Pergamon

Tetrahedron, Vol. 53, No. 38, pp. 12855-12866, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00815-6

A Stereoselective Synthesis of 1α-(3′-Carboxypropyl)-4-androsten-17β-ol-3-one: Preparation of Immunoreagents for Quantification of Testosterone by Fluorescence Polarization Immunoassay

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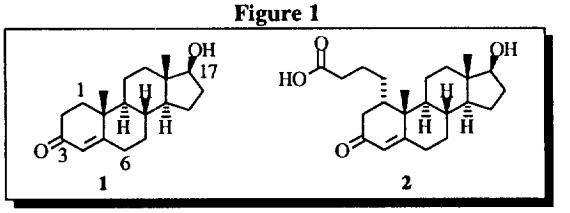
Abstract: *1α-(3′-Carboxypropyl)-4-androsten-17β-ol-3-one (2), a novel hapten, was prepared from boldenone (3) via addition of 4-pentenyl magnesium bromide in the presence of CuBr, as a key step in >95% epimeric excess and 13.8% overall yield. Two immunogens (7,8) and three fluorescent tracers (11,18,19) were prepared from acid (2) for the development of an immunoassay for testosterone (1).*

4-Androsten-17β-ol-3-one (1) commonly known as testosterone (Figure 1), is a hormone and is essential for the development of secondary sexual characteristics (voice, hair, body shape, etc.) and function of the reproductive system in males.[1] Testosterone (1) is secreted by the Leydig or interstitial cells of the testes and mainly controlled in adulthood by pituitary luteinising hormone (LH). Approximately 99% of testosterone (1) is bound to plasma proteins (i.e. sex hormone binding globulin and albumin), the remainder (about 1%) is freely circulated in blood and is primarily metabolized in the liver. In females, testosterone (1) is normally present in much lower levels than in males.[1] The presence of an abnormal level of testosterone (1) can lead to serious defects in the development of sexual characteristics and function of the reproductive system. Common causes of decreased amounts of testosterone (1) in males include hypergonadism, orchidectomy, Klinefelter's syndrome, hyperpituitarism, testicular feminization and hepatic cirrhosis. In females, the common causes of increased levels of 1 include polycystic ovaries (Stein-Leventhal syndrome), ovarian tumors and adrenal hyperplasia.[1] Therefore, the accurate measurement of 1 in body fluids is extremely important for diagnosis and treatment of various testosterone related illnesses.[1b,2]

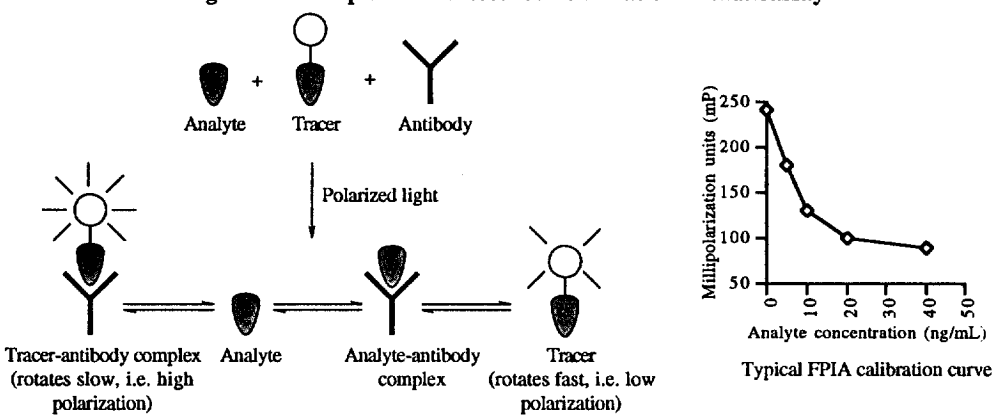
Figure 1



Quantification of testosterone (1): A variety of methods such as gas chromatography/mass spectrometry,[3] isotope dilution mass spectrometry,[4] thin layer chromatography (TLC),[5] high-performance liquid chromatography (HPLC),[6] chemiluminescence,[7] enzyme-linked immunoassay,[8] and radioimmunoassay (RIA)[9] have been reported for quantification of testosterone (1) in various body fluids. The ideal method for the quantification of a clinical analyte (e.g. testosterone, 1) should be accurate, reliable, fast, and inexpensive. Although the radioimmunoassay (RIA) method[10] revolutionized clinical diagnostics because of its simplicity, sensitivity and speed, this method suffers from disadvantages such as waste disposal, regulatory requirements

and short reagent half-life.[2] In contrast to the RIA method, recently introduced homogeneous immunoassay techniques such as the fluorescence polarization immunoassay (FPIA)[11] have gained wide spread use, particularly for the measurement of low molecular weight (<2000 Da) compounds. FPIA (Figure 2) is based on the principle that a fluorescent molecule when excited with polarized light, will emit the fluorescence depending on the rate of its rotation. Small molecules (e.g. tracer) rotate faster in solution than large molecules (e.g. tracer-antibody complex) hence, when polarized light excites the small molecule, the polarization decreases more than for large molecules. In a competitive FPIA, the analyte competes with the tracer for a limited number of antibody binding sites. Thus, with increasing concentration of analyte, more tracer becomes unbound, therefore the fluorescence polarization signal decreases. A calibration curve (Figure 2) is generated by measuring the fluorescence polarization of an analyte (e.g. testosterone, **1**) with known concentration. Using such calibration curve one can measure the amount of analyte present in an unknown sample.[12] Therefore, the development of fluorescence polarization immunoassay requires antibodies for binding and a signal generating material (tracer) for detection. The antibodies can be generated by immunization of an animal with immunogen, which can be prepared by conjugating the hapten (modified analyte) to carrier protein. The tracer is usually similar in structure to the analyte and contains a fluorescent signal generating moiety (e.g. fluorescein).

