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Synthesis, *In Vivo* Antileukemic Evaluation and Comparative Study of Novel 5α-7-Keto Steroidal Esters of Chlorambucil and its Active Metabolite

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Abstract. *Recent structure-antileukemic activity studies showed that the steroidal part of complex molecules containing DNA alkylators does not play only the role of the "biological carrier". New such compounds designed to possess an allylic 7-ketone showed enhanced antileukemic potency compared with derivatives with a simple steroidal skeleton. In order to investigate whether the enhancement of the antileukemic potency is attributed to the introduction of the 7-ketone or to the Δ^5-7-keto conjugated steroidal system we decided to reduce the Δ^5 double bond. The 5α-7-keto-steroidal skeletons synthesized were tethered to chlorambucil and phenyl acetic acid's nitrogen mustard and studied against leukemia P338 in vivo. The reduction of the double bond had a negative impact on the antileukemic potency since the comparative study of the novel derivatives showed that a series of very potent Δ^5-7-keto-steroidal esters were converted by this modification to compounds with marginally accepted activity.*

Improving drug selectivity and minimizing toxicity is of considerable importance in modern chemotherapy. Therapeutic strategies, such as using drug precursors, is one of the solutions that relies on the accumulation of the drug at its site of action. Such derivatives usually constitute a carrier moiety that, in addition to targeting the agent may have or generate an activity of its own. There is ongoing research regarding derivatization of chlorambucil (CHL) with carrier molecules (*e.g.* nucleoside, amidine, steroidal analogs) (1, 2). Regarding hormone conjugates of CHL, prednimustine (prednisolone ester of chlorambucil) has been used in clinical practice against hematological malignancies (3-6), while in a recent study a chlorambucil nitrogen mustard moiety connected to a steroidal androgen provided evidence supporting a novel mechanism for selective toxicity in androgen receptor-positive cancer cells, presumably through sterically blocking the access of DNA-repair enzymes or holding the receptor away from important regulatory sites (7-9).

Our previous work, concerning the clastogenic and antileukemic potency of a series of CHL and phenylacetic acid nitrogen mustard (PHE, CHL active metabolite) steroidal esters showed that the steroidal carrier, in addition to changing the physicochemical parameters of the alkylating congener, has a propensity for altering the activity of the nitrogen mustard, depending on the chemical structure of the steroid used. Esters comprising a modified steroidal moiety showed enhanced antileukemic activity and reduced toxicity compared with the non-derivatized nitrogen mustards or their conjugates with a simple steroidal skeleton (10-13). Specifically, it has been found that steroidal skeletons that carry a -NHCO- moiety either as a D-lactam (14-16) or as a 17β-acetamidic substituent (17, 18) are more appropriate modules, while the insertion of an allylic 7-ketone in simple and D-modified steroids is crucial for the potency of these hybrid toxins (19, 20).

In order to elucidate whether the enhancement of the antileukemic potency is attributed to the introduction of the oxygen at the 7-position of the steroidal skeleton or to the Δ^5-7-keto-conjugated steroidal system, we decided to reduce the Δ^5 double bond. This study deals with the synthesis of 3β-hydroxy-5α-androstan-7,17-dione [**7**] and two new steroidal skeletons, namely 3β-hydroxy-17α-aza-D-homo-5α-androstan-7,17-dione [**8**] and 3β-hydroxy-17α-acetamido-5α-androstan-7-one [**9**], as well as the synthesis of their esters with CHL and PHE. The final esteric derivatives [**7a-9a**] were evaluated against leukemia P388 *in vivo* and compared with the corresponding Δ^5-7-keto-conjugated ones [**7b-9b**].

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Materials and Methods

General procedure for the reduction of the steroidal skeletons. A solution of the corresponding Δ^5-7-keto steroid (4 mmol) in ethyl acetate or methanol was hydrogenated under two atmospheres of hydrogen in the presence of palladium on activated charcoal 10% (680 mg) for 2 or 4 hours at room temperature. After the removal of the catalyst by filtration through a Celite pad, the filtrate was concentrated to dryness. The residue was chromatographed over silica gel/dichloromethane, and the product was eluted with dichloromethane/methanol.

