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Short communication

Synthesis and preliminary *in vitro* biological evaluation of 7α-testosterone–chlorambucil hybrid designed for the treatment of prostate cancer

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ABSTRACT

The synthesis of 7α-testosterone–chlorambucil hybrid is reported. This compound is made from testosterone in a 6 step reaction sequence and with 23% overall yield. An alternative convergent reaction sequence yielded the same hybrid through a Grubbs metathesis reaction between chlorambucil allyl ester and 7α-allyltestosterone with 35% overall yield. MTT assays showed that the hybrid is selective towards hormone-dependent prostate cancer cell line (LNCaP (AR+)) and shows similar activity than the parent drug, chlorambucil. Thus, the new hybrid shows promising potential for drug targeting of hormone-dependent prostate cancer through its capacity of delivering chlorambucil directly to the site of treatment. This could extend the use of chlorambucil to prostate cancer in the future.

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1. Introduction

In Canada, prostate cancer is the most frequently diagnosed cancer in men accounting for 28% of all new cancer cases [1]. However, prostate cancer ranks third with 10% of all cancer deaths in men, after lung and colorectal cancers with 28% and 12% of all cancer deaths, respectively [1]. Nonetheless, it is a serious health problem, not only in Canada but worldwide [2].

The androgens, testosterone (T) and dihydrotestosterone (DHT) are implicated in the development and normal functions of prostate cells. They are also involved in male sexual organ growth and sexual function. Testosterone is the principal androgen in the blood while DHT is the most potent androgen in the cells [3]. In order to induce their biological effects, androgens have to bind to the androgen receptor (AR): the hormone–receptor complex binds DNA and modulates gene expression [4]. Upon androgen stimulation, the proliferation of prostate cells is increased and a malignant tumour can develop [4]. In addition, the androgen receptor level is higher in prostate cancer cells compared to normal cells [4]. Consequently, androgens are involved not only in prostate tumorigenesis, but also in hormone-dependent cancer progression, supporting the use of androgen deprivation therapy in prostate cancer patients [5]. However, one limitation is that most tumours become resistant to this type of therapy and so, additional options of treatments are required to care for the patient. This letter reports on the development of such alternate treatment for prostate cancer.

Chlorambucil is an alkylating agent of the nitrogen mustard group and is used as cytostatic drug in cancer therapy [6]. In general, alkylating agents are both mutagenic and genotoxic [7]. The alkylating agents form adducts with DNA. However, they also form adducts with RNA and protein which contribute to the overall cytotoxicity [7]. The main side effects of chlorambucil are bone marrow suppression, anaemia and weak immune system [8,9].

Recently, we reported a series of estradiol–chlorambucil hybrids as anticancer drugs for site-directed chemotherapy of breast cancer [10]. The new hybrids showed moderate to significant cytotoxic activity in hormone-dependent (MCF-7) and hormone-independent (MDA-MB-436 and MDA-MB-486) breast cancer cell lines. Unfortunately, the hybrids were not selective towards the hormone-dependent breast cancer cell line MCF-7. Despite this, we sought to apply this type of strategy to the development of testosterone–chlorambucil hybrid for the treatment of prostate cancer. Therefore, this study was undertaken to verify if that

Abbreviations: T, testosterone; DHT, dihydrotestosterone; AR, androgen receptor; AR^+, androgen receptor positive; AR^-, androgen receptor negative; T-CHL, 7α-testosterone–chlorambucil hybrid.

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particular combination could lead to a selective hybrid towards hormone-dependent prostate cancer *in vitro*.

In order to increase the potential for selectivity towards the androgen receptor, the known 7α-allyltestosterone was selected as the starting material. Position 7 is considered the site of choice as it is located midway between the two functional groups found on testosterone (ketone and hydroxyl) that interact with the AR. These functional groups should remain intact and free of steric hindrance to favour AR binding [11–13]. Furthermore, it was decided to link the testosterone moiety to chlorambucil via an ester bound. It was speculated that the ester linkage would be sufficiently stable to reach the target cells and would have the potential to be hydrolyzed within the acidic environment of the cancer, releasing the anticancer agent chlorambucil. If successful, this approach would broaden the use of chlorambucil to hormone-dependent prostate cancer.

The current manuscript describes the synthesis of 7α-testosterone–chlorambucil hybrid (**1**) (Fig. 1) using two synthetic methods. The steroid and the nitrogen-mustard alkylating parts are linked together by a *trans*-but-2-enyl tether chain. The manuscript also presents preliminary biological evaluation of the novel hybrid on two prostate cancer cell lines; LNCaP (androgen receptor positive; AR^+) and PC3 (androgen receptor negative; AR^-).

