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Synthesis and evaluation of new steroidal lactam conjugates with aniline mustards as potential antileukemic therapeutics

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ABSTRACT

Alkylating agents are still nowadays one of the most important classes of cytotoxic drugs, which display a wide range of therapeutic use for the treatment of various cancers. We have synthesized and tested four hybrid homo-azasteroidal alkylating esters for antileukemic activity against five sensitive to alkylating agents human leukemia cell lines *in vitro* and against P388 murine leukemia *in vivo*. Comparatively, melphalan and 3-(4-(bis(2-chloroethyl)amino)phenoxy)propanoic acid (POPAM) were also examined. All the homo-aza-steroidal alkylators showed relatively lower acute toxicity, very promising and antileukemic activity both *in vitro* and *in vivo*.

1. Introduction

Alkylating agents, classical nitrogen mustards, nitro-nitrosoureas soureas and non-classical alkylators, such as platinum complexes, are still one of the most important classes of cytotoxic drugs, which display a wide range of therapeutic use for the treatment of various cancers including Hodgkin's disease, non-Hodgkin's lymphoma, leukemias, lung, ovarian, breast cancer, etc [1]. Chlorambucil [2], melphalan [3] and mechlorethamine [4,5], are some of the alkylating agents (nitrogen mustards) that are currently in clinical use. Their healing properties are derived from their capacity to block the DNA replication by creating permanent and stable bonds (cross-links) between the double-stranded DNA chains [6]. These bonds can be internal (inter-strand cross-links), which are located in two antiparallel DNA strands or external (intra-strand cross-links), which are made between bases of the same strand of DNA [7].

Major disadvantage of using alkylating agents in cancer chemotherapy is their systemic toxicity and efficacy [6,8,9], as they are non-selective and kill both cancerous and healthy cells. In order to reduce toxicity, steroids have been used as carrier molecules to deliver the alkylators to a specific target tissue by interacting with steroid hormone receptors [10,11]. These conjugates improve the physicochemical properties of the drugs such as the lipophilicity and the solubility. Usually, the alkylating agent is attached on the steroid with an ester bond. Representative examples are the steroidal alkylating agents Estramustine (ester of estradiol and mechlorethamine, **(1)** and Prednimustine (ester of prednisolone and chlorambucil **(2)**, which are currently applied in cancer therapy on the treatment of prostate cancer and lymphoproliferative malignancies, respectively (Fig. 1) [12–14]. Homo-azasteroids, incorporating a lactam moiety (-NH-CO-) into steroidal ring/s, in their esterified forms have also been introduced as carrier molecules of alkylating agents with significant therapeutic efficacy in preclinical studies against leukemia and several other neo-plastic malignancies both *in vivo* and *in vitro* [15–23]. Herein, we report the synthesis and the evaluation of novel steroidal lactam derivatives of type **3**, which are conjugated with 3-(4-(bis(2-chloroethyl)amino)phenoxy)propanoic acid (POPAM) in the expectation of improving its biological activity.

The new homo-aza-steroidal alkylating esters of POPAM were designed with the ultimate goal of improving therapeutic efficacy of treatment of resistant tumors to classical and non-classical alkylating drugs. These hybrid compounds were aimed at: i) to link a well-balanced acting alkylator due to concerns of its toxicity and therapeutic effect with a modified aza-steroid which will assist with transportation of the alkylating moiety inside the cell and direct it to the targeted biomolecules in the nucleus, ii) to diminish

Abbreviations: POPAM, 3-(4-(bis(2-chloroethyl)amino)phenoxy)propanoic acid; L-PAM, L-phenylalanine mustard.

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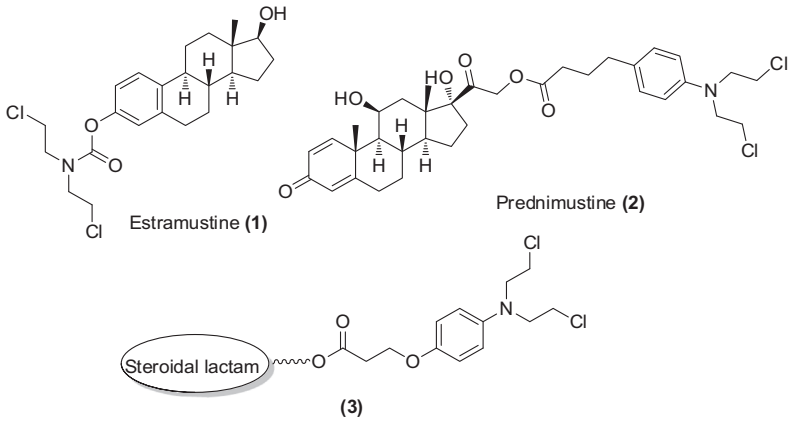


Fig. 1. Representative known steroidal alkylating agents **(1, 2)** and the general type of the steroidal lactam alkylators **(3)** studied in this work.

systemic toxicity and increase anticancer activity significantly resulting to improved therapeutic ratio, iii) to produce a multi-targeted antitumor effect and interfere with crucial biochemical molecular pathways and DNA repair mechanisms of the cancer cells such as the protein kinase C (PKC) signaling pathway [22,23].

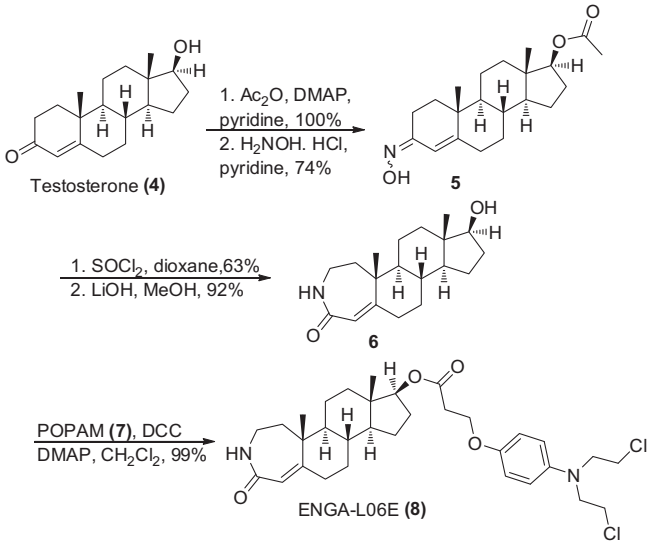
2. Results and discussion

Four new ester conjugates of steroidal lactams with POPAM were synthesized. The steroidal lactams (aza-homo steroids) used in the present study, carry one or more amide functionalities at the rings of the basic steroidal framework. It is known that such steroidal lactams can be synthesized from a ketosteroid via the corresponding oximes and Beckman rearrangement [24–26]. The first conjugate was prepared starting from testosterone **4** via the lactam **6** according to the known procedure of Catsoulacos and Camoutsis (Scheme 1) [27]. Testosterone was acetylated by acetic anhydride in pyridine and the corresponding acetate was condensed with hydroxylamine to give the *E*- and *Z*-oximes **5**. Next, the Beckman rearrangement of the mixture of **5** with $SOCl_2$ in dioxane was attempted. It is known that in a typical Beckman rearrangement the group *anti* to the OH group migrates from C to N. In the case of oximes **5** and in accordance to previously published data a single lactam **6** was isolated in 63% yield most likely due to *E/Z* isomerization prior to alkyl migration [25,28]. After deprotection of the acetyl moiety by basic hydrolysis (92%), the esterification of hydroxyl group was carried out by POPAM **(7)**, DMAP and DCC in CH_2Cl_2 yielding the desired conjugate ENGA-L06E **(8)** in quantitative yield.

