New testosterone derivatives as semi-synthetic anticancer agents against prostate cancer: synthesis and preliminary biological evaluation

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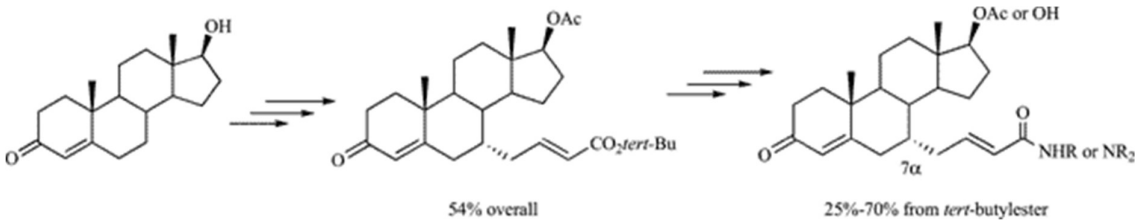
Abbreviation list: AR, androgen receptor; AR+, androgen-dependent cancer; AR-, androgen-independent cancer; CRPC, castration-resistant prostate cancer; PC, prostate cancer.

Graphical abstract



Abstract

Prostate cancer (PC) is a major health issue in the world. Treatments of localized PC are quite efficient and usually involve surgery, radiotherapy and/or hormonal therapy. Metastatic PC is however rarely curable to this day. Treatments of metastatic PC involve radiotherapy, chemotherapy and hormonal treatment such as orchiectomy, antiandrogens and luteinizing hormone-releasing hormone agonists. The suppression of tumor growth by hormonal treatment is efficient but overtime resistance still occurs and the disease progresses. Thus, more urgently than ever there is a need for discovery of new treatment options for castration-resistant PC (CRPC). Hence, we designed and tested a series of amide derivatives located at position 7α of testosterone as prospective "natural" or "semi-synthetic" anticancer agents against CRPC with the goal of discovering therapeutic alternatives for the disease. This manuscript describes an efficient path towards the target molecules that are made in only 6 or 7 chemical steps from testosterone in good overall yields. This strategy can be used to make several compounds of interest that present higher biological activity than the classic antiandrogen; cyproterone acetate (**3**). The best testosterone-7 α-amide was the *N*-2-pyridylethylamide (**25**) which was as active as the antiandrogen cyproterone acetate (**3**) on androgen-dependent LNCaP cells and 2.7 times more active on androgen-independent PC3 prostate cancer cells. The results obtained show the synthetic feasibility and the potential for future development of this unique class of semi-synthetic anticancer agents that offer the premise of new treatment modalities for patients afflicted with CRPC.

Keywords. Testosterone-7α-linked amides, prostate cancer, anticancer agents.

INTRODUCTION

Prostate cancer (PC) afflicts thousands of men in the world. This disease is ranked first amongst all cancer cases diagnosed in men and it is the second most deadly after lung cancer [1]. In the US, recent estimates show that during 2014, about 233 000 cases of PC will be detected and that 29 480 Americans will not survive the disease [1]. Much work has been dedicated towards the treatment and prevention of PC. Although efficient treatment strategies exist for localized PC, the treatments of advanced (or metastatic) diseases still need to be improved. The main strategies for the treatment of localized PC include: surgery or radiation therapies that can be used alone or in combination. Hormone therapy may be given following these treatments. For advanced diseases the gold standard strategy is achieved by hormone ablation treatments that are also followed by surgery and/or radiation therapies. The various treatments for PC were recently reviewed [2, 3]. Despite the fact that several patients benefited from a positive response to hormonal treatments and experience prolonged remission, 10-20% of patients developed resistance and this inevitably led to castration-resistant prostate cancer (CRPC). CRPC occurs when the cancer progresses in spite of the use of androgen-deprivation therapy [4-6]. Largely, recent research clearly shows that in CRPC the androgen receptor (AR) still plays an important role in regulating and promoting cancer growth [4-6]. The natural ligands for the AR are testosterone (**1**) and dihydrotestosterone (**2**) (Fig. 1). These hormones play an important role in the initiation and development of PC and are also essential for male sexual growth and development [4-6]. Unmistakably, the AR is a key therapeutic target for the treatment of the disease. There are two main approaches by which AR can be targeted, either directly or indirectly. Drugs that target AR directly are classified as androgen antagonist such as cyproterone acetate (**3**) and bicalutamide (**4**) and, those targeting the AR indirectly are classified as androgen synthesis inhibitors such as ketoconazole (**5**) (Fig. 1) [7].

