C-3 GLUCOSIDURONATES OF METABOLITES OF ADRENAL STEROIDS

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ABSTRACT

On treatment with methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucuronate and silver carbonate, tetrahydrocortisone* 21-acetate gave the corresponding 3-glucosiduronate triacetyl methyl ester. This product was converted into the 20-semicarbazone which, by treatment with alkali to hydrolyze the ester functions and acid to hydrolyze the semicarbazone moiety, gave tetrahydrocortisone 3-glucosiduronic acid. The acid was converted into the crystalline barium salt and into the methyl ester. An analogous series of reactions was carried out on tetrahydrocortexolone 21-acetate. Treatment of the 20-semicarbazone of tetrahydrocortisone 3-glucosiduronic acid with potassium borohydride reduced the 11-oxo function to an 11β hydroxyl group; acid-catalyzed removal of the semicarbazone group produced tetrahydrocortisol 3-glucosiduronic acid which also was obtained as the barium salt and the methyl ester.

INTRODUCTION

Among the metabolites of cortisol which occur in human urine are two steroidal glucosiduronic acids, 15 and 21. The former substance (15) has been isolated as an amorphorus sodium salt [1] and identified by conversion into the methyl ester tetraacetate (6) which was prepared synthetically [2]. The latter metabolite (21) has been isolated as a purified fraction and characterized [3,4,5,6] by chromatographic and hydrolytic procedures. More recently this substance has been prepared chemically [7] via the Koenigs-Knorr reaction. An additional steroidal C-3 glucosiduronic acid which has

*Tetrahydrocortisone = 3α,17,21-trihydroxy-5β-pregnane-11,20-dione; tetrahydrocortisol = 3α,11β,17,21-tetrahydroxy-5β-pregnan-20-one; tetrahydrocortexolone = 3α,17,21-trihydroxy-5β-pregnan-20-one.

been shown to be present in human urine is compound **16**. This acid has been characterized chromatographically and converted into a crystalline tetraacetyl methyl ester **8** [4]. The present paper describes the chemical synthesis of steroidal glucosiduronic acids and salts **14**, **15**, **16**, **17**, **20**, **21** and several derivatives of these substances.

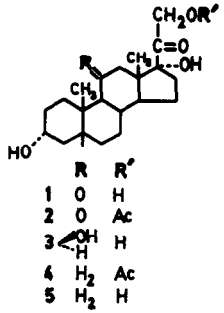
We obtained a 24% yield of **6**, along with a considerable amount of starting material by treating **2** with 3 equivalents of methyl acetobromoglucuronate; with 6 equivalents the yield was raised to 54%.

Also formed in the foregoing reaction was orthoacetate **9** which was obtained in 17% yield. Although glucosiduronate **6** may be separated satisfactorily from orthoacetate **9** by chromatography, it is also possible to treat the crude reaction mixture of **6** and **9** briefly with methanolic hydrogen chloride and thereby hydrolyze orthoacetate **9** to produce **2**. The glucosiduronate (**6**) and the free steroid (**2**) can be separated easily.

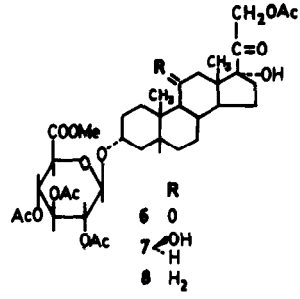
The structure of orthoacetate **9** follows from its analysis, its facile hydrolysis by acid [8] to produce steroid **2**, its recovery unchanged after treatment with acetic anhydride-pyridine, and its nmr spectrum which shows a signal at 1.75 ppm δ that is characteristic [9] of an orthoacetate with a methyl group endo to the pyranose ring.

The acetyl groups were removed from glucosdiduronate **6** by transesterification and, after purification by chromatography, glucosiduronic ester **13** was obtained in 50% yield.

