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Preparation of Haptens for Use in Immunoassays of Tetrahydro-11-deoxycortisol and Its Glucuronides[1)]

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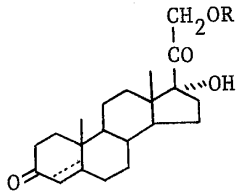
For the purpose of developing immunoassays of tetrahydro-11-deoxycortisol or its glucuronides, various haptenic derivatives were synthesized. The 3-hemisuccinate (**15**), 21-hemisuccinate (**8**), 3-hemiglutarate (**16**), 21-hemiglutarate (**9**) and glucuronides (**19**, **23**, **25**) of tetrahydro-11-deoxycortisol were prepared starting from 11-deoxycortisol 21-acetate (**1**). Then, antisera were elicited in two rabbits by immunization with the conjugate of tetrahydro-11-deoxycortisol 3-glucuronide (**19**) with bovine serum albumin. It was found that the antisera had binding affinities to enzyme-labeled antigens prepared from **15**, **16** and **19**; the binding was inhibited by the glucuronide (**19**) in the enzyme immunoassay procedure.

Keywords——tetrahydro-11-deoxycortisol; tetrahydro-11-deoxycortisol glucuronide; hapten for immunoassay; tetrahydro-11-deoxycortisol hemisuccinate; tetrahydro-11-deoxycortisol hemiglutarate; anti-tetrahydro-11-deoxycortisol 3-glucuronide antiserum; tetrahydro-11-deoxycortisol 3-glucuronide-β-galactosidase conjugate

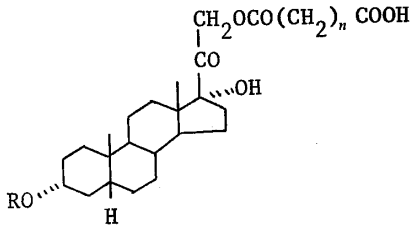
11-Deoxycortisol, an intermediate in cortisol biosynthesis in the human adrenal cortex, is metabolized by the liver to tetrahydro-11-deoxycortisol (THS) and excreted in the urine mainly as conjugates with glucuronic acid. The drug metyrapone inhibits the last step in cortisol biosynthesis, causing an increase in 11-deoxycortisol secretion in the presence of adrenocorticotropic hormone. Thus, immunoassays of 11-deoxycortisol in plasma or THS in urine are useful in the metyrapone test,[2)] assessment of pituitary-adrenal reserve. Various radioimmunoassay[3)] and enzyme immunoassay[4)] systems for the determination of 11-deoxycortisol have been developed. In the case of THS, a radioimmunoassay method for the estimation of THS glucuronide equivalent in human urine has been reported.[5)] Enzyme immunoassay is an attractive method, particularly if a direct assay procedure to measure the glucuronides can be developed. Such an assay should have high sensitivity and specificity, and hence, the use of appropriate haptenic derivatives for antibody production and enzyme labeling is necessary.[6)] This paper deals with the synthesis of the hemisuccinates, hemiglutarates, and glucuronides of THS. Production of anti-THS 3-glucuronide antisera was also carried out.

11-Deoxycortisol 21-acetate (**1**) was the starting material for our synthesis. Conversion of **1** into THS 21-acetate (**3**), a key intermediate, involves the stereochemistry of reduction of the Δ^4-3-keto group. Harnik has reported on the hydrogenation of **1** in ethyl acetate in the presence of palladium-on-charcoal;[7)] this gives 5β-dihydro-11-deoxycortisol 21-acetate (**2**) and the 5α-isomer in the ratio of *ca.* 3 : 2. Previously, we found that the saturation of the 4,5-double bond in cortisol 21-acetate by the method of Combe *et al.*[8)] led to the predominant formation of the corresponding 5β-compound.[9)] This was also the case in the present work. When **1** was hydrogenated with palladium-on-calcium carbonate in pyridine, the reduction proceeded in the desired fashion, yielding **2**. The formation of the 5α-isomer was less than 5%, as judged by thin-layer chromatography. Then, the Raney nickel hydrogenation of **2** was carried out

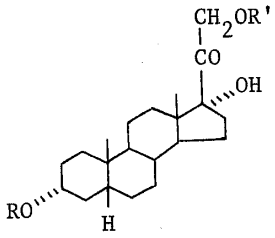
according to the method of Harnik.[7] Separation of THS 21-acetate (**3**) from the 3β-epimer was achieved efficiently by column chromatography on silica gel. This compound was used for the preparation of the 21-hemisuccinate (**8**), 21-hemiglutarate (**9**), and 3-glucuronide (**19**).