Figure 2: Principle of Fluorescence Polarization Immunoassay

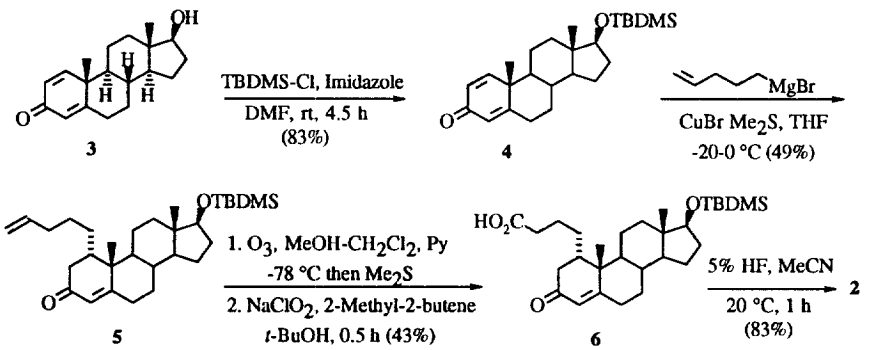


Selection of appropriate functionality and the site of substitution play an important role in identifying suitable reagents for assaying testosterone (**1**). Fluorescein and biotin are two common labels that have been incorporated at the C3,[13] C6,[14] C7,[15] and C15,[16] positions of testosterone for use as probes. However, the 1-position in testosterone (**1**) is a novel and very desirable site for label attachment, particularly if the sp^3 nature of the C1-carbon is retained, so as to preserve the native conformation of the A-ring. Additionally, α-stereochemistry at C1 is preferred because it places the label in a *trans* position to the C17 hydroxyl group as well as to the C18 and C19 methyl groups, thus minimizing steric interference with the critical binding determinants and allowing the probe to interact selectively with binding proteins. Moreover, immunogens prepared from the 1-position of testosterone (**1**) should trigger the animals immune response to generate antibodies of high selectivity to testosterone (**1**), since this position is not involved in metabolism. Production

of antibodies with high selectivity for testosterone (**1**) is critical for assay development. In this paper, we describe a stereoselective synthesis of a novel 1α-(3′-carboxypropyl)-4-androsten-17β-ol-3-one (**2**) and its application to the preparation of immunogens (**7,8**) and fluorescent tracers (**11,18,19**) which are critical components for the development of an fluorescence polarization immunoassay for testosterone (**1**).

Synthesis of acid (2): The strategy for the synthesis of acid (**2**) (Scheme 1) involves the stereoselective addition of 4-pentenylmagnesium bromide in the presence of CuBr catalyst to a suitably protected boldenone (**3**) and subsequent functionalization of the side chain. Thus, boldenone (1,4-androstadien-17β-ol-3-one, **3**) was protected as its TBDMS ether (**4**) using *t*-butyldimethylsilyl chloride in DMF in 83% yield.[17] The silyl ether (**4**) was then treated with the copper reagent prepared from 4-pentenylmagnesium bromide and CuBr-Me_2S complex at -20 °C and the mixture was stirred at 0 °C.[18] After 4 h, the reaction was quenched with 9N HCl and the crude product purified by silica gel column chromatography to afford the corresponding 1-α-pentenyl derivative (**5**) in 49% yield and >99% epimeric excess as determined by ^1H NMR and HPLC. The addition of the copper reagent took place from the sterically less hindered α-face of the α,β-unsaturated ketone system in the steroidal molecule (**4**) and thus gave the compound (**5**) in excellent stereoselectivity. The next step in the synthesis of acid (**2**) was fuctionalization of olefinic side chain in **5**. Accordingly, the compound (**5**) was subjected to ozonolysis in a mixture of MeOH-CH_2Cl_2, followed by oxidation of the resulting crude aldehyde with sodium chlorite to afford the acid (**6**) in 43% overall yield after silica gel column chromatography. Finally, the silyl ether in compound **6** was cleaved using 5% HF in acetonitrile[19] and purified by silica gel column chromatography to afford 1-α-(3′-carboxypropyl)-4-androsten-17β-ol-3-one (**2**) in 83% yield.

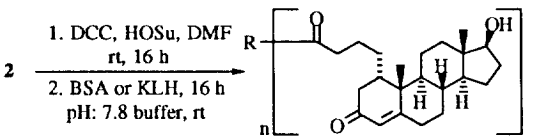
Scheme 1



Preparation of Immunogens: Small haptens such as acid (**2**) (molecular weight <2000 Da) do not as such induce the formation of antibodies, since their low molecular weight and simplicity is not sufficient to trigger the recipients immune system. However, it is possible to elicit an immune response to such haptens by conjugating them to a carrier protein, thus forming an immunogen. Bovine serum albumin (BSA, molecular weight: 66,430 Da) or keyhole limpet hemocyanin (KLH, molecular weight: 450 kDa – 13000 kDa) are two commonly used carrier proteins for the preparation of immunogens.[20] BSA is well suited as a carrier protein due to its solubility in various aqueous buffers and high content of available primary amines (59 lysines and the terminal amine) for conjugation of hapten. Additionally, BSA is available from several different manufacturers in reasonable cost and high purity. Immunogens prepared using KLH as carrier protein have also been used

