

Synthesis of new immunogens for the development of radioimmunoassay of levonorgestrel and its 3-oximes

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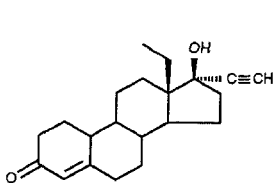
Abstract

The synthesis, from 13 β -ethyl-17 β -hydroxy-3-methoxygon-1,3,5(10),8-tetraene, of new immunogens for levonorgestrel and its 3-*syn* and *anti*-oximes by coupling the steroids to bovine serum albumin (BSA) via an 11 β -carboxymethoxymethyl group is reported. In addition, the preparation of homologous histamine conjugates, which serve as substrates for the synthesis of ¹²⁵I-labelled ligands required for radioimmunoassay (RIA), is reported. The 11 β -carboxymethoxymethyl group was constructed through an Rh₂(OAc)₄-catalyzed carbene insertion reaction into the hydroxyl bond of an 11 β -hydroxymethyl derivative.

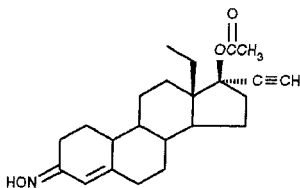
Key words: Radioimmunoassay, levonorgestrel, immunogen synthesis.

1. Introduction

Levonorgestrel is a potent synthetic progestin widely used in the oral contraceptive pill and in Norplant as a subdermal implant¹. Some structurally related new derivatives of levonorgestrel such as Norgestimate are also being used in oral contraceptive formulations².



Levonorgestrel



Norgestimate

For monitoring the levels of these hormones in body fluids, a sensitive and specific radioimmunoassay (RIA) is necessary. RIA methods developed earlier^{3,4} for levonorgestrel and some of its derivatives were not able to distinguish between the 3-oxo function and the 3-oxime and the 3-oxime derivatives exhibited high cross-reaction. Steroid hormones can be rendered immunogenic by covalent coupling to a macromolecule such as bovine serum albumin (BSA) to obtain immunogens capable of generating specific anti-steroid sera. We have shown earlier^{5,6} that the site of attachment on the steroid nucleus and the type of 'chemical handle' employed in coupling to the protein are very critical in terms of specificity. We presented evidence that an ether linkage from the β -side of the steroid molecule, remote from the existing functional groups, presents itself in a more favourable conformation to generate monospecific antisera.

We report in this publication the synthesis of new immunogens for levonorgestrel and its 3-*syn* and *anti*-oximes by coupling the steroids to BSA through the 11 β -position which is remote from the structurally important Δ^4 -3-oxo region (Scheme 1). We also described the preparation of homologous histamine conjugates which serve as substrates for the preparation of ¹²⁵I-labelled ligands required for RIA (Scheme 2).

2. Results and discussion

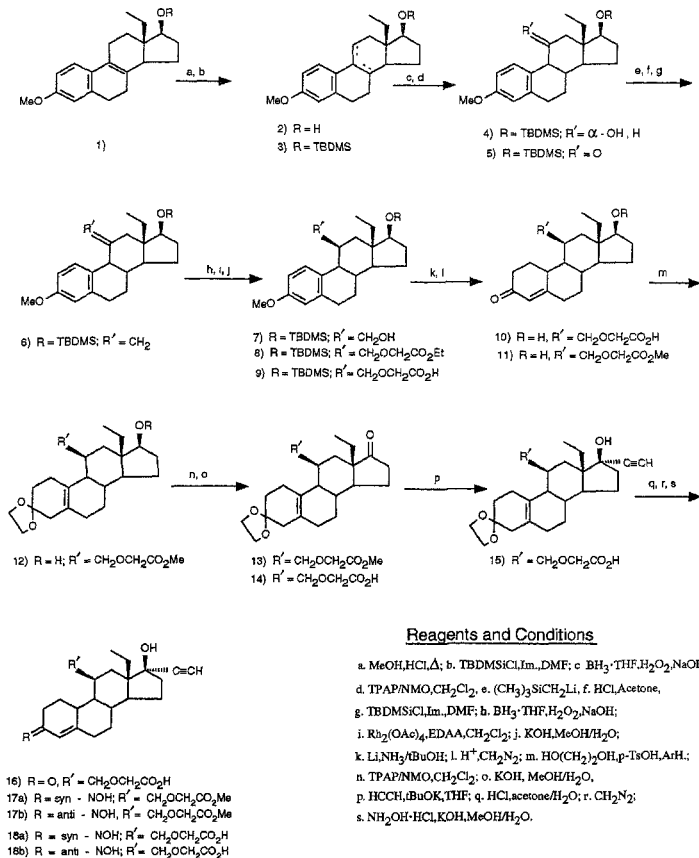
13 β -Ethyl-17 β -hydroxy-3-methoxygon-1,3,5(10),8-tetraene (**1**) served as the starting material and was obtained from Berlichem, Wayne, NJ. Treatment of (**1**) with concentrated HCl in refluxing methanol gave a mixture (**2**) consisting of $\Delta^8/\Delta^9(11)$ compounds as a 7:3 equilibrium mixture⁷. Fractional recrystallization resulted in partial removal of (**1**) from the mixture (**2**). NMR analysis of (**2**) showed it to be approximately a 1:1 $\Delta^8/\Delta^9(11)$ isomeric mixture.

The 17 β -hydroxyl function in mixture (**2**) was protected as the *t*-butyldimethyl silyl ether⁸ (TBDMS) to afford mixture (**3**). Hydroboration/oxidation gave 11 α -alcohol (**4**) and the unreacted Δ^8 material. Alcohol (**4**) was isolated from the mixture *via* preparative HPLC (80% yield based on $\Delta^8/\Delta^9(11)$ ratio). Recovered Δ^8 material was saved for future double-bond isomerization reactions.

11 α -Alcohol (**4**) was oxidized with tetrapropylammonium perruthenate (TPAP) in the presence of N-methyl morpholine N-oxide (NMO) as we have described earlier⁷ to give C-11 ketone (**5**) in 63% yield. Methenylation of ketone (**5**) to give (**6**) proceeded smoothly under Peterson reaction conditions with trimethylsilylmethyl lithium to give an intermediate hydroxy silane in high yield. Subsequent acid hydrolysis and reprotection of the 17 β -hydroxyl as the TBDMS afforded (**6**) in 96% yield.

