Steroidal esters of the aromatic nitrogen mustard 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoic acid (2-PHE-BU): synthesis and in-vivo biological evaluation

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On the basis of the results of in-silico predictions and in an effort to extend our structure–activity relationship studies, the aromatic nitrogen mustard 2-[4-*N*,*N*-bis(2-chloroethyl) amino-phenyl]butanoic acid (2-PHE-BU) was synthesized and conjugated with various steroidal alcohols. The resulting steroidal esters were evaluated for their in-vivo toxicity and antileukemic activity in P388-leukemia-bearing mice. The new derivatives showed significantly reduced toxicity and marginally improved antileukemic activity compared with free 2-PHE-BU. Nevertheless, they did not prove to be superior either to the template steroidal ester used for in-silico predictions or to previously synthesized steroidal esters of aromatic nitrogen mustards. The results obtained indicate that in-silico design predictions may guide the design and synthesis of new bioactive steroidal esters, but further parameters should be considered aiming at the discovery of compounds with optimum activity. *Anti-Cancer Drugs* 24:52–65 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2013, **24**:52–65

Keywords: antileukemic activity, aromatic nitrogen mustards, in-silico drug design, (±)-2-phenylbutyric acid, steroidal esters

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Received 19 February 2012 **Revised form accepted** 10 July 2012

Introduction

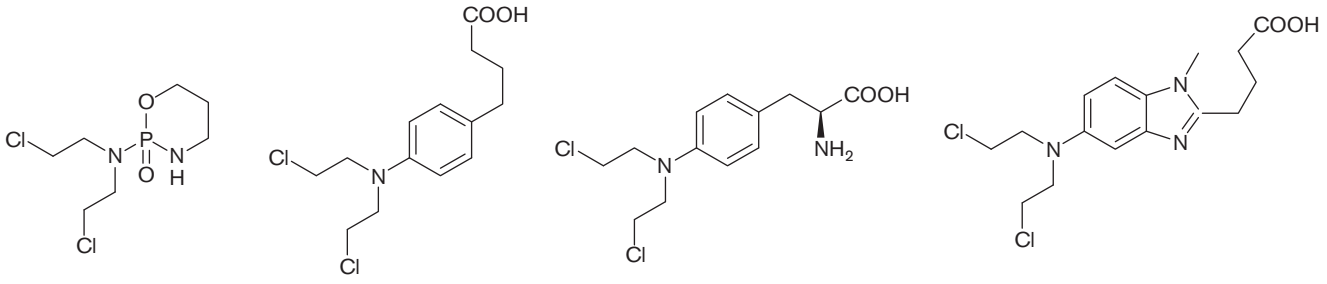
Several chemotherapeutics act as DNA-damaging agents, resulting in cell cycle arrest and cell death of the uncontrollably proliferating cancer cells [1]. Among them, bifunctional DNA alkylating agents [2], such as the nitrogen mustards cyclophosphamide, chlorambucil (CHL), and melphalan, have been used for years in the clinic, whereas recently, the US FDA approved bendamustine [3] (Fig. 1) for the treatment of chronic lymphocytic leukemia and indolent B-cell non-Hodgkin's lymphoma [4,5]. They have an *N*,*N*-bis(2-chloroethyl)amine functional group and act as reactive electrophiles through their intramolecular transformation into aziridinium cations. As a consequence, they bind covalently to electron-rich atoms of DNA bases, forming either intrastrand or interstrand crosslinks, and inhibit DNA replication and transcription [6]. Although these drugs remain some of the most commonly prescribed chemotherapies for the treatment of various solid and hematological malignancies, particularly in combination with other classical or targeted therapeutics in multiagent regimens [7,8], severe side effects on normal tissues represent drawbacks of their use. These are attributed to their low alkylation selectivity of DNA bases because of their high inherent reactivity, resulting in the nonspecific alkylation of other cellular nucleophilic species such as amino acid residues or low-molecular-weight thiols [9].

Several approaches have been explored to reduce the toxicity and increase the therapeutic efficacy of nitrogen mustards. Among them, the generation of DNA-directed alkylating agents through the chemical linkage of nitrogen mustards with molecules of increased DNA-binding affinity [10] and the synthesis of nitrogen mustard prodrugs [11] led to interesting results. Another strategy utilizes the conjugation of nitrogen mustards with steroids, exploiting the role of the nuclear receptors in the effective transport and localization of the alkylating agents into the nucleus or the preferential sensitivity of hormone-dependent tumors to relevant hormone-drug conjugates [12,13]. Estramustine and prednimustine are steroidal derivatives of alkylating agents that have been used in combination therapies for the treatment of various types of cancer [14].

In this direction, our group has published a series of studies related to steroidal esters of aromatic nitrogen mustards as antineoplastic, especially antileukemic, agents [15]. Our efforts have succeeded in the identification of potent and promising derivatives (Fig. 2) with enhanced activity and reduced toxicity compared with the corresponding nitrogen mustards against in-vitro and in-vivo experimental tumors [15,16]. Extensive structure–activity relationship (SAR) studies have shown unique structural features of both the steroidal part and the nitrogen mustard, which contribute positively to the bioactivity of the target steroidal esters [16–20]. Furthermore, recent 3D QSAR/CoMFA and CoMSIA studies have led to the generation of related models that indicate the influence of stereoelectronic and

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328357f687

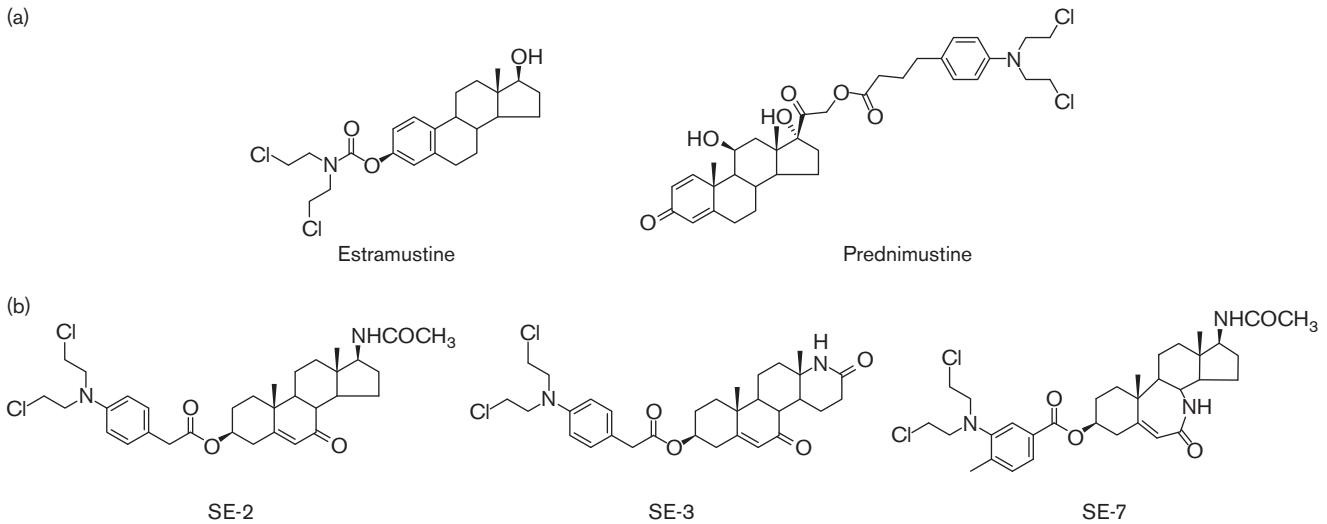
Fig. 1



Cyclophosphamide Chlorambucil Melphalan Bendamustine

DNA alkylating agents.

