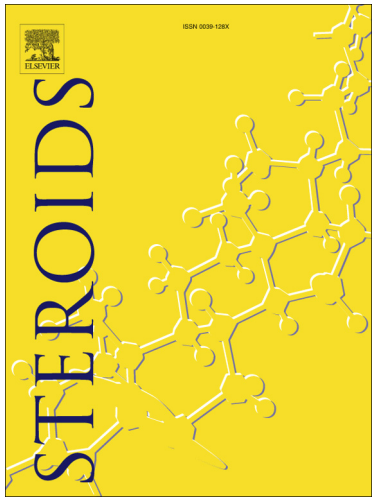

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PII: S0039-128X(17)30089-2
DOI: http://dx.doi.org/10.1016/j.steroids.2017.05.011
Reference: STE 8108

To appear in: *Steroids*

Received Date: 14 April 2017
Revised Date: 12 May 2017
Accepted Date: 18 May 2017

Please cite this article as: Lao, K., Sun, J., Wang, C., Lyu, W., Zhou, B., Zhao, R., Xu, Q., You, Q., Xiang, H., Design, Synthesis and Biological Evaluation of Novel Androst-3,5-diene-3-carboxylic Acid Derivatives as Inhibitors of 5α-Reductase Type 1 and 2, *Steroids* (2017), doi: http://dx.doi.org/10.1016/j.steroids.2017.05.011

Design, Synthesis and Biological Evaluation of Novel Androst-3,5-diene-3-carboxylic Acid Derivatives as Inhibitors of 5α-Reductase Type 1 and 2

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Abstract

5α-Reductase is a key enzyme responsible for dihydrotestosterone biosynthesis and has been recognized as an important target for discovering new drugs against benign prostatic hyperplasia (BPH). In this study, a series of novel steroidal androst-3,5-diene-3-carboxylic acids have been designed and synthesized. Biological evaluations were performed on their 5α-reductase inhibitory activities by both *in vitro* enzyme inhibition assay and *in vivo* by prostate weighing method. Results showed that most of them displayed excellent 5α-reductase inhibitory potency. Detailed evaluation indicated that most of the compounds displayed slightly higher inhibition potency towards type 2 isozyme. Among all the compounds, **16a** was found to be the most potential inhibitor with the IC_{50} of 0.25μM and 0.13μM against type 1 and 2 isozymes respectively. *In vivo* 5a-reductase inhibitory evaluation of **16a** also showed a more significant reduction effect ($p<0.001$) in rat prostate weight than epristeride. Furthermore, the results of *in silico* ADME study indicated that compound **16a** exhibited good pharmacokinetic properties. Thus, **16a** could serve as promising lead candidates for further study.

Keywords：benign prostatic hyperplasia (BPH); 5α-reductase inhibitors; steroidal 3-carboxylic acids; epristeride

1. Introduction

Benign prostatic hyperplasia (BPH), the leading disorder in aging males, is characterized by a progressive enlargement of prostatic tissue, resulting in obstruction of the proximal urethra and causing urinary flow disturbances [1]. Nearly half of men aged over 50 years have histological evidence of BPH and, after the age of 70, the proportion increases to 80% [2]. It has long been established that prostatic growth is stimulated by androgens. Testosterone (T), which is

biosynthesized in testicles and adrenal glands, can be further converted to more potent dihydrotestosterone (DHT) by 5α-reductase in prostate. 5α-Reductase is a membrane-bound, NADPH-dependent enzyme that irreversibly catalyzes the reduction of 4-ene-3-oxosteroids to the corresponding 5α-3-oxosteroids (**Figure 1**). The 5α-reductase family is composed of three isozymes. 5α-Reductase type 1 (5αR-1) is mainly expressed in skin and liver with the optimum pH range of 6.0~8.5, while the 5α-reductase type 2 (5αR-2) with the optimum pH 5.5, is mainly expressed in prostate and other genital tissues [3]. More recently, type 3 isozyme was identified in castration-resistant prostate cancer cells as well as other tissues such as pancreas, brain, skin and adipose tissues [4, 5]. 5α-Reductase has emerged as a target for the pharmaceutical treatment of BPH as abnormally high activity of the enzyme in human results in excessive DHT levels. Thus the suppression of DHT biosynthesis by 5α-reductase inhibitors is a logical treatment for BPH [6].

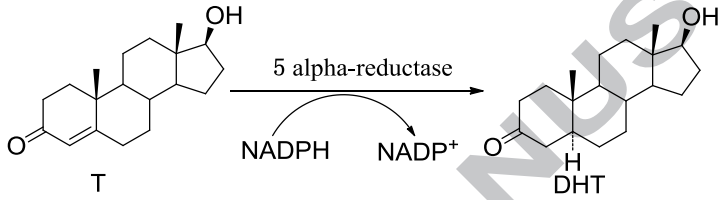


Fig. 1 The reaction catalyzed by 5α-reductase.

Finasteride (**1**) and dutasteride (**2**), the two widely used 5α-reductase inhibitors in clinical practice, are both 4-azasteriods. However, in spite of their success in improving the quality of life in men suffering from BPH symptoms, a substantial body of evidence exists which points to serious and potentially ill-health effects including loss or reduced libido, erectile dysfunction, orgasmic and ejaculatory dysfunction, development of high grade prostate tumors, potential negative cardiovascular events, and depression, which was suspicious of the presence of two nitrogen atoms at C-4 and C-20 positions [7-8]. Steroidal 3-carboxylic acids are a kind of novel 5α-reductase inhibitors, among which, epristeride (**3**) was the first one launched in 2000 as a therapy for BPH[9].

