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SCIENCE DIRECT®
Steroids 68 (2003) 629–639
Steroids
www.elsevier.com/locate/steroids

Syntheses and ligand-binding studies of 1α- and 17α-aminoalkyl dihydrotestosterone derivatives to human sex hormone-binding globulin

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Received 24 February 2003; received in revised form 23 April 2003; accepted 28 April 2003

Abstract

We report on the syntheses of 1α- and 17α-aminoalkyl dihydrotestosterone (DHT) derivatives and the particularly high binding affinity of the 1α-aminohexyl ligand for human sex hormone-binding globulin (SHBG). The two 17α-aminopropyl-17β-hydroxy-5α-androstan-3-one (**1**) and 17α-aminocaproylamidoethyl-17β-hydroxy-5α-androstan-3-one (**2**) derivatives were synthesized via a 17β-spirooxirane intermediate in high yields. The 1α-aminohexyl-17β-hydroxy-5α-androstan-3-one compound (**3**) was obtained in a seven step synthesis using a copper-catalyzed conjugate addition of a ω-silyloxyhexyl Grignard reagent to 17β-benzoyloxy-5α-androst-1-en-3-one. All structures were elucidated based on ^{1}H NMR spectroscopy and mass spectral analyses. The three aminosteroid derivatives were tested as ligands for SHBG by competition experiments with tritiated testosterone as tracer under equilibrium conditions. The association constants of the two 17α-DHT derivatives were approximately $1 \times 10^7\ M^{-1}$, whereas the 1α-DHT derivative showed a remarkably high binding affinity to SHBG with an association constant of $1.40 \times 10^9\ M^{-1}$.

These aminoalkyl derivatives, substituted either at the D-ring or the A-ring of the steroid skeleton, can be easily coupled onto a carboxymethylated solid state surface of a biosensor. Such a device lends itself to kinetic and thermodynamic studies aimed to provide a better understanding of the biospecific interaction of steroids with SHBG.

Keywords: 1α-Aminohexyl-17β-hydroxy-5α-androstan-3-one; 17α-Aminopropyl-17β-hydroxy-5α-androstan-3-one; 17α-Aminocaproylamidoethyl-17β-hydroxy-5α-androstan-3-one; Human sex hormone-binding globulin; Steroid; ^{1}H NMR

1. Introduction

Human sex hormone-binding globulin (SHBG), a 93.4-kDa homodimeric glycoprotein produced by hepatocytes, is the major sex steroid-binding protein in plasma [1] that binds testosterone (T), 5α-dihydrotestosterone (DHT), 17β-estradiol (E2), and related steroids with different affinities [2–5]. The glycoprotein is a product of a single gene located on the short arm of chromosome 17 [6,7]. Glycosylation of the monomeric protein forms one O-linked oligosaccharide at Thr-7 and two N-linked oligosaccharides at Asn-351 and -367 in the C-terminal region of the molecule [8,9]. The two monomers of SHBG interact very strongly with each other even in the absence of ligand [10].

The major physiological role of SHBG is the regulation of bioavailability of T and E2 by controlling their respective metabolic clearance rates [11–14]. SHBG has also been reported to exert cellular influences on the uptake of sex steroids [15–17] in target cells and on signal transduction [18,19]. Plasma SHBG levels vary considerably between individuals and are influenced by hormonal, metabolic, and nutritional factors [1]. It is of particularly high clinical interest that low serum levels of SHBG are found in women suffering from disorders characterized by androgen excess [1] and are also considered as a prognostic indicator for the onset of type II diabetes mellitus, hyperthyroidism, and cardiovascular disease [20,21]. Thus, the knowledge of factors regulating SHBG levels, the understanding of the interaction between steroid ligands and SHBG, and the measurement

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0039-128X/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0039-128X(03)00092-8

of protein-bound and free steroid fractions [22] are of great endocrinological significance.

Optimal binding of steroids to SHBG is known to require a planar C_{19} steroid with a 17β-hydroxyl group and an electronegative functional group at C-3 [23]. The recent crystal structure of the N-terminal recombinant human SHBG ligand-binding domain (LBD) complexed with steroidal ligands [24] has revealed that each monomer contains an LBD for a steroid molecule within the N-terminal laminin G-like domain (G-domain). The C-3 oxygen atom of bound DHT is anchored in the interior of the protein, while rings A and B are completely buried. Substitutions are well tolerated at the positions C-4, C-12α, and C-17α, as expected from previous binding experiments [25–27]. Other modifications at several other ring positions have been shown to significantly reduce the relative binding affinities (RBA) [3]. However, the 1α position seems to be qualified as a potential candidate for introducing a substitution, which may be deduced from the fact that mesterolone (17β-hydroxy-1α-methyl-5α-androstan-3-one) has a high affinity for SHBG [26]. Moreover, recent site-directed mutagenesis experiments have shown that dimerization-deficient SHBG monomeric variants still contain a LBD with an affinity and specificity indistinguishable from wild-type SHBG [24,28].

To provide further physicochemical data on the thermodynamics and kinetics of the interaction of androgen ligands with SHBG by the way of highly sensitive real time biosensor methodologies, our aim was first to synthesize and determine the binding characteristics of DHT derivatives modified at the 1α and 17α position by alkylamine spacers sufficiently long enough to allow an unhampered access of SHBG to the steroidal ligand after covalent coupling onto a carboxymethylated solid state surface of a biosensor device.

2. Experimental

2.1. Chemicals and reagents

Con A-Sepharose was purchased from Amersham Pharmacia Biotech, Freiburg, Germany. DHT (17β-hydroxy-5α-androstan-3-one), 5α-androstan-3,17-dione, T (17β-hydroxy-4-androsten-3-one), and 3-amino-9-ethyl-carbazole (AEC) were from Sigma, Deisenhofen, Germany. 9-Fluorenylmethoxycarbonyl (Fmoc)-ε-aminocaproic acid was purchased from Bachem Biochemica, Heidelberg, Germany. [1,2,6,7-^{3}H]-T (^{3}H-T, specific activity: 2.92 TBq/mmol) was from Amersham Pharmacia Biotech. For column chromatography, silica gel (grain 0.063–0.200) from Merck and neutral Al_2O_3 (aluminia N Super I, W 200) or basic Al_2O_3 (aluminia B Super I, W 200) from Woelm Pharma (Bad Honnef, Germany) were used. Thin layer chromatography (TLC) was performed using silica gel 60 F_{254} (0.2 mm) or neutral Al_2O_3 60 F254 type E (0.2 mm), both materials from Merck. All other laboratory chemicals were from Sigma.

The polyclonal rabbit anti-SHBG antibody was from Dako A/S, Glostrup, Denmark, and the secondary horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG polyclonal antibody (pab) was from Amersham Pharmacia. The gel filtration LMW and HMW calibration kits and the Superdex 200 HR10/30 column for size-exclusion chromatography were from Amersham Pharmacia. PVDF membranes (Immobilon-P, pore size 0.45 μm) and Microcon® centrifugal filters were from Millipore, Bedford, MA, USA.