2. Results and discussion

2.1. Chemistry

Testosterone (**2**) was initially functionalized using a known three-step reaction sequence (Scheme 1). The 7α-allyltestosterone (**3**) was obtained with 48% overall yield as described earlier [11–13]. Of note, this reaction sequence can be performed with up to 63% overall yield.

Scheme 1 illustrates the synthesis of testosterone–chlorambucil hybrid (**1**) by a S_N2 type substitution reaction. First, the 7α-allyltestosterone derivative (**3**) was subjected to an olefin cross-metathesis reaction. For this purpose, derivative **3** and allyl chloride were treated with Hoveyda-Grubbs catalyst 2nd generation in dichloromethane (DCM) at reflux for 8–10 h [14,15]. This reaction yielded 7α-(4-chloro-but-2-enyl) testosterone (**4**) in 90% yield as a mixture of *cis* and *trans* isomers (1:9). Hydrolysis of the acetate was performed in the presence of aqueous hydrochloric acid (HCl) in methanol (CH_3OH) under mild reflux to yield derivative **5** (94%, *cis*:*trans*, 1:9). Then, the substitution reaction was done using the allyl chloride **5**, chlorambucil and sodium bicarbonate in a mixture of DMF and water at reflux for 9 h. The testosterone–chlorambucil hybrid (**1**) was obtained with 58% yield (*cis*:*trans*, 15:85). Through purification, the mixture was slightly enriched into the *cis* isomer. Starting from testosterone, the complete sequence of reaction uses only 6 chemical steps and gave hybrid **1** with 23% yield.

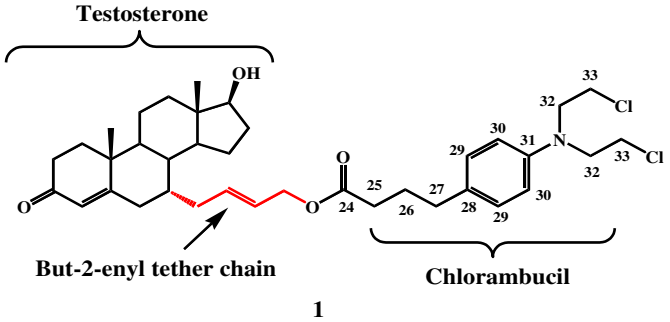


Fig. 1. Structure of 7α-testosterone–chlorambucil hybrid (**1**).

Scheme 2 illustrates an alternative synthesis of hybrid **1** by an olefin cross-metathesis reaction. Initially, derivative **3** was hydrolyzed with 10% aqueous HCl in methanol under a light reflux to yield 7α-allyltestosterone (**6**) with 95% yield. Secondly, chlorambucil was transformed into 4-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-butyric acid allyl ester (**7**) upon treatment with oxalyl chloride followed by reaction with allyl alcohol and pyridine in dichloromethane with 95% yield. Derivatives **6** and **7** (excess) were treated with Hoveyda-Grubbs catalyst 2nd generation in DCM at reflux for 24 h. This reaction yielded the testosterone–chlorambucil hybrid (**1**) with 78% yield (*cis*:*trans*, 15:85). It is noteworthy, that the same reaction provided the dimer **8** (as a side product which was easily isolated by flash column chromatography). The latter is also an anticancer tetraalkylating agent. Of note, the dimer **8** could be easily hydrolyzed to chlorambucil which could be recycled to the allyl ester **7** and coupled to 7α-allyltestosterone **6** in order to avoid the loss of starting material. Derivative **8** was also synthesized by self condensation of **7** upon treatment with Hoveyda-Grubbs catalyst 2nd generation in DCM at reflux for 15 h with 75% yield. All compounds were fully characterized by the use of infrared (IR), nuclear magnetic resonance spectroscopy (NMR) and their respective high resolution mass analysis.

