Rational Design, Synthesis and *In Vitro* Evaluation of Three New Alkylating Steroidal Esters

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Abstract: Recent studies have indicated that minor functional changes on the steroidal part of complex molecules, comprising of an alkylating moiety and a steroidal congener, lead to compounds with enhanced biological activity. The observed induction of the genotoxic, cytotoxic and antileukemic effects suggest a determinative role of the steroidal congener on the mechanism of action. In order to further elucidate the structural requirements responsible for this, we designed and synthesized a new modified steroid, carrying a 17β-acetamide substituent and a B lactamic ring, and studied the ability of its esters with three potent nitrogen mustards to induce sister chromatid exchange (SCEs) and to inhibit cell proliferation in normal human lymphocytes *in vitro*. The role of the steroidal skeleton was clearly stated by the results of the *in vitro* evaluation of the final compounds, as all three derivatives proved better inducers of SCE (58-102 SCE/cell) and cell division delays (1.18-1.25 PRI) than the simple nitrogen mustards (24-38 SCE/cell and 1.51-1.62 PRI). Obviously, the steroidal module enhances the formation of DNA adducts that cannot be repaired by excision repair enzymes probably through the induction of the interaction of these complex compounds with different base sequences or by disabling the repair mechanisms through the blockage of the enzymes responsible for excision repair. On the other hand, it seems that these compounds also act through a parallel site of action responsible for cell death when their primary binding site becomes saturated, as in higher concentrations two of the derivatives tested showed enhanced cytotoxicity while their ability to induce SCE stabilized.

Key Words: Steroidal esters, B steroidal lactam, nitrogen mustards, SCE, SAR, proliferation rate.

INTRODUCTION

In the past years, researchers had speculated that the steroidal part of complex molecules, which contains DNA alkylators tethered to steroidal skeletons, played the role of the "biological platform" on which the alkylator could easily been transported through biological barriers and approach the site of action [1-4]. Recently, new molecules of this category, which were designed to possess small but effective differentces (e.g. a simple keto group on specific position of the steroidal skeleton), proved that the observed significant differentiations on the antileukemic potency of these compounds could not be explained only on the basis of the change of the physicochemical parameters (e.g. lipophylicity) [5-6].

Over the past years a number of studies on the mechanism of action of congener compounds have been carried out, in order to gain additional data about the specific sites of interaction responsible for the positive responses of certain tumors to these multifunctional compounds. These studies have revealed that some hormone-linked antineoplastic agents are highly effective in receptor positive experimental tumors and are superior to mixtures of unlinked alkylating agents with hormones [7-9]. Moreover, indications for a relative enrichment of DNA damaging effects in the tumor tissue and for reduced toxicity have been obtained with specific hormone conjugates [10-11], while in a recent study a rationally designed hormone-linked genotoxicant was found to possess the ability to block repairing enzymes by camouflaging the DNA-adducts formed [12].

At present, prednimustine (prednisolone ester of chlorambucil) and estramustine phosphate (oestradiol-3-mustard carbamate-17-phosphate) are hormone-linked antitumor compounds that carry clinical promise [13-14]. Studies concerning estramustine have shown that it has the ability to disrupt interphase and mitotic microtubule network in cells, while it has been found to bind to a specific protein at the prostate tissue [15-19].

Our previous work, concerning the genotoxic and cytostatic evaluation of a series of nitrogen mustards tethered to steroidal skeletons, has shown that in order to design molecules with enhanced activity there are some structural requirements that must be taken into consideration. Chlorambucil (CHL) [20] and its active metabolite (PHE, phenylacetic acid's nitrogen mustard) [21] have successfully been used in the past as the alkylating congener [5,6,22-23], while from a series of steroidal esters of N,N-bis(2-chloroethyl) aminobenzoic acid isomers tested [24], the 4-methyl-3-N,N-bis (2-chloroethyl)amino benzoate's (4-Me-CABA) derivatives proved the most potent [22-25]. Concerning the steroidal part of congener compounds, structure-activity relationship studies have established the importance of the presence of

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functional groups on the steroidal module. Specifically, it has been found that steroidal skeletons that carry a –NHCO- moiety are more appropriate modules than the common or non-modified steroids, since their esters with chlorambucil and its analogs are much more effective genotoxic agents. A number of studies in this field have established the importance of the presence of this amidic group on the steroidal congener either as a D- lactam [26-28] or as a 17β-acetamidic substituent [5,22,25]. Moreover, recent studies have indicated that functional changes in the B steroidal ring (e.g. 7-keto group and B-lactam) had considerable effects on the final molecules' genotoxic and cytostatic activity [5-6,23], leading us to assume that its modification is fundamental for the design of more effective molecules. Among these compounds two 7-oxidized steroidal derivatives of PHE showed clastogenic and aneuploidogenic effects, while they were capable of inducing DNA excision repair [29].