General procedure for the hydrolysis of the steroidal skeletons. A solution of the corresponding 3β-acetoxy steroid (3.5 mmol) in 25 ml methanol was treated with LiOH 1N (24 ml) and the mixture was stirred at room temperature for 1 hour. The mixture was then poured into saturated NaCl and extracted with dichloromethane. The organic layer was washed with saturated NaCl, and dried over Na_2SO_4. Evaporation of the solvent gave the corresponding steroidal alcohols.

General procedure for the preparation of the steroidal esteric derivatives of CHL and PHE via asymmetric anhydrides. A solution of the corresponding nitrogen mustard (1.8 mmol) in 15 ml of dry toluene was treated with 2,4,6-trichlorobenzoyl chloride (2.16 mmol) and triethylamine (2.16 mmol) and refluxed under Ar for 1.5 h for CHL as starting material and 2 h for PHE. In the above mixtures, a solution of the steroidal alcohol (1.5 mmol) in dry toluene and 4-dimethylaminopyridine (1.5 mmol) were added. The reaction mixture was refluxed under Ar for 2.5 h. The solvent was evaporated, the residue dissolved in dichloromethane, washed successively with 5% aq. HCl, water, 5% aq. $NaHCO_3$, water and then dried over Na2SO4. The solvent was removed under reduced pressure and the residue chromatographed on silica gel/dichloromethane. Elution with dichloromethane/ methanol (99:1 v/v) for the esters of **7a**, **7b** and dichloromethane/methanol (98:2 v/v) for the esters of 8a, 8b and **9a**, **9b** gave the desired compounds.

Tables I-III show the physicochemical and spectroscopic characteristics of the compounds.

In vivo experiments.

Compounds. For intraperitoneal (*i.p.)* 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.

Tumours. Leukemia P388-bearing BDF1 (DBA/2 x C57BL) mice were used to evaluate the cytostatic effect. Lymphocytic P388 leukemia was 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.

Estimation of acute toxicity. The acute toxicity of the compounds was determined following a single *i.p.* 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 was equal to $LD_{10}/2$ value.

Antileukemic evaluation. For the survival experiments, the antileukemic activity of the tested compounds against the above-mentioned murine tumours was assessed from the oncostatic parameter T/C%, *i.e.* the increased 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 tumour inoculation. Each drug-treated group consisted of 6 mice while the tumour control group included 8 mice; in each group, equal numbers of male and female mice were used. Experiments were initiated by implanting mice with tumour cells according to the protocol of the National Cancer Institute, USA (21). Treatments were given as an intermittent dose ($LD_{10}/2$ x 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.

Results

The double bond of 3β-acetoxy-androst-5-en-7,17-dione [**1**] was successfully reduced with catalytic hydrogenation using H2/Pd-C 10% in ethyl acetate. The reaction time did not exceed 2 hours using a pressure of 2 Atm, in contrast to the method described in the literature where the pressure applied was 1 Atm with a reaction time of 4 hours (22, 23), giving **4** with 98% yield. Application of the same reductive system to steroidal skeletons **2** and **3** did not satisfactorily yield the desired aliphatic ketones **5** and **6** correspondingly, even when the reaction time was prolonged to 10 hours. The best results were obtained using methanol as a solvent instead of ethyl acetate with a concurrent prolongation of the reaction time to 4 hours. The 5α-7-keto steroids **5** and **6** were obtained in 96% and 94% yield correspondingly.

Compounds **1-6** were hydrolyzed under mild basic conditions to give the corresponding alcohols **7a-9a** and **7b-9b** which were esterified *via* the asymmetric anhydride procedure with 4-*N,N-bis*(2-chloroethyl)aminophenylbutyric acid (CHL) and 4-*N,N-bis*(2-chloroethyl)amino phenyl acetic acid (PHE) to the final steroidal derivatives (see Figures 2 and 3).

Table I shows the toxicity values of the 5α-7-keto steroidal esters, as well as those of the alkylating agents and the parent Δ^5-7-keto derivatives (Figure 3). The LD_{50} values showed that in all cases the chemical linkage of the nitrogen mustards with the 7-keto steroids resulted in a reduction of the toxic effects induced by the alkylating agents. PHE was more toxic compared with CHL, as well as the derivatives of PHE in comparison to the corresponding steroidal esters of CHL. No significant differentiation was observed from the reduction of the Δ^5 double bond.

