SYNTHESIS OF MONO- AND DIGLUCOSIDURONATES OF METABOLITES OF DEOXYCORTICOSTERONE AND CORTICOSTERONE AND ANALYSIS BY A NEW MASS SPECTROMETRIC TECHNIQUE

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ABSTRACT

By condensing 3α,21-dihydroxy-5β-pregnan-20-one, or its appropriate monoacetate, with methyl 2,3,4-tri-O-acetyl-1-bromo-1-deoxy-α-D-glucuronate in the Koenigs-Knorr reaction β-D-glucosiduronates 10, 4, and 7 were obtained as polyacetate methyl esters. Alkaline hydrolysis of these substances cleaved the ester groups and gave the corresponding steroidal glucosiduronic acids 12, 6 and 8. Upon treatment with diazomethane, these acids produced the equivalent methyl esters.

The C-3, the C-21 and the C-3,21 glucosiduronates of 3α,21-dihydroxy-5β-pregnan-11,20-dione were prepared by previously reported methods and converted into the corresponding C-20 semicarbazones (14, 20 and 26). With C-20 stabilized by the semicarbazone group against reduction, it was possible to reduce the 11-oxo function in these substances to an 11β-hydroxyl group; after removal of the semicarbazone moiety from these products at pH 2.0, glucosiduronic acids 18, 22 and 28 were obtained.

The mass spectra of a representative group of the mono- and diglucosiduronic acids and esters were determined by utilizing fast atom bombardment and monitoring ions in both positive and negative modes of operation.

INTRODUCTION

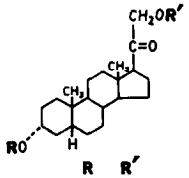
An important route in the metabolism of corticosteroids in man involves the reduction of 3-oxo-4-pregnenes to the corresponding 3α-hydroxy-5β-pregnanes and subsequent conjugation of the reduced products with glucuronic acid. Although conjugation at C-3 is usually the dominant process, conjugation at C-21 also occurs [1,2,3]. In addition, conjugation at both C-3 and C-21 to produce a diglucosiduronate [4,5] may take place to a small extent. This paper describes

the chemical synthesis of the C-3, the C-21, and the C-3,21 glucosiduronates of 3α,21-dihydroxy-5β-pregnan-20-one and 3α,11β,21-trihydroxy-5β-pregnan-20-one which are metabolites of deoxycorticosterone and corticosterone, respectively. These synthetic steroidal glucosiduronic acids should be invaluable for use as standards in studies involving gas chromatography, mass spectrometry, radioimmunoassay, etc., of this class of compounds.

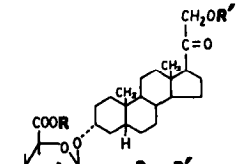
The molecular weight of a number of the intact underivatized compounds was determined by mass spectrometry using the new technique of fast atom bombardment (FAB-MS).

Conjugates of 3α,21-dihydroxy-5β-pregnan-20-one. Treatment of 21-acetoxy-3α-hydroxy-5β-pregnan-20-one (2), Fig. 1, with methyl acetobromoglucuronate and silver carbonate gave the C-3 conjugate (4) in 41% yield. This substance was converted into the corresponding C-20 semicarbazone (16) in order to protect the alkali-sensitive ketol group during subsequent hydrolysis of the acetate and the methyl ester groups [6]. After these functions had been hydrolyzed the semicarbazone group was removed at pH 2.0, the glucosiduronic acid (6) was separated from the newly-formed semicarbazide by use of Amberlite XAD-2 [6] and obtained crystalline in 74% yield from 16. Esterification of glucosiduronic acid 6 with diazomethane gave the corresponding ester (5) which could be acetylated to give tetra-acetate ester 4.

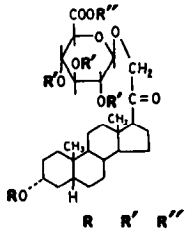
To prepare the corresponding C-21 glucosiduronate (7), compound 3 was subjected to the Koenigs-Knorr procedure, utilizing cadimum carbonate [7] as the bromide acceptor; compound 7 was obtained in 70% yield. This substance, which has the ketolic side