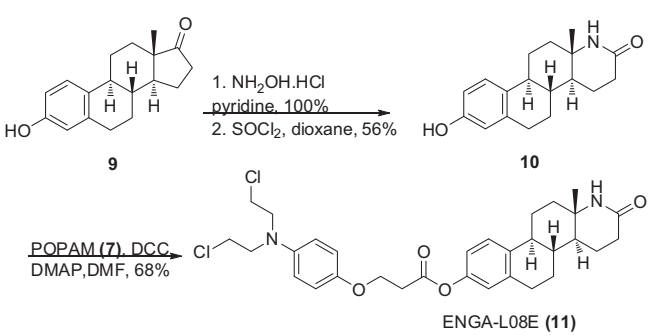
The second conjugate, ENGA-L08E **(11)** is a derivative of estrone **9** and was synthesized from the esterification of lactam **10** with acid **7** in the presence of DCC and DMAP in DMF (Scheme 2). Lactam 17*a*-aza-*D*-homoestrone **10** was synthesized according to the methodology of Liao and coworkers [29].

The other two steroidal lactam alkylators were prepared from adrenosterone via the novel monolactam **16** and dilactam **20**. The preparation of monolactam **16** was accomplished as presented in Scheme 3. Regioselective reduction of 17-keto group of the *D*-ring of adrenosterone was performed according to Morreal [30–32] with $NaBH_4$/MeOH to give the desired alcohol with traces of another product, which was used in the next step without further purification. Subsequent protection of hydroxyl group yielded 17-acetate **13** in 90% yield. Following the established route, compound **13** was selectively converted to oximes **14** after treatment with $NH_2OH.HCl$ in pyridine. Beckman rearrangement of the mixture of **15** gave the desired monolactam **16**, the structure of which was determined by comprehensive analyses of its ^1H and ^{13}C NMR, COSY, HSQC, and HMBC spectroscopic data. As shown in Fig. 2 key HMBC interactions of the protons H-2 with C-10, C-19 and C-3 indicate the NH group to be positioned between C-2 and C-3. This is further supported by the COSY correlations of NH with H-4 and H-2. Finally, deprotection of the hydroxyl group of **16** followed by esterification with acid **7** afforded conjugate ENGA-L07E **(17)** in 96% yield.

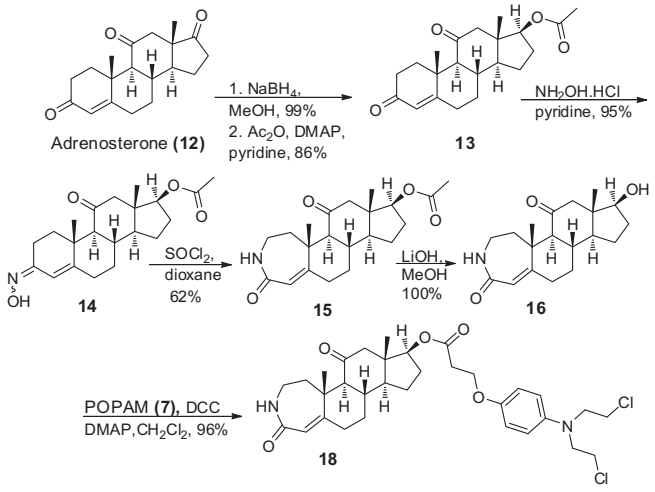
The bislactam alkylator **21** was prepared in a similar fashion by the synthetic route illustrated in Scheme 4. Oximes **18** were subjected to Beckmann rearrangement and the bislactam **19** was iso-



Scheme 1. Synthesis of ENGA-L06E **(8)**.



Scheme 2. Synthesis of ENGA-L08E **(11)**.



Scheme 3. Synthesis of ENGA-L07E (**17**).

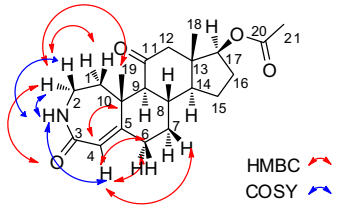
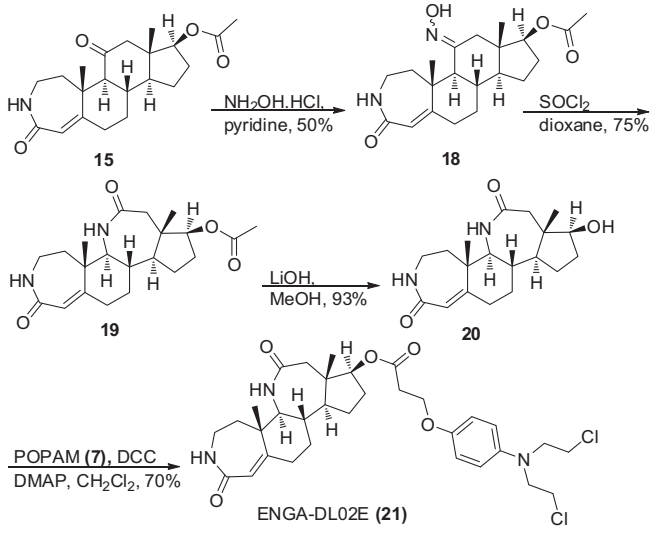


Fig. 2. Key COSY and HMBC interactions of **16**.



Scheme 4. Synthesis of ENGA-DL02E (**21**).

lated in 75% yield. The structure of **19** has been confirmed by its 1D- and 2D-NMR spectra.

We have tested the new hybrid homo-aza-steroidal alkylators against five sensitive to alkylating agents human leukemia cell lines *in vitro*, in comparison with the well-established cancer therapeutics alkylator, L-PAM and the azasteroid esters' alkylating moiety, POPAM (Fig. 3). As it is presented in Tables 1 and 2, L-PAM was significantly more active than POPAM. On the other hand, all the four conjugates displayed significantly higher antileukemic activity, cytostatic and cytotoxic effect on the tested human leukemia cell lines than the non-steroidal alkylators L-PAM and POPAM.

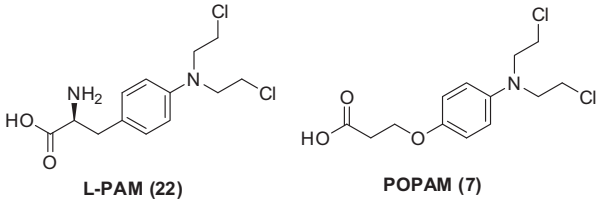


Fig. 3. Melphalan (L-phenylalanine mustard, L-PAM) and POPAM.

Table 1
Growth inhibition/cytostatic (GI_{50} and TGI μM) and cytocidal/cytotoxic (IC_{50} μM) antileukemic effects induced by L-PAM, POPAM and ENGA-L06E on MOLT-4, K562, CCRF-CEM, JURKAT and SUP-B15 human leukemia cell lines.

