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Synthesis and biological evaluation of novel daunorubicin-estrogen conjugates

Konstantinos M. Kasiotis[a], Prokopios Magiatis[b], Harris Pratsinis[b], Alexios-Leandros Skaltsounis[b], Vasiliki Abadji[c], Avgui Charalambous[c], Paraskevi Moutsatsou[b], Serkos A. Haroutounian[a,*]

[a]*Chemistry Lab., Agric. University of Athens, Iera odos 75, Athens 11855, Greece*
[b]*Dept. of Pharmacy and Medical School, University of Athens, Zografou 15771, Greece*
[c]*ELPEN SA, Pharmaceutical Company, Pikermi 19009, Greece*

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Abstract

The synthesis of two novel daunorubicin-estrogen conjugates with a steroidal and a non-steroidal ligand was undertaken in an attempt to target the cytotoxicity of anthracycline to estrogen-receptor positive cells. These conjugates (**3** and **4**), in contrast to their corresponding ligands, displayed weak binding affinities of 0.079 and 0.851 for the estrogen receptor. Conjugate **3** was consistently more cytotoxic than **4**, which however showed some selectivity to estrogen receptor positive cell lines. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Daunorubicin conjugates; Steroidal and non-steroidal estrogen ligand; Synthesis; Estrogen receptor binding; Cytotoxic activity

1. Introduction

The lack of selectivity of several antitumor agents as well as their acute toxicity toward rapidly proliferating tissues, constitute the major drawbacks in their use for the treatment of human cancer. In order to circumvent this problem, one approach is to couple these agents to carriers which have shown selectivity towards the tumors [1,2], or the tissues from which the tumors are derived [3–5]. This might enable the selective delivery of a cytotoxic agent to cells or tissues with high concentration of binding sites for the carrier. In this context, an estrogen can be used to target breast cancers, since it is well established that most of breast cancers exhibit detectable levels of estrogen receptor (ER) (estimated range = 5000 to 50 000 receptor molecules/cell) [6]. Thus, estrogens are well fitted to serve as effective carriers of DNA-directed cytotoxic agents into the nuclei of ER-rich cells, since it is widely accepted that the steroidal hormones bind to a receptor dimer-DNA complex within the nucleus, which subsequently alters the rate of transcription by an unknown mechanism [7].

Todate, several estrogen cytotoxic agents have been prepared by linking steroidal or non-steroidal moieties to a chemical function with known cytotoxic activity (nitrogen mustards, nitrosoureas and carbamates, aziridines, vinca alkaloids, dichloroplatinum, ellipticine etc) [8–13]. In order for this approach to be successful, the conjugates should exhibit antitumor activity and also possess sufficient binding affinity to the ER, which would allow their selective accumulation in ER-rich cells [11,13]. Previous findings based on the number of receptors per cell and the possible drug concentration have suggested that the relative binding affinity (RBA) value should be at least 1% comparing to estradiol (100%) [14].

Daunorubicin **1**, belongs to the anthracycline antibiotics family and is very commonly used as a broad-spectrum antitumor agent for the treatment of advanced cancers, either as a single agent or in combination chemotherapy [15]. Despite its cardiotoxicity (associated with its ability to form free radicals) [16], daunorubicin is one of the preferred treatments for progressive metastatic breast cancers [17]. Thus, we were interested to link this cytotoxic compound to a potent estrogen like estradiol. Such conjugate should exhibit antitumor activity and can potentially bind to ER. The initial estrogen-anthracycline (daunorubicine and doxorubicine) conjugates **2**, which have been reported and patented

* Corresponding author. Tel: +30-1-5294247; fax: +30-1-5294265.
E-mail address: sehar@aua.gr (S.A. Haroutounian).

