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Rational design, synthesis, and in vivo evaluation of the antileukemic activity of six new alkylating steroidal esters

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Abstract—The synthesis and the in vivo evaluation against leukemias P388 and L1210 of six new alkylating steroidal esters are described. The esteric derivatives incorporating the 17β-acetamido-B-lactamic steroidal skeleton exhibited increased antileukemic activity and lower toxicity, compared to the 17β-acetamido-7-keto analogs. Among the 17β-acetamido-B-lactamic steroidal esters, the most potent compound afforded four out of six cures in leukemia P388 and was measured to be almost non-toxic, producing significant low levels of toxicity.

1. Introduction

Alkylating agents are a heterogeneous group of drugs used in the treatment of many neoplastic diseases. The common feature of these drugs is their ability to alkylate nucleophilic sites in cellular macromolecules, favoring nitrogen, oxygen, and sulfur. Although these agents damage many cellular components, their biological effects, which include cytotoxicity, mutagenesis, and clastogenesis are attributed to their ability to damage DNA.[1] Among them nitrogen mustards are an important group which exert their biological activity by inducing interstrand cross-links in the major groove of DNA, which represents the most toxic of all alkylation events.[2–4] Despite their long history in cancer treatment only a few members of these compounds remain in clinical use today (e.g., chlorambucil and melphalan), due to their high toxicity and lack of selectivity caused by their high inherent chemical reactivity.[5,6]

Among the various approaches used to minimize these undesirable effects, drug derivatization has been demonstrated as an important means of improving drug efficacy.[7] The chemical linkage of nitrogen mustards to carrier molecules (e.g., hormones) with affinity for specific binding sites (hormone receptors) has been used in order to achieve a more selective and less toxic antineoplastic treatment.[8–10] Studies in this field revealed that some hormone-linked antineoplastic agents are highly effective in receptor positive experimental tumors.[11–13] Indications for a relative enrichment of DNA damaging effects in the tumor tissue and for reduced myelotoxicity have been obtained with specific hormone conjugates.[14] Estramustine and Prednimustine are typical representatives of this category of compounds used in clinical practice against several types of cancers.[15,16]

Our ongoing studies in this field have demonstrated that steroidal esteric derivatives of aromatic nitrogen mustards increase the damaging effects on specific DNA sequences and achieve better selectivity and reduced toxicity compared to nitrogen mustards themselves.[17–20] Steroidal skeletons, which incorporate a –NHCO-moiety proved to be more appropriate modules than the common or non-modified steroids, since their esters with chlorambucil and its analogs were much more effective antileukemic agents. Structure–activity relationship (SAR) studies established the importance of the presence of this amidic group on the steroidal part of these compounds either as a D-lactam[21–23] or as a 17β-acet-

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amido substituent.[24–26] Moreover, recent studies that indicated the insertion of a keto group in the B steroidal ring produced surprisingly positive effects on the final potency,[25,27,28] which led us to assume that this modification may be considered fundamental for the design of more effective molecules.

Based on these results, we studied a new modified steroidal skeleton (**10**), which incorporates a 17β-acetamido group and a lactamic B-ring. Additionally, we focused on the observation that the introduction of a second –NHCO-group at the 17-position of a A-lactamic steroid resulted in a derivative with inherent cytostatic activity which inhibited DNA synthesis reversibly, bound to nucleic acids, and increased the life span of treated P388-bearing animals by 48–58%.[29]

This study describes the preparation of steroidal ligand **10** as well as the synthesis and the in vivo antileukemic evaluation of its esters with chlorambucil (CHL), phenylacetic acid mustard (chlorambucil's active metabolite, PHE),[30,31] and a benzoic acid mustard analog (4-Me-CABA).[19,24] In order to gain supplementary data for SAR studies, we designed and synthesized three more analogs, which consist of the same nitrogen mustards tethered to 7-keto-17β-acetamido-steroidal skeleton **9**. The six final steroidal derivatives, as well as the three alkylating agents were studied against leukemias P388 and L1210 after the subsequent estimation of their toxicity in vivo.

2. Results

For the synthesis of the target compound 3β-acetoxy-7α-aza-B-homo-17β-acetamido-androst-5-en-7-one **4**, two synthetic routes were designed using 3β-acetoxy-pregnenolone **1** as starting material (see Scheme 1). The first procedure was preferable because it had less synthetic steps and the repetition of the Beckmann rearrangement[32,33] could be avoided. The allylic oxidation of **1** was accomplished in good yields (>80%) using the biphasic oxidative system developed in our laboratory,[34] with *tert*-butyl hydroperoxide (TBHP) as the oxidant, CuI as the catalyst and tetra-butyl ammonium bromide (TBAB) as the phase transfer catalyst. 3β-Acetoxy-pregn-5-en-7,20-dioxime **3** was synthesized using classical conditions and subjected to Beckmann rearrangement with $SOCl_2$/THF at 0 °C. Surprisingly, neither the desired product was obtained from this reaction nor was the starting material recovered. The formation of a series of by-products was observed which could not be isolated after column chromatography. TLC analysis indicated that extensive degradation of the molecule had occurred. Subsequent reactions under modified conditions using other acid catalysts (e.g., phosphorus oxychloride) and/or solvents (e.g., 1,4-dioxane), were unsuccessful.