2.2. Apparatus

Melting points (uncorrected) were determined on a Reichert Thermovar and on a Büchi 510. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ and C_5D_5N on a Bruker Avance 400 (400 MHz), using Me_4Si as the reference signal. Data are reported in the following form: chemical shift (multiplicity, coupling constant in Hz if available, number of protons, position of proton). Infrared (IR) spectra (in KBr or neat) were obtained on a Bio-Rad Excalibur FTS 3000 MX spectrophotometer, only characteristic IR bands (cm^{-1}) are reported. Optical rotation values were determined on a Perkin Elmer 241 polarimeter, *c* is given in g per 100 cm^{-3}. Mass spectrometry (MS) analyses were performed on Finnigan MAT 95, MAT SSQ 700, as well as on Varian MAT 112S and 311A mass spectrometers. For size-exclusion chromatography, the ÄKTA*FPLC* system from Amersham Pharmacia was used. Gel electrophoresis was performed using the Mini-Protean 3 Cell, whereas protein transfer was realized using the Mini Trans-Blot Electrophoretic Transfer Cell; both cells were purchased from BioRad, Hercules, CA, USA.

2.3. Syntheses of the 1α- and 17α-DHT derivatives

For general organic chemical procedures, see Mappus et al. [29] and Hauptmann et al. [30].

2.3.1. 17α-Aminopropyl-17β-hydroxy-5α-androstan-3-one (*1*)

A solution of 3,3-(dimethoxy)-17α-aminopropyl-DHT precursor (30 mg, 0.076 mmol) in 10 ml of CH_2Cl_2 containing 0.1 ml of aqueous 1 M HCl was stirred for 2 h at 22 °C. Then, the reaction mixture was cooled to 4 °C, washed with 1 ml of a saturated aqueous solution of $NaHCO_3$ and three times with 2 ml of water, and finally evaporated under reduced pressure. Purification of the crude residue by TLC on silica gel (ethyl acetate, then $CHCl_3$/MeOH/NH_4OH, 25:10:1) led to the final product **1** as a pale yellow oil (16 mg, 60% yield). ν_{max} ($CHCl_3$): 1706 (3-CO); ^{1}H NMR (C_5D_5N). 0.93 (s, 3H, 19-CH_3), 1.11 (s, 3H, 18-CH_3), 3.45 (m, 2H, –CH_2NH_2); MS (ESI^+, *m/z*, relative intensity %): 348 (MH^+ 100%), 330 ($M^+ - H_2O$, 59%); high-resolution MS (CI): found 348.2903, $C_{22}H_{37}NO_2$ (MH) requires 348.2903; $[]_D = +0.1^\circ$ ($CHCl_3$, $c = 0.1$).

2.3.2. 17α-Aminocaproylamidoethyl-17β-hydroxy-5α-androstan-3-one (2)

The 3,3-(dimethoxy)-17α-aminoethyl-DHT derivative **1b** was synthesized from 5α-androstan-3,17-dione, according to [29] with the following three key steps: epoxidation, leading to 17β-(2′-oxiran)-3,3-(dimethoxy)-5α-androstane, nucleophilic opening of the epoxide with cyanide anion leading to 17β-hydroxy-17α-(cyanomethyl)-3,3-(dimethoxy)-5α-androstan-3-one, reduction with $LiAlH_4$, leading to the 17-ethylamine intermediate. **1b** was purified by liquid–solid chromatography (LSC) on silica gel using a CH_2Cl_2/EtOH/NH_4OH mixture (10:10:1, v/v/v) as eluent, giving the free amine as a pale resin in 69% yield.

The 17-amino derivative **1b** (100 mg, 0.26 mmol) was suspended in 2 ml of dry CH_2Cl_2. Subsequently, the Fmoc-protected 6-aminocaproic acid (101 mg, 0.29 mmol), *p*-dimethylaminopyridine (DMAP) (6 mg, 0.05 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (60 mg, 0.29 mmol) were added to the reaction mixture and stirred at room temperature for 24 h. The solvent was removed under reduced pressure. The product was dissolved in a mixture of CH_2Cl_2 and MeOH (9:1, v/v) and purified by LSC on silica gel (CH_2Cl_2/MeOH, 9:1, v/v). The Fmoc protected amide derivative **2a** was obtained as a pale resin (162 mg, 87% yield). ν_{max} (KBr): 3411, 3331 (OH, NH), 1704 (urethane CO), 1648 (amide I), 1539 (amide II), 1110–1050 (H_3C–O–C–); ^{1}H NMR ($CDCl_3$) δ: 0.84 (s, 3H, 18-CH_3), 0.90 (s, 3H, 19-CH_3), 2.14 (2H, t: J = 7.3 Hz), 3.13 (s, 3H, CH_3–O) and 3.19 (s, 3H, CH_3–O), 3.16–3.32 (m, 6H, –C*H*$_2$–NH–CO–CH_2–O; CH_3–O–C– and –CH*H*$_A$–NHCO–), 3.59–3.71 (m, 1H, –CH*H*$_B$–NHCO–), 4.21 (1H, t: J = 7.0 Hz, 9H of the 9-alkylfluorene group), 4.39 (2H, d: J = 7.0 Hz, O–CH_2-fluorene), 4.89–4.94 (m, 1H, –N*H*–$COCH_2$–), 6.48 (s broad, 1H, –CH_2–N*H*–$COCH_2$–), 7.40–7.77 (m, 8 H, aromatic protons); MS (ESI, *m*/*z*, relative intensity %): 737 ($M + Na^+$, 100%), 695 ($M^+ - 18$, 56%); elemental analysis: $C_{44}H_{62}N_2O_6$ (714.98), calcd C, 73.91, H, 8.74, N 3.92; found C, 73.73, H, 8.86, N 3.70; $[\alpha]_D = -1.4^\circ$ (CH_2Cl_2, $c = 1.22$).

For cleavage of the Fmoc group, the amide derivative **2a** (120 mg, 0.17 mmol) was dissolved in a mixture of 1 ml CH_2Cl_2 and 1 ml ethanolamine. After 1 h at room temperature, the mixture was diluted with 5 ml CH_2Cl_2, and ethanolamine was eliminated by three successive extractions with 5 ml water. The combined organic layers were dried with Na_2SO_4 and evaporated under reduced pressure. The product was analyzed by TLC and then chromatographed on silica gel (CH_2Cl_2/MeOH/NH_4OH, 10:10:1, v/v/v). After freeze drying, the amine intermediate **2b** was obtained (66 mg, 79% yield). ν_{max} (KBr): 3350 (OH, NH), 1644 (amide I), 1556 (amide II), 1110–1050 (H_3C–O–C–); ^{1}H NMR ($CDCl_3$) δ: 0.80 (s, 3H, 19-CH_3), 0.84 (s, 3H, 18-CH_3), 2.17 (2H, t: J = 7.3 Hz, –C*H*$_2$–CO–NH–), 2.70 (2H, t: J = 6.6 Hz, –C*H*$_2$–NH_2), 3.14 (s, 3H, CH_3–O), 3.19 (s, 3H, CH_3–O), 3.21–3.32 (m, 1H, –CH*H*$_A$–NHCO–), 3.62–3.72 (m, 1H, –CH*H*$_B$–NHCO–), 6.67 (m, 1H, –NH–CO–); MS (ESI, *m*/*z*, %): 493 ($M + H^+$, 100%), 461 ($M^+ - 32$, 58%); high-resolution MS (DCI, CH_4): found 493.4014, $C_{29}H_{53}N_2O_4$ (MH) requires 493.4005; $[\alpha]_D = -0.2^\circ$ (CH_2Cl_2, $c = 0.85$).