frequently for the production of antibody, however, they are difficult to characterize due to the poor solubility and variable mass (450 kDa - 13000 kDa) of the KLH. Generally, the hapten is conjugated to a carrier protein through a site on the hapten that is remote from the critical binding determinants (unique features) to achieve selective antibodies. We prepared (Scheme 2) two immunogens (**7**, **8**) by conjugating the acid (**2**) to BSA and KLH. Accordingly, the acid (**2**) was treated with *N*-hydoxysuccinimide (HOSu) in the presence of dicyclohexylcarbodiimide (DCC) and the resulting succinimidyl ester was reacted with BSA in buffer solution (pH: 7.8). After 16h, the crude mixture was purified by dialysis and lyophilized to afford the immunogen (**7**) as a powder. The degree of hapten incorporation in immunogen (**7**) was estimated to be 52% [average 31 haptens (**2**) per BSA molecule] which represents the percentage of substitution of available amino groups for conjugation, by titration with trinitrobenzenesulfonic acid (TNBS).[21] Similarly, treatment of the succinimidyl ester of acid (**2**) with KLH in buffer solution (pH: 7.8) and subsequent dialysis and lyophilization afforded the immunogen (**8**). As mentioned above, determination of hapten incorporation in the KLH drived immunogens (e.g. **8**) is problematic and unreliable due to the variable mass of KLH, however, the immunogen (**8**) was succesfully used for generation of antibodies.

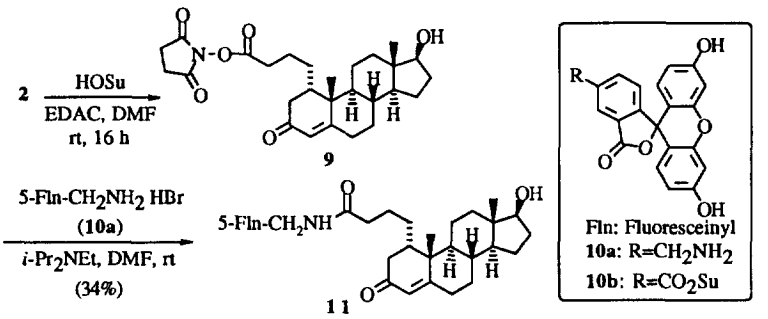
Scheme 2



7: R=BSA
8: R=KLH

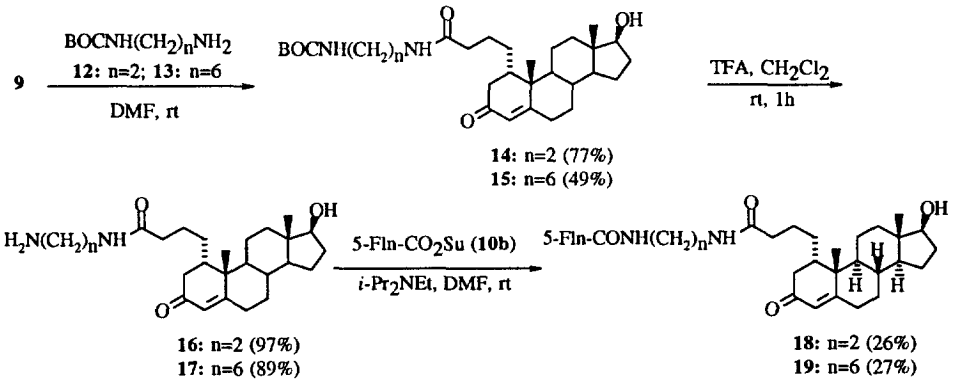
Preparation of Tracers: The selection and preparation of complementary labeled hapten (tracer) is also a critical step for the development of an immunoassay.[12,20] Generally, when preparing a tracer, the fluorescent label is attached to a hapten through the same site to which the carrier protein was conjugated in preparing the immunogen. The immunogen and tracer prepared by such design are called homologous. One reason for choosing the homologous approach in immunoreagent design is that the carrier protein sterically blocks access of the immune system to that part of the hapten closest to the point of attachment. In addition, the length of the spacer arm used for attachment of hapten to the fluorescent moiety can also play a critical role in establishing the right competition between the tracer and analyte for antibody binding sites. We prepared three fluorescent tracers (**11**,**18**,**19**) (Scheme 3 and 4) from acid (**2**). Accordingly, the acid (**2**) was treated (Scheme 3) with HOSu and EDAC in DMF to form the succinimidyl ester (**9**). The crude activated ester (**9**) was then treated with 5-aminomethylfluorescein hydrobromide (**10a**, 5-Fln-CH_2NH_2 HBr)[22] in the presence of diisopropylethylamine in DMF to afford the fluorescent tracer (**11**) in 34% yield. For the study of steric effects of spacer arm on binding parameters in an immunoassay system, we prepared two tracers (**18**, **19**) with additional two and six carbon units respectively between the acid (**2**) and fluorescent moiety. Thus, the succinimidyl ester (**9**) (Scheme 4) was treated with *tert*-butyl-*N*-(2-aminoethyl)carbamate (**12**) in DMF to afford **14** in 77% yield. The BOC group in **14** was removed using trifluoroacetic acid in CH_2Cl_2 to give the amine (**16**) in 97% yield, which was then treated with carboxyfluorescein succinimidyl ester (**10b**, 5-Fln-CO_2Su)[24] in the presence of diisopropylethylamine in DMF to afford tracer (**18**) in 26% yield. Similarly, the succinimidyl ester (**9**) was treated with *tert*-butyl-*N*-(6-aminohexyl)carbamate (**13**)[23] in DMF to afford **15** in 49% yield. The

Scheme 3



BOC compound (**15**) was then treated with trifluoroacetic acid in CH_2Cl_2 to afford the amine (**17**) in 89% yield. Finally the amine (**17**) was reacted with 5-carboxyfluorescein succinimidyl ester (**10b**)[24] in the presence of diisopropylethylamine to afford the tracer (**19**) in 27% yield and >99% purity.