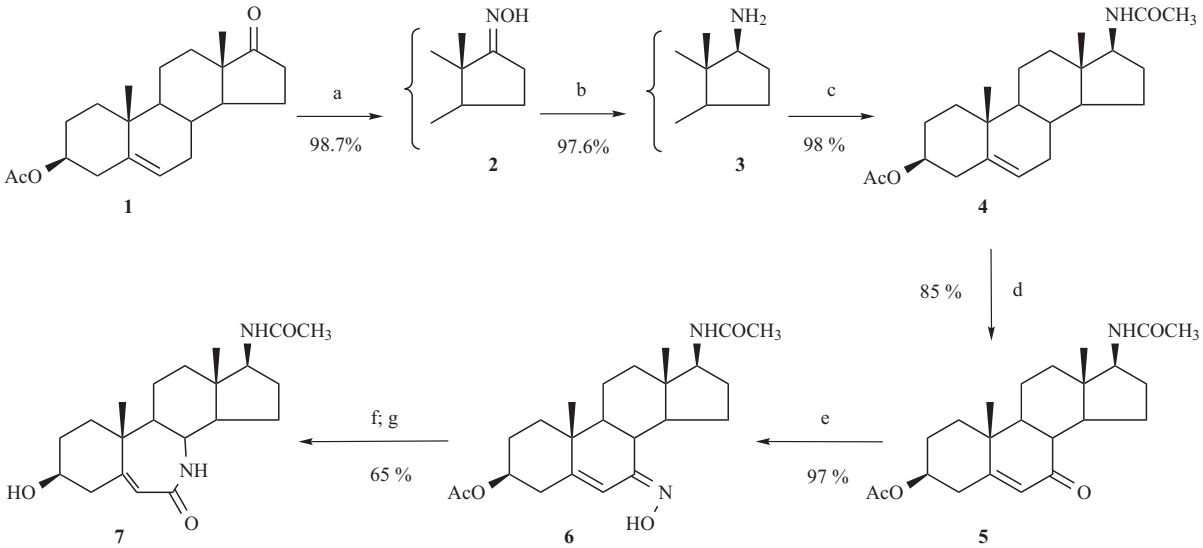
Based on these SAR data, we decided to design, synthesize and study a new steroidal skeleton (**7**) which combines two of the afore-mentioned structural requirements in order to be used as an appropriate module, videlicet an acetamidic substituent at the 17-position and a B-lactamic ring.

This study concerns the synthesis of compound **7**, as well as the synthesis of its esters with CHL, PHE, and 4-Me-CABA. The final esteric derivatives were evaluated for their ability to induce sister chromatid exchange (SCE) and the reduction of the proliferation rate index (PRI) in normal human lymphocytes *in vitro*, in order to study their potential genotoxic and cytostatic activity. Moreover, the purpose of this study was to elucidate the role of this steroidal skeleton on the mechanism of action of these multifunctional compounds.

RESULTS AND DISCUSSION

The steroid (**1**) easily converted to the 17-ketoxime (**2**), which subsequently reduced to 17β-amino derivative (**3**). The reduction of this ketoxime, using the Na/n-BuOH system, mainly affords the 17β- amino diastereomer, but we isolated small amounts (~ 5%) of the 17α-amino diastereomer. Any effort to further decrease the percentage of the 17α- amino derivative, using different alcohols or modifying the reaction conditions proved unsuccessful. The reverse of the yields of these diastereomers was achieved when we applied the catalytic (PtO_2) hydrogenation as reducing agent, mainly affording the 17α-amino diastereomer. We chose this synthetic strategy (Fig. **1**) in order to avoid a second Beckmann rearrangement (the 17β-acetamido-3β-acetoxy-androst-5-ene could be prepared from the 3β-acetoxy-pregn-5-ene, applying a Beckmann rearrangement on the corresponding 20-ketoxime), which is a reaction with generally moderate yields [30,31]. For the allylic oxidation of **4** the t-BuOOH/ CuI-TBAB biphasic oxidizing method was applied [32] and the produced 7-keto-Δ^5-steroidal skeleton **5** was converted, using classical conditions [33], to its 7-ketoxime **6**. This compound was subjected to Beckmann rearrangement by the use of $SOCl_2$/THF at 0°C, to afford the final steroidal skeleton **7** in 65% yield after removal of the 3β-acetoxy group with mild conditions. The latter experiment was repeated in order to acquire the necessary amount for the following experiments.

The alcohol **7** was esterified *via* the assymetric anhydrides procedure [25] with 4-N,N-bis(2-chloroethyl)amino phenylbutyric acid (CHL), 4-N,N-bis(2-chloroethyl)amino phenylacetic acid (PHE) and 4-methyl-3-N,N-bis(2-chloro-



Conditions: a) $H_2NOH.HCl$/ EtOH/ $NaHCO_3$, r.t., stirred, 4 hr; b) Na/n-BuOH, reflux, 3hr; c) Ac_2O/Pyridine, r.t., overnight; d) TBHP/ CuI/ TBAB/ DCM, reflux, 4 hr; e) $H_2NOH.HCl$/ EtOH/ pyridine, reflux, 2 hours; f) $SOCl_2$/ THF, stirred, 0 °C, 4 hr; g) Na_2CO_3/MeOH, stirred, 20 hr;

Fig. (1). Synthetic procedures for the preparation of 17β-acetamido-B-lactamic derivative.

ethyl)amino benzoic acid (4-Me-CABA) to give the final steroidal derivatives **7a, 7b** and **7c**. See Fig (**2**) and (**3**).