Hydroboration/oxidation of (**6**) gave (**7**) in 80% yield. NMR data indicated that hydroboration was exclusively from the α -face of the 11-methylene to give a β -hydroxymethyl group at C-11. Elaboration of the side chain through the 11 β -hydroxymethyl group was effected by reacting (**7**) with ethyldiazoacetate in the presence of catalytic rhodium acetate^{5,6} to give (**8**). Hydrolysis of ethyl ester (**8**) to the acid (**9**), followed by lithium/ammonia reduction with subsequent acid hydrolysis gave (**10**).

Conversion of (**10**) to methyl ester (**11**) with diazomethane, followed by protection

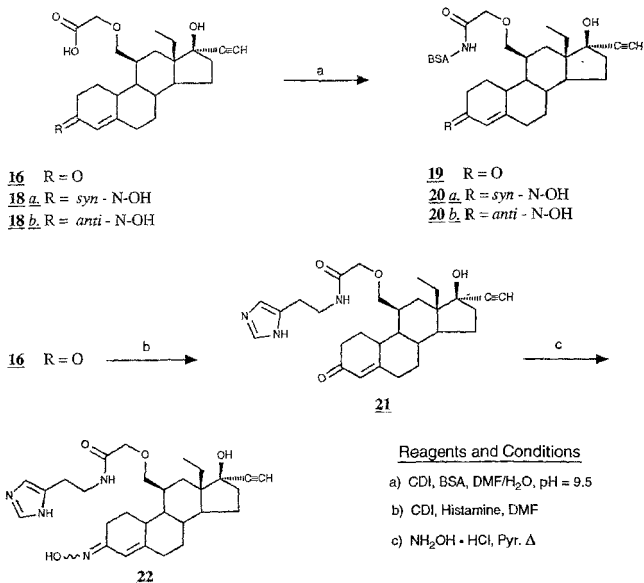


Reagents and Conditions

- a. MeOH, HCl, Δ ; b. TBDMSiCl, Im., DMF; c. $\text{BH}_3 \cdot \text{THF}, \text{H}_2\text{O}_2, \text{NaOH}$;
d. TPAP/NMO, CH_2Cl_2 ; e. $(\text{CH}_3)_3\text{SiCH}_2\text{Li}$, f. HCl, Acetone,
g. TBDMSiCl, Im., DMF; h. $\text{BH}_3 \cdot \text{THF}, \text{H}_2\text{O}_2, \text{NaOH}$;
i. $\text{Rh}_2(\text{OAc})_4$, EDAA, CH_2Cl_2 ; j. KOH, MeOH/ H_2O ;
k. $\text{Li}, \text{NH}_3/\text{tBuOH}$; l. $\text{H}^+, \text{CH}_2\text{N}_2$; m. $\text{HO}(\text{CH}_2)_2\text{OH}, p\text{-TsOH}, \text{ArH}$;
n. TPAP/NMO, CH_2Cl_2 ; o. KOH, MeOH/ H_2O ;
p. $\text{HCCH}, t\text{BuOK}, \text{THF}$; q. HCl, acetone/ H_2O ; r. CH_2N_2 ;
s. $\text{NH}_2\text{OH} \cdot \text{HCl}, \text{KOH}, \text{MeOH}/\text{H}_2\text{O}$.

SCHEME 1. Synthesis of steroidal haptens.

of the 3-ketone as the 3-ethylene ketal gave (12). The 17-hydroxyl group in (12) was oxidized with TPAP-NMO⁹ to afford 17-ketone (13). Hydrolysis of keto-ester (13) to keto-acid (14) and subsequent ethynylation using potassium acetylide, generated



SCHEME 2. Synthesis of steroid protein conjugates and the histamine derivatives.

in situ from uncomplexed potassium *t*-butoxide and dissolved acetylene gas in THF¹⁰ gave (**15**) in 65% yield.

Deketalization of (**15**) with aqueous acetone containing 10% HCl gave the levonorgestrel hapten (**16**) with a carboxymethoxymethyl at C-11 position. Treatment of (**16**) with diazomethane followed by reaction with hydroxylamine hydrochloride in pyridine gave the methyl ester oxime as a mixture of *syn* and *anti* isomers (**17a** and **b**) in the ratio of 3:7. Chromatographic separation of this isomeric mixture and subsequent base hydrolysis gave the individual 3-oxime derivatives (**18a** and **18b**) of levonorgestrel hapten (**16**).

Using steroid haptens (**16**, **18a** and **18b**), we were able to prepare three steroid hapten bovine serum albumen (BSA) conjugates (**19**, **20a** and **20b**) to serve as the immunogens. The reaction involved activation of the carbonyl moiety of the side chain *via in situ* generation of an imidazolium intermediate followed by subsequent reaction with BSA at a pH of 9.5–10.0 (Scheme 2). The BSA conjugates were

characterized by quantitative UV spectroscopy¹¹ and quantitative ninhydrin¹². Over the years we observed that the UV determination usually gives a higher value compared to the ninhydrin procedure. We attributed this due to nonspecific binding of traces of the steroid hapten to the protein.

Similarly, reaction of (16) with N, N-carboxydimidazole in DMF, followed by the addition of histamine (free base) afforded histamine conjugate (21). Attempts to prepare individual *syn* and *anti*-oxime histamine conjugates were not successful. However, we were able to produce oxime histamine conjugate (22) by converting histamine conjugate (21) to the 3-oxime as a 2:3 *syn/anti* mixture, as determined by NMR. Unfortunately, the *syn* and *anti* isomers were inseparable by HPLC.

The synthetic sequence described in the preparation of the steroidal immunogens appears to be very straight forward. However, we would like to discuss some of the problems encountered before we were able to come up with a practical method for preparing the desired products. Oxidation of 11 α -alcohol (4) to 11-ketone (5) proved to be somewhat difficult. A number of standard oxidation procedures such as Collins¹³, Sarett¹⁴, PCC¹⁵, Oppenauer¹⁶, and Swern¹⁷ were attempted and in every case the yield of ketone (5) was less than 25%. In addition, there was appreciable decomposition of substrate (4) and ketone (5) that was obtained was shown to be an epimeric mixture at C-9. Ketone (5) is analogous to 11-keto estrogens, which are known to undergo facile epimerization at C-9, with the 9 β -epimer being 1.47 kcal/mole more stable than the 9 α -epimer¹⁸. As stated earlier, we were able to obtain ketone (5) in 63% yield using the TPAP/NMO oxidation procedure described earlier by our group⁹.