Scheme 4



The immunogens (**7**,**8**) were used for inoculation of mice to generate monoclonal antibodies, according to the standard protocol.[25] Evaluation of these monoclonal antibodies showed high specificity for testosterone (**1**) and negligible cross-reactivity to the potentially interfering endogenous substances such as 5α-hydroxytestosterone, DHEA, DHEA-s, androstenedione, progesterone, 11-deoxycortisole, estradiol, and norethisterone. The development of a clinically relevant immunoassay for testosterone (**1**) is currently underway and the performance will be reported in due course. In summary, 1α-(3'-carboxypropyl)-4-androsten-17β-ol-3-one (**2**), a novel hapten was prepared from boldenone (**3**) via a stereoselective addition of 4-pentenylmagnesium bromide in the presence of CuBr, as a key step in 13.8% overall yield and excellent stereoselectivity. The immunoreagent, two immunogens (**7**,**8**) and four fluorescent tracers (**11**,**18**,**19**) were prepared from acid (**2**), which are critical reagents for the development of an fluorescent polarization immunoassay for testosterone (**1**).

Experimental

General Procedure: ^1H NMR and ^{13}C NMR spectra were recorded at 300 MHz on a Varian Gemini spectrometer or 70 MHz Varian instrument. Mass spectra were obtained on a Nermang 3010 MS-50, JEOL

SX102-A mass spectrometers or Perkin-Elmer Sciex API 100 electrospray mass spectrometer. Thin layer chromatography was performed on pre-coated Whatman MK6F silica gel 60 Å plates (layer thickness: 250 μm) and were visualized with UV light and/or using a $KMnO_4$ solution [$KMnO_4$ (1.0 g) and NaOH (8.0 g) in water (200 mL)] or phosphomolybdic acid reagent (20 wt% solution in ethanol), unless otherwise noted. Column chromatography was performed on silica gel, Merck grade 60 (230–400 mesh). THF was freshly distilled from a purple solution of sodium and benzophenone and CH_2Cl_2 was freshly distilled from CaH_2 under nitrogen. All reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and used without further purification, except where noted. All the solvents employed were of HPLC grade purchased from EM Science (Gibbstown, NJ) and used as received. Analytical normal phase HPLC was performed on a Waters μPorasil/125 Å, 10 μ, 3.9× 300 mm column eluting with ethyl acetate/hexane [ratio v/v reported]. Analytical reverse phase HPLC was performed using a Waters μBondapak C18 10 μ (8 mm × 100 mm) reversed phase column eluting with MeCN/0.1% aqueous formic acid [ratio v/v reported] with UV detection (see experimental for wave length). Preparative HPLC was performed using a Waters μBondapak C18 10μ (25 mm × 100 mm) reversed phase column eluting at 9 mL/min with MeCN/0.1% aqueous formic acid [ratio v/v reported] with UV detection (see experimental for wave length).

1,4-Androstadien-17β-*t*-butyldimethylsilyloxy-3-one (4): 1,4-Androstadien-17β-ol-3-one (boldenone, **3**) (10.2 g, 35.0 mmol) and imidazole (9.52 g, 140.0 mmol, 4.0 equiv.) were added to a stirred solution of *t*-butyldimethylsilyl chloride (11.0 g, 72.9 mmol, 2.08 equiv.) in anhydrous DMF (150 mL) at room temperature under nitrogen. The progress of the reaction was monitored by TLC (silica gel, 10% EtOAc in *n*-hexane) and after completion of the reaction in 4.5 h, the colorless precipitate was diluted with pentane (300 mL). The mixture was washed with ice cold 5% HCl solution (2 × 100 mL), 5% $NaHCO_3$ solution (2 × 100 mL), brine (2 × 100 mL) and dried ($MgSO_4$). The solvent was removed on a rotary evaporator and the resulting viscous oil was purified by silica gel column chromatography (10% EtOAc in *n*-hexane) to afford 11.6 g of **4** in 83% yield. Analytical HPLC: [ethyl acetate/hexane (10/90), 1.0 mL/min at 260 nm] R_t 3.93 min, >99%; ^1H NMR ($CDCl_3$): δ 7.05 (d, 1 H, J = 10.3 Hz), 6.22 (dd, 1 H, J = 10.3, 1.8 Hz), 6.06–6.00 (m, 1 H), 3.55 (t, 1 H, J = 8.3 Hz), 2.60–2.30 (m, 2 H), 2.00–0.70 (m, 13 H), 1.24 (s, 3 H), 0.88 (s, 9 H), 0.78 (s, 3 H), 0.00 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 186.3, 169.2, 155.8, 127.5, 123.8, 81.4, 52.7, 49.8, 43.6, 43.5, 36.7, 35.7, 33.2, 32.8, 30.8, 25.8, 23.7, 22.6, 18.8, 18.07, 11.4, -4.5, -4.8; MS (DCI, NH_3): m/z 401 $(M + H)^+$, 418 $(M + NH_4)^+$.

1α-(4′-Pentenyl)-4-androstadien-17β-*t*-butyldimethylsilyloxy-3-one (5): Magnesium turnings (4.30 g, 177.0 mmol, 1.05 equiv.) were placed in an oven dried three necked round bottom flask equipped with a magnetic stir bar, argon inlet and an addition funnel (250 mL) and heated with heat gun. After cooling the magnesium to room temperature, anhydrous THF (30 mL) and a crystal of iodine were added. After the iodine brown color disappeared, a solution of 5-bromo-1-pentene (25.03 g, 168.0 mmol) in dry THF (50 mL) was added dropwise over 2.5 h. The mixture was then stirred at room temperature for 2 h and dry THF (70 mL) was added to dissolve the precipitated reagent. The resulting solution of 4-pentenylmagnesium bromide was cannulated under argon into a dry, septum-capped brown bottle (200 mL) and stored at room temperature.