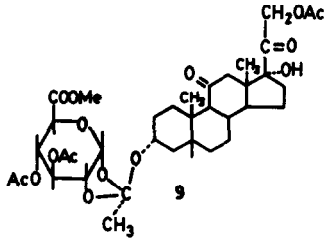
Treatment of glucosiduronate **6** with semicarbazide produced semicarbazone **10** which was convertible into starting material **6** by treatment with pyruvic acid or into the unacetylated methyl ester



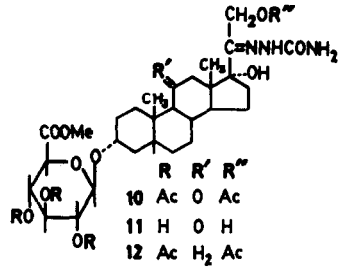
	R	R′
1	O	H
2	O	Ac
3	OH (α)	H
4	H_2	Ac
5	H_2	H



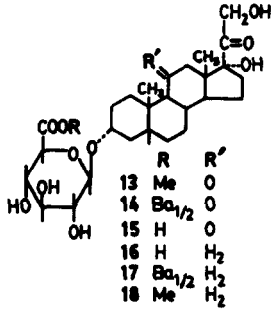
	R
6	O
7	OH (α)
8	H_2



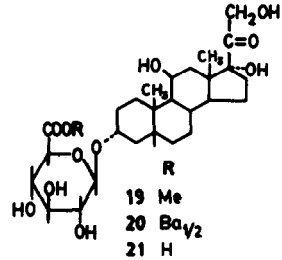
9



	R	R′	R‴
10	Ac	O	Ac
11	H	O	H
12	Ac	H_2	Ac



	R	R′
13	Me	O
14	$Ba_{1/2}$	O
15	H	O
16	H	H_2
17	$Ba_{1/2}$	H_2
18	Me	H_2



	R
19	Me
20	$Ba_{1/2}$
21	H

semicarbazone 11 by treatment with dilute alkali in methanol. With the alkali-sensitive dihydroxyacetone function stabilized through formation of a semicarbazone [10], the ester group of compound 11 was hydrolyzed with alkali; without isolating the intermediate product, the semicarbazone group was removed by acidic hydrolysis and crude glucosiduronic acid 15 was formed. After separating the semicarbazide from the glucosiduronic acid by use of a column [11] of Amberlite XAD-2 and chromatographing the conjugate, amorphous acid 15 was obtained in 79% yield from 10. Attempts to crystallize this homogeneous product as the free acid, the sodium salt, the thallium salt, and the ammonium salt were unsuccessful. However, a crystalline barium salt (14) was obtained in 51% yield from 10.

Both the amorphous acid (15) and the barium salt (14) underwent hydrolysis upon treatment with β-glucuronidase (Ketodase) and 3α,17,21-trihydroxy-5β-pregnane-11,20-dione (1) was formed, thereby demonstrating that no rearrangement of the steroid molecule had occurred during the synthesis of conjugate 15 from 2.

To prepare the 11β-hydroxy steroidal glucosiduronic acid 21, the acetyl groups were removed from semicarbazone 10 by transesterification and, without isolating the intermediate product (11), the solution was treated with potassium borohydride. This treatment reduced the 11-oxo group to an 11β-ol and hydrolyzed the methyl ester group [4]. After removal of the semicarbazone function by acidic hydrolysis and desalting the product on Amberlite XAD-2, crystalline glucosiduronic acid 21 was obtained from 10 in a 76% yield. A crystalline barium salt (20) also was obtained. Both

glucosiduronic acid 21 and barium salt 20 underwent glycosidic cleavage in the presence of β-glucuronidase (Ketodase). Upon treatment with diazomethane, acid 21 gave the corresponding ester (19) which was acetylated to produce tetraacetate ester 7. The latter compound, upon oxidation with chromic oxide in pyridine, was converted into the 11-oxo derivative (6).

Tetrahydrocortexolone 21-acetate (4) was converted into its C-3 glucosiduronic acid derivatives 8, 12, 16, 17 and 18 by the same procedures that were employed for making the corresponding derivatives from tetrahydrocortisone (1).

The chromatographic properties of compounds 14, 15, 16, 17, 20, 21 (acids or salts), 13, 18, 19 (methyl esters), and a group of related steroidal glucosiduronates have been determined in several solvent systems [12, 13, 14].