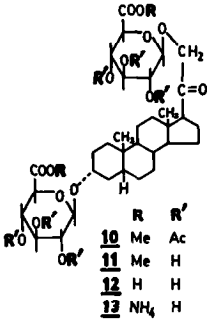
	R	R′
1	H	H
2	H	Ac
3	Ac	H



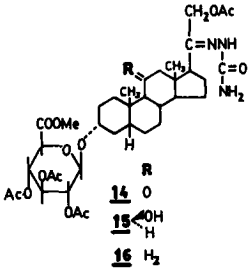
	R	R′
4	Me	Ac
5	Me	H
6	H	H



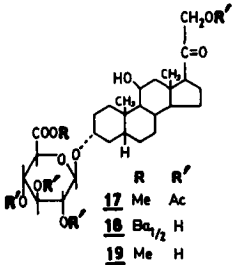
	R	R′	R″
7	Ac	Ac	Me
8	H	H	H
9	H	H	Me



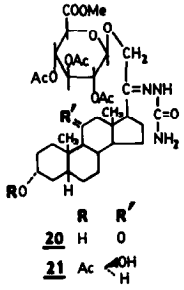
	R	R′
10	Me	Ac
11	Me	H
12	H	H
13	NH_4	H



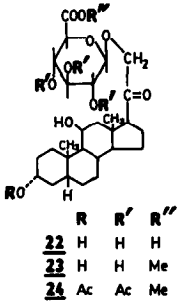
	R
14	O
15	<OH H
16	H_2



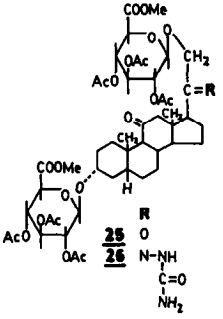
	R	R′
17	Me	Ac
18	$Ba_{1/2}$	H
19	Me	H



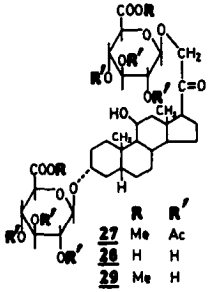
	R	R′
20	H	O
21	Ac	<OH H



	R	R′	R″
22	H	H	H
23	H	H	Me
24	Ac	Ac	Me



	R
25	O
26	N-NH C=O NH_2



	R	R′
27	Me	Ac
28	H	H
29	Me	H

Fig 1.

chain stabilized by the glucosyluronate group at C-21, can be hydrolyzed to the free acid (8) in good yield without protecting C-20 with a semicarbazone group.

The 3,21-diglucosiduronic acid (12) was prepared by treating the corresponding dihydroxy compound (1) with methyl acetobromoglucosiduronate and removing the acetate and ester groups from the product. The acid was converted into the crystalline ammonium salt (13) and the methyl ester (11).

By analogy with previous results from the Koenigs-Knorr reaction [6], it is probable that during preparation of compounds 4, 7 and 10 significant amounts of cyclic orthoacetates between the glucuronate and steroid moieties were formed; however, no attempt was made to isolate and characterize these postulated by-products.

Conjugates of 3α,11β,21-trihydroxy-5β-pregnan-20-one. In a previous paper we reported the synthesis of the 3-mono-, the 21-mono- and the 3,21-diglucosiduronate of 3α,21-dihydroxy-5β-pregnane-11,20-dione [6]. The availability of considerable amounts of these conjugates indicated to us that we should prepare the 11β-hydroxy analogues by reducing the 11-oxo group with borohydride after having protected C-20 with a semicarbazone group. Accordingly, this mode of synthesis was chosen in preference to application of the Koenigs-Knorr reaction to the appropriate precursor.

To prepare barium C-21 glucosiduronate 18, the corresponding 11-oxo-20-semicarbazone (14) was treated with alkali to hydrolyze the ester groups and the product was treated with borohydride to reduce the 11-oxo group. Finally, the 20-semicarbazone group was hydrolyzed at pH 2.0, the glucosiduronic acid was separated from the semicarbazide

by use of a column of Amberlite XAD-2 and crystalline barium glucosiduronate 18 was obtained. The amorphous acid, which corresponds to barium salt 18, was converted into ester 19 by use of diazomethane and the ester was acetylated to give tetraacetate ester 17. Oxidation of compound 17 with chromium trioxide converted the 11-hydroxyl group to a ketone group and produced methyl (21-acetoxy-11,20-dioxo-5β-pregnan-3α-yl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)-uronate [6] which was prepared previously. Since the latter compound is known to contain a β-glycosidic linkage it follows that the configuration of the anomeric carbon was not altered during reduction of the 11-oxo group on the pathway to preparation of 18.

The 11β-hydroxy 21-mono- and 3,21-diglucosiduronic acids (22 and 28) were synthesized from precursors 20 and 26, respectively, by the same general approach as was employed to prepare compound 18.

The various unesterified glucosiduronates (6, 8, 18, 22 and 28) were hydrolyzed by treatment with β-glucuronidase and each conjugate produced its respective aglycone.

Recently a new technique for mass spectrometric ionization of relatively nonvolatile and thermolabile compounds was described [8,9]. With this technique a straightforward analysis of polar and high molecular weight biomolecules can often be performed without the necessity of preparing derivatives such as trimethylsilyl ethers to increase volatility. The technique, called fast atom bombardment mass spectrometry (FAB-MS), is extremely useful for the analysis of

synthetic steroidal glucosiduronic acids and esters [10]. Thus, we have used this technique to confirm the molecular weight of a number of the glucosiduronates reported in this paper.

When the steroidal glucosiduronic acids were examined by positive ion FAB-MS a protonated molecular ion (MH^+) was identifiable for some, but not all, of the conjugates. Also clearly identifiable were prominent ions associated with the aglycone. For illustrative purposes, the positive ion FAB-MS of 3α-hydroxy-11,20-dioxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid [6], a precursor of compound 20, is reproduced in Fig. 2a. The ion at mass to charge ratio (m/z) 525 is the protonated molecular ion of the compound; the ion at m/z 507 is produced by loss of water from the MH^+ ion. The major peaks in the spectrum at m/z 349 and 331 represent the protonated aglycone ion (AH^+), and this species minus water, respectively. This spectrum, with peaks for the protonated molecular ion, the protonated aglycone ion, and the two species minus water, was representative of many of the glucosiduronic acids, including compound 8. However, the protonated molecular ion was often weak or absent from the spectrum of the free acid, as was the case for glucosiduronic acids 6, 12 and 22.