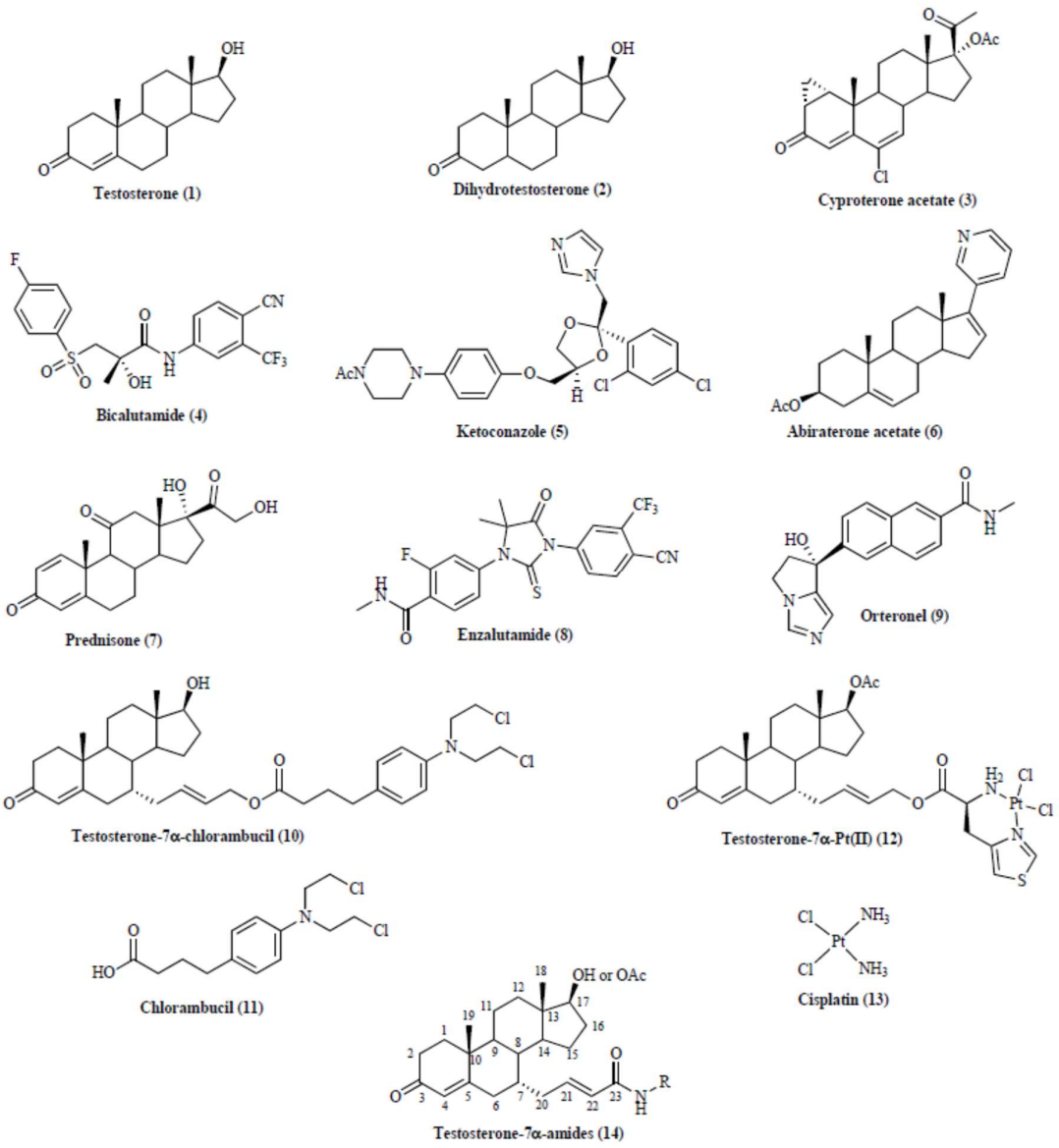


Fig. 1. Molecular structures of testosterone (**1**), dihydrotestosterone (**2**), cyproterone acetate (**3**), bicalutamide (**4**), ketoconazole (**5**), abiraterone acetate (**6**), prednisone (**7**), enzalutamide (**8**), orteronel (**9**), testosterone-7α-chlorambucil (**10**), chlorambucil (**11**), testosterone-7□-Pt(II) (**12**), cisplatin (**13**) and general structure of testosterone-7□-amides assessed in this study (**14**). Derivative **14** displays the carbon numbering used for the proton and carbon NMR spectral assessments.

Novel therapeutic agents for the treatment of CRPC and a description of their targets were recently reviewed [8, 9]. Amongst the newest strategies described in the reviews, there are innovative drugs targeting AR activity such as: 1) abiraterone acetate (**6**, Zytiga®, approved in 2011 in combination with prednisone (**7**) for post chemotherapy treatment of CRPC), 2) a selective cytochrome P450-17 inhibitor, enzalutamide (**8**, Xtandi®, approved in 2012 for post chemotherapy treatment of CRPC), 3) a selective AR antagonist and, orteronel (**9**, TAK-700), an androgen synthesis inhibitor currently tested in phase III studies in combination with prednisone (**7**) (Fig. 1) [8, 9]. Much progress has been made to manage CRPC and work is still continuing in this area of research [8-10].

Clearly there is a need to discover better antitumor agents for the management of both PC and CRPC. Hence, we have reported an interesting testosterone-chlorambucil conjugate (**10**) that showed selectivity towards AR+ cancer cell line (LNCaP) and had comparable activity to chlorambucil (**11**), the reference drug [11]. Moreover, we recently developed several platinum(II) complexes conjugated at position 7α of 17β-acetyl-testosterone as new combi-molecules against PC [12]. The best derivative bearing a thiazolyl platinum(II) moiety (**12**) showed the highest antiproliferative activity on several PC cell lines and was up to five times more potent than cisplatin (**13**) itself (Fig. 1) [12].

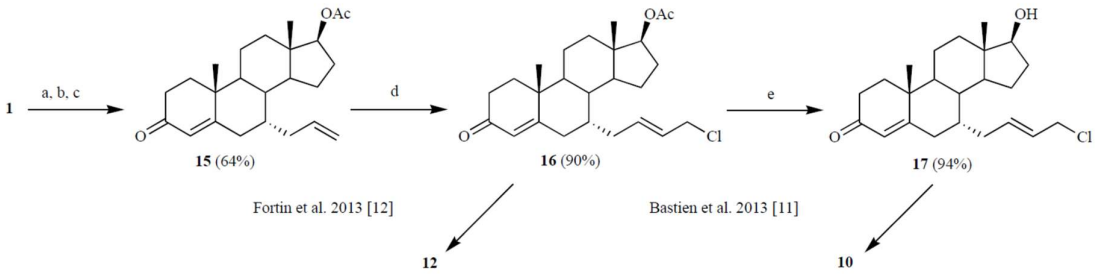
In the current study, we designed, synthesized and characterized a series of testosterone amide derivatives (**14**) by efficient functionalization of position 7α of the steroid nucleus (Fig. 1). Rationale for molecular design was to make simpler, less intricate molecules in comparison to the testosterone-chlorambucil (**10**) and testosterone platinum(II) molecules (**12**) that can potentially act as androgen antagonist (or by other mechanisms of action) and to extend the chemical arsenal available against PC. Furthermore, this work was undertaken to study the biological effect of relatively plain and ordinary amides at position 7α of the testosterone

nucleus on prostate cancer cells. The novel derivatives were also evaluated for their antiproliferative activity on two human PC cell lines; LNCaP and PC3, androgen-dependent and androgen-independent cancers, respectively. This manuscript presents our initial findings concerning these novel testosterone-7α-amide derivatives and precursor analogs.

RESULTS AND DISCUSSION

Chemistry

We recently reported that the functionalization of testosterone (**1**) at position 7α can be readily performed through a combination of the Sakurai reaction (to yield **15**) and the Grubbs cross-metathesis reaction (to yield **16**). This reaction sequence led to the synthesis of derivative **16**, obtained in 58% overall yield from testosterone, that can be further transformed into hybrid anticancer molecules such as **10** (via the intermediate **17**) and **12** (Scheme 1) [11, 12].