The final esteric derivatives **7a-7c**, as well as the three nitrogen mustards used (CHL, PHE, 4-Me-CABA) were evaluated for their ability to induce sister chromatid exchange (SCE) and the reduction of the proliferation rate index (PRI) in normal human lymphocytes *in vitro*. SCEs have been frequently used as highly sensitive indicators of DNA damage and/or subsequent repair [34-35]. Non-repaired damage expressed as SCEs in normal cells, caused by certain chemicals, might indicate inability for the repair of the damage induced by the same chemicals in cancer cells. There are findings indicating that the effectiveness in SCE induction by potential antitumor agents in cancer cells *in vitro* and *in vivo* [36] is positively correlated with *in vivo* tumor response to these agents. This suggests that the SCE assay could be used to predict both the sensitivity of human tumor cells to chemotherapeutics and the heterogeneity of drug sensitivity of individual tumors [37]. Other studies investigating a relationship between SCE induction and other expressions of genotoxicity have also been shown as a positive relationship between SCE and reduced cell survival and alteration in cell cycle kinetics [38]. In the present study a good correlation ($P<0.02$) between SCE enhancement and PRI suppression was observed. The results of the *in vitro* experiments are illustrated in Table **1**.

All compounds were tested at the concentrations of 0.4 μM and 1.0 μM and induced statistically a significant increase in SCE rates. The steroidal esteric derivatives, **7a-7c** showed a notable induction of the genotoxic effects when the dose was almost doubled (especially derivatives **7b** and **7c**), while the corresponding nitrogen mustard congeners' (CHL, PHE, and 4-MeCABA) genotoxic activity was not significantly altered between the two concentrations. Thus, in order to evaluate how the genotoxic effects induced by these new compounds are correlated with the concentration used,

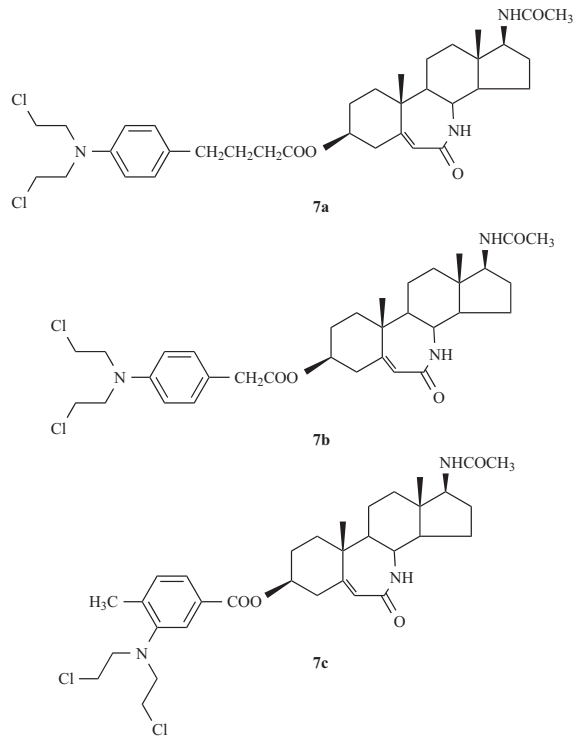
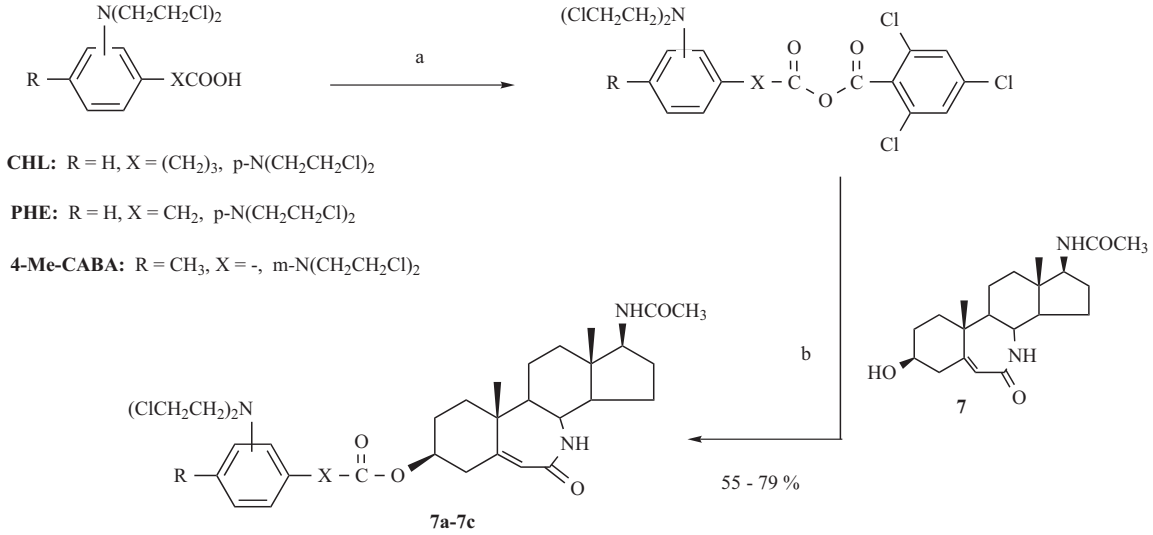


Fig. (3). Chemical structures of the steroidal esteric derivatives of CHL, PHE, and 4-Me-CABA.

the steroidal esters were also tested at 1.6 μM and 2.0 μM. Concerning the induction of SCEs, Fig. (**4**), derivative **7a** showed a dose-dependent genotoxic behavior, while the induction observed for derivatives **7b** and **7c** gave a peak at 1.6 μM and diminished at higher concentration. A possible explanation for this might be that the latter steroidal esters



Conditions: a) 2,4,6-trichlorobenzoyl chloride/ Et_3N/ toluene, reflux, 1-2 hr; b) 4-DMAP/ toluene, reflux, 2-2.5 hr

Fig. (2). General procedure for the preparation of the final esteric derivatives.