When the protonated molecular ion of the acid was of inadequate intensity it was possible to confirm the molecular weight of the glucosiduronic acid by the addition of sodium ion (Na^+) to the sample on the probe. The Na^+ can be added as essentially any sodium salt; e.g., sodium acetate. In this way the steroidal glucosiduronic acid is converted in part into its sodium salt. The salt is desorbed from the probe along with another Na^+ ion to generate a charged entity which

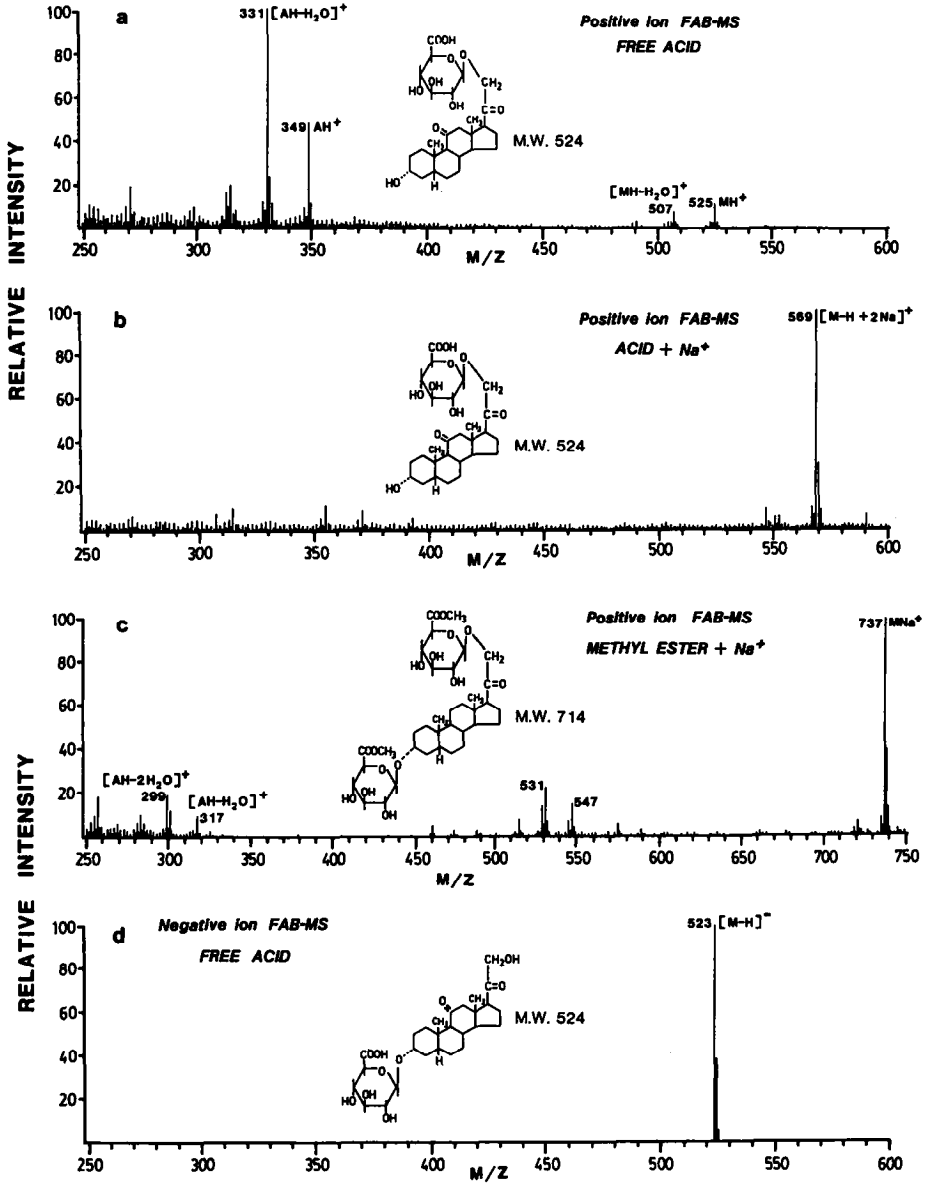


Fig. 2. Mass spectra of steroidal glucosiduronates

a,b. 3α-Hydroxy-11,20-dioxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid
c. 20-Oxo-5β-pregnan-3α,21-ylene bis[methyl(β-D-glucopyranosid)uronate]
d. 21-Hydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-glucopranosiduronic acid

can be analyzed by the mass spectrometer. Thus, the ion detected, namely $[M-H+2Na]^+$, is equivalent to the free acid, minus a proton, plus two sodium ions. With this technique, an intense ion is produced for steroidal glucosiduronic acids and the molecular weight of the intact conjugate can be deduced readily from the mass of the ion. Fig. 2b shows the positive ion FAB mass spectrum of 3α-hydroxy-11,20-dioxo-5β-pregnan-21-yl β-D-glucopyranosiduronic acid [6] and reveals an intense ion of m/z 569 from which the molecular weight is calculated as 524. The sodium addition ion, namely $[M-H+2Na]^+$, is more stable and thus is more abundant than the protonated molecular ion (MH^+). The molecular weights of compounds 6 and 22 were confirmed by sodium ion addition to the free acid on the probe. Although we used Na^+ to generate the ion associated with the molecular weight of the molecule, other ions such as Li^+ or K^+ can be used.