EXPERIMENTAL SECTION

Microanalyses were performed by Mr. J. F. Alicino, Metuchen, New Jersey. Unless indicated otherwise, samples were dried at 100° *in vacuo* immediately before being analyzed. Melting points were taken on a Fisher-Johns apparatus and are corrected. Solutions were evaporated under reduced pressure in a rotary evaporator at a bath temperature below 40°. Optical rotations were taken at 26 ± 1° (C ∿1.0). Infrared spectra were taken on a Beckman IR-18 spectrophotometer; ultraviolet spectra were obtained on a Beckman DU instrument. Bis(pyridine)chromium oxide was obtained from Eastman Organic Chemicals, Rochester, NY 14650.

For column chromatography, Celite 545 was used as received from Johns-Manville, impregnated with 40% its weight of stationary (more polar) phase in the presence of mobile phase and packed as a slurry using a Martin packer. The elution volume of a compound is expressed as hold-back volume (HBV), the volume of mobile phase present in the packed portion of the column. Paper chromatograms (pc) were run as described previously [11]; ratios of solvents in the systems are by volume. Zaffaroni technique was used with systems S1-S3 and Bush technique with systems S4-S7. Semicarbazones were detected by viewing chromatograms under 254 nm radiation or by treating the dried chromatograms with 5% ethanolic phosphomolybdic acid; ketolic compounds were detected by treating the chromatograms with alkaline tetrazolium blue [15].

Chromatography systems:

S1. Cyclohexane-benzene (1:2) - formamide
S2. Cyclohexane-benzene (3:1) - formamide
S3. Chloroform - formamide
S4. Toluene-ethyl acetate-methanol-water (9:1:5:5).
S5. Butyl acetate-butyl alcohol-water (9:1:10).
S6. Butyl acetate-butyl alcohol-water-acetic acid (9:1:9:1).
S7. Butyl acetate-butyl alcohol-water-acetic acid (8:2:9:1).
S8. Butyl acetate-toluene-methanol-water-acetic acid (8:2:5:4.5:0.5)
S9. Toluene-butyl acetate-methanol-water (1:1:1:1).

Thin layer chromatography (tlc) was run on plates containing silica gel G, using isooctane-ethyl acetate (15:85 v/v) as solvent; compounds were detected by spraying with water-sulfuric acid (1:1) and charring.

3α,17,21-Trihydroxy-5β-pregnane-11,20-dione (1).--One mg amounts of amorphous glucosiduronic acid **15** and crystalline Ba salt **14** were hydrolyzed separately by β-glucuronidase as described previously [16] and in each case the product was identified as **1** by pc in solvent systems S3 and S4.

3α,17,21-Trihydroxy-5β-pregnane-11,20-dione 21-Acetate (2).--To 200 mg of orthoacetate **9** in 20 ml of benzene was added 2.0 ml of 0.1 N hydrochloric acid in methanol. After 10 min the solution was washed with 1.0 N sodium carbonate, with water, and taken to dryness. Crystals (56 mg, 51%, mp 213-215°) were obtained from benzene and identified as **2** (mmp, tlc and ir).

3α,11β,17,21-Tetrahydroxy-5β-pregnan-20-one (3).--Compound **21** (5 mg) was hydrolyzed by treatment [16] with Ketodase and the aglycone was identified as **3** (pc in S3, S4 and ir).

3α,17α,21-Trihydroxy-5β-pregnan-20-one (5).--Compound **16** was hydrolyzed with Ketodase and the product was identified as **5** (pc in S3 and tlc).