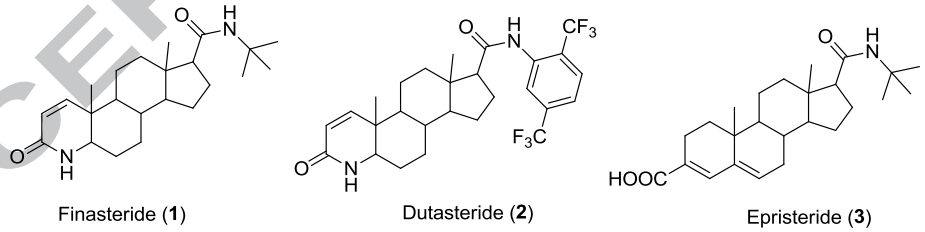


Fig. 2 The structures of the finasteride (**1**), dutasteride (**2**), and epristeride (**3**).

Since the 5α-reductase isozymes have not been purified and crystalized due to their unstable nature, the design of novel 5α-reductase inhibitor mainly relies on structure-based optimization approach. The ring A of the 3-carboxylic acids was designed to mimic the putative enzyme-bound enolate intermediate by incorporating sp^2-hybridized centers at C-3 and C-4 and, most critically, an anionic carboxylic acid at C-3 as a charged replacement for the enolate oxyanion. Because of this presumably favorable electrostatic interaction between the carboxylate and the positively charged oxidized cofactor, the acrylate preferentially binds in a ternary complex with enzyme and NADP+, which leads to the noncompetitive kinetic mechanism [9-12]. The 3D-QSAR study of 3-carboxysteroids indicated that presence of bulky electronegative substitutions at 17-C was

favorable for maximal activity, and around position C-3 of steroid skeleton, electronegative groups and small steric substituents were required for optimal inhibitory activity [13].

Interestingly, considerable attention has been also focused on the modifications of substitutions at the C-17 position of steroid skeleton. Among them, aniline moiety has been widely used as functional groups in compounds targeting androgen axis as exemplified in **Figure 3** [14-17]. A series of androstene 17β-carboxamides were reported by C.Amaral et al. as 5α-reductase inhibitors with anti-proliferate activities. In our previous study, several androstene-17β-carboxamides has been disclosed for their strong inhibition of 5α-reductase inhibition and modulations of androgen receptor (**Figure 3**) [18].

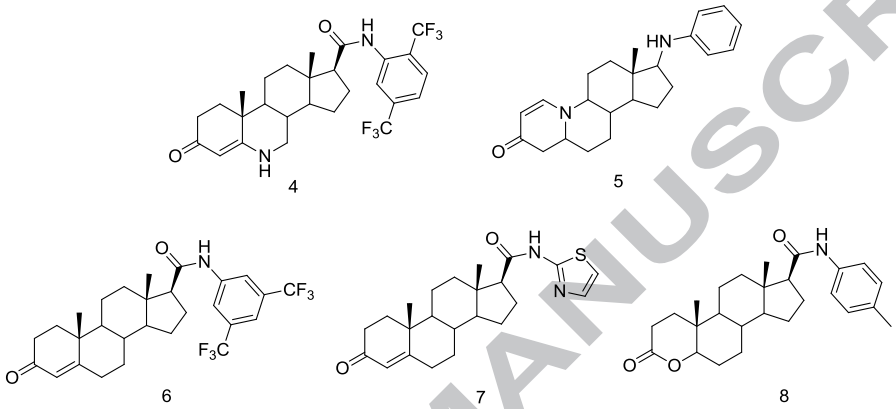


Fig.3 Structures of known compounds bearing aniline substitutions

Inspired by the promising research results mentioned above, we set out to design a number of 3-carboxylic acids with various substituted 17β-N-phenylcarbamoyl groups. Their 5α-reductase inhibitory activities *in vitro* toward 5α-reductase type 1 and 2 isozymes was evaluated. The *in vivo* 5α-reductase inhibitory activity of the most active compound **16a** was further assessed by prostate weighting method. *In silico* ADME analysis was also performed to predict important pharmacokinetic parameters.

2. Experimental

2.1. Chemistry

2.1.1. General procedure

Melting points of compounds were measured on a RY-1 melting point apparatus and were uncorrected. Nuclear magnetic resonance (1H NMR) spectra were recorded on a Bruker AV-300 (300 MHz) spectrometer as deuterochloroform ($CDCl_3$) solutions using tetramethylsilane (TMS) as an internal standard ($\delta = 0$) unless noted otherwise. Electron impact mass spectral (EI-MS) data were obtained on a SHIMADZU GCMS-QP2010 system. All chemicals were purchased from commercial sources and were used without further purification unless otherwise noted. The solvents (such as MeOH, EtOAc, EtOH, CH_2Cl_2 and others) were C.P. grade purchased from Nanjing Chemical Co., Ltd. and used without further purification. Column chromatography (CC) was carried out on silica gel (200–300 mesh, Qingdao Ocean Chemical Company, China). Thin-layer chromatography (TLC) analyses were carried out on silica gel GF254 (Qingdao Ocean Chemical Company, China) glass plates (2.5 cm × 10 cm with 250 μm layer). Concentration and evaporation of the solvent after reaction or extraction was carried out on a rotary evaporator operated at reduced pressure.

2.1.2. Synthesis of 3β-hydroxyl-5-pregnen-20-one(**10**)

To the solution of 3β-hydroxyl-5,16-Pregnadien-20-one-3-acetate (6g, 16.8mmol) in 180ml EtOAc, 0.6g Raney Ni was slowly added at 0℃. The reaction mixture was stirred at 25℃for 1h. After filtration, KOH (0.94g, 16.8mmol) was added. The reaction mixture was refluxed for another 2h. After cooled to room temperature, the resulting precipitate was filtered, washed with water, and dried to give white solide 4.85g, yield 92.81%. ^{1}H NMR ($CDCl_3$, 300MHz) δ: 5.3 (s, 1H, 6-H), 3.5 (s, 1H, 3-H), 2.5 (1H, t, 17-H), 2.1(s, 3H, 21-H), 1.0 (s, 3H, 19-CH_3), 0.6 (s, 3H, 18-CH_3) ppm; ESI-MS *m/z*: 339$[M+Na]^+$.