Fast atom bombardment of steroidal glucosiduronic esters and monitoring for positive ions did not produce useful spectra unless Na^+ was added to the sample on the probe; with Na^+ present an intense MNa^+ species was generated. Only one sodium atom is involved in this ion since there is no highly-acidic proton available to be replaced by Na^+ as with the free acids. An example of such a spectrum is shown in Fig. 2c for the dimethyl ester of a steroidal diglucosiduronate (11). An intense MNa^+ ion at m/z 737 allows calculation of the molecular weight of the methyl ester as 714. In this spectrum ions at m/z 529, 531 and 547 result from the loss of one glucuronic acid methyl ester moiety from the MNa^+ ion. Ions at m/z 317 and 299 result from the loss of one and two molecules of water, respectively, from the protonated aglycone fragment (AH^+), which is

not observed in this case. This technique (adding sodium acetate to the ester on the probe) was used to confirm the molecular weights of methyl esters 5, 9, 11, 19, 23 and 29. Acetyl derivatives and barium salts of steroidal glucosiduronates did not give useful positive ion FAB mass spectra.

Positive ion FAB-MS on the free acid, the sodium salt of the acid or the sodium addition complex with the ester allowed confirmation of the molecular weight of the glucosiduronates of interest in this study. However, it has been found that some steroidal glucosiduronates, particularly those which are highly hydroxylated, do not produce unambiguous molecular weight information in any positive ion FAB-MS mode [10]. In contrast, the negative ion FAB mass spectra of all steroidal glucosiduronic acids examined reveal intense $[M-H]^-$ ions, usually with no observable fragmentation. For illustrative purposes the negative ion FAB-MS of 21-hydroxy-11,20-dioxo-5β-pregnan-3α-yl β-D-glucopyranosiduronic acid [6], a precursor of compound 14, is shown in Fig. 2d. Equipment for measurement of negative ions is available in only a few mass spectrometry laboratories at the present time. However, it is recommended that for obtaining the maximal amount of FAB-MS information from intact steroidal glucosiduronates, the spectra should be recorded whenever possible on the same sample and in the order 1) negative ion, 2) positive ion, and 3) positive ion plus Na^+.

EXPERIMENTAL SECTION

The general laboratory procedures which were employed have been described previously [6]. Systems for thin layer chromatography (t.l.c.): Solvent system A, benzene-ether (1:1); system B, ethyl acetate-methanol (24:1). Compounds were detected by spraying with 1:1 alcohol-sulfuric acid and charring.

Systems for paper and column chromatography:
S1 Isooctane with carbitol-formamide (2:1)
S2 Benzene-isooctane (1:2) - carbitol-formamide (1:2)
S3 Benzene-isooctane (1:3) - carbitol-formamide (1:2)
S4 Benzene-cyclohexane (1:5) - carbitol-formamide (2:1)
S5 Chloroform-formamide
S6 Ethyl acetate-acetic acid (98:2) - ethylene glycol
S7 Butyl acetate-butyl alcohol-water-acetic acid (5:5:9:1)
S8 Butyl acetate-butyl alcohol-water-acetic acid (9:1:9:1)
S9 Butyl acetate-toluene-methanol-water-acetic acid (50:50:50:45:5)
S10 Butyl acetate-toluene-methanol-water (1:1:1:1)

Where indicated below, FAB mass spectrometry was used to confirm the molecular weight (M.W.) of a number of the glucosiduronates. Those compounds which required addition of Na^+ to the sample for obtaining an unambiguous molecular weight are indicated by the presence of Na^+ in the molecular ion of the substance. The integral weight equivalent to that calculated by using 12, 1, and 16 for C, H, and O, respectively is used in expressing the calculated M.W. and that determined by FAB-MS. The calculated values are for the unsolvated substances.

Mass spectra were obtained by operating a Kratos model MS50-DS55 mass spectrometer/computer system at 6 KV accelerating potential. The mass scale was calibrated by the data system using polyethylene glycol of average molecular weight 600 (Aldrich Chemical Co., Milwaukee, WI). A primary atom beam of Xe was produced by using a FAB11NF saddle field ion source (Ion Tech Ltd., Teddington, England) operating with a tube current of 1.5 mA at an energy of 7 KeV. Samples to be analyzed were dissolved, or suspended, in glycerol and deposited on a copper-tipped insertion probe. The sample and mass spectrometer source were held at ambient temperature (27°C).

Preparations:

3α,21-Dihydroxy-5β-pregnan-20-one (1).--Treatment of 10.0 mg amounts of 6 and 8 in separate flasks with 25,000 units of β-glucuronidase (Ketodase) under conditions described previously [11] gave 1 [12] which was identified by mp, mmp and ir.

3α-Acetoxy-21-hydroxy-5β-pregnan-20-one (3).--A solution of 1.88 g of 3α,21-diacetoxy-5β-pregnan-20-one [13] in 188 ml of methanol was mixed with a solution of 1.88 g of potassium bicarbonate in 62 ml of water and after the mixture had stood at 25° for 4 hr a slight excess of acetic acid was added and the methanol was removed in vacuo. The aqueous residue was extracted with chloroform, the organic solution was washed with water and evaporated to dryness. The residue was chromatographed on Celite in system S1 (HBV = 1.9). The product (3) crystallized from methanol in 54% yield; mp 112.5-114°; $[\alpha]_D$ + 106 ± 2° ($CHCl_3$); ir (KBr) 3500 (OH), 1727 (acetate C=O), 1700 (C-20 C=O) and 1235 cm^{-1} (acetate C-O). Anal. Calcd for $C_{23}H_{36}O_4$: C, 73.36; H, 9.63. Found: C, 72.49; H, 9.83.