Hydrolysis of the acetal group of compound **2b** (50 mg, 0.1 mmol) was performed with 200 μl of 5N HCl in a mixture of 2 ml tetrahydrofuran (THF) and 1 ml MeOH. The mixture was stirred at room temperature for 3 h. After evaporation of the solvent under reduced pressure, the deprotected amine product **2** was recovered quantitatively as a hydrochloride by lyophilization. The synthesis of **2** was performed in an overall high yield of 68%. ν_{max} (KBr): 3335 and 3191 (OH, NH), three broad bands at 3030, 2500, 2100 (NH_3-), 1704 (3-CO), 1649 (amide I), 1551 (amide II); ^{1}H NMR (C_5D_5N) δ: 0.92 (s, 3H, 19-CH_3), 1.10 (s, 3H, 18-CH_3), 2.45 (2H, t: J = 7.3 Hz, CH_3–CO–), 3.23 (2H, t: J = 7.3 Hz, –C*H*$_2$–NH_3^+), 3.87–4.05 (m, 2H, –C*H*$_2$–NH–CO), 11.1 (s broad, 3H, –NH_3^+); MS (FAB, *m*/*z*, %): 447 ($MH^+ - Cl$, 100%), 429 ($MH^+ - Cl - H_2O$, 42%); elemental analysis as monohydrate: $C_{27}H_{49}ClN_2O_4$ (501.14), calcd C, 64.71, H, 9.86, N 5.59; found C, 63.95, H, 9.75, N 5.29; $[\alpha]_D = +0.7^\circ$ ($CHCl_3$, $c = 2.60$).

2.3.3. 1α-Aminohexyl-17β-hydroxy-5α-androstan-3-one (3)

2.3.3.1. 1α-TBSO-hexyl-3-oxo-5α-androstane-17β-yl benzoate (3b). Synthesis started from the known compound 5α-androst-1-en-3-one-17β-yl benzoate (**3a**), which was prepared according to the protocol from Pelc [31]. The Grignard reagent dimethyl-*t*-butyl-silyloxy-hexyl-MgBr (TBSO-*n*-hexyl MgBr) was synthesized as previously described [32,33] by adding dropwise a solution of TBSO-*n*-hexyl bromide (10 Eq.) in anhydrous THF to Mg (11 Eq.) in THF under a N_2 atmosphere at 50 °C. The resulting clear solution was further diluted with solvent, and after 1 h, cooled to −40 to −30 °C in an isopropanol/dry ice-bath. A slurry of freshly prepared CuI (2 Eq.) in THF was added. After 30 min, the enone **3a** (200 mg, 0.51 mmol, 1 Eq.) in anhydrous THF was added dropwise over a period of 50 min to the solution, which was maintained at −45 to −35 °C, giving a light lemon yellow suspension. After stirring for a further 60 min, the color disappeared, and the reaction mixture was quenched with glacial acetic acid at −30 °C and then basified with a saturated $NaHCO_3$ solution. The solvent was removed, and the residue extracted with methyl-*tert*-butyl ether (MTBE) and washed with an aqueous saturated NH_4Cl solution. The crude oily extract was first chromatographed on silica gel using CH_2Cl_2 as eluent to separate the aliphatic byproducts and then subjected to a LSC using petroleum ether (PE)/MTBE (1:1, v/v) as eluent. The 1-silyloxyhexyl derivative **3b** was isolated as a colorless oil (199 mg, 64% yield). ν_{max} (film): 1717 (3-CO), 1100, 836, 767 (all three Si–R); ^{1}H NMR

($CDCl_3$) δ: 0.04 (s, 6H, Me_2–Si), 0.88 (s, 9H, *tert*-butyl-Si), 0.95 (s, 3H, 18-CH_3), 1.14 (s, 3H, 19-CH_3), 2.04–2.58 (m, 5H, 2α, 2β, 4α, 4β, 16α H), 3.58 (2H, t: $J = 6.7$ Hz, –CH_2–Si), 4.87 (1H, dd: $J = 7.9$ Hz and 9.1 Hz, 17α-H), 7.4–7.6 (m, 5H, aromatic H); MS (DCI, NH_3, *m/z*, %): 609 (MH^+, 100%), 626 ($M + NH_4^+$, 62%); high-resolution MS (DCI, CH_4): found 609.4334, $C_{38}H_{61}O_4Si$ (MH) requires 609.4339; $[\alpha]_D = +51.5^\circ$ (CH_2Cl_2, $c = 5.55$).

*2.3.3.2. 1α-Hydroxyhexyl-3-oxo-5α-androstane-17β-yl benzoate (**3c**).* The silyl ether **3b** (150 mg, 0.25 mmol, 1 Eq.) was dissolved under N_2 in 2 ml anhydrous THF, and 1 ml of a tetrabutylammonium fluoride solution (TBAF) in THF (4 Eq., 1 mmol/l) was added. The mixture was stirred at room temperature for 2 h. Then, a saturated NaCl solution was added, and the product was extracted three times with MTBE. After removal of the solvent, purification by LSC, using MTBE as eluent, led to the 1-hydroxyhexyl derivative **3c** as colorless crystals, recrystallized from *n*-heptane (117.5 mg, 95% yield). Melting point (m.p.): 118–120 °C. ν_{max} (KBr): 3463 (OH), 1715 (3-CO), 1276 (C–O–C); ^{1}H NMR ($CDCl_3$) δ: 0.95 (s, 3H, 18-CH_3), 1.14 (s, 3H, 19-CH_3), 2.05–2.58 (m, 5H, 2α, 2β, 4α, 4β, 16α H), 3.63 (2H, t: $J = 6.6$ Hz, –CH_2–OH), 4.88 (1H, dd: $J = 7.8$ Hz and 9.1 Hz, 17α-H), 7.42–7.59 (m, 5H, aromatic H); MS (CI, NH_3, *m/z*, %): 495 (MH^+, 100%), 512 ($M + NH_4^+$, 95%); high-resolution MS (DCI, CH_4): found 495.3472, $C_{32}H_{47}O_4$ (MH) requires 495.3474; $[\alpha]_D = +51.5^\circ$ (CH_2Cl_2, $c = 4.53$).