Table I. *Physicochemical and analytical data of the steroidal skeletons.*

Compound	Yield (%)	Recrystallisation solvent	m.p. (˚C)	IR (cm^{-1})	^1H NMR ($CDCl_3$) δ	Elemental analysis					
						Calculated (%)			Found (%)		
						C	H	N	C	H	N
4	98	Methanol (39, 40)	166-7	1732, 1703, 1240	4.69 m, 2.04 s, 1.14 s, 0.88 s	72.8	8.73	-	72.82	8.74	-
5	96	Methanol	265-7	3234, 1728, 1709, 1666, 1244	6.03 s, 4.66 m, 2.04 s, 1.17 s, 1.12 s	69.78	8.64	3.87	69.80	8.64	3.88
6	94	Methanol	230-2	3196, 1738, 1710, 1641, 1238	5.21 d, 4.67 m, 3.90 m, 2.02 s, 1.97 s, 1.09 s, 0.66 s	70.92	9.06	3.60	70.92	9.03	3.61
7a	98.8	Methanol (39)	197-8	1700	3.62 m, 1.10 s, 0.86 s	74.96	9.27	-	74.95	9.28	-
7b	97	Methanol (41)	236-7	1726, 1653	5.93 s, 3.69 m, 1.15 s, 0.86 s	75.46	8.67	-	75.44	8.61	-
8a	90	Methanol	313-5	3236, 1710, 1666	5.93 s, 3.62 m, 1.16 s, 1.08 s	71.44	9.15	4.38	71.45	9.17	4.38
8b	98.2	Methanol	300	3137-3083, 1674, 1653	10.96 s, 6.92 s, 5.87 s, 3.59 m, 1.07 s, 0.93 s	71.98	8.57	4.41	72.09	8.49	4.31
9a	92	Methanol	267-9	3196, 1714, 1645	5.25 d, 3.92 m, 3.61 m, 1.98 s, 1.07 s, 0.66 s	72.58	9.57	4.03	72.58	9.58	4.01
9b	94	Methanol	270-2	3210-3184, 1666, 1663	10.12 s, 6.65 d, 5.84 s, 3.67 m, 3.91 m, 2.01 s, 0.97 s, 0.72 s	73.01	9.04	4.05	73.12	9.09	4.12

m.p.: melting point.

Table II. *Physicochemical and analytical data of the steroidal esteric derivatives of CHL.*

Compound	Yield (%)	Recrystallisation solvent	m.p. (˚C)	IR (cm^{-1})	^1H NMR ($CDCl_3$) δ	Elemental analysis					
						Calculated (%)			Found (%)		
						C	H	N	C	H	N
7a-CHL	90	Ethyl acetate-hexane	128-9	1736, 1700, 1249, 804	7.07d, 6.61d, 4.69m, 3.70t, 3.62t, 2.54t, 2.30t, 1.86m, 1.11s, 0.86s	67.11	7.68	2.37	67.12	7.70	2.38
7b-CHL	90	Ethyl acetate-hexane	120-1	1736, 1725, 1681, 1245, 806	7.21d, 6.61d, 5.71s, 4.63m, 3.71t, 3.68t, 2.56t, 2.31t, 1.98m, 1.21s, 0.91s	67.34	7.36	2.38	67.33	7.35	2.34
8a-CHL	94	Ethyl acetate-hexane	234-5	3198, 1725, 1711, 1657, 1248, 804	7.08d, 6.62d, 6.42s, 4.70m, 3.71t, 3.63t, 2.55t, 2.29t, 1.89m, 1.17s, 1.11s	65.44	7.66	4.63	65.44	7.68	4.66
8b-CHL	53.4	Ethyl acetate-hexane (11)	182-3	3500-3050, 1730, 1672, 1650, 1246, 804	7.12d, 6.60d, 6.82s, 5.64s, 4.58m, 3.70t, 3.66t, 2.51t, 2.29t, 1.85m, 1.22s, 1.11s	65.66	7.35	4.64	65.64	7.30	4.76
9a-CHL	97	Ethyl acetate-hexane	221-2	3306, 1732, 1714, 1651, 1248, 804	7.07d, 6.65d, 5.24d, 4.69m, 3.91m, 3.68t, 3.63t, 2.25t, 2.29m, 1.98s, 1.09s, 0.66s	66.34	7.95	4.42	66.35	7.98	4.43
9b-CHL	66.5	Ethyl acetate-hexane	158-161	3306, 1732, 1670, 1651, 1248, 804	7.09d, 6.82s, 6.61d, 5.64s, 4.61m, 3.89m, 3.72t, 3.67t, 2.49t, 2.23t, 1.83m, 2.01s, 1.21s, 0.71s	66.55	7.66	4.43	66.45	7.71	4.53

m.p.: melting point.