Methyl (21-Acetoxy-20-oxo-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (4).--One mmol of 2 [13] was treated

with 4 mmoles of silver carbonate and 6 mmol of methyl acetobromoglucuronate by the usual procedure [14]. After the solution had been filtered and evaporated to dryness the residue was dissolved in 70 ml of benzene, 7.0 ml of 0.1 N HCl in t-butyl alcohol was added and the solution stood for 10 min to hydrolyze steroidal 3-glucuronosyl orthoacetate which is commonly a by-product [6]. The solution was washed with cold sodium bicarbonate solution, water and taken to dryness. Chromatography of the residue are a column of Celite in system S4 (HBV = 1.8) and crystallization from benzene-cyclohexane gave 295 mg (41% yield) of 4; m.p. 140.5-141°; $[\alpha]_D$ + 47 ± 2° ($CHCl_3$); ir (KBr) 1760 (ester C=O), 1738 (acetate C=O), 1722 (C-20 C=O) and 1230 cm^{-1} (acetate C-O). Anal. Calcd for $C_{36}H_{52}O_{13}$: C, 62.41; H, 7.56. Found: C, 62.69; H, 7.34.

Methyl (21-Hydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosid)-uronate (5).--Compound 6 was esterified with diazomethane and the product was chromatographed on a column in system S5 (HBV = 4.4). The appropriate fractions were combined, washed with water and evaporated in vacuo. An amorphous powder was obtained from methanol-benzene; ir (KBr) 3420 (OH), 1750 (ester C=O) and 1710 cm^{-1} (C-20 C=O). Anal. Calcd for $C_{28}H_{44}O_9$: C, 64.09; H, 8.45. Found: C, 63.72; H, 8.36.

21-Hydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosiduronic Acid (6).--Compound 16 (1.0 mmol) was treated by a previously described [6] procedure to hydrolyze the ester groups and the semicarbazone group. Crystals were obtained from water in a yield of 74%; mp 147-148.5°; $[\alpha]_D$ + 54 ± 2° (CH_3OH); ir (KBr) 3420 (OH) and 1710 cm^{-1} (C=O). M. W. Calcd: 510. Found: $[M-H+2Na]^+$ = 555; M = 510. Anal. Calcd for $C_{27}H_{42}O_9.H_2O$: C, 61.34; H, 8.39. Found: C, 61.14; H, 8.36.

Methyl (3α-Acetoxy-20-oxo-5β-pregnan-21-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (7).--A mixture containing 1.0 mmol of 3 and 2 mmol of $CdCO_3$ in 20 ml of dry toluene was treated with 3 mmol of methyl acetobromoglucuronate using a previously-described procedure [14]; reaction time was 3 hours. Upon chromatography on a Celite column in solvent system S3 (HBV = 1.9) and crystallization from benzene-isooctane, compound 7 was obtained in 70% yield; mp 190-191°; $[\alpha]_D$ + 12 ± 2° ($CHCl_3$); ir (KBr) 1757 and 1744 (acetate C=O), 1733 (ester C=O), 1718 (C-20 C=O), 1240 and 1215 cm^{-1} (acetate C-O). Anal. Calcd for $C_{36}H_{52}O_{13}$: C, 62.40; H, 7.56. Found: C, 62.50; H, 7.64.

3α-Hydroxy-20-oxo-5β-pregnan-21-yl β-D-Glucopyranosiduronic Acid (8).--Compound 7 (0.5 mmol) was hydrolyzed by a previously-described two step procedure [14] and crystals were obtained from aqueous methanol. A small amount of impurity (probably the 3-acetate) was removed by chromatographing the product in system S6 (HBV = 8.3). Crystals were obtained from aqueous methanol; yield 52%, mp 187-188°, $[\alpha]_D$ + 36° ± 2° (CH_3OH); ir (KBr) 3385 (OH), 1762 (carboxyl C=O), and 1693 (C-20 C=O). M. W. Calcd: 510. Found: $[M-H+2Na]^+$ = 555; M = 510. Anal. Calcd for $C_{27}H_{42}O_9.H_2O$: C, 61.34; H, 8.39. Found: C, 61.79; H, 8.18.

When Compound 8 was crystallized rapidly from a concentrated solution of methanol-water and dried at 100°, the ir spectrum had five sharp peaks in the carbonyl region (1672, 1689, 1736, 1747, and 1760 cm^{-1}); when the same sample of material crystallized slowly from a dilute solution of the same solvent mixture peaks at 1689, 1736 and 1747 cm^{-1} were missing. Samples prepared by the two modes of crystallization showed no differences in chromatographic mobility or mp and the mp of a mixture of crystals from the two sources was the same as that of the individual samples.

