and distillation of the residue gave ethyl diazomalonyl chloride (9.87 g, 58%) as a pale yellow oil: b.p. 49–54° (0.07 mm) [lit[23] b.p. 35° (0.03 mm)].

Testosterone Ethyl Diazomalonate. Ethyl diazomalonyl chloride (0.35 g, 2.0 mmoles) followed by ether (1.0 ml) was added with swirling to a solution of testosterone (288 mg, 1.00 mmole) in dry pyridine (1 ml). After 1 hour, the mixture was diluted with ether (20 ml), washed with

[23] D. J. Brunswick and B. S. Cooperman, *Proc. Nat. Acad. Sci. U.S.* **68**, 1801 (1971).

dilute hydrochloric acid and with water, and dried. The solvent was removed under reduced pressure and the residue was dissolved in benzene and chromatographed on silica gel. Elution with 4:1 benzene–ether gave the diazo ester (0.31 g, 72%) as a syrup, pure by TLC (1:1 benzene–ether). This syrup was crystallized with difficulty from ethanol–water to give pure testosterone ethyl diazomalonate as white needles: m.p. 80–82°; NMR δ 0.86 (s, 3, C-18 H), 1.20 (s, 3, C-19 H), 1.32 (t, 3, $J = 7$ Hz, OCH_2CH_3), 4.29 (q, 2, $J = 7$ Hz, OCH_2CH_3), 4.73 (m, 1, C-17 H), and 5.74 ppm (s, 1, C-4 H); IR 2145 cm^{-1} (C=N=N); UV_{max} 242 nm (ϵ 25,400). Recrystallization from methanol–water gave the analytical sample, m.p. 81–83°.[21]

6β-Hydroxyprogesterone Ethyl Diazomalonate. Ethyl diazomalonyl chloride (1.06 g, 6.00 mmoles) was added with swirling to a solution of 6β-hydroxyprogesterone (198 mg, 0.599 mmole) in dry pyridine (3.0 ml), and the reaction mixture was kept at room temperature for 16 hours. Work-up as described for testosterone ethyl diazomalonate gave a syrup (0.61 g) which was chromatographed on silica gel. Elution with 9:1 benzene–ether gave 6β-hydroxyprogesterone ethyl diazomalonate (235 mg, 84%) as a gum which solidified and was pure by TLC (1:1 benzene–ether): NMR δ 0.71 (s, 3, C-18 H), 1.31 (t, 3, $J = 7$ Hz, OCH_2CH_3), 1.32 (s, 3, C-19 H), 2.15 (s, 3, C-21 H), 4.32 (q, 2, $J = 7$ Hz, OCH_2CH_3), 5.65 (m, 1, C-6 H), and 6.02 ppm (s, 1, C-4 H); IR 2120 cm^{-1} (C=N=N). A benzene solution of the diazo ester was chromatographed a second time on silica gel. Elution with 9:1 benzene–ether gave a syrup. Lyophilization of a benzene solution of this syrup gave the analytical sample as an amorphous, white powder.[21]

11α-Hydroxyprogesterone Ethyl Diazomalonate. Ethyl diazomalonyl chloride (1.77 g, 10.0 mmoles) was added dropwise with stirring to 11α-hydroxyprogesterone (662 mg, 2.00 mmoles) in pyridine (3.6 ml). The mixture was allowed to stand overnight. Work-up was similar to that described for testosterone ethyl diazomalonate except that methylene chloride was the organic solvent instead of ether. The crude product was chromatographed on silica gel. Elution with 8:1 benzene–ether gave the diazo ester (861 mg, 92%) which was recrystallized from ethyl acetate–hexane to give pure 11α-hydroxyprogesterone ethyl diazomalonate (402 mg, 43%): m.p. 155–156° dec; $[\alpha]^{24}D$ +50.7° (*c* 3.02, dioxane); IR 2120 cm^{-1} (C=N=N).[21] The NMR spectrum was similar to that of 6β-hydroxyprogesterone ethyl diazomalonate except for the positions of the C-11 and C-4 protons at 5.52 and 5.84 ppm, respectively.