Fig. 2



Estramustine Prednimustine

SE-2 SE-3 SE-7

LD_{50} (mg/kg) = 75
[a]T/C_{P388} % = 314
[b]Cures: 0/6

LD_{50} (mg/kg) = 90
T/C_{P388} % = 333
Cures: 3/6

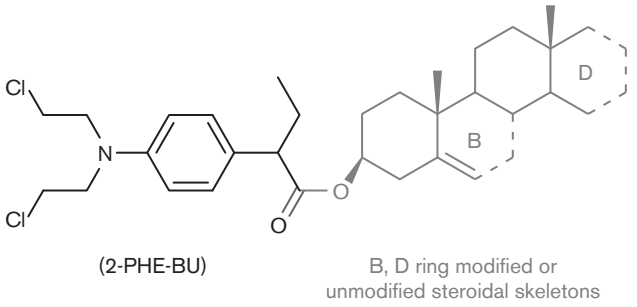
LD (mg/kg) = 280
T/C_{P388} % = 300
Cures: 4/6

(a) Structures of estramustine and prednimustine. (b) In-vivo toxicity and antileukemic activity of representative steroidal esters of aromatic nitrogen mustards on P388-leukemia-bearing mice. [a]T/C%, the percent increase median life span of the drug-treated animals (T) versus corn-oil-treated animals (C) × 100; [b]Cure, the number of long-term survival defined as mice alive for 90 days after tumor inoculation.

physicochemical parameters on the antileukemic activity of target compounds [21]. Particularly, these studies have indicated that the steric effect and the hydrophobic/hydrophilic balance especially in the steroidal part of the molecules probably determined their activity. Notably, it was observed that the orientation of the alkylating agent toward the surface of the B ring of the steroidal part was correlated with increased activity. Moreover, the presence of hydrophobic groups in rings B and D of the steroidal skeleton and the insertion of hydrophilic/H-bond acceptor groups at positions 7 and 17 of the steroidal core enhanced the antileukemic activity. The reliability of both CoMFA and CoMSIA models was evaluated and their predictive ability on the activity of a test set of compounds was found to be satisfactory. Furthermore, on the basis of the CoMFA model proposed and using the de-novo ligand design routine LeapFrog of SYBYL [22], a series of candidate molecules with potentially optimal bioactivity have been proposed, creating new challenges in further investigation of this class of compounds.

On the basis of the aforementioned results and extending our SAR studies, we aimed to examine whether the incorporation of an in-silico predictive nitrogen mustard [21]

Fig. 3



New steroidal esters of 2-PHE-BU. 2-PHE-BU, 2-[4-*N,N*-bis (2-chloroethyl)amino-phenyl]butanoic acid.

into various simple and modified steroids might yield steroidal esters with improved antineoplastic activity. The selection of the nitrogen mustard that was synthesized and used in this study was made on the basis of the predictive antileukemic activity of its corresponding steroidal esters and the synthetic accessibility of the intermediate and target compounds. Thus, we designed and synthesized a new series of steroidal esters containing the aromatic nitrogen mustard 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoic acid (2-PHE-BU) [23] (Fig. 3). 2-PHE-BU is structurally related to other aromatic nitrogen mustards that have been used extensively in our previous studies such as CHL, 4-methyl-3-*N*,*N*-bis(2-chloroethyl)amino-benzoic acid (4-Me-CABA), and especially with 4-*N*,*N*-bis(2-chloroethyl)amino-phenylacetic acid (PHE). In an effort to evaluate the predictive reliability of the Leapfrog routine and compare the biological activity of structurally related compounds, a variety of simple or modified steroidal skeletons were conjugated with 2-PHE-BU, yielding a diverse library of new steroidal esters. Herein, we report a straightforward synthesis of this alkylating agent and selected target steroidal esters as well as results of the evaluation of their in-vivo acute toxicity and antileukemic activity.

Materials and methods

Chemicals

All reagents were obtained commercially from Alfa Aesar (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany), Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany), or Merck (Merck KGaA, Darmstadt, Germany) and used without further purification. Reactions involving moisture-sensitive reactants were run in flame-dried glassware under an atmosphere of argon. Benzene and toluene were purchased in anhydrous forms and used without further purification. Analytical TLC was performed on Merck Silica gel 60 F_{254} on precoated silica gel plates, with visualization under UV light (254 and 365 nm) or/and by exposure to iodide vapors. Flash column chromatography was performed on a silica gel (SDS 60A, 40–63 μm). Melting points were determined on an Electrothermal IA9200 digital melting point apparatus (Electrothermal, Rochford, Essex, UK) in capillary tubes and are uncorrected. The infrared (IR) spectra were recorded on an FT-IR Jasco spectrophotometer (JASCO Inc., Easton, Maryland, USA). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using a Brucker DPX 400 MHz spectrometer (Bruker, Karlsruhe, Germany). Chemical shifts are in parts per million (δ) relative to $CHCl_3$ (7.26 and 77.0 for ^{1}H and ^{13}C-NMR, respectively) as the internal standard and the following abbreviations are used: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of doublets (dd), and quartet of doublets (qd). Electron-spray ionization (ESI) mass spectra were recorded at 30 V on a Micromass-Platform LC spectrometer (Waters Corporation, Milford, Massachusetts, USA) using methanol or acetonitrile as a solvent.

3β-Hydroxy-androst-5-en-17-one was purchased from Steraloids Inc. (Newport, Rhode Island, USA). 3β-Hydroxy-cholest-5-en-7-one [24], 3β-hydroxy-androst-5-en-7,17-dione [25], 3β-hydroxy-17β-acetamide-androst-5-en-7-one [15], 3β-hydroxy-17α-aza-D-homo-androst-5-en-7,17-dione [26], 3β-hydroxy-7α-aza-B-homo-cholest-5-en-7-one [27], 3β-hydroxy-7α-aza-B-homo-androst-5-en-7,17-dione [28], 3β-hydroxy-7α-aza-B-homo-17β-acetamide-androst-5-en-7-one [15], 3β-hydroxy-7α,17α-diaza-B,D-dihomoandrost-5-en-7,17-dione [28], 3β-hydroxy-17β-acetamide-androst-5-en [29], and 3β-hydroxy-17α-aza-D-homo-androst-5-en-17-one [30] were prepared according to the procedures reported in the literature.

2-(4-Nitrophenyl)butanoic acid *(2)*

A mixture (19.08 ml) of concentrated nitric (9.54 ml) and sulfuric acid (9.54 ml) was added slowly to (±)-2-phenylbutyric acid **1** (20 g, 121.80 mmol) at 0°C with stirring. After completion of the addition, the cooling bath was removed and the mixture was stirred vigorously at room temperature for 2 h. Then, it was poured into an aqueous solution of sodium hydrogen sulfate 20% (100 ml) and extracted with dichloromethane (4 × 50 ml). The combined organic phases were washed with brine, dried over sodium sulfate anhydrous, filtered, and evaporated under reduced pressure to yield **2** as a crude oil (23.94 g), which was used without further purification.

Ethyl 2-(4-nitrophenyl)butanoate *(3)*

To a crude solution of **2** (23.94 g) in absolute ethanol (64 ml), concentrated sulfuric acid (2.6 ml) was added and the mixture was refluxed for 6 h under argon. Then, the solvent was removed under reduced pressure and the crude residue was diluted with dichloromethane and extracted (4 × 50 ml). The combined organic extracts were washed with brine, dried over sodium sulfate anhydrous, filtered, and evaporated under reduced pressure to yield **3** as a crude oil (25.93 g), which was used without further purification.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.90 (t, J = 7.3 Hz, 3H), 1.22 (t, J = 7.1 Hz, 3H), 1.74–1.88 (m, 1H), 2.05–2.22

(m, 1H), 3.56 (t, $J = 7.7$ Hz, 1H), 4.04–4.22 (m, 2H), 7.49 (d, $J = 8.6$ Hz, 2H), 8.18 (d, $J = 8.6$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.02, 14.09, 26.81, 53.32, 61.12, 123.73(2 × C), 128.94 (2 × C), 146.53, 147.17, 172.74.

Ethyl 2-(4-aminophenyl)butanoate *(4)*

A crude solution of **3** (25.93 g) in methanol (354 ml) was hydrogenated over Pd 10% on activated carbon (338 mg) at 50 psi for 1 h. Filtration of the catalyst through a celite pad and evaporation of the filtrate under reduced pressure yielded **4** as crude oil (21.52 g), which was used without further purification.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.07 (t, $J = 7.4$ Hz, 3H), 1.40 (t, $J = 7.1$ Hz, 3H), 1.86–2.00 (m, 1H), 2.23 (qt, $J = 15.1$, 7.4 Hz, 1H), 3.52 (t, $J = 7.7$ Hz, 1H), 3.80 (br s, 2H), 4.21–4.38 (m, 2H), 6.84 (d, $J = 8.5$ Hz, 2H), 7.29 (d, $J = 8.4$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.08, 14.12, 26.68, 52.64, 60.38, 115.26 (2 × C), 128.72 (2 × C), 129.33, 145.06, 174.49.