An oven dried 250 mL round bottom flask equipped with stir bar and argon inlet was charged with 4-pentenylmagnesium bromide in THF (75.0 mL, 75.0 mmol, 6.8 equiv.) and cooled to -20 °C. To this rapidly stirred solution, CuBr-Me_2S complex (1.52 g, 7.4 mmol, 0.67 equiv.) was added in one portion. After 3 min,

1,4-androstadien-17β-*t*-butyldimethylsilyloxy-3-one (**4**) (4.42 g, 11.03 mmol) in THF (7.0 mL) was added over 5 min. The flask was warmed to 0 °C in an and stirred for 15 min. The resulting blood-red solution was cooled to -78 °C (dry ice-acetone) and slowly quenched with 9 N HCl (8.1 mL) (deoxygenated by purging with argon) to give a yellow mixture. The cooling bath was removed and the mixture was diluted with ether (150 mL). The organic layer was separated and washed successively with aq. 10% $NaHCO_3$ solution (100 mL), brine (2 × 100 mL) and dried ($MgSO_4$). The solvent was removed on a rotary evaporator and the crude product was purified by silica gel column chromatography (10% EtOAc in hexane) to afford 2.52 g of **5** in 49% yield. Analytical HPLC: [ethyl acetate/hexane (5/95), 1.0 mL/min at 260 nm] R_t 4.45 min, >99%; ^1H NMR ($CDCl_3$): δ 6.00–5.50 (m, 2 H), 5.10–4.90 (m, 2 H), 3.58 (t, 1 H, J = 8.3 Hz), 2.68–2.28 (m, 4 H), 2.04–0.70 (m, 20 H), 1.30 (s, 3 H), 0.88 (s, 9 H), 0.76 (s, 3 H), 0.00 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 199.1, 168.8, 138.5, 123.7, 114.6, 81.6, 50.4, 46.5, 43.2, 41.9, 41.7, 37.9, 36.7, 35.6, 33.7, 32.9, 30.9, 30.6, 26.7, 26.6, 25.8, 23.5, 20.5, 19.9, 18.1, 11.4, -4.5, -4.8; MS (DCI, NH_3): m/z 471 $(M + H)^+$, 488 $(M + NH_4)^+$.

1α-(3′-Carboxypropyl)-4-androstadien-17β-*t*-butyldimethylsilyloxy-3-one (6): To the solution of 1-α-(4′-pentenyl)-4-androstadien-17β-*t*-butyldimethylsilyloxy-3-one (**5**) (1.918 g, 4.07 mmol) in a mixture of CH_2Cl_2 (200 mL) and MeOH (100 mL) in a 500 mL round bottom flask was added pyridine (2.0 mL) and Sudan III solution (0.1% in ethanol) (2.0 mL). The resulting light pink solution was cooled to -78 °C (dry ice-acetone) with stirring and a stream of ozone [generated at 90 V, 7.5 psi of O_2 and 0.2 slpm, (standard liters per minute)] passed through an inlet just below the surface of the solution. After the disappearance of the Sudan III indicator color (about 30–40 min) the ozone flow was stopped and nitrogen was bubbled gently into the solution to displace any excess ozone present in the reaction mixture. After addition of dimethylsulfide (4.0 mL, 54.3 mmol), the cooling bath was removed and mixture was allowed to warm to room temperature and stirred over night. The solvent was removed on a rotary evaporator and the residue was co-evaporated twice using toluene to remove any trace amount of methanol. The TLC (silica gel, 20% EtOAc in *n*-hexane) of the crude mixture showed complete consumption of starting material **5**.

The resulting crude aldehyde was dissolved in a mixture of *t*-BuOH (30 mL) and 2-methyl-2-butene (6.7 mL, 63.0 mmol), and a freshly prepared oxidant solution [prepared by dissolving sodium chlorite (0.920 g, 8.14 mmol) in phosphate buffer solution (pH 3.3, 0.20 M, 5.0 mL)] was added slowly at room temperature. After stirring the mixture for 30 min, the solvent was removed to dryness on a rotary evaporator and the residue was dissolved in ethyl acetate (300 mL) and brine (300 mL). The solution was adjusted to pH 3 using 1N HCl. The organic layer was separated and washed with 2% aq. sodium sulfite solution (pH 4.0, 300 mL) and dried ($MgSO_4$). The solvent was removed on a rotary evaporator and the crude product was purified by silica gel column chromatography (5% MeOH in $CHCl_3$) to give 0.863 g of **6** in 43% yield. Analytical reverse phase HPLC: [MeCN/ 0.1% aqueous formic acid (90:10), 2.0 mL/min at 240 nm] R_t 7.77 min, 97.1%; ^1H NMR ($CDCl_3$): δ 5.70 (s, 1 H), 3.58 (t, 1 H, J = 8.1 Hz), 2.70–2.30 (m, 2 H), 2.32 (t, 2 H, J = 6.9 Hz), 2.00–1.68 (m, 4 H), 1.64–0.80 (m, 17 H), 1.31 (s, 3 H), 0.88 (s, 9 H), 0.76 (s, 3 H), 0.00 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 198.8, 178.5, 168.8, 123.7, 82.5, 50.3, 46.5, 43.2, 41.8, 41.7, 37.9, 36.6, 35.6, 33.9, 32.9, 30.9, 30.6, 26.8, 25.9, 23.5, 22.7, 20.5, 19.9, 18.1, 11.4, -4.5, -4.8; MS (DCI, NH_3): m/z 489 $(M + H)^+$, 506 $(M + NH_4)^+$.