*2.3.3.3. 1α-Methylsulfonyloxyhexyl-3-oxo-5α-androstane-17β-yl benzoate (**3d**).* 115 mg (0.23 mmol, 1 Eq.) of alcohol **3c** were dissolved in 5 ml CH_2Cl_2, and freshly distilled NEt_3 (2 Eq.) was added at room temperature. A solution of 30 mg mesyl chloride (0.25 mmol, 1.1 Eq.) in 2 ml CH_2Cl_2 was added dropwise. After stirring at room temperature for 60 min, a saturated $NaHCO_3$ solution was added, and the reaction mixture was extracted three times with CH_2Cl_2. The remaining yellow oil was further purified by LSC using MTBE as the eluent. The mesylate **3d** was isolated as colorless crystals (119 mg, 90% yield). Recrystallization was performed from *n*-heptane/CH_2Cl_2, m.p.: 113–116 °C. ν_{max} (KBr): 1716 (3-CO), 1354 and 1175 (–SO_2–O–); 1277 (C–O–C); ^{1}H NMR ($CDCl_3$) δ: 0.95 (s, 3H, 18-CH_3), 1.14 (s, 3H, 19-CH_3), 2.05–2.58 (m, 5H, 2α, 2β, 4α, 4β, 16α H), 3.00 (s, 3H, –O–SO_2–CH_3), 4.21 (2H, t: $J = 6.6$ Hz), 4.89 (1H, dd: $J = 7.8$ and 9.1 Hz, 17α-H), 7.42–7.58 (m, 5H, aromatic H); MS (CI, NH_3, *m/z*, %): 573 (MH^+, 31%), 590 ($M + NH_4^+$, 100%); high-resolution MS (DCI, CH_4): found 573.3255 (MH), $C_{33}H_{49}O_6S$ (MH) requires 573.3250; $[\alpha]_D = +54.7^\circ$ (CH_2Cl_2, $c = 2.30$).

*2.3.3.4. 1α-Azidohexyl-3-oxo-5α-androstane-17β-yl benzoate (**3e**).* 155 mg (0.27 mmol, 1 Eq.) of the mesylate **3d** was dissolved in 3 ml hexamethyl phosphoric acid triamide (HMPT) at room temperature. Eighty-eight milligrams (1.35 mmol, 5 Eq.) of solid NaN_3 were added, and the suspension was stirred for 4 h. The light yellow reaction mixture was diluted with 10 ml H_2O and extracted three times with MTBE. The organic layer was dried with Na_2SO_4 and further purified by LSC using a PE/MTBE mixture (2:1, v/v) as the eluent. The azidohexyl derivative **3e** was isolated as a pale yellow oil (124 mg, 89% yield). ν_{max} (film): 2096 (N_3), 1716 (3-CO), 1276 (C–O–C); ^{1}H NMR ($CDCl_3$) δ: 0.95 (s, 3H, 18-CH_3), 1.14 (s, 3H, 19-CH_3), 2.05–2.59 (m, 5H, 2α, 2β, 4α, 4β, 16α H), 4.88 (1H, dd: $J = 7.9$ and 9.1 Hz, 17α-H), 7.41–7.59 (m, 5H, aromatic H); MS (EI, 70 eV, *m/z*, %): 519 ($M^{\bullet+}$, 5%), 491 ($M^{\bullet+} - N_2$, 58%), 476 ($M^{\bullet+} - HN_3$, 90%); high-resolution MS (EI, 70 eV): found 519.3461, $C_{32}H_{45}N_3O_3$ requires 519.3459; $[\alpha]_D = +55.4^\circ$ (CH_2Cl_2, $c = 1.90$).

*2.3.3.5. 1α-Azidohexyl-3,3-ethylenedioxy-5α-androstane-17β-yl benzoate (**3f**).* 123 mg (0.24 mmol, 1 Eq.) of the azidohexyl derivative **3e** were dissolved in 20 ml benzene. Then, 300 mg of ethyleneglycol p.a. (4.8 mmol, 20 Eq.) and PTSA (2 mg) were added. The emulsion was refluxed for 2 h using a Dean-Stark water separator. The reaction mixture was treated with one drop Et_3N and washed three times with water. The benzene phase was dried with Na_2SO_4, evaporated under reduced pressure, and further purified by LSC using a PE/MTBE mixture (1:1, v/v) as the eluent. The 3,3-ethylene acetal derivative **3f** was isolated as a viscous oil (23 mg, 91% yield). ν_{max} (film): 1716 (ester), 1277 (ester C–O–C); ^{1}H NMR ($CDCl_3$) δ: 0.93 (s, 3H, 18-CH_3), 1.14 (s, 3H, 19-CH_3), 3.26 (2H, t: $J = 7.0$ Hz, –CH_2–N_3), 3.83–4.0 (m, 4H, –O–CH_2–CH_2–O), 4.87 (1H, dd: $J = 7.7$ and 9.1 Hz, 17α-H), 7.42–7.58 (m, 5H, aromatic H); MS (DCI, NH_3, *m/z*, %): 564 (M^+, 100%), 581 ($M + NH_4^+$, 83%); high-resolution MS (DCI, CH_4): found 563.3714, $C_{34}H_{49}N_3O_4$ requires 563.3723; $[\alpha]_D = +23.3^\circ$ (CH_2Cl_2, $c = 1.25$).

*2.3.3.6. 1α-Aminohexyl-3,3-ethylenedioxy-17β-hydroxy-5α-androstane (**3g**).* $LiAlH_4$ (115 mg, 15 Eq.) was added under a N_2 atmosphere to 10 ml of anhydrous THF. A suspension of the azide **3f** (116 mg, 0.2 mmol, 1 Eq.) in 3 ml THF was added slowly to the $LiAlH_4$ mixture at room temperature and stirred for 20 h. The reaction mixture was cooled in an ice-bath, and the excess of $LiAlH_4$ was destroyed by dropwise addition of 1 ml of 1N NaOH under a nitrogen atmosphere and vigorous stirring. The supernatant was decanted, the remaining slurry was extracted three times with a total of 15 ml THF, and the solvent was evaporated. The crude residue was dissolved in 1 ml methanol, diluted with 3 ml of benzene, and the resulting emulsion was freeze dried. The viscous crude product was dissolved in MeOH/CH_2Cl_2 (1:1, v/v) and then subjected to LSC using a CH_2Cl_2/MeOH/NH_4OH mixture (10:10:1, v/v). The 1-aminohexyl derivative **3g** was isolated as a resin (54 mg, 62% yield). ν_{max} (film): 3364 and 3297 (NH_2, OH);

^{1}H NMR ($CDCl_3$) δ: 0.74 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 2.68 (2H, t: $J = 7.0$ Hz, –CH_2–NH_2), 3.64 (1H, t: $J = 8.5$ Hz, 17α-H), 3.82–3.98 (m, 4H, –O–CH_2–CH_2–); MS (DCI, NH_3, *m/z*, %): 434 (MH^+, 100%), high-resolution MS (EI, 70 eV): found 433.3552, $C_{27}H_{47}NO_3$ requires 433.3556; $[\alpha]_D = +18.0^\circ$ (CH_2Cl_2, $c = 1.15$).