The second procedure (Scheme 1) where pregnenolone acetate **1** was converted to its oxime and subjected to Beckmann rearrangement with $POCl_3$/pyridine[35] to give 3β-acetoxy-17β-acetamido-androst-5-en **6** was followed. The allylic oxidation of **6** using the biphasic system[34] gave 3β-acetoxy-17β-acetamido-androst-5-en-7-one **7**, which was then converted easily to its corresponding oxime **8**. The Beckmann rearrangement of **8** resulted in the formation of the desired steroidal skeleton **4** in 65% yield.

Compounds **7** and **4** were hydrolyzed under mild basic conditions to the corresponding alcohols **9** and **10**, which were esterified via the asymmetric anhydrides procedure[24] with 4-*N*,*N*-bis(2-chloroethyl)aminophenylbutyric acid (CHL), 4-*N*,*N*-bis(2-chloroethyl)amino phenyl-acetic acid (PHE) and 4-methyl-3-*N*,*N*-bis(2-chloroethyl)amino benzoic acid (4-Me-CABA) to give the final steroidal derivatives **9a**–**9c** and **10a**–**10c** correspondingly (see Scheme 2 and Fig. 1).

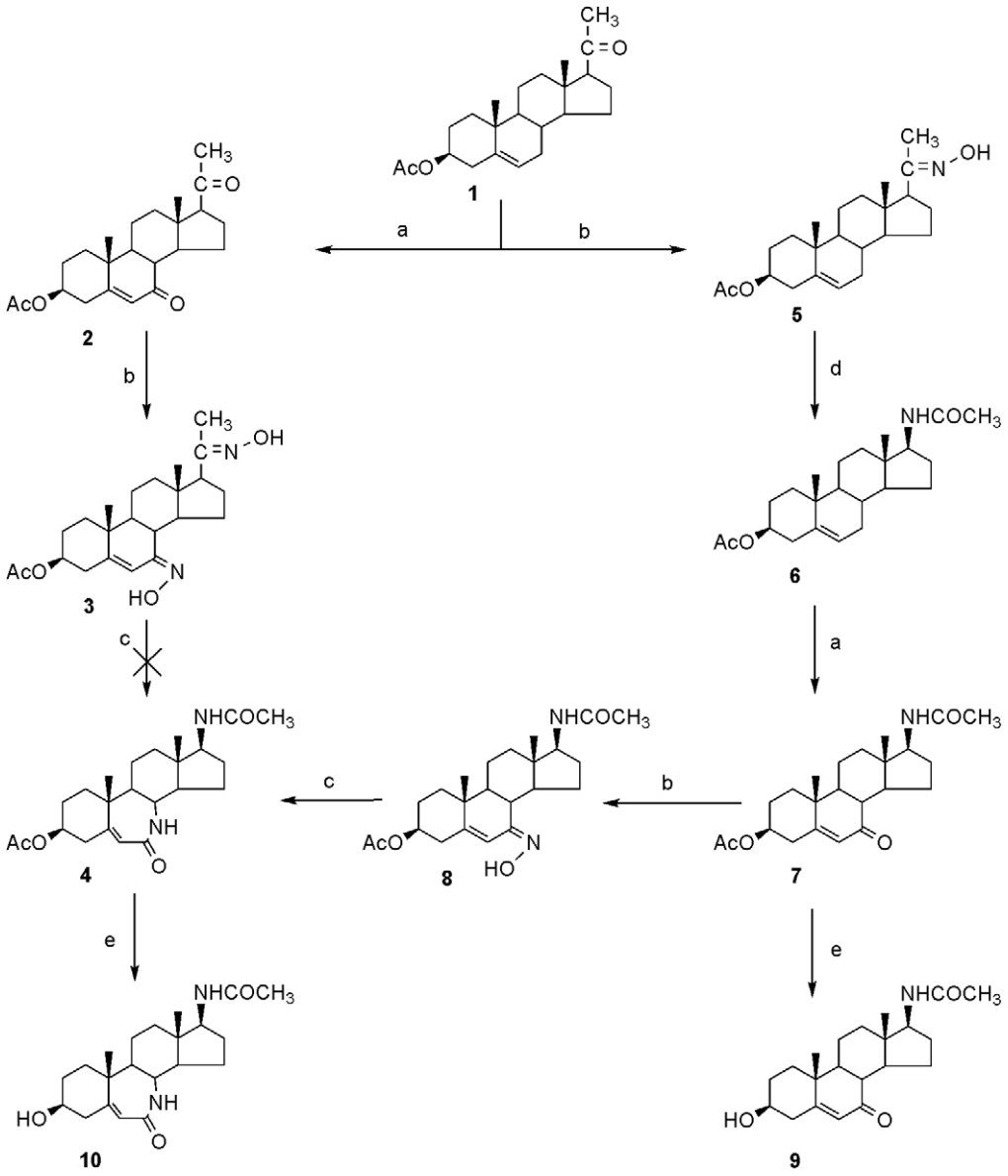
Table 1 illustrates the toxicity values of the final compounds, as well as those of the alkylating agents. The LD_{50} values proved that in all cases the chemical linkage of the nitrogen mustards with the modified steroids resulted in a reduction of the toxic effects induced by the corresponding free alkylating agents. The derivatives of PHE (**9b**, **10b**) proved to be the most toxic among all, while the derivatives of 4-Me-CABA (**9c**, **10c**) proved to be the least toxic.