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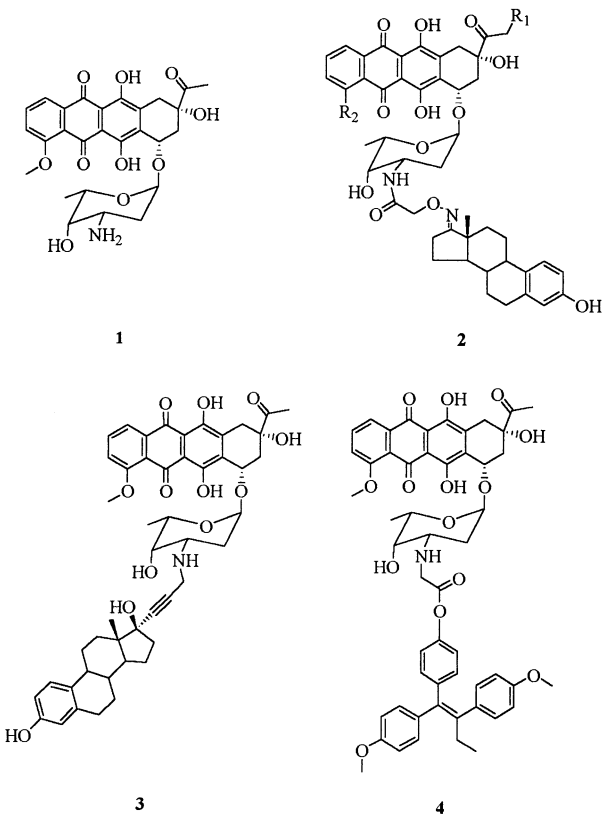


Fig. 1. Daunorubicine **1** and daunorubicin-estrogen conjugates **2–4**.

earlier by Hartman et al. [18,19], have been substituted at carbon 17 by an imine functionality which is attached to the anthracycline moiety. As a consequence, they show negligible binding affinity for the ER and no selectivity in the treatment of receptor positive tumors. Since it is established by structure-activity relationship studies, that the presence of the 17β-OH group is essential for high binding affinity of a steroidal estrogen to the ER [20], we have synthesized a novel daunorubicine-estradiol conjugate **3** with a long 17α-linker chain, which would potentially locate the cytotoxic molecule away from the critical for binding to ER 17β-OH group. Thus, we hoped that the conjugate might retain some affinity to the ER. In addition, we have also prepared a similar non-steroidal conjugate of daunorubicin (**4**). The present report discusses the synthesis of conjugates **3** and **4**, their binding affinity to ER and their in vitro cytotoxicity against a variety of human cell lines.

2. Experimental

2.1. General remarks

All anhydrous reactions were carried out under argon atmosphere. Solvents were dried by distillation prior to use. Solvent mixtures employed in chromatography were reported as volume to volume ratios. Starting materials were purchased from Aldrich (analytical reagent grades) and used without further purification. Analytical thin-layer chromatography (TLC) was conducted on Merck glass plates coated with silica gel 60 F_{254} and spots were visualized with UV light or/and an alcohol solution of anisaldehyde. Flash column chromatography was performed using Merck silica gel 60 (230–400 mesh ASTM). When analyses are indicated by symbols of the elements, analytical results obtained for those elements were $\pm$0.4% of their theoretical values.

2.2. Apparatus

Melting points were determined on a Büchi melting point apparatus and are uncorrected. ^{1}H and 2D NMR spectra were recorded at 400 MHZ on a Bruker DRX-400 spectrometer in the indicated solvents. The coupling constants are recorded in Hertz (Hz) and the chemical shifts are reported in parts per million (δ, ppm) downfield from tetramethylsilane (TMS), which was used as an internal standard (by asterisk are indicated the overlapped peaks). Infrared spectra were obtained on a Nicolet Magna 750, series II spectrometer.

2.3. 17α-(Hydroxypropargyl)-3,17β-estradiol (**5**)

To an ice-cold solution of estrone (0.5 g, 1.84 mmol) and potassium ethoxide (2.25 g, 26.8 mmol) in anhydrous THF (10 ml) was added propargyl alcohol (0.85 ml, 14.8 mmol). The reaction was allowed to reach the room temperature and stirred for 2 h. Then the reaction mixture was poured into ice water, neutralized with acetic acid and extracted with EtOAc (2 $\times$ 20 ml). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under vacuum. Flash column chromatography (EtOAc/hexane 3:7) gave 0.42 g (70%) of the title compound as an off white solid: mp 200–202°C; IR (neat) ν_{max} 3380, 2225 cm^{-1}; ^{1}H and ^{13}C NMR data are identical to those reported in the literature [21]. Anal ($C_{21}H_{26}O_3$) C, H.