Deoxycorticosterone Ethyl Diazomalonate. Ethyl diazomalonyl chloride (0.35 g, 2.0 mmoles) was added to a swirled solution of deoxycorticosterone (330 mg, 1.00 mmole) in pyridine (2.0 ml), and the mixture

was kept 8 hours at room temperature. Work-up as described for testosterone ethyl diazomalonate gave the crude product (0.31 g) as a syrup which was chromatographed on silica gel. Elution with 85:15 benzene–ether gave deoxycorticosterone ethyl diazomalonate (0.27 g, 58%), pure by TLC (1:1 benzene–ether), as a gum. Lyophilization of a benzene solution of this gum gave the analytical sample as an amorphous, light tan powder.[21] Its NMR spectrum was similar to that of 11α-hydroxyprogesterone ethyl diazomalonate except for the absence of the C-11 proton multiplet and the appearance of the C-21 protons as 2 doublets (J = 18 Hz) at 4.69 and 4.95 ppm.

Testosterone Diazoacetate. *N*-Carboxyglycine anhydride[19,20] (101 mg, 1.00 mmole) was added to a solution of testosterone (288 mg, 1.00 mmole) in ethyl acetate (2 ml) containing dry hydrogen chloride. The mixture was stirred 15 minutes at 60° and then kept 24 hours at room temperature. The precipitate was collected by filtration and was washed three times with hot ethyl acetate. This crude testosterone glycinate hydrochloride (229 mg) was dissolved in water (2 ml), methylene chloride (4 ml) was added, and the mixture was cooled (—1°) in an acetone–dry ice bath. Sodium nitrite (70 mg, 1.01 mmoles) was added to the stirred mixture, the bath temperature was lowered to —9°, and 5% sulfuric acid (2 ml) was added dropwise. After 10 minutes, the mixture was allowed to come to room temperature and was stirred for an additional 30 minutes. The methylene chloride layer was separated, washed with 5% sodium bicarbonate and with water, and dried. Removal of the solvent and crystallization from methanol gave testosterone diazoacetate (27 mg, 8% based on testosterone) as pale yellow prisms: m.p. 163–165°; NMR δ 0.65 (s, 3, C-18 H), 1.23 (s, 3, C-19 H), 4.86 (m, 1, C-17 H), 4.87 (s, 1, $N_2CHC{=}O$), and 5.93 ppm (s, 1, C-4 H); IR 2100 cm^{-1} (C=N=N).[21]

C. Sulfur-Containing Steroids

For the preparation of sulfur-containing derivatives of testosterone such as steroidal sulfenyl thiolcarbonates[24] (steroid—S—S—CO_2R) steroidal sulfenyl thiocyanates[25] (steroid—S—SCN), and steroidal alkyl or aryl disulfides[26] (steroid—S—S—R), which might react with cysteine to form a disulfide bond at the active site of a receptor protein,[10] the synthesis of 4-androstene-17β-thiol-3-one was investigated in some detail.

[24] S. J. Brois, J. F. Pilot, and H. W. Barnum, *J. Amer. Chem. Soc.* **92**, 7629 (1970).
[25] R. G. Hiskey and W. P. Tucker, *J. Amer. Chem. Soc.* **84**, 4789 (1962).
[26] E. E. Reid, "Organic Chemistry of Bivalent Sulfur," Vol. III. Chem. Publ. Co., New York, 1960.