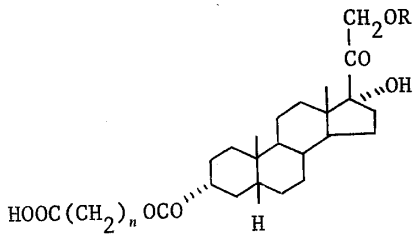
1: Δ^4, R=Ac
2: 5β-H, R=Ac
10: 5β-H, R=H
11: 5β-H, R=*tert*-BDMS



6: R=*tert*-BDMS, n=2
7: R=*tert*-BDMS, n=3
8: R=H, n=2
9: R=H, n=3



THS: R=R′=H
3: R=H, R′=Ac
4: R=*tert*-BDMS, R′=Ac
5: R=*tert*-BDMS, R′=H
12: R=H, R′=*tert*-BDMS



13: R=*tert*-BDMS, n=2
14: R=*tert*-BDMS, n=3
15: R=H, n=2
16: R=H, n=3
tert-BDMS=$-Si(CH_3)_2C(CH_3)_3$

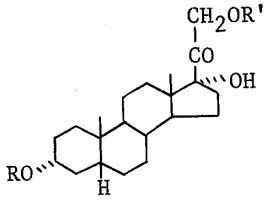
Chart 1

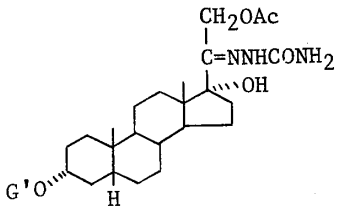
Treatment of **3** with *tert*-butyldimethylsilyl chloride and imidazole in dimethylformamide–pyridine gave the 3-silyl ether (**4**). Deacetylation of **4** with sodium methoxide in methanol afforded the THS 3-silyl ether (**5**) in good yield. On treatment with succinic anhydride in pyridine, **5** was converted into the hemisuccinate (**6**). Removal of the silyl group at C-3 in **6** with sulfuric acid in acetone furnished the desired THS 21-hemisuccinate (**8**). In a similar manner, THS 21-hemiglutarate (**9**) was prepared.

The preparation of the 3-hemisuccinate (**15**) and hemiglutarate (**16**) was then undertaken. For this purpose, 5β-dihydro-11-deoxycortisol (**10**), obtained from **2**, was derivatized into the 21-silyl ether (**11**). Selective reduction of the carbonyl group at C-3 in **11** was done by hydrogenation with Raney nickel as a catalyst, providing THS 21-*tert*-butyldimethylsilyl ether (**12**) and the 3β-epimer in the ratio of *ca.* 2 : 1. The stereochemistry at C-3 was determined on the basis of the proton nuclear magnetic resonance (^{1}H-NMR) spectral data. The C-3 proton signal of **12** appeared at 3.65 ppm as a multiplet with the half-band width of *ca.* 20 Hz, showing the axial nature of this proton, whereas the 3β-epimer exhibited the signal of $W_{1/2}$ = *ca.* 10 Hz at 4.08 ppm. On treatment with succinic anhydride or glutaric anhydride, followed by desilylation, the silyl ether (**12**) was transformed into the desired haptens **15** and **16**.

The 3- and 21-monoglucuronides (**19**, **23**) can also be used as haptens as well as standard samples in immunoassays. Therefore, the preparation of the glucuronides was carried out. First, introduction of the glucuronyl residue into **3** was achieved by using the Koenigs–Knorr reaction with methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate in toluene in the presence of silver carbonate, yielding the glucuronide acetate-methyl ester (**17**). Prior to

saponification of **17**, the alkali-sensitive side chain at C-17 was protected by derivatization into the 20-semicarbazone (**18**). Sequential removal of the protecting groups in **18** was carried out by treatment with methanolic sodium hydroxide, and then with pyruvic acid–acetic acid to give the desired THS 3-glucuronide (**19**) in a satisfactory yield.[10] Next, the preparation of THS 21-glucuronide (**23**) was carried out. Acetylation of **12** with acetic anhydride in pyridine gave the acetate-silyl ether (**20**), which, on removal of the silyl group with sulfuric acid in acetone yielded THS 3-acetate (**21**). The Koenigs–Knorr reaction of **21**, followed by simultaneous removal of the protecting groups in both the steroid and sugar moieties by treatment with methanolic potassium hydroxide, gave **23**. The 3,21-diglucuronide (**25**) was also synthesized through the sequence of reactions THS→**24**→**25**. In the ^{1}H-NMR spectra of **19** and **23**, the signal due to the anomeric proton was observed as a doublet of $J=7$ Hz at 4.32 and 4.37 ppm, respectively, showing β-configuration of the anomeric center.