2.2. Synthesis of 3-oxo-4-pregnen-20-one(**11**)

To the solution of **10** (7g, 22mmol) in 112ml totuene, cyclohexanone(21ml, 0.2mol) was added. After circumfluence to repel water for 2h, aluminium isopropoxide (1.05g, 5.1mmol) was added, and then refluxed for another 1.5g. The resulting mixture was poured into 10ml cold 10% NaOH aqueous solution. The solvent was evaporated by steam distillation to provide yellow solid 6.3g, yield 90.03%. ^{1}H NMR ($CDCl_3$, 300MHz) δ: 5.8 (s, 1H, 4-H), 2.6 (1H, t, 17-H), 2.4 (m, 2H, 2-H), 2.1(s, 3H, 21-H), 1.2 (s, 3H, 19-CH_3), 0.7 (s, 3H, 18-CH_3) ppm; ESI-MS *m/z*: 315$[M+H]^+$.

2.3. Synthesis of17β-carboxy-androst-4-ene-3-one(**12**)

To the solution of NaOH (5.9g, 143mmol) in 50ml water, Br_2 (1.9ml, 37mmol) was slowly added at 0℃ to provide NaOBr aqueous solution. Resolving **11** (3.5g, 11mmol) in 105ml dioxane and 36ml water, the NaOBr aqueous solution was added. After stirred at room temperature for 1.5h, 19ml 10% Na_2SO_3 was added and refluxed for 15min. After cooled to room temperature, 2N hydrochloric acid was added to adjust pH to 2 with in ice bath. Dioxane was evaporate and The resulting precipitate was filtered, washed with water, and dried to give crude product. Recrystallized by acetone to provide white solid 2.6g, yield 75.48%. ^{1}H NMR ($CDCl_3$, 300MHz) δ: 5.7 (s, 1H, 4-H), 2.4-2.6 (5H, m, 2-H, 6-H, 17-H), 1.2 (s, 3H, 19-CH_3), 0.9 (s, 3H, 18-CH_3) ppm; ESI-MS *m/z*: 315$[M-H]^-$.

2.3.1. Synthesis of 3-Bromo-androsta-5,16-dien-17β-carboxylic acid (**13**)

To solution of **12**(10g, 32mmol) in 52ml acetic acid, PBr_3 (4.6ml,) was added dropwise. The resultant mixture was then stirred at room temperature for 1h, poured into water and filtered to get white solid **5** (7.8g, 65%). ESI-MS m/z: 378$[M-H]^-$.

2.3.2. General procedures of synthesis of **14a~14j**

To a solution of 3-Bromo-androsta-5,16-dien-17β-carboxylic acid (**13**) (1g, 3.16mmol), anhydrous pyridine (0.32ml, 4.12mmol) in 15ml anhydrous toluene, oxalyl chloride (0.34ml, 3.56mmol) in 7ml anhydrous toluene was added drop wise at 0℃. The resulting solution was stirred at room temperature 1.5h then corresponding amine (7.9mmol) was added. The mixture was stirred at room temperature for another 8h. 30ml water was added and the reaction mixture was extracted with ethyl acetate. It was dried with sodium sulfate and the solvent was removed in vacuum. The compound was purified by column chromatography (PE /EA, 9:1).

2.3.3. 2.3.2. General procedures of synthesis of **16a~16j**

To a solution of **14a~14j** (1.35mmol) in 15ml DMF, CuCN (0.24g, 2.7mmol) was added. The resultant mixture was then stirred at 180℃ for 12 h. After cooled to room temperature, the residue was removed by filtration. The filtrate was quenched by 75ml water and filtered to get dark yellow powder without further purification. To a solution of **15a~15j** (0.84mmol) in 10ml ethanol, 0.17ml 20% NaOH aqueous solution was added. The resultant mixture was then refluxed for 6h, cooled to room temperature and removed the ethanol under vacuum. Adjust pH to 2 with 2N hydrochloric acid in ice bath. The resulting precipitate was filtered, washed with water, and dried to give crude

product. The compound was purified by column chromatography (PE/Ethyl acetate, 4:1).

2.3.4. 17β-[N-[2,5-bis(trifluoromethyl)phenyl]amide]-androsta-3,5-dien-3-carboxylic acid (**16a**)

Yield 43%; m.p.: 280-284℃; ^{1}H NMR (DMSO-d6, 300MHz) δ: 12 (s, 1H, COOH), 9.4 (s, 1H, NH), 8.0~7.7 (m, 3H, Ar-H), 6.8 (s, 1H, 4-H), 5.9 (s, 1H, 6-H), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR (DMSO-d6, 75 MHz) δ: 175.04, 168.77, 141.20, 137.79, 131.37, 126.52, 55.97,54.99, 48.02, 43.52, 38.11, 34.64, 33.50, 32.12, 31.78, 24.40, 23.73, 22.02, 20.96, 19.13, 13.50;IR (KBr) ν:3434, 2930, 1725, 1384, 1097 cm^{-1}; ESI-MS m/z:554[M-H]$^{+}$; Anal.calcd for $C_{29}H_{31}F_6NO_3$: C 62.70, H 5.62, N 2.52; Found: C 62.84, H 5.384, N 2.318.