2.3.3.7. 1α-Aminohexyl-17β-hydroxy-5α-androstan-3-one hydrochloride (3). Formation of the hydrochloride and cleavage of the 3-acetal group were performed in two successive steps. First, the 3-ethylenedioxy-1-aminohexylderivative **3g** (52 mg, 0.12 mmol, 1 Eq.) was dissolved in 1 ml MeOH, and 6N HCl (1.5 Eq.) was added. Then, 3 ml of benzene were added, and the solution was immediately freeze dried. Secondly, the resulting dry foam was dissolved in 5 ml anhydrous acetone and maintained in the presence of catalytic amounts of HCl gas at 40 °C for 24 h. Solvent and the formed acetone ethylene acetal were removed in the high vacuum. The 3-oxo-1-hexylamine hydrochloride end product **3** was recovered quantitatively as a colorless rigid resin (51 mg, 27% overall yield). ν_{max} (KBr): 3655 and 3383 (OH), 3030, 2400 and 2100 (NH_3), 1705 (C=O); ^{1}H NMR ($CDCl_3$) δ: 0.76 (s, 3H, 18-CH_3), 1.13 (s, 3H, 19-CH_3), 2.92–3.06 (m, 2H, –CH_2–NH_3^+), 3.67 (m, 1H, 17-H), 8.2 (s broad, 3H, –NH_3); MS (DCI, NH_3, *m/z*, %): 390 (MH^+, 100%), 372 ($MH^+ - H_2O$, 7%), high-resolution MS (DCI, CH_4): found 390.3376, $C_{25}H_{44}NO_2$ ($MH^+ - Cl$) requires 390.3372; $[\alpha]_D = +20.0^\circ$ (CH_2Cl_2, $c = 1.22$).

2.4. Size-exclusion chromatography of affinity purified homodimeric SHBG

Purified human SHBG (lot #A0011105, isolated by affinity chromatography from human pregnancy plasma) was purchased from Fitzgerald Industries International, Concord, MA, USA. A concentrated SHBG solution (5 mg/ml) was prepared in bidistilled water from the supplied lyophilized material. Subsequently, analytical size-exclusion chromatography was performed on a Superdex 200 HR10/30 column using the ÄKTA*FPLC* system, preequilibrated with buffer solutions containing 5 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 50 mM K_2SO_4 and 1 mM $CaCl_2$. Sample size was 0.25 ml at a flow rate of 0.2 ml/min, and UV monitoring was performed at 280 nm. The column was calibrated with gel filtration LMW and HMW standard proteins, which were run under identical conditions. Gel chromatography fractions of SHBG were run on a discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [34]. A stacking gel and a 10% polyacrylamide separation gel were applied under reducing conditions using a discontinuous buffer system (0.1 M Tris base, pH 8.25, 0.1 M Tricine and 0.1% SDS at the cathode and 0.2 M Tris, pH 8.9 at the anode). The protein was electrotransferred to a PVDF membrane (pore size 0.45 μm), probed with the specific anti-SHBG pab, and visualized with HRP-conjugated secondary pab using 3-amino-9-ethyl-carbazole (AEC) as dye substrate. SHBG-positive fractions were pooled and concentrated using Microcon centrifugal filters with a molecular weight cut-off of 30 kDa. After adjustment to a concentration of 0.5 mg/ml, the purified SHBG was stored in aliquots at −20 °C until analysis.

2.5. Relative binding affinities of the 1α- and 17α-DHT derivatives to SHBG

RBA of 1α- and 17α-DHT derivatives to SHBG were determined under equilibrium conditions at 37 °C using Con A-Sepharose as solid phase and ^{3}H-T as tracer according to the method of Dunn et al. [25]. The competition curves were obtained in the presence of increasing concentrations of each ligand. The purified homodimeric SHBG was used at a constant concentration of 100 nM. SHBG was first preincubated for 30 min with the Con A-Sepharose slurry at room temperature. After centrifugation at 3000 × *g*, the pellet was washed extensively and resuspended in PBS buffer. Then, ^{3}H-T (approximately 50,000 dpm) was added together with various final concentrations (10–5000 nM) of the three radioinert 1- and 17-aminoalkyl ligands. After incubation for 90 min at 37 °C with shaking, the samples were centrifuged at 3000 × *g*, and the supernatants were counted for β-radioactivity using opti-Fluor scintillation fluid (Packard Instruments, Groningen, The Netherlands) in a 1219 Rackbeta liquid scintillation counter from Wallac. Specific binding of the competitor was expressed as a percentage of the maximum binding that was calculated by subtracting non-specific binding (estimated in the presence of 100-fold excess of T) from total binding. RBA to SHBG were calculated according to the following equation:

RBA = $(C_{0.5,T}/C_{0.5,competitor}) \times 100$ (with $C_{0.5}$ representing the concentration of radioinert competitor required to reduce ^{3}H-T specific binding by 50%; RBA value for T was set at 100%).

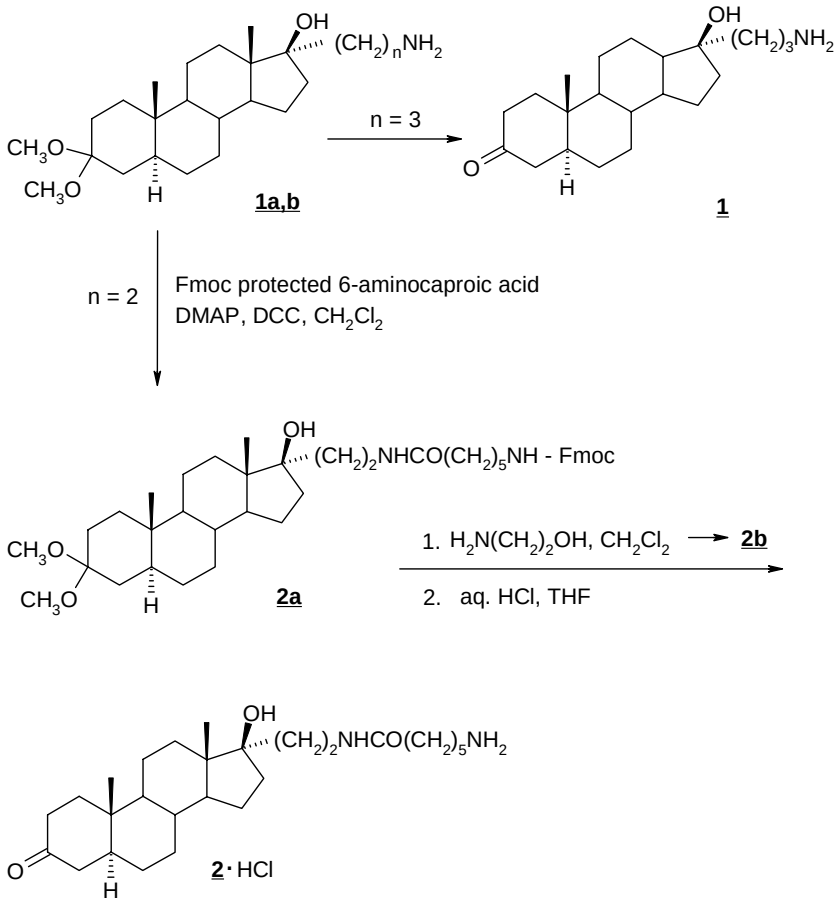
Determination of the association constant (K_a) of SHBG for binding T was performed by adding unlabeled T in final concentrations from 1 to 1000 nM. Subsequently, K_a was evaluated from the slope of the Scatchard plot ($y =$ dpm supernatant/dpm total, $x =$ nM ^{3}H-T bound) and corresponded to a value of 3.19 ± 0.32 (S.D.) $\times 10^9$ M^{-1} ($n = 5$). From this, the association constants for the 1α- and 17α-DHT derivatives were calculated using the following equation:

$K_{a_{competitor}} = K_{a_T}/((1 + R)/RBA) - R$ (with $R = B/B_0$ at 50% displacement) [26].