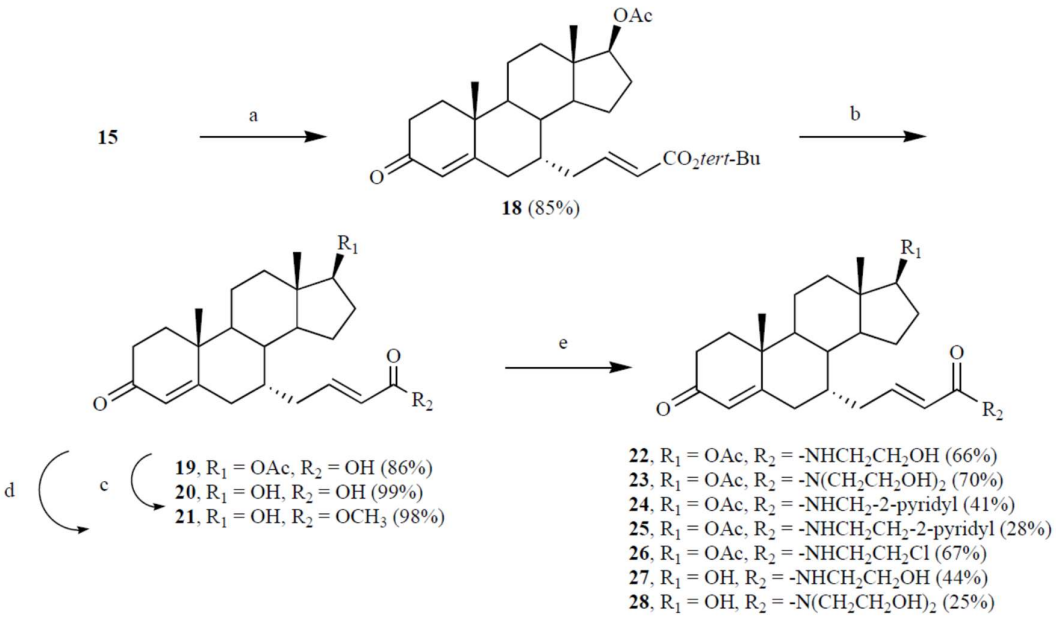


Reagents and conditions [11, 12]: a) AcCl, Ac_2O, Pyr, reflux, 4 h, (96%); b) 1. NBS, DMF, 0 °C, 1.5 h; 2. Li_2CO_3, LiBr, 92 °C, 2 h, (85%); c) 1. $TiCl_4$, Pyr, CH_2Cl_2, -78 °C, 5 min; 2. Allyltrimethylsilane, -30 °C, 1.5 h, (78%); d) Allyl chloride, 2nd generation Hoveyda-Grubbs catalyst, CH_2Cl_2, reflux, 8 h, (90%); e) 5 N HCl, MeOH, mild reflux, 2 h, (94%).

Scheme 1. 7α-Allyl testosterone as a key intermediate for the construct of hybrid anticancer molecules: testosterone-7 α-Pt(II) (**12**) [12] and testosterone-7 α-chlorambucil (**10**) [11].

The intermediate **15** can also be efficiently transformed

into the α,β-unsaturated ester **18**, yet again using a Grubbs cross-metathesis reaction with *tert*-butyl acrylate in excellent yield (85%) (Scheme 2) [11, 13, 14]. This compound is now ready for further chemical transformations into the 17β-acetoxy testosterone 7α-but-2-enoic acid (**19**) and 17β-hydroxy-testosterone 7α-but-2-enoic acid (**20**) that in turn can provide the targeted testosterone-7α-amides **22**-**28**. Hence, upon treatment with trifluoroacetic acid in dichloromethane (CH_2Cl_2) the *tert*-butyl ester **18** gave derivative **19** in 86% yield. Further hydrolysis of the 17β-acetyl function was performed in a mixture of sodium hydroxide in tetrahydrofurane and water to give derivative **20** in 99% yield. In addition, the acidic treatment of **19** in methanol afforded the methyl ester analog **21** in 98% yield. This product was made for additional biological comparison along with the amide derivatives. The amides were made from the acids **19** or **20** by condensation of the relevant amine under standard reaction conditions (1-hydroxybenzotriazole, dicyclohexylcarbodiimide (HOBt, DCC)) to give compounds **22**-**28** in 25–70% yield. The detailed experimental conditions are found in the materials and methods section. The new testosterone derivatives were characterized with infrared (IR), nuclear magnetic resonance spectroscopy (^{1}H NMR and ^{13}C NMR) and with mass spectrometry.



Reagents and conditions: a) *tert*-Butyl acrylate, Grubbs catalyst 2nd generation, CH_2Cl_2, reflux, 8 h, (85%); b) TFA, CH_2Cl_2, 15 min, (86%); c) NaOH, H_2O/THF, 10 h, (99%); d) HCl, CH_3OH, reflux, 7 h. (98%); e) 1. **19** or **20**, HOBt, DCC, dry DMF, 22 °C, 1 h; 2. Add appropriate primary or secondary amine (RNH_2 or $(R)_2NH$), (from 25%-70%).

Scheme 2. Functionalization of testosterone at position 7α.

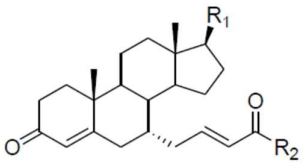
Antiproliferative Activity on Prostate Cancer Cell Lines

The next objective of this study was to measure the antiproliferative activity of the various derivatives on androgendependent (LNCaP, AR+) and androgen-independent (PC3, AR-) human PC cell lines using the MTT cell proliferation assay [15, 16]. The MTT assay was executed over a 72 h incubation period. The various testosterone derivatives were tested along with two control compounds: cyproterone acetate (**3**) and chlorambucil (**11**) for further comparison with a known antiandrogen and a nitrogen mustard derivative, respectively.

Table 1 shows the result of the antiproliferative activity of all the derivatives. Cyproterone acetate (**3**) presents an IC_{50} of 17 and 16 μM on LNCaP and PC3 cell lines, respectively, while chlorambucil (**11**) shows an IC_{50} of 52 and 56 μM on the same cells. This result indicates that the antiandrogen is about 3 times more active than the nitrogen mustard *in vitro*.