Methyl (21-Acetoxy-17-hydroxy-11,20-dioxo-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (6). A. From 2.--To 2.4 g (5.0 mmol) of **2** suspended in 200 ml of benzene was added 12.4 g (45 mmol) of freshly-prepared silver carbonate and the mixture was treated [11] with 30 mmol of methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucuronate. The product was chromatographed on 700 g of Celite in system S1. The effluent (19.4 ml/fraction) was monitored by transferring 10 µl aliquots from alternate fractions to paper and dipping in a 1:9 mixture (v/v) 0.4% tetrazolium blue and 3 N sodium hydroxide. In addition, an aliquot (10 µl) from each fraction was mixed with 5 µl of 0.1 N methanolic hydrogen chloride and after 10 min the solvent was removed in an air stream; the residue was dissolved in 10 µl of 1:1 chloroform-methanol and chromatographed by tlc using compound **2** as standard. The methanolic hydrogen chloride served to convert orthoacetate **9** to starting material **2** and distinguish the band of

orthoacetate in the column effluent from that of glucosiduronate **6**. (Fractions 91-150 (HBV = 2.2) were combined, washed with water and taken to dryness; see a later paragraph for **9** from **2**). Fractions 151-241 (HBV = 3.5) were combined, washed with water and concentrated; crystals of **6** were obtained when the solution was refrigerated; 1.89 g, 52%, mp 210-211°; 104 mg, 3%, mp 198-203°; $[\alpha]_D$ + 35° ± 2° ($CHCl_3$); (lit [2] mp 209-212°; $[\alpha]_D$ 37.8° $CHCl_3$); ir (KBr) 3415 (OH), 1756 (acetate C=O), 1740sh (ester C=O), 1715 (C-20 C=O), 1702 (C-11 C=O), and 1210 cm^{-1} (acetate C-O).

The foregoing preparation of **6** from **2** was repeated with two modifications: 1) the freshly prepared silver carbonate was dried by being washed with acetone, then with benzene and finally enough benzene was distilled from a magnetically stirred suspension of the silver carbonate to free it of water; 2) the orthoacetate **9** present in the reaction product was hydrolyzed before the crude mixture was chromatographed to separate glucosiduronate **6** from the other components of the mixture. After 2.04 g of **2** had been treated with silver carbonate and methyl 2,3,4,-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucuronate the solution was filtered, the volume was diluted to 250 ml with benzene, the solution was brought to 25° and 25 ml of 0.1 N hydrochloric acid (made by diluting conc aqueous hydrochloric acid with methanol) was added. After 5 min the solution was washed with water, twice with 0.1 M sodium carbonate solution and finally with three portions of water and taken to dryness. The residue was chromatographed as described previously; only a trace of orthoacetate **9** was present. Glucosiduronate **6** (1.56 g, mp 213-214°; 0.411 g, mp 200-205°) was obtained in 54% yield.

B. From **7**.--To 50 mg of **7** in 2.5 ml of pyridine was added 50 mg of bis(pyridine)chromium oxide in 2.5 ml of pyridine. The mixture stood at room temperature for one hour, water was added and the mixture was extracted with three portions of chloroform. The extract was washed twice with sodium bisulfite solution, three times with dilute hydrochloric acid, twice with water, and taken to dryness. Crystals (13 mg, mp 210-211°) were obtained from methanol and identified as **6** (mmp and ir).

C. From **10**.--Treatment [11] of **10** (100 mg) with 80% pyruvic acid gave a product which crystallized from methanol (63 mg, mp 211-212°) and was identified as **6** (mmp and ir).

D. From **13**.--Compound **13** (100 mg) was acetylated in 1:1 acetic anhydride-pyridine during 5 hrs and the product (88 mg, mp 214-216°) was identified as **6** (tlc, mmp, and ir).

Methyl 21-Acetoxy-11β,17-dihydroxy-20-oxo-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosiduronate (**7**).--Compound **21** (200 mg) was esterified with diazomethane, the product was acetylated in 1:1 acetic anhydride-pyridine (4 hrs) and crystals (199 mg) were obtained from methanol. The sample for analysis was homogeneous by tlc; it melted at 137-138°, recrystallized spontaneously and remelted at 187-188° dec. (lit [7] mp 191-194° dec.); $[\alpha]_D$ + 25 ± 2°

($CHCl_3$), (lit [7] $[\alpha]_D$ + 29° ($CHCl_3$); ir (KBr) 3470 (OH), 1752 (acetate C=O), 1736sh (ester C=O), 1720sh (C-20 C=O), and 1218 cm^{-1} (acetate C-O). Anal. Calcd for $C_{36}H_{52}O_{15}$: C, 59.65; H, 7.23; CH_3O, 4.28; CH_3CO, 23.75. Found: C, 59.47; H, 7.22; CH_3O, 4.14; CH_3CO, 24.18.