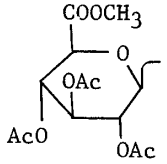




18

17: R=G′, R′=Ac
19: R=G, R′=H
20: R=Ac, R′=*tert*-BDMS
21: R=Ac, R′=H
22: R=Ac, R′=G′
23: R=H, R′=G
24: R=R′=G′
25: R=R′=G

G′=



G =

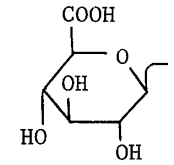


Chart 2

Production of antibodies to THS 3-glucuronide was then carried out. The *N*-succinimidyl ester prepared from **19** by condensation with *N*-hydroxysuccinimide in the presence of a water-soluble carbodiimide was covalently linked to bovine serum albumin (BSA). Spectrometric analysis showed that a satisfactory number of steroid molecules had been incorporated into the BSA molecule. Anti-THS 3-glucuronide antisera were elicited in two rabbits by immunization with this conjugate. On the other hand, the activated ester was reacted with β-galactosidase to give an enzyme-labeled antigen. It was found that the labeled antigen showed a satisfactory immunoreactivity to the antisera prepared. Useful dose–response curves could be constructed by incubating 0—1 ng of unlabeled THS 3-glucuronide and a fixed amount (10 ng) of the labeled antigen with appropriately diluted antisera; for example, the inhibition of enzymic activity of immune precipitate by 1 ng of the unlabeled antigen was 80% at 1 : 6000 dilution of one of the antisera. With assay systems using enzyme-labeled antigens prepared from compounds **15** and **16**, similar dose–response curves were obtained.

The antisera, haptenic derivatives, and glucuronides prepared in this work may be useful in the development of practical immunoassays for THS glucuronides in human urine.

Experimental

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were determined in $CHCl_3$ unless otherwise specified. ^{1}H-NMR spectra were measured with a JEOL JNM-FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard.

5β-Dihydro-11-deoxycortisol 21-Acetate (2)——A solution of 11-deoxycortisol 21-acetate (**1**) (5 g) in pyridine (25 ml) was stirred under a hydrogen gas stream for 8 h at atmospheric pressure in the presence of palladium-on-calcium carbonate (10 g). After addition of acetone (60 ml) followed by removal of the catalyst by filtration, the filtrate was concentrated to one-third of its initial volume under reduced pressure. Upon addition of H_2O to the residue, a precipitate was formed; this was collected by filtration and dried. Recrystallization of the crude product from EtOH gave **2** (3.5 g) as colorless leaflets. mp 193—195 °C (lit. mp 192—194 °C).[7]

THS 21-Acetate (3)——A solution of **2** (3 g) in dioxane (15 ml) was hydrogenated for 8 h in the presence of Raney Ni (W-2) (*ca.* 6 g). The filtrate was evaporated down under reduced pressure. The residue obtained was purified by column chromatography on silica gel. Elution with benzene–ether (1 : 1) and recrystallization of the product from AcOEt gave **3** (1.5 g) as colorless needles. mp 229—232 °C (lit. mp 221—224 °C).[7]

21-Acetoxy-3α,17α-dihydroxy-5β-pregnan-20-one 3-*tert*-Butyldimethylsilyl Ether (4)——A solution of **3** (200 mg), imidazole (400 mg), and *tert*-butyldimethylsilyl chloride (200 mg) in pyridine (0.5 ml)–dimethylformamide (1 ml) was stirred at room temperature for 1 h. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. Recrystallization of the crude product from EtOH gave **4** (230 mg) as colorless needles. mp 188—189 °C. $[\alpha]_D^{20}$ +55° (c=0.30). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (6H, s, 3-OSi$(CH_3)_2$), 0.67 (3H, s, 18-CH_3), 0.90 (12H, s, 19-CH_3 and 3-OSi-*tert*-Bu), 2.16 (3H, s, 21-$OCOCH_3$), 3.58 (1H, m, 3β-H), 4.80 and 5.05 (each 1H, d, J=18 Hz, 21-H). *Anal.* Calcd for $C_{29}H_{50}O_5Si$: C, 68.73; H, 9.95. Found: C, 68.43; H, 10.08.