Table 2 summarizes the results of the activity of the compounds studied, against leukemias P388 and L1210. Since the treatment schedule D/2 × 3 was proved to be superior to the single dose treatment in the previous studies,[24,25,28] we chose to test the final compounds using this schedule.

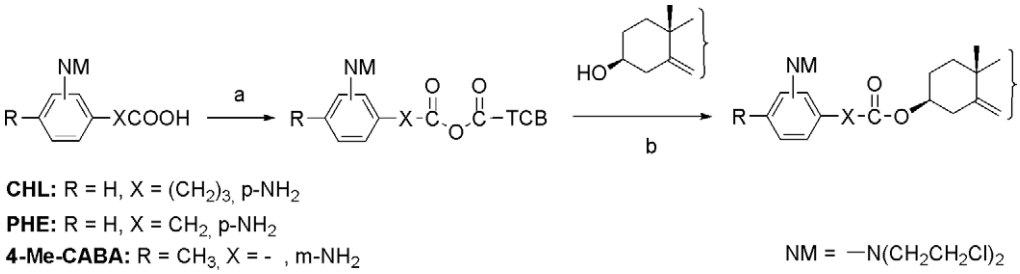
The results obtained herein support evidence from previous studies[24–28] that the derivatization of nitrogen mustards with steroidal skeletons bearing –NHCO-moieties leads to compounds with enhanced antileukemic activity, since all six esters tested gave better T/C% values than their corresponding free nitrogen mustards, except for compound **9c** in leukemia L1210. Among them **9b** gave the best T/C% values in both leukemias tested and had lower but marginally accepted toxicity. Compound **10c** proved almost equipotent with **9b** (comparing the T/C% values) but afforded additionally four out of six cures in leukemia P388. It is important to remark that this potent compound was measured to be almost non-toxic, producing significant low levels of toxicity. Concerning steroidal esters bearing a 7-keto group (**9a**–**9c**), we can see that there is a straight correlation between toxicity levels and antileukemic potency while the opposite correlation is observed concerning the derivatives with a B-lactamic ring (**10a**–**10c**) where the less toxic a compound was found, the more potent it proved showing in that way a selectivity of the latter compounds toward leukemic cells.

3. Discussion

Concerning the chemistry part of this study, we confronted the failure of 7,20-dioxime **3** to simultaneously rearrange under the conditions studied. A possible



Scheme 1. Synthetic procedures for the preparation of B-lactamic-17-acetamide. Reagents and conditions: (a) TBHP/CuI/TBAB/DCM, reflux, 4 h; (b) $H_2NOH{\cdot}HCl$/EtOH/pyridine, reflux, 2 h; (c) $SOCl_2$/THF, stirred, 0 °C, 4 h; (d) $POCl_3$/pyridine, stirred, 2 h; (e) Na_2CO_3/MeOH, stirred, rt, 20 h.



Scheme 2. General procedure for the preparation of the final esteric derivatives. Reagents and conditions: (a) 2,4,6-trichlorobenzoyl chloride (TCB–Cl)/Et_3N/toluene, reflux, 1–2 h; (b) 4-DMAP/toluene, reflux, 2–2.5 h.

explanation might be an intramolecular long-range effect induced between B and D steroidal rings, as similar reactions carried out by other research groups gave the desirable product **13** in 25% yield[33,36] (see Scheme 3).

This observation further elucidates the results of an earlier study describing our endeavors to synthesize a steroidal B,D-bilactamic derivative,[32] where intramolecular effects induced by the presence of a D-lactamic