In our initial attempts we subjected compound (7) to lithium/ammonia reduction with subsequent transformation of the 1,4-dihydro system to the 3-ketal. However, it was soon discovered that this approach was not successful. Dissolving metal reduction of (7) with subsequent ketalization initially appeared to have given the correct product, but mass-spectral data indicated that ring-A had been over reduced (M^+ was two more than calculated). Furthermore, acid hydrolysis of the product from this reaction failed to give the expected Δ^4 -3-oxo compound.

In retrospect, the problem lies in the proximity of the 11 β -hydroxymethyl group to ring A. It is plausible that the 11-hydroxymethyl group is participating in an internal protonation/deprotonation which allows the initially formed 1,4-dihydro system to become conjugated and undergo further reduction. Previous work in these laboratories has shown that similar systems containing the 11-hydroxymethyl group can be successfully subjected to dissolving metal conditions to afford the 1,4-dihydro system by first protecting the free hydroxyl¹⁹.

Accordingly, we resolved this problem of over-reduction of ring A by first protecting the free hydroxyl at C-11 *via* elaboration of the side chain and then carrying out the metal-ammonia reduction. This procedure²⁰, indeed, gave the 1,4-dihydro intermediate, which was identified by NMR, MS, and the characteristic IR bands at 1696 and 1666 cm^{-1} . The rest of the synthetic procedure was carried out without any major problems.

3. Experimental

Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Proton-NMR spectra were recorded on a Varian EM-390 (90 MHz) spectrometer in deuteriochloroform, unless indicated otherwise, using tetramethylsilane (TMS) as an internal standard ($\delta=0.0$). Infrared spectra were recorded on a Perkin-Elmer 1600 FT-instrument equipped with a diffuse reflectance accessory using a potassium bromide (KBr) matrix. Optical rotations were measured on a Rudolph Research Autopol II automatic polarimeter using 1.0 dm cell. Mass spectral analyses (EI) were conducted by Dr Susan Weintraub of the University of Texas Health Science Center at San Antonio using a Finnigan-MAT model 4615. Combustion analyses were performed by Midwest Microlabs Ltd, Indianapolis, Indiana.

All reagents were ACS reagent grade or better. Tetrahydrofuran (THF) was distilled from lithium aluminum hydride immediately before use. All other solvents were purified or dried by standard laboratory methods.

13 β -Ethyl-17 β -hydroxy-3-methoxygona-1,3,5(10),8-tetraene (**1**) was purchased from Berlichem, Wayne, NJ.

Preparative HPLC separations were conducted using a Waters Prep LC/System 500. Analytical HPLC analyses were conducted using a Waters HPLC system equipped with a model 6000 \AA pump, model U6K injector, Model 481 variable wavelength detector, and model 730 data module.

3.1. 13 β -Ethyl-17 β -hydroxy-3-methoxygona-1,3,5(10),9(11)-tetraene(2)

A methanol (300 ml) solution of 13 β -ethyl-17 β -hydroxy-3-methoxygona-1,3,5(10),8-tetraene (**1**, 12.5 g, 41.9 mmol) under nitrogen was treated with concentrated HCl (61 ml). The solution was heated at reflux for 45 min, cooled to room temperature and most of the methanol was evaporated *in vacuo*. The mixture was diluted with brine and extracted with ether (3x). The ether layers were sequentially washed with water, saturated sodium bicarbonate solution, and brine. The combined ether layers were dried over sodium sulfate and evaporation of the solvent gave 12.6 g as a stable foam. Fractional recrystallization of the material from ether/hexane (1:1) gave a solid 4.48 g that was predominantly Δ^8 double bond starting material. Evaporation and recrystallization of the mother liquor afforded an additional amount of Δ^8 starting material (1.38 g). Analysis of the mother liquor by NMR showed it to be a 1:1 mixture of $\Delta^8/\Delta^9(11)$ compounds and accounted for 7.57 g.

NMR δ 1.06 (t, $J=6$ Hz, 13 β -CH₂CH₃), 3.79(s,-OCH₃), 3.96(m,17 α -H), 6.16(br. d, 11-H), 6.71(m, C-2 and C-4 H), 7.19(m, C-1 H of 8(9)), 7.57(d, $J=9$ Hz, C-1 H of 9(11))ppm.

3.2. 13 β -Ethyl-3-methoxy-17 β -*t*-butyldimethylsilyloxygona-1,3,5(10),9(11)-tetraene(3)

To a solution of alcohol mixture (**2**, 14.7 g, 49.26 mmol) in dry N,N-dimethylformamide (100 ml) was added imidazole (22.14 g, 325.1 mmol) and *t*-butyldimethylsilyl chloride (22.27 g, 147.8 mmol). The reaction mixture was stirred under anhydrous

conditions at room temperature overnight, then poured into ice water and extracted with hexanes. The hexane extract was washed with water (2x) and brine (1x), dried over sodium sulfate, and concentrated *in vacuo*. The *t*-butyldimethylsilyl ether (**3**) was isolated by preparative HPLC (1 PrePak, 0.25% EtOAc/Hex.) to give 18.75 g (92%) as a double-bond isomeric mixture.

NMR δ 0.93(s, *t*-butyl), 1.03(t, $J=6\text{Hz}$, $13\beta\text{-CH}_2\text{CH}_3$), 3.80(s, OCH_3), 3.80(m, $17\alpha\text{-H}$), 6.13(m, 11-H), 6.70(m, C-2 and C-4 H), 7.17(C-1 H of 8(9)), 7.56(d, $J=9\text{ Hz}$, C-1 H of 9(11))ppm.

