On the Formation of Estrone Lactam Esters of *N,N*-Bis(2-chloroethyl)aminocinnamic Acid Isomers, *p*-*N,N*-Bis(2-chloroethyl)aminophenylbutyric Acid and Their Antitumor Activity

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New estrone-lactam esters, 17α-aza-D-homo-$\Delta^{1,3,5(10)}$-estratrien-3-ol-17-one-*N,N*-bis(2-chloroethyl)-aminocinnamic isomers and *p*-*N,N*-bis(2-chloroethyl)aminophenylbutyrate have been synthesized and tested in P388 leukemia *in vivo* and P388 and L1210 leukemias *in vitro*. The effect of these compounds on SCE rates and on cell kinetics in cultured human lymphocytes was studied also.

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A particular aspect is the effort to develop hybrid compounds, agents which combine in one molecule two, such as steroidal-lactam esters of carboxylic derivatives of *N,N*-bis(2-chloroethyl)aniline. The presence of the characteristic group (-NHCO-) of the lactam molecule is important in order to lower systemic toxicity and improve specificity in cancer research.

Most steroidal alkylating agents have proved inactive in L1210 leukemia [1], while most homoazasteroidal esters gave satisfactory results in P388 and L1210 leukemias [2-4] and solid tumors [5-6], with substitution in A or D-ring of the steroid nucleus by an easily cleaved ester bond.

The idea to design azasteroids as biological platform systems arose from the observation that the biological action of lactam characterized by the amide group may be structurally specific and therefore more prolonged as the result of the multiple interactions of such a group with similar groups that exist in proteins and nucleic acids. On the other hand the lactam nucleus is probably transformed by a metabolic process to the active species which could attack the contents of cancer cells [7].

In the course of screening a number of congeners, it was found of interest to study the activity against P388 leukemia *in vivo* and P388 and L1210 leukemias *in vitro* of *ortho*-, *meta*- and *para*-*N,N*-bis(2-chloroethyl)amino-cinnamic acid esters and *p*-*N,N*-bis(2-chloroethyl)amino-phenylbutyric acid ester of 17α-aza-D-homo-$\Delta^{1,3,5(10)}$-estratrien-3-ol-17-one, as a biological platform [8].

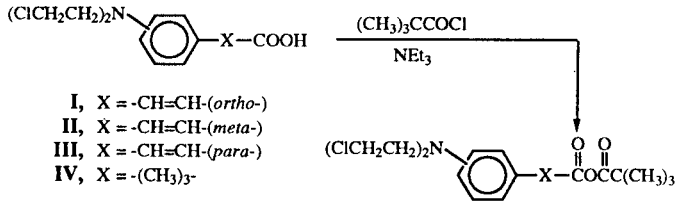
EXPERIMENTAL

Chemistry.

General Procedure for the Synthesis of Estrone Lactam Esters of Cinnamic Acid Isomers, of *N,N*-Bis(2-chloroethyl)aniline and Chlorambucil with Mixed Anhydrides.

a. Synthesis of Mixed Anhydrides.

One half gram (1.65 mmoles) of the corresponding acid was



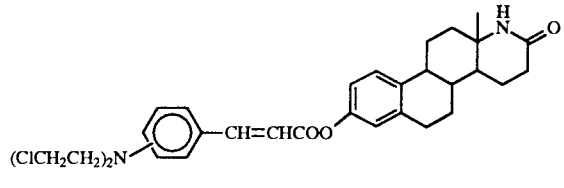
I, X = -CH=CH-(*ortho*-)
II, X = -CH=CH-(*meta*-)
III, X = -CH=CH-(*para*-)
IV, X = -$(CH_3)_3$-

Ia, X = -CH=CH-(*ortho*-)
IIa, X = -CH=CH-(*meta*-)
IIIa, X = -CH=CH-(*para*-)
IVa, X = -$(CH_3)_3$-

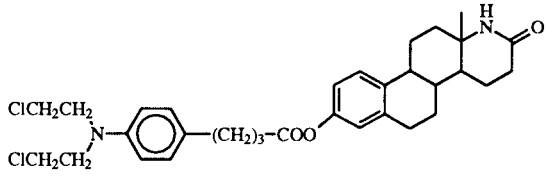
diluted in 20 ml of xylene and the solution was cooled in a water-ice bath. To the previous solution 1.65 mmoles (0.203 ml) of pivaloyl chloride and 1.65 mmoles (0.230 ml) of triethylamine were added and the mixture was refluxed for 2 hours. After the reaction time the mixture was cooled and the triethylamine hydrochloride was filtered off. The solutions of the mixed anhydrides **Ia**, **IIa**, **IIIa** and **IVa** were used in the next stage.

b. Synthesis of Steroidal Esters.

The corresponding anhydride of the previous stage was added in a solution of 1.65 mmoles (0.47 g) of estrone lactam, 1.65



Ib, (*ortho*-)
IIb, (*meta*-)
IIIb, (*para*-)



IVb, Chlorambucil Ester

mmoles (0.230 ml) of triethylamine, 1.65 mmoles (0.2 g) of 4-dimethylaminopyridine in 300 ml of xylene, and the mixture was refluxed for 2 days.