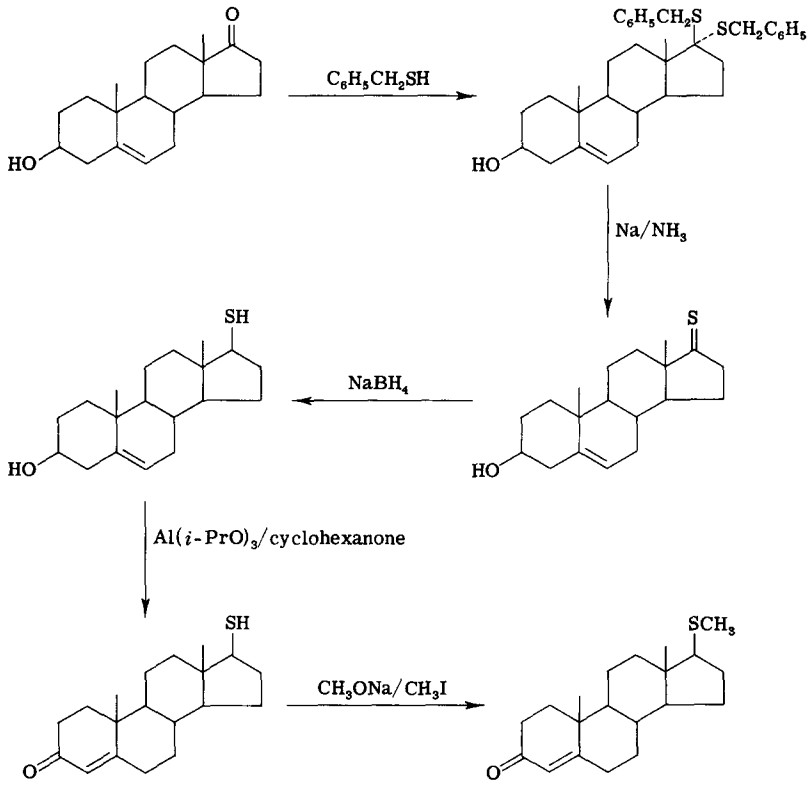

FIG. 7

The synthetic scheme was outlined earlier[27,28] (Fig. 7), but it lacked many experimental details. As shown in Fig. 7, a sulfur atom was substituted for the carbonyl oxygen atom of 5-androsten-3β-ol-17-one by reduction of its dimercaptal derivative. Reduction of 5-androsten-3β-ol-17-thione gave 5-androstene-17β-thiol-3β-ol, which was oxidized to the desired thiol analog of testosterone. Treatment of the sodium salt of the thiol with methyl iodide gave 17β-methylthio-4-androsten-3-one.

5-Androsten-3β-ol-17-one Dibenzyl Thioketal. A solution of 5-androsten-3β-ol-17-one (10.0 g, 34.7 mmoles), benzyl mercaptan (10.0 ml, 85.2 mmoles), and *p*-toluenesulfonic acid (1.0 g) in glacial acetic acid (50 ml) was allowed to stand at room temperature for two days. The precipitate which formed was collected and washed several times with water

[27] R. M. Dodson and P. B. Sollman, U.S. Patent 2,763,669 (1956); *Chem. Abstr.* **51**, 5134a (1957).

[28] C. Djerassi and D. Herbst, *J. Org. Chem.* **26**, 4675 (1961).

and then was boiled 2 hours in 1 *N* ethanolic potassium hydroxide (500 ml). The mixture was poured into ice water (about 400 ml) and acidified with glacial acetic acid. The precipitate was collected and recrystallized from 95% ethanol to give 5-androsten-3β-ol-17-one dibenzyl thioketal (12.4 g, 69%) as white needles: m.p. 184–186° (lit[29] 184–186°); NMR δ 1.05 (s, 3, C-19 H), 1.11 (s, 3, C-18 H), 3.62 (m, 1, C-3 H), 4.06 (s, 4, SCH_2), 5.52 (m, 1, C-6 H), and 7.52 ppm (m, 10, aromatic H); IR 700 cm^{-1} (phenyl out of plane bending).

5-Androsten-3β-ol-17-thione. 5-Androsten-3β-ol-17-one dibenzyl thioketal (5.19 g, 10.0 mmoles) was added to a stirred solution of sodium (2.6 g, 0.11 g-atom) in twice-distilled liquid ammonia (about 300 ml) and anhydrous ether (100 ml). After 0.5 hours additional sodium (2.6 g, 0.11 mole) was added, and the deep blue reaction mixture was stirred for an additional 4 hours. Additional anhydrous ether (100 ml) was then added, the excess sodium was destroyed by careful addition of absolute ethanol, and the ammonia was allowed to evaporate, leaving a clear, colorless solution. Ice was added, the mixture was stirred 0.5 hour, and then extracted with ether. The aqueous layer was acidified with glacial acetic acid and extracted with chloroform. The chloroform layer was dried and evaporated to give crude 5-androsten-3β-ol-17-thione (2.75 g, 90%) as an orange solid with no IR absorption at 700 cm^{-1}.

This material was usually used without purification. Chromatography on silica gel (eluted with 9:1 benzene–ether) gave material homogeneous to TLC (9:1 benzene–ethyl acetate) with an unchanged IR spectrum.

5-Androstene-17β-thiol-3β-ol. Excess sodium borohydride was added with stirring to a solution of 5-androsten-3β-ol-17-thione (2.75 g, 9.03 mmoles) in dry methanol (100 ml) until a clear, colorless solution was obtained. The mixture was acidified with glacial acetic acid and reduced to near dryness under reduced pressure. Ice and water were added, and the mixture was extracted with methylene chloride. The methylene chloride solution was dried, and the methylene chloride removed to give crude 5-androstene-17β-thiol-3β-ol (2.42 g, 87%), which was chromatographed on silica gel. Elution with 9:1 benzene–ether gave the thiol (1.95 g, 70%) as a white amorphous solid, pure by TLC (9:1 benzene–ethyl acetate). Crystallization from hexane afforded the thiol as white needles: m.p. 169–171° (lit[27] 174.5–175.5°); NMR δ 0.75 (s, 3, C-18 H), 1.05 (s, 3, C-19 H), 3.62 (m, 1, C-3 H), and 5.49 ppm (m, 1, C-6 H); IR 2540 (SH, weak) and 3250 cm^{-1} (OH).