2.3.5. 17β-[N-[3,5-bis(trifluoromethyl)phenyl]amide]-androsta-3,5-dien-3-carboxylic acid (**16b**)

Yield 23%; m.p.: 140-142℃; ^{1}H NMR (DMSO, 300MHz) δ: 10.2 (s, 1H, COOH), 8.4 (s, 1H, NH), 7.73~7.68 (m, 3H, Ar-H), 7.3 (1H, s, Ar-H), 6.8 (s, 1H, 4-H), 5.7 (s, 1H, 6-H), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR (DMSO-d6, 75 MHz) δ: 175.1, 168.85, 141.21, 137.76, 131.46, 126.50, 55.95, 54.94, 48.00, 43.56, 38.08, 34.67, 33.48, 32.12, 31.79, 24.42, 23.69, 21.79, 20.92, 19.17, 13.57; IR (KBr) ν:3357, 2941, 1670, 1436, 1134 cm^{-1}; ESI-MS m/z:554[M-H]$^{+}$; Anal.calcd for $C_{29}H_{31}F_6NO_3$: C 62.70, H 5.62, N 2.52; Found: C 62.95, H 5.298, N 2.309.

2.3.6. 17β-[N-[4-carboxy-3-methylphenyl]amide]-androsta-3,5-dien-3- carboxylic acid (**16c**)

Yield 35%; m.p.: 294-296℃; ^{1}H NMR (DMSO, 300MHz) δ: 9.0 (s, 1H, NH), 7.8~7.6 (m, 3H, Ar-H), 6.9 (s, 1H, 4-H), 5.9 (s, 1H, 6-H), 2.3 (s, 3H, Ar-CH_3), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 141.41, 132.31, 132.25, 129.58, 87.84, 58.50, 56.53, 53.05, 34.52, 32.77, 29.68, 24.34, 18.97, 17.73, 13.29; IR (KBr) ν: 3439, 2967, 2200, 1681, 1292 cm^{-1}; ESI-MS m/z:476[M-H]$^{+}$; Anal.calcd for $C_{29}H_{35}NO_5$: C 72.93, H 7.39, N 2.93; Found: C 73.27, H 7.71, N 2.54.

2.3.7. 17β-[N-[4-methoxypheny]amide]-androsta-3,5-dien-3- carboxylic acid (**16d**)

Yield 24%; m.p.: 194-196℃; ^{1}H NMR (DMSO, 300MHz) δ: 9.4 (s, 1H, NH), 7.5 (d, 2H, *J*=8.67Hz, 2',6'-H), 6.7 (d, 2H, *J*=8.67Hz, 3',5'-H), 6.7 (s, 1H, 4-H), 5.7 (s, 1H, 6-H), 3.7 (s, 3H, -OCH_3), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 170.90, 170.27, 156.29, 140.84, 134.88, 131.25, 130.70, 127.58, 121.78, 114.13, 57.84, 56.59, 55.51, 48.06, 44.30, 38.42, 34.75, 33.47, 32.15, 31.76, 24.42, 23, 69, 21.99, 21.03, 18.95, 13.30; IR (KBr) ν: 3414, 2940, 1661, 1511, 1234 cm^{-1}; ESI-MS m/z:450[M+H]$^{+}$; Anal.calcd for $C_{28}H_{35}NO_4$: C 74.80, H 7.85, N 3.12; Found: C 74.55, H 8.117, N 2.755.

2.3.8. 17β-[N-[4-chloro-3-trifluoromethylphenyl]amide]-androsta-3,5-dien-3-carboxylic acid (**16e**)

Yield 13%; m.p.: 158-164℃; ^{1}H NMR (DMSO, 300MHz) δ: 10.0 (s, 1H, COOH), 8.2 (s, 1H, NH), 7.9 (d, 1H, *J*=8.7Hz, 2'-H), 7.7 (d, 1H, *J*=8.67Hz, 5'-H), 7.3 (m, 1H, 6'-H), 6.7 (s, 1H, 4-H), 5.7 (s, 1H, 6-H), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 171.44, 170.22, 140.81, 136.96, 134.96, 131.93, 130.71, 127.56, 123.66, 58.07, 56.64, 48.00, 44.53, 38.40, 34.74, 33.49, 32.09, 31.74, 30.88, 29.68, 23.69, 21.99, 21.00; IR (KBr) ν: 3339. 2940, 1659, 1413, 1173 cm^{-1}; ESI-MS m/z:523[M+H]$^{+}$; Anal.calcd for $C_{28}H_{31}ClF_3NO_4$: C 64.43, H 5.99, N 2.68; Found: C 64.55, H 5.776, N 2.556.

2.3.9. 17β-[N-[3-trifluoromethylphenyl]amide]-androsta-3,5-dien-3-carboxylic acid (**16f**)

Yield 17%; m.p.: 140-142℃; ^{1}H NMR (DMSO, 300MHz) δ: 9.9 (s, 1H, NH), 8.1 (s, 1H, 2'-H), 7.8 (d, 1H, *J*=8.01Hz, 6'-H), 7.5~7.3 (m, 1H, 4',5'-H), 6.7 (s, 1H, 4-H), 5.7 (s, 1H, 6-H), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR (DMSO-d6, 75 MHz) δ: 171.53, 142.92,139.99, 139.47, 132.70, 129.74, 122.79, 120.10, 119.16, 115.35, 105.99, 56.27, 55.7, 47.27, 44.06, 37.35,

33.83, 32.19, 31.50, 31.26, 31.06, 24.08, 23.63, 23.34, 20.22, 18.60, 13.38; IR (KBr) ν: 3342. 2937, 1661, 1333, 698 cm^{-1}; ESI-MS m/z:523$[M+H]^+$; Anal.calcd for $C_{28}H_{32}F_3NO_3$: C 68.98, H 6.62, N 2.87; Found: C 68.56, H 7.173 N 2.952.