Methyl (21-Acetoxy-17-hydroxy-20-oxo-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (8).--A solution of methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucuronate (1.19 g, 3.0 mmol) in 20 ml of toluene was added dropwise to a mixture of compound 4 392 mg, 1.0 mmol) and $CdCO_3$ (345 mg, 2.0 mmol) in 20 ml of boiling toluene according to a previously-described technique [11]. The product was chromatographed on a column of Celite (system S2) and the band which emerged at 10 HBV gave crystalline 8 in 34% yield from ethyl acetate-isooctane; mp 171.5-174.5°, (lit [4] 165-167°); $[\alpha]_D$ + 19 ± 2° ($CHCl_3$); ir (KBr) 3490 (OH), 1755, 1730 (carbonyl CO), 1722 (C-20 CO) and 1211 cm^{-1} (acetate C-O). Anal. Calcd for $C_{36}H_{52}O_{14}$: C, 61.00; H, 7.40. Found: C, 61.06; H, 7.27.

(1S)-Methyl α-D-Glucopyranuronate Cyclic 1,2-(Hydrogen Orthoacetate) 3,4-Diacetate 3-Ester with 21-Acetoxy-3α,17-dihydroxy-5β-pregnane-11,20-dione (9).--The residue corresponding to HBV = 2.2 (See 6 from 2) was crystallized from methanol-water (614 mg, 17%, mp 100-106°). After being recrystallized twice from methanol-water the product (9) melted at 107-110°; $[\alpha]_D$ + 48° ± 2° ($CHCl_3$); nmr ($CDCl_3$) 0.59 (C-18 CH_3), 1.13 (C-19 CH_3), 1.75 (orthoacetate CH_3 endo to pyranose ring), 2.09 (3', 4' acetate groups), 2.15 (C-21 acetate), 3.12 (C-17 OH), 3.76 (CH_3 of ester), 4.92-5.42 (H_2', H_3', H_4', H_5'), and 5.80 ppm (doublet, H_1', $J_{1,2}$ = 5Hz); ir (KBr) 3510 (OH), 1755 (acetate C=O), 1738sh (ester C=O), 1720sh (C-20 C=O), 1705sh (C-11 C=O), and 1215 cm^{-1} (acetate C-O). Anal. Calcd for $C_{36}H_{50}O_{15}\cdot 1\ 1/2\ H_2O$: C, 57.67; H, 7.13; CH_3O, 4.14; CH_3CO, 22.96. Found: C, 57.68; H, 6.94; CH_3O, 4.54; CH_3CO, 24.57.

Treatment of orthoacetate 9 with Ac_2O.--To 25 mg of orthoacetate 9 in 2.5 ml of dry pyridine was added 1.25 ml of acetic anhydride. After 2 hrs, the solvent was removed completely in an air current. Crystals of unchanged starting material 9 (21 mg, mp 105-110°) were obtained from aqueous methanol and identified (mmp, tlc, and ir).

Methyl (21-Acetoxy-17-hydroxy-11-oxo-20-semicarbazono-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (10).--One mmol of 6 was treated with semicarbazide by a previously-described [10] procedure to obtain 10 (756 mg, 97%, mp 155-157°). A sample, recrystallized twice from ethanol, melted at 161-162°; λ_{max}^{MeOH} 238 nm (ε = 12,300, shoulder 223 nm); ir (KBr) 3640, 3500, 3380, 3270 (NH + OH), 1758 (acetate C=O), 1746 (ester C=O), 1700 (C-11 C=O), 1690 (amide C=O), 1580 (amide), 1224 and 1212 cm^{-1} (acetate C-O). Anal. Calcd for $C_{37}H_{54}O_{15}N_3\cdot H_2O$: C, 55.63; H, 7.07; N, 5.26. Found: C, 55.45; H, 6.90; N, 5.44.