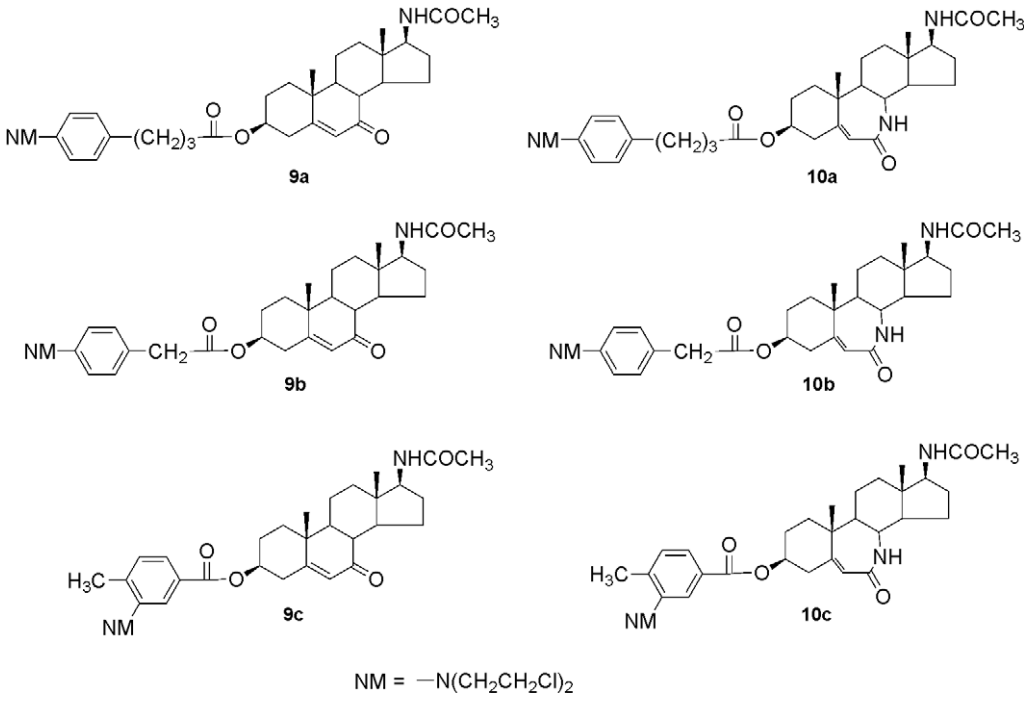


Figure 1. Chemical structures of the steroidal esteric derivatives of CHL, PHE, and 4-Me-CABA.

Table 1. Toxicity of the compounds studied

Compound	LD_{50}[a] (mg/kg)	LD_{10} (mg/kg)
CHL	24	15
9a	87	44
10a	180	100
PHE	20	10
9b	75	25
10b	80	60
4-Me-CABA	18	10
9c	600	400
10c	280	240

[a] LD_{50} values were estimated graphically, where the percentage of deaths due to the toxicity of each dose is shown in the ordinate, while the administered doses are indicated on the abscissae on semilogarithmic paper. For chemotherapy testing, the dose used was $LD_{10}/2$. Therefore, the drugs in the following experiments were compared at equitoxic doses.

ring (endocyclic –NHCO-moiety) hindered the Beckmann rearrangement of the B steroidal ring. A similar effect must be induced between the two steroidal rings, involving the long-range interaction between the two intermediate carbenions formed during Beckmann rearrangement of dioxime **3**, since neither the starting material was recovered nor other possible rearranged steroidal skeletons were produced.

Nitrogen mustards are highly reactive compounds with an inherent chemical affinity toward the nucleophilic sites of several biomolecules such as plasma proteins, while at the same time they are rapidly hydrolyzed and in some cases are deactivated by cellular resistance mechanisms.[37–41] These disadvantages result in a significant loss of the active drug before reaching the DNA target and on the other hand increase the toxic effects. The conjugation of nitrogen mustards with steroidal skeletons may increase their lipophilicity and alter their physicochemical properties resulting in an easier and more quantitative penetration through the cellular and nuclear membranes and consequently improve their antileukemic activity and reduce their toxic effects.

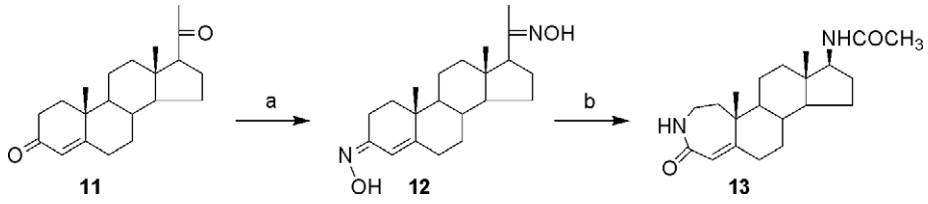
In order to increase the antileukemic potency, the modification of the steroidal part of these complex molecules is essential. As mentioned before, previous studies showed that the presence of a –NHCO-moiety at the 17-position of steroidal skeleton in axial orientation[24–28] and the insertion of a 7-keto group[25,27,28] afforded a positive influence on the antileukemic potency. The comparison of the antileukemic activity and the toxicity of the 7-keto-17-acetamide steroidal esters of the nitrogen mustards selected herein, with the corresponding B-lactamic-17-acetamide ones showed that the introduction of a second –NHCO-moiety resulted in an enhancement of the selectivity toward leukemic cells especially in leukemia P388. This observation further establishes the antileukemic effectiveness of the amidic moiety. As previously reported,[42,43] the antineoplastic effects of these derivatives could result from the multiple interactions with similar groups present in proteins and DNA; thus a possible explanation for the improvement of the biological profile of the B-lactamic molecules studied herein might be that these interactions favor mechanistic pathways responsible for the expression of antileukemic activity and/or produce differences in absorption, distribution or metabolism of these compounds.