THS 3-*tert*-Butyldimethylsilyl Ether (5)——A solution of **4** (220 mg) and NaOMe (*ca.* 200 mg) in dry MeOH (10 ml) was stirred at room temperature for 15 min under a nitrogen gas stream. After neutralization with AcOH, the resulting solution was diluted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue obtained was chromatographed on silica gel (10 g). Elution with hexane–AcOEt (1 : 2) and recrystallization of the product from ether–hexane gave **5** (160 mg) as colorless needles. mp 187—189 °C. $[\alpha]_D^{18}$ +24° (c=0.33). ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s, 3-OSi$(CH_3)_2$), 0.63 (3H, s, 18-CH_3), 0.90 (12H, s, 19-CH_3 and 3-OSi-*tert*-Bu), 3.58 (1H, m, 3β-H), 4.29 and 4.65 (each 1H, d, J=19 Hz, 21-H). *Anal.* Calcd for $C_{27}H_{48}O_4Si$: C, 69.78; H, 10.41. Found: C, 69.64; H, 10.15.

3α-*tert*-Butyldimethylsilyloxy-17α,21-dihydroxy-5β-pregnan-20-one 21-Hemisuccinate (6)——A solution of **5** (80 mg) and succinic anhydride (240 mg) in pyridine (1 ml) was allowed to stand at 40 °C for 10 h. After addition of H_2O, the resulting mixture was extracted with AcOEt. The organic layer was dried over anhydrous Na_2SO_4 and evaporated down. Recrystallization of the crude product from aqueous MeOH gave **6** (80 mg) as colorless needles. mp 160—162 °C. $[\alpha]_D^{18}$ +55° (c=0.38). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (6H, s, 3-OSi$(CH_3)_2$), 0.65 (3H, s, 18-CH_3), 0.90 (12H, s, 19-CH_3 and 3-OSi-*tert*-Bu), 2.78 (4H, s, –$COCH_2CH_2CO$–), 3.60 (1H, m, 3β-H), 4.87 and 5.17 (each 1H, d, J=18 Hz, 21-H). *Anal.* Calcd for $C_{31}H_{52}O_7Si$: C, 65.92; H, 9.28. Found: C, 65.52; H, 9.37.

3α-*tert*-Butyldimethylsilyloxy-17α,21-dihydroxy-5β-pregnan-20-one 21-Hemiglutarate (7)——A solution of **5** (480 mg) and glutaric anhydride (900 mg) in pyridine (5 ml) was allowed to stand at 40 °C for 10 h. After addition of H_2O, the mixture was extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue obtained was chromatographed on silica gel (10 g). Elution with hexane–AcOEt (1 : 1) and recrystallization of the product from ether–hexane gave **7** (450 mg) as colorless leaflets. mp 160—162 °C. $[\alpha]_D^{17}$ +48° (c=0.38). ^{1}H-NMR ($CDCl_3$) δ: 0.07 (6H, s, 3-OSi$(CH_3)_2$), 0.65 (3H, s, 18-CH_3), 0.90 (12H, s, 19-CH_3 and 3-OSi-*tert*-Bu), 2.4—2.7 (4H, –$COC\underline{H}_2CH_2C\underline{H}_2CO$–), 3.60 (1H, m, 3β-H), 4.83 and 5.11 (each 1H, d, J=18 Hz, 21-H). *Anal.* Calcd for $C_{32}H_{54}O_7Si$: C, 66.40; H, 9.40. Found: C, 66.04; H, 9.63.

THS 21-Hemisuccinate (8)——A solution of **6** (70 mg) and 30% H_2SO_4 (0.1 ml) in acetone (5 ml) was stirred at room temperature for 30 min. The resulting solution was diluted with AcOEt, washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The crude product obtained was purified by preparative silica gel thin-layer chromatography using $CHCl_3$–MeOH (10 : 1) as developing solvent and recrystallized from aqueous MeOH to give **8** (35 mg) as colorless needles. mp 175—177 °C. $[\alpha]_D^{20}$ +53° (c=0.28, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 2.76 (4H, s, –$COCH_2CH_2CO$–), 3.65 (1H, m, 3β-H), 4.87 and 5.21 (each 1H, d, J=18 Hz, 21-H). *Anal.* Calcd for $C_{25}H_{38}O_7 \cdot 5/4H_2O$: C, 63.47; H, 8.63. Found: C, 63.37; H, 8.53.