Methyl (17,21-Dihydroxy-11-oxo-20-semicarbazono-5β-pregnan-3α-yl β-D-Glucopyranosid)uronate (11). A. From 10.--To a solution of 10 (0.50 mmol) in 5.0 ml $CHCl_3$ was added 2.5 ml methanol and 2.5 ml 0.04 N NaOH in dry methanol. After 45 min, 4.5 ml of 0.05 N acetic acid in methanol was added and the solution was evaporated to dryness immediately to prevent acid-catalyzed removal of the semicarbazone group. Two 5 ml portions of alcohol were removed in vacuo. Crystals (300 mg, 97%) were obtained from methanol-butyl acetate. Traces of impurities were removed by chromatographing† this material in system S5 (HBV = 1.6) and crystallizing the product from butyl alcohol-butyl acetate; mp 211-213; λ_{max}^{MeOH} 223 nm (ε = 11,600, shoulder 238 nm); ir (KBr) 3500-3330 (OH + NH), 1740 (ester C=O), 1700sh (C-11 C=O), 1685 (amide C=O), and 1575 cm^{-1} (amide). Anal. Calcd for $C_{29}H_{45}O_{11}N_3 \cdot H_2O$: C, 55.31; H, 7.53. Found: C, 55.38; H, 7.51.

B. From 13.--Ester 13 (20 mg) was converted into semicarbazone 11 by the procedure described under 10 from 6. The reaction mixture was taken to dryness, dissolved in water and freed of excess semicarbazide and salts by use of Amberlite XAD-2 (10 g). The alcoholic effluent was taken to dryness and 11 (8.3 mg, λ_{max}^{MeOH} 223 nm;) was obtained from methanol-butyl acetate and identified by pc (S7) and ir.

Methyl (21-Acetoxy-17-hydroxy-20-semicarbazono-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (12). To a solution of 1.79 mmol of 8 in a mixture of 9 ml of chloroform and 90 ml of methanol was added a solution of 17.9 mmol of semicarbazide hydrochloride and 10.7 mmol of sodium bicarbonate in 3.6 ml of water. After 64 hr water was added, the solution was concentrated in vacuo and crystals of 12 were obtained; yield 93%. Recrystallized (MeOH-H_2O) product melted at 145-148°; λ_{max}^{MeOH} 236 nm (ε = 12,400); ir (KBr) 3515 (NH, OH), 1758 (ester C=O), 1693 (amide C=O) 1583 (amide) and 1217 cm^{-1} (acetate C-O). Anal. Calcd for $C_{37}H_{55}O_{14}N_3 \cdot H_2O$: C, 56.69; H, 7.33. Found: C, 56.74; H, 7.35.

Methyl (17,21-Dihydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-Glucopyranosid)uronate (13). A. From 6.--To 723 mg (1.0 mmol) of 6 in a mixture of 28 ml of chloroform and 15 ml of methanol was added 15 ml of 0.04 N sodium hydroxide in methanol. The solution stood at room temperature for 45 minutes, and was acidified with dilute acetic acid in methanol and taken to dryness. The product was purified by chromatography on 100 g of Celite using solvent S7. The band corresponding to HBV = 6.0 yielded crystals (280 mg, 50%, mp 150-155°) of 13. The sample for analysis was recrystallized from ethyl acetate-cyclohexane; homogeneous by paper chromatography in system S9; mp 150-155°; $[\alpha]_D$ + 36° ± 2° (MeOH); ir (KBr) 3420 (OH), 17401718sh (ester C=O), and 1703 cm^{-1} (C-11 + C-20 C=O). Anal. Calcd for $C_{28}H_{43}O_{11} \cdot H_2O$: C, 58.62; H, 7.91; CH_3O, 5.41. Found: C, 58.75; H, 7.68; CH_3O, 5.18.

† Partial hydrolysis of the semicarbazone group of compound 11 occurs during chromatography of the substance on paper in an acidic system such as S6.