2.3.10. 17β-[N-phenylamide]-androsta-3,5-dien-3-carboxylic acid (**16g**)

Yield 24%; m.p.: 138-140℃; ^{1}H NMR (DMSO, 300MHz) δ: 9.5 (s, 1H, NH), 7.6 (d, 2H, *J*=7.89Hz, 2',6'-H), 7.3 (t, 2H, *J*=7.53Hz, 3',5'-H), 7.0 (t, 1H, *J*=7.26Hz, 4'-H), 6.7 (s, 1H, 4-H), 5.7 (s, 1H, 6-H), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 171.01, 138.02, 134.97, 130.74, 128.99, 127.46, 124.10, 119.84, 56.62, 48.05, 44.37, 38.51, 34.76, 33.47, 32.14, 31.77, 29.68, 24.41, 23.63, 21.98, 21.04, 18.95, 14.08, 13.28; IR (KBr) ν: 3474, 2936, 1661, 1439, 753 cm^{-1}; ESI-MS m/z:420$[M+H]^+$; Anal.calcd for $C_{27}H_{33}NO_3$: C 77.29, H7.93, N 3.34; Found: C 77.20, H 7.66 N 3.74.

2.3.11. 17β-[N-phenylamide]-androsta-3,5-dien-3-carboxylic acid (**16h**)

Yield 15%; m.p.: 175-180℃; ^{1}H NMR (DMSO, 300MHz) δ: 10.0 (s, 1H, COOH), 11.9 (s, 1H, NH), 8.1 (d, 1H, *J*=7.4Hz, 6'-H), 7.8 (m, 1H, 5'-H), 7.6 (d, 1H, *J*=7.52Hz, 3'-H), 7.4 (m, 1H, 4'-H), 6.9 (s, 1H, 4-H), 5.9 (s, 1H, 6-H), 0.85 (s, 3H, 19-CH_3), 0.6 (s, 3H, 18-CH_3) ppm; ^{13}C NMR (DMSO-d6, 75 MHz) δ: 175.08, 169.63, 168.84, 161.99, 141.24, 137.75, 134.85, 132.99, 131.39, 129.01, 126.53, 121.30, 56.59, 54.93, 48.01, 45.85, 43.53, 38.07, 37.40, 34.64, 33.61, 31.97, 29.89, 24.39, 23.69, 22.07, 20.92, 19.14, 13.66; IR (KBr) ν: 3457, 2938, 1672, 1610, 774 cm^{-1}; ESI-MS m/z:423$[M-H]^+$; Anal.calcd for $C_{28}H_{32}N_2O_3 \cdot 1/4CH_3OH$: C 74.97, H 7.35, N 6.19; Found: C 75.06, H 7.831 N 5.986.

2.3.12. 17β-[N-[2,5-dimethylphenyl]amide]-androsta-3,5-dien-3-carboxylic acid (**16i**)

Yield 28%; m.p.: 200-205℃; ^{1}H NMR (DMSO, 300MHz) δ: 12.1 (s, 1H, COOH), 8.9 (s, 1H, NH), 7.15 (s, 1H, 6'-H), 7.08 (d, 1H, *J*=7.68Hz, 3'-H), 6.9 (s, 1H, 4-H), 5.85 (s, 1H, 6-H), 2.3 (s, 3H, Ar-CH_3), 2.2 (s, 3H, Ar-CH_3), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR (DMSO-d6, 75 MHz) δ: 171.17, 141.23, 137.70, 137.03, 135.26, 132.99, 131.43, 130.41, 129.58, 129.32, 126.47, 56.44, 49.04, 48.17, 44.20, 37.96, 34.70, 33.51, 31.98, 29.89, 24.65, 22.01, 20.99 , 19.14, 18.27, 13.88; IR (KBr) ν: 2963, 2360, 1669, 1284, 804 cm^{-1}; ESI-MS m/z:446$[M-H]^+$; Anal.calcd for $C_{29}H_{37}NO_3$: C 77.82, H 8.33, N 3.13; Found: C 77.71, H 7.895 N 3.056.

2.3.13. 17β-[N-[2-methoxyphenyl]amide]-androsta-3,5-dien-3-carboxylic acid (**16j**)

Yield 26%; m.p.: 232-235℃; ^{1}H NMR (DMSO, 300MHz) δ: 12.1 (s, 1H, COOH), 8.4 (s, 1H, NH), 7.9 (d, 1H, *J*=8.07Hz, 6'-H), 7.1~6.9 (m, 3H,3',4',5'-H), 6.9 (s, 1H, 4-H), 5.85 (s, 1H, 6-H), 3.8 (s, 3H, -OCH_3), 0.9 (s, 3H, 19-CH_3), 0.8 (s, 3H, 18-CH_3) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 147.71, 141.26, 132.91, 127.90, 123.33, 121.16, 119.80, 109.88, 58.35, 6.58, 55.79, 48.08, 44.15, 38.13, 34.78, 33.39, 32.30, 31.92, 29.69, 24.41, 23.42, 22.68, 21.43, 19.03, 14.10, 13.19; IR (KBr) ν: 3425, 2946, 1673, 14833, 741 cm^{-1}; ESI-MS m/z:448$[M-H]^+$; Anal.calcd for $C_{28}H_{35}NO_4 \cdot 1/2H_2O$: C 74.06, H 7.88, N 3.08; Found: C 73.68, H 8.135 N 2.738.

2.4. Biological activity

2.4.1. In vitro rat 5α-reductase inhibitory activity against type 1 and type 2 5α-reductase enzyme

Preparation of tissue. Rat prostatic enzyme was prepared according to the method of Liang et al.with slight modifications[19]. Male rats were killed and prostates were taken within 5 min and put in ice cold 0.9% NaCl solution. All the following operations were performed at 0±4℃. The prostates were dissected free from fat and connective tissue, cut into pieces and weighed. Per 1 g of tissue, 3 mL of 20 mM phosphate buffer, pH 6.5, were added. The tissue was homogenized, centrifuged for 30 min at 10,000 g. The supernatant was obtained and the centrifugation was

repeated at 100,000 for 1h. The final pellet resuspended in a minimum volume of phosphate buffer and stored in 300μL portions at -70℃.