After the reaction time the solvent was removed under reduced pressure and the residue was diluted in toluene which is also removed under reduced pressure. The procedure was repeated with benzene until all traces of xylene are removed from the solution. Finally the residue was diluted in chloroform, washed with a 5% solution of hydrochloric acid and neutralized with a saturated solution of sodium bicarbonate. The chloroform solution was dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure and the residue which remained was chromatographed on silica gel. Elution with chloroform yielded the corresponding esters as follows:

17α-Aza-D-homo-$\Delta^{1,3,5(10)}$-estratrien-3-ol-17-one-2-*N,N*-bis(2-chloroethyl)aminocinnamate Ester (**Ib**).

This compound was obtained in 52% yield, mp 165° as a powder (ethyl acetate/diethyl ether); ir (potassium bromide): 3780, 3050 (NH), 1725 (COO), 1655-1625 (NHCO), 755 (Ar ring).

Anal. Calcd. for $C_{31}H_{36}N_2O_3Cl_2$: C, 67.02; H, 6.48; N, 5.04. Found: C, 67.30; H, 6.30; N, 5.00.

17α-Aza-D-homo-$\Delta^{1,3,5(10)}$-estratrien-3-ol-17-one-3-*N,N*-bis(2-chloroethyl)aminocinnamate Ester (**IIb**).

This compound was obtained in 58% yield, mp 149% from ethyl acetate/diethyl ether; ir (potassium bromide): 3200, 3060 (NH), 1730 (CO), 1660-1635 (NHCO), 780 (Ar ring).

Anal. Calcd. for $C_{31}H_{36}N_2O_3Cl_2$: C, 67.02; H, 6.48; N, 5.04. Found: C, 67.00; H, 6.49; N, 5.00.

17α-Aza-D-homo-$\Delta^{1,3,5(10)}$-estratrien-3-ol-17-one-4-*N,N*-bis(2-chloroethyl)aminocinnamate Ester (**IIIb**).

This compound was obtained in a yield of 68%, mp 134°, from ethyl acetate-diethyl ether; ir (potassium bromide): 3190, 3040 (NH), 1720 (COO), 1665-1625 (NHCO), 740 (Ar ring).

Anal. Calcd. for $C_{31}H_{36}N_2O_3Cl_2$: C, 67.02; H, 6.48; N, 5.04. Found: C, 67.30; H, 6.55; N, 5.09.

17α-Aza-D-homo-$\Delta^{1,3,5(10)}$-estratrien-3-ol-17-one-4-*N,N*-bis(2-chloroethyl)aminophenylbutyrate Ester (**IVb**).

This compound was obtained in 72% yield as a powder, mp 202-203°, from ethyl acetate/diethyl ether; ir (potassium bromide): 3190, 3060 (NH), 1750 (COO), 1655-1615 (NHCO), 750 (Ar ring).

Anal. Calcd. for $C_{32}H_{40}N_2O_3Cl_2$: C, 67.25; H, 7.00; N, 4.90. Found: C, 67.50; H, 7.12; N, 4.80.

Biological Data.

In vivo Experiments of **Ib-IVb**.

Mice-Tumors.

BALB/C, DBA/2 and BDF_1 mice of both sexes, 20-22 g, 2-4 weeks old, were used for toxicity studies and antileukemic testing. Mice were obtained from the Experimental Laboratory of Theagenion Cancer Hospital.

Lymphocytic P388 leukemia was maintained in ascitic form in DBA/2 mice, by injection of 10^6 cells, at 7-day intervals, into the peritoneal cavity.

Drug compounds **Ib-IVb** were dissolved in DMSO, suspended in corn oil and administered i.p.

Antitumor Evaluation.

Antileukemia experiments were initiated by implanting BDF_1 with the appropriate number of P388 cells. Drug treatment consisted of i.p. injections given on days 1, 5 and 9 (D/2 x 3). The antitumor activity was assessed by the percentage increase in median life-span over the controls (T/C %). The results are reported in Table II.

Estimation of Acute Toxicity.

Estimation is performed following single i.p. injection into BALB/C mice (groups of 10 mice/dose). The mice were observed for 30 days and the LD_{50} values were estimated graphically (Table I).

In vitro Experiments.

L1210 leukemia cells were grown in RPMI-1640 medium. P388 cells were grown in Dulbecco's medium. All media were supplemented with 10% calf serum, streptomycin, penicillin and 42 m*M* HEPES.

Treatment with the Compounds.

The experiments were carried out in a cell suspension with 1 x 10^6 cells/ml. The compounds were dissolved in DMSO and then culture medium was added. The final concentration of DMSO was not more than 0,5%. The incubation time was 60 minutes at a concentration of 1,6 x 10^{-6}*M*.

Incorporation of [Methyl-^{3}H]Thymidine.

DNA synthesis was determined after 30 minutes incubation of the cells with the radioactive precursor of DNA. The cell suspension was placed on Whatman No. 41 filters and the wet filters were soaked in 5% cold TCA for 10 minutes. The filters were further washed twice with 5% TCA, twice with 96% alcohol, once with mixture of ether:ethanol (1:1) and once in ether. After drying, the filters were placed in scintillation fluid and the radioactivity was determined (Table III).