Human cancer cell lines	L-PAM (μM)			POPAM (μM)			ENGA-L06E (μM)		
	GI_{50}	TGI	IC_{50}	GI_{50}	TGI	IC_{50}	GI_{50}	TGI	IC_{50}
MOLT-4	2	25	>100	20	75	>100	<1	20	95
K562	40	>100	>100	75	>100	>100	5	65	>100
CCRF-CEM	5	95	>100	50	>100	>100	<1	15	>100
JURKAT	7	75	>100	60	>100	>100	<1	25	>100
SUP-B15	20	>100	>100	65	>100	>100	2	30	>100

Table 2
Growth inhibition/cytostatic (GI_{50} and TGI μM) and cytocidal/cytotoxic (IC_{50} μM) antileukemic effects induced by ENGA-L07E, ENGA-L08E and ENGA-DL02E on MOLT-4, K562, CCRF-CEM, JURKAT and SUP-B15 human leukemia cell lines.

Human cancer cell lines	ENGA-L07E (μM)			ENGA-L08E (μM)			ENGA-DL02E (μM)		
	GI_{50}	TGI	IC_{50}	GI_{50}	TGI	IC_{50}	GI_{50}	TGI	IC_{50}
MOLT-4	<1	15	90	<1	10	80	<1	20	90
K562	<1	40	>100	6	50	>100	15	65	>100
CCRF-CEM	<1	5	65	<1	15	>100	7	25	80
JURKAT	<1	10	80	<1	15	90	<1	25	90
SUP-B15	<1	20	85	<1	25	>100	10	40	>100

ENGA-L07E was the most potent among the other three aza-steroidal alkylators.

The steroidal esters were also evaluated for their *in vivo* acute toxicity. The toxicity values of the compounds tested are illustrated in Table 3. In all cases the esterification of the alkylating agent with the steroidal moiety resulted in an increase of the LD_{50} values. As indicated by the LD_{50} and LD_{10} values in Table 3, the lactam steroidal esters induced significantly decreased acute toxicity effects (LD_{10} > 80 mg/kg) in comparison with the non-steroidal alkylators POPAM (LD_{10} = 14 mg/kg) and L-PAM (LD_{10} = 15 mg/kg).

The *in vivo* antileukemic activity of the new esters was assessed using the oncostatic parameter T/C%. The results on the antileukemic activity of the compounds against leukemia P388 are shown in Table 4. The non-steroidal alkylators POPAM showed significant antileukemic activity with a T/C equal to 223%. The value of T/C = 125% is considered as the limit for a potential chemotherapeutic agent according to the National Cancer Institute criteria. However, the majority of the steroidal esters showed improved

Table 3
Acute toxicity of compounds in BDF1 mice.

Compounds	LD_{50} (mg/kg)	LD_{10} (mg/kg)
POPAM	20	15
L-PAM	20	14
ENGA-L06E	150	120
ENGA-L07E	140	110
ENGA-L08E	95	80
ENGA-DL02E	180	140

Table 4
Comparison of antitumor activity of POPAM, L-PAM with the steroidal esters on P388-leukemia-bearing mice.

Compounds	Treatment schedule	Dosage (mg/kg)	MST[a] ± SD (days)	T/C[b] (%)	Cures
ENGA-L06E	Day-1	120	31,1 ± 2	324	2/6
ENGA-L08E	Day-1	80	29,8 ± 2.7	310	2/6
ENGA-L07E	Day-1	110	34,2 ± 3	356	2/6
ENGA-DL02E	Day-1	140	33,4 ± 2,9	347	2/6
POPAM	Day-1	15	21,4 ± 2	223	0/6
L-PAM	Day-1	14	39,2 ± 6	408	3/6
CONTROL	Day-1	Saline	9,6 ± 2	100	0/8

[a] MST = Mean Survival Time.
[b] T/C = the percent increase median life span of the drug-treated animals (T) versus corn-oil-treated animals (C).

antileukemic activity compared with their alkylating component alone (POPAM). As it is indicated by the corresponding T/C% values of the steroidal alkylators, it is confirmed that the chemical linkage between the nitrogen mustard (POPAM) and the lactam-steroids not only reduces the toxic effects and acute toxicity, but also leads to compounds with very improved bioactivity and antitumor effects. The mice treated with ENGA-L06E, ENGA-L07E, ENGA-L08E, ENGA-L02E demonstrated a significantly prolonged life span compared with control mice (Table 4). Moreover, two out of six cures were also recorded for the treated P388-bearing mice.

As shown in the Table 4 the compound L-PAM, which is currently a widely used antitumor and specifically antileukemic drug, presented significantly better antileukemic activity versus P388 rodent leukemia *in vivo* in comparison to POPAM and slightly better than the tested steroidal alkylators. However, all four new compounds exhibited a closely similar antileukemic activity with 2/5 cures compared to the L-PAM, which ends with 3/5 cures on our experiments.

3. Conclusions

To conclude, all new ester conjugates of steroidal lactams with POPAM performed important antileukemic activity *in vitro* and *in vivo*, together with relatively very low acute toxicities, producing a high therapeutic ratio. Based on the above mentioned encouraging preliminary results, further studies are required for the development of these molecules as anticancer agents.

4. Experimental section

All reactions were carried out under an atmosphere of Ar unless otherwise specified. Commercial reagents of high purity were purchased and used without further purification, unless otherwise noted. Reactions were monitored by TLC and using UV light as a visualizing agent and aqueous ceric sulfate/phosphomolybdic acid, ethanolic 4-anisaldehyde solution, potassium permanganate solution, and heat as developing agents. The ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, and 300 and 75 MHz with tetramethylsilane as an internal standard. Chemical shifts are indicated in δ values (ppm) from internal reference peaks (TMS ^{1}H 0.00; $CDCl_3$ ^{1}H 7.26, 13C 77.00; DMSO-d_6 1H 2.50, ^{13}C 39.51, pyridine-d_5 ^{1}H 8.74, 7.58, 7.22; ^{13}C 150.35, 135.91, 123.87). Optical rotations were measured with a sodium lamp and are reported as follows: $[\alpha]_D^{\circ C}$ (c = g/100 mL, solvent). Melting points (mp) are uncorrected. High-resolution mass spectra (HRMS) were recorded by direct injection of 2 μl of a 2 μM solution of the compounds in water–acetonitrile (1/1; v/v) and 0.1% formic acid on a mass spectrometer (hybrid ion trap-orbitrap mass spectrometer) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 °C) with resolution R = 60.000 at m/z = 400 (mass range = 150–2000) and dioctylphthalate (m/z = 391.28428) as the "lock mass".