Notwithstanding its low antiproliferative activity, chlorambucil (**11**) still remains a clinically useful anticancer drug for the treatment of leukemia. Discrepancies can be observed between *in vitro* and *in vivo* biological assays [17, 18]. Assays revealed that the *tert*-butyl ester bearing a 17β-acetyl function, compound **18** is less active than both reference derivatives (**3** and **11**) with an IC_{50} of 65 μM on the cells tested. This ester might be too lipophilic for higher biological activity. Interestingly, the corresponding acid analog **19** is not active on LNCaP (IC50 > 160 μM) but is 5.4 times more active than derivative 18 on the AR- PC3 cells (IC_{50} = 12 μM). In addition, the acid **19** is 13 times more potent on AR- PC3 cells compared to the AR+ LNCap PC cells. Derivative **20** bearing a 17β-hydroxyl function and the but-2-enoic acid function is 3.4 times less active than the corresponding 17β-acetyl analog derivative **19**, on PC-3 cells. The methyl ester analog, derivative **21** presents essentially the same activity as the acid **20** on both cells tested with IC_{50} of > 160 μM and 46 μM on LNCap and PC3 cells, respectively.

Table 1. Antiproliferative activity of precursor derivatives **18**, **19**, **20**, of methyl ester analog **21** and of novel testosterone-7α-amides (compounds **22**-**28**), along with control derivatives cyproterone acetate (**3**) and chlorambucil (**11**) on androgen-dependent (LNCaP) and androgen-independent (PC3) human prostate adenocarcinoma cell lines.



18-28

Compd	LNCaP (AR+) IC_{50}* (μM)	PC3 (AR-) IC_{50} (μM)	Substituents	
			R_1	R_2
3	17	16	---	---
11	52	56	---	---
18	67	65	-OAc	-O-*tert*-Bu
19	> 160	12	-OAc	-OH
20	> 160	41	-OH	-OH
21	> 160	46	-OH	$-OCH_3$
22	> 160	> 160	-OAc	$-NHCH_2CH_2OH$
23	> 160	> 160	-OAc	$-N(CH_2CH_2OH)_2$
24	74	17	-OAc	$-NHCH_2Pyr$
25	24	6	-OAc	$-NHCH_2CH_2Pyr$
26	NT†	NT	-OAc	$-NHCH_2CH_2Cl$
27	> 160	46	-OH	$-NHCH_2CH_2OH$
28	> 160	> 160	-OH	$-N(CH_2CH_2OH)_2$

* Inhibitory concentration (IC_{50}) is the concentration of drug inhibiting cell growth by 50%. The experiments were performed at least twice in triplicate. The IC_{50} assay was considered valid when the relative standard deviation was less than 10%. † NT = Not tested. This derivative was unstable and rapidly decomposed.

Interesting results were obtained for several of the testosterone-7α-amide derivatives as some of them were active while others were not. In fact, we observed that the ethanolamide **22** and the diethanolamide **23** molecules were completely inactive (IC_{50} > 160 μM) on both types of cells. Furthermore, for the corresponding analogs (**27** and **28**) bearing an hydroxyl function at position 17β instead of the acetyl group, only the ethanolamide derivative **27** is active on androgen-independent PC cell with a IC_{50} of 46 μM. This value is essentially similar to that of

derivatives **11**, **18**, **20** and **21**. The chloroethyl amide (**26**, analog of **22**) was made but was too unstable to be tested in vitro. Of note, the bis-(2-chloroethyl) amide was not obtained (structure not shown) using a similar protocol as for the synthesis of **26** with bis-(2-chloroethyl)amine hydrochloride instead of 2-chloroethylamine hydrochloride. It was most likely formed but was too unstable to be isolated. Contrarily, the *N*-2-pyridylmethylamide (**24**) and *N*-2-pyridylethylamide (**25**) were active on both types of cells. Derivative **24** displayed higher activity on androgen-independent PC3 cancer cells with an IC_{50} of 17 μM in comparison with 74 μM for the androgen-dependent cancer cells, LNCaP. The best testosterone-7α-amide was the *N*-2-pyridylethylamide (**25**). It exhibited an activity similar to that of the antiandrogen cyproterone acetate (**3**) on LNCaP, with IC_{50} of 24 μM compared to 17 μM, respectively. It is worth noting that derivatives **24** and **25** are 4.4 and 4.0 times more active on the AR- PC3 cancer cell line when compared to the AR+ LNCaP cancer cell line, respectively. Furthermore, the testosterone amide **25** is about 2.7 times more active than the reference antiandrogen **3**, the former showing an IC_{50} of 6 μM versus 16 μM for **3**.

CONCLUSION

In this study we designed, prepared and characterized several new testosterone-7α-amide derivatives. They were synthesized from testosterone (**1**) in 6 to 7 steps through an efficient Grubbs cross-metathesis reaction between **15** and *tert*-butyl acrylate, giving the key α,β-unsaturated ester (**18**) that, in turn yielded the final testosterone-7α-amides (compounds **22-28**). The results showed that the precursor derivative **19**, a relatively simple testosterone analog, was 13 times more active on PC3 AR- PC cells (12 μM) compare to the LNCaP AR+ PC cells (> 160 μM) and thus might be of potential interest for the treatment of CRPC. Furthermore, derivative **19** was essentially as active as the antiandrogen cyproterone acetate

(**3**) on AR- PC cells. Cyproterone acetate (**3**) was, however active on both types of cells; AR+ and AR-. The corresponding 17β-hydroxyl analog derivative **20** was still selective on AR- PC cell but was less potent, about 3.4 times less than analog **19**. The 17β-hydroxyl methyl ester derivative **21** was also selective on AR- PC cell line but less potent than **19**, around 3.8 times less. Surprising results were obtained for the testosterone-7α-amides derivatives. The various ethanolamide derivatives (**22** and **27**) and bisethanolamide derivatives (**23** and **28**) were not very active. Unfortunately, the chloroethylamide **26** was too unstable to be tested. The most interesting amide was the pyridylethylamide derivative **25** being 2.7 times more active than cyproterone acetate (**3**) and 4 times more active on AR- PC cells. Amongst the testosterone-7α-amides, derivatives **24** and **25** showed promising antiproliferative activity for the treatment of CRPC and warrant further investigation. Additionally, the precursor acid **19** must be further investigated considering its potency and significant selectivity on AR- PC cells. It is noteworthy that the key α,β-unsaturated ester (**18**) is easily accessible from testosterone in 54% overall yield. This compound could be used to build chemical libraries of testosterone derivatives. The results indicate that both pyridyl derivatives **24** and **25** are more active and selective towards CRPC. In the future, the synthesis of several heteroaromatic amides from acids **19** and **20** could provide compounds with even higher biological activity and higher selectivity against CRPC.