Ethyl 2-[4-*N*,*N*-bis(2-hydroxyethyl)amino-phenyl]butanoate *(5)*

Ethylene oxide (24.2 ml, 484.55 mmol) was added to an ice-cooled solution of **4** (21.52 g, 103.8 mmol) in a mixture of glacial acetic acid (9.7 ml) and water (48.4 ml) in an autoclave. The mixture was allowed to warm up slowly at room temperature with stirring for 48 h. After cooling in an ice bath, the autoclave was opened and the mixture was exposed to the air with stirring overnight. The concentrated solution was poured into ice-water and the mixture was extracted with dichloromethane (5 × 50 ml). The combined organic phases were washed with saturated aqueous sodium hydrogen carbonate (1 × 50 ml) and brine (1 × 50 ml), dried over sodium sulfate anhydrous, filtered, and evaporated under reduced pressure. The crude residue was purified by silica gel flash column chromatography (0–2% MeOH in dichloromethane) to yield **5** (24.6 g, 68% over four steps) as an oil.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.88 (t, $J = 7.3$ Hz, 3H), 1.21 (t, $J = 7.1$ Hz, 3H), 1.67–1.79 (m, 1H), 2.04 (ddd, $J = 22.5$, 14.3, 6.7 Hz, 1H), 3.33 (t, $J = 7.7$ Hz, 1H), 3.54 (dd, $J = 11.0$, 6.4 Hz, 4H), 3.81 (dd, $J = 9.7$, 4.9 Hz, 4H), 4.00–4.18 (m, 2H), 6.64 (d, $J = 8.6$ Hz, 2H), 7.15 (d, $J = 8.7$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.00, 13.98, 26.54, 52.27, 55.10 (2 × C), 60.35 (3 × C), 112.18 (2 × C), 126.94, 128.51 (2 × C), 146.73, 174.66; MS (ESI$^+$) (*m*/*z*): 334.24 $[M + K]^+$, 318.30 $[M + H]^+$.

Ethyl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(6)*

To an ice-cooled solution of **5** (24.6 g, 83.29 mmol) in dry benzene (86 ml), a solution of freshly distilled phosphorus oxychloride (18 ml, 193.11 mmol) in dry benzene (10.5 ml) was added dropwise. After the completion of the addition, the mixture was refluxed for 1 h protected from moisture with a calcium chloride trap. Then, it was carefully poured into ice-water with stirring and the two phases were separated. The aqueous phase was washed with benzene (3 × 50 ml). The combined organic phases were washed with brine (1 × 50 ml), dried over sodium sulfate anhydrous, filtered, and evaporated under reduced pressure to yield **6** (22.76 g, 82%) as an oily residue, which was used without further purification; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.89 (t, $J = 7.4$ Hz, 3H), 1.22 (t, J = 7.1 Hz, 3H), 1.68–1.81 (m, 1H), 2.0–2.11(m, 1H), 3.35 (t, J = 7.7 Hz, 1H), 3.59–3.66 (m, 4H), 3.67–3.74 (m, 4H), 4.02–4.20 (m, 2H), 6.66 (d, J = 8.8 Hz, 2H), 7.20 (d, J = 8.8 Hz, 2H); MS (ESI$^+$) (*m*/*z*): 393.38 $[M + Na + K]^+$, 332.28 $[M + H]^+$.

2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoic acid *(2-PHE-BU)*

A solution of **6** (22.76 g, 68.5 mmol) in hydrochloric acid 37% (50 ml) was refluxed for 5 h. Ice water was added and the mixture was extracted with dichloromethane (4 × 50 ml). The combined organic phases were washed with saturated aqueous sodium carbonate (1 × 50 ml) and brine (1 × 50 ml), dried over sodium sulfate anhydrous, filtered, and evaporated under reduced pressure to give 2-PHE-BU (17.71 g, 85%) as an oil; IR (KBr): ν_{max} 1703, 1612, 1518, 815, 738 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.91 (t, $J = 7.4$ Hz, 3H), 1.70–1.83 (m, 1H), 2.00–2.13 (m, 1H), 3.36 (t, $J = 7.7$ Hz, 1H), 3.62 (t, $J = 7.3$ Hz, 4H), 3.71 (t, $J = 6.6$ Hz, 4H), 6.64 (d, $J = 8.8$ Hz, 2H), 7.20 (d, $J = 8.8$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.09, 26.10, 40.38 (2 × C), 52.18, 53.48 (2 × C), 111.98 (2 × C), 127.33, 129.32 (2 × C), 145.34, 180.53; MS (ESI$^+$) (*m*/*z*): 304.44 $[M + H]^+$, 268.41 $[M - Cl]^+$.

General procedure for the synthesis of steroidal esters of 2-PHE-BU *(7a–k)*

A solution of 2-PHE-BU (3 mmol) in dry toluene or dry benzene (15 ml) was treated with 2,4,6-trichlobenzoyl chloride (3.6 mmol) and triethylamine (3.6 mmol) and refluxed for 1.5 h under argon. In the above mixture, a solution of the appropriate steroidal alcohol (2.5 mmol) in dry toluene or dry benzene (15 ml) and 4-dimethylaminopyridine (2.5 mmol) was added. The reaction mixture was refluxed additionally for the indicated time under argon. The solvent was removed under reduced pressure and the residual oil was dissolved in dichloromethane and washed successively with 5% aqueous HCl (1 × 50 ml), water (1 × 50 ml), 5% aqueous $NaHCO_3$ (1 × 50 ml), and water (1 × 50 ml). The organic phase was dried over sodium sulfate anhydrous, filtered, and evaporated under reduced pressure. The crude residue was purified by silica gel flash column chromatography using mixtures of MeOH in dichloromethane to yield the desired steroidal ester.

Cholest-5-en-7-one-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7a)*

The title compound was obtained using toluene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (1–2% MeOH in dichloromethane) to yield **7a** (370 mg, 62%), white solid; mp 61°C; IR (KBr): ν_{max} 2951, 1730, 1672, 1265, 814, 738 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.68 (s, 3H), 0.86 (d, J = 1.9 Hz, 3H), 0.87 (d, J = 1.8 Hz, 3H), 0.90–0.93 (m, 6H), 1.19 (s, 3H), 3.33 (t, J = 7.7 Hz, 1H), 3.61–3.65 (m, 4H), 3.70–3.73 (m, 4H), 4.64–4.77 (m, 1H), 5.68 (d, J = 15.3, 1H), 6.64 (d, J = 8.7 Hz, 2H), 7.18 (d, J = 8.8 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 11.94, 12.16, 17.26, 18.85, 21.15, 22.52, 22.77, 23.81, 26.28, 26.81, 27.39, 27.96, 28.50, 35.68, 36.16, 38.29, 38.66, 39.45, 40.38 (2 × C), 43.10, 45.39, 49.78, 49.96, 52.51, 53.58 (2 × C), 54.78, 72.25, 112.12 (2 × C), 125.11, 126.56, 128.18, 129.09 (2 × C), 145.09, 163.78, 173.59, 201.84; MS (ESI$^+$) (*m*/*z*): 710.35 $[M + K]^+$.

Androst-5-en-7,17-dione-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7b)*

The title compound was obtained using toluene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (0–0.5% MeOH in dichloromethane) to yield **7b** (520 mg, 80%); oil; IR (KBr): ν_{max} 2961, 1736, 1670, 1614, 1261, 815, 736 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.87 (s, 3H), 0.88–0.93 (m, 3H), 1.21 (s, 3H), 2.74–2.84 (m, 1H), 3.32 (t, J = 7.7 Hz, 1H), 3.59–3.63 (m, 4H), 3.68–3.71 (m, 4H), 4.64–4.77 (m, 1H), 5.72 (d, J = 15.4 Hz, 1H), 6.62 (d, J = 8.7 Hz, 2H), 7.17 (d, J = 8.7 Hz, 2H); ^{13}C-NMR (100 MHz, CD_3OD): δ 12.14, 13.67, 17.32, 20.45, 24.08, 26.75, 27.24, 30.58, 35.55, 35.86, 37.59, 37.75, 38.36, 40.31 (2 × C), 44.24, 45.61, 47.76, 49.81, 52.38, 53.48 (2 × C), 71.94, 112.03 (2 × C), 126.39, 128.12, 129.03 (2 × C), 144.96, 164.76, 173.55, 200.63; MS (ESI$^+$) (*m*/*z*): 588.42 $[M]^+$, 552.39 $[M - Cl]^+$.