4.1. Testosterone-17β-acetate (S1)

Testosterone **4** (500 mg, 1.73 mmol) was dissolved in 2.5 mL acetic anhydride. Then, 4 mg (0.035 mmol) of DMAP and 0.25 mL of dry pyridine were added. The mixture was stirred at room temperature for 24 h. The reaction was quenched with water and the mixture was extracted with ethyl acetate (3 × 30 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was used to the next step without further purification (567 mg, 100% yield). The NMR spectral data were accordance with those reported in the literature [33]. **S1**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.73 (s, 1H), 4.60 (dd, J = 9.0, 8.0 Hz, 1H), 2.48–2.25 (m, 4H), 2.18 (m, 1H), 2.04 (s, 3H), 2.03–1.98 (m, 1H), 1.77–1.87 (m, 2H), 1.75–1.62 (m, 2H), 1.61–1.47 (m, 3H), 1.46–1.26 (m, 2H), 1.21–1.16 (m, 1H), 1.19 (s, 3H), 1.05–0.9 (m, 3H), 0.84 (s, 3H).

4.2. E- and Z-testosterone 17-β-acetate oximes, 5

Testosterone 17-β-acetate **S1** (914 mg, 2.77 mmol) was dissolved in 10 mL of dry pyridine. Hydroxylamine hydrochloride (461 mg, 6.64 mmol) was added and the solution was stirred under reflux for 6 h. The solution was poured into water, and the mixture was extracted with ethyl acetate (3 × 30 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product that was further purified by chromatography on SiO_2 (eluent; hexane:ethyl acetate = 4:1) to afford 675 mg of the *E*- and *Z*-oximes (*Z*-/*E*- = 1/3, 74% yield) as white solids [27]. ***E*-5:** ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.76 (s, 1H), 4.59 (t, J = 8.4 Hz, 1H), 2.36–2.07 (m, 4H), 2.04 (s, 3H), 1.90 (d, J = 13.7 Hz, 1H), 1.77 (m, 2H), 1.72–1.43 (m, 5H), 1.43–1.28 (m, 3H), 1.17 (m, 1H), 1.07 (s, 3H), 1.05–0.84 (m, 3H), 0.82 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.2, 157.2, 155.3, 117.3, 82.6, 53.6, 50.4, 42.4, 38.0, 36.7, 35.6, 34.6, 32.3, 31.7, 27.5, 23.5, 21.2, 20.8, 18.6, 17.8, 12.0; ***Z*-5:** ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.47 (s, 1H), 4.59 (t, J = 8.4 Hz, 1H), 2.46–2.10 (m, 4H), 2.04 (s, 3H), 1.89 (d, J = 13.1 Hz, 1H), 1.85–1.72 (m, 2H), 1.70–1.43 (m, 5H), 1.42–1.14 (m, 4H), 1.11 (s, 3H), 1.08–0.83 (m, 3H), 0.83 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.2, 159.2, 154.2, 110.2, 82.6, 53.9, 50.3, 42.5, 38.9, 36.7, 36.2, 35.5, 32.9, 32.0, 27.5, 24.7, 23.5, 21.2, 20.7, 18.1, 12.0; FT-IR (KBr) 3245, 2956, 2927, 2893, 2868, 2848, 1735, 1714, 1239, 1041, 1020; HRMS m/z for $C_{21}H_{32}NO_3$ $[M+H]^+$ calcd 346.2382, found 346.2383.

4.3. 3-Aza-17β-acetoxy-A-homo-4α-androsten-4-one, S2

E- and *Z*-testosterone-17-acetate oximes **5** (100 mg, 0.29 mmol) were dissolved in 5.1 mL of dry dioxane. The mixture was cooled to 0 °C and thionyl chloride (0.6 mL) was added dropwise. The mixture was allowed to warm to room temperature and stirred for 3 h. The reaction was quenched with aq. $NaHCO_3$ and the mixture was extracted with ethyl acetate (3 × 20 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product that was further purified by chromatography on SiO_2 (ethyl acetate) to afford 63 mg of 3-aza-17β-acetoxy-A-homo-4α-androsten-4-one (74%) as a yellowish solid [27]. **S2**: mp = 228–231 °C; $[\alpha]_D^{23}$ −6.0 (*c* 1.67, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.44 (s, 1H), 5.73 (s, 1H), 4.59 (t, J = 8.5 Hz, 1H), 3.37–3.04 (m, 2H), 2.48 (td, J = 13.5, 4.5 Hz, 1H), 2.29–2.08 (m, 2H), 2.02 (s, 3H), 2.00–1.68 (m, 4H), 1.66–1.44 (m, 5H), 1.45–1.20 (m, 3H), 1.14 (s, 3H), 1.12–0.92 (m, 2H), 0.81 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.9, 169.9, 160.4, 119.2, 82.5, 53.3, 50.4,

44.5, 42.7, 42.1, 36.9, 36.7, 36.2, 35.5, 33.2, 27.6, 23.5, 21.4, 21.3, 21.0, 12.1. FT-IR (KBr): 3453, 2930, 2848, 1732, 1653, 1606, 1480, 1446, 1374, 1249, 1130, 1041, 875; HRMS *m/z* for $C_{21}H_{32}NO_3$ $[M+H]^+$ calcd 346.2382, found 346.2384.

4.4. 3-Aza-17β-hydroxy-A-homo-4α-androsten-4-one, **6**

3-Aza-17β-acetoxy-A-homo-androsten-4-one, **S2** 117 mg (0.341 mmol) was dissolved in 4.9 mL MeOH and LiOH (1 N, 2 mL) were added dropwise. The mixture was stirred at room temperature for 1 h. The reaction was quenched with NH_4Cl and the mixture was extracted with dichloromethane (3 × 10 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford 87 mg of 3-aza-17β-hydroxy-A-homo-4α-androsten-4-one **6** in 92% yield [27]. **6**: mp = 283–286 °C; $[\alpha]_D^{23}$ −16.4 (*c* 0.58, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.20 (s, 1H), 5.73 (s, 1H), 3.64 (t, *J* = 8.5 Hz, 1H), 3.23 (m, 1H), 3.14 (m, 1H), 2.48 (td, *J* = 13.2, 4.0 Hz, 1H), 2.14 (m, 1H), 2.07 (m, 1H), 1.98 (dd, *J* = 14.6, 8.0 Hz, 1H), 1.90–1.77 (m, 2H), 1.67–1.53 (m, 4H), 1.50–1.40 (m, 1H), 1.37 (dd, *J* = 13.0, 4.0 Hz, 1H), 1.29 (ddd, *J* = 17.5, 11.7, 5.2 Hz, 2H), 1.15 (s, 3H), 1.10–0.82 (m, 3H), 0.77 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.9, 160.9, 119.0, 81.6, 53.4, 50.5, 44.5, 42.1, 42.1, 36.7, 36.5, 36.3, 35.6, 33.2, 30.5, 23.3, 21.4, 21.4, 11.1; FT-IR (KBr): 3313, 2960, 2928, 2846, 1654, 1648, 1611, 1467, 1258, 1125, 1055; HRMS *m/z* for $C_{19}H_{30}NO_2$ $[M+H]^+$ calcd 304.2277, found 304.2279.