Table 1. Induction of SCEs and Cell Division Delays by CHL, PHE, 4-MeCABA, and Their Steroidal Esters in Human Lymphocytes

Compound	Concentration (μM)	SCE/cell ± SE	PRI
Control	-	11.09 ± 0.59	1.89
CHL	0.4	30.72 ± 1.21	1.60
	1.0	37.22 ± 0.81	1.51
7a	0.4	29.45 ± 0.81	1.70
	1.0	42.12 ± 1.31	1.58
	1.6	49.02 ± 1.07	1.22
	2.0	57.91 ± 2.00	1.24
PHE	0.4	24.30 ± 1.14	1.66
	1.0	38.32 ± 0.72	1.51
7b	0.4	56.35 ± 1.12	1.44
	1.0	85.69 ± 1.69	1.48
	1.6	102.49 ± 2.66	1.25
	2.0	102.33 ± 2.49	1.19
4-Me-CABA	0.4	17.38 ± 0.73	1.62
	1.0	24.32 ± 0.82	1.55
7c	0.4	19.02 ± 0.64	1.75
	1.0	69.71 ± 1.82	1.51
	1.6	84.09 ± 2.16	1.18
	2.0	79.98 ± 2.46	1.28

SCEs have been correlated with corresponding PRI values ($r=-0.51$, $t=2.63$ and $P<0.02$)

induce SCEs through their binding to specific sites in the cell, which become saturated over a specific dose. Comparing these two derivatives with **7a** we can conclude that they are more effective inducers of SCEs, which further contributes to the fact that they have the ability to bind in a more selective manner and generate specific non-repairable lesions in human lymphocytes. This kind of DNA-damaging behavior renders derivatives **7b** and **7c** potentially effective antileukemic agents.

The role of the steroidal skeleton is clearly stated among all derivatives tested, since the observed induction of SCEs was increased through the conjugation of the nitrogen mustards to the modified steroidal congener. Especially, concerning the dose of 1.0 μM, when PHE and 4-MeCABA were tethered with the steroid the induction was 2 to 3-fold higher in relation to the genotoxic effect induced by the nitrogen mustards alone. It seems that the steroidal part alters the mechanism of action since the DNA damage caused is less repairable. This kind of behavior suggests that these multifunction compounds have the ability either to induce the binding of the agent at specific DNA sequences (probably different than those of the nitrogen mustards') which cannot be repaired by excision repair enzymes, or block the repairing enzymes from performing their action. The latter hypothesis is further substantiated by a recent study [12], showing the ability of a steroidal alkylating agent to block excision repair mechanisms by binding to a specific receptor and camouflaging in such a way that the DNA adducts produced from the enzymes responsible for the maintenance of the nucleic acids' integrity when exposed to certain chemicals.

Concerning the reduction of the proliferating rate index (PRI) there is a good correlation between SCEs induction and PRIs depression, Fig. (**5**). Compounds **7b** and **7c** achieved

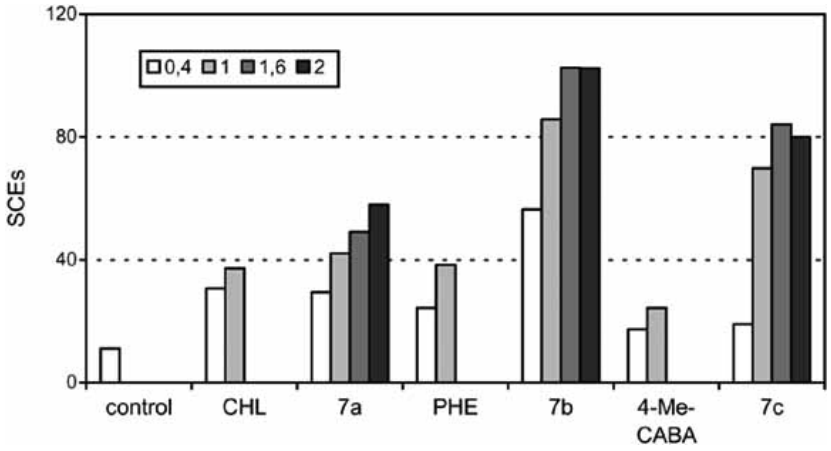


Fig. (4). Induction of sister chromatid exchange (SCEs) in normal human lymphocytes *in vitro* by the compounds studied.

better cell division delays than the corresponding nitrogen mustards at the concentration of 1μM, as it was expected; while **7a** proved almost equipotent with CHL as it was also previously observed with its effectiveness towards SCEs induction. All three steroidal derivatives (**7a-7c**) further reduced the proliferation rate index in higher doses. However, such an impressive increment of cell division delays, cannot be explained only on the basis of the induction of the DNA damaging effects, especially in the case of derivatives **7b** and **7c** which showed a plateau over the dose of 1.6 μM concerning their ability to induce SCEs. This observation suggests the existence of another parallel mechanism of action of these compounds that results in cell death, which contributes to their cytostaticity when the primary site of action has been saturated. As mentioned above, estramustine [15-18] is a typical example of this category of compounds that has been found to also act through the disruption of interphase and mitotic microtubule network in cells and thus achieving cell death. Concerning these studies, we can assume that an analogous site of action related to the induction of cell death or to the activation of apoptosis must render the steroidal derivatives **7b** and **7c** which are better cytotoxic agents at higher doses. Nevertheless, additional studies on the mechanism of action of these promising compounds will be carried out in order to elucidate their biological profile.