THS 21-Hemiglutarate (9)——Desilylation of **7** (400 mg) with 30% H_2SO_4 was carried out in the manner described for **8**. After usual work-up, the crude product obtained was recrystallized from aqueous MeOH to give **9** (150 mg) as colorless needless. mp 138—139 °C. $[\alpha]_D^{25}$ +69° (c=0.43, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 2.4—2.7 (4H, –$COC\underline{H}_2CH_2C\underline{H}_2CO$–), 3.65 (1H, m, 3β-H), 4.84 and 5.14 (each 1H, d, J=18 Hz, 21-H). *Anal.* Calcd for $C_{26}H_{40}O_7 \cdot 1/2H_2O$: C, 65.93; H, 8.73. Found: C, 65.87; H, 8.61.

5β-Dihydro-11-deoxycortisol 21-*tert*-Butyldimethylsilyl Ether (11)——Silylation of 5β-dihydro-11-deoxycortisol (**10**) (1.5 g), prepared from **2**, with *tert*-butyldimethylsilyl chloride was carried out in the manner described for **4**. After usual work-up, the crude product obtained was recrystallized from ether–hexane to give **11** (1.8 g) as colorless

needles. mp 160—161 °C. $[\alpha]_D^{20}$ +31° (c=0.80). ^{1}H-NMR ($CDCl_3$) δ: 0.12 (6H, s, 21-OSi(CH_3)$_2$), 0.72 (3H, s, 18-CH_3), 0.93 (9H, s, 21-OSi-*tert*-Bu), 1.05 (3H, s, 19-CH_3), 4.48 and 4.60 (each 1H, d, J=18 Hz, 21-H). *Anal.* Calcd for $C_{27}H_{46}O_4Si$: C, 70.08; H, 10.02. Found: C, 69.98; H, 9.76.

THS 21-*tert*-Butyldimethylsilyl Ether (12)——Hydrogenation of **11** (2.2 g) with Raney Ni was carried out in the manner described for **3**. After usual work-up, the residue obtained was chromatographed on silica gel. Elution with benzene–ether (1 : 2) and recrystallization of the product from benzene gave 3β,17α,21-trihydroxy-5β-pregnan-20-one 21-*tert*-butyldimethylsilyl ether (500 mg) as colorless leaflets. mp 180—182 °C. $[\alpha]_D^{20}$ +20° (c=0.30). ^{1}H-NMR ($CDCl_3$) δ: 0.12 (6H, s, 21-OSi(CH_3)$_2$), 0.66 (3H, s, 18-CH_3), 0.93 (9H, s, 21-OSi-*tert*-Bu), 0.96 (3H, s, 19-CH_3), 4.08 (1H, m, 3α-H), 4.40 and 4.52 (each 1H, d, J=18 Hz, 21-H). *Anal.* Calcd for $C_{27}H_{48}O_4Si$: C, 69.78; H, 10.41. Found: C, 69.71; H, 10.66. Further elution and recrystallization of the product from ether–benzene gave **12** (1.1 g) as colorless needles. mp 187—188 °C. $[\alpha]_D^{20}$ +19° (c=0.20). ^{1}H-NMR ($CDCl_3$) δ: 0.12 (6H, s, 21-OSi(CH_3)$_2$), 0.68 (3H, s, 18-CH_3), 0.95 (12H, s, 19-CH_3 and 21-OSi-*tert*-Bu), 3.65 (1H, m, 3β-H), 4.48 and 4.60 (each 1H, d, J=18 Hz, 21-H). *Anal.* Calcd for $C_{27}H_{48}O_4Si$: C, 69.78; H, 10.41. Found: C, 69.86; H, 10.81.

21-*tert*-Butyldimethylsilyloxy-3α,17α-dihydroxy-5β-pregnan-20-one 3-Hemisuccinate (13)——Hemisuccinoylation of **12** (200 mg) with succinic anhydride was carried out at 80 °C in the manner described for **6**. After usual work-up, the crude product was recrystallized from aqueous MeOH to give **13** (120 mg) as colorless leaflets. mp 148—149 °C. $[\alpha]_D^{18}$ +37° (c=0.37). ^{1}H-NMR ($CDCl_3$–CD_3OD (4 : 1)) δ: 0.12 (6H, s, 21-OSi(CH_3)$_2$), 0.64 (3H, s, 18-CH_3), 0.93 (12H, s, 19-CH_3 and 21-OSi-*tert*-Bu), 2.63 (4H, s, –$COCH_2CH_2CO$–), 4.43 and 4.72 (each 1H, d, J=18 Hz, 21-H), 4.7 (1H, m, 3β-H). *Anal.* Calcd for $C_{31}H_{52}O_7Si \cdot 1/2H_2O$: C, 64.89; H, 9.31. Found: C, 64.94; H, 9.09.