Incubation Procedure. The assay was performed as described with modifications[19]. All values were run in duplicate. The incubation was carried out for 10 min at 37°C in a total volume of 150μL. In the case of rat enzyme preparation, phosphate buffer (40 mM, pH 6.6 for type 1) or citrate buffer (40 mM, pH 5.5 for type 2) was used. The incubation mixture contained approximately 200μM NADPH, 0.21 μM T, and 2% DMSO with or without test compound (10μM). The absorbance at 340nm was tested at beginning and 10min.

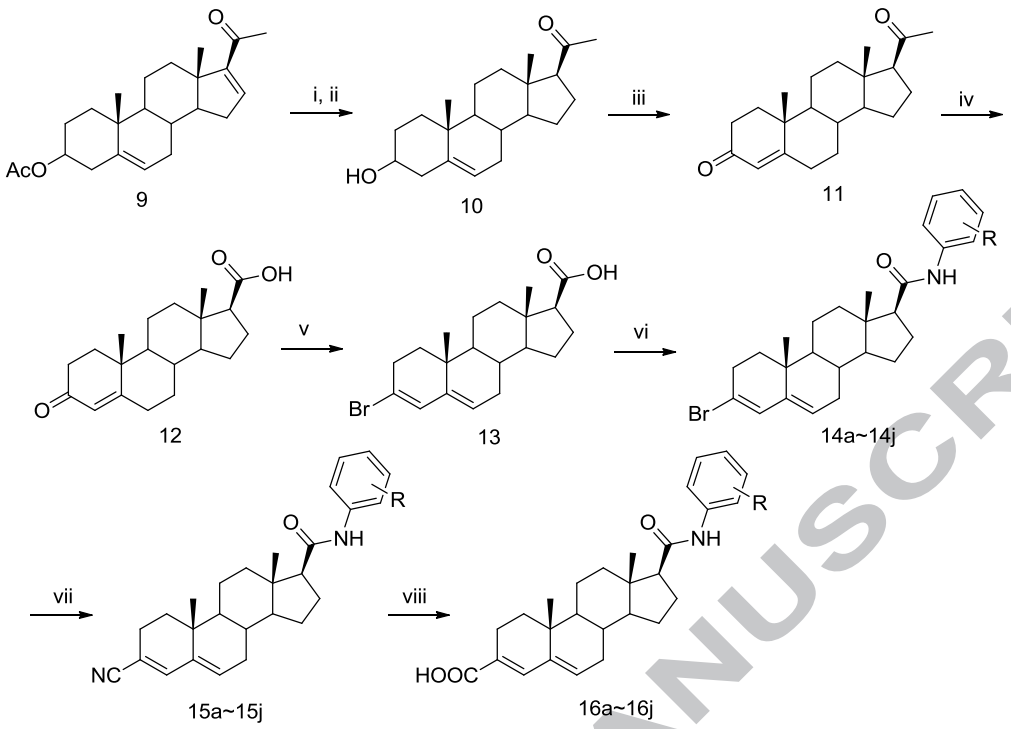
2.4.2. In vivo 5α-reductase inhibitory activity

Experiments were performed on SD male rats weighing from 180 to 220g. After gonadectomy, the rats were was administered **16a** orally once daily with the dose of 0.1mg/ml, 0.3mg/ml, 0.6mg/ml respectively. The rats in positive control group were received an orally once daily of 0.3mg/ml epristeride Two groups of gonadectomized animals were kept as control; one was injected with 1ml/100g of 2.5% Tween 80, the second with 1 ml/100g of testosterone. After 30 days' treatment, the animals were sacrificed. The prostate and seminal vesicles of each animal were dissected and weighed.

3. Results and discussion

3.1. Chemistry

Taking commercial available compound 16-dehydropregnenolone acetate (**9**) as starting material, the compounds **16a**~**16j** can be easily prepared through 8 steps (**Scheme 1**). Compound **9** could be selective reduced with Raney Ni in EtOH. After filtration, the hydrolysis of the acetate group at C-3 was produced by directly adding KOH to resulting filtrate to obtain compound **10**. The resulting secondary alcohol at C-3 was oxidized to the α,β-unsaturated ketone compound **11** by Oppenauer oxidation using aluminum isopropoxide and cyclohexanone in refluxing toluene. Compound **11** then reacted with bromine and sodium hydroxide to form the carboxylic acid compound **12**. The resulting compound **12** was treated with PBr_3 in HOAc to afford the key intermediate compound **13**. The 17-amide derivatives **14a~14k** were achieved by combining different amides with key intermediate compound **13.** The cyanidation of **14a**~**14j** in DMF at 180℃ gave **15a~15j**. The compound **16a~16j** was eventually prepared form their corresponding 3-CN compounds by hydrolysis in ethanol containing 20% NaOH.



Reagents and conditions: i) Ni, H_2, EtOH; ii) KOH, EtOH; iii) cyclohexanone, Al(O-i-Pr)$_3$; iv) NaOBr, Na_2SO_3; v) PBr_3, HOAc, r.t.; vi) $(COCl)_2$, Py, toluene, amide,r.t.; vii) CuCN, DMF,170℃;viii) NaOH, EtOH,r.f.