Methyl (3α-Hydroxy-20-oxo-5β-pregnan-21-yl β-D-Glucopyranosid)-uronate (9).--Esterification of 8 with diazomethane and crystallization of the product from methanol-ethyl acetate gave 9, mp 208-210°; $[\alpha]_D$ + 36 ± 2° (CH_3OH); ir (KBr) 3470 and 3410 (OH), 1754 and 1749 (ester C=O) and 1713 cm^{-1} (C-20 C=O). Anal. Calcd for $C_{28}H_{44}O_9$: C, 64.09; H, 8.45. Found: C, 63.95; H, 8.71.

20-Oxo-5β-pregnan-3α,21-ylene Bis[methyl (2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate] (10).--One mmol of 1 [12] in 20 ml of toluene was treated with 4 mmol of $CdCO_3$ and 4 mmol of methyl acetobromoglucuronate in the usual manner [6]. The product was chromatographed in system S2 and crystalline 10 was obtained from ethanol-water in 48% yield; mp 166-168°; $[\alpha]_D$ + 7 ± 2° ($CHCl_3$); ir (KBr) 1755 (carbonyl C=O) and 1210 cm^{-1} (acetate C-O). Anal. Calcd for $C_{47}H_{66}O_{21}$: C, 58.37; H, 6.88. Found: C, 58.28; H. 7.00.

20-Oxo-5β-pregnan-3α,21-ylene Bis[methyl (β-D-Glucopyranosid)-uronate] (11).--One-half mmol of 12 in methanol was esterified with diazomethane. An amorphous product (69%) which by pc (system S9) contained a trace of impurity was obtained from acetone-ethyl acetate. Column chromatography in system S9 gave a homogeneous product which could not be crystallized; mp 141-144°, $[\alpha]_D$ + 14 ± 2° (CH_3OH); ir (KBr) 3430 (OH), 1745 (ester C=O) and 1715 cm^{-1} (C-20 C=O). M.W. Calcd: 714. Found: MNa^+ = 737; M = 714. Anal. Calcd for $C_{35}H_{54}O_{15}.H_2O$: C, 57.36; H, 7.70. Found: C, 57.61; H, 7.78.

20-Oxo-5β-pregnan-3α,21-ylene Bis(β-D-glucopyranosiduronic Acid) (12).--One-half mmol of 10 was treated with sodium hydroxide in a two step procedure to hydrolyze the ester groups [14] and the hydrolysate was desalted on Amberlite XAD-2. A small amount of impurity was removed by chromatographing the product in system S7 (HBV = 1.6). It was not possible to crystallize acid 12. Chromatographic evidence for the presence of two carboxyl groups in acid 12 was obtained when the substance was chromatographed on paper in the presence of a liquid ion exchanger and different concentrations [15] of counterion.

20-Oxo-5β-pregnan-3α,21-ylene Bis[ammonium (β-D-Glucopyranosid)-uronate] (13).--Amorphous acid 12 (102 mg) was dissolved in 4 ml of 1 N NH_4OH, the solution was taken to dryness in vacuo, 5 ml of alcohol was added and removed in vacuo and the residue was crystallized

from water-acetone; yield 74 mg; mp 165° dec. $[\alpha]_D$ + 7 ± 2° (CH_3OH). Anal. Calcd for $C_{33}H_{56}O_{15}N_2.3H_2O$: C, 51.14; H, 8.06. Found: C, 51.28; H, 8.21.

Methyl (21-Acetoxy-11β-hydroxy-20-semicarbazono-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (15).--Treatment of 200 mg of 17 with semicarbazide and sodium bicarbonate gave crude 15 (mp 142-145°) in 96% yield. Recrystallized material melted at 148-149°; λ_{max}^{MeOH} 236 nm, ε = 12,800; ir (KBr) 3480, 3390 (OH, NH), 1750 (ester C=O), 1694 (amide C=O), 1568 (amide) and 1215 cm^{-1} (acetate C-O). Anal. Calcd for $C_{37}H_{55}O_{14}N_3$: C, 58.02; H 7.24; N, 5.49. Found: C, 57.57; H, 7.08; N, 5.48.

Methyl (21-Acetoxy-20-semicarbazono-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (16).--Compound 4 (1.50 g) was converted into the semicarbazone in the usual manner [14] and the product was recrystallized from chloroform-methanol-water to give 1.25 g of 16, mp 125-126°; λ_{max}^{MeOH} 238 nm, ε = 11,500; ir (KBr) 3440 (NH), 1760 (carbonyl C=O), 1693 (amide C=O), 1580 (amide) and 1215 cm^{-1} (acetate C-O). Anal. Calcd for $C_{37}H_{55}O_{13}N_3$: C, 59.26; H, 7.39. Found: C, 59.62; H, 7.32.

Methyl (21-Acetoxy-11β-hydroxy-20-oxo-5β-pregnan-3α-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate. (17). Barium salt 18 (1.17 mmol) was dissolved in 80 ml of water, the pH was adjusted to 2.0 with hydrochloric acid and the solution was poured into a column which contained 40 g of Amberlite XAD-2. The column was eluted stepwise with four bed volumes of water; the final bed volume contained no barium. (The barium in the aqueous effluent was precipitated as the sulfate and quantified gravimetrically; the barium salt 18 contained 17.1% Ba). The glucosiduronic acid (corresponding to barium salt 18) was eluted form the column with six bed volumes of ethanol, and the solution was taken to dryness. Treatment of the residue with diazomethane followed by acetylation of the ester in acetic anhydride-pyridine and crystallization from methanol gave 17 (787 mg, mp 162-163°); $[\alpha]_D$ + 50 ± 2° ($CHCl_3$); ir (KBr) 3565 (OH), 1754 (acetate C=O), 1736 (ester C=O), 1722 (C-20 C=O) and 1225 cm^{-1} (acetate C-O). Anal. Calcd for $C_{36}H_{52}O_{14}$: C, 61.01; H, 7.40; CH_3O, 4.38, CH_3CO 24.29. Found: C, 61.02; H, 7.08; CH_3O, 4.47; CH_3CO 23.20.