4.5. Conjugate ENGA-L06E, **8**

3-Aza-17β-hydroxy-A-homo-4α-androsten-4-one, **6** 87 mg (0.287 mmol) was dissolved in 28 mL of dry dichloromethane. Then, 3-(4-(bis(2-chloroethyl)amino)phenoxy)propanoic acid (106 mg, 0.573 mmol), DCC (119 mg, 0.574 mmol) and a catalytic amount of DMAP were added. After the resulting solution was stirred at room temperature for 24 h the solvent was evaporated and the residue was purified by flash column chromatography on SiO_2 (eluent; hexane:ethyl acetate = 1:2) to give conjugate **8** (191 mg, 99%). **8**: mp = 53–56 °C; $[\alpha]_D^{23}$ +10.5 (*c* = 0.91 $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.92 (s, 1H), 6.83 (d, *J* = 8.8 Hz, 2H), 6.66 (d, *J* = 8.6 Hz, 2H), 5.72 (s, 1H), 4.66 (t, *J* = 8.4 Hz, 1H), 4.17 (t, *J* = 6.0 Hz, 2H), 3.63 (m, 4H), 3.59 (m, 4H), 3.32–3.04 (m, 2H), 2.75 (t, *J* = 6.1 Hz, 2H), 2.48 (m, 1H), 2.27 (m, 1H), 2.15 (m, 2H), 1.50–1.98 (m, 10H), 1.33 (m, 2H), 1.14 (s, 3H), 1.05 (m, 1H), 0.80 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.0, 170.4, 161.3, 151.3, 140.8, 118.8, 116.3, 114.4, 82.7, 64.4, 54.2, 53.2, 50.2, 44.5, 42.7, 41.9, 40.7, 36.7, 36.2, 35.3, 33.8, 33.1, 27.5, 25.6, 24.9, 23.4, 21.3, 12.1; FT-IR (KBr): 3450, 2925, 1731, 1651, 1607, 1512, 1469, 1353, 1238, 1181, 1041, 869, 813; HRMS *m/z* for $C_{32}H_{45}Cl_2N_2O_4$ $[M+H]^+$ calcd 591.2756, found 591.2760.

4.6. Estrone oxime, **S3**

To a solution of estrone **9** (100 mg, 0.37 mmol) in 2.2 mL absolute ethanol was added hydroxylamine hydrochloride (62 mg, 0.88 mmol) and pyridine (1.2 mL). The mixture was refluxed for 6 h. Then, water was added and the mixture was extracted with ethyl acetate (3 × 10 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product that was further purified by chromatography on SiO_2 (eluent; hexane:ethyl acetate = 3:1) to afford 105 mg of estrone oxime **S3** (100%) as a white solid. The spectral data were accordance with those reported in the literature [34]. **S3:** mp = 250–253 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.08 (s, 1H), 9.00 (s, 1H), 7.05 (d, *J* = 8.2 Hz, 1H), 6.51 (d, *J* = 8.2 Hz, 1H), 6.44 (s, 1H), 4.04 (s, 2H), 3.57 (s, 1H), 2.73 (m, 2H), 2.44–2.21 (m, 2H), 2.16 (m, 1H), 1.96–1.73 (m, 2H), 1.58–1.43 (m, 1H), 1.45–1.17 (m, 4H), 0.85 (s, 3H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 167.9, 154.9, 137.0, 130.1, 125.9, 114.9, 112.7, 52.5, 43.6, 43.3, 37.9, 34.2, 29.0, 26.8, 25.9, 24.8, 22.5, 17.3; FT-IR (KBr): 3413, 3261, 2959, 2925, 2851, 1655, 1560, 1455, 1238, 1117, 1107; HRMS *m/z* for $C_{18}H_{24}NO_2$ $[M+H]^+$ calcd 286.1807, found 286.1810.

4.7. Lactam 17a-aza-D-homoestrone, **10**

Estrone oxime **S3** (108 mg, 0.378 mmol) were dissolved in 6.4 mL of dry dioxane. The mixture was cooled to 0 °C and thionyl chloride (0.7 mL) was added dropwise. The mixture was allowed to reach room temperature and stirred for 24 h. The reaction was quenched with $NaHCO_3$ and the mixture was extracted with dichloromethane (3 × 20 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product that was further purified by chromatography on SiO_2 (eluent; hexane: ethyl acetate = 2:1) to afford 42 mg of lactam **10** (56% based on recovered starting material) accompanied by recovered starting material [32 mg of starting material (0.112 mmol)]. The spectral data were in accordance with those reported in the literature [29,35]. **10**: mp > 300 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.01 (s, 1H), 7.53 (s, 1H), 7.05 (d, *J* = 8.0 Hz, 1H), 6.51 (d, *J* = 8.4 Hz, 1H), 6.44 (s, 1H), 2.72 (m, 2H), 2.38–2.11 (m, 4H), 2.06–1.75 (m, 4H), 1.60–1.32 (m, 2H), 1.21 (m, 3H), 1.07 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 169.9, 154.9, 136.9, 130.0, 125.9, 114.6, 112.8, 53.6, 45.9, 42.8, 39.1, 39.06, 30.6, 29.3, 26.4, 25.7, 21.8, 19.5; FT-IR (KBr): 3386, 2933, 2865, 1631, 1584, 1500, 1455, 1383, 1293, 1255, 1176, 1159, 1135, 972; HRMS *m/z* for $C_{18}H_{24}NO_2$ $[M+H]^+$ calcd 286.1807, found 286.1810.

4.8. ENGA-L08E (**11**)

Lactam **10** (42 mg, 0.147 mmol) was dissolved in 14 mL of dry DMF. Then, 3-(4-(bis(2-chloroethyl)amino)phenoxy)propanoic acid (90 mg, 0.293 mmol), DCC (61 mg, 0.293 mmol) and a catalytic amount of DMAP were added. After the resulting solution was stirred at room temperature for 24 h the solvent was evaporated and the residue was purified by flash column chromatography on SiO_2 (eluent; dichloromethane:acetone = 2:1) to give conjugate **11** (56 mg, 68%). **11**: glass, $[\alpha]_D^{23}$ +73.5 (*c* 0.90, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.25 (d, *J* = 6.0 Hz, 1H), 6.89 (d, *J* = 8.8 Hz, 2H), 6.85 (s, 1H), 6.82 (s, 1H), 6.68 (d, *J* = 8.8 Hz, 2H), 6.31 (s, 1H), 4.30 (t, *J* = 6.1 Hz, 2H), 3.62 (dt, *J* = 29.2, 6.6 Hz, 8H), 2.97 (dd, *J* = 15.1, 9.0 Hz, 2H), 2.88 (m, 2H), 2.58–2.36 (m, 4H), 2.23–2.00 (m, 2H), 1.92–1.66 (m, 3H), 1.60–1.29 (m, 4H), 1.19 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.7, 169.8, 151.3, 148.5, 141.0, 137.8, 137.2, 126.1, 121.3, 118.7, 116.5, 114.5, 64.4, 54.4, 54.2, 46.6, 43.4, 40.7, 39.9, 38.9, 34.9, 30.5, 29.5, 26.5, 25.9, 22.1, 19.8; FT-IR (KBr): 3329, 2927, 2850, 1757, 1626, 1577, 1512, 1437, 1311, 1244, 1157, 1088, 1045, 892; HRMS *m/z* for $C_{31}H_{39}Cl_2N_2O_4$ $[M+H]^+$ calcd 574.2287, found 574.2289.