3.3. *13 β -Ethyl-11 α -hydroxy-3-methoxy-17 β -*t*-butyldimethylsilyloxygona-1,3,5(10)-triene (4)*

A cold (0°C) THF (500 ml) solution of (**3**, 18.75 g, 45.4 mmole) was treated dropwise with borane THF complex (1.0 M in THF, 68.15 ml) under nitrogen. The reaction was kept at 0°C for 4.0 h, then allowed to warm to room temperature and stirred an additional 4.0 h. The reaction was chilled in an ice bath and carefully treated with 10% aqueous sodium hydroxide (56.0 ml) followed by treatment with 30% hydrogen peroxide (72.0 ml). The mixture was allowed to stir overnight. The reaction mixture was diluted with water (750 ml) and the aqueous mixture was extracted with ethyl acetate (3x). The ethyl acetate layers were sequentially washed with 10% aqueous sodium sulfite (1x), water (1x), and brine (1x). The combined ethyl acetate layers were dried over sodium sulfate and evaporation of the solvent gave 20.74 g of a stable foam. Purification by preparative HPLC (5% EtOAc/Hex.) gave 9.76 g (49.8%) of alcohol (**4**) and 6.9 g (36.8%) of the unreacted Δ^8 double bond material as a stable foam.

NMR δ 0.90(s, Si-(*t*-Bu)), 1.0(t, $J=3\text{ Hz}$, $13\beta\text{-CH}_2\text{CH}_3$), 3.73(s on top of t, 3-OCH_3 and $17\alpha\text{-H}$), 4.03(m, 6 lines, $11\beta\text{-H}$), 6.70(m, C-2 and C-4 methines), 7.83(d, $J=9\text{ Hz}$, C-2 methine)ppm. Analysis Calcd for $\text{C}_{26}\text{H}_{42}\text{O}_3\text{Si}$: C, 72.50; H, 9.83. Found: C, 72.52; H, 9.83.

FTIR(KBr, diffuse reflectance: ν_{max} 3350, 2995, 1609, and 1572 cm^{-1} .
MS(m/z): M^+ =430.

3.4. *13 β -Ethyl-11-oxo-3-methoxy-17 β -*t*-butyldimethylsilyloxygona-1,3,5(10)-triene(5)*

A mixture of alcohol (**4**, 3.11 g, 7.22 mmol), 4-methyl morpholine N-oxide (1.69 g, 14.44 mmol), and 4 Å molecular sieves (16.3 g) in dry methylene chloride (125 ml) was prepared and stirred under nitrogen for 20 min. Tetrapropylammonium perruthenate (TPAP) (0.254 g, 10 mole %) was added and the mixture was stirred under nitrogen for 1.5 h. The mixture was filtered and the filtrate was evaporated to near dryness. The residue was taken up in hexanes with a trace of methylene chloride and loaded on to a pre-equilibrated flash column (5% EtOAc/Hex.). The column was developed normally with 5% EtOAc/Hex. to afford 1.93 g (62.4%) of ketone (**5**) as a white solid. Recrystallization of a small sample from ether/pentane afforded an analytical sample, mp = 150–151°C.

NMR δ 0.90(s, $-\text{Si}-(t\text{-Bu})$), 1.03(t, $J=3\text{Hz}$, $13\beta\text{-CH}_2\text{CH}_3$), 3.40(br. d, $9\alpha\text{-H}$), 3.73(s, 3-OCH_3), 3.97(br. t, $17\alpha\text{-H}$), 6.59(d, $J=3\text{Hz}$, C-4 H), 6.73(d of d, $J=9\text{Hz}$, $J'=3\text{Hz}$,

C-2 H), 7.33(d, $J=9\text{Hz}$, C-1 H)ppm; FTIR (KBr, diffuse reflectance): ν_{max} 2980. 1712, 1609, 1505 cm^{-1} .

MS (m/z): $M^+ = 428$.

Analysis Calcd for $\text{C}_{26}\text{H}_{40}\text{O}_3\text{Si}$: C, 72.84; H, 9.41. Found: C, 72.90; H, 9.46.

3.5. 13 β -Ethyl-11-methylene-3-methoxy-17 β -*t*-butyldimethylsilyloxygona-1,3,5(10)-triene(6)

A cold (-78°C) THF (165.0 ml) solution of ketone (5, 4.57 g, 10.57 mmol) was treated dropwise with trimethylsilylmethyl lithium (0.74 *M* in pentane, 26 ml). The solution was stirred at -78°C for 2 h, allowed to warm to 0°C , and stirred an additional 2 h. The reaction mixture was poured into ice-cold ammonium chloride solution (14 g $\text{NH}_4\text{Cl}/300\text{ ml H}_2\text{O}$) and the aqueous mixture was extracted with methylene chloride (3x). The methylene chloride extracts were washed with water and brine. The combined methylene chloride extracts were dried over sodium sulfate and evaporation of the solvent gave 5.62 g of the hydroxy silane. TLC (15% EtOAc/Hex.), showed the crude material to be homogeneous.

NMR δ 0.90(s, Si-(*t*-Bu)), 3.63(t, 17 α -H), 3.73(s, 3-OCH₃), 6.70(m, C-2 and C-4 methines), 7.85(d, $J=9\text{Hz}$, C-1 methine)ppm.

The crude hydroxy silane (5.62 g) was dissolved in acetone (60 ml) under nitrogen and treated with concentrated HCl (6.1 ml). The reaction was stirred at room temperature until all the starting material was consumed as evidenced by TLC (ca. 1.5–2.0 h). The HCl was neutralized with solid sodium acetate and the mixture was diluted with water. The aqueous mixture was extracted with ether (3x). The ether extracts were sequentially washed with saturated sodium bicarbonate (1x), water (1x), and brine (1x). The combined ether extracts were dried over sodium sulfate and evaporation of the solvent gave 3.76 g of 11-methylene. NMR analysis and TLC mobility showed that the 17-silyl-protecting group had been lost. The crude material was treated with *t*-butyldimethylsilyl chloride according to the standard procedure. After extractive workup and chromatography, 4.28 g (96.6% yield from (5)) of (6) was obtained as an oil.

NMR δ 0.90(s, -Si-(*t*-Bu)), 1.0(t, 13 β -CH₂CH₃), 3.77(s, 3-OCH₃), 3.80(t, 17 α -H), 4.90(d, $J=9\text{Hz}$, C-11 exo CH₂), 6.70(m, C-2 and C-4 methine), 7.27(d, $J=9\text{Hz}$, C-1 methine)ppm.

The material was used in the subsequent reaction without further characterization.