Barium Bis[(11β,21-dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosid)uronate] (18).--One mmol of compound 14 [6] was added to 50 ml of 0.04 N NaOH in methanol and the mixture was stirred at 25° until homogeneous (approx. 10 min). After an additional 20 min a solution of 1.08 g of KBH_4 in 50 ml H_2O was added and the solution stood at 25° for 18 hr. The solution was concentrated in vacuo to ∿25 ml to remove the methanol, diluted to 50 ml with water, adjusted to pH 2.1 with 5 N H_2SO_4 and allowed to stand one hr. The solution was diluted to 80 ml with H_2O, poured onto a column of 40 g of Amberlite XAD-2, and the resin was washed with 2 x 80 ml 0.01 N H_2SO_4, 4 x 80 ml H_2O and 3 x 80 ml ethanol. [Titration of aliquots of the effluent with iodine [16] showed that all of the semicarbazide emerged in the

first 240 ml of outflow from the column]. The alcoholic effluent was taken to dryness in vacuo. It was not possible to crystallize this product as the carboxylic acid. Crystals (318 mg) of the corresponding barium salt (18) were obtained by dissolving the residue in 10 ml of 75% ethanol and adding 1 mmol of barium acetate in 10 ml of 75% ethanol. Additional crystalline material (164 mg) was obtained from the mother liquor; mp, > 240° dec; ir (KBr) 3380 (OH), 1714 (C-20 C=O), 1588 and 1408 cm^{-1} (ionized carboxyl). Anal. Calcd for $C_{27}H_{41}O_{10}.Ba.C_2H_3O_2.H_2O$: C, 47.05; H, 6.26; CH_3CO, 5.86; Ba, 18.50. Found: C, 47.25; H, 6.28; CH_3CO, 4.38; Ba, 15.62.

Methyl (11β,21-Dihydroxy-20-oxo-5β-pregnan-3α-yl β-D-Glucopyranosid)uronate (19).--Treatment of 340 mg of amorphous acid (identical to that from which barium salt 18 was prepared) with diazomethane followed by chromatography of the ester in system S10 (HBV = 3.6) and crystallization of the main band from ethyl acetate-cyclohexane gave 229 mg of 19; mp 134-137°; $[\alpha]_D$ + 40 ± 2° (CH_3OH); ir (KBr) 3420 (OH), 1742 (ester C=O) 1708 cm^{-1} (C-20 C=O). M.W. Calcd: 540. Found: MNa^+ = 563; M = 540. Anal. Calcd for $C_{28}H_{44}O_{10}$: C, 62.20; H, 8.21; CH_3O, 5.74. Found: C, 61.97; H, 7.94; CH_3O, 5.43.

Methyl (3α-Acetoxy-11β-hydroxy-20-semicarbazono-5β-pregnan-21-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (21).--Compound 24 (0.28 mmol) was converted into semicarbazone 21 in the usual way [6]; yield 93%; mp 200-202°; λ_{max}^{MeOH} 236 nm, ε = 12,800; ir (KBr) 3560, 3425, 3395 (OH + NH), 1784, 1755 (ester C=O), 1698 (amide C=O) and 1220 cm^{-1} (acetate C-O). Anal. Calcd for $C_{37}H_{55}O_{14}N_3$: C, 58.02; H, 7.24; N, 5.49. Found: C, 57.69; H, 7.08; N 5.46.

3α,11β-Dihydroxy-20-oxo-5β-pregnan-21-yl β-D-Glucopyranosiduronic Acid (22).--A solution of 1.44 g (2.0 mmol) of 20 in 100 ml of 0.04 N sodium hydroxide in methanol [6] was allowed to stand 30 min to remove the acetate groups. A solution of 2.16 g of potassium borohydride in 100 ml of water was added and the mixture stood for 18 hr. The methanol was removed in vacuo, the pH was brought to 2.1 with sulfuric acid and after the mixture had stood for one hour the mixture was processed on a column of Amberlite XAD-2 to remove the semicarbazide. The product was recovered, chromatographed on a column in system S8 (HBV = 2.4) and crystallized from methanol; yield 620 mg; mp 233-236° dec.; $[\alpha]_D$ + 46 ± 2° (CH_3OH); ir (KBr) 3400 (OH), 1783 (monomer carboxyl C=O); 1755 (dimer carboxyl C=O), and 1708 cm^{-1} (C-20 C=O). M.W. Calcd: 526. Found: MH^+, no peak; $[M-H+2Na]^+$ = 571; M = 526. Anal. Calcd for $C_{27}H_{42}O_{10}$: C, 61.58; H, 8.04. Found: C, 61.13; H, 8.00.