MATERIALS AND METHODS

Biological Method

Human PC androgen-dependent (LNCaP, AR+) and androgen-independent (PC3, AR-) cell lines were obtained from ATCC, Rockville, MD. The cells were maintained in RPMI medium

containing 10% bovine growth serum containing 50 μg/mL gentamycin, at 37 °C in a moisture-saturated atmosphere containing 5% CO_2.

Antiproliferative Activity Assay

Cells were plated in 96-well plates for a period of 48 h before the assay. Stock solutions of the derivatives were prepared by dissolving them in *N*,*N*-dimethylformamide (DMF). Cells were treated for 72 h with serial dilution of the drugs between 160 and 0.3 μM in a total volume of 100 μL per well. A double dilution (or half dilution) was used to perform the test up to the lowest concentration. The final concentration of DMF in the culture media was 0.1% and was kept constant in all experiment conditions. After an incubation period of 68 h, 10 μL of the MTT (5 mg/mL, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well and left reacting for a period of 4 h. Afterwards, the solubilization solution (100 μL of 10% SDS in 0.01 M HCl) was added and the plate was incubated overnight (37 °C, 5% CO_2). The optical density was read with Fluostar OPTIMA BMG (BMG LABTECH inc., Durham, NC) at 550 nm.

Chemistry

Anhydrous reactions were performed under an inert atmosphere of nitrogen. The starting material, reactant and solvents were obtained commercially and were used as such or purified and dried by standard means [19]. The organic solutions were dried over $MgSO_4$, filtered and evaporated on a rotary evaporator under reduced pressure. The reactions were followed by TLC revealed by UV fluorescence or staining with iodine. Commercial TLC plates were Sigma T 6145 (polyester silica gel 60 Å, 0.25 mm). Preparative TLC was performed on 1 mm silica gel 60 Å, 20 X 20 plates (Whatman, 4861 840). Flash column chromatography was

performed on Merck grade 60 silica gel, 230-400 mesh [20]. The chromatographic solvents were distilled.

The melting points (MP) were recorded on an Electrothermal apparatus and are uncorrected. The infrared spectra (IR) were taken on a Nicolet Impact 420 FT-IR spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian 200 MHz NMR apparatus. Samples were dissolved in deuterochloroform ($CDCl_3$) for data acquisition using tetramethylsilane or chloroform as internal standard (TMS, δ 0.0 ppm for ^{1}H NMR and $CDCl_3$ δ 77.16 ppm for ^{13}C NMR). Chemical shifts (δ) are expressed in parts per million (ppm), the coupling constants (J) are expressed in hertz (Hz). Multiplicities are described by the following abbreviations: s for singlet, d for doublet, dd for doublet of doublets, t for triplet, q for quartet, m for multiplet, #m for several multiplets and, br s for broad singlet. Mass spectral assays were carried out using a MS model 6210, Agilent technology instrument and the high-resolution mass spectra (HRMS) were obtained by TOF (time-of-flight) using ESI (electrospray ionization) in the positive mode (ESI+) at the "Plateforme analytique pour molécules organiques" located at the Université du Québec à Montréal.

Note: The nomenclature of the testosterone derivatives described in this manuscript was based on the androgen skeleton (4-androsten-17β-ol-3-one) for better clarity to the reader in the field.

Synthesis of Precursor Derivatives (18-20), of Methyl Ester Analog (21) and of Amides at Position 7α of Testosterone (Compounds 22-28)

Synthesis of *trans*-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic acid *tert*-butyl Ester (18)

The synthesis and spectral data for 7α-allyl-4-androsten-17β-ol-3-one acetate (**15**) were reported in the literature [21-23]. Under an atmosphere of nitrogen, 7α-allyl-4-androsten-17β-ol-3-one acetate (**15**) (467 mg, 1.26 mmol) was dissolved in dry CH_2Cl_2 (10 mL). The Hoveyda-Grubbs catalyst 2nd generation (11.1 mg, 17.7 x 10^{-6} mol) dissolved in dry CH_2Cl_2 (2 mL) was added to the steroid solution. Then excess *tert*-butyl acrylate (1.9 mL, 12.9 mmol) was added to the mixture. The solution was stirred at reflux for 9 h (or until the disappearance of the starting material **15** as detected by TLC). Then the solvents were evaporated under reduced pressure. The crude product was purified by flash chromatography with hexanes/acetone (9.25:0.75) as the eluent to give the desired material **18** (504 mg) in 85% yield. MP: 169-172 °C; IR (ATR, ν max, cm^{-1}): 1738 (C=O, acetate), 1709 (C=O, α,β-unsaturated ester), 1667 (C=O, enone), 1617 (C=C), 1246 (C-O); ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 6.68 (1H, m, 21-CH), 5.69 (1H, s, 4-CH), 5.69 (1H, d, J=15.3 Hz, 22-CH), 4.58 (1H, t, J=7.5 Hz, 17-CH), 2.01 (3H, s, 17-OAc), 1.45 (9H, s, 3 x CH_3), 1.18 (3H, s, 19-CH_3), 0.82 (3H, s, 18-CH_3), 1.00-2.50 (20H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 198.9 (C-3), 171.1 (17-OAc), 168.4 (C-23), 165.7 (C-5), 145.9 (C-21), 126.4 (C-4), 124.9 (C-22), 82.3 (C-17), 80.3, 47.0, 46.1, 42.6, 38.6, 38.2, 36.4, 35.9, 35.8, 33.9, 28.5, 28.1 (C-25), 27.3, 22.8, 21.1, 20.7, 17.9, 11.9; ESI+ HRMS: $(M+Na)^+$ calculated for $C_{29}H_{42}NaO_5$ = 493.2924; found = 493.2924.