17β-Acetamide-androst-5-en-7-one-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7c)*

The title compound was obtained using toluene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (0–2% MeOH in dichloromethane) to yield **7c** (560 mg, 70%), white solid; mp 106°C; IR (KBr): ν_{max} 3306, 2949, 1728, 1668, 1614, 1292, 814, 736 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.67 (s, 3H), 0.88 (t, J = 7.4 Hz, 3H), 1.18 (s, 3H), 1.96 (s, 3H), 3.31 (t, J = 7.7 Hz, 1H), 3.59–3.63 (m, 4H), 3.74–3.65 (m, 4H), 3.88 (q, J = 9.1 Hz, 1H), 4.74–4.63 (m, 1H), 5.48 (d, J = 9.0 Hz, 1H), 5.67 (d, J = 12.9 Hz, 1H), 6.61 (d, J = 8.8 Hz, 2H), 7.16 (d, J = 8.8 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 11.98, 12.16, 17.30, 20.74, 23.55, 25.78, 26.78, 27.29, 28.66, 35.85, 37.56, 37.77, 38.31, 40.41 (2 × C), 43.55, 45.32, 46.48, 49.72, 52.45, 53.46 (2 × C), 57.92, 72.10, 111.92 (2 × C), 126.43, 128.02, 129.05 (2 × C), 145.13, 164.49, 169.94, 173.58, 201.15; MS (ESI$^+$) (*m*/*z*): 653.16 $[M + Na]^+$, 631.18 $[M + H]^+$, 595.09 $[M - Cl]^+$.

17α-Aza-D-homo-androst-5-en-7,17-dione-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7d)*

The title compound was obtained using toluene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (0–2% MeOH in dichloromethane) to yield **7d** (448 mg, 56%), yellow oil; IR (KBr): ν_{max} 3435, 2962, 1728, 1683, 1653, 1273, 817, 734; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.88 (t, J = 7.4 Hz, 3H), 1.17 (s, 3H), 1.20 (s, 3H), 2.67–2.77 (m, 1H), 3.32 (t, J = 7.7 Hz, 1H), 3.60–3.63 (m, 4H), 3.69–3.72 (m, 4H), 4.63–4.75 (m, 1H), 5.71 (d, J = 15.5 Hz, 1H), 6.62 (d, J = 8.5 Hz, 2H), 7.05 (br s, 1H), 7.17 (d, J = 8.7 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.15, 16.73, 20.55, 21.36, 22.58, 26.78, 27.16, 30.58, 35.69, 37.28, 38.23, 38.89, 40.40 (2 × C), 41.29, 44.55, 48.95, 52.40, 53.43 (2 × C), 53.79, 72.05, 111.89 (2 × C), 126.30, 127.91, 129.03 (2 × C), 145.14, 163.14, 172.89, 173.58, 200.15; MS (ESI$^+$) (*m*/*z*): 641.26 $[M + K]^+$.

7α-Aza-B-homo-cholest-5-en-7-one-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7e)*

The title compound was obtained using benzene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (0–2% MeOH in dichloromethane) to yield **7e** (378 mg, 63%), orange foam; mp 85°C; IR (KBr): ν_{max} 3447, 2955, 1730, 1660, 1267, 814, 738 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.68 (s, 3H), 0.86 (d, J = 2.1 Hz, 3H), 0.87 (d, J = 2.0 Hz, 3H), 0.89–0.91 (m, 6H), 1.23 (s, 3H), 3.25–3.38 (m, 2H), 3.61–3.64 (m, 4H), 3.70–3.73 (m, 4H), 4.64–4.77 (m, 1H), 5.69 (s, 1H), 5.79 (d, J = 18.3 Hz, 1H), 6.63 (d, J = 8.0 Hz, 2H), 7.17 (d, J = 8.7 Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 11.40, 12.17, 18.54, 19.35, 22.52, 22.78, 23.23, 23.78, 25.05, 26.80, 27.78, 27.97, 35.21, 35.52, 35.93, 38.58, 39.41, 40.47 (2 × C), 41.40, 41.57, 41.87, 43.66, 48.95, 51.78, 52.51, 53.53 (2 × C), 55.45, 55.72, 72.40, 111.98 (2 × C), 122.44, 128.19, 129.10 (2 × C), 145.14, 156.39, 167.30, 173.56; MS (ESI$^+$) (*m*/*z*): 739.38 $[M + K]^+$, 723.39 $[M + Na]^+$, 701.34 $[M + H]^+$.

7α-Aza-B-homo-androst-5-en-7,17-dione-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7f)*

The title compound was obtained using benzene as a solvent. After the addition of the steroidal alcohol, the

reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (0–4% MeOH in dichloromethane) to yield **7f** (637 mg, 80%), yellow oil; IR (KBr): ν_{max} 3454, 2962, 1736, 1660, 1614, 1267, 819, 736; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.86–0.90 (m, 6H), 1.25 (s, 3H), 3.31 (t, $J = 7.7$ Hz, 1H), 3.60–3.64(m, 4H), 3.69–3.72 (m, 4H), 4.66–4.76 (m, 1H), 5.81 (d, $J = 18.8$ Hz, 1H), 6.13 (br s, 1H), 6.62 (d, $J = 7.4$ Hz, 2H), 7.16 (d, $J = 8.8$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 11.97, 12.95, 19.06, 22.28, 22.40, 26.43, 26.55, 26.66, 30.24, 35.04, 35.46, 40.30 (2 × C), 43.90, 46.57, 49.46, 49.78, 50.76, 52.20, 53.20 (2 × C), 72.18, 111.70 (2 × C), 122.31, 127.68, 127.76, 128.81 (2 × C), 144.91, 167.72, 173.27, 218.32; MS (ESI$^+$) (*m/z*): 641.20 [M + K]$^+$, 625.20 [M + Na]$^+$, 603.28 [M + H]$^+$.

7α-Aza-B-homo-17β-acetamide-androst-5-en-7-one-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7g)*

The title compound was obtained using benzene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (2–7% MeOH in dichloromethane) to yield **7g** (460 mg, 65%), yellow solid; mp 185°C; IR (KBr): ν_{max} 3273, 2962, 1728, 1660, 1612, 1273, 814, 740 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.69 (s, 3H), 0.88 (t, $J = 7.4$ Hz, 3H), 1.24 (s, 3H), 1.98 (s, 3H), 3.31 (t, $J = 7.6$ Hz, 1H), 3.60–3.64 (m, 4H), 3.69–3.72 (m, 4H), 4.66–4.77 (m, 1H), 5.32 (d, $J = 8.9$ Hz, 1H), 5.80 (d, $J = 19.4$ Hz, 1H), 6.15 (br s, 1H), 6.62 (d, $J = 8.8$ Hz, 2H), 7.16 (d, $J = 8.7$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 11.51, 12.24, 19.44, 22.99, 23.49, 24.25, 26.84, 28.06, 35.27, 35.83, 40.52 (2 × C), 41.52, 42.43, 44.00, 49.15, 51.56, 52.08, 52.51, 53.53 (2 × C), 58.33, 72.34, 111.99 (2 × C), 121.83, 127.94, 128.07, 129.12 (2 × C), 145.18, 167.76, 170.16, 171.00, 173.62; MS (ESI$^+$) (*m/z*): 684.28 [M + K]$^+$, 668.28 [M + Na]$^+$, 646.17 [M + H]$^+$.

7α,17α-Diaza-B,D-dihomoandrost-5-en-7,17-dione-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7h)*

The title compound was obtained using benzene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 2 h. The crude residue was purified by silica gel flash column chromatography (2–7% MeOH in dichloromethane) to yield **7h** (452 mg, 61%), gray oil; IR (KBr): ν_{max} 3470, 2962, 1730, 1653, 1625, 1269, 844, 738 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.88 (t, $J = 8.2$ Hz, 3H), 1.15 (s, 3H), 1.23 (s, 3H), 3.31 (t, $J = 7.7$ Hz, 1H), 3.60–3.64 (m, 4H), 3.69–3.72 (m, 4H), 4.64–4.72 (m, 1H), 5.81 (d, $J = 18.8$ Hz, 1H), 6.59 (br s, 1H), 6.63 (d, $J = 8.8$ Hz, 2H), 7.16 (d, $J = 8.7$ Hz, 2H), 7.38 (br s, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.17, 19.12, 21.42, 21.84, 21.88, 26.82, 30.80, 35.26, 38.06, 40.43 (2 × C), 41.58, 44.45, 46.78, 49.08, 51.10, 52.48, 53.04, 53.56 (2 × C), 72.64, 112.09 (2 × C), 121.70, 121.75, 127.96, 129.08 (2 × C), 145.12, 168.43, 168.74, 171.94, 173.47; MS (ESI$^+$) (*m/z*): 640.25 [M + Na]$^+$, 618.33 [M + H]$^+$.