It has been reported from earlier studies[17,18,24–28] that both the chemical structure of the nitrogen mustard and the configuration of the steroidal moiety determine the antineoplastic effect of these compounds. This study

Table 2. Antitumor activity of CHL, PHE, 4-Me-CABA, and their steroidal esters on P388- and L1210-bearing mice leukemia, using doses based on toxicity studies

Compound	Dosage[a] (mg/kg/day)	P388			L1210		
		MST ± SEM[b] (days)	T/C[c] (%)	Cures	MST ± SEM[b] (days)	T/C[c] (%)	Cures
Control	Corn oil	9.5 ± 0.42	100	0/8	10.8 ± 0.36*	100	0/8
CHL	7.5	14.7 ± 0.49*	155	0/6	12.3 ± 0.49*	114	0/6
9a	22	18.8 ± 0.47*,†	198	0/6	16.2 ± 0.40*,†	150	0/6
10a	50	22.0 ± 0.73*,†	231	0/6	15.2 ± 0.60*,†	140.7	0/6
PHE	5	10.7 ± 0.80*	113	0/6	12.4 ± 0.71*	115	0/6
9b	12.5	29.8 ± 0.30*,†	314	0/6	26.3 ± 0.66*,†	243	0/6
10b	30	21.0 ± 0.44*,†	221	**1/6**	15.6 ± 0.61*,†	144.4	0/6
4-Me-CABA	5	13.7 ± 0.66*	144	0/6	13.9 ± 1.13*	129	0/6
9c	200	14.6 ± 0.49*,†	153	0/6	13.7 ± 0.67*	127	0/6
10c	120	28.5 ± 0.5	300	**4/6**	20.5 ± 0.43*,†	189.8	0/6

[a] Treatment was given as an intermittent dose equal to $LD_{10}/2$ on days 1, 4, and 7.
[b] MST ± SEM = Mean survival time of mice inoculated with lymphocytic leukemia P388 or lymphoid leukemia L1210 cells and treated with compounds ± Standard error of mean.
[c] T/C = The percent increase in median life span of the drug-treated animals (T) versus corn oil-treated animals (C).
* Significant increase ($P < 0.05$, Wilcoxon test) compared with the control.
† Significant increase ($P < 0.05$, Wilcoxon test) compared with their corresponding nitrogen mustards.



Scheme 3. Preparation of A-lactamic-17β-acetamido steroidal derivative. Reagents and conditions: (a) $H_2NOH{\cdot}HCl$/ EtOH/ pyridine; (b) $SOCl_2$/1,4-dioxane.

confirms the results of earlier reports[25,27,28] which showed that the 7-keto steroidal group favors the activity of the derivatives of PHE and on the other hand we conclude once more[44] that the B-lactamic steroidal ring ameliorates the antileukemic potency of the esters of 4-Me-CABA. Although the exact mechanism of action of steroid alkylators is still unknown, we speculate that the improved potency caused by these structurally different derivatives derives from different mechanistic pathways.

The derivative **10c** proved to be the more selective antileukemic agent among all compounds studied herein given its greater antitumor activity (4/6 cures) and its low toxicity. As it has been reported for analog compounds, the alkylator and the steroid are liberated in the cellular microenvironment by a rate-limiting hydrolysis procedure.[45] We consider that the esteric bond of this molecule is slowly hydrolyzed, because of the stereochemical hindrance, resulting in an increased concentration of the agent at its binding site and consequently to a more effective antileukemic response.

This study further substantiates the concept that steroidal skeletons carrying –NHCO-moieties can be used as efficient and proper modules as they obviously contribute to the antileukemic activity. In parallel, since the structural changes on the B steroidal ring afforded an impressive differentiation on the antileukemic potency, the B-ring can be considered to play a key role on the mechanism of action of these compounds and its further modification seems to be fundamental for the design of more effective antineoplastic derivatives.

4. Conclusion

The above presented study resulted in some interesting information for the steroids chemistry, for the SAR of the in vivo activity against leukemias P388 and L1210 of six steroidal esters of nitrogen mustards and, therefore, for the design and synthesis of new more potent antileukemic derivatives.

Initially, it was found that the simultaneous Beckmann rearrangement of the steroidal 7,20-dioxime **3** in order to produce the desired bis-modified skeleton **10** is impossible using already known methods and conditions, and for this reason we designed, studied and successfully synthesized the target molecule **10** using the described alternative chemical route.

Later on, the already known SAR studies indicated that the presence of an amidic moiety on the D-ring of the steroidal skeleton in complex molecules consisting of a DNA-damaging agent tethered to a steroidal carrier improves the antileukemic activity compared with non-modified steroids. Recent studies reported that the configuration of the B-ring of the steroid had remarkable effects on the antileukemic activity. As a part of our ongoing research, we focused on the influ-

ence of the steroidal moiety on the expression of the antileukemic activity and we were prompted to design and synthesize a new modified steroidal skeleton, which possesses a 17β-acetamido group and a lactamic moiety in the B-ring, and to study the antileukemic potency of its esters with three active and well-studied nitrogen mustards. Subsequently, we synthesized three more esteric compounds incorporating the same nitrogen mustards on the 17β-acetamido-7-keto steroidal moiety.