Synthesis of *trans*-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic Acid (19)

Trifluoroacetic acid (0.65 mL, 8.75 mmol) was added to a solution of the ester (**18**) (393 mg, 0.83 mmol) dissolved in 3 mL CH_2Cl_2. The mixture is stirred at room temperature until de disappearance of the starting material as detected by TLC. Afterwards, the solvents were evaporated under reduced pressure to a viscous liquid. Then ether and hexanes were added to the oil and the mixture evaporated again to a solid. This operation might be repeated several

times to obtain the crude solid. The product was purified by flash chromatography with hexanes/acetone (8:2) as the eluent to give **19** (296 mg) in 86% yield. MP: 203-206 °C; IR (ATR, ν max, cm^{-1}): 3150-2800 (COOH), 1730 (C=O, acetate), 1713 (C=O, α, β-unsaturated acid), 1645 (C=O, enone), 1245 (CO); ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 6.91 (1H, m, 21-CH), 5.83 (1H, d, J=15.7 Hz, 22-CH), 5.74 (1H, s, 4-CH), 4.62 (1H, t, J=7.4 Hz, 17-CH), 2.05 (3H, s, 17-OAc), 1.21 (3H, s, 19-CH_3), 0.85 (3H, s, 18-CH_3) 1.00-2.60 (21H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 199.5 (C-3), 171.3 (17-OAc), 170.7 (C-23), 168.9 (C-5), 149.9 (C-21), 126.3 (C-4), 122.7 (C-22), 82.3 (C-17), 47.0, 46.1, 42.6, 38.7, 38.2, 36.4, 36.3, 35.9, 33.8, 29.7, 28.8, 27.3, 22.8, 21.1, 20.7, 17.9, 11.9; ESI+ HRMS: $(M+H)^+$ calculated for $C_{25}H_{35}O_5$ = 415.2479; found = 415.2476.

Synthesis of *trans*-4-(4-androsten-17β-hydroxy-3-one-7α-yl)-but-2-enoic Acid (20)

Trans-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic acid (**19**) (118 mg, 0.29 mmol) was dissolved in THF (6 mL) and water (3 mL). The steroid solution was treated with aquous NaOH (3.35 mL, 0.01 g/mL) and stirred at reflux until the starting material dissapeared as shown by TLC (about 6 hours). The THF was evaporated and the aquous solution acidified with HCl. The mixture was diluted with ethyl acetate (40 mL) and washed with water (4 x 10 mL). The organic phase was dried, filtered and everaporated under vacuum. The crude material was homogeneous by TLC and used as such in the next step. The acid **20** was obtained in 99% yield (106 mg). MP: 95-102 °C; IR (ATR, ν max, cm^{-1}): 3700-3100 (COOH, OH), 1695 (C=O, acid), 1651 (C=O, enone), 1240 (C-O); ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 6.91 (1H, m, 21-CH), 5.81 (1H, d, J=15.6 Hz, 22-CH), 5.80 (2H, br s, -COOH and OH), 5.73 (1H, s, 4-CH), 3.67 (1H, t, J=7.9 Hz, 17-CH), 1.20 (3H, s, 19-CH_3), 0.80 (3H, s, 18-CH_3), 1.00-2.60 (20H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, CDCl3, δ ppm): 199.5 (C-3), 170.4 (C-23), 169.0 (C-5), 149.8 (C-21), 126.2 (C-22), 122.7 (C-4), 81.5

(C-17), 47.2, 46.2, 42.9, 38.7, 38.4, 36.5, 36.1, 35.9, 35.8, 33.9, 30.1, 28.9, 22.7, 20.8, 17.9, 11.0; ESI+ HRMS: $(M+H)^+$ calculated for $C_{23}H_{33}O_4$ = 373.2373; found = 373.2372.

Synthesis of *trans*-4-(4-androsten-17β-hydroxy-3-one-7α-yl)-but-2-enoic Acid Methyl Ester (21)

Trans-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic acid (**19**) (118 mg, 0.29 mmol) dissolved in methanol (5 mL) was treated with a 5N solution of hydrochloric acid (350 µL, 11.40 mmol). The reaction mixture was stirred at reflux during 7 h. The solution was diluted with diethylether (50 mL). The organic phase was washed successively with a saturated sodium bicarbonate solution and with water (4 x 10 mL). The organic phase was dried, filtered and evaporated under vacuum. The crude product was purified by flash chromatography with chloroform/methanol (99:1) as the eluent to give **21** (104 mg) in 98% yield. MP: 136-141 °C; IR (ATR, ν max, cm^{-1}): 3455 (OH), 1720 (C=O, ester), 1655 (C=O, enone), 1607 (C=C), 1244 (C-O); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 6.83 (1H, m, 21-CH), 5.80 (1H, d, J=15.6 Hz, 22-CH), 5.71 (1H, s, 4-CH), 3.73 (3H, s, $-CO_2CH_3$) 3.66 (1H, t, J=8.2 Hz, 17-CH), 1.21 (3H, s, 19-CH_3), 0.81 (3H, s, 18-CH_3), 1.00-2.60 (21H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 199.0 (C-3), 168.5 (C-23), 166.8 (C-5), 147.7 (C-21), 126.3 (C-22), 122.8 (C-4), 81.5 (C-17), 51.5, 47.1, 46.2, 42.9, 38.7, 38.4, 36.4, 36,2, 36.0, 35.8, 34.0, 30.2, 28.8, 22.7, 20.8, 17.9, 11.0; ESI+ HRMS: $(M+H)^+$ calculated for $C_{24}H_{35}O_4$ = 387.2530; found = 387.2526.

General Procedure for the Synthesis of Testosterone-7α-Amides

The appropriate testosterone acid derivative (**19** or **20**) (0.36 mmol) was dissolved in DMF (1 mL). Separately, the relevant amine (0.38 mmol) was dissolved in DMF (1 mL). If the amine was purchased as its hydrochloride salt, it was neutralized with triethylamine. Then

dicyclohexylcarbodiimide (DCC) (0.40 mmol), 1-hydroxybenzotriazole (HOBt) (0.41 mmol) and the amine solution were added to the acid solution. The mixture was stirred at room temperature until the complete disapperance of the acid (**19** or **20**) as detected by TLC. Afterwards, the mixture was diluted with ethyl acetate (30 mL) and washed four times with water (4 x 10 mL). The organic phase was dried, filtered and everaporated under vacuum.