Finally, among all compounds studied **7b** proved to be the most effective genotoxic and cytotoxic agent. In a previous study [5] the corresponding 17β-acetamide-Δ^5-steroidal ester of PHE tested at the concentration of 0.4μM, proved less potent compound than the 7-keto steroidal analog of PHE, leading to the conclusion that small but effective structural differences (e.g. a simple keto group on specific position of the steroidal skeleton), play a significant role in the mechanism of action of compounds of this category. Comparing the results obtained from this study with the previous one [5] it can be concluded that the modification of the B steroidal ring to lactamic (**7b**) further increased the effectiveness of the final compound. Subsequently, the present study further supports the notion that the steroidal congener alters the whole biological profile of the final molecule, not only on the basis of its physicochemical characteristics, but also from a mechanistic point of view. Particularly, the B steroidal skeleton seems to play a crucial role in the induction of the

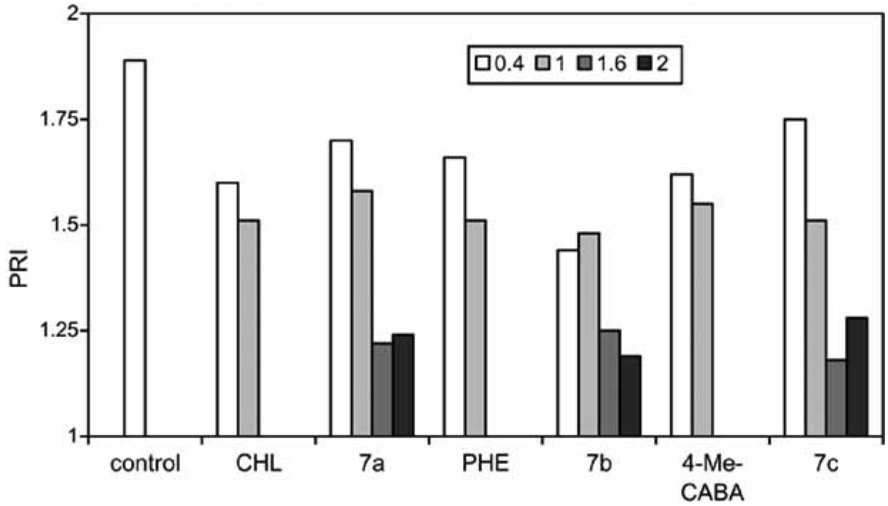


Fig. (5). Reduction of the proliferation rate index (PRI) in normal human lymphocytes *in vitro* by the compounds studied.

mechanisms of action responsible for the enhancement of the biological response.

CONCLUSION

The conjugation of the three nitrogen mustards to the newly synthesized B,D-modified steroidal skeleton lead to compounds with enhanced genotoxic and cytotoxic activity. Through analyzing the results of this study, it is obvious that the effectiveness of these steroidal esteric derivatives can be attributed to the ability of the steroidal congener to enhance the formation of DNA adducts that cannot easily be repaired by excision repair mechanisms. On the other hand, it seems that these compounds can also act through a parallel site of action responsible for cell death if their primary binding site becomes saturated. Among them, the steroidal derivative of phenylacetic acid mustard (PHE) proved to be the most potent, while through its comparison with previously studied analogous steroidal derivatives of PHE the role of the B steroidal ring configuration on the mechanism of action was clearly found to be determinative. All these data suggest that the steroidal skeleton not only alters the physicochemical character of the final molecule but also has an active role in the mechanism of action.

EXPERIMENTAL

1. Synthetic Procedures

General

Melting points were measured on a Kallenkamp melting point apparatus in capillary tubes and are uncorrected. IR spectra were recorded on a FT-IR Jasco spectrophotometer, and ^{1}H NMR spectra were recorded on a Brucker 400 spectrometer in $CDCl_3$. The chemical shifts are given as δ values (ppm) with tetramethylsilane as the internal standard and only characteristic peaks are listed. Elemental analyses were performed on a Carlo Erba, CHN Analyzer. Thin layer-chromatography (TLC) was performed on E. Merck pre-coated silica gel plates (Kieselgel 60 F_{254}). Visualization was accomplished by exposure to iodide vapors and/or under UV light (254 nm). Column chromatography was performed with silica gel (E. Merck, 70-230 mesh).

3β-acetoxy-androst-5-en-17-one (**1**) and 4-N,N-bis(2-chloroethyl)amino phenylbutyric acid (CHL) were purchased from Steraloids (Newport, RI) and Sigma, Germany. 4-N,N-bis(2-chloroethyl)amino phenylacetic acid (PHE) [26], and 4-methyl-3-N,N-bis(2-chloroethyl)amino benzoic acid (4-Me-CABA) [39-40] were prepared according to procedures in the literature. The t-BuOOH/ CuI-tetrabutyl ammonium bromide biphasic oxidizing method was applied for the allylic oxidation of **4** [31], while the final esteric steroidal derivatives were synthesized according to the asymmetric anhydrides procedure [25].