4.9. 17α-Hydroxyandrost-4-ene-3,11-dione, **S4**

A solution of adrenosterone (500 mg, 1.65 mmol) in 100 mL in MeOH was cooled down to 0 °C. To this solution, 50 mg (1.32 mmol) $NaBH_4$ were slowly added. Then, the reaction mixture was allowed to reach room temperature. After 1 h, the solvent was removed and the mixture was extracted with ethyl acetate (3 × 50 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. Ethyl acetate was added and the precipitate was collected by filtration to give 497 mg of **S4** as a white solid (99% yield) [30]. **S4**: mp = 146–149 °C; $[\alpha]_D^{23}$ +184.1 (*c* = 1.67 $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.57 (s, 1H), 3.97 (d, *J* = 7.0 Hz, 1H), 3.72 (t, *J* = 8.3 Hz, 2H), 3.37 (s, 1H), 2.60 (d, *J* = 13.1 Hz, 1H), 2.45–1.96 (m, 5H), 1.67 (ddd, *J* = 77.4, 27.8,

18.5 Hz, 7H), 1.26 (d, *J* = 14.3 Hz, 3H), 1.12 (s, 2H), 0.60 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 209.1, 199.7, 169.2, 123.9, 79.0, 62.3, 54.4, 49.2, 46.6, 37.9, 37.1, 34.3, 33.3, 31.9, 31.3, 29.9, 22.4, 16.9, 11.5; FT-IR (KBr): 3420, 3302, 2964, 2932, 2851, 1704, 1666, 1612, 1347, 1370, 1323, 1267, 1242, 1192, 1133, 1064, 943, 866; HRMS *m*/*z* for $C_{19}H_{27}O_3$ $[M+H]^+$ calcd 303.1960, found 303.1963.

4.10. 17α-Acetoxyandrost-4-ene-3,11-dione, ***13***

17α-Hydroxyandrost-4-ene-3,11-dione **S4** (484 mg, 1.59 mmol) was dissolved in 2.2 mL acetic anhydride. Then, 4 mg (0.037 mmol) of DMAP and 0.25 mL of dry pyridine were added. The mixture was stirred at room temperature for 24 h. The reaction was quenched with water and the mixture was extracted with ethyl acetate (3 × 30 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product that was further purified by chromatography on SiO_2 (eluent; hexane: ethyl acetate = 6:1) to afford 472 mg of 17α-acetoxyandrost-4-ene-3,11-dione in 90% yield. **13**: mp = 162–164 °C; $[\alpha]_D^{23}$ +148.0 (*c* 1.68, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.69 (s, 1H), 4.76 (t, *J* = 8.6 Hz, 1H), 2.83–2.69 (m, 1H), 2.54–2.20 (m, 6H), 2.01 (d, *J* = 1.2 Hz, 3H), 1.92 (m, 3H), 1.85–1.55 (m, 4H), 1.51–1.41 (m, 1H), 1.44–1.34 (m, 3H), 1.32–1.10 (m, 2H), 0.85–0.69 (m, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 208.3, 199.5, 170.8, 168.3, 124.6, 80.2, 62.6, 54.8, 49.4, 46.2, 38.2, 37.0, 34.7, 33.7, 32.1, 31.7, 27.6, 22.9, 20.9, 17.2, 12.8; FT-IR (KBr): 3443, 2958, 2935, 2850, 1732,1702, 1677, 1618, 1426, 1373, 1360, 1343, 1271, 1238, 1224, 1045, 1027; HRMS *m*/*z* for $C_{21}H_{29}O_4$ $[M+H]^+$ calcd 345.2066, found 345.2069.

4.11. 17α-Acetoxyandrost-4-ene-3,11-dione 3-oximes, ***14***

To a solution of 17α-acetoxyandrost-4-ene-3,11-dione **13** (465 mg, 1.35 mmol) in 8 mL absolute ethanol was added hydroxylamine hydrochloride (109 mg, 1.58 mmol) and dry pyridine (2.6 mL). The mixture was stirred at room temperature for 24 h. Then, water was added and the mixture was extracted with ethyl acetate (3 × 40 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product that was further purified by chromatography on SiO_2 (eluent; dichloromethane:ethyl acetate = 20:1) to afford 461 mg of the oximes **14** (99%). **14**: FT-IR (KBr): 3274, 2966, 2924, 1741, 1704, 1631, 1454, 1431, 1376, 1243, 1047; HRMS *m*/*z* for $C_{21}H_{30}NO_4$ $[M+H]^+$ calcd 360.2175, found 360.2178.

4.12. 3-Aza-17a-acetoxy-A-homo-4α-androsten-4,11-dione, ***15***

Oximes **14** (264 mg, 0.74 mmol) were dissolved in 13 mL of dry dioxane. The mixture was cooled to 0 °C and thionyl chloride (1.4 mL) was added dropwise. The mixture was allowed to reach room temperature and stirred for 24 h. The reaction was quenched with $NaHCO_3$ and the mixture was extracted with ethyl acetate (3 × 20 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product that was further purified by chromatography on SiO_2 (eluent; ethyl acetate:methanol = 1:0.03) to afford 163 mg of lactam **15** in 62% yield. **15**: mp = 270–272 °C; $[\alpha]_{23}^D$ +160 (c 1.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.39 (s, 1H), 5.75 (s, 1H), 4.78 (t, *J* = 8.6 Hz, 1H), 3.35–3.18 (m, 1H), 3.09 (dt, *J* = 14.7, 7.2 Hz, 1H), 2.67 (dd, *J* = 14.9, 8.2 Hz, 1H), 2.48 (td, *J* = 13.6, 3.9 Hz, 1H), 2.34–2.20 (m, 3H), 2.14 (dd, *J* = 9.3, 6.5 Hz, 1H), 2.03 (s, 3H), 2.01–1.86 (m, 2H), 1.83–1.53 (m, 5H), 1.48–1.37 (m, 1H), 1.38 (s, 3H), 1.28–1.08 (m, 1H), 0.76 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 209.0, 170.8, 169.5, 158.4, 120.1, 80.1, 62.4, 55.1, 49.9, 46.7, 43.6, 40.4, 36.8, 36.8, 35.5, 33.2, 27.6, 22.8, 21.1, 20.9, 12.8; FT-IR (KBr): 3428, 2971, 2920, 2878, 2364, 2341, 1736, 1701, 1664, 1639, 1599, 1444, 1375, 1339, 1245, 1127, 1089, 1046; HRMS *m*/*z* for $C_{21}H_{30}NO_4$ $[M+H]^+$ calcd 360.2175, found 360.2178.