Scheme 1. Synthesis of **16a~16j**

3.2. Biological evaluation

3.2.1. *In vitro* 5α-reductase inhibition assay

To evaluate the inhibitory potencies of the synthesized compounds, we first tested their activities towards 5α-reductase 1 and 2 isozymes altogether and the result was shown in **Table 1**. It is obvious that androsta-3,5-diene-3-carboxylic acid is a favorable skeleton for 5α-reductase inhibitors. Generally speaking, most of the compounds (**16a**, **16b**, **16g~16j**) showed the inhibition rate more than 50%, especially compound **16a** with the IC_{50} of 0.21μM, which was better than the positive controls. Different amide substituents were also investigated. The high potencies observed among **16a** and **16h~16j** with nanomolar IC_{50} (0.21-0.61μM) indicated that the substitution at 2'-aniline might be beneficial. The compounds with 3'-subsititution, such as **16c**, **16e** and **16f**, presented a slightly weaker potency than the unsubstituted compound **16g** with the inhibition rate lower than 60% at 50μM. Meanwhile, it seemed that compounds **16d** and **16e** were much more inferior to the others, which might be blamed to the electron donor substitution at 4'-aniline.

Table 1

The 5α-reductase inhibitory activities of compounds **16a~16j**

Compound	R	Inhibition%（50μM）	IC_{50}(μM)
16a	2,5-CF_3	80	0.21
16b	3,5-CF_3	60.7	6.51
16c	3-CH_3, 4-COOH	45	-

16d	4-OCH_3	2.7	-
16e	4-Cl, 3-CF_3	1.7	-
16f	3-CF_3	40.7	-
16g	H	98.4	27.29
16h	2-CN	100	0.65
16i	2,5-CH_3	100	0.89
16j	2-OCH_3	100	0.61
Finasteride(**1**)	-	100	0.59
Epristeride(**3**)	-	100	0.27

To further investigate the inhibitory potency, the inhibition assay was performed on compounds **16a**, **16h**, **16i** and **16j** towards the 1 and 2 isozymes respectively. As shown in **Table 2**, all of the tested compounds displayed good inhibitory activities to both type 1 and type 2 isozymes, in which compound **16a** was the most potential one with the IC_{50} of 0.25μM and 0.13μM respectively. For most compounds, the inhibitory activities towards the type 2 isozyme were slightly higher than type 1 only except **16i**.

Table 2

The inhibitory activities towards the two 5α-reductase isozymes.

Compound	Type 1 (pH 6.6)		Type 2 (pH 5.5)	
	Inhibition% (1μM)	IC_{50}(μM)	Inhibition% (1μM)	IC_{50}(μM)
16a	70.96	0.25	71.32	0.13
16h	79.28	0.40	72.03	0.29
16i	70.77	0.58	79.41	0.98
16j	71.64	0.48	66.22	0.41
Finasteride(**1**)	73.69	1.042	70.04	0.04

3.2.2. *In vivo* inhibitory activity (changes in rat prostate and seminal vesicles weight)

As the most active 5α-reductase inhibitor *in vitro*, **16a** was assessed for its *in vivo* activity using prostate weighing method. For each inhibitor to be evaluated, mature male rats were domesticated and were given once daily with different dose of compounds under test for 30 days and the inhibitory effects on the growth of prostate and seminal vesicles were examined on 31th day (24h after last dose). After 30 days' treatment, the prostatic/body weight ratio of the control group was 129 mg/100g. The prostatic/body weight ratio in group that underwent induced prostatic hyperplasia by testosterone injection was 491 mg/100g, which was significantly heavier than that of the control group. As shown in **Figure 3**, compound **16a** produced weight reduction effects in a dose-response manner on both prostate and seminal vesicles weight, while epristeride, the positive control, only on prostate. At 6mg/kg, compound **16a** displayed a significant weight reduction effect with the prostatic/body weight ratio of 352mg/100g, better than that of epristeride (381mg/100g).

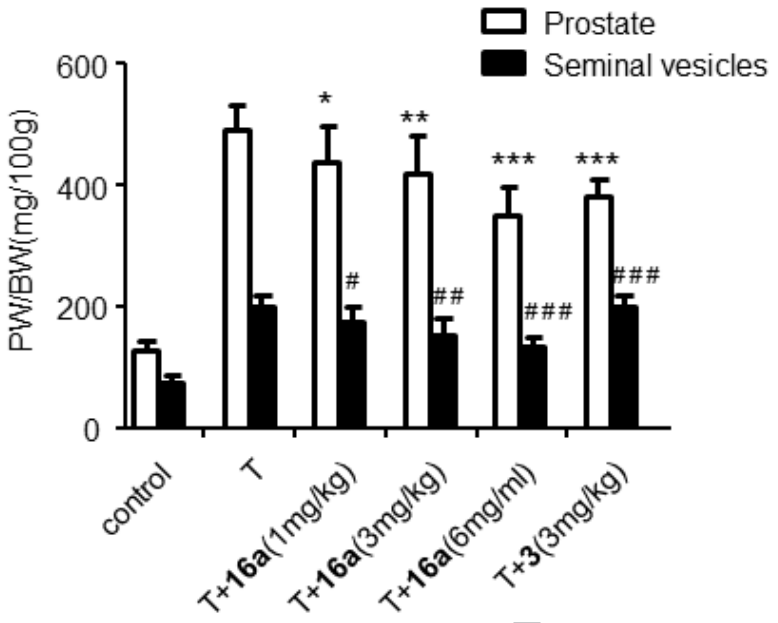


Fig. 3 Prostatic/body weight ratio of prostate and seminal vesicles glands of castrated rats receiving treatments for 30 days. The control group was treated with vehicle only. The pharmacological experiment was carried out in duplicate. Values are mean ± SD (n=10). *P < 0.05, **P < 0.01, ***P < 0.001 vs. prostate of testosterone group. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 vs. seminal vesicles of testosterone group.