3. Results and discussion

3.1. Syntheses

Syntheses of the 17α-alkylamine derivatives of DHT followed the synthetic pathway described in [29], whereas for the 1α-hexylamine DHT derivative, a route with a copper-catalyzed conjugate addition of a Grignard reagent



Scheme 1. Synthetic pathway for 17α-aminopropyl- and 17α-aminocaproylamidoethyl-17β-hydroxy-5α-androstan-3-one.

to a 1-en-3-one functional group as the key reaction was established.

*3.1.1. 17α-Aminopropyl-17β-hydroxy-5α-androstan-3-one (**1**)*

The synthesis started from 5α-androstan-3,17-dione, which was converted to 3,3-(dimethoxy)-5α-androstan-17-one. Condensation with the dimethylsulfonium methylide reagent [35,36] led to the formation of a 17β-spirooxirane intermediate as the major product with more than 90% yield [37]. The epoxide ring was opened by an acetonitrile carbanion to give a 17α-ethylcyanide group, which was reduced by $LiAlH_4$ to the 3,3-(dimethoxy)-17α-aminopropyl-DHT compound **1a**. Acid hydrolysis of the 3-dimethoxy acetal group of **1a** led to the 17α-aminopropyl-DHT derivative **1** (Scheme 1).

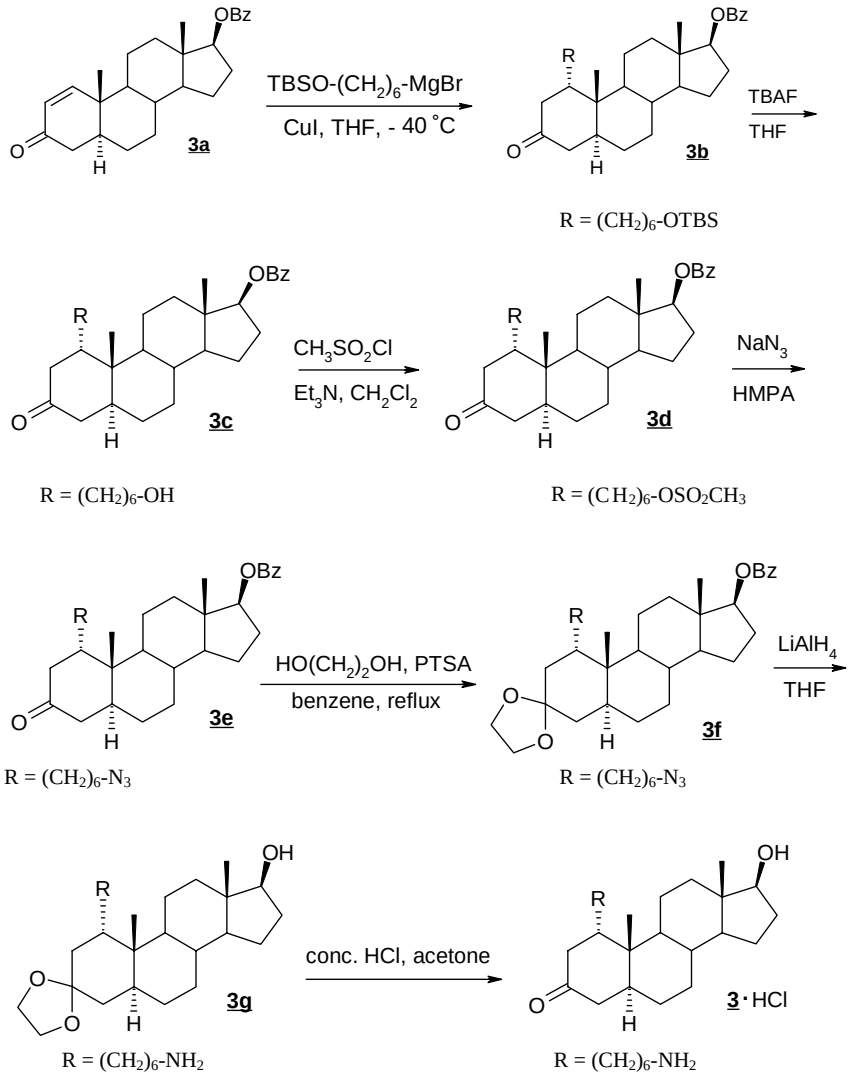
*3.1.2. 17α-Aminocaproylamidoethyl-17β-hydroxy-5α-androstan-3-one (**2**)*

The 3,3-(dimethoxy)-17α-aminoethyl-DHT precursor **1b** was synthesized from 5α-androstan-3,17-dione according to the procedure given in [29]. Condensation of the 17α-ethylamine group with Fmoc-protected 6-aminocaproic acid was accomplished using the carbodiimide method to provide the Fmoc protected amide derivative **2a**. The Fmoc group was then cleaved to give the amine derivative **2b**. Hydrolysis of the 3-dimethoxy acetal protecting group led to the final 17α-substituted amine derivative **2**, which was isolated as the hydrochloride salt in a high overall yield (Scheme 1).

In a ^{1}H NMR NOSY experiment on compound **2** (data not shown), a NOE of the C-18β methyl to the 5′-methylene protons of the 17α-side chain was observed, suggesting the existence of a hydrogen bond between the C-17β hydroxyl and the amide carbonyl functions. Therefore, under these experimental conditions (nonpolar organic solvent), the aminocaproyl-amidoethyl side chain was assumed to have a conformation in which it remained in close proximity to the steroidal D-ring of compound **2**, thus potentially inducing steric hindrance around the 17-OH group and restricting the through-space distance between C-17 and the terminal amine.