4.13. 3-Aza-17a-hydroxy-A-homo-4α-androsten-4,11-dione, ***16***

Lactam **15** 76 mg (0.21 mmol) was dissolved in 3 mL MeOH and LiOH (1 N, 1.2 mL) were added dropwise. The mixture was stirred at room temperature for 1 h. The reaction was quenched with NH_4-Cl and the mixture was extracted with dichloromethane (3 × 5 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford 67 mg of lactam **16** (100% yield). **16**: mp = 269–271 °C; $[\alpha]_{23}^D$ +57.6 (c 1.7, $CHCl_3$); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.72 (s, 1H), 5.51 (s, 1H), 4.66 (d, *J* = 4.7 Hz, 1H), 3.66 (dd, *J* = 13.4, 8.4 Hz, 1H), 3.08–2.90 (m, 2H), 2.47–(12.32 (m, 2H), 2.29 (d, *J* = 11.5 Hz, 1H), 2.21 (d, *J* = 11.2 Hz, 1H), 2.14–2.01 (m, 2H), 2.01–1.78 (m, 3H), 1.74–1.50 (m, 3H), 1.40 (m, 1H), 1.28 (s, 3H), 1.23 (s, 1H), 1.15–1.02 (m, 1H), 0.55 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 210.2, 167.8, 157.3, 120.3, 78.1, 61.0, 54.5, 48.8, 47.1, 43.1, 40.4, 36.8, 35.5, 34.9, 33.1, 29.9, 22.3, 20.9, 11.8; FT-IR (KBr): 3423, 3262, 2952, 2923, 2853, 1693, 1647, 1609, 1458, 1407, 1375, 1353, 1261, 1062; HRMS *m*/*z* for $C_{19}H_{28}NO_3$ $[M+H]^+$ calcd 318.2069, found 318.2071.

4.14. ENGA-L07E, ***17***

Lactam **16** (30 mg, 0.084 mmol) was dissolved in 8.2 mL of dry DCM. Then, 3-(4-(bis(2-chloroethyl)amino)phenoxy)propanoic acid (51 mg, 0.17 mmol), DCC (51 mg, 0.25 mmol) and a catalytic amount of DMAP were added. After the resulting solution was stirred at room temperature for 24 h the solvent was evaporated and the residue was purified by flash column chromatography on SiO_2 (eluent; ethyl acetate) to give conjugate **17** (48.5 mg, 96%). **17:** mp = 148–150 °C; $[\alpha]_{23}^D$ +10.5 (*c* 0.91 $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.83 (d, *J* = 9.0 Hz, 2H), 6.67 (d, *J* = 9.0 Hz, 2H), 6.11 (s, 1H), 5.76 (s, 1H), 4.86 (t, *J* = 8.6 Hz, 1H), 4.17 (t, *J* = 6.2 Hz, 2H), 3.61 (m, 8H), 3.25 (m, 1H), 3.18–3.01 (m, 1H), 2.84–2.59 (m, 2H), 2.59–2.38 (m, 1H), 2.37–2.25 (m, 3H), 2.16 (m, 1H), 2.10–1.89 (m, 3H), 1.87–1.55 (m, 4H), 1.39 (s, 3H), 1.26 (m, 2H), 1.12 (m, 1H), 0.76 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 208.9, 170.9, 169.5, 158.7, 151.3, 140.9, 119.9, 116.2, 114.5, 80.4, 64.2, 62.4, 55.1, 54.3, 49.9, 46.8, 43.6, 40.7, 36.8, 35.5, 34.9, 33.9, 27.6, 25.6, 24.9, 22.8, 21.2, 12.8; FT-IR (KBr): 3432, 3328, 2927, 2850, 1733, 1701, 1664, 1626, 1599, 1513, 1444, 1389, 1369, 1310, 1273, 1243, 1179, 1087, 1041, 999; HRMS *m*/*z* for $C_{32}H_{43}Cl_2N_2O_5$ $[M+H]^+$ calcd 605.2549, found 605.2552.

4.15. 3-Aza-17a-acetoxy-A-homo-4α-androsten-4,11-dione 11-oximes, ***18***

To a solution of **15** (100 mg, 0.28 mmol) in 1.5 mL absolute ethanol in a sealed tube was added hydroxylamine hydrochloride (21 mg, 0.31 mmol) and dry pyridine (0.9 mL). The mixture was heated at 140 °C for 7 days. Then, water was added and the mixture was extracted with ethyl acetate (3 × 5 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford a crude product, which was used to the next step without further purification.

4.16. 3,11-Diaza-17a-acetoxy-A,C-dihomo-4α,11a-androsten-4,11-dione, ***19***

The crude oximes **18** described above (0.28 mmol) were dissolved in 4.9 mL of dry dioxane. The mixture was cooled to 0 °C and thionyl chloride (0.54 mL) was added dropwise. The mixture was allowed to reach room temperature and stirred for 24 h. The reaction was quenched with $NaHCO_3$ and the mixture was

extracted with ethyl acetate (3 × 20 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure to afford the crude product that was further purified by chromatography on SiO_2 (eluent; ethyl acetate:methanol = 1:0.1) to afford 52 mg of lactam in 50% yield. **19:** mp = 221–224 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.00 (s, 1H), 5.77 (s, 1H), 5. 59 (s, 1H), 4.61 (t, J = 8.3 Hz, 1H), 3.20 (m, 2H), 3.02 (dd, J = 9.6, 5.0 Hz, 1H), 2.48–2.40 (m, 2H), 2.31 (d, J = 13.7 Hz, 1H), 2.16 (m, 2H), 2.09–2.0–1.97 (m, 5H), 1.74–1.84 (m, 2H), 1.51–1.40 (m, 2H), 1.35–1.30 (m, 1H),1.24 (s, 3H), 1.23 (m, 1H), 1.08 (m, 1H), 0.95 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.1, 170.9, 169.4, 156.3, 120.4, 80.1, 64.2, 55.5, 45.2, 44.6, 41.0, 40.8, 38.0, 36.3, 34.4, 31.0, 25.4, 25.2, 21.9, 21.0, 11.7; FT-IR (KBr): 3392, 2960, 2925, 2869, 1748, 1736, 1715, 1653, 1556, 1455, 1248, 1160; HRMS m/z for $C_{21}H_{30}N_2NaO_4$ $[M + Na]^+$ calcd 397.2103, found 397.2103.

4.17. 3,11-Diaza-17a-hydroxy-A,C-dihomo-4α,11a-androsten-4,11-dione, **20**

Ester **19** 31 mg (0.08 mmol) was dissolved in 1.2 mL MeOH and LiOH (1 N, 0.5 mL) were added dropwise. The mixture was stirred at room temperature for 1 h. The reaction was quenched with NH_4-Cl and the mixture was extracted with ethyl acetate (3 × 5 mL). The organic layers were dried (Na_2SO_4) and concentrated under reduced pressure. The crude product that was further purified by chromatography on SiO_2 (eluent; ethyl acetate: methanol = 1: 0.1) to afford 26 mg of lactam **20** in 93% yield. **20**: mp = 315–317 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.75 (s, 1H), 6.13 (d, J = 3.9 Hz, 1H), 5.53 (s, 1H), 4.66 (d, J = 5.3 Hz, 1H), 3.40 (m, 2H), 3.11–2.93 (m, 2H), 2.44–2.34 (m, 1H), 2.29 (s, 2H), 2.04–1.72 (m, 5H), 1.65 (m, 2H), 1.24 (m, 3H), 1.20 (s, 3H), 0.89 (m, 1H), 0.66 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 174.6, 167.7, 156.1, 120.1, 78.1, 69.8, 63.4, 44.9, 44.2, 41.2, 40.4, 37.7, 35.3, 33.7, 31.3, 27.7, 24.4, 20.9, 10.7; FT-IR (KBr): 3574, 2914, 2854, 2791, 1748, 1651, 1553, 1403, 1311, 1151; HRMS m/z for $C_{19}H_{29}N_2O_3$ $[M+H]^+$ calcd 333.2178, found 333.2178.