3β-acetoxy-androst-5-en-17-ketoxime (2)

3β-acetoxy-androst-5-en-17-one (**1**) (16.2mmol, 5.34g) was diluted in 25 ml of absolute ethanol and 15 ml of pyridine. Hydroxylamine hydrochloride (21 mmol, 1.46g) was added to this solution, and the reaction mixture was refluxed for 2 h. After completion of the reaction, the mixture was poured into ice water, and the white precipitate was filtered under vacuum, was washed with water, and dried. The oxime was obtained in 98.7% yield (16 mmol, 5.52g). The m.p. 179-181°C (methanol; 178-180°C, [41]). IR ν (cm^{-1}): 3360(N-OH), 1707 (C=O acetate), 1267 (C-O acetate). ^{1}H NMR ($CDCl_3$) δ: 7.41 (s, 1H), 6.58 (s, 1H), 5.42 (d, 1H), 4.62 (m, 1H), 2.05 (s, 3H), 1.06 (s, 3H), 0.95 (s, 3H). Analysis calculated for $C_{23}H_{34}N_2O_4$ (M = 345.48): C, 73.01; H, 9.04; N, 4.05; found: C, 72.99; H, 9.00; N, 4.06.

3β-hydroxy-17β-amino-androst-5-ene (3)

3β-acetoxy-androst-5-en-17-ketoxime (**2**) (16 mmol, 5.52 g) was diluted in 400 ml of absolute n-butanol. Sodium (1.5 mol, 34.5g) was then added portionwise in continuous stirring of the mixture at room temperature. After dilution, the mixture was refluxed for 1h. After completion of the reaction, the mixture was poured into ice water, and the white precipitate was filtered under vacuum, was washed with water, and dried. 3β-hydroxy-17β-amino-androst-5-ene. (**3**) was obtained in 97.6% yield (15.6 mmol, 4.5g). The m.p. 165-167°C (methanol; 164-167°C [42]). IR ν (cm^{-1}): 3390-3318(-OH), 3210-3184 (-NH-). ^{1}H NMR ($CDCl_3$) δ: 10.12(s, 1H), 7.35 (s, 2H), 3.91 (m, 1H), 3.01 (m, 1H), 0.99 (s, 3H), 0.74 (s, 3H). Analysis calculated for $C_{19}H_{31}NO$ (M = 289.46): C, 78.84; H, 10.79; N, 4.84; found: C, 78.81; H, 10.75; N, 4.80.

3β-acetoxy-17β-acetamido-androst-5-ene (4)

3β-hydroxy-17β-amino-androst-5-ene. (**3**) (15.6 mmol, 4.5g) was diluted in 20 ml of acetic anhydride and 10 ml of pyridine. After stirring at room temperature over night the reaction mixture was poured into ice water, and the white precipitate was filtered under vacuum, was washed with water, and dried. 3β-acetoxy-17β-acetamido-androst-5-ene (**4**) was obtained in 98% yield (15.3 mmol, 5.7g). The m.p. 189-190°C (ethyl acetate; 187-190°C [31]). IR ν (cm^{-1}): 3400-3300 (N-H), 1730 (C=O acetate), 1650 (-C=O amide), 1244 (C-O acetate). ^{1}H NMR ($CDCl_3$) δ: 5.38 (d, 1H), 4.60 (m, 1H), 3.87 (m, 1H), 2.02 (s, 3H), 1.98 (s, 3H), 0.98 (s, 3H), 0.70 (s, 3H). Analysis calculated for $C_{23}H_{35}NO_3$ (M = 373.53): C, 73.96; H, 9.44; N, 3.75; found: C, 73.98; H, 9.42; N, 3.78.

3β-acetoxy-17β-acetamido-androst-5-en-7-one (5)

The t-butyl hydroperoxide (TBHP) 70% in water (153 mmol, 19.7ml), copper iodide (10.4 mmol, 1.98g) and tetra-n-butylammonium bromide (TBAB) (1.83 mmol, 0.59g) were added to a solution of 3β-acetoxy-17β-acetamido-androst-5-ene (**4**) (15.3 mmol, 5.7g) in 20 ml of dichlomethane. The reaction mixture was refluxed, and additional TBHP (153 mmol, 19.7ml) was added 1.5 and 3 h after the beginning of the reaction. Reflux was continued for another 1h (TLC monitoring). Then, the reaction mixture was poured into water and extracted with dichloromethane. The organic layer was washed successively with an aqueous solution of HCl 5%, an aqueous solution of $NaSO_3$, and water, dried over anhydrous sodium sulphate, and evaporated. 3β-acetoxy-17β-acetamido-androst-5-en-7-one (**5**) was isolated using column chromatography (absorbent: silica gel, eluent: dichloromethane/ methanol (98:2 v/v)) in 85% yield (13 mmol, 5g). The m.p. 230-232°C (methanol; 231-232°C [43]). IR ν (cm^{-1}): 3210-3235 (N-H), 1734 (C=O acetate), 1688 (^{7} C=O),

1666 (-C=O amide), 1244 (C-O acetate). ^{1}H NMR ($CDCl_3$) δ: 5.98 (s, 1H), 5.78 (s, 1H), 4.60 (m, 1H), 3.87 (m, 1H), 2.02 (s, 3H), 1.98 (s, 3H), 0.98 (s, 3H), 0.71 (s, 3H). Analysis calculated for $C_{23}H_{35}NO_3$ (M = 387.51): C, 71.29; H, 8.58; N, 3.61; found: C, 71.30; H, 8.58; N, 3.64.