Table III. *Physicochemical and analytical data of the steroidal esteric derivatives of PHE.*

Compound	Yield (%)	Recrystallisation solvent	m.p. (°C)	IR (cm^{-1})	^1H NMR ($CDCl_3$) δ	Elemental analysis					
						Calculated (%)			Found (%)		
						C	H	N	C	H	N
7a-PHE	82	Ethyl acetate-hexane	156-7	1736, 1709, 1253, 808	7.16d, 6.66d, 4.69m, 3.70t, 3.63t, 3.49s, 1.13s, 0.87s	66.18	7.35	2.49	66.20	7.33	2.49
7b-PHE	81.4	Ethyl acetate-hexane(19)	154-6	1736, 1670, 1248, 806	7.21d, 6.62d, 5.73s, 4.62m, 3.72t, 3.63t, 3.42s, 1.22s, 0.91s	66.42	7.01	2.50	66.43	7.01	2.48
8a-PHE	52	Ethyl acetate-hexane	261-2	3230, 1734, 1709, 1651, 1257, 808	7.17d, 6.65d, 6.59s, 4.70m, 3.71t, 3.65t, 3.50s, 1.18s, 1.12s	64.46	7.33	4.85	64.49	7.35	4.89
8b-PHE	71.3	Ethyl acetate-hexane(19)	183-5	3196, 1729, 1668, 1650, 1256, 801	7.13d, 6.92s, 6.62d, 5.63s, 4.59m, 3.65t, 3.67t, 3.48s, 1.21s, 1.07s	64.69	7.00	4.87	64.56	7.12	4.89
9a-PHE	65	Ethyl acetate-hexane	235-7	3300, 1734, 1712, 1652, 1249, 808	7.15d, 6.66d, 5.22d, 4.68m, 3.90m, 3.73t, 3.62t, 3.48s, 1.98s, 1.09s, 0.66s	65.44	7.66	4.63	65.46	7.67	4.63
9b-PHE	64.1	Ethyl acetate-hexane(19)	143-5	3310, 1734, 1670, 1639, 1249, 806	7.14d, 7.03s, 6.63d, 5.63s, 4.59m, 3.91m, 3.71t, 3.69t, 3.48s, 2.02s, 1.17s, 0.72s	65.66	7.35	4.64	65.49	7.33	4.66

m.p.: melting point.