4-Androstene-17β-thiol-3-one. A mixture of 5-androstene-17β-thiol-3β-ol (500 mg, 1.63 mmoles), aluminum isoproxide (500 mg, 2.45

[29] R. H. Levin and J. L. Thompson, *J. Amer. Chem. Soc.* **70**, 3140 (1948).

mmoles), cyclohexanone (5 ml), and toluene (10 ml) was boiled for 16 hours. The cooled mixture was then acidified with glacial acetic acid, filtered through Celite, and the Celite was washed with benzene. The solvents were removed from the combined filtrate and washings under reduced pressure yielding a pale yellow liquid (4.75 g) which was chromatographed on silica gel. Elution with benzene gave 2-cyclohexenyl cyclohexanone (~2.22 g).[30] Elution with 9:1 benzene–ether gave 4-androstene-17β-thiol-3-one (466 mg, 94%) as a syrup, pure by TLC (9:1 benzene–ethyl acetate), which was rechromatographed on silica gel to give 323 mg (65%) of syrup which crystallized: m.p. 99–109° (lit[27] 118–119°); IR 2520 (SH, weak) and 1665 cm^{-1} (conjugated C=O).

In a similar preparation using 400 mg of 5-androsten-3β-ol-17β-thiol, the reaction was worked up by addition of a solution of potassium sodium tartrate (4.0 g) in water (25 ml). The mixture was stirred for 1 hour and then steam-distilled to remove toluene and cyclohexanone. The aqueous residue was extracted with methylene chloride and the extract was evaporated under reduced pressure to a syrupy residue (400 mg) which was chromatographed on silica gel. Elution with 9:1 benzene–ether afforded pure 4-androstene-17β-thiol-3-one (0.21 g, 53%) which crystallized from methanol–water as white needles: m.p. 110–113°.

17β-Methylthio-4-androsten-3-one. Sodium (90 mg, 3.9 mg-atoms) dissolved in dry methanol (50 ml) was added with swirling to 4-androsten-17β-thiol-3-one (690 mg, 2.27 mmoles), and the mixture was heated on a steam plate for 10 minutes. Excess methyl iodide (about 2 ml) was then added, and the mixture was heated for an additional 10 minutes. It was then poured on ice, acidified with glacial acetic acid, and extracted with methylene chloride. The extract was dried, the solvent was removed under reduced pressure, and the residue (0.66 g) was chromatographed on silica gel. Elution with 9:1 benzene–ether gave crystalline 17β-methylthio-4-androsten-3-one (319 mg, 44%), pure by TLC (9:1 benzene–ethyl acetate). Recrystallization from ethanol, followed by sublimation at 95° (0.02 mm) afforded the compound as white needles: m.p. 128–130° (lit[27] m.p. 131.0–132.5°); NMR δ 0.81 (s, 3, C-18 H), 1.20 (s, 3, C-19 H), 2.12 (s, 3, SCH_3), and 5.73 ppm (s, 1, C-4 H).

D. Mercurated Steroids

A previous report of affinity labeling of an estradiol receptor protein using 4-acetoxymercuri-estradiol[12] suggested the synthesis of mercurated

[30] R. A. Abramovitch and A. R. Vinutha, *J. Chem. Soc. C* p. 2104 (1969).