3β-acetoxy-17β-acetamido-androst-5-en-7-ketoxime (6)

3β-acetoxy-17β-acetamide-androst-5-en-7-one (**5**) (13m mol, 5g) was diluted in 25 ml of absolute ethanol and 15 ml of pyridine. Hydroxylamine hydrochloride (17 mmol, 1.18g) was added to this solution, and the reaction mixture was refluxed for 2 h. After completion of the reaction, the mixture was poured into ice water, and the white precipitate was filtered under vacuum, was washed with water, and dried. The oxime was obtained in 97% yield (12.6 mmol, 5.06g). The m.p. 273-274 (methanol). IR ν (cm^{-1}): 3317(N-OH), 3196 (-NH-), 1738 (C=O acetate), 1641 (^{20}C=O), 1238 (C-O acetate). ^{1}H NMR ($CDCl_3$) δ: 6.76 (s, 1H), 6.58 (s, 1H), 5.27 (d, 1H), 3.99 (m, 1H), 4.70 (m, 1H), 2.05 (s, 3H), 1.98 (s, 3H), 1.13 (s, 3H), 0.71 (s, 3H). Analysis calculated for $C_{23}H_{34}N_2O_4$ (M = 402.53): C, 68.63; H, 8.51; N, 6.96; found: C, 68.59; H, 8.40; N, 6.98.

3β-hydroxy-7a-aza-B-homo-17β-acetamido-androst-5-en-7-one (7)

Freshly distilled thionylchloride (39 mmol, 2.8ml) in 9 ml of freshly distilled tetrahydrofuran (THF) was added dropwise to a solution of 3β-acetoxy-17β-acetamide-androst-5-en-7-ketoxime (**6**) (2.4 mmol, 0.95g) in 12 ml freshly distilled THF cooled to 0°C. After the addition, the reaction mixture was stirred at 0°C for 4h. The mixture was then poured into ice-water, neutralized with an aqueous solution of NH_3 (pH=7), and extracted with dichloromethane. The organic layer was washed with water and dried over Na_2SO_4. Evaporation of the solvent gave an oily residue, which was chromatographed over silica gel/ dichloromethane, and the product was eluted with dichloromethane / methanol (97:3) to give 3β-acetoxy-7a-aza-B-homo-17β-acetamido-androst-5-en-7-one in 65% yield (1.56 mmol, 0.63g). This experiment was repeated in order to acquire the necessary amount for the following experiments. The m.p. 248-249 (methanol). IR ν (cm^{-1}): 3285 (-NH-), 1732 (C=O acetate), 1658 (^{20}C=O and ^{7}C=O), 1243 (C-O acetate). ^{1}H NMR ($CDCl_3$) δ: 5.84 (s, 1H), 5.56 (s, 1H), 5.28 (d, 1H), 4.71 (m, 1H), 3.98 (m, 1H), 3.31 (t, 1H), 2.17 (s, 3H), 1.99 (s, 3H), 1.27 (s, 3H), 0.70 (s, 3H). Analysis calculated for $C_{23}H_{34}N_2O_4$ (M = 402.53): C, 68.63; H, 8.51; N, 6.96; found: C, 68.65; H, 8.51; N, 6.94. 3β-acetoxy-7a-aza-B-homo-17β-acetamide-androst-5-en-7-one (8 mmol, 3.2g) was diluted in 40 ml methanol. Na_2CO_3 (11.2 mmol, 1.2g) was added to this solution, and the mixture was stirred at room temperature for 20h. The reaction mixture was filtered under vacuum to remove the remaining salt. Evaporation of the solvent gave **7** in 95% yield (7.6 mmol, 2.7g). The m.p. 253-254 (methanol). IR ν (cm^{-1}): 3429(-OH), 3300-3150 (-NH-), 1657 (^{20}C=O and ^{7}C=O). ^{1}H NMR ($CDCl_3$) δ: 5.84 (s, 1H), 5.58 (s, 1H), 5.27 (d, 1H), 4.70 (m, 1H), 3.99 (m, 1H), 3.29 (t, 1H), 2.10 (s, 3H), 1.27 (s, 3H), 0.70 (s, 3H). Analysis calculated for $C_{21}H_{32}N_2O_3$ (M = 360.49): C, 69.97; H, 8.95; N, 7.77; found: C, 69.99; H, 8.86; N, 7.73.

General Procedure for the Preparation of the Esteric Derivatives of 7 with CHL, PHE, and 4-MeCABA via Assymetric Anhydrides

A solution of the corresponding nitrogen mustard (3 mmol, 0.9g for CHL and 0.83g for PHE and 4-Me-CABA) in 15 ml of dry toluene was treated with 2,4,6-trichlobenzoyl chloride (3.6 mmol, 0.56ml) and triethylamine (3.6 mmol, 0.5ml) and refluxed under Ar for 1.5h for CHL as starting material, 2h for PHE, and 1h for 4-Me-CABA. In the above mixtures a solution of the steroidal alcohol (2.5 mmol, 0.9g) **7**, in dry toluene and 4-dimethylaminopyridine (2.5 mmol, 0.3g) were added. The reaction mixture was refluxed under Ar for 2.5 h. The solvent was evaporated, the residue dissolved in DCM, washed successively with 5% aq. HCl, water, 5% aq. $NaHCO_3$, water and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue chromatographed on silica gel/DCM. Elution with DCM/ methanol 99:1 gave the desired compounds.