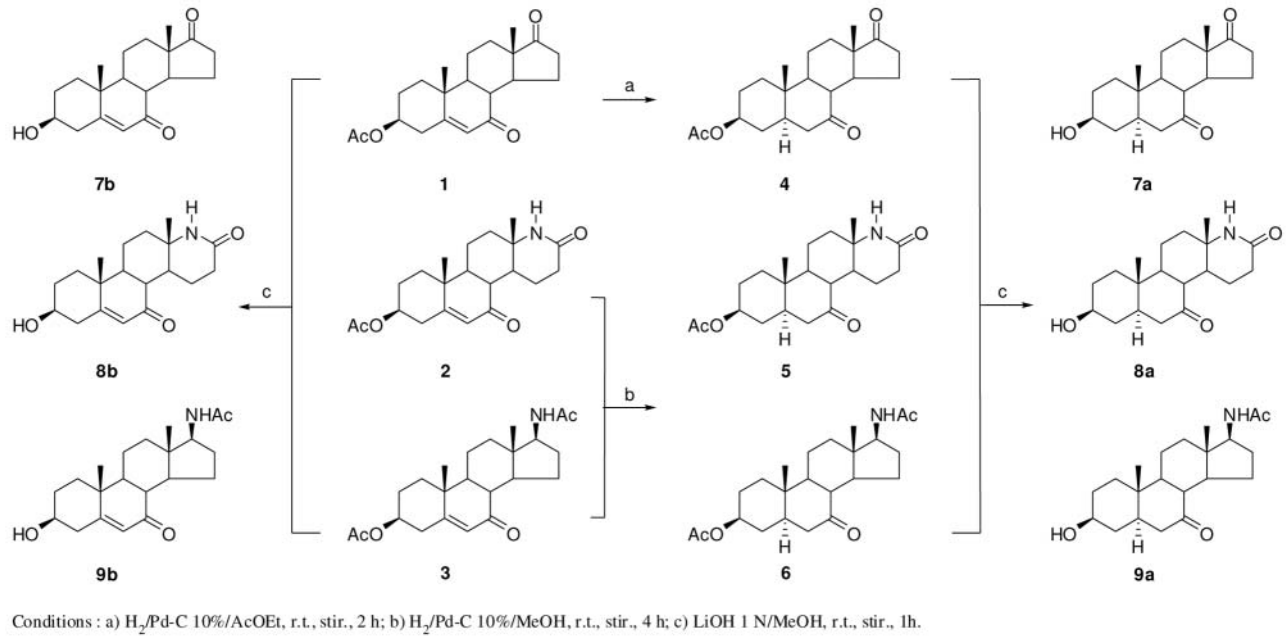

Figure 1. *Catalytic reduction of Δ5-7-keto steroids and hydrolysis.*

Table II summarizes the results of the activity of the compounds studied, against leukemia P388. In all cases examined the steroidal esteric derivatives of PHE had better antileukemic activity compared to the corresponding derivatives of CHL. The conjugation of CHL with 5α-7-keto steroids did not have a significant impact on their antileukemic potency. On the other hand, the corresponding Δ^5-7-keto steroids considerably enhanced the activity of the

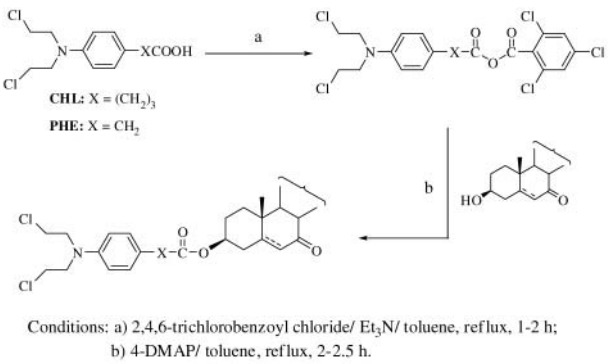

Figure 2. *General procedure for the preparation of the final esteric derivatives.*

nitrogen mustard, as previous studies had also shown (11). In the case of PHE, its derivatization with the aliphatic 7-keto steroids increased the antileukemic activity to a greater extent in comparison to the corresponding aliphatic 7-keto steroidal esters of CHL, but not to the degree observed when PHE was tethered to the steroids bearing an allylic 7-keto group.

It is obvious that the effect of the 7-ketone on the antileukemic potency of CHL and PHE steroidal esters in P388 leukemia is positively expressed only when this keto group is conjugated with a double bond. The reduction of the Δ^5 double bond resulted in an almost half reduction of the T/C value in all cases while no curable effect was found among the 5α-7-keto steroidal derivatives in contrast to the Δ^5-7-keto ones, where 1/6 cures was observed for **8b-CHL** and 3/6 cures for **8b-PHE** (Figure 4). Especially in the case of the derivatives of PHE this small alteration in the chemical structure of the steroid resulted in a remarkable diminuation of the potency, rendering three very active molecules marginally active.