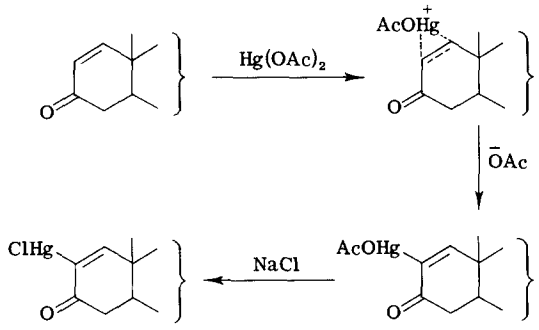

FIG. 8

steroids, in expectation that these might form mercaptide bonds with cysteine residues of androgen and progestagen receptors. The aim was to introduce mercury into the testosterone or progesterone molecule adjacent to the C-3 carbonyl group.[31] In contrast to a number of acyclic model compounds, α,β-unsaturated 3-keto steroids do not react with mercuric acetate in methanol at room temperature. 3-Keto steroids with a readily abstractable allylic proton, such as progesterone, appear to be oxidized on heating of the steroid in methanol and in acetic acid with mercuric acetate but no well-defined products were isolated. 3-Keto steroids without this structural feature and with a C-1 double bond react in boiling acetic acid with addition of acetoxymercuri occurring at the C-1 double bond. The proton at C-2 is abstracted, and the 2-acetoxymercuri-1-en-3-one is formed (Fig. 8). For ease of purification, the acetate ion was replaced with a chloride ion. Steroidal 1,4-dien-3-ones and 1,4,6-trien-3-ones also react similarly and more readily, the latter being the most reactive.

It is possible to follow the conversion of the steroidal substrate to the 2-acetoxymercuri derivative using NMR spectroscopy. On mercuration, the doublet at 7.1 ppm (J = 10 Hz) assigned to the C-1 proton in the substrate spectrum collapses to a singlet and is shifted to a slightly lower field.

The mercurated steroids are extremely difficult to purify since even after purification they tend to form gums. They also appear to retain solvents tenaciously as indicated by the NMR spectra of purified compounds which showed additional protons characteristic of the solvent used. For these reasons the yield of pure mercurated compound in some

[31] R. G. Smith, H. E. Ensley, and H. E. Smith, *J. Org. Chem.* **37**, 4430 (1972).

cases was low although conversion to the mercurated steroid was high.

2-Chloromercuri-5α-androst-1-ene-3,17-dione. 5α-Androst-1-ene-3,17-dione (200 mg, 0.698 mmole) and mercuric acetate (1.00 g, 3.14 mmoles) in glacial acetic acid (5.0 ml) were boiled for 24 hours. After cooling the solution was poured into a stirred saturated solution of sodium chloride (50 ml). The precipitated gum was taken up in chloroform and the solution was washed with water until the wash water was neutral. Evaporation of the dried chloroform solution left a gum which on trituration with ether gave a white solid. Four recrystallizations from chloroform–ether gave pure 2-chloromercuri-5α-androst-1-ene-3,17-dione as white microcrystals (40 mg, 11%): m.p. 180° dec; NMR δ 7.25 ppm (s, 1, C-1 H); IR 1640 (conjugated C=O) and 1730 cm^{-1} (C=O).[31] This compound retained chloroform so tenaciously that it was never obtained completely free of chloroform.[31]

2-Chloromercuri-1,4-androstadiene-3,17-dione. 1,4-Androsta-1,4-diene-3,17-dione (2.00 g, 7.03 mmoles) and mercuric acetate (10.0 g, 31.4 mmoles) were boiled in glacial acetic acid (125 ml) for 6 hours. After cooling, the mixture was poured into a saturated sodium chloride solution. The resulting yellow precipitate was taken up in chloroform. Careful addition of hexane to this solution precipitated the mercurated steroid. Reprecipitation on cooling from 95% ethanol gave 2-chloromercuri-1,4-androstadiene-3,17-dione (0.16 g, 4%) as a white amorphous solid: m.p. 282–283° dec; NMR δ 6.20 (s, 1, C-4 H), and 7.17 ppm (s, 1, C-1 H); IR 1640 (conjugated C=O) and 1725 cm^{-1} C=O).[31]

2-Chloromercuri-1,4,6-androstatriene-3,17-dione. 1,4,6-Androstatriene-3,17-dione (5.00 g, 17.7 mmoles) and mercuric acetate (13.0 g, 40.8 mmoles) were boiled in glacial acetic acid for 30 minutes. Dilution of the reaction mixture with water (250 ml) containing sodium chloride (15 g) precipitated a solid which was washed with water and extracted with hot acetone (400 ml). This solution was reduced in volume to 75 ml, and dilution with hexane (100 ml) precipitated a white solid. Crystallization of this solid from acetone gave 2-chloromercuri-1,4,6-androstatriene-3,17-dione (0.867 g, 9.5%) as white needles: m.p. 280–285° dec; $[\alpha]^{25}_{D}$ +27° (*c* 0.84, chloroform); NMR δ 6.05–6.45 (m, 3, C-4, C-6, and C-7 H), 7.28 (s, 1, C-1 H), and 7.28 ppm (d, about 0.2, J = 280 Hz, C-1 H coupled to ^{199}Hg).[31]