21-*tert*-Butyldimethylsilyloxy-3α,17α-dihydroxy-5β-pregnan-20-one 3-Hemiglutarate (14)——Hemiglutaroylation of **12** (350 mg) was carried out at 80 °C in the manner described for **7**. After usual work-up, the residue obtained was chromatographed on silica gel (5 g). Elution with hexane–AcOEt (1 : 2) and recrystallization of the product from ether–hexane gave **14** (200 mg) as colorless leaflets. mp 88—89.5 °C. $[\alpha]_D^{20}$ +39° (c=0.23). ^{1}H-NMR ($CDCl_3$) δ: 0.12 (6H, s, 21-OSi(CH_3)$_2$), 0.63 (3H, s, 18-CH_3), 0.92 (12H, s, 19-CH_3 and 21-OSi-*tert*-Bu), 2.2—2.5 (4H, –$COCH_2CH_2CH_2CO$–), 4.42 and 4.72 (each 1H, d, J=18 Hz, 21-H), 4.7 (1H, m, 3β-H). *Anal.* Calcd for $C_{32}H_{54}O_7Si$: C, 66.40; H, 9.40. Found: C, 66.78; H, 9.80.

THS 3-Hemisuccinate (15)——Desilylation of **13** (120 mg) with 30% H_2SO_4 was carried out in the manner described for **8**. After usual work-up, the crude product obtained was purified by preparative thin-layer chromatography using $CHCl_3$–MeOH (10 : 1) as the developing solvent and recrystallized from aqueous MeOH to give **15** (63 mg) as colorless needles. mp 168—171 °C. $[\alpha]_D^{22}$ +48° (c=0.13). ^{1}H-NMR ($CDCl_3$–CD_3OD (4 : 1)) δ: 0.63 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 2.63 (4H, s, –$COCH_2CH_2CO$–), 4.30 and 4.68 (each 1H, d, J=19 Hz, 21-H), 4.7 (1H, m, 3β-H). *Anal.* Calcd for $C_{25}H_{38}O_7 \cdot H_2O$: C, 64.08; H, 8.60. Found: C, 64.33; H, 8.71.

THS 3-Hemiglutarate (16)——Desilylation of **14** (400 mg) with 30% H_2SO_4 was carried out in the manner described for **8**. After usual work-up, purification was carried out in the manner described for **15**. Recrystallization of the product from acetone–hexane gave **16** (200 mg) as colorless leaflets. mp 111—113 °C. $[\alpha]_D^{22}$ +48° (c=0.40). ^{1}H-NMR (CD_3OD) δ: 0.62 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 2.2—2.5 (4H, –$COCH_2CH_2CH_2CO$–), 4.24 and 4.64 (each 1H, d, J=19 Hz, 21-H), 4.7 (1H, m, 3β-H). *Anal.* Calcd for $C_{26}H_{40}O_7$: C, 67.21; H, 8.68. Found: C, 67.09; H, 8.56.

Methyl (21-Acetoxy-17α-hydroxy-20-oxo-5β-pregnan-3α-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (17)——Freshly prepared Ag_2CO_3 (2 g) and methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (1.5 g) were added to a solution of **3** (500 mg) in toluene (50 mg), and the suspension was stirred at room temperature for 12 h. After addition of AcOEt, the resulting solution was passed through Florisil (10 g) on a sintered-glass funnel, and the filtrate was evaporated down. The residue was chromatographed on silica gel (70 g). Elution with benzene–ether (1 : 2) and recrystallization of the product from acetone–hexane gave **17** (330 mg) as colorless needles. mp 169—170 °C (lit. mp 171.5—174.5 °C).[10] $[\alpha]_D^{20}$ +23° (c=0.20). ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 2.04, 2.08 and 2.19 (12H, –$OCOCH_3$), 3.66 (1H, m, 3β-H), 3.78 (3H, s, –$COOCH_3$), 4.06 (1H, m, 5′-H), 4.68 (1H, d, J=7 Hz, 1′-H), 4.87 and 5.11 (each 1H, d, J=18 Hz, 21-H), 4.9—5.4 (3H, 2′-, 3′-, 4′-H). *Anal.* Calcd for $C_{36}H_{52}O_{14} \cdot 1/2H_2O$: C, 60.24; H, 7.44. Found: C, 60.31; H, 7.27.