*3.1.3. 1α-Aminohexyl-17β-hydroxy-5α-androstan-3-one (**3**)*

The synthetic route chosen for access to the 1α-alkyl DHT derivatives was established from the 17β-benzoyloxy-5α-androst-1-en-3-one precursor (**3a**) [31]. Using a copper(I)-mediated conjugate addition [38–40] of the ω-silyloxyhexyl Grignard reagent $TBSO(CH_2)_6MgBr$, the 1α-alkyl-substituted derivative **3b** was obtained as the only product in 74%



Scheme 2. Synthetic pathway for 1α-aminohexyl-17β-hydroxy-5α-androstan-3-one.

yield. After removing the silyl group, mesylation of the terminal hydroxyl group led to compound **3d**. Substitution with sodium azide gave the azide **3e**. The 3-oxo group of this latter compound was then protected as the ethylene acetal giving the compound **3f**. Reduction of the azide with $LiAlH_4$ provided the amine derivative **3g**. Finally, the protecting acetal group was removed by transacetalization using gaseous HCl and acetone, which also converted the target 1α-aminohexyl DHT molecule **3** into the hydrochloride salt (Scheme 2).

In the ^{1}H NMR analyses for all 1α-DHT derivatives, we observed a typical signal pattern for the 2α, 2β, 4α, and 4β protons with deducible coupling constants. The chemical shifts of these protons are in agreement with data described for a series of 5α-androstanes [41,42]. Kirk et al. [41] found for 5α-androstan-3-one the following chemical shifts (δ, ppm): 1α H 1.34, 1β H 2.01, 2α H 2.27, 2β H 2.37, 4α H 2.06, 4β H 2.24. In our 1α-DHT derivatives, we found similar δ-values (e.g. for **3d**: 2α H 2.31, 2β H 2.55, 4α H 2.08, 4β H 2.23). The chemical shift of the 1β proton could be assigned by H–H correlated NMR spectroscopy in the multiplet at 1.78–1.86 ppm. The coupling constants of the A-ring protons (2α, 2β, 4α, and 4β, together with the 5α H) could be identified; the proton system may be considered as 2 *ABX* spectra (1β, 2α, 2β and 4α, 4β, 5α), which were resolved at first order, as shown in Fig. 1 for **3d**. The corresponding *J* values are summarized in Table 1.

3.2. Size-exclusion chromatography of affinity purified SHBG

We used commercially available human SHBG, affinity purified from pooled pregnancy sera. Although the purity of this SHBG was high (>98% by SDS–PAGE), a further gel chromatography was performed in order to further improve the purity and to remove possible oligomeric forms as well as bound steroidal ligands (mainly E2) in the SHBG preparation [43]. As shown in Fig. 2, a PAGE under denaturing conditions showed the presence of two distinct monomers at

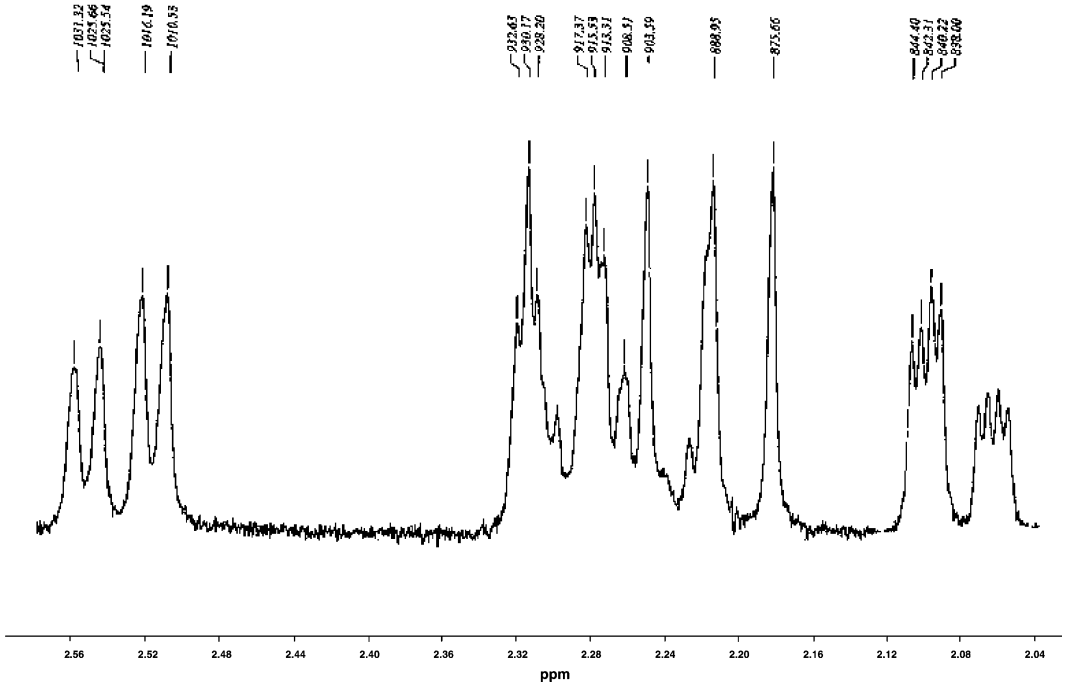


Fig. 1. ^{1}H NMR spectrum (400 MHz; downfield portion) of 17β-benzoyloxy-1α-methyl-sulfonyloxyhexyl-5α-androst-3-one (**3d**), giving a representative overview of the signal pattern of the 2α (2.31 ppm), 2β (2.55), 4α (2.08) and 4β (2.23) protons of ring A.

molecular weights of approximately 50 and 47 kDa, as expected from the well established size heterogeneity of SHBG attributed to variations in carbohydrate content [44]. Traces of dimeric protein were seen at 90 kDa, while no other impurities could be detected.

3.3. Determination of binding affinities

The equilibrium measurements shown in Fig. 3 and data presented in Table 2 depict that the two 17α-DHT derivatives **1** and **2** with either a short or a long spacer interact almost identically with SHBG. The calculated K_a values were 1.25×10^7 and 1.50×10^7 M^{-1}, respectively. The 1α-DHT derivative **3**, however, showed a much higher binding affinity to SHBG, with a K_a value of 1.40×10^9 M^{-1}. This rep-

Table 1
Splitting patterns and *J* values for the 2α, 2β, 4α, and 4β protons of the derivatives **3b**–**3d**, obtained by ^{1}H NMR analysis (for details, see Section 3.1)

Proton	Compound	δ (ppm)	Splitting pattern	*J* (Hz)
2α	**3b**	2.33	dd	15.0/2.0
	3c	2.32	dd	15.0/2.0
	3d	2.31	dd	15.0/2.0
	3e	2.32	dd	15.0/2.3
2β	**3b**	2.54	dd	15.0/5.6
	3c	2.55	dd	15.0/5.6
	3d	2.55	dd	15.0/5.7
	3e	2.55	dd	15.0/5.6
4α	**3b**	2.10	ddd	15.0/4.3/2.0
	3c	2.09	ddd	15.0/4.4/2.0
	3d	2.08	ddd	15.0/4.4/2.1
	3e	2.08	ddd	15.0/4.5/2.2
4β	**3b**	2.22	dd	15.0/13.0
	3c	2.22	dd	15.0/13.2
	3d	2.22	dd	15.0/13.0
	3e	2.22	dd	15.0/13.4