17α-Methyl-1,4,6-androstatrien-17β-ol-3-one. A solution of 17α-methyl-5-androstene-3β,17β-diol (6.1 g, 20 mmoles) and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (13.9 g, 61 mmoles) in dry dioxane (300 ml) was boiled under nitrogen for 16.5 hours. The mixture was allowed to cool, filtered to remove the hydroquinone, and evaporated at reduced pressure to yield a brown gum. The gum was dissolved in ethyl

acetate and chromatographed on alumina (neutral, Brockmann activity I) (160 g). Elution with ethyl acetate gave the crude, crystalline product. Recrystallization from ethyl acetate gave pure 17α-methyl-1,4,6-androstatrien-17β-ol-3-one (1.2 g, 20%): m.p. 136–138° (lit[32] m.p. 139–140°); NMR δ 5.92–6.36 (m, 4, C-2, C-4, C-6, and C-7 H) and 7.08 ppm (d, 1, J = 10 Hz, C-1 H).

2-Chloromercuri-17α-methyl-1,4,6-androstatrien-17β-ol-3-one. A solution of 217α-methyl-1,4,6-androstatrien-17β-ol-3-one (0.813 g, 2.72 mmoles) and mercuric acetate (4.00 g, 12.6 mmoles) in glacial acetic acid was boiled for 15 minutes. After cooling, the solution was poured into a stirred, saturated sodium chloride solution (250 ml). The precipitated crude product was washed with water and recrystallized twice from 95% ethanol to give 2-chloromercuri-17α-methyl-1,4,6-androstatrien-17β-ol-3-one (0.370 g, 25%) as pale yellow microcrystals: m.p. 180–181° dec (lit[33] 155–162°); NMR δ 6.0–6.3 (m, 3, C-4, C-6, and C-7 H) and 7.29 ppm (s, 1, C-1 H); IR 1585 (C=C) and 1612 cm^{-1} (C=O).[31]

1,4,6-Pregnatriene-3,20-dione. Using the procedure as described for the preparation of 17α-methyl-1,4,6-androstatrien-17β-ol-3-one, 5-pregnen-3β-ol-20-one was dehydrogenated with DDQ to give 1,4,6-pregnatriene-3,20-dione (28%), eluted from an alumina (neutral, Brockmann activity I) column with ethyl acetate, and recrystallized from ethyl acetate: m.p. 148–149° (lit[34] m.p. 150–152°); NMR δ 5.91–6.37 (m, 4, C-2, C-4, C-6, and C-7 H) and 7.08 ppm (d, 1, J = 10 Hz, C-1 H).

2-Chloromercuri-1,4,6-pregnatriene-3,20-dione. 1,4,6-Pregnatriene-3,20-dione (4.40 g, 14.2 mmoles) and mercuric acetate (22.0 g, 69.0 mmoles) in glacial acetic acid (40 ml) were boiled for 30 minutes. Dilution of the cooled reaction mixture with saturated sodium chloride precipitated a solid, which was washed with water and then extracted into chloroform and dried. Evaporation of the chloroform left a gum which on heating in 95% ethanol and then cooling gave solid 2-chloromercuri-1,4,6-pregnatriene-3,20-dione (2.5 g, 32%): m.p. (135° softens) 150° dec. An analytical sample was obtained by heating a suspension of the solid in ethanol for a few minutes, allowing the mixture to cool to 50°, decanting the ethanolic solution from a gummy residue, and allowing crystallization to proceed at 0° for 24 hours. Two additional recrystallizations in this manner gave 2-chloromercuri-1,4,6-pregnatriene-3,20-dione (0.510 g, 7%) as off-white microcrystals: m.p. (137° shrinks) 145–150° dec;

[32] G. O. Weston, D. Burn, D. N. Kirk, and V. Petrow, British Patent 854,343 (1960); *Chem. Abstr.* **55**, 18813f (1961).
[33] M. Kocor and M. Gumulka, *Tetrahedron Lett.* p. 3067 (1969).
[34] S. K. Pradhan and H. J. Ringold, *J. Org. Chem.* **29**, 601 (1964).

$[\alpha]^{25}_{D}$ +58° (*c* 1.0, chloroform); NMR δ 5.94–6.36 (m, 3, C-4, C-6, and C-7 H) and 7.28 ppm (s, 1, C-1 H).[31]

Acknowledgments

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