2.2. Antiproliferative activity

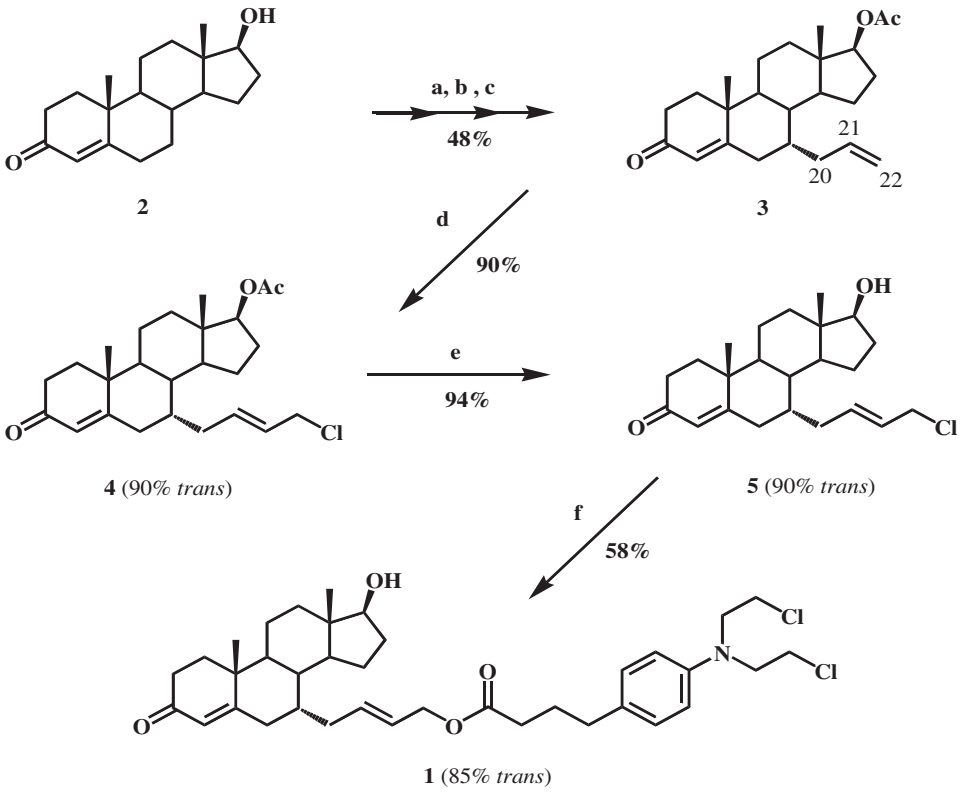
The second objective of the present study was to determine the cytotoxic effect of the hybrid along with controls (chlorambucil and cyproterone acetate) on both androgen-dependent (AR^+) and androgen-independent (AR^-) human prostate cancer cells. The biological activity of the compounds was evaluated *in vitro* using the MTT cell proliferation assay [16,17]. The MTT assay was performed over an incubation period of 72 h.

As shown by the MTT assays (Table 1), the new 7α-testosterone–chlorambucil hybrid (**1**) showed differential toxicity towards the two human prostate cancer cell lines used in our study (LNCaP (AR^+) and PC3 (AR^-)) compared to chlorambucil itself. Hence, hybrid (**1**) exhibited an IC_{50} of 101.0 μM for LNCaP cell line and was completely inactive towards the PC3 cell line at the maximum dose tested (160 μM, see Table 1). Chlorambucil was active on both types of cells with an IC_{50} of 124.3 μM and of 131.3 μM for, respectively, LNCaP and PC3 cell lines. Of note, despite the low activity of chlorambucil *in vitro*, it remains a very useful anticancer drug used daily in clinics. So, one should always be aware of the known discrepancies between *in vitro* and *in vivo* results [18–20]. The ability of chemosensitivity assays cannot always accurately predict the activity of a new drug *in vivo*. This study shows that the hybrid (**1**) is as active as chlorambucil *in vitro* which prelude favourably for its potential *in vivo*. Furthermore, the selectivity of hybrid (**1**) may be useful in the treatment of hormone-dependent prostate cancer reducing toxicities associated with chemotherapy. The hybrid (**1**) and chlorambucil are less cytotoxic than cyproterone acetate (CPA), a clinically used steroid-based antiandrogen. Of course, the mechanism of action of a nitrogen-mustard based drug is quite different from that of an antiandrogen.

While the affinity for the androgen receptor (AR) is an important data to take into account, and bearing in mind the relatively low activity of the final hybrid (**1**), it was decided not to measure the AR affinity at the moment.

3. Conclusion

This manuscript presents two efficient syntheses of 7α-testosterone–chlorambucil hybrid (**1**). Derivative **1** is readily available from testosterone either in a 6 step sequence with 23% overall yield or by following a more convergent 5 step sequence with 35% overall



Scheme 1. Synthesis of testosterone–chlorambucil hybrid (1) via an S_N2 substitution reaction. Reagents and conditions: (a) AcCl, Ac_2O, Pyr, reflux, 4 h, (b) 1. NBS, DMF, 0 °C, 1.5 h. 2. Li_2CO_3, LiBr, DMF, 92 °C, 4 h (c) 1. $TiCl_4$, Pyr, DCM, −78 °C, 5 min, 2. allyltrimethylsilane, −30 °C; 1.5 h (d) Allyl chloride, Hoveyda-Grubbs catalyst 2nd generation, CH_2Cl_2, reflux, 8 h (e) 5 N HCl, MeOH, mild reflux, 2 h. (f) Chlorambucil, $NaHCO_3$, DMF, H_2O, reflux, 9 h.