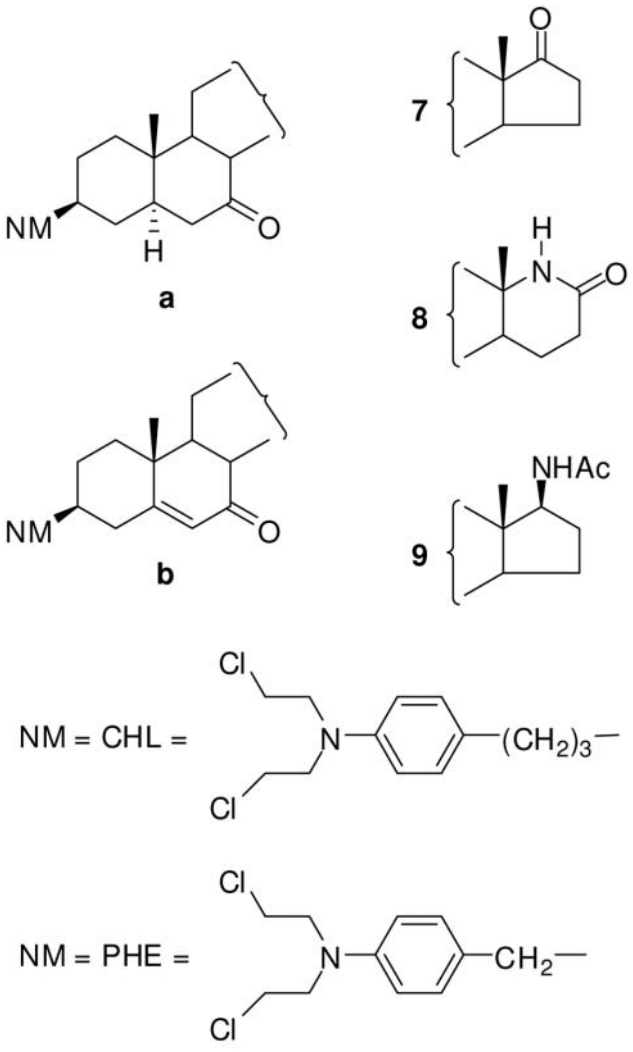

Figure 3. *Steroidal esters of CHL and PHE prepared and tested.*

Discussion

Concerning the chemistry part of this study, the selective reduction of the Δ^5-7-keto steroidal double bond was achieved using the Birch reduction in the presence of Li/liquid NH_3 in tetrahydrofuran for squalamine and cholesterol analogs (24-26). Our efforts to reduce the Δ^5-7-keto double bond of the steroids studied herein using Birch reduction were not as successful as those described in the literature. In the case of steroidal skeleton 1 the yield reached ~40%, and in the case of steroidal skeletons 2 and 3 the starting material was recovered.

Hydrogenation using PtO_2 as catalyst (27, 28) was then applied but the starting material was recovered in all cases. Substitution of the catalyst with Pd on activated charcoal 10%, in ethyl acetate as a solvent, selectively reduced the Δ^5 double bond of **1** with 98% yield without giving any 7-

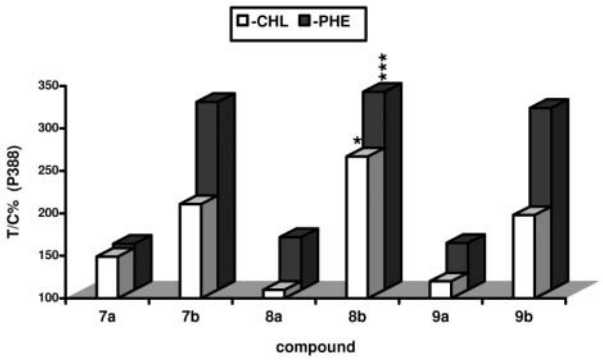

Figure 4. *The effect of 7-ketone on the antileukemic potency of CHL and PHE steroidal esters in P388 leukemia. *Cures (The number of long-term survivors defined as mice alive for 90 days after tumour inoculation).*

Table IV. *Toxicity of CHL and PHE and their steroidal esters.*

Compound	LD_{50}[a] mg/kg	LD_{10} mg/kg
CHL	24	15
7a-CHL	120	60
7b-CHL	110	70
8a-CHL	87	48
8b-CHL	62	43
9a-CHL	87	23
9b-CHL	87	44
PHE	20	10
7a-PHE	87	20
7b-PHE	65	37
8a-PHE	78	30
8b-PHE	90	56
9a-PHE	71	24
9b-PHE	75	25

[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 abscissa on semi logarithmic paper. For chemotherapy testing, the dose used was $LD_{10}/2$. Therefore the drugs in the following experiments were compared at equitoxic doses.