1α-(3′-Carboxypropyl)-4-androstadien-17β-ol-3-one (2): A freshly prepared solution of 5% HF (48%) in MeCN (v/v) (10 mL) was added to the stirred suspension of 1-α-(3′-carboxypropyl)-4-androstadien-17β-*t*-butyldimethylsilyloxy-3-one (**6**, 0.841 g, 1.76 mmol) in MeCN (50 mL) at room temperature. The mixture gradually became homogeneous and continued stirring the mixture for an additional 1 hr. The reaction mixture was poured into brine (300 mL) and extracted with ethyl acetate (2 × 200 mL). The combined organic layers were washed with brine (2 × 300 mL) and dried ($MgSO_4$). The solvent was removed on a rotary evaporator and the crude product was purified by silica gel column chromatography (10% MeOH in $CHCl_3$) to afford 0.553 g of **2** in 83% yield. Analytical reverse phase HPLC: [MeCN/0.1% aqueous formic acid (60/40), 1.0 mL/min at 230 nm] R_t 6.06 min, 98.6%; ^1H NMR ($CDCl_3$): δ 5.70 (s, 1 H), 5.45 (br s, 1 H), 3.67 (t, 1 H, J = 8.5 Hz), 2.70–2.30 (m, 2 H), 2.31 (t, 2 H, J = 6.9 Hz), 2.15–0.80 (m, 21 H), 1.31 (s, 3 H), 0.80 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 199.1, 178.4, 168.9, 123.7, 81.5, 50.7, 46.4, 42.8, 41.8, 41.6, 37.9, 36.2, 35.5, 33.9, 32.9, 30.5, 30.3, 26.7, 23.4, 22.6, 20.4, 19.9, 11.1; MS (DCI, NH_3): m/z 375 $(M + H)^+$, 392 $(M + NH_4)^+$.

Immunogen (7): To the stirred solution of 1-α-(3′-carboxypropyl)-4-androstadien-17β-ol-3-one (**2**) (0.064 g, 0.17 mmol) in anhydrous DMF (1.0 mL) was added *N*-hydroxysuccinimide (HOSu, 0.024 g, 0.21 mmol, 1.24 equiv.) followed by *N,N*-dicyclohexylcarbodiimide (DCC, 0.035 g, 0.17 mmol, 1.0 equiv.) in one portion at room temperature under N_2. The resulting homogeneous mixture became cloudy after 30 min. After stirring at room temperature for 16 h, the reaction mixture was filtered (a disposable pipette filled with cotton) and the solid was washed with anhydrous DMF (1.0 mL). A solution of bovine serum albumin (BSA, 0.293 g) in phosphate buffer (0.1 M, pH 7.80, 5.0 mL) was added dropwise at room temperature to the DMF solution of the crude succinimidyl ester of **2**. The mixture was stirred at room temperature for 16 h and transferred to a dialysis membrane tubing (Spectra/Por* 2,797925; catalogue No. D1614-12; size: 25 nm; diameter: 15.9 nm) and dialyzed against phosphate buffer (0.1 M, pH 7.80, 4.0 L) for 6 h. The dialysis continued against distilled water (4.0 L) for 3.5 days by replacing the water approximately every 12 h. The resulting solution was lyophilized to afford 0.265 g of immunogen (**7**) as a powder. Trinitrobenzenesulfonic acid (TNBS) titration[21] showed 52% [average 31 haptens (**2**) per BSA] substitution of amino groups in BSA.

Immunogen (8): HOSu (0.024 g, 0.21 mmol, 1.24 equiv.) and DCC) 0.035 g, 0.17 mmol, 1.0 equiv.) were added to the stirred solution of 1-α-(3′-carboxypropyl)-4-androstadien-17β-ol-3-one (**2**) (0.064 g, 0.17 mmol) in anhydrous DMF (1.0 mL) at room temperature under nitrogen atmosphere. After stirring the reaction mixture at room temperature for 16 h, it was then filtered (a disposable pipette filled with cotton) and the solid was washed with anhydrous DMF (1.0 mL). A solution of keyhole limpet hemocyanin (KLH, 0.292 g) in phosphate buffer (0.1 M, pH 7.80, 5.0 mL) was added dropwise at room temperature to the DMF solution of crude succinimidyl ester of **2**. The mixture was stirred at room temperature for 16 h and transferred to a dialysis membrane tubing (Spectra/Por* 2,797925) and dialyzed against phosphate buffer (0.1M, pH 7.80, 4.0 L) for 6 h. Dialysis continued against distilled water (4.0 L) for 3.5 days by replacing the water approximately for every 12 h The resulting solution was lyophilized to afford 0.215 g of immunogen (**8**).

1α-(3′-Carboxypropyl)-4-androstadien-17β-ol-3-one-succinimidyl ester (9): HOSu (0.092 g, 0.8 mmol, 2.0 equiv.) and 1-ethyl-3-(3-dimethylpropyl)carbodiimide (EDAC, 0.153 g, 0.8 mmol, 2.0 equiv.) were added to a solution of 1-α-(3′-carboxy propyl)-4-androstadien-17β-ol-3-one (**2**, 0.149 g, 0.4 mmol) dissolved in

anhydrous CH_2Cl_2 (6.0 mL) at room temperature under nitrogen atmosphere. The resulting mixture was stirred for 17 h, diluted with water (30 mL) and the pH was adjusted to 4.0 with 1 M HCl. The mixture was extracted with ether (3 × 30 mL) and the combined extracts were washed with brine (30 mL) and dried (Na_2SO_4). The solvent was removed on a rotary evaporator to afford 0.172 g of the succinimidyl ester (**9**). Analytical reverse phase HPLC: [MeCN/0.1% aqueous formic acid (60/40), 1.0 mL/min at 230 nm] R_t 7.8 min, 94%; ^1H NMR ($CDCl_3$): δ 5.68 (s, 1 H), 3.65 (t, 1 H, J = 8.0 Hz), 2.82 (s, 4 H), 2.70–2.26 (m, 6 H), 2.14–0.80 (m, 19 H), 1.30 (s, 3 H), 0.78 (s, 3 H); ES/MS: 472 $(M + H)^+$, 489 $(M + NH_4)^+$.