The results of their in vivo biological tests showed that almost all of the esteric products proved much less toxic than the free nitrogen mustards, while their antileukemic potency was significantly improved. The most potent compound **10** had significantly decreased toxicity and impressive potency (4/6 cures against leukemia P388), but these biological properties diminished when the B-ring was transformed to simple 7-ketone **9c**. Changing the incorporated alkylator, the results were inverted and the most potent compound proved the **9b**, which bears at the B-ring the 7-ketone. Subsequently, from the results we noticed that as the potency of the B-lactamic derivatives is increased so their toxicity is decreased. This fact may intimate a kind of selectivity towards leukemic cells.

These observations lead us to conclude that the antileukemic effects caused by steroid alkylators are strongly determined by the combination of the proper modules (steroid and alkylator), and that further studies are needed in order to establish that the B steroidal ring play a crucial role on the mechanism of action of such compounds.

5. Experimental

5.1. Synthetic procedures

5.1.1. 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 the following abbreviations are used: singlet (s), doublet (d), triplet (t), and multiplet (m). Elemental analyses were performed on a Carlo Erba, CHN Analyzer. Liquid Chromatography–Mass Spectrometry (LC–MS) was carried out on Waters Micromas 2696 instrument running in positive ion electrospray mode, employing a Lichrospher RP-8 column (5 μm; 4 × 150 mm) and a gradient elution from 30% to 100% acetonitrile in water within 30 min (flow rate 1 mL/min). Thin layer-chromatography (TLC) was performed on E. Merck precoated 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-pregn-5-en-20-one (**1**) and 4-*N*,*N*-bis(2-chloroethyl)amino phenylbutyric acid (CHL) were purchased from Aldrich and Sigma, Germany. 3β-Acetoxy-17β-acetamide-androst-5-en (**6**),[35] 4-*N*,*N*-bis(2-chloroethyl)amino phenylacetic acid (PHE),[21] and 4-methyl-3-*N*,*N*-bis(2-chloroethyl)amino benzoic acid (4-Me-CABA)[46,47] were prepared according to the procedures in the literature. The *t*-BuOOH/CuI-tetra-butyl ammonium bromide biphasic oxidizing method was applied for the allylic oxidation of **1** and **6**,[34] while the final esteric steroidal derivatives were synthesized according to the asymmetric anhydrides procedure.[24]

5.1.1.1. 3β-Acetoxy-pregn-5-en-7,20-dioxime (3). 3β-Acetoxy-pregn-5-en-7,20-dione (**2**) (1 mmol, 372.5 mg) was diluted in absolute ethanol (5 mL) and pyridine (3 mL). Hydroxylamine hydrochloride (2.5 mmol, 173.8 mg) 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; yield 95%; mp 234–235 °C (methanol); IR (KBr) ν (cm^{-1}): 3312 (N–OH), 1732 (C=O acetate), 1244 (C–O acetate); ^{1}H NMR ($CDCl_3$) δ: 8.05 (s, 1H), 7.55 (s, 1H), 6.67 (s, 1H), 4.78 (m, 1H), 2.13 (s, 3H), 1.98 (s, 3H), 1.22 (s, 3H), 0.76 (s, 3H). Anal. Calcd for $C_{23}H_{34}N_2O_4$: C, 68.63; H, 8.51; N, 6.96. Found: C, 68.59; H, 8.40; N, 6.98; LC–MS (ESI+) *m*/*z* 403.10 [M+H$^+$].

5.1.1.2. 3β-Acetoxy-17β-acetamide-androst-5-en-7-oxime (8). 3β-Acetoxy-17β-acetamide-androst-5-en-7-one (**7**) (15 mmol, 5.8 g) was diluted in absolute ethanol (25 mL) and pyridine (15 mL). Hydroxylamine hydrochloride (18 mmol, 1.25 g) 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; yield 97%; mp 273–274 °C (methanol). IR (KBr) 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). Anal. Calcd for $C_{23}H_{34}N_2O_4$: C, 68.63; H, 8.51; N, 6.96. Found: C, 68.59; H, 8.40; N, 6.98; LC–MS (ESI+) *m*/*z* 403.12 [M+H$^+$].

5.1.1.3. 3β-Acetoxy-7α-aza-B-homo-17β-acetamide-androst-5-en-7-one (4). Freshly distilled thionylchloride (39 mmol, 2.8 mL) in freshly distilled tetrahydrofuran (THF, 9 mL) was added dropwise to a solution of 3β-acetoxy-17β-acetamide-androst-5-en-7-oxime (**8**) (2.4 mmol, 965 mg) in freshly distilled THF (12 mL) cooled at 0 °C. After the addition, the reaction mixture was stirred at 0 °C for 4 h. The mixture was then poured into ice-water, neutralized with an aqueous solution of NH_3 (pH 7), and extracted with dichloromethane (DCM). 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/DCM, and the product was eluted with DCM/methanol (97: 3 v/v); yield 65%; mp 248–249 °C (methanol); IR (KBr) 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). Anal. Calcd for $C_{23}H_{34}N_2O_4$: C, 68.63; H, 8.51; N, 6.96. Found: C, 68.65; H, 8.51; N, 6.94; LC–MS (ESI+) *m/z* 403.12 [M+H$^+$].