Androst-5-en-17-one-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7i)*

The title compound was obtained using toluene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (dichloromethane) to yield **7i** (470 mg, 78%), yellow foam; mp 59°C; IR (KBr): ν_{max} 2962, 1734, 1612, 1267, 814, 736; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.88–0.92 (m, 6H), 1.03 (s, 3H), 2.45 (dd, $J = 19.2$, 8.8 Hz, 1H), 3.32 (t, $J = 7.7$ Hz, 1H), 3.61–3.65 (m, 4H), 3.69–3.72 (m, 4H), 4.56–4.66 (m, 1H), 5.38 (dd, $J = 14.8$, 4.8 Hz, 1H), 6.63 (d, $J = 7.9$ Hz, 2H), 7.19 (d, $J = 8.4$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.18, 13.52, 19.34, 20.30, 21.85, 26.89, 27.74, 30.75, 31.40, 31.45, 35.81, 36.72, 37.85, 38.09, 40.41 (2 × C), 47.48, 50.12, 51.69, 52.64, 53.58 (2 × C), 73.76, 112.06 (2 × C), 121.79, 128.60, 129.12 (2 × C), 139.91, 145.01, 173.76, 220.89; MS (ESI$^+$) (*m/z*): 612.35 [M + K]$^+$, 596.29 [M + Na]$^+$, 574.37 [M + H], 538.35 [M − Cl]$^+$.

17β-Acetamide-androst-5-en-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7j)*

The title compound was obtained using toluene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (0–1% MeOH in dichloromethane) to yield **7j** (438 mg, 73%), brown oil; IR (KBr): ν_{max} 3445, 2962, 1726, 1649, 1267, 814, 736 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.69 (s, 3H), 0.89 (t, $J = 7.52$ Hz, 3H), 1.01 (s, 3H), 1.98 (s, 3H), 3.32 (t, $J = 7.73$ Hz, 1H), 3.60–3.65 (m, 4H), 3.69–3.72 (m, 4H), 4.55–4.63(m, 1H), 5.26 (d, $J = 9.01$ Hz, 1H), 5.35 (dd, $J = 14.91$, 4.67 Hz, 1H), 6.64 (d, $J = 8.62$ Hz, 2H), 7.19 (d, $J = 8.81$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 11.94, 12.19, 19.35, 20.54, 23.52, 23.63, 26.91, 27.55, 27.76, 28.68, 31.50, 32.03, 36.65, 36.74, 37.87, 38.11, 40.35 (2 × C), 42.66, 49.97, 52.66, 52.76, 53.68 (2 × C), 58.90, 73.91, 112.25 (2 × C), 122.18, 128.19, 129.16 (2 × C), 139.75, 169.97, 173.72; MS (ESI$^+$) (*m/z*): 617.45 [M + H], 605.42 [M + Na + H − Cl]$^+$.

17α-Aza-D-homo-androst-5-en-17-one-3β-yl 2-[4-*N*,*N*-bis(2-chloroethyl)amino-phenyl]butanoate *(7k)*

The title compound was obtained using benzene as a solvent. After the addition of the steroidal alcohol, the reaction mixture was refluxed for additional 1.5 h. The crude residue was purified by silica gel flash column chromatography (0–2% MeOH in dichloromethane) to yield **7k** (414 mg, 69%), yellow foam; mp 111°C (dec.); IR (KBr): ν_{max} 3200, 2964, 1726, 1653, 1263, 814, 736 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.87

(t, $J = 7.5$ Hz, 3H), 0.97 (s, 3H), 1.13 (s, 3H), 3.30 (t, $J = 7.7$ Hz, 1H), 3.58–3.61 (m, 4H), 3.66–3.70 (m, 4H), 4.53–4.61 (m, 1H), 5.33 (d, $J = 16.8$ Hz, 1H), 6.60 (d, $J = 8.6$ Hz, 2H), 6.86 (br s, 1H), 7.17 (d, $J = 8.6$ Hz, 2H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.00, 18.98, 19.70, 20.61, 21.63, 26.67, 27.43, 30.42, 30.96, 31.98, 36.54, 37.37, 37.61, 39.06, 40.27 (2 × C), 47.58, 49.10, 52.37, 53.27 (2 × C), 53.90, 73.43, 111.75 (2 × C), 121.46, 128.18, 128.87 (2 × C), 139.34, 144.82, 171.93, 173.51; MS (ESI$^+$) (*m/z*): 627.28 $[M + K]^+$, 589.36 $[M + Na]^+$.

In-vivo experiments

Compounds

For an intraperitoneal 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. This concentration by itself produced no observable toxic effects.

Mice: BALB/c, DBA/2, and BDF1 mice of both sexes, weighing 20–23 g, 6–8 weeks old, were used for toxicity studies and antitumor evaluation. Mice obtained from the experimental section of the Research Center of Theagenion Anticancer Hospital (Thessaloniki, Greece) were kept under conditions of constant temperature and humidity, in sterile cages, with water and food *ad libitum*. The animals used in this study were cared for in accordance with the Declaration of Helsinki and the guidelines of the local ethics committee.

Tumors

Leukemia P388-bearing BDF1 (DBA/2 × C57BL) mice were used to evaluate the cytostatic effect. Lymphocytic P388 leukemia was maintained in an ascitic form by an injection of 10^6 cells at 7-day intervals into the peritoneal cavity of DBA/2 mice.

Estimation of acute toxicity

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

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%, which is the increase median life span of the drug-treated animals (T) excluding long-term survivors versus corn-oil-treated controls (C), expressed as a percentage. The other index of antileukemic activity used was the number of long-term survivors defined as mice alive for 90 days after tumor inoculation. Each drug-treated group included six mice, whereas the tumor control group included eight mice; in each group, equal numbers of male and female mice were used. Experiments were initiated by implanting mice with tumor cells according to the protocol of the National Cancer Institute (USA) [31]. Treatments were given as a single dose (LD_{10}, day 1) and as an intermittent dose ($LD_{10}/2 \times 3$, days 1, 3 and 6). The experiments were terminated on day 90. Statistical evaluation of the experimental data was carried out using the Wilcoxon test.

Results and discussion

Chemistry

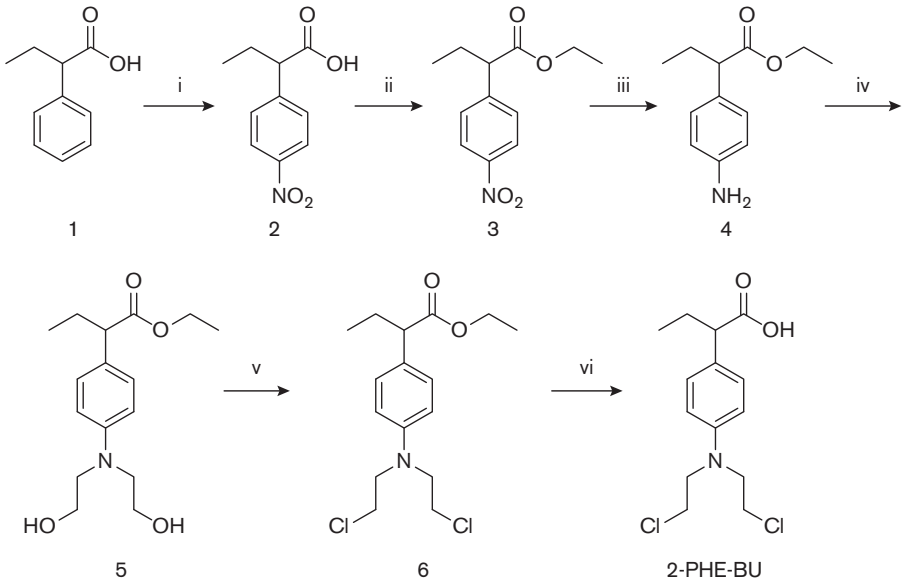
2-PHE-BU was synthesized using the synthetic route, which is shown in Scheme 1. Commercially available (±)-2-phenylbutyric acid **1** was nitrated by treatment with a mixture of concentrated nitric and sulfuric acid. The desired *p*-nitro-derivative **2** was obtained in more than a 70% yield, as estimated by ^{1}H-NMR analysis of the crude mixture. The latter was subsequently transformed into the ethyl ester **3** [32], which was immediately hydrogenated over a Pd catalyst to the corresponding 4-aminophenyl derivative **4** [32]. The installation of the *N*,*N*-bis-chloroethyl moiety on the aniline was carried out following a standard two-step procedure. Particularly, the crude mixture of **4** obtained upon prolonged treatment with ethylene oxide in an autoclave yielded the *N*,*N*-bis-hydroxyethyl precursor **5** in a 68% overall yield over four steps. The latter was chlorinated by means of phosphorus oxychloride to the *N*,*N*-bis-chloroethyl intermediate **6**. Finally, acidic hydrolysis of the ethyl ester **6** provided the desired aromatic nitrogen mustard 2-PHE-BU in a very good yield.