1α-[3'-(5-Carboxamidomethylfluorescein)propyl]-4-androstadien-17β-ol-3-one (11): 5-amino methylfluorescein hydrobromide(**10a**)[22] (5-AMF HBr, 0.033 g, 0.074 mmol, 1.0 equiv.) and diisopropylethylamine (DIEA, 0.025 mL, 0.15 mmol, 2.0 equiv.) were added to the solution of succinimidyl ester (**9**, 0.034 g, 0.0736 mmol) dissolved in anhydrous DMF (1.0 mL) at room temperature under nitrogen atmosphere. After stirring the mixture for 17 h, the solvent was remove on a rotary evaporator. The resulting residue was dissolved in a mixture of MeCN and 0.1% aqueous acetic acid (90:10 ratio, 2.0 mL) and purified by preparative reverse phase HPLC (25 mm × 100 mm) [MeCN/0.1% aqueous acetic acid (45/55) at 9.0 mL/min]. The solvent was removed on a rotary evaporator and the final trace amount of water was removed azeotropically using a mixture of toluene and methanol (1:1 ratio) (3 × 10 mL) to afford 0.0216 g of fluorescent tracer (**11**) in 41% yield. Analytical reverse phase HPLC: [MeCN/0.1% aqueous acetic acid (45/55), 1.0 mL/min at 230 nm] R_t 13.99 min, 99.5%; ^1H NMR (CD_3OD): δ 7.87 (s, 1 H), 7.65 (dd, 1 H, J = 1.0, 8.0 Hz), 7.15 (d, 1 H, J = 8.1 Hz), 6.67–6.49 (m, 6 H), 5.64 (s, 1 H), 4.51 (q, 2 H, J =10.0, 11.1 Hz), 3.53 (t, 1 H, J = 8.1 Hz), 2.78–2.66 (m, 1 H), 2.50–2.22 (m, 6 H), 2.08–0.85 (m, 17 H), 1.33 (s, 3 H), 0.77 (s, 3 H); ES/MS: 719 $(M + H)^+$.

1α-[3'-(2-*N*-*tert*-Butoxycarbonylaminoethyl)carboxamidopropyl]-4-androstadien-17β-ol-3-one (14): The succinimidylester (**9**) (0.172 g, 0.365 mmol) was dissolved in anhydrous DMF (4.0 mL) and a solution of *tert*-butyl-*N*-(2-aminoethyl)carbamate (**12**, 0.064 g, 0.40 mmol, 1.2 equiv.) in anhydrous DMF (2.0 mL) was added at room temperature under nitrogen. To this mixture was added diisopropylethylamine (0.077 mL, 0.44 mmol, 1.2 equiv.) stirred for 2.5 h. The solvent was then removed on a rotary evaporator and the crude compound was purified by silica gel column chromatography (2-4% MeOH in CH_2Cl_2) to afford 0.146 g of **14** in 77% yield. Analytical reverse phase HPLC: [MeCN/0.1% aqueous acetic acid (60:40), 1.0 mL/min at 230 nm] R_t, 6.84 min, 97.1% ; ^1H NMR ($CDCl_3$): δ 6.17 (br s, 1 H), 5.68 (s, 1 H), 4.99 (br s, 1 H), 3.66 (q, 1 H, J = 8.0, 14.2 Hz), 3.40–3.20 (m, 4 H), 2.80–0.90 (m, 25 H), 1.43 (s, 9 H), 1.30 (s, 3 H), 0.79 (s, 3 H); ES/MS: 517 $(M+H)^+$, 534 $(M + NH_4)^+$.

1α-[3'-(6-*N*-*tert*-Butoxycarbonylaminohexyl)carboxamidopropyl]-4-androstadien-17β-ol-3-one (15): A solution *tert*-butyl-*N*-(6-aminohexyl)carbamate[23] (**13**, 0.058 g, 0.27 mmol, 1.1 equiv.) in anhydrous DMF (1.0 mL) was added to succinimidylester (**9**, 0.114 g, 0.244 mmol) dissolved in anhydrous DMF (3.0 mL) at room temperature under N_2. To this mixture, diisopropylethylamine (DIEA, 0.051 mL, 0.29 mmol, 1.2 equiv.) was added. After stirring for 4.5 h, the solvent was removed on a rotary evaporator and the crude compound was purified by silica gel column chromatography (2-5% MeOH in CH_2Cl_2) to afford 0.068 g of **15** in 49% in yield. Analytical reverse phase HPLC: [MeCN/0.1% aqueous acetic acid (60/40), 1.0 mL/min at 230

nm] R_t 9.74 min, 98.1% ; ^1H NMR ($CDCl_3$): δ 5.70 (s, 1 H), 5.58 (br s, 1 H), 4.61 (br s, 1 H), 3.53 (t, 1 H, J = 7.9 Hz), 3.28–3.10 (m, 4 H), 2.68–2.60 (m, 1 H), 2.46–0.90 (m, 32 H), 1.43 (s, 9 H), 1.30 (s, 3 H), 0.80 (s, 3 H); ES/MS: 573 $(M + H)^+$.