Methyl (3α,11β-Dihydroxy-20-oxo-5β-pregnan-21-yl β-D-Glucopyranosid)uronate (23).--Esterification of 22 with diazomethane and crystallization of the product from ethyl acetate gave 23, mp 195-196°; $[\alpha]_D$ + 47 ± 2° (CH_3OH); ir (KBr) 3400 (OH), 1756 (ester C=O) and 1716 cm^{-1} (C-20 C=O). Anal. Calcd for $C_{28}H_{44}O_{10}$: C, 62.20; H, 8.20; CH_3O, 5.74. Found: C, 61.77; H, 8.21; CH_3O, 5.76.

Methyl (3α-Acetoxy-11β-hydroxy-20-oxo-5β-pregnan-21-yl 2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate (24).--One mmol of 20 was transesterified, reduced with borohydride, treated with acid to remove the semicarbazone group and processed through Amberlite XAD-2 as described under the preparation of 22 from 20. The residue from the alcoholic eluate was dissolved in 50 ml of methanol, and esterified with diazomethane. The ester was acetylated in acetic anhydride-pyridine and homogeneous (t.l.c. in system A) 24 was obtained in 50% yield by crystallization from ethanol; mp 239-240°; $[\alpha]_D$ + 49 ± 2° ($CHCl_3$); ir (KBr) 3550 (OH), 1746 (ester C=O) 1719 (C-20 C=O) and 1230 cm^{-1} (acetate C-O). Anal. Calcd for $C_{36}H_{52}O_{14}\cdot 1/2H_2O$: C, 60.24; H, 7.44; CH_3O. 4.31; CH_3CO, 23.98. Found: C, 60.21; H, 7.12; CH_3O, 4.44; CH_3CO, 23.33.

11β-Hydroxy-20-oxo-5β-pregnan-3α,21-ylene Bis[methyl (2,3,4-Tri-O-acetyl-β-D-glucopyranosid)uronate] (27).--To 3.11 g (3.0 mmol) of 26 [6] in 22.5 ml of chloroform was added 22.5 ml of 0.04 N NaOH in methanol. After 45 min (during which the acetyl groups of 20 were removed), 90 ml of methanol and 42 ml of 1.0 N aqueous sodium hydroxide were added; the mixture stood at 25° for 1.5 hr. The solution was concentrated in vacuo to approx 25 ml and wo 50 ml portions of water were removed in vacuo to displace all organic solvent. The solution was diluted to 180 ml with water and 2.27 g (60 mmol) of $NaBH_4$ was added. After 63 hr the pH was adjusted to 2.1 with sulfuric acid and the solution stood for one hr (to hydrolyze the semicarbazone). Salts were removed on a column of Amberlite XAD-2 and the conjugate was chromatographed on a column in system S7; (HBV = 5.0). The acid was esterified with diazomethane, the ester was acetylated with acetic anhydride-pyridine and 27 was crystallized (yield 36%) from chloroform-methanol; mp 219.5-222°; $[\alpha]_D$ + 16 ± 2° (CH_3OH); ir (KBr) 3570 (OH), 1753 (carbonyl C=O) 1718 (C-20 C=O) and 1213 cm^{-1} (acetate C-O). Anal. Calcd for $C_{47}H_{66}O_{22}$: C, 57.42; H, 6.76. Found: C, 57.20; H, 6.76.

Compound 27 was converted into the known [6] 11-oxo analog 25 by oxidation with chromium trioxide - pyridine using previously-described conditions [14]; 25 was identified by its ir spectrum.

11β-Hydroxy-20-oxo-5β-pregnan-3α,21-ylene Bis(β-D-glucopyranosiduronic Acid) (28).--Compound 27 (469 mg) was hydrolyzed by a previously described procedure [6], processed through Amberlite XAD-2 to remove the salts and the glucosiduronic acid was chromatographed in system S7 (HBV = 5.4). A trace of debris from the Celite column was removed by Amberlite XAD-2 and acid 28 was obtained as crystals (132 mg, 40%) from methanol; mp 177.5-179°; $[\alpha]_D$ + 27 ± 2° (MeOH); ir (KBr) 3430 (OH) and 1719 cm^{-1} (carboxyl C=O and C-20 C=O). Anal. Calcd for $C_{33}H_{50}O_{16}\cdot 2H_2O$: C, 53.64; H, 7.36. Found: C, 53.48; H, 7.37.

Hydrolysis of conjugates 18, 22 and 28 by treatment with β-glucuronidase under previously-described conditions [11] gave, in each case, 3α,11β,21-trihydroxy-5β-pregnan-20-one which was identified by chromatography in two solvent systems.

11β-Hydroxy-20-oxo-5β-pregnan-3α,21-ylene Bis[methyl (β-D-Gluocpyranosid)uronate] (29).--Compound 28 (slightly impure, solvent system B) was esterified with diazomethane and the ester was freed of a less polar impurity by chromatographing it on a column of silica gel in ethyl acetate:methanol (24:1). The ester (29) crystallized from methanol; yield 46%; mp 243° dec; $[\alpha]_D$ + 23 ± 2° (CH_3OH); ir (KBr) 3385 (OH), 1737 (ester C=O) and 1717 cm^{-1} (C-20 C=O). M.W. Calcd: 730. Found: MNa^+ = 753; M = 730. Anal. Calcd for $C_{35}H_{54}O_{16}$: C, 57.52; H, 7.44. Found: C, 57.36; H, 7.53.

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