Synthesis of *trans*-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic acid *N*-2-ethanolamide (22)

The general procedure for the synthesis of amide was performed as described above with the following quantities: acid (**19**) (151 mg, 0.36 mmol), ethanolamine (23 µL, 0.38 mmol), DCC (83 mg, 0.40 mmol), HOBt (55 mg, 0.41 mmol). The crude product was purified by flash chromatography with hexanes/acetone (3:2) as the eluent to give **22** (111 mg) in 66% yield. MP: 83-100 °C; IR (ATR, ν max, cm^{-1}): 3326 (OH and NH), 1730 (C=O, acetate), 1663 (C=O, amide and enone), 1627 (C=C), 1242 (C-O); ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 6.73 (1H, m, 21-CH), 6.29 (1H, t, J=5.5 Hz, NH) 5.77 (1H, d, J=14.9 Hz, 22-CH), 5.64 (1H, s, 4-CH), 4.61 (1H, t, J=7.4 Hz, 17-CH), 3.76 and 3.49 (4H, 2 x m, (-$CONHCH_2CH_2$-), 3.13 (1H, br s, OH), 2.04 (3H, s, 17-OAc), 1.21 (3H, s, 19-CH_3), 0.85 (3H, s, 18-CH_3) 1.00-2.60 (20H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 199.4 (C-3), 171.1 (C-17-OAc), 169.1 (C-23), 166.6 (C-5), 143.5 (C-21), 126.1 (C-22), 125.3 (C-4), 82.3 (C-17), 62.4, 47.2, 46.1, 42.6, 38.7, 38.2, 36.4, 36.0, 35.6, 34.0, 28.7, 27.3, 22.8, 22.6, 21.1, 20.7, 17.9, 11.9; ESI+ HRMS: $(M+H)^+$ calculated for $C_{27}H_{40}NO_5$ = 458.2901; found = 458.2897.

Synthesis of *trans*-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic acid *N*,*N*-bis-2-ethanolamide (23)

The general procedure for the synthesis of amide was performed as described above with the following quantities: acid (**19**) (207 mg, 0.50 mmol), diethanolamine (86 mg, 1.07 mmol), DCC (125 mg, 0.60 mmol), HOBt (81 mg, 0.60 mmol). The crude product was purified by flash chromatography with hexanes/acetone (3:2) as the eluent to give derivative **23** (174 mg) in 70% yield. MP: 58-62 °C; IR (ATR, ν max, cm^{-1}): 3390 (OH), 1730 (C=O, acetate), 1662 (C=O, amide and enone), 1602 (C=C), 1242 (C-O); ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 6.72 (1H, m, 21-CH), 6.28 (1H, d, J=15.2 Hz, 22-CH), 5.67 (1H, s, 4-CH), 4.59 (1H, t, J=7.5 Hz, 17-CH), 4.07 (1H, OH), 3.67 (8H, m, 2 x -CH_2CH_2OH), 2.02 (3H, s, 17-OAc), 1.18 (3H, s, 19-CH_3), 0.83 (3H, s, 18-CH_3) 1.00-2.60 (21H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 199.9 (C-3), 171.1 (17-OAc), 169.5 (C-23), 168.3 (C-5), 145.0 (C-21), 126.0 (C-22), 122.8 (C-4), 82.3 (C-17), 61.9 and 60.5 (2 x -CH_2OH), 51.8, 51.2, 47.3 and 46.1 (-CH_2NCH_2-), 42.6, 38.6, 38.3, 36.6, 36.4, 36.1, 35.2, 33.9, 29.6, 27.3, 22.8, 21.1, 20.7, 18.1, 11.9; ESI+ HRMS: $(M+H)^+$ calculated for $C_{29}H_{44}NO_6$ = 502.3163; found = 502.3160.

Synthesis of *trans*-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic acid *N*-2-pyridylmethylamide (24)

The general procedure for the synthesis of amide was performed as described above with the following quantities: acid (**19**) (108 mg, 0.26 mmol), 2-(aminomethyl)pyridine (57 mg, 0.53 mmol), DCC (56 mg, 0.27 mmol), HOBt (38 mg, 0.28 mmol). The crude product was purified by flash chromatography with hexanes/acetone (7.5:2.5) as the eluent to give amide **24** (64 mg) in 41% yield. MP: 190-193 °C; IR (ATR, ν max, cm^{-1}): 3334 (NH), 1736 (C=O, acetate), 1659 (C=O, amide and enone), 1615 (C=C), 1230 (C-O); ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 8.55 (1H, d, J=4.7 Hz, -CH), 7.66 (1H, t, J=7.6 Hz, -CH), 7.25 (2H, m, 2 x -CH), 6.84 (2H, m, 21-CH and NH), 5.88 (1H, d, J=15.2 Hz, 22-CH), 5.71 (1H, s, 4-CH), 4.63 (2H, d, J=4.6 Hz, -$NHCH_2$), 4.62 (1H, m, 17-CH), 2.05 (3H, s, 17-OAc), 1.20 (3H, s, 19-CH_3), 0.85

(3H, s, 18-CH_3), 1.00-2.60 (20H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 199.1 (C-3), 171.1 (17-OAc), 168.7 (C-5), 165.5 (C-23), 156.1, 149.0 (C-21), 143.2, 136.9, 126.3 (C-22), 125.4, 122.4 (C-4), 122.2, 82.3 (C-17), 47.1, 46.1, 44.4, 42.6, 38.6, 38.2, 36.4, 36.3, 36.0, 35.7, 34.0, 29.7, 27.3, 22.8, 21.1, 20.7, 17.9, 11.9; ESI+ HRMS: $(M+H)^+$ calculated for $C_{31}H_{41}N_2O_4$ = 505.2988; found = 505.3051.

Synthesis of *trans*-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic acid *N*-2-pyridylethylamide (25)

The general procedure for the synthesis of amide was performed as described above with the following quantities: acid (**19**) (103 mg, 0.25 mmol), 2-(2-aminoethyl)pyridine (64 mg, 0.52 mmol), DCC (61 mg, 0.30 mmol), HOBt (41 mg, 0.30 mmol). The crude product was purified by flash chromatography with CH_2Cl_2/methanol (97:3) as the eluent to give compound **25** (64 mg) in 28 % yield as an oil. IR (ATR, ν max, cm^{-1}): 3292 (NH), 1729 (C=O, acetate), 1668 (C=O, amide and enone), 1593 (C=C), 1245 (C-O); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 8.54 (1H, d, J=4.3 Hz, -CH), 7.61 (1H, t, J=7.6 Hz, -CH), 7.17 (1H, d, J=7.5 Hz, -CH), 7.16 (1H, m, -CH), 6.63 (2H, m, 21-CH and NH), 5.72 (1H, d, J=15.2 Hz, 22-CH), 5,66 (1H, s, 4-CH), 4.60 (1H, t, J=8.4 Hz, 17-CH), 3.74 (2H, m, -$NHCH_2$), 3.01 (2H, t, J=6.3 Hz, -CH_2Pyr), 2.03 (3H, s, 17-OAc), 1.19 (3H, s, 19-CH_3), 0.84 (3H, s, 18-CH_3), 1.00-2.60 (20H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 199.1 (C-3), 171.1 (17-OAc), 168.9 (C-5), 165.4 (C-23), 159.6, 149.2 (C-21), 142.5, 136.7, 126.0 (C-22), 125.8, 123.5 (C-4), 121.6, 82.3 (C-17), 47.1, 46.1, 42.6, 38.7, 38.6, 38.1, 36.8, 36.4, 36.3, 35.9, 35.6, 33.9, 29.7, 27.3, 22.8, 21.1, 20.7, 17.9, 11.9; ESI+ HRMS: $(M+H)^+$ calculated for $C_{32}H_{43}N_2O_4$ = 519.3142; found = 519.3215.