yield. The key features of the syntheses involved a) the known selective addition of an allyl moiety at position 7α of the steroid nucleus to give **3**, b) an efficient cross-metathesis reaction with allyl chloride to give the intermediate **4**, c) a S_N2 type substitution reaction of the allyl chloride **5** with chlorambucil sodium carboxylate to yield the desired hybrid **1** and, e) alternatively, a cross-metathesis reaction between 7α-allyltestosterone **6** and chlorambucil allyl ester **7** to yield the same hybrid. MTT assays were performed on an androgen-dependent and androgen-independent prostate cancer cell lines, LNCaP and PC3 respectively. Hybrid **1** shows promising selectivity towards hormone-dependent prostate cancer cells. Interestingly, the reference chemotherapeutic drug; chlorambucil is slightly less active and shows no selectivity towards the tested cells. The objective of efficiently linking testosterone to chlorambucil at the strategic 7α position of the steroid nucleus was reached using two different approaches. The biological *in vitro* results, akin to the reference drug (chlorambucil), anticipate selectivity and good *in vivo* activity in an animal model. Further research will be necessary to evaluate the complete biological potential of this novel molecule, to design even more powerful testosterone–nitrogen mustard hybrids and to evaluate the AR binding affinity.

4. Experimental protocols

4.1. Biological method

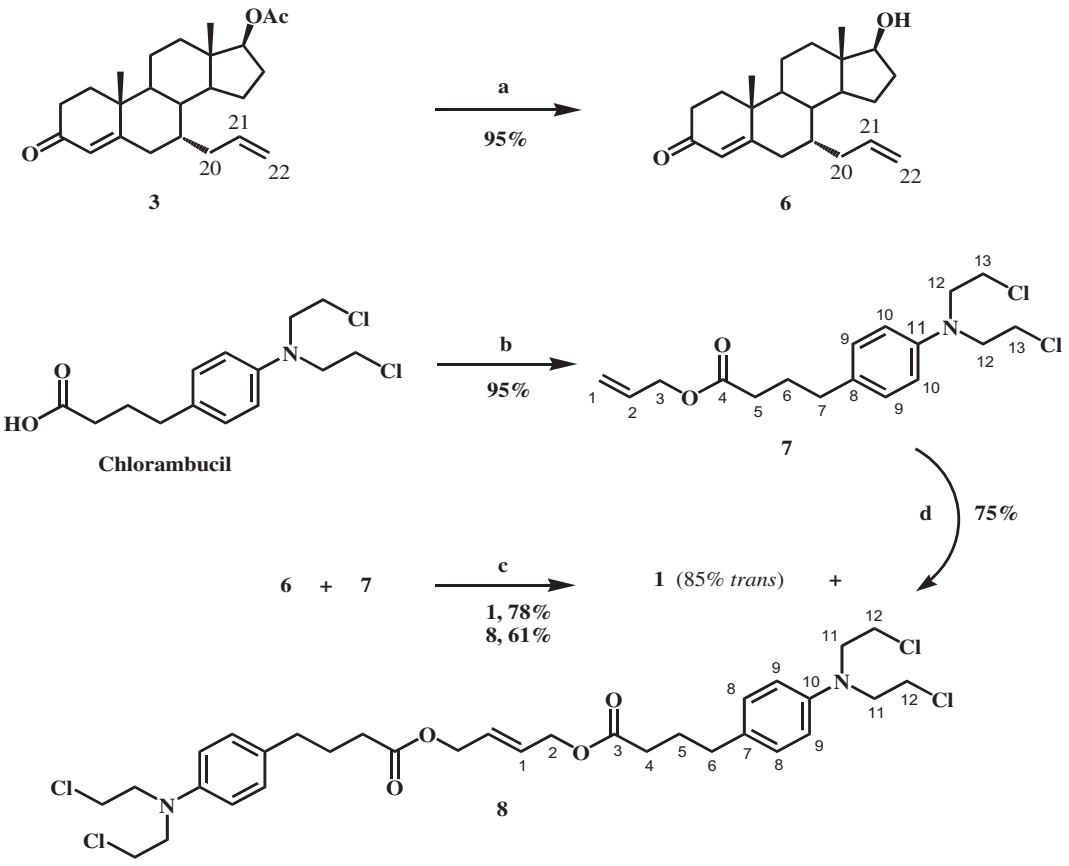
Human prostate cancer cell lines LNCaP (AR+) and PC3 (AR−) were purchased from ATCC, Maryland and maintained in RPMI medium containing 10% bovine growth serum containing 50 mg/mL gentamycin. The cells were maintained at 37 °C in a moisture-saturated atmosphere containing 5% CO_2.