THS 3-Glucuronide (19)——A mixture of **17** (500 mg), semicarbazide·HCl (342 mg), and $NaHCO_3$ (146 mg) in 90% MeOH (10 ml)–$CHCl_3$ (2 ml) was stirred at room temperature for 2 d. Upon addition of H_2O a precipitate was formed, and this was collected by filtration and dried. The crude product was chromatographed on silica gel with $CHCl_3$–MeOH (20 : 1) as an eluent to give the 20-semicarbazone (**18**). To a solution of **18** (500 mg) in MeOH (5 ml)–$CHCl_3$ (10 ml), 2% methanolic NaOH (15 ml) was added, and the reaction mixture was allowed to stand at room temperature for 2 h. After addition of H_2O followed by neutralization with AcOH, the resulting solution was evaporated down under reduced pressure. The oily residue was dissolved in 80% pyruvic acid (3.8 ml)–AcOH (2.5 ml)–$CHCl_3$ (10 ml), and the solution was stirred at room temperature for 12 h. After removal of the organic solvent followed by addition of H_2O, the resulting solution was subjected to column chromatography on Amberlite XAD-2. Elution with MeOH gave the crude product, which was chromatographed on silica gel with $CHCl_3$–MeOH–H_2O–AcOH (100 : 20 : 2 : 0.1) as an eluent, and then on Amberlite XAD-2, yielding **19** (210 mg) as colorless semi-

crystals. ^{1}H-NMR ($CDCl_3$–CD_3OD (4 : 1)) δ: 0.62 (3H, s, 18-CH_3), 0.94 (3H, s, 19-CH_3), 4.32 and 4.64 (each 1H, d, J=19 Hz, 21-H), 4.32 (1H, d, J=7 Hz, 1′-H). The barium salt was obtained according to the method of Mattox *et al.*[11] mp >230 °C. $[\alpha]_D^{22}$ +17° (c=0.35, AcOH–MeOH (1 : 19)). *Anal.* Calcd for $C_{27}H_{41}O_{10}Ba_{1/2}\cdot 2H_2O$: C, 51.45; H, 7.20. Found: C, 51.21; H, 7.42.

3α-Acetoxy-17α,21-dihydroxy-5β-pregnan-20-one 21-*tert*-Butyldimethylsilyl Ether (20)——A solution of **12** (1.1 g) and acetic anhydride (7.5 ml) in pyridine (15 ml) was allowed to stand at room temperature for 12 h. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. Recrystallization of the crude product from ether–hexane gave **20** (1.0 g) as colorless needles. mp 184—186 °C. $[\alpha]_D^{19}$ +56° (c=0.50). ^{1}H-NMR ($CDCl_3$) δ: 0.12 (6H, s, 21-OSi(CH_3)$_2$), 0.68 (3H, s, 18-CH_3), 0.93 (12H, s, 19-CH_3 and 21-OSi-*tert*-Bu), 2.04 (3H, s, 21-OCOCH_3), 4.40 and 4.55 (each 1H, d, J=18 Hz, 21-H), 4.69 (1H, m, 3β-H). *Anal.* Calcd for $C_{29}H_{50}O_5Si$: C, 68.73; H, 9.95. Found: C, 68.51; H, 10.16.

THS 3-Acetate (21)——Desilylation of **20** (900 mg) with 30% H_2SO_4 was carried out in the manner described for **8**. After usual work-up, the crude product obtained was recrystallized from acetone to give **21** (600 mg) as colorless needles. mp 194—195 °C (lit. mp 196—197.5 °C).[12] ^{1}H-NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 2.04 (3H, s, 3-OCOCH_3), 4.28 and 4.64 (each 1H, d, J=19 Hz, 21-H), 4.69 (1H, m, 3β-H).

Methyl (3α-Acetoxy-17α-hydroxy-20-oxo-5β-pregnan-21-yl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranosid)uronate (22)——The Koenigs–Knorr reaction of **21** (580 mg) was carried out in the manner described for **17**. After usual work-up, the residue was chromatographed on silica gel with benzene–ether (2 : 1) as an eluent. Recrystallization of the product from ether–hexane gave **22** (200 mg) as colorless leaflets. mp 108—110 °C. $[\alpha]_D^{25}$ +32° (c=0.26, MeOH). ^{1}H-NMR ($CDCl_3$) δ: 0.63 (3H, s, 18-CH_3), 0.94 (3H, s, 19-CH_3), 2.02 and 2.11 (12H, –OCOCH_3), 3.75 (3H, s, –COOCH_3), 4.02 (1H, m, 5′-H), 4.66 (1H, d, J=7 Hz, 1′-H), 4.43 and 4.80 (each 1H, d, J=18 Hz, 21-H), 4.65 (1H, m, 3β-H), 4.95—5.40 (3H, 2′-,3′-,4′-H). *Anal.* Calcd for $C_{36}H_{52}O_{14}$: C, 61.00; H, 7.40. Found: C, 61.01; H, 7.11.