1α-[3′-*N*-(2-Aminoethyl)carboxamidopropyl]-4-androstadien-17β-ol-3-one (16): Trifluoroacetic acid (4.0 mL) was added to **14** (0.145 g, 0.281 mmol) dissolved in anhydrous CH_2Cl_2 (4.0 mL) at room temperature. The mixture was stirred for 1.0 h and the solvent was removed on a rotary evaporator. The resulting residue was azeotropically dried using mixture of toluene and methanol (1:1, v/v) (8 × 10 mL). The resulting gummy oil was triturated with ether (15 drops) and dried on a vacuum pump to afford 0.145 g (97%) of amine (**16**) as colorless foam. Analytical reverse phase HPLC: [MeCN/0.1% aqueous trifluoroacetic acid (35/65) 1.0 mL/min at 230 nm] R_t 6.46 min, 93%; ^1H NMR (CD_3OD): δ 5.68 (s, 1 H), 3.57 (t, 1 H, J = 7.8 Hz), 3.42 (t, 2 H, J = 6.0 Hz), 3.02 (t, 2 H, J = 6.4 Hz), 2.80–2.70 (m, 1 H), 2.54–2.12 (m, 4 H), 2.60–1.96 (m, 2 H), 1.90–0.80 (m, 23 H); ES/MS: 417 $(M+ H)^+$.

1α-[3′-*N*-(6-Aminohexyl)carboxamidopropyl]-4-androstadien-17β-ol-3-one (17): The compound **15** (0.068 g, 0.12 mmol) was dissolved in anhydrous CH_2Cl_2 (2.0 mL) and added trifluoroacetic acid (2.0 mL) at room temperature. The mixture was stirred for 1.0 h and the solvent was removed on a rotary evaporator. The resulting residue was azeotropically dried on a rotary evaporator using mixture of toluene and methanol (1:1, v/v) (6 × 10 mL) to afford 0.062 g of amine (**16**) in 89% yield. Analytical reverse phase HPLC: [MeCN/0.1% aqueous trifluoroacetic acid (40/60), 1.0 mL/min at 230 nm] R_t 5.49 min, 92%; ^1H NMR (CD_3OD): δ 5.67 (s, 1H), 3.58 (t, 1 H, J = 8.1 Hz), 3.20–3.12 (m, 2 H), 2.90–3.00 (m, 2 H), 2.78–2.66 (m, 1 H), 2.52–2.18 (m, 4 H), 2.17–1.90 (m, 4 H), 1.89–0.80 (m, 29 H); ES/MS: 473 $(M + H)^+$.

1α-[3′-*N*-(2-[5-Carboxamidofluorescein]ethyl)carboxamidopropyl]-4-androstadien-17β-ol-3-one (18): 5-Carboxyfluorescein succinimidyl ester (**10b**)[24] (0.066 g, 0.14 mmol, 1.0 equiv.) and diisopropylethylamine (0.061 mL, 0.35 mmol, 2.5 equiv.) were added to the amine (**16**, 0.073 g, 0.138 mmol) dissolved in anhydrous DMF (2.0 mL) at room temperature under nitrogen. The flask was covered with aluminum foil and stirred for 18 h. The solvent was removed on a rotary evaporator under vacuum and the crude compound was purified by preparative reverse phase HPLC [MeCN/0.1% aqueous acetic acid (45/55), 9 mL/min, 230 nm]. The solvent was removed on a rotary evaporator and the residue was azeotropically dried using mixture of toluene and methanol (1/1, v/v, 3 × 10 mL) to afford 0.028 g of fluorescent tracer (**18**) in 26% yield. Analytical reverse phase HPLC: [MeCN/0.1% aqueous acetic acid (45/55), 1.0 mL/min at 230 nm] R_t, 9.97 min, 99.8%; ^1H NMR (CD_3OD): δ 8.44 (s, 1 H), 8.18 (d, 1 H, J = 8.2 Hz), 7.30 (d, 1 H, J = 8.1 Hz), 6.70–6.50 (m, 6 H), 5.63 (s, 1 H), 3.60–3.38 (m, 5 H), 2.68 (dd, 1 H, J = 4.5, 16.8 Hz), 2.78–0.90 (m, 23 H), 1.31 (s, 3 H), 0.77 (s, 3 H); ES/MS: 476 $(M + H)^+$.

1α-[3′-*N*-(6-[5-Carboxamidofluorescein]hexyl)carboxamidopropyl]-4-androstadien-17β-ol-3-one (19): The amine (**17**, 0.026 g, 0.045 mmol) was dissolved in anhydrous DMF (1.0 mL) and added 5-carboxyfluorescein succinimidyl ester (**10b**)[24] (0.021 g, 0.045 mmol), 1.0 equiv.) followed by diisopropylethylamine (0.016 mL, 0.092 mmol, 2.0 equiv.) at room temperature under nitrogen atmosphere. The flask was covered with aluminum foil and the mixture was stirred for 17 h. The solvent was removed on a

rotary evaporator and the crude compound was purified by preparative reversed phase HPLC [MeCN/0.1% aqueous acetic acid (45/55), 9 mL/min, 225 nm). The solvent was removed on a rotary evaporator and the residue was azeotropically dried using mixture of toluene and methanol (1/1, v/v, 3 × 10 mL) to afford 0.0099 g of fluorescent tracer (**19**) in 26% yield. Analytical reverse phase HPLC: [MeCN/0.1% aqueous acetic acid (50:50), 1.0 mL/min at 230 nm] R_t, 9.1 min, 99.6%; ^{1}H NMR (CD_3OD): δ 8.43 (s, 1 H), 8.16 (dd, 1 H, J = 1.5, 8.1 Hz), 7.30 (d, 1 H, J = 8.1 Hz), 6.7–6.50 (m, 6 H), 5.66 (s, 1 H), 3.57 (t, 1 H, J = 8.4 Hz), 3.43 (t, 2 H, J = 6.9 Hz), 3.10–3.20 (m, 2 H), 2.70 (dd, 1 H, J = 4.2, 16.5 Hz), 2.50–0.90 (m, 31 H), 1.33 (s, 3 H), 0.77 (s, 3 H); ES/MS: 832 $(M + H)^+$.

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(*Received in USA* 13 *June* 1997; *accepted* 15 *July* 1997)