3.6. 13 β -Ethyl-11 β -hydroxymethyl-17 β -*t*-butyldimethylsilyloxygona-1,3,5(10)-triene(7)

A cold (0°C) THF (125 ml) solution of (6, 3.79 g, 8.88 mmol) under nitrogen was treated dropwise with borane THF complex (1.0 *M* in THF, 31.1 ml). The reaction was stirred at 0°C for 2 h and for an additional 2 h at room temperature. The reaction was cooled in an ice bath and treated carefully with a 10% sodium hydroxide solution (21 ml), followed by the addition of 30% hydrogen peroxide (25 ml). The aqueous mixture was stirred overnight. The reaction mixture was diluted with water

(400 ml) and extracted with ethyl acetate (3x). The ethyl acetate layers were sequentially washed with 10% sodium sulfite (1x), water (1x), and brine (1x). The combined ethyl acetate layers were dried over sodium sulfate and evaporation of the solvent gave 3.91 g of a stable foam. Flash chromatography (10% EtOAc/Hex.) afforded 3.0 g (80%) of (7).

NMR δ 0.90(s, -Si-(*t*-Bu)), 1.10(t, 13β -CH₂CH₃), 3.53(d, $J=3\text{Hz}$, -CH₂OH), 3.67(t, 17α -H), 3.70(s, 3-OCH₃), 6.16(d, $J=3\text{Hz}$, C-4 methine), 6.70(d of d, $J=9\text{Hz}$, $J'=3\text{Hz}$, C-2 methine), 7.23(d, $J=9\text{Hz}$, C-1 methine)ppm.

MS(m/z): M^+ =444.

3.7. 13 β -Ethyl-11 β -ethoxycarbonylmethoxymethyl-3-methoxy-17 β -*t*-butyldimethylsilyloxygona-1,3,5(10)-triene (8)

To a methylene chloride (25 ml) solution of (7, 1.31 g, 2.96 mmol) was added rhodium acetate dimer (65 mg, 0.15 mmol) and the mixture was stirred under nitrogen until homogeneous. A methylene chloride solution of ethyldiazoacetate (EDAA), (25.87 ml, 0.2 M/CH₂Cl₂) was added dropwise over a 4 h period using a syringe pump. Evaporation of the solvent and flash chromatography (Si, 10% EtOAc/Hex.) of the residue afforded 1.13 g (75%) of (8, R=Et) as an oil along with the recovery of 250 mg of starting material (7).

NMR δ 0.90(s, -Si-(*t*-Bu)), 1.17(t, 13β -CH₂CH₃), 1.23(t, OCH₂CH₃), 3.25-3.50(m, 11β -CH₂O-), 3.73(t, 17α -H), 3.77(s, 3-OCH₃), 3.97(d, -OCH₂CO₂Et), 4.20(q, OCH₂CH₃), 6.63(d, $J=3\text{Hz}$, C-4 H), 6.77(d of d, $J=9\text{Hz}$, $J'=3\text{Hz}$, C-2 H), 7.30(d, $J=9\text{Hz}$, C-1 H)ppm.

MS(m/z): M^+ =530.

3.8. 13 β -Ethyl-11 β -carboxymethoxymethyl-3-methoxy-17 β -*t*-butyldimethylsilyloxygona-1,3,5(10)-triene (9)

A methanol (250 ml) solution of ester (8, 4.41 g, 8.32 mmol) was treated with aqueous potassium hydroxide (1.0 M, 16.64 ml) and the solution was stirred overnight. Most of the methanol was removed *in vacuo* at room temperature and the residue was diluted with ice/water. The pH of the aqueous solution was adjusted to 2-3 with HCl. The mixture was extracted with methylene chloride (3x). The methylene chloride extracts were washed with water and brine, combined, and dried over sodium sulfate. Evaporation of the solvent gave 4.06 g (96%) of the acid (9) as a stable foam.

NMR δ 0.90(s, -Si-(*t*-Bu)), 1.13(br. t, $J=3\text{Hz}$, 13β -CH₂CH₃), 3.43(br. d, $J=6\text{Hz}$, 11β -CH₂OCH₂CO₂H), 6.67(d, $J=2\text{Hz}$, C-4 H), 6.77(d of d, $J=9\text{Hz}$, $J'=2\text{Hz}$, C-2 H), 7.27(d, $J=9\text{Hz}$, C-1 H), 8.47(br. s, -CO₂H)ppm.

FTIR (KBr, diffuse reflectance): ν_{max} 3000 strong, broad -OH, 1732, 1609, 1574 cm⁻¹.

3.9. 13 β -Ethyl-17 β -hydroxy-11 β -methoxycarbonylmethoxymethylgon-4-en-3-one(11)

To redistilled liquid ammonia (\approx 100 ml) was added 9, (2.0 g, 3.98 mmole) as a solution in THF (50.0 ml) and *t*-butanol (30.0 ml, 318 mmol). While stirring

vigorously. lithium metal (552 mg, 80.0 mmol), cut into small pieces, was added over a 5-min period. The reaction was stirred at reflux (-32°C) for 3 h at which time the reaction had started to lose its blue color. The reaction was quenched with the addition of methanol (30.0 ml). The ammonia was allowed to evaporate under a stream of nitrogen. The residue was taken up in water and the pH of the very basic solution was adjusted to 2–3. The aqueous mixture was extracted with methylene chloride (3x). The methylene chloride extracts were washed with water and brine, combined, and dried over sodium sulfate. Evaporation of the solvent gave 2.08 g of (**10**) as a stable foam. The crude reaction product was treated with ethereal diazomethane to afford the methyl ester. TLC analysis at this time showed two major products visible under a UV lamp, indicating complete hydrolysis of the intermediate 1,4-dihydro system as well as partial hydrolysis of the silyl group at 17. The crude methyl ester was dissolved in methanol (125 ml) and concentrated HCl (20 drops) was added. After 2 h, only one product was evident by TLC. After workup and flash chromatography, (65% EtOAc/Hex.) 0.8 g of (**11**) was obtained as a stable foam.

NMR δ 1.11(t, $13\beta\text{-CH}_2\text{CH}_3$), 3.33–3.95(m, $11\beta\text{-CH}_2\text{O-}$ and $17\alpha\text{-H}$), 3.77(s, $-\text{CO}_2\text{CH}_3$), 4.10(d, $-\text{OCH}_2\text{CO}_2\text{Me}$), 5.86(br. s, C-4 H)ppm.