Synthesis of *trans*-4-(4-androsten-17β-acetoxy-3-one-7α-yl)-but-2-enoic acid *N*-2-chloroethylamide (26)

The general procedure for the synthesis of amide was performed as described above with the following quantities: acid (**19**) (113 mg, 0.27 mmol), 2-chloroethylamine hydrochloride (39 mg, 0.33 mmol) with triethylamine (50 μL, 0.36 mmol), DCC (68 mg, 0.33 mmol), HOBt (46 mg, 0.34 mmol). The crude product was purified by flash chromatography with hexanes/acetone (3:2) as the eluent to give the *N*-2-chloroethylamide **26** (87 mg) in 67% yield. MP: 76-81 °C; IR (ATR, ν max, cm^{-1}): 3300-3100 (NH, amide), 1731 (C=O, acetate), 1660 (C=O, amide and enone), 1610 (C=C), 1246 (C-O); ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 6.70 (1H, m, 21-CH), 6.08 (1H, br s, NH), 5.77 (1H, d, J=15.3 Hz, 22-CH), 5.69 (1H, s, 4-CH), 4.60 (1H, t, J=8.6 Hz, 17-CH), 3.66 (4H, m, $-CH_2CH_2Cl$), 2.03 (3H, s, 17-OAc), 1.20 (3H, s, 19-CH_3), 0.84 (3H, s, 18-CH_3), 1.00-2.60 (20H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 199.2 (C-3), 171.1 (C-17-OAc), 168.7 (C-23), 165.5 (C-5), 143.9 (C-21), 126.2 (C-22), 125.1 (C-4), 82.3 (C-17), 47.1, 46.1, 43.9, 42.6, 41.2, 38.7, 38.2, 36.4, 36.0, 35.6, 34.0, 30.9, 28.6, 27.3, 22.8, 21.1, 20.7, 17.9, 11.9; The HRMS is unavailable because the product is unstable.

Synthesis of *trans*-4-(4-androsten-17β-hydroxy-3-one-7α-yl)-but-2-enoic acid *N*-2-ethanolamide (27)

The general procedure for the synthesis of amide was performed as described above with the following quantities: acid (**20**) (102 mg, 0.27 mmol), ethanolamine (21 mg, 0.34 mmol), DCC (66 mg, 0.32 mmol), HOBt (41 mg, 0.30 mmol). The crude product was purified by flash chromatography with hexanes/acetone (5.5:4.5) as the eluent to give the amide **27** (50 mg) in 44% yield as an oil. IR (ATR, ν max, cm^{-1}): 3650-3100 (OH and NH), 1657 (C=O, amide and enone), 1220 (C-O); ^{1}H NMR (200 MHz, $CDCl_3$, δ ppm): 6.72 (1H, m, 21-CH), 6.41 (1H, t, J=5.5 Hz, NH), 5.77 (1H, d, J=14.1 Hz, 22-CH), 5.67 (1H, s, 4-CH), 3.75 (2H, t, J=4.7 Hz, -

CH_2OH), 3,66 (1H, t, J=8.2 Hz, 17-CH), 3.47 (2H, m, -$NHCH_2$), 1.21 (3H, s, 19-CH_3), 0.80 (3H, s, 18-CH_3), 1.00-2.60 (22H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 199.6 (C-3), 169.5 (C-23), 166.6 (C-5), 143.5 (C-21), 126.0 (C-22), 125.3 (C-4), 81.5 (C-17), 62.4, 47.6, 46.3, 43.0, 42.6, 38.7, 38.4, 36.4, 36.2, 36.0, 35.5, 34.0, 30.3, 28.7, 22.7, 20.8, 18.0, 11.0: ESI+ HRMS: $(M+H)^+$ calculated for $C_{25}H_{38}NO_4$ = 416.2795; found = 416.2791.

Synthesis of *trans*-4-(4-androsten-17β-hydroxy-3-one-7α-yl)-but-2-enoic acid *N,N*-bis-2-ethanolamide (28)

The general procedure for the synthesis of amide was performed as described above with the following quantities: acid (**20**) (130 mg, 0.35 mmol), diethanolamine (40 mg, 0.38 mmol), DCC (81 mg, 0.39 mmol), HOBt (52 mg, 0.38 mmol). The crude product was purified by flash chromatography with hexanes/acetone (5.5:4.5) as the eluent to give *N,N*-bis-2-ethanolamide **28** (87 mg) in 25% yield as an oil. IR (ATR, νmax, cm^{-1}): 3414 (OH), 1681 (C=O, amide), 1650 (C=O, enone), 1619 (C=C), 1222 (C-O); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 6.77 (1H, m, 21-CH), 6.30 (1H, d, J=14.5 Hz, 22-CH), 5.70 (1H, s, 4-CH), 3.64 (9H, m, 2 x -CH_2CH_2OH and 17-CH), 1.21 (3H, s, 19-CH_3), 0.81 (3H, s, 18-CH_3), 1.00-2.60 (23H, # m, remaining steroid protons); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): 200.1 (C-3), 169.7 (C-23), 168.4 (C-5), 145.2 (C-21), 125.9 (C-22), 122.8 (C-4), 81.5 (C-17), 62.2 and 60.5 (2 x -CH_2OH), 51.7 and 51.1 (-CH_2NCH_2-), 47.5, 46.3, 43.0, 38.7, 38.6, 36.6, 36.2, 36.1, 35.2, 34.0, 30.3, 29.6, 22.7, 20.9, 18.1, 11.0; ESI+ HRMS: $(M+H)^+$ calculated for $C_{27}H_{42}NO_5$ = 460.3057; found = 460.3050.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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