THS 21-Glucuronide (23)——A solution of **22** (500 mg) in 2% methanolic KOH (20 ml) was stirred at room temperature for 2 h. After addition of H_2O followed by neutralization with AcOH, the resulting solution was evaporated down under reduced pressure. The crude product obtained was subjected to column chromatography on Amberlite XAD-2. The eluate was purified in the manner described for **19**. Recrystallization of the product from MeOH–AcOEt gave **23** (200 mg) as colorless leaflets. mp 175 °C (dec.). $[\alpha]_D^{18}$ +9.3° (c=0.33, MeOH). ^{1}H-NMR (CD_3OD) δ: 0.61 (3H, s, 18-CH_3), 0.94 (3H, s, 19-CH_3), 4.37 (1H, d, J=7 Hz, 1′-H), 4.48 and 4.96 (each 1H, d, J=19 Hz, 21-H). *Anal.* Calcd for $C_{27}H_{42}O_{10}\cdot 5/4H_2O$: C, 59.05; H, 8.12. Found: C, 58.95; H, 7.98.

THS 3,21-Diglucuronide (25)——The Koenigs–Knorr reaction of THS (340 mg) was carried out in the manner described for **17**, yielding a mixture of **24** and a sugar derivative. Separation of these products was achieved after acetylation of the latter compound. Purification by chromatography on silica gel with benzene–ether (2 : 1) as an eluent and recrystallization of the product from MeOH gave **24** (760 mg) as colorless leaflets. mp 169—170 °C. $[\alpha]_D^{18}$ −3.1° (c=0.48). *Anal.* Calcd for $C_{47}H_{66}O_{22}$: C, 57.42; H, 6.76. Found: C, 57.11; H, 6.68. Saponification of **24** (740 mg) with methanolic KOH and purification by Amberlite XAD-2 chromatography were carried out in the manner described for **23**, yielding **25** (280 mg) as a colorless powder. mp 170 °C (dec.). $[\alpha]_D^{16}$ −2.0° (c=0.71, MeOH). *Anal.* Calcd for $C_{33}H_{50}O_{16}\cdot 3/2H_2O$: C, 54.31; H, 7.32. Found: C, 54.64; H, 7.47.

Preparation of Immunogen——1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (64 mg) and *N*-hydroxysuccinimide (18 mg) were added to a solution of **19** (57 mg) in 95% dioxane (0.5 ml), and the resulting solution was allowed to stand at room temperature for 1 h. The reaction mixture was extracted with AcOEt, washed with H_2O, and dried over anhydrous Na_2SO_4. The organic solution was passed quickly through an Al_2O_3 (2 g) layer on a sintered-glass funnel, and the filtrate was evaporated down to give the *N*-succinimidyl ester of THS 3-glucuronide (25 mg). BSA (90 mg) in 0.05 M phosphate buffer (pH 7.3) (1 ml)–pyridine (1 ml) was added and the whole was stirred overnight at 4 °C. The resulting solution was dialyzed against cold running H_2O overnight. After addition of acetone, the suspension was centrifuged at 2000 rev./min for 10 min. This procedure was repeated until free steroid was completely removed. The precipitate was lyophilized to give the steroid–BSA conjugate (*ca.* 90 mg) as a fluffy powder. The number of steroid molecules linked to a BSA molecule was 16, as determined by a spectrometric method (425 nm) using 83% H_2SO_4 as a color-reaction reagent.

Preparation of Antisera——Immunization of two rabbits with the immunogen was carried out in the manner described previously.[13] The antisera obtained were stored at 4 °C in 0.1% NaN_3. They were diluted with 0.05 M phosphate buffer containing 0.1% gelatin, 0.9% NaCl, and 0.1% NaN_3, when required for enzyme immunoassay.

Enzyme Immunoassay Procedures——Preparation of enzyme-labeled antigens and enzyme immunoassay were carried out in the manner described previously.[14] In short, the *N*-succinimidyl esters of **19** obtained above and of the haptenic derivatives **15** and **16** were reacted with β-galactosidase at a molar ratio (steroid to enzyme) of 6 to give labeled antigens. In the enzyme immunoassay, the enzymic activity of the immune precipitate formed by a double antibody method was determined spectrophotometrically with *o*-nitrophenyl β-D-galactopyranoside as a substrate.

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