FTIR (KBr, diffuse reflectance): ν_{max} 3450(strong $-\text{OH}$), 2950, 1753, 1667, 1620 cm^{-1} .

MS (m/z): $M^- = 390$.

Analysis Calcd for $\text{C}_{23}\text{H}_{34}\text{O}_5$: C, 70.74; H, 8.78. Found: C, 70.71; H, 8.75.

3.10. *13 β -Ethyl-11 β -methoxycarbonylmethoxymethyl-3,3-ethylenedioxy-17 β -hydroxygon-5(10)-ene*(**12**)

A mixture of en-one (**11**, 101.0 mg, 0.26 mmole), ethylene glycol (0.3 ml, 5.2 mmole), and *p*-toluene sulfonic acid (5.0 mg, 0.026 mmol) in dry benzene was stirred at reflux in a flask equipped with a Dean–Stark trap for azeotropic water removal. The mixture was refluxed for 3 h. The mixture was chilled in an ice bath and diluted with a saturated sodium bicarbonate solution. The aqueous mixture was extracted with methylene chloride (3x). The methylene chloride extracts were washed with water and brine, combined, and dried over sodium sulfate. Evaporation of the solvent gave 113.6 mg of a stable foam. The crude material was dissolved in methanol (5 ml) and treated with aqueous potassium hydroxide (1.0 N, 0.5 ml). The reaction mixture was stirred until no starting material was evident by TLC. The reaction mixture was diluted with cold water and the pH was adjusted to 2–3 with aqueous HCl. The aqueous mixture was extracted with methylene chloride and the methylene chloride extracts were washed with water and brine, combined and dried over sodium sulfate. Evaporation of the solvent gave a stable foam which was converted to the methyl ester with ethereal diazomethane and accounted for 104.1 mg (93%) of ketal (**12**).

NMR δ 1.11(br. t, $13\beta\text{-CH}_2\text{CH}_3$), 3.30–3.50(m, $11\beta\text{-CH}_2\text{O-}$), 3.60–3.85(observed t, $17\alpha\text{-H}$), 3.77(s, $-\text{OCH}_2\text{CH}_2\text{O-}$), 4.00(s, $-\text{CO}_2\text{CH}_3$), 4.10(d, $J=6\text{Hz}$, $-\text{OCH}_2\text{CO}_2\text{CH}_3$)ppm.

FTIR (KBr, diffuse reflectance): ν_{max} 3484 (strong OH), 1756, 1479, 1134 cm^{-1} .

MS (m/z): $M^+ = 434$ (base).

Analysis Calcd for $C_{25}H_{38}O_6$: C, 69.09; H, 8.81. Found: C, 69.19; H, 8.87.

3.11. 13 β -Ethyl-11 β -carbonylmethoxymethyl-3,3-ethylenedioxygon-5(10)-en-17-one (14)

A mixture of alcohol (**12**, 342 mg, 0.79 mmol), 4-methyl morpholine N-oxide (184.4 mg, 157 mmol) and 4 \AA molecular sieves (2.0 g) was prepared and stirred under nitrogen for 20 min. Tetrapropylammonium perruthenate (TPAP) (27.4 mg, 0.08 mmol) was added and the mixture was stirred for 30 min. TLC analysis at this time showed no starting alcohol remaining. The mixture was filtered and the filtrate was evaporated to near dryness. The residue was taken up in ether containing a trace of methylene chloride and passed through a short column of neutral alumina. The column was eluted with 5 passes of 100% ether and evaporation of the solvent gave 333.5 mg (98%) of the ketone (**13**) as a white solid. This material was homogeneous by TLC and recrystallization of a small sample (from ether/Hex.) gave a crystalline material, mp=111–113°C.

NMR δ 0.90(t, $J=6$ Hz, 13 β -CH₂CH₃), 3.33–3.57(m, 11 β -CH₂O–), 3.77(s, –OCH₂-CH₂O–), 3.97(–CO₂CH₃), 4.08(s, –OCH₂CO₂CH₃)ppm.

FTIR (KBr, diffuse reflectance): ν_{max} no-OH, 1752, 1725, 1427, 1372, 1128 cm⁻¹.

MS (m/z): $M^+ = 432$ (base).

Analysis Calcd for $C_{25}H_{36}O_6$: C, 69.42; H, 8.39. Found: C, 69.32; H, 8.39.

$[\alpha]_D^{27} = 134.58^\circ$ ($c = 1.07$, CHCl₃).

The keto ester from the above (333.5 mg) was hydrolyzed with methanolic potassium hydroxide. Most of the methanol was evaporated and the mixture was diluted with water. The aqueous mixture was chilled in an ice bath and the pH of the mixture was carefully adjusted to 2–3 with aqueous HCl. The aqueous mixture was extracted with methylene chloride. The methylene chloride extracts were washed with water and brine, combined, and dried over sodium sulfate. Evaporation of the solvent gave 322 mg (100%) of keto-acid (**14**). The material was homogeneous by TLC (10% MeOH/CH₂Cl₂ with trace AcOH).

NMR δ 0.87(br. t, 13 β -CH₂CH₃), 3.47(br. d, 11 β -CH₂O–), 4.0(s, –OCH₂CH₂O–), 4.10(s, –OCH₂CO₂H), 10.17(br. s, –CO₂H concentration dependent)ppm.

$[\alpha]_D^{27} = 125.36^\circ$ ($c = 1.03$, CHCl₃).

3.12. 13 β -Ethyl-17 α -ethynyl-17 β -hydroxy-11 β -carboxymethoxymethyl-3,3-ethylene-dioxygon-5(10)-ene (15)

Acetylene gas, dried by passing through two traps at –78°C, was bubbled through ice-cold THF (50 ml) for 15 min. Potassium *t*-butoxide (1.0 M/THF, 12.5 ml) was added to give a white slurry. Continued bubbling acetylene through the slurry for an additional 30 min, before keto-acid (**14**, 716 mg, 1.71 mmol) dissolved in THF (15.0 ml), was added dropwise over a 10-min period, followed by a THF (5.0 ml) rinse of the syringe. The reaction mixture was stirred for 1.5 h while maintaining a constant stream of acetylene. The reaction mixture was poured into a cold ammonium chloride solution (5.12 g, NH₄Cl/200 ml, H₂O). The pH of the aqueous mixture was

adjusted to 2–3 with aqueous HCl and then extracted with methylene chloride. The methylene chloride extracts were washed with water and brine, combined, and dried over sodium sulfate. Evaporation of the solvent gave 769 mg. Flash chromatography (Si, 3% MeOH/CH₂Cl₂ with 1% AcOH) afforded 600 mg of (15). Recrystallization of this material from ether/pentane gave 494 mg (65%) of (15), mp=166–168°C.