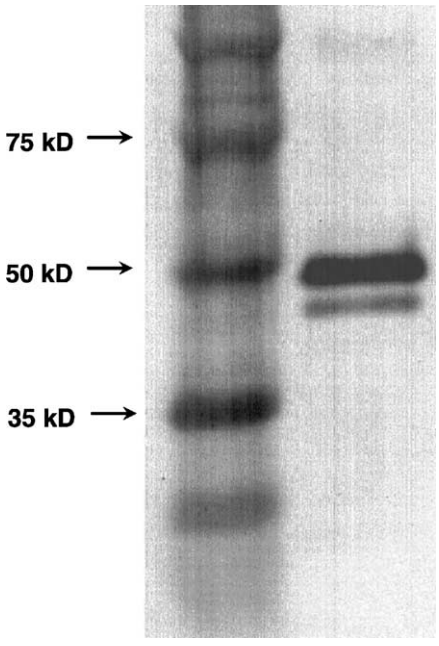


Fig. 2. Western blot of affinity-purified native human SHBG after gel electrophoresis in a 10% SDS polyacrylamide gel. Primary antibody: rabbit anti-human SHBG pab; secondary antibody: HRP-labeled goat anti-rabbit IgG pab; dye: 3-amino-9-ethylcarbazole. Left lane: molecular weight marker; right lane: SHBG gel filtration preparation.

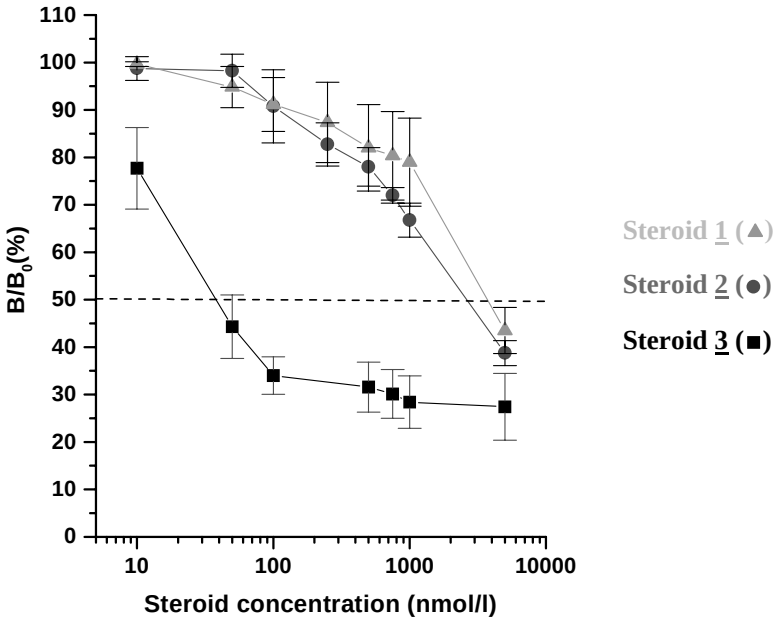


Fig. 3. Displacement of ^{3}H-testosterone by the three unlabeled 1α- and 17α-aminoalkyl DHT derivatives from SHBG. The results are plotted as percent ^{3}H-T bound to SHBG vs. unlabeled steroid concentrations; values are given as means ± 1 S.D. (n = 4, 4, 6). **1** (▲), **2** (●), **3** (■).

Table 2
Binding affinities of the DHT derivatives **1**–**3** to SHBG, measured under equilibrium conditions

1 (n = 4)			**2** (n = 4)			**3** (n = 6)			T (n = 5)
RBA (%)	R	K_a (10^7 M^{-1})	RBA (%)	R	K_a (10^7 M^{-1})	RBA (%)	R	K_a (10^7 M^{-1})	K_a (10^7 M^{-1})
0.62 ± 0.05	0.435 ± 0.024	1.25 ± 0.79	0.60 ± 0.01	0.395 ± 0.006	1.50 ± 0.10	52.0 ± 11.2	0.423 ± 0.041	140 ± 32	319.4 ± 31.7

Results are given as means ± 1 S.D. RBA = relative binding activity; $R = B/B_0$ at 50% displacement.

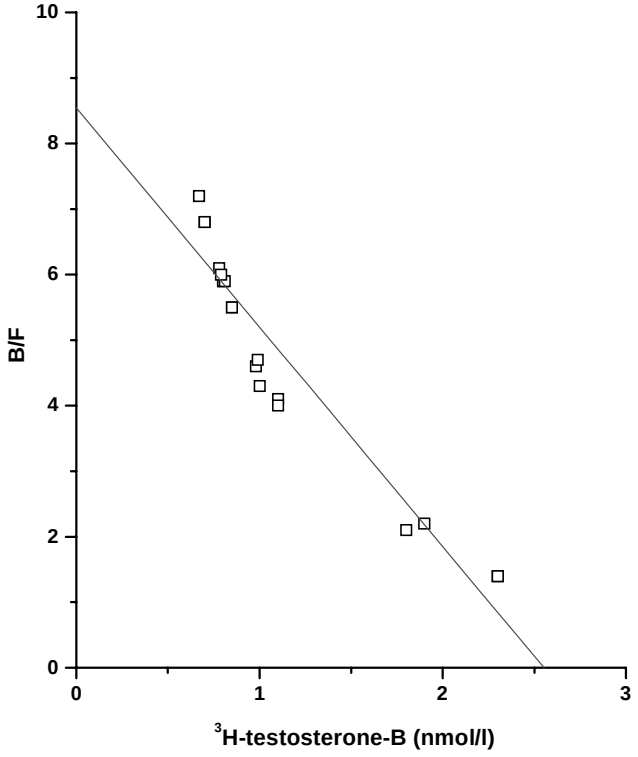


Fig. 4. Scatchard analysis of ^{3}H-testosterone binding to SHBG at 37 °C.

resents about half of the K_a value (3.19×10^9 M^{-1}) determined in parallel for unsubstituted T (see Scatchard analysis in Fig. 4), which is in good agreement with values given in the literature [25,45–47].

These relatively high association constants, in particular, the unexpectedly high affinity of the 1α-aminohexyl DHT compound **3**, which had a much larger 1-substituent than the previously known 1α-methyl substituted mesterolone ligands (vide infra), encouraged us to establish a biosensor system for ligand-binding studies of the interaction of these 17α- and 1α-alkylamine DHT derivatives with SHBG. These investigations using a surface plasmon resonance (SPR) biosensor are presented elsewhere [48]. The approach is to immobilize the amino DHT derivatives covalently onto a carboxymethyl dextran-coated surface of the system's flow cell by the *N*-ethyl-*N*′-(3-diethyl-aminopropyl)-carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) technique. Such biosensor measurements may shed light on the thermodynamics and kinetics of the interaction of steroids and other potential ligands of the SHBG binding site. The access to immobilized 1α- and 17α-substituted DHT derivatives, displaying freely available D- and A-rings, respectively, provides a potentially useful tool for studying the interaction of the unsubstituted parts of the steroid skeleton and for evaluating the role of

conformations of the covalent linker arms, which might influence steroid positioning in the SHBG binding site.

Acknowledgements

The authors are indebted to Ms. Manuela Meyer and Ms. Monika Söder for their excellent technical assistance.

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