4.1.1. Antiproliferative activity MTT assay

Cells were plated in 96-well plates 48 h before the assay. Stock solutions of the compounds were prepared by dissolving them in cremophor EL™:ethanol (1:1). Cells were treated for 72 h with serial dilution of the drugs between 160 and 1.25 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 cremophor EL™:ethanol (1:1) in the culture media was 0.1% and was kept constant in all experiment conditions. After 68 h of incubation, 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) was added to each well. Then, 4 h later, 100 μL of the solubilization solution (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.

4.2. Chemistry

Anhydrous reactions were performed under an inert atmosphere; the setup was assembled and cooled under nitrogen. Unless otherwise noted, starting material, reactant and solvents were obtained commercially and were used as such or purified and dried by standard means [21]. Organic solutions were dried over magnesium sulfate ($MgSO_4$), filtered and evaporated on a rotary evaporator under reduced pressure. All reactions were monitored 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 × 20 plates (Whatman, 4861 840). Flash column chromatography was performed according to the method of Still et al. on Merck grade 60 silica gel, 230–400 mesh [22]. All solvents used in chromatography were distilled.



Scheme 2. Synthesis of testosterone–chlorambucil hybrid (1) via olefin cross-metathesis reaction. Reagents and conditions: a) 10% aqueous HCl, MeOH, light reflux, 2 h; b) 1. $(COCl)_2$, 15 min. 2. allyl alcohol, pyridine, CH_2Cl_2, 30 min; c) Hoveyda-Grubb's catalyst 2nd generation, CH_2Cl_2, 24 h; d) Hoveyda-Grubb's catalyst 2nd generation, CH_2Cl_2, 15 h.

The melting points (MP) were recorded on an Electrothermal apparatus and are uncorrected. The infrared spectra were taken on a Nicolet Impact 420 FT-IR spectrophotometer. Mass spectral assays were obtained using a MS model 6210, Agilent technology instrument. The high resolution mass spectra (HRMS) were obtained by TOF (time of flight) using ESI (electrospray ionization) using the positive mode (ESI+) (Université du Québec à Montréal). 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 ^1H NMR and $CDCl_3$ δ 77.0 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. Note: Only the chemical shifts of the major *trans* isomer are given for the ^{13}C NMR of derivatives **1**, **4** and **5**.

Table 1
Inhibitory concentration[a] of cyproterone acetate, chlorambucil and 7α-testosterone–chlorambucil hybrid (**1**) on both AR^+ and AR^- prostate cancer cell lines.

Compounds	LNCaP (AR^+) IC_{50}, μM[a]	PC3 (AR^-) IC_{50}, μM[a]
Cyproterone acetate	43.0 ± 2.5	32.3 ± 3.7
Chlorambucil	124.3 ± 7.5	131.3 ± 3.1
T-CHL hybrid (1)	101.0 ± 12.7	NR

NR: Not reached.
[a] Inhibitory concentration (IC_{50}, μM) as obtained by the MTT assay. Experiments were performed in duplicates and the results represent the mean ± SEM of three or four independent experiments. The cells were incubated for a period of 72 h.

4.3. Synthesis of 7α-testosterone–chlorambucil hybrid (1) via a S_N2 substitution reaction (Scheme 1)

The synthesis and spectral data for 7α-allyl-4-androsten-17β-ol-3-one acetate (**3**) were already reported in the literature [11–13].

4.3.1. Synthesis of 7α-(4-chloro-but-2-enyl)-4-androsten-17β-ol-3-one acetate (**4**)

Under a nitrogen atmosphere, the steroid **3** (0.65 g, 1.75 mmol) was dissolved in dry CH_2Cl_2 (6 mL). The Hoveyda-Grubbs 2nd generation catalyst (0.11 g, 0.17 mmol) dissolved in dry CH_2Cl_2 (1 mL) was added to the steroid solution. Finally, excess allyl chloride (1.15 mL, 13.9 mmol) was added to the mixture. The resulting solution was stirred at reflux for 9 h. Afterwards, the mixture was evaporated to dryness. The crude brownish product was purified by flash chromatography with hexanes/acetone (95:5) as the eluent to give the pure steroid **4** (0.64 g) with 90% yield (90% *trans*). MP: 87–91 °C; IR(NaCl, ν_{max}, cm^{-1}): 1728 (C=O, acetate), 1669 (C=O, enone), 1614 (C=C), 1250 (C–O); ^1H NMR ($CDCl_3$, δ ppm): 5.69 (1H, s, 4-CH), 5.59 (2H, m, 21-CH and 22-CH), 4.58 (1H, t, J = 8.2 Hz, 17-CH), 4.00 (2H, m, 23-CH_2), 2.02 (3H, s, –OAc), 1.18 (3H, s, 19-CH_3), 0.82 (3H, s, 18-CH_3); ^{13}C NMR ($CDCl_3$, δ ppm): 199.2 (C-3), 171.3 (17-OAc), 169.1 (C-5), 134.1 (C-21), 128.5 (C-22), 126.5 (C-4), 82.6 (C-17), 47.2, 46.2, 45.2 (C-23), 42.8, 38.8, 38.5, 36.6, 36.4, 36.3, 36.1, 34.2, 28.7, 27.5, 23.1, 21.4, 20.9, 18.2, 12.1; ESI + HRMS:

$(M + H)^+$ calculated for $C_{25}H_{36}ClO_3 = 419.2348$; found = 419.2346 $(M - H)^+$.

4.3.2. Synthesis of 7α-(4-chloro-but-2-enyl)-4-androsten-17β-ol-3-one (**5**)

The steroid **4** (0.65 g, 1.55 mmol) was initially dissolved in methanol (12 mL). To this solution, 5 N aqueous HCl (4 mL) was added and the mixture was gently heated for 2 h. Of note, the reaction can occur at room temperature but must be stirred for a longer period of time. The reaction mixture was cooled down and transferred into a separatory funnel with ether (50 mL) and water (25 mL). The ethereal phase was washed once with a saturated sodium bicarbonate ($NaHCO_3$) solution (10 mL) and with water (4 × 10 mL). The organic solution was dried, filtered and evaporated to a solid. The final product **5** (0.54 g) was obtained with 94% yield (90% *trans*) and excellent purity. As a result, it was used without purification at the next step. MP: 142–145 °C; IR(NaCl, ν_{max}, cm^{-1}): 3423 (O–H), 1663 (C=O), 1611 (C=C); ^1H NMR ($CDCl_3$, δ ppm): 5.69 (1H, s, 4-CH), 5.61 (2H, m, 21-CH and 22-CH), 3.99 (2H, m, 23-CH_2), 3.63 (1H, t, J = 8.2 Hz, 17-CH), 1.19 (3H, s, 19-CH_3), 0.78 (3H, s, 18-CH_3); ^{13}C NMR ($CDCl_3$, δ ppm): 199.3 (C-3), 169.4 (C-5), 134.2 (C-21), 128.4 (C-22), 126.4 (C-4), 81.8 (C-17), 47.4, 46.4, 45.2 (C-23), 43.2, 38.9, 38.8, 36.9, 36.5, 36.4, 36.2, 34.2, 30.5, 28.8, 23.0, 21.1, 18.2, 11.2; ESI + HRMS: $(M + H)^+$ calculated for $C_{23}H_{34}ClO_2 = 377.2242$; found = 377.2240 $(M + H)^+$.

4.3.3. Synthesis of 7α-(4-(4-(bis-(2-chloro-ethyl)-amino)-phenyl-propylcarbonyloxy)-but-2-enyl)-4-androsten-17β-ol-3-one (**1**)

Steroid **5** (50 mg, 0.133 mmol) was initially dissolved in DMF (1 mL) in a pear shaped flask. In another flask, chlorambucil (50 mg, 0.164 mmol) and $NaHCO_3$ (10 mg, 0.16 mmol) were dissolved in DMF (4 mL) and water (0.15 mL). The resulting mixture was warmed up to help dissolution. Then, the steroid solution was added and, the resulting mixture, stirred at reflux for 9 h. Afterwards, the solution was cooled down, transferred into a separatory funnel with ether (30 mL) and water (10 mL). The ethereal phase was washed with water (6 × 20 mL), dried, filtered and evaporated to a crude solid. Flash chromatography with hexanes/acetone (95:5) as the eluent gave the final pure hybrid **1** (50 mg) with 58% yield (85% *trans*). MP: 47–50 °C; IR(NaCl, ν_{max}, cm^{-1}): 3422 (O–H), 1730 (C=O, ester), 1664 (C=O, enone), 1242 (C–O); ^1H NMR ($CDCl_3$, δ ppm): 7.07 (2H, d, J = 8.6 Hz, 29-CH), 6.60 (2H, d, J = 8.6 Hz, 30-CH), 5.68 (1H, s, 4-CH), 5.54 (2H, m, 21-CH and 22-CH), 4.48 (2H, d, J = 4.7 Hz, 23-CH_2), 3.65 (9H, m, 17-CH, 32-CH_2, 33-CH_2), 1.19 (3H, s, 19-CH_3), 0.78 (3H, s, 18-CH_3); ^{13}C NMR ($CDCl_3$, δ ppm): 199.3 (C-3), 173.6 (C-24), 169.52 (C-5), 144.5 (C-31), 134.3 (C-21), 129.9 (C-28, C-29), 126.5 (C-22), 126.4 (C-4), 112.5 (C-30), 81.8 (C-17), 64.9 (C-23), 53.9, 47.4, 46.4, 43.1, 40.7, 38.9, 38.7, 36.5, 36.4, 36.2, 34.2, 33.8, 30.5, 28.9, 26.9, 22.9, 21.1, 18.2, 11.2; ESI + HRMS: $(M + H)^+$ calculated for $C_{37}H_{52}Cl_2NO_4 = 644.3268$; found = 644.3260 $(M + H)^+$.