5.1.1.4. 3β-Hydroxy-17β-acetamide-androst-5-en-7-one (9) and 3β-hydroxy-7α-aza-B-homo-17β-acetamide-androst-5-en-7-one (10). 3β-Acetoxy-17β-acetamide-androst-5-en-7-one (**7**) (8 mmol, 3.1 g) or 3β-acetoxy-7α-aza-B-homo-17β-acetamide-androst-5-en-7-one (**4**) (8 mmol, 3.2 g) was diluted in methanol (40 mL). Na_2CO_3 (11.2 mmol, 1.18 g) was added to this solution, and the mixture was stirred at room temperature for 20 h. The reaction mixture was filtered under vacuum to remove the remaining salt. Evaporation of the solvent gave **9** or **10.**

5.1.1.5. 3β-Hydroxy-17β-acetamide-androst-5-en-7-one (9). Yield 98%; mp 270–271 °C (methanol); IR (KBr) *v* (cm^{-1}): 3390–3318 (–OH), 3210–3184 (–NH–), 1666 (^{7}C=O), 1663 (^{20}C=O); ^{1}H NMR ($CDCl_3$) δ: 10.12 (s, 1H), 5.84 (s, 1H), 5.58 (d, 1H), 3.91 (m, 1H), 3.67 (m, 1H), 2.01 (s, 3H), 0.97 (s, 3H), 0.72 (s, 3H). Anal. Calcd for $C_{21}H_{31}NO_3$: C, 73.01; H, 9.04; N, 4.05. Found: C, 73.12; H, 9.09; N, 4.12; LC–MS (ESI+) *m/z* 346.02 [M+H$^+$].

5.1.1.6. 3β-Hydroxy-7α-aza-B-homo-17β-acetamide-androst-5-en-7-one (10). Yield 95%; mp 253–254 °C (methanol); IR (KBr) *v* (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). Anal. Calcd for $C_{21}H_{32}N_2O_3$: C, 69.97; H, 8.95; N, 7.77. Found: C, 69.99; H, 8.86; N, 7.73, LC–MS (ESI+) *m/z* 361.04 [M+H$^+$].

5.1.2. General procedure for the preparation of the esteric derivatives of 9 and 10 with CHL, PHE, and 4-Me-CABA via asymmetric anhydrides. A solution of the corresponding nitrogen mustard (3 mmol) in dry toluene (15 mL) was treated with 2,4,6-trichlorobenzoyl chloride (3.6 mmol) and triethylamine (3.6 mmol) and refluxed under Ar for 1.5 h for CHL as starting material, 2 h for PHE, and 1 h for 4-Me-CABA. In the above mixtures, a solution of the steroidal alcohol (2.5 mmol) **9** or **10**, in dry toluene (15 mL) and 4-dimethylaminopyridine (2.5 mmol) 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 v/v) for the esters of **9**, and DCM/methanol (97: 3 v/v) for the esters of **10**, gave the desired compounds.

5.1.2.1. 3β-Hydroxy-17β-acetamide-androst-5-en-7-one-4-*N*,*N*-bis(2-chloroethyl)amino phenylbutyrate (9a). Yield 66.5%; mp 158–161 °C (ethyl acetate–hexane). IR (KBr) *v* (cm^{-1}): 3306 (–NH–), 1732 (C=O), 1670 (^{7}C=O), 1651 (^{20}C=O), 1248 (C–O acetate), 804 (=C–H); ^{1}H NMR ($CDCl_3$) δ: 7.09 (d, 2H), 6.82 (s, 1H), 6.61 (d, 2H), 5.64 (s, 1H), 4.61 (m, 1H), 3.89 (m, 1H), 3.72 (t, 4H), 3.67 (t, 4H), 2.49 (t, 2H), 2.23 (t, 2H), 1.83 (m, 2H), 2.01 (s, 3H), 1.21 (s, 3H), 0.71 (s, 3H). Anal. Calcd for $C_{35}H_{48}N_2O_4Cl_2$: C, 66.55; H, 7.66; N, 4.43. Found: C, 66.45; H, 7.71; N, 4.53; LC–MS (ESI+) *m/z* 631.19 [M+H$^+$].

5.1.2.2. 3β-Hydroxy-17β-acetamide-androst-5-en-7-one-4-*N*,*N*-bis(2-chloroethyl)amino phenylacetate (9b). Yield 64.1%; mp 143–145 °C (ethyl acetate–hexane); IR (KBr) *v* (cm^{-1}): 3310 (–NH–), 1734 (C=O), 1670 (^{7}C=O), 1639 (^{20}C=O), 1249 (C–O acetate), 806 (=C–H); ^{1}H NMR ($CDCl_3$) δ: 7.14 (d, 2H), 7.03 (s, 1H), 6.63 (d, 2H), 5.63 (s, 1H), 4.59 (m, 1H), 3.91 (m, 1H), 3.71 (t, 4H), 3.69 (t, 4H), 3.48 (s, 2H), 2.02 (s, 3H), 1.17 (s, 3H), 0.72 (s, 3H). Anal. Calcd for $C_{33}H_{44}N_2O_4Cl_2$: C, 65.66; H, 7.35; N, 4.64. Found: C, 65.49; H, 7.33; N, 4.66; LC–MS (ESI+) *m/z* 603.20 [M+H$^+$].