NMR δ 1.13(br. t, 13 β -CH₂CH₃), 2.63(s, -C=CH), 3.43(br. d, 11 β -CH₂O-), 3.97(s, -OCH₂CH₂O-), 4.10(br. d, 11 β -CH₂OCH₂CO₂H), 6.47(br. s, -CO₂H, concentration dependent)ppm.

FTIR (KBr, diffuse reflectance): ν_{\max} 3514, 3305, 2886, 1723, 1138 cm⁻¹.

MS (*m/z*): M⁺=444, M⁺-345=99(base).

Analysis Calcd for C₂₆H₃₆O₆: C, 70.24; H, 8.16. Found: C, 69.97; H, 8.08.

$[\alpha]_D^{27} = 80.82^\circ$ (c=1.03, CHCl₃).

3.13. 13 β -Ethyl-17 α -ethynyl-17 β -hydroxy-11 β -carboxymethoxymethylgon-4-en-3-one (16)

Steroid (15, 750 mg, 1.69 mmol) was dissolved in 10% aqueous acetone and the solution was treated with concentrated HCl (0.25 ml). The reaction mixture was stirred at 55–60°C for 4 h and diluted with ice/water and extracted with methylene chloride (3x). The methylene chloride extracts were washed with water and brine, combined, and dried over sodium sulfate. Evaporation of the solvent gave 697.5 mg of a stable foam. Attempted crystallization of this material from a variety of solvents was unsuccessful. Thus, a center cut of a flash chromatography afforded 482 mg (71%) of pure en-one (16). HPLC analysis (NovaPak C₁₈, 60% MeOH/40% 0.05 M KH₂PO₄ pH=3 buffer, 0.5 ml/min, UV=240 nm) showed the material to be pure.

NMR δ 1.11(br. t, 13 β -CH₂CH₃), 2.63(s, -C=CH), 3.35–4.0(m, 3H), 4.13(br. d, -OCH₂CO₂H), 5.92(br. s, C-4 H), 7.5(br. s, -CO₂H concentration dependent)ppm.

FTIR (KBr, diffuse reflectance): ν_{\max} 3256, 2938, 1742, 1657, 1129 cm⁻¹

MS (*m/z*): M⁺=400, M⁺-309=91(base).

Analysis Calcd for C₂₄H₃₂O₅: C, 71.97; H, 8.05. Found: C, 71.11; H, 8.30.

$[\alpha]_D^{27} = 17.56^\circ$ (c=1.08, CHCl₃).

3.14. 13 β -Ethyl-17 α -ethynyl-17 β -hydroxy-11 β -carboxymethoxymethylgon-4-en-3-syn/anti-oxime (18a and 18b)

A mixture of the methyl ester of the steroid (16, 442.4 mg, 1.07 mmol) and hydroxylamine hydrochloride (370.8 mg, 5.3 mmole) in pyridine (10.0 ml) was stirred at 100°C for 6 min. TLC analysis (15% acetone/CH₂Cl₂) showed no starting en-one remaining and the appearance of two products. The reaction mixture was chilled in an ice bath and diluted with cold water. The aqueous mixture was extracted with ethyl acetate (3x). The ethyl acetate layers were washed with water and brine, combined, and dried over sodium sulfate. Evaporation of the solvent gave 458.4 mg of a white powder. Flash chromatographic (55% EtOAc/Hex.) separation of the two products gave 225 mg of the more mobile component and 145 mg of the more polar component. Also, 25.3 mg of a mixture was recovered. By NMR, the more mobile component was determined to be the *anti*-oxime (17b) and the more polar material was

determined to be the *syn*-oxime (**17a**). Furthermore, the *anti*-oxime (**17b**) was crystalline, mp=185–188°C, whereas the *syn*-oxime (**17a**) exists as an amorphous foam.

Syn-oxime (**17a**): NMR (CDCl₃/d₅-Pyr) δ 1.20 (br. t, 13β-CH₂CH₃), 2.63(s, -C=CH), 3.73(s, -CO₂CH₃), 4.08(d, *J*=3Hz, -OCH₂CO₂CH₃), 6.73(br. s, C-4 H)ppm.

FTIR (KBr, diffuse reflectance): ν_{\max} 3479, 3270, 2102, 1753, 1639, 1132 cm⁻¹.

MS (*m/z*): M⁺=429, M⁺-338=91(base).

HPLC analysis (NovaPak C₁₈, 30% H₂O/MeOH, 0.5 ml/min, UV=240 nm): R_T(*anti*) =7.45 min; R_T(*syn*)=8.08 min.

Anti-oxime (**17b**): NMR (CDCl₃/d₅-Pyr) δ 1.23(br. t, 13β-CH₂CH₃), 2.63(s, -C=CH), 3.73(s, -CO₂CH₃), 4.10(d, *J*=3Hz, -OCH₂CO₂CH₃), 6.06(br. s, C-4 H)ppm.

FTIR (KBr, diffuse reflectance): ν_{\max} 3416, 3309, 2117, 1723, 1638, 1126 cm⁻¹.

MS (*m/z*): M⁺=429, M⁺-338=91(base).

[α]_D²⁵ =126.99° (c=1.09, CHCl₃).

3.15. Ester hydrolysis

The ester-oximes (**17**) were dissolved in 15% aqueous methanol and treated with aqueous sodium hydroxide (1.0 M, 1.2 eq). The reaction was stirred at room temperature until no starting material was evident by TLC (ca. 4 h). Most of the methanol was removed *in vacuo* and the residue was diluted with cold water. The pH of the aqueous mixture was adjusted to 1–2 with aqueous HCl and the mixture was extracted with ethyl acetate (3x). The ethyl acetate layers were washed with water and brine, combined, and dried over sodium sulfate. Evaporation of the solvent gave the oximes as the free acids (**18a** and **18b**) in 95–100% yield. Recrystallization of each isomer from ethyl acetate/hexanes provided crystalline material, mp (*anti*) (**18b**)=191–192.5°C (dec.); mp (*syn*) (**18a**)=172–175°C (dec.)