4.4. Synthesis of 7α-testosterone–chlorambucil hybrid (1) via an olefin cross-metathesis reaction (Scheme 2)

4.4.1. Synthesis 7α-allyl-4-androsten-17β-ol-3-one (**6**)

The steroid **3** (0.71 g, 1.9 mmol) was initially dissolved in methanol (15 mL). To this solution, 5 N aqueous HCl (5 mL) was added and the mixture was gently heated for 2 h. The reaction mixture was cooled down and transferred into a separatory funnel with ether (100 mL) and water (25 mL). The ethereal phase was washed once with a saturated sodium bicarbonate ($NaHCO_3$) solution (10 mL) and with water (4 × 10 mL). The organic solution was dried, filtered and evaporated to a solid. The final product **6** (0.60 g) was obtained with 95% yield and excellent purity. As a result, it was used without purification at the next step. MP: 195–200 °C; IR(NaCl, ν_{max}, cm^{-1}): 3449 (O–H), 1656 (C=O), 1615 (C=C), 1077 (C–O); ^1H NMR ($CDCl_3$, δ ppm): 5.69 (1H, s, 4-CH), 5.60 (1H, m, 21-CH), 5.02–4.94 (2H, dd, J = 3.1 Hz and J = 1.2 Hz, 22-CH_a and dt, J = 11.3 Hz and J = 2.0 Hz, 22-CH_b), 3.64 (1H, t, J = 16.8 Hz, 17-CH), 1.19 (3H, s, 19-CH_3), 0.78 (3H, s, 18-CH_3); ^{13}C NMR ($CDCl_3$, δ ppm): 199.4 (C-3), 169.8 (C-5), 137.1 (C-21), 126.4 (C-4), 117.0 (C-22), 81.8 (C-17), 47.4, 46.4, 43.1, 38.9, 38.7, 36.5, 36.3, 36.2, 36.1, 34.3, 30.5, 30.4, 22.9, 21.1, 18.2, 11.2; MS (m/e): 328 (M^+), 287 ($M^+ - C_3H_5$). exact mass: calculated for $C_{22}H_{32}O_2 = 328.2402$; found = 328.2395.

4.4.2. Synthesis of 4-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-butyric acid allyl ester (**7**)

Chlorambucil (350 mg, 1.15 mmol) was added into a 10 mL pear shaped flask. Oxalyl chloride ($(COCl)_2$, 3.0 g, 2 mL, 23.6 mmol) was then added to the solid, and the mixture stirred for a period of 15 min at room temperature (20 °C), under an inert atmosphere of nitrogen. Afterwards, the excess $(COCl)_2$ was evaporated using a flow of dry nitrogen. The resulting oil was dissolved in CH_2Cl_2 (2 mL) and evaporated once more with nitrogen. The acid chloride intermediate was treated with a solution of allyl alcohol (667 mg, 0.78 mL, 11.5 mmol) dissolved in DCM (2 mL) and pyridine (1.82 g, 1.86 mL, 23.0 mmol). The resulting solution was stirred at room temperature for 30 min. Then, the reaction mixture was transferred into a separatory funnel with diethyl ether (40 mL) and washed several times with water (5 × 10 mL). The organic phase was dried, filtered and evaporated to a crude solid. Flash chromatography with hexanes/acetone (4:1) as the eluent gave the final pure ester **7** (373 mg) with 95% yield (see numbering system on Scheme 2). IR(NaCl, ν_{max}, cm^{-1}): 1734 (C=O, ester); 1617 (C=C); ^1H NMR ($CDCl_3$, δ ppm): 7.08 (2H, d, J = 8.5 Hz, 9-CH), 6.63 (2H, d, J = 8.5 Hz, 10-CH), 5.90 (1H, m, 2-CH), 5.25 (2H, m, 1-CH_2), 4.58 (2H, apparent d, J = 2.3 Hz, 3-CH_2), 3.66 (8H, m, 12-CH_2 and 13-CH_2), 2.57 (2H, t, J = 7.4 Hz, 7-CH_2), 2.36 (t, 2H, J = 7.4 Hz, 5-CH_2), 1.94 (2H, q, J = 7.4 Hz, 6-CH_2); ^{13}C NMR ($CDCl_3$, δ ppm): 173.3 (C-4), 144.5 (C-11), 132.5 (C-2), 130.7 (C-9), 129.9 (C-8), 118.3 (C-1), 112.4 (C-10), 65.1 (C-3), 53.8 (C-12), 40.7 (C-13), 34.1 (C-7), 33.7 (C-5), 26.9 (C-6); ESI + HRMS: $(M + H)^+$ calculated for $C_{17}H_{24}Cl_2NO_2 = 344.1179$; found = 344.1182 $(M + H)^+$.