Target steroidal esters of 2-PHE-BU were obtained using the method of mixed anhydrides [33], which has been used successfully by our group in the synthesis of related compounds. Accordingly, the treatment of 2-PHE-BU with 2,4,6-trichlobenzoyl chloride in the presence of triethylamine in refluxing toluene or benzene yielded the intermediate mixed anhydride (Scheme 2). Following a one-pot procedure, the mixed anhydride was treated subsequently with the appropriate steroidal alcohol in the presence of 4-dimethylamino pyridine, producing the target steroidal esters **7a–k** in a very good yield.

Biological evaluation

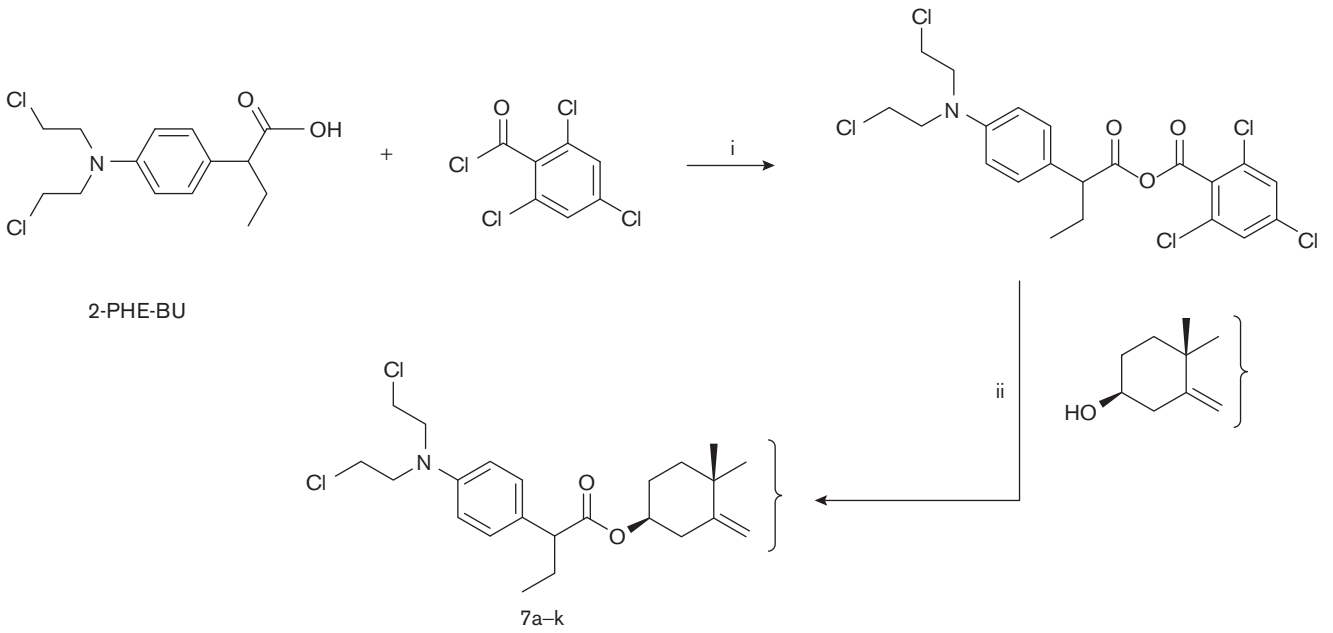
The results of the biological evaluation of the newly synthesized esters of 2-PHE-BU with various 7-keto, B-lactam, and slightly modified steroidal alcohols are presented in Table 1. The newly synthesized steroidal esters were first evaluated for their in-vivo acute toxicity. As indicated by the LD_{50} and LD_{10} values in Table 1, the target steroidal esters **7a–k** ($LD_{50} = 250$ to >500 mg/kg) induced significantly decreased toxicity effects compared with the free nitrogen mustard 2-PHE-BU ($LD_{50} = 160$ mg/kg). Among the tested compounds, derivative **7g** was

Scheme 1



Reagents and conditions: (i) HNO_3, H_2SO_4, 0°C to rt, 2 h; (ii) CH_3CH_2OH, H_2SO_4, reflux, 6 h; (iii) H_2, Pd 10% on activated C, CH_3CH_2OH, 1 h; (iv) ethylene oxide, CH_3COOH, rt, 72 h, 68% (over four steps); (v) $POCl_3$, C_6H_6, reflux, 1 h, 82%; (vi) HCl 9N, reflux, 5 h, 85%.

Scheme 2



Reagents and conditions: (i) NEt_3, dry toluene or benzene, reflux, 1.5 h; (ii) 4-DMAP, dry toluene or benzene, reflux, 1.5–2 h, 56–80%. 4-DMAP, 4-dimethylaminopyridine

the most toxic; derivatives **7a**, **7b**, **7d**, **7h**, **7i**, **7j**, and **7k** showed moderate toxicity, whereas derivatives **7c**, **7e**, and **7f** showed low toxicity values. These results are in agreement with those of previous studies [19,20,33–35], indicating that the conjugation of aromatic nitrogen mustards either on simple or modified steroidal skeletons results in the limitation of their undesired or toxic effects, possibly because of the enhancement of their lipophilicity and effective transport through the cell membranes to the nucleus, close to DNA-specific sites.

Table 1 Toxicity and antitumor activity of 2-PHE-BU and steroidal esters 7a–k on P388-leukemia-bearing mice