2.4. 17α-(Bromopropargyl)-3,17β-estradiol (**6**)

To an ice-cold stirred solution of **5** (0.2 g, 0.61 mmol) and triphenylphosphine (0.32 g, 1.22 mmol) in anhydrous THF (2 ml), carbon tetrabromide (0.31 g, 0.91 mmol) was added in one batch. The mixture was warmed to room temperature, stirred for 2 h and concentrated under reduced pressure. Flash chromatographic purification (EtOAc/hexane 3:7) produced 0.155 g (65%) of **6** as a white solid. Mp 68–70°C; IR (neat) ν_{max} 3350, 2218 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ, 0.97 (s, 3H, CH_3), 1.35–1.57 (m, 5H, H-8, H-11, H-15), 1.74 (m, 1H, H-14), 1.77–1.84 (m, 4H, H-6, H-12), 2.01 (dt, J = 4, 7 Hz, 1H, H-16α), 2.20 (m, 1H, H-9), 2.25–2.36 (m, 3H, H-11, H-16β), 2.78–2.81 (m, 2H, H-7), 4.02 (s, 2H, H-21), 6.55 (d, J = 2.4 Hz, 1H, H-4), 6.62 (dd, J = 2.5, 8.1 Hz, 1H, H-2), 7.14 (d, J = 8.1 Hz, 1H, H-1); ^{13}C NMR ($CDCl_3$) δ, 12.79 (C-18), 14.82 (C-21), 22.83

(C-15), 26.36 (C-11), 27.16 (C-6), 29.59 (C-7), 32.79 (C-12), 38.70 (C-16), 39.36 (C-8), 43.48 (C-9), 47.63 (C-13), 49.54 (C-14), 80.01 (C-17), 81.15 (C-20), 90.52 (C-19), 112.68 (C-2), 115.21 (C-4), 126.53 (C-1), 132.34 (C-10), 138.18 (C-5), 153.40 (C-3). Anal ($C_{21}H_{25}BrO_2$) C, H.

2.5. Daunorubicin-3′-NH-17α-(propargyl)-3,17β-estradiol (3)

To a stirred solution of daunorubicin hydrochloride (0.6 mmol) and triethylamine (0.1 ml, 0.71 mmol) in anhydrous DMF (0.5 ml) was added dropwise a solution of compound **6** (0.06 g, 0.15 mmol) in DMF (1 ml). The reaction mixture was stirred for 6 h and the solvent was evaporated under reduced pressure. Purification by flash chromatography (CH_2Cl_2/MeOH 99.5:0.5) furnished 0.005 g (10%) of the desired conjugate **3** as a red solid, while 71% of the unreacted steroid was recovered. ^{1}H NMR (MeOD) δ, *anthracycline* 2.10 (dd, J = 15, 1 Hz, 1H, H-8eq), 2.31 (dd, J = 15, 4 Hz, 1H, H-8ax), 2.33 (s, 3H, H-14), 2.78 (d, J = 18 Hz, 1H, H-10eq), 2.96 (d, J = 18 Hz, 1H, H-10ax), 3.95 (s, 3H, OCH_3), 5.03 (dd, J = 4, 1 Hz, 1H, H-7), 7.45 (d, J = 8 Hz, 1-H, H-3), 7.75 (t, J = 8 Hz, 1H, H-2), 7.78 (d, J = 8 Hz, 1H, H-1); *sugar* 1.27 (s, 3H, CH_3), 1.80* and 1.99* (2H, H-2′), 3.27 (m, 1H, H-3′), 3.69 (br s, 1H, H-4′), 4.21 (m, 1H, H-5′), 5.40 (br s, 1H, H-1′); *steroid* 0.69 (s, 3H, H-18″), 1.11* (2H, H-15″), 1.47* (2H, H-6″), 1.48* (1H, H-14″), 1.66* (2H, H-12″), 1.80* (2H, H-16″), 1.82* (1H, H-9″), 2.08* (2H, H-11″), 2.10* (1H, H-8″), 2.43* (2H, H-7″), 3.60 (s, 2H, H-21″), 6.24 (s, 1H, H-4″), 6.37 (d, J = 8 Hz, H-2″), 6.80 (d, J = 8 Hz, H-1″); ^{13}C (MeOD) δ, *anthracycline* 24.60 (C-14), 33.38 (C-10), 36.55 (C-8), 56.97 (OCH_3), 71.63 (C-7), 77.66 (C-9), 112.5 (C-6a), 112.40 (C-10a), 120.15 (C-3), 120.56 (C-1), 121.70 (C-4a), 135.55 (C-12a), 135.92 (C-11a), 136.22 (C-5a), 137.13 (C-2), 156.14 (C-11), 157.42 (C-6), 162.35 (C-4), 187.56 (C-12), 187.70 (C-5), 213.40 (C-13); *sugar* 17.32 (CH_3), 30.61 (C-2′), 52.73 (C-3′), 68.49 (C-4′), 68.64 (C-5′), 102.16 (C-1′); *steroid* 13.17 (C-18″), 23.50 (C-15″), 27.46 (C-11″), 28.33 (C-6″), 30.42 (C-7″), 33.96 (C-12″), 35.24 (C-21″), 39.73 (C-16″), 40.75 (C-8″), 44.65 (C-9″), 48.00 (C-13″), 50.54 (C-14″), 80.46 (C-17″), 82.19 (C-20″), 89.61 (C-19″), 113.61 (C-2″), 115.89 (C-4″), 127.02 (C-1″), 132.17 (C-10″), 138.49 (C-5″), 155.77 (C-3″).