B. From 15.--A solution of 15 (104 mg) in methanol was esterified with ethereal diazomethane to give crystals of 13 (62 mg, 59% from ethyl acetate-cyclohexane); identified by mmp and ir.

Barium bis[(17,21-Dihydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-Glucopyranosid)uronate] (14).--To 200 mg of acid 15 in 5 ml of 50% ethanol was added 5 ml of 0.1 M barium acetate in 50% ethanol containing 1 drop of 1:10 acetic acid-ethanol. The solution was concentrated in vacuo, and crystals (129 mg, 51%; mp >250°) were obtained. The sample for analysis was recrystallized from water; mp dec > 250°; ir (KBr) 3410 (OH), 1705 (C-11 + C-20 C=O), 1600 and 1419 cm^{-1} (ionized carboxyl). Anal. Calcd for $C_{27}H_{39}O_{11}Ba_{1/2}\cdot 4H_2O$: C, 47.66, H, 6.96; Ba, 10.10. Found: C, 47.69; H, 6.54; Ba, 10.74.

An aliquot of the foregoing analytical sample was dried at 140° for 12 hours. Loss in wt = 10.7%; calcd for 4 H_2O = 10.6%. Anal. Calcd for $C_{27}H_{39}O_{11}Ba_{1/2}$: C, 53.22; H, 6.67. Found: C, 53.44; H, 6.71.

A sample of amorphous sodium (17,21-dihydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-glucopyranosid)uronate which had been isolated from human urine by Dr. John J. Schneider, was converted into the glucosiduronic acid by use of a column [11] of Amberlite XAD-2 and the amorphous acid was converted into the crystalline barium salt by the procedure just described. The ir spectra of the barium salts derived from the two sources were identical.

17,21-Dihydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-Glucopyranosiduronic Acid (15).--To 756 mg of 10 in a mixture of 15.0 ml of chloroform and 7.0 ml of methanol was added 7.0 ml of 0.04 N sodium hydroxide in methanol, and the solution stood at room temperature for 45 minutes. To this mixture was then added 30.0 ml of methanol and 14.0 ml of 1.0 N aqueous sodium hydroxide, and it remained at room temperature for 30 minutes. The mixture was then acidified to pH 4 with dilute sulfuric acid and filtered to remove Na_2SO_4. The filtrate was concentrated in vacuo until the chloroform and methanol had been removed and then diluted to 80 ml with water. The pH was adjusted to 2.0 with dilute sulfuric acid, the solution was allowed to stand at room temperature for one hour, and the mixture was desalted [11] on 40 g of Amberlite XAD-2. Since the salt-free product contained an uv absorbing impurity (pc in system S7) which did not reduce tetrazolium blue, but absorbed maximally at ∿200 mμ, it was purified by chromatography on 100 g of Celite using solvent system S7. The dry residue from the principal band (HBV = 2.0) weighed 416 mg (79% yield). When a solution of the product (15) in acetone-benzene (1:1) was concentrated in vacuo in a rotary evaporator an amorphous friable substance (homogeneous in system S9) separated, mp 167-179° dec. A sample which had been dried to constant wt. at 105° was hygroscopic and gained 3.8% (3.33% = 1 H_2O) when equilibrated with air at 43% relative humidity. Anal. The rehydrated sample was analyzed. Calcd for $C_{27}H_{40}O_{11}\cdot H_2O$: C, 58.05; H, 7.58. Found: C, 58.22; H, 7.64.

17,21-Dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosiduronic Acid (16).--Compound 12 (367 mg) was converted into the free acid (16) by the procedure used for preparing 15 from 10, but with the following variation: a) the solution stood at pH 2.1 for 18 hr to remove the semicarbazone group; b) the crude glucosiduronic acid was chromatographed on a column in system S8 (HBV = 1.9) which yielded 141 mg of amorphous acid (16). This acid was not obtained in crystalline form.