4.4.3. Synthesis of 7α-(4-(4-(bis-(2-chloro-ethyl)-amino)-phenyl-propylcarbonyloxy)-but-2-enyl)-4-androsten-17β-ol-3-one (**1**) and of 4-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-butyric acid 4-(4-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-butyryloxy)-but-2-enyl ester (**8**)

Under a nitrogen atmosphere, the 7α-allyltestosterone **6** (20 mg, 6.1×10^{-5} mol) was dissolved in dry CH_2Cl_2 (0.5 mL). The Hoveyda-Grubbs 2nd generation catalyst (4 mg, 6.3×10^{-6} mol) dissolved in dry CH_2Cl_2 (0.5 mL) was added to the steroid solution. Finally, excess allyl ester **7** (40 mg, 1.16×10^{-4} mol) was added to the mixture. The resulting solution was stirred at reflux for 24 h. Afterwards, the mixture was evaporated to dryness. The crude brownish product was purified by flash chromatography with hexanes/acetone (4:1) as the eluent to give the pure steroid **1** (30 mg) with 78% yield (85% *trans*). This yield is based on derivative **6** initially used. Along with the desired cross-coupling derivative **1**, the dimer **8** was also isolated (23 mg) as a side product with 61% yield. The latter yield is based on derivative **7** used in the reaction and the maximum quantity of dimer this could produce. The spectral data for compound **1** are identical as those previously described above in Section 4.3.3. The spectral data for compound **8** (see numbering system on Scheme 2): IR(NaCl, ν_{max}, cm^{-1}): 1734 (C=O, ester), 1615 (C=C); ^1H NMR ($CDCl_3$, δ ppm): 7.08 (2H, d, J = 8.2 Hz, 8-CH), 6.64 (2H, d, J = 8.2 Hz, 9-CH), 5.86 (1H, s, 1-CH), 4.60 (2H, s, 2-CH_2), 3.67 (8H, m, 11-CH_2 and 12-CH_2), 2.57 (2H, t, J = 7.4 Hz, 6-CH_2), 2.36 (t, 2H, J = 7.4 Hz, 4-CH_2), 1.94 (2H, apparent p, J = 7.4 Hz, 5-CH_2); ^{13}C NMR ($CDCl_3$, δ ppm): 173.2 (C-3), 144.4 (C-10), 130.8 (C-7), 129.9 (C-8), 128.3 (C-1), 112.4 (C-9), 63.9 (C-2),

53.8 (C-11), 40.7 (C-12), 34.1 (C-6), 33.6 (C-4), 26.8 (C-5); ESI + HRMS: $(M + H)^+$ calculated for $C_{32}H_{43}Cl_4N_2O_4 = 661.1947$; found = 661.1952 $(M + H)^+$.

4.4.4. Direct synthesis of 4-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-butyric acid 4-(4-{4-[bis-(2-chloro-ethyl)-amino]-phenyl}-butyryloxy)-but-2-enyl ester (**8**)

The allyl ester **7** (307 mg, 8.95×10^{-4} mol) was initially dissolved in CH_2Cl_2 (9 mL) and the solution maintained under a nitrogen atmosphere. The Hoveyda-Grubbs 2nd generation catalyst (56 mg, 8.9×10^{-5} mol) dissolved in dry CH_2Cl_2 (1 mL) was added to the solution. The solution was stirred at reflux for 15 h (or until the disappearance of the starting material **7** as detected by TLC). Afterwards, the solvent was evaporated to dryness. The crude product was purified by flash chromatography with hexanes/acetone (95:5) as the eluent to give the desired dimer **8** (221 mg) with 75% yield. The spectral data for compound **8** are identical as those previously described above in Section 4.4.3.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.04.027.

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