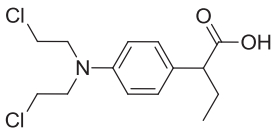
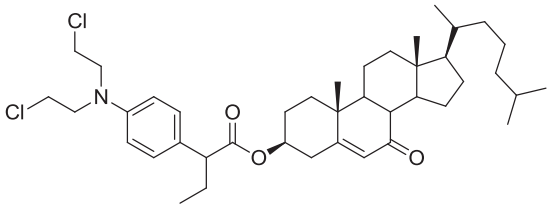
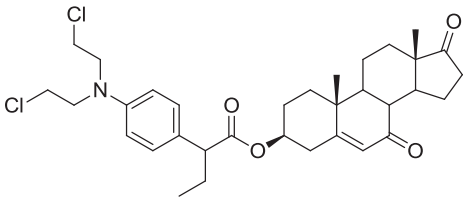
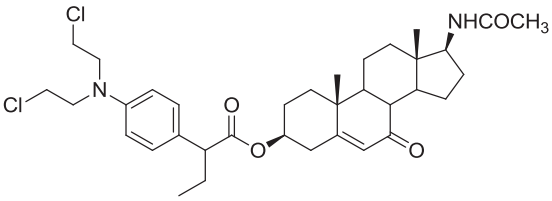
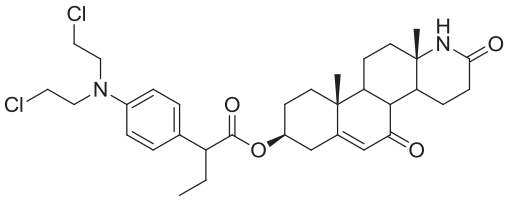
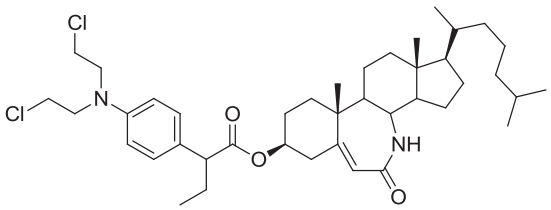
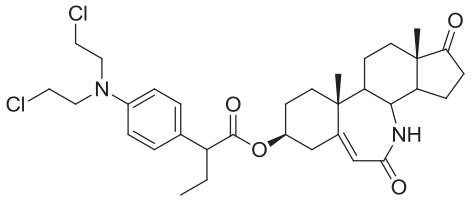
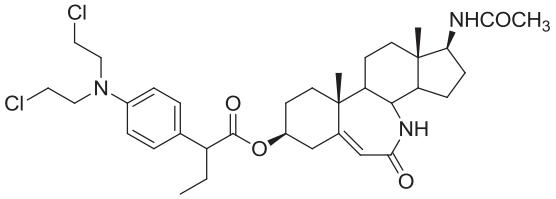
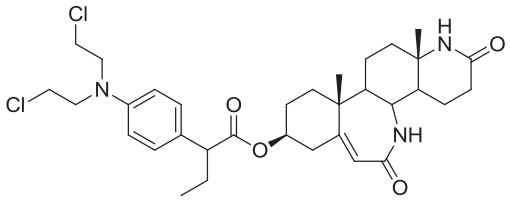
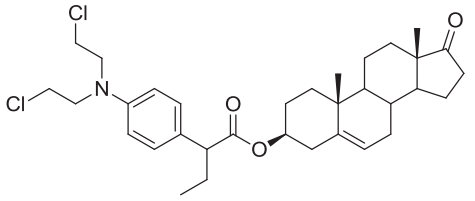
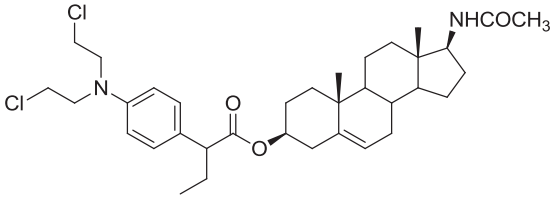
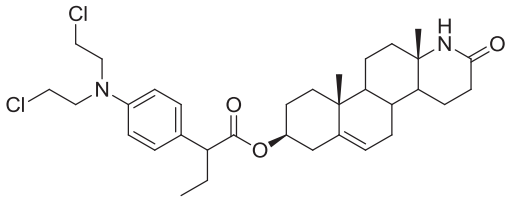
Compounds	LD_{50}[a] (mg/kg)	LD_{10} (mg/kg)	Treatment schedule (days)	Dosage (mg/kg/day)	T/C[b] (%)
Control	–	–	–	Corn oil	100
 **2-PHE-BU**	160	50	1 1, 3, 6	50 25	120 128
 **7a**	390	240	1 1, 3, 6	240 120	118 125
 **7b**	420	240	1 1, 3, 6	240 120	135 144
 **7c**	450	450	1 1, 3, 6	450 225	114 144
 **7d**	410	320	1 1, 3, 6	320 160	114 140
 **7e**	460	270	1 1, 3, 6	270 135	115 122

Table 1 (*continued*)

Compounds	LD_{50}[a] (mg/kg)	LD_{10} (mg/kg)	Treatment schedule (days)	Dosage (mg/kg/day)	T/C[b] (%)
 **7f**	>500	280	1 1, 3, 6	280 140	128 118
 **7g**	250	175	1 1, 3, 6	175 87.5	100 105
 **7h**	310	220	1 1, 3, 6	220 110	128 147
 **7i**	410	250	1 1, 3, 6	250 125	110 118
 **7j**	320	210	1 1, 3, 6	210 105	125 130
 **7k**	310	180	1 1, 3, 6	180 90	135 138

[a]LD_{50} values were estimated graphically, where the percentage of deaths because of the toxicity of each dose is shown in the ordinate, whereas the doses administered are indicated on the abscissae on semilogarithmic paper. For chemotherapy testing, the highest dose used for a single treatment was LD_{10}. Therefore, the drugs in the following experiments were compared at equitoxic doses.

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

Next, the in-vivo antileukemic activity of the newly synthesized compounds was assessed using the oncostatic parameter T/C%. P388 lymphocytic leukemia-bearing mice were treated with the tested compounds following two different schedule treatments as presented in Table 1. However, the intermittent administration of the compounds at doses equal to $LD_{10}/2$ on days 1, 3, and 6 after the implantation of the tumor cells was found to be superior in all cases.

The free nitrogen mustard 2-PHE-BU showed marginally accepted antileukemic activity with a T/C equal to 128%, slightly above the value of 125%, which is considered as the limit for a potential chemotherapeutic agent according to the National Cancer Institute criteria. However, the majority of the steroidal esters showed improved antileukemic activity compared with 2-PHE-BU, as indicated by the corresponding T/C% values, confirming that this type of chemical linkage between an aromatic nitrogen mustard and steroids not only reduces the toxic effects but may also lead to compounds with improved bioactivity.

Furthermore, the B,D-bilactam derivative **7h** was found to be the most potent among the tested compounds. As we have reported elsewhere, the modification of the B ring into a seven-membered lactam ring induces conformational changes in the steroidal part, which enhances the genotoxic and cytotoxic activity [36] as well as the in-vivo antileukemic potency [15] of the target steroidal esters. Especially, B,D-bilactam derivatives have shown diverse in-vivo toxicity and moderate to excellent antileukemic activity depending on the structural features of the conjugated nitrogen mustard [16,20,37]. These results possibly indicate an alternative mechanism of action for the B-lactamic steroidal esters that does not involve indispensably the interaction of the endocyclic –NHCO– group in the B-ring with cellular sites crucial for tumor growth, but with other specific binding sites that are able to induce antileukemic responses. Nevertheless, as can be observed from the T/C% values of derivatives **7e**, **7f**, and **7g**, the presence of a B-seven-membered lactam ring is not adequate to improve the antileukemic activity of 2-PHE-BU as they appeared less active than compound **7h**. Obviously, the conjugation of an aromatic nitrogen mustard with a properly modified steroidal skeleton that evidently contributes to the enhancement of the antileukemic potency may lead to steroidal esters with improved antileukemic potency.

However, steroidal esters **7b** and **7c** with an allylic 7-keto group showed an equipotent, but slightly decreased antileukemic activity compared with compound **7h**. Particularly, comparing the T/C% values for the following pairs of steroidal esters **7b–7i**, **7c–7j**, and **7d–7k**, it is obvious that the introduction of a keto group at position 7 of the steroidal skeleton clearly enhances the antileukemic activity. This observation confirms our previous studies [19,33], which point to the fundamental role of the 7-keto group in the enhancement of the antineoplastic activity of these compounds, possibly through the interaction of the conjugated steroidal enone system with specific sites of the DNA that induce effective genotoxic and cytotoxic effects.

As mentioned previously, the number and structural diversity of steroidal skeletons that have been tethered to 2-PHE-BU were used to evaluate the predictive reliability of the Leapfrog routine. Some of them were simple steroids, whereas others had privileged structural features that have been shown to be essential for the antineoplastic activity of many alkylating steroidal esters, such as a transformed D-six-membered lactam ring, an axial 17-acetamido group, a conjugated 7-keto moiety, or an expanded B-seven-membered lactam ring. Nevertheless, the steroidal esters of 2-PHE-BU present low to moderate activity, as determined by the corresponding T/C% values, indicating a reduced antineoplastic potency of the nitrogen mustard. This is also confirmed by the quite low potency of the free nitrogen mustard (T/C 128%) compared with CHL (T/C 155%) [37] or PHE (T/C 113%) [16] as has already been reported in comparable studies. Furthermore, 2-PHE-BU ($LD_{50} = 160$ mg/kg) was found to be approximately one order of magnitude less toxic than CHL ($LD_{50} = 24$ mg/kg) [37] or other aromatic nitrogen mustards such as PHE ($LD_{50} = 20$ mg/kg) [17] and 4-Me-CABA ($LD_{50} = 18$ mg/kg) [38], which have been used in our earlier works. These findings suggest that although tethering of low potency alkylating agents on steroid skeletons leads to steroidal esters with improved antineoplastic activity, the unique structural features of the nitrogen mustard determine crucially the efficacy of the target steroidal esters. As we have reported earlier, the type of chemical linkage between the steroidal part and the nitrogen mustard is crucial for the activity of the target compounds. An easily hydrolyzed chemical bond between the alkylating agent and the steroidal skeleton is essential for the bioactivity of the target steroidal esters, as noneasily hydrolyzed conjugates such as amide conjugates have been proved to be nonpotent derivatives [18].