Barium bis[(17,21-Dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosid)uronate] (17).--Treatment of 94 mg of amorphous compound 16 with barium acetate (as described under the preparation of 14) gave 95 mg of crystalline 17 from water-ethanol; mp > 220° dec; ir (KBr) 3400 (OH), 1714 (C-20 C=O), 1601 and 1423 cm^{-1} (ionized carboxyl). Anal. Calcd for $C_{27}H_{41}O_{10}Ba_{1/2}\cdot 1\ 1/2\ H_2O$: C, 52.19; H, 7.14. Found: C, 52.04; H, 7.33.

Methyl 17,21-Dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosiduronate (18).--Esterification of 47 mg of acid 16 with diazomethane followed by chromatography of the product on a column of Celite in system S9 (HBV = 1.8) gave 34 mg of amorphous powder from acetone-benzene; mp 131-134°; $[\alpha]_D$ + 22° ± 2° (MeOH); ir (KBr) 3450 (OH), 1745 (ester C=O) and 1714 cm^{-1} (C-20 C=O). Anal. Calcd for $C_{28}H_{44}O_{10}$: C, 62.20; H, 8.20. Found: C, 62.09; H, 8.26.

Methyl 11β,17,21-Trihydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosiduronate (19).--To 100 mg of 21 in methanol was added an excess of diazomethane in ether and crystals (93 mg, 90%, mp 160-165°) of 19 were obtained from ethyl acetate-cyclohexane; homogeneous in solvent system S7; $[\alpha]_D$ + 34° ± 2° (MeOH); ir (KBr) 3430 (OH), 1737 (ester C=O), and 1714 cm^{-1} (C-20 C=O). Anal. Calcd for $C_{28}H_{45}O_{11}\cdot H_2O$: C, 58.42; H, 8.23; CH_3O, 5.39. Found: C, 58.83; H, 7.90; CH_3O, 5.20.

Barium bis[(11β,17,21-Trihydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosid)uronate] (20).--To 83 mg of 21 in 1.25 ml of 50% ethanol was added 1.25 ml of 0.1 M barium acetate in 50% ethanol containing 1 drop of 1:10 acetic acid-ethanol. Hygroscopic crystals (64 mg, 66%, mp above 230° dec.) were obtained from water; ir (KBr) 3420 (OH), 1710 (C-20 C=O), 1600 and 1414 cm^{-1} (ionized carboxyl). Anal. Calcd for $C_{27}H_{41}O_{11}Ba_{1/2}\cdot H_2O$: C, 51.61; H, 6.90; Ba, 10.93. Found: C, 51.15; H, 7.01; Ba, 10.88.

11β,17,21-Trihydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosiduronic Acid (21).--To 780 mg (1.0 mmol) of 10 was added 50.0 ml of 0.04 N sodium hydroxide in methanol, and the solution was allowed to sit at room temperature for 30 minutes. This treatment produced the 20-semicarbazone of compound 13. To the solution was added 1.08 g (20.0 mmol) of potassium borohydride in 50 ml of water and the mixture stood at room temperature for 18 hours. The methanol was removed in vacuo, the pH was taken to 2.1 with 1 N sulfuric acid, and the mixture stood at room temperature for 1 hour during which time the semicarbazone function was hydrolyzed. The solution was poured onto a column

of Amberlite XAD-2 resin (40 g) which was then washed with two 80 ml portions of 0.01 N sulfuric acid, and two 80 ml portions of water. The steroid was eluted with four 80 ml portions of ethanol and the ethanolic eluates were combined and taken to dryness. The residue was purified on a column of 100 g of Celite using solvent system S7. The eluate corresponding to 2.3 HBV gave hygroscopic crystals (348 mg, 76%) from acetone-ethyl acetate; mp 175° dec; (lit [7] mp 192-198° dec.); $[\alpha]_D$ + 34° ± 2° (MeOH), (reported $[\alpha]_D$ = 16.6° (MeOH): ir (KBr) 3440 (OH), 1725 (carboxyl C=O), and 1712 cm^{-1} (C-20 C=O). Anal. Calcd for $C_{27}H_{42}O_{11} \cdot 1/2\ H_2O$: C, 58.79; H, 7.86. Found: C, 58.67; H, 7.90.

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