3.3. *In silico* ADME property study

Employing Schrödinger software, compounds **16a** were subjected to the analyses of several physiochemical properties (ADME) related to pharmacokinetics[20]. **16a** follow Lipinski's rule of five for good bioavailability. With the calculated LogP values less than 6.5, it presented good hydrophilicity with moderate lipophilicity and hence should be able to gain access to membrane surfaces. The aqueous solubility (LogS) of a compound is a major driving force that leads to good absorption and distribution characteristics. The calculated LogS value of **16a** was within the acceptable range. Other calculations related to solubility, serum protein binding, blood–brain barrier (LogBB and apparent MDCK cell permeability), gut-blood barrier (Caco-2 cell permeability), number of Primary Metabolites, skin permeability (Kp), and human oral absorption in the gastrointestinal tract showed these values of **16a** within the standard ranges for good bioavailable drugs (**Table 3**).

Table 3

Various physicochemical (ADME) parameters calculated for 33d and 33e

Parameter	calculated values	Stand. range*
log S for aqueous solubility	-5.570	(-6.5/0.5)
log P for octanol/water	5.969	(-2.0/6.5)
log K hsa Serum Protein Binding	1.440	(-1.5/1.5)
log BB for brain/blood	-0.116	(-3.0/1.2)
No. of Primary Metabolites	4	(1.0 / 8.0)

Apparent Caco-2 Permeability (nm/s)	221	(<25 poor, >500 great)
Apparent MDCK Permeability (nm/sec)	1212	(<25 poor, >500 great)
log Kp for skin permeability	-2.964	(-8.0 to -1.0, Kp in cm/h)
% Human Oral Absorption in GI	84	<25% is poor

* Note: for 95% of known drugs based on Schrödinger, QikProp v4.4 (2015) software results.

4. Conclusion

In this study, a series of novel steroidal androst-3, 5-diene-3-carboxylic acid derivatives have been designed and synthesized in a simple and convenient route. Biological evaluations were performed on their 5α-reductase inhibitory activity in vitro against 5α-reductase type 1 and 2 and in vivo by prostate weighing method. Results showed that most of them displayed good 5α-reductase inhibitory activities. It is obvious that the compounds bearing 2'- substituted aniline (compound **16a**, **16h~16j**) presented more promising potencies with nanomolar IC_{50} (0.21-0.61μM). In the detailed evaluation of their inhibition activities against type 1 and 2 respectively, most compounds displayed slightly higher inhibition potency towards the type 2 isozyme than type 1 only except **16i**. Among all the compounds, **16a** was found to be the most potential inhibitor with the IC_{50} of 0.25μM and 0.13μM respectively. In vivo study, compound **16a** displayed a significant weight reduction effects in a dose-response manner on both prostate and seminal vesicles weight. *In silico* studies showed that compound **16a** possessed good physiochemical properties (ADME) related to pharmacokinetics. Overall, based on biological activities data, **16a** has been identified as a potential 5α-reductase inhibitor lead molecule which might be of therapeutic importance for the treatment of BPH.

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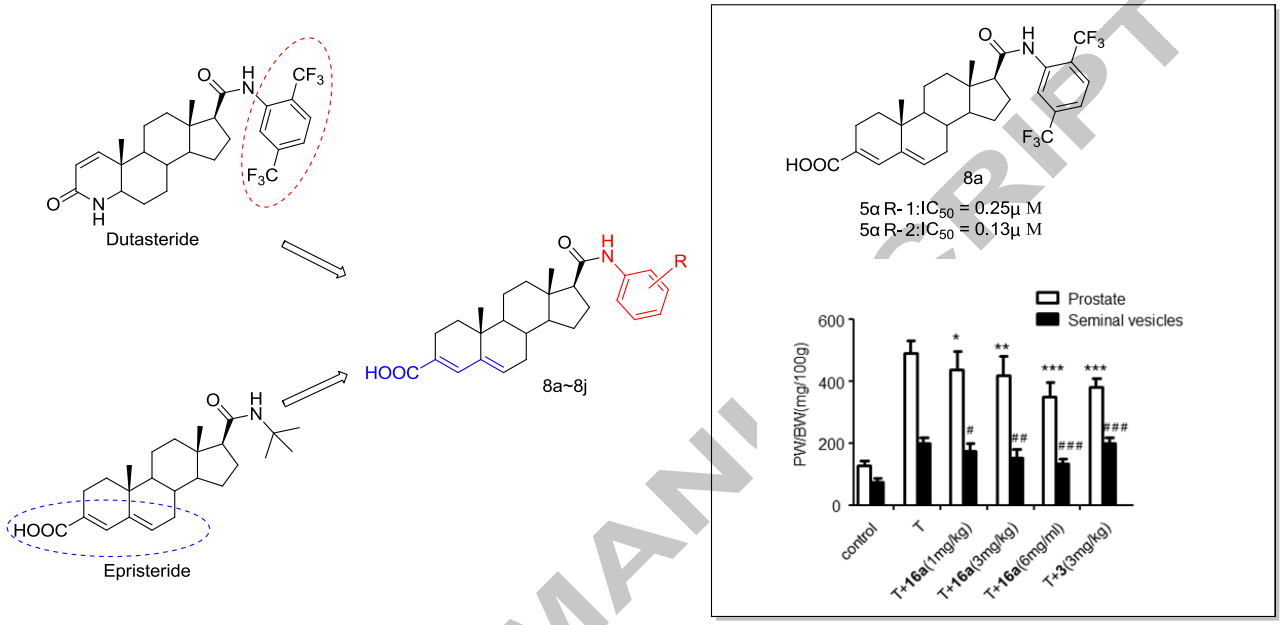
Graphical Abstract



Highlights：

- A series of novel steroidal androst-3,5-diene-3-carboxylic acids have been designed and synthesized.
- Most of the synthesized compounds displayed excellent 5α-reductase inhibitory potency.
- **16a** was found to be the most potential inhibitor against type 1 and 2 isozymes.
- **16a** exhibited a significant reduction effect in rat prostate weight *in vivo* as well as good pharmacokinetic properties *in silico.*