Furthermore, according to recent conformational analysis studies [21], the structure of the side chain between the carboxylic group and the aromatic ring of the nitrogen mustard affects the orientation of the alkylating agent toward the B-ring of steroidal skeleton, which is considered an important stereochemical feature for the antineoplastic activity. Possibly, this part of the molecule plays a pivotal role in the bioactivity, as it might influence the effective enzymatic hydrolysis of steroidal ester and the release of nitrogen mustard *in vivo*. In this context, comparing the more active steroidal esters of 2-PHE-BU with the corresponding esters of PHE (**SE-1 – SE-6**) (Table 2), it is obvious that the presence of the ethyl group around the ester bond is detrimental for the bioactivity, although this chemical modification was

Table 2 Comparison of toxicity and antitumor activity of 2-PHE-BU, PHE, and their steroidal esters on P388-leukemia-bearing mice

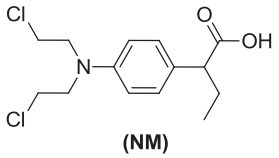
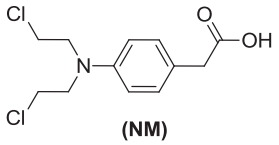
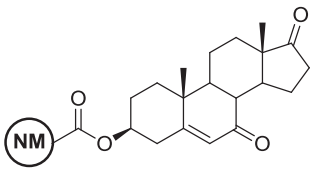
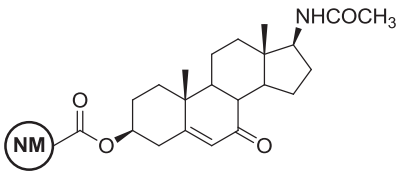
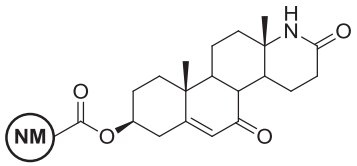
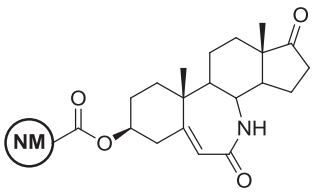
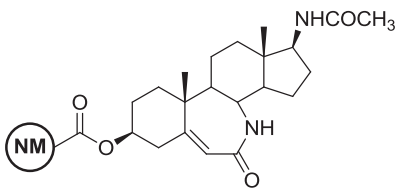
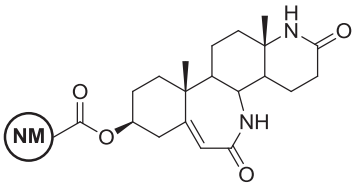
Structure	Compounds	LD_{50}[a] (mg/kg)	T/C[b] (%)	Cures
 (NM)	**2-PHE-BU**	160	128[c]	0/6
 (NM)	**PHE**	20	113[d]	0/6
	**7b** **SE-1**	420 65	144[c] 321[d]	0/6 0/6
	**7c** **SE-2**	450 75	144[c] 314[d]	0/6 0/6
	**7d** **SE-3**	410 90	140[c] 333[d]	0/6 3/6
	**7f** **SE-4**	>500 64	118[c] 133[d]	0/6 0/6
	**7g** **SE-5**	250 80	105[c] 221[e]	0/6 1/6

Table 2 (*continued*)

Structure	Compounds	LD_{50}[a] (mg/kg)	T/C[b] (%)	Cures
	**7h**	310	147[c]	0/6
	SE-6	90	153[d]	0/6

2-PHE-BU, 2-[4-*N*,*N*-bis(2-chloroethyl) amino-phenyl]butanoic acid; PHE, 4-*N*,*N*-bis(2-chloroethyl)amino-phenylacetic acid.
[a]LD_{50} values were estimated graphically, where the percentage of deaths because of the toxicity of each dose is shown in the ordinate, whereas the doses administered 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.
[b]T/C = the percent increase median life span of the drug-treated animals (T) versus corn-oil-treated animals (C).
[c]Treatment was given as an intermittent dose equal to $LD_{10}/2$ on days 1, 3, and 6 for 2-PHE-BU and its steroidal esters.
[d]Treatment was given as an intermittent dose equal to $LD_{10}/2$ on days 1, 5, and 9 for PHE and its steroidal esters.
[e]Treatment was given as an intermittent dose equal to $LD_{10}/2$ on days 1, 4, and 7 for PHE and its steroidal esters.

pioneered by the LeapFrog prediction. This provides evidence that the steric hindrance around the ester bond might prevent the enzymatic hydrolysis and the release of the active nitrogen mustard *in vivo*. It is also in accordance with the low toxicity of 2-PHE-BU and can lead to the assumption that a huge percentage of the administered dose either of the steroidal ester or even of the free nitrogen mustard bypasses the metabolic activation without playing its role *in vivo*.

Notably, steroidal ester **SE-3** was used as a template compound in the LeapFrog routine with restraining of the nitrogen mustard groups [$-N(CH_2CH_2Cl)_2$], whereas stereoelectronic contour maps obtained by the CoMFA analysis were inserted as input for the generation of a hypothetical receptor cavity [21]. Operating on the OPTIMIZE mode, which suggests further improvement of the existing leads and after repeated structural changes of the template, new optimized compounds were found with lower binding energy to the receptor in comparison with compound **SE-3**. Interestingly, after initial structural modifications of **SE-3**, compound **7d** arose and ranked first among other optimized compounds. Despite this promising prediction, compound **7d** was found to be almost more than two times less potent and significantly less toxic than the parent compound **SE-3** (Table 2). These findings show that the stereoelectronic parameters that are taken into account by the in-silico design are probably not adequate to ensure reliable wide predictions, as perhaps other issues such as metabolic modifications interfere and contribute substantially towards the bioactivity of the target compounds. Furthermore, aspects related to the construction and initial validation of the CoMFA model should be reconsidered. For example, the number and the chemical diversity of the inserted molecules in both the training and the test set of compounds should be enriched, aiming at the generation of a revised model with optimum predictive reliability in the design of more potent analogues. Thus, further studies that are ongoing and considering the aforementioned results may shed more light on the design and synthesis of new steroidal esters with improved bioactivity.

Conclusion

In continuation of our studies in the field of antineoplastic agents, new steroidal esters of 2-PHE-BU were designed and synthesized on the basis of the results of our extended SAR studies and guided by in-silico predicted simulations derived from a recent 3D QSAR analysis. Particularly, 2-PHE-BU was synthesized from commercially available (±)-2-phenylbutyric acid following a straightforward approach in six steps and an overall yield of 47%. Subsequently, 2-PHE-BU was conjugated to various modified or unmodified steroidal alcohols using the well-established method of mixed anhydrides, producing the steroidal esters **7a–k** in very good yields (56–80%).

In general, the new alkylating steroidal esters showed reduced toxicity and slightly improved antileukemic activity against P388-leukemia-bearing mice compared with the 2-PHE-BU. Nevertheless, they were not superior to the already synthesized and structurally similar steroidal esters of PHE, indicating that the enzymatic hydrolysis and the release of the nitrogen mustard *in vivo* are possibly disfavored because of the steric hindrance of the ethyl group around the ester bond formed. This becomes apparent on comparing the toxicity obtained and the T/C% values for derivatives **7d** and **SE-3**. It is worth noting that **SE-3** was used initially as a template in de-novo design by the LeapFrog routine, whereas **7d** was generated during the optimization process as a candidate compound with improved bioactivity. Nevertheless, the biological results obtained in the present study clearly indicate that except for the important stereoelectronic requirements that are considered in and incorporated as

data into the LeapFrog routine, other parameters may possibly be implicated and determine the bioactivity of the target steroidal esters.

We expect that a careful revision of the implicated parameters such as the chemical diversity and the number of the used compounds will lead to the generation of new CoMFA and CoMSIA models that will subsequently contribute toward the higher predicted reliability of the in-silico design by software packages such as LeapFrog. Relevant studies are under way, and the results will be reported in due course.

Acknowledgements

I. Papaconstantinou was supported by a fellowship from the 'Cyprus State Scholarship Foundation'. The authors thank Dr D. Vachliotis and Dr G. Magoulas (Center of Instrumental Analysis, University of Patras, Greece) for recording NMR and MS spectra.

Conflicts of interest

There are no conflicts of interest.

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