3β-hydroxy-7a-aza-B-homo-17β-acetamide-androst-5-en-7-one-4-N,N-bis(2-chloroethyl)Amino Phenylbutyrate (7a)

79% yield. The m.p. 171-172 (ethyl acetate). IR ν (cm^{-1}): 3292 (-NH-), 1728 (C=O), 1658 (^{7}C=O), 1612 (^{20}C=O), 1267 (C-O acetate), 806, 736 (=C-H). ^{1}H NMR ($CDCl_3$) δ: 7.10 (d, 2H), 6.66 (d, 2H), 6.64 (s, 1H), 5.86 (s, 1H), 5.28 (d, 1H), 4.74 (m, 1H), 3.99 (m, 1H), 3.72 (t, 4H), 3.64 (t, 4H), 3.34(t, 1H), 2.57 (t, 2H), 2.32 (t, 2H), 1.94 (m, 2H), 2.02 (s, 3H), 1.29 (s, 3H), 0.72 (s, 3H). Analysis calculated for $C_{35}H_{49}N_3O_4Cl_2$ (M = 647): C, 65.00; H, 7.64; N, 6.50; found: C, 65.02; H, 7.63; N, 6.48.

3β-hydroxy-7a-aza-B-homo-17β-acetamide-androst-5-en-7-one-4-N,N-bis(2-chloroethyl)Amino Phenylacetate (7b)

55% yield. The m.p. 148-150 (ethyl acetate). IR ν (cm^{-1}): 3292 (-NH-), 1728 (C=O), 1658 (^{7}C=O), 1612 (^{20}C=O), 1271 (C-O acetate), 819, 736 (=C-H). ^{1}H NMR ($CDCl_3$) δ: 7.17 (d, 2H), 6.64 (d, 2H), 6.49 (s, 1H), 5.83 (s, 1H), 5.40 (d, 1H), 4.73 (m, 1H), 3.96 (m, 1H), 3.72 (t, 4H), 3.65 (t, 4H), 3.50 (s, 2H), 3.32 (t, 1H), 2.00 (s, 3H), 1.27 (s, 3H), 0.70 (s, 3H). Analysis calculated for $C_{33}H_{45}N_3O_4Cl_2$ (M = 619): C, 64.07; H, 7.33; N, 6.79; found: C, 64.06; H, 7.31; N, 6.77.

3β-hydroxy-7a-aza-B-homo-17β-acetamide-androst-5-en-7-one-4-methyl-3-N,N-bis(2chloroethyl) Amino Benzoate (7c)

60% yield. The m.p. 160-162 (ethyl acetate). IR ν (cm^{-1}): 3290 (-NH-), 1712 (C=O), 1658 (^{7}C=O), 1606 (^{20}C=O), 1257 (C-O acetate), 763, 736 (=C-H). ^{1}H NMR ($CDCl_3$) δ: 7.80 (s, 1H), 7.70 (d, 1H), 7.3 (d, 1H), 6.70 (s, 1H), 5.90 (s, 1H), 5.27 (d, 1H), 4.98 (m, 1H), 4.00 (m, 1H), 3.40 (m, 8H), 3.30 (t, 1H), 2.40 (s, 3H), 2.03 (s, 3H), 1.36 (s, 3H), 0.74 (s, 3H). Analysis calculated for $C_{33}H_{45}N_3O_4Cl_2$ (M = 619): C, 64.07; H, 7.33; N, 6.79; found: C, 64.05; H, 7.35; N, 6.80.

2. *IN VITRO* SCE AND PRI ASSAY [35]

Lymphocyte cultures were set up by adding 11 drops of heparinized whole blood from three normal subjects to 5 ml of chromosome medium 1A (RPMI 1640, Biochrom, Berlin). For SCEs demonstration 5 μg/ml 5-Bromodeoxyuridine (Brd Urd) and the chemicals were added at the beginning of culture life. Throughout, all cultures were maintained in the

dark to minimize photolysis of BrdUrd. The cultures were incubated for 72 h at 37° C. Metaphases were collected during the last 2 h with colchicines at 0.3 μg/ml. Air-dried preparations were made stained by the FPG procedure [44]. The preparations were scored for cells in their first mitosis (both chromatids dark staining), second mitosis (1 chromatid of each chromosome dark staining) and third and subsequent divisions (a portion of chromosomes with both chromatids light staining). 20 suitably spread 2^{nd} division cells from each culture were blindly scored for SCEs. For Proliferation Rate Indices (PRIs), 100 cells at least were scored. For the statistical evaluation of the experimental data, the x^2 test was performed for the cell kinetic comparisons. For the SCE frequencies the Student's t test was used. We also calculated the correlation between SCEs and PRI values. The formula for the Pearson product moment correlation coefficient r was applied. Then a criterion for testing whether r differed significantly from zero was applied, whose sampling distribution is Student's t-test with n-2 d.f.

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