Table V. *Antitumor activity of CHL, PHE and their steroidal esters on P388-bearing mice leukemia, using doses based on toxicity studies.*

Compound	Dosage mg/kg/day	P388		
		MST[a] (days)	T/C[b] (%)	Cures
Control	Corn oil	12.1	100	0/6
CHL	7.5	13.8	114	0/6
7a-CHL	30.0	18.0	149	0/6
7b-CHL	35.0	25.5	211	0/6
8a-CHL	24.0	13.3	110	0/6
8b-CHL	21.5	32.3	267	1/6
9a-CHL	11.5	14.5	120	0/6
9b-CHL	22.0	24.0	198	0/6
PHE	5.0	13.9	115	0/6
7a-PHE	10.0	18.6	154	0/6
7b-PHE	18.5	38.8	321	0/6
8a-PHE	15.0	19.6	162	0/6
8b-PHE	28.0	40.3	333	3/6
9a-PHE	12.0	18.8	155	0/6
9b-PHE	12.5	38.0	314	0/6

[a]MST, Mean survival time of mice inoculated with lymphocytic leukemia P388 cells and treated with compounds; [b]T/C, The increased median life-span of the drug-treated animals (T) *versus* that of corn-oil-treated animals (C); *Treatment schedule doses days 1, 4 and 7.

hydroxy byproducts as reported in the literature for the catalytic hydrogenation with Pd/C 5% of a squalamine analog (29). The same reductive system proved unsuccessful for skeletons **2** and **3**, maybe because these two skeletons were not fully dissolved in the reaction solvent (AcOEt). For this reason these two skeletons were subjected to catalytic hydrogenation with Pd/C 10%/ $NaNO_2$ in EtOH/ H_2O (30). Despite the full dissolution of the starting material in this combination of solvents neither the desired skeletons 5 and 6 were obtained nor were the corresponding starting materials, **2** and **3** recovered. As the dissolution seemed to be the main problem of the initial reductive system used (H_2/Pd-C 10%/AcOEt), we decided to use methanol instead of ethyl acetate. This alteration with a concurrent prolongation of the reaction time to 4 hours proved suitable and gave the desired 5α-7-keto steroids **5** and **6** in good yields.

Prodrugs are described as pharmacologically inactive chemical derivatives that could be used to alter the physicochemical properties of drugs, in a temporary manner, to increase their usefulness and/ or to decrease associated toxicity (31). Steroids offer the opportunity of targeting nitrogen mustards to the DNA through the exploitation of nuclear hormone receptors (32). However, a number of studies suggest that the steroidal part is not only the transporter of the DNA-damaging agent but also generates an activity of its own depending on the structure of the steroid used. Specifically the introduction of a 7-keto group had such an impressive influence on the antileukemic activity which cannot be based solely upon the improvement of its physicochemical parameters (10, 11, 19, 20).

In a continuation of our research concerning the role of the 7-keto group we designed the hybrid toxins presented herein. The comparative study of the allylic 7-keto steroidal derivatives with the corresponding 5α-7-keto ones establishes the significance of the presence of the conjugated Δ^5-7-keto system on the steroidal part of these hybrid toxins and further supports the notion that the steroid influences the mechanism of action of these compounds. The reduction of the 5-double bond not only reduces the electron density on the 7-keto group but also changes the stereochemistry of the B steroidal ring. Apparently these changes affect the capability of the steroidal skeleton to interfere with essential binding sites for the expression of antileukemic activity.

Our studies concerning the mechanism of action of a series of Δ^5-7-keto steroidal alkylating agents have shown their ability to induce *in vitro* sister chromatid exchanges (SCEs) and excision-repairable lesions on lymphocytes (19, 20, 33) compared with the non-derivatized nitrogen mustards or with derivatives bearing simple steroidal skeletons. These findings suggest that the DNA target is

reached by the whole molecule, not only by the nitrogen mustard, and consequently it is the modified steroidal skeleton which differentiates the results obtained. Nuclear hormone receptors can be considered as the main binding site of the steroidal part of these molecules and recent studies have focused on the exploitation of the ability of these multifunctional compounds to bind androgen or estrogen receptors and inhibit their transcriptional activity and at the same time conceal DNA from repair enzymes (34-36). On the other hand, there is a continually increasing interest concerning the non-genomic effects of hormones and many studies implicate these pathways in oncogenesis (37, 38). Δ^5-7-Keto steroidal damaging agents may bear these characteristics and a possible explanation for their ability to induce antileukemic potency might be their affinity to one of the afore-mentioned binding sites.

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