5.1.2.3. 3β-Hydroxy-17β-acetamide-androst-5-en-7-one-4-methyl-3-*N*,*N*-bis(2-chloroethyl) amino benzoate (9c). Yield 85.5%; mp 160–161 °C (ethyl acetate); IR (KBr) *v* (cm^{-1}): 3281 (–NH–), 1712 (C=O), 1672 (^{7}C=O), 1650 (^{20}C=O), 1255 (C–O acetate), 763, 733 (=C–H); ^{1}H NMR ($CDCl_3$) δ: 7.81 (s, 1H), 7.72 (d, 1H), 7.31 (d, 1H), 5.72 (s, 1H), 4.93 (m, 1H), 3.92 (m, 1H), 3.40 (m, 8H), 2.40 (s, 3H), 2.01 (s, 3H), 1.21 (s, 3H), 0.81 (s, 3H). Anal. Calcd for $C_{33}H_{44}N_2O_4Cl_2$: C, 65.66; H, 7.35; N, 4.64. Found: C, 65.72; H, 7.13; N, 4.66; LC–MS (ESI+) *m/z* 603.20 [M+H$^+$].

5.1.2.4. 3β-Hydroxy-7α-aza-B-homo-17β-acetamide-androst-5-en-7-one-4-*N*,*N*-bis(2-chloroethyl)amino phenylbutyrate (10a). Yield 79%; mp 171–172 (ethyl acetate). IR (KBr) *v* (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). Anal. Calcd for $C_{35}H_{49}N_3O_4Cl_2$: C, 65.00; H, 7.64; N, 6.50. Found: C, 65.02; H, 7.63; N, 6.48, LC–MS (ESI+) *m/z* 646.19 [M+H$^+$].

5.1.2.5. 3β-Hydroxy-7α-aza-B-homo-17β-acetamide-androst-5-en-7-one-4-*N*,*N*-bis(2-chloroethyl)amino phenylacetate (10b). Yield 55; mp 148–150 °C (ethyl acetate); IR (KBr) *v* (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). Anal. Calcd for $C_{33}H_{45}N_3O_4Cl_2$: C, 64.07; H, 7.33; N, 6.79. Found: C, 64.06; H, 7.31; N, 6.77, LC–MS (ESI+) *m/z* 618.07 [M+H$^+$].

5.1.2.6. 3β-Hydroxy-7α-aza-B-homo-17β-acetamide-androst-5-en-7-one-4-methyl-3-*N*,*N*-bis(2-chloroethyl) amino benzoate (10c). Yield 60%; mp 160–162 °C (ethyl

acetate). IR (KBr) 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). Anal. Calcd for $C_{33}H_{45}N_3O_4Cl_2$: C, 64.07; H, 7.33; N, 6.79. Found: C, 64.05; H, 7.35; N, 6.80; LC–MS (ESI+) m/z 618.07 [M+H$^+$].

5.2. In vivo experiments

5.2.1. Compounds. For intraperitoneal (ip) treatment, stock solutions of the compounds used in this study were prepared immediately before use. They were suspended in corn oil in the desired concentration following initial dissolution in 5% dimethylsulfoxide (DMSO). This concentration by itself produced no observable toxic effects.

Mice: BALB/c, DBA/2 and BDF1 mice of both sexes, weighting 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.

5.2.2. Tumors. Leukemia P388- and L1210-bearing BDF1 (DBA/2 × C57BL) mice were used to evaluate the cytostatic effect. Lymphocytic P388 and lymphoid L1210 leukemias were maintained in ascitic form by injection of 10^6 and 10^5 cells, respectively, at 7-day intervals, into the peritoneal cavity of DBA/2 mice.

5.2.3. Estimation of acute toxicity. The acute toxicity of the compounds was determined following a single ip injection into BALB/C in groups of 10 mice per dose at three different dosages. The mice were observed for 30 days and the therapeutic dose of the compounds was determined after graphical estimation of the LD_{50} (30-day curves). The dose used for a single treatment was equal to the $LD_{10}/2$.

5.2.4. Antileukemic evaluation. 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%, that is, the increase in median life span of the drug-treated animals (T) excluding long-term survivors versus corn oil-treated controls (C) was expressed as a percentage. The other index of the antileukemic activity used was the number of long-term survivors defined as mice alive for 90 days after tumor inoculation. Each drug-treated group consisted of six mice while 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.[48] Treatments were given as an intermittent dose ($LD_{10}/2 \times 3$, days 1, 4, and 7). The experiments were terminated on day 90. Statistical evaluation of the experimental data was made by the Wilcoxon test.

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