Syn-oxime (**18a**): NMR (CDCl₃/d₄-MeOH) δ 1.13(br. t, 13β-CH₂CH₃), 2.73(s, -C=CH), 3.4–4.0(br. m, 3H), 4.10(d, *J*=3Hz, -OCH₂CO₂H), 6.60(br. s, C-4 H)ppm.

MS (*m/z*): M⁺=415.

UV: λ_{max}(0.05 M *tris* buffer, pH=8.5)=248 nm, log ε = 4.10.

HPLC analysis (NovaPak C₁₈, 60% MeOH/40% 0.05 M KH₂PO₄, pH=3.0, 0.75 ml/min, UV=240 nm): R_T(*anti*-oxime)=9.12 min; R_T(*syn*-oxime)=10.50 min.

Anti-oxime (**18b**): NMR (d₆-DMSO) δ 1.13(br. t, 13β-CH₂CH₃), 3.30(s, C=CH), 4.0(br. s, -OCH₂CO₂H), 5.80(br. s, C-4 H)ppm.

FTIR (KBr, diffuse reflectance): ν_{\max} 3584, 3398, 1739, 1711, 1627, 1243 cm⁻¹.

MS (*m/z*): M⁺=415.

UV: λ_{max}(0.05 M *tris* buffer, pH=8.5)=244 nm, log ε=4.19.

3.16. General experimental for the synthesis of BSA conjugate (**19**) and (**20**)

The steroid hapten (**16**) or (**18a** and **18b**) was dissolved in DMF (2 ml/50 mg of hapten) under nitrogen and the solution was treated with 1, 1'-carbonyldiimidazole (CDI) (2.0 eq.) and the solution was stirred for 30 min at room temperature. Bovine

serum albumen (BSA) (100 mg BSA/50 mg hapten) was dissolved in water (while stirring) (8.0 ml/100 mg BSA) and the pH was carefully adjusted to 9.5–10.0 with the addition of triethylamine. The imidazolium complex from the above was added dropwise to the DMF/H₂O solution of BSA while maintaining the pH at 9.5–10.0 with the addition of triethylamine. After the change in pH had stabilized, the reaction was allowed to stir overnight. The reaction mixture was transferred to dialysis tubing and the material was dialyzed against cold (4°C) running water overnight (16–18 h). The contents of the dialysis tubing were then freeze-dried to afford the steroid hapten BSA conjugate as a foam.

Quantitation of the moles of steroid bound per mole of protein was obtained by ultraviolet absorption spectrometry¹¹ and by determination of free amino groups in the conjugate by a minor modification of the quantitative ninhydrin procedure¹² (Table I).

Table I

Moles of steroid per mole of conjugate

Steroid	UV method ¹¹	Ninhydrin method ¹²
19	39	21
20a	27	13
20b	22	15

3.17. 13 β -Ethyl-17 α -ethynyl-17 β -hydroxy-11 β -carboxymethoxymethylgon-4-en-3-one histamine conjugate (21)

A stirred DMF (4.0 ml) solution of steroid (16, 144.9 mg, 0.36 mmol), under nitrogen was treated with CDI (88.0 mg, 0.54 mmol) and the mixture was stirred for 30 min at room temperature. Histamine (free base) (120.6 mg, 1.08 mmol) was added and the reaction was stirred for 24 h at room temperature. The reaction mixture was diluted with water (50 ml) and the aqueous mixture was extracted with methylene chloride (3x). The methylene chloride extracts were washed with 1.0 M sodium carbonate, water and brine. The combined methylene chloride extracts were dried over sodium sulfate, and evaporation of the solvent gave 197 mg of a red oil. The histamine conjugate (21) was isolated via preparative TLC (Si, 20×20 cm, 1000 μ plate thickness, 15% MeOH/CH₂Cl₂ with 0.5% TEA). The band of interest (R_f=0.45) was cut out and the material was eluted from the Si gel using 25% MeOH/CH₂Cl₂ with 0.5% TEA. Evaporation of the solvent afforded 97 mg (54.3%) of an off-white stable foam. HPLC analysis (Whatman Partisil 10 ODS-3, 30% H₂O/MeOH with 0.1% TEA, 0.5 ml/min. UV=240 nm) showed the material (R_T=10.91 min) to be approximately 93% pure.

NMR δ 1.1(br. t, 13 β -CH₂CH₃), 2.67(s, C \equiv CH), 3.95(br. s, OCH₂CON-), 5.89 (br. s, C-4 H), 6.9(br. s, 1 H), 6.9–7.2(br. m, NH), 7.69(br. s, 1 H), 7.80–8.25 (br. m, NH)ppm.

FTIR (KBr, diffuse reflectance): ν_{\max} 3288, 2949, 2883, 2099, 1665, 1563, 1110 cm⁻¹.

MS (*m/z*): M⁺=493, 94 (base).

3.18. *13β-Ethyl-17α-ethynyl-17β-hydroxy-11β-carboxymethoxymethylgon-4-en-3-syn/anti-oxime histamine conjugate (22)*

Norgestrel histamine conjugate (**21**, 22.0 mg, 0.04 mmole), dissolved in pyridine (1.0 ml) under nitrogen, was treated with hydroxylamine hydrochloride (15.5 mg, 0.22 mmole). The mixture was heated at 100°C for 6 min. The pyridine was removed under a stream of nitrogen and the residue was taken up in water (10.0 ml). The aqueous mixture was extracted with ethanol/ethyl acetate (1:9) (3x). The organic extracts were washed with water and brine. The combined organic extracts were dried over sodium sulfate and evaporation of the solvent gave 23.0 mg of a stable foam.

NMR (d_4 -MeOH): δ 1.1(br. m, 13β - CH_2CH_3), 2.75(s, $-\text{C}\equiv\text{CH}$), 3.75(br. s, $-\text{OCH}_2\text{CONH}-$), 5.70(br. s, *anti*-C-4 H), 6.38(br. s, *syn*-C-4 H), 6.78(br. s, 1H), 7.6(br. s, 1H)ppm.

FTIR (KBr, diffuse reflectance): ν_{max} 3268, 2928, 1665, 1112 cm^{-1} .

MS (m/z): M^+ =508, M^+-17 =491, 94(base).

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