4.18. Conjugate ENGA-DL02E, 21

Lactam **20** (30 mg, 0.09 mmol) was dissolved in 9 mL of dry DCM. Then, 3-(4-(bis(2-chloroethyl)amino)phenoxy)propanoic acid (67 mg, 0.218 mmol), DCC (60 mg, 0.293 mmol) and a catalytic amount of DMAP were added. After the resulting solution was stirred at room temperature for 24 h the solvent was evaporated and the residue was purified by flash column chromatography on SiO_2 (eluent; DCM:acetone = 2:1) to give conjugate **21** (39 mg, 70%). **21**: mp = 119–121 °C; $[\alpha]_D^{23}$ −72.7 (c 0.975, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.85 (d, J = 9.0 Hz, 2H), 6.76 (s, 1H), 6.65 (d, J = 9.0 Hz, 2H), 5.79 (s, 1H), 5.46 (s, 1H), 4.68 (t, J = 8.3 Hz, 1H), 4.17 (t, J = 6.2 Hz, 2H), 3.60 (m, 4H), 3.51–3.42 (m, 1H), 3.18 (m, 2H), 3.02 (dd, J = 9.5, 5.0 Hz, 1H), 2.85–2.67 (m, 2H), 2.51 (d, J = 13.8 Hz, 1H), 2.44 (dd, J = 13.6, 10.1 Hz, 1H), 2.33 (d, J = 13.8 Hz, 1H), 2.25–2.06 (m, 2H), 2.07–1.73 (m, 5H), 1.73–1.28 (m, 5H), 1.25 (s, 3H), 1.17–1.03 (m, 2H), 0.96 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.7, 170.9, 169.1, 155.6, 151.3, 140.9, 120.7, 116.3, 114.4, 80.5, 64.2, 64.1, 55.5, 54.2, 45.3, 44.5, 41.1, 40.7, 38.1, 36.3, 34.8, 33.9, 31.0, 25.6, 25.5, 24.9, 21.9, 11.8; FT-IR (KBr): 3410, 3330, 2926, 2850, 1734, 1654, 1627, 1577, 1513, 1445, 1349, 1273, 1243, 1180, 1133, 1110, 1087, 1044, 890; HRMS m/z for $C_{32}H_{44}Cl_2N_3O_5$ $[M+H]^+$ calcd 620.2658, found 620.2658.

4.19. In vitro anticancer activity

Five well established human leukemia cell lines, MOLT-4 (Human acute T-lymphoblastic leukemia), K562 (Human chronic myelogenous leukemia), CCRF-CEM (Human acute T-lymphoblastic leukemia), JURKAT (Human acute T-cell leukemia) and SUP-B15 (Human acute B-lymphoblastic leukemia) were treated with newly synthesized compounds at concentrations of 1–100 μM to test their cytostatic and cytotoxic activity. The cell lines were obtained from the American Type Culture Collection (ATCC) and were grown in different culture medium according to the instructions. The MTT ((3-(4, 5-imethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay is a well-established and standard method for evaluating the cytostatic and cytotoxic activity of drugs and chemicals [17,20,22]. Briefly, the cells were plated in 96-well plate at a density of 3×10^4 cells/ml per well and maintained for 72 h at 37 °C in a 5% CO_2 incubator and grown as monolayers or suspensions. After 24 h, cells were treated with 1–100 μmol/l of the compounds for 48 h. The viability of cultured cells was estimated MTT (Sigma, St Louis, Missouri, USA) metabolic assay as described previously. Absorbance of the converted dye was measured at a wavelength of 540 nm on an ELISA reader (Versamax, Orleans, USA). The mean concentrations of each drug that generated 50% or total (100%) growth inhibition (GI_{50} and TGI, respectively) as well as the drug concentrations that produced cytotoxicity against 50% of the cultured cells [(half maximal cytotoxic concentration (IC_{50})] were calculated using the linear regression method. Using seven absorbance measurements [time 24 h (Ct24), control growth 72 h (Ct72), and test growth in the presence of drug at five concentration levels (Tt72x)], the percentage of growth was calculated at each level of the drug concentrations. The percentage growth inhibition was calculated according to National Cancer Institute (NCI) as: [(Tt72x)–(Ct24)/(Ct72)–(Ct24)] × 100 for concentrations for which Tt72x>Ct24 and [(Tt72x)–(Ct24)/Ct24] × 100 for concentrations for which Tt72x<Ct24; GI_{50} was calculated from [(Tt72x)-(Ct24)/(Ct72)–(Ct24)] × 100 = 50,TGI from [(Tt72x)–(Ct24)/(Ct72)–(Ct24)] × 100 = 0, and IC_{50} from [(Tt72x)–(Ct24)/Ct24] × 100 = 50. All the experiments were carried out in triplicate.

4.20. Mice

BALB/c, DBA/2, and BDF1 mice of both sexes, weighing 20–23 g, 6–8 weeks old, were used for toxicity studies and antitumor evaluation. Mice obtained from the experimental section of the Research Center of Theagenion Anticancer Hospital (Thessaloniki, Greece) were kept under conditions of constant temperature and humidity, in sterile cages, with water and food ad libitum. Animal experiments were performed in accordance with European Directive 86/609/EEC guidelines for the care and use of laboratory animals.

4.21. Tumors

Leukemia P388-bearing BDF1 (DBA/2xC57BL) mice were used to evaluate the cytostatic effect. Lymphocytic P388 leukemia was maintained in an ascitic form by an injection of 10^6 cells at 7-day intervals into the peritoneal cavity of DBA/2 mice.

4.22. In vivo acute toxicity

The acute toxicity of the compounds was determined following a single intraperitoneal injection into BALB/C in groups of 10 mice/dose at three different dosages. The mice were observed for 30 days and the therapeutic dose of the compounds was determined after a graphical estimation of the LD_{50} (30-day curves). The highest dose used for a single treatment was equal to the LD_{10} value.

4.23. Treatment

Experimental groups of 6 mice for each drug tested and a control group of 8 mice were used. Experiments were initiated by implanting mice with the tumor cells. Mice were treated by intraperitoneal (i.p.) bolus injection on day 1 after tumor implantation, using the LD_{10} doses of all compounds tested.

4.24. In vivo antileukemic activity

For the survival experiments, the antileukemic activity of the tested compounds against the above-mentioned murine tumors was assessed from the oncostatic parameter T/C%, which is the increase median life span of the drug-treated animals (T) excluding long-term survivors versus corn-oil-treated controls (C), expressed as a percentage. The other index of antileukemic activity used was the number of long-term survivors defined as mice alive for 90 days after tumor inoculation (cured). Each drug treated group included six mice, whereas the tumor control group included eight mice; in each group, equal numbers of male and female mice were used. Experiments were initiated by implanting mice with tumor cells according to the protocol of the National Cancer Institute (USA) [36]. Treatments were given as a single dose (LD_{10} on day 1). The experiments were terminated on day 90. Statistical evaluation of the experimental data was carried out using the *t*-test.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2016.07.009.

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