In vitro SCE Assay.

Lymphocyte cultures were prepared by adding four drops of heparinized whole blood from normal subjects to 4 ml of chromosome medium. The cultures were incubated for 96 hours at 37°. Metaphases were collected during the last 2 hours with colchicine treatment of 0.3 μg/ml. Treatment with the chemicals started 18 hours after the initiation of the culture. For the demonstration of SCE, 5-bromodeoxyuridine (BDU) at 4 μg/ml was added 24 hours after initiation of the culture. All cultures were kept in the dark to minimize photolysis of BDU. Air-dried preparations were made and stained by the fluorescence plus Giemsa procedure. The preparations were scored for cells in their, 1st, 2nd, 3rd and subsequent divisions with criteria previously described and suitably spread second-division cells were scored for SCE. A minimum of 50 cells was scored for each culture. For proliferation rate indices (PRIs), at least 100 cells were scored. SCEs and PRIs were scored after the slides had been coded. For statistical evaluation of the experimental data the X^2 test was used for the cell kinetic comparisons, whereas for the SCE frequencies student's t-test was performed to determine whether any values deviated significantly from the controls (Table IV).

Results.

Table I

Toxicity	LD50	LD10
IVb	120	70
Ib	55	35
IIb	220	150
IIIb	220	150

Table II

Antitumor Activity of **Ib-IVb** on Leukemia P388

Compound	Treatment Schedule (days)	Dosage mg/kg	MST (days)	T/C %
Control	—	Saline	11.6	100
IVb	1, 5, 9	35	17.2	148
Ib	1, 5, 9	17.5	12.8	110
IIb	1, 5, 9	75	12	103
IIIb	1, 5, 9	75	12	103

Table III

Cytostatic Effect of **Ib-IVb** on DNA Synthesis in Leukemias P388 and L1210

Compound	Mean % Inhibition of [Methyl-^{3}H]thymidine Incorporation P388	L1210
IVb	71	59
Ib	78	73
IIb	47	60
IIIb	62	67

Table IV

SCEs and Cell Division Delays Induced by Compounds **Ib-IVb** in Human Lymphocytes

Compound	Concentration μM	SCEs/cell±SE	PRI
Control	—	9.68 ±0.41	2.40
IVb	3.4	91.64 ±4.02	1.42
	6.8	93.0 ±8.23	1.05
Ib	3.4	48.11 ±2.88	1.60
	6.8	59.07 ±3.06	1.56
IIb	not tested		
IIIb	3.4	41.86 ±5.48	1.96
	6.8	43.58 ±4.02	1.80

PRIs were calculated as $(1M_2+2M_2+3M_3+)/100$, where M_1 is the percent value of cells in the 1st, M_2 in the 2nd and M_3+ in the 3rd and higher divisions. The PRIs were correlated with corresponding SCEs: $r = -0.93$, $p < 0.01$.

Compounds **Ib**, **IIIb** and **IVb** (**IIb** was not tested) induced statistically significant ($p < 0.01$) increase in SCE rates and cell division delays, at all concentrations used. Compound **IVb** was the most effective agent. Next in order of effectiveness was **Ib** with **IIIb** following. The results in Table IV ($r = -0.93$, $p < 0.01$) seem to show that a correlation, the higher the PRI value the lower the SCE frequency, exists. In the present study for the four chemicals used, a correlation between cell division delay, SCE induction and antitumor activity, was observed.

Discussion.

Compounds **Ib-IIIb** were inactive in P388 leukemia. The ester **IVb** produced T/C value 148% (Table II). (A ≥125% T/C is usually required as a minimum for activity.) In a previous communication, the antineoplastic activity of 3β-hydroxy-13α-amino-13,17-seco-5α-androstan-17-oic-13,17-lactam-*N,N*-bis(2-chloroethyl)aminocinnamates isomers in lymphoid leukemia P388 were studied [9]. Of the three isomers tested the *ortho* was active. A good correlation exists between, antitumor activity and chemical reactivity assessed by determining the percent hydrolysis of the nitrogen mustard moiety. The higher chemical reactivity of the *ortho*-substituted nitrogen mustards compared with the *meta* and *para* isomers is not uncommon and has been attributed to steric hindrance of mesomerism. Steric repulsion exerted by the *ortho* substituent twists the nitrogen out of conjugation with the π-electrons of the ring and increases its basicity.

The activity of **Ib** is better than the two other isomers (Table III).

Concerning the synthesis of DNA, *in vitro* experiments, the activity of **Ib** is better than the other three compounds, while the cytogenetic study (SCEs and PRI) showed better activity for **IVb**, followed by **Ib**.

The reduction of resonance between the nitrogen atom of the mustard moiety and the carboxyl group of the ester, by the interference of a saturated carbon chain between the aromatic ring and carbonyl group, leads to compounds with significantly increased activity in cancer chemotherapy [2-6].

REFERENCES AND NOTES

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