2.6. (E)-1-(p-Benzyloxyphenyl)-1,2-bis-(p-methoxyphenyl)-1-butene (7)

A stirred solution of magnesium (0.1 g, 4.14 mmol) and traces of iodine in anhydrous diethylether (40 ml) was warmed to 40°C and p-benzyloxyphenylbromide (1.05 g, 4.14 mmol) was added dropwise. After 2 h of stirring at that temperature, a solution of p-methoxy-2-(p-methoxyphenyl)-butyrophenone (0.4 g, 1.38 mmol) in anhydrous diethylether (10 ml) was added via syringe. The resulting mixture was stirred at room temperature for an additional 4 h. Then the reaction was quenched with 1N HCl (10 ml) and subsequently extracted with EtOAc (2× 20 ml). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The resulting residue was refluxed for 90 min in a mixture of ethanol (8 ml) and 30% HCl (2 ml). The solvent was evaporated under vacuum and the residue was partitioned in diethylether (20 ml) and water (20 ml). Workup of the reaction mixture gave 69% of the product as a 3:1 mixture (by ^{1}H NMR) of the *E/Z* diastereomers. Flash chromatographic separation (EtOAc/hexane 1:9) and recrystallization from methylene chloride-petroleum ether resulted in the separation of the pure *E* isomer as a pale yellow solid. Mp 163–164°C; ^{1}H NMR ($CDCl_3$) δ, 1.12 (t, J = 7.1 Hz, 3H, H-4), 2.68 (q, J = 7.2 Hz, 2H, H-3), 3.80 (s, 3H, $OC\underline{H}_3$), 3.87 (s, 3H, $OC\underline{H}_3$), 5.17 (s, 2H, $C\underline{H}_2$-Bn), 6.68–7.62 (m, 17H, H_{ar}); ^{13}C NMR ($CDCl_3$) δ, 13.63 (C-4), 28.85 (C-3), 54.87 ($O\underline{C}H_3$), 69.72 ($\underline{C}H_2$-Bn), 112.65, 114.56 (C-3_{ar}, C-5_{ar}), 126.76, 131.83 (C-2_{ar}, C-6_{ar}), 134.51 (C-1_{Bn}), 135.62, 137.20 (C-1_{ar}, C-1), 140.28 (C-2), 157.10 (C-4_{ar}). Anal. ($C_{31}H_{30}O_3$) C, H.

The minor (*Z*) diastereomer has similar spectroscopic data except the peaks at 3.72 (s, 3H, $OC\underline{H}_3$), 3.81 (s, 3H, $OC\underline{H}_3$) and 5.03 (s, 2H, $C\underline{H}_2$-Bn) of ^{1}H NMR and 54.69 ($O\underline{C}H_3$), 69.50 ($\underline{C}H_2$-Bn) of ^{13}C NMR.

2.7. (E)-1-(p-Chloroacetylphenyl)-1,2-bis-(p-methoxyphenyl)-1-butene (8)

Alkene **3** (0.5g, 1.13 mmol) was dissolved in ethyl acetate (15 ml) and hydrogenated over 10% Pd/C (0.05 g) under 1 atmosphere pressure for 40 min in the absence of sunlight. The mixture was filtered over Celite, dried over $MgSO_4$, and evaporated furnishing (*E*)-1-(p-hydroxyphenyl)-1,2-bis-(p-methoxyphenyl)-1-butene (0.37 g, 95%) as a pale yellowish oil. Subsequently, 0.15 g of this phenolic intermediate (0.43 mmol) was dissolved in ice-cold anhydrous diethylether (1 ml) and of pyridine (7 μl, 0.084 mmol) and chloroacetylchloride (44 μl, 0.51 mmol) were added. The reaction was run under stirring at that temperature for 2 h, then diluted with EtOAc (5 ml). The organic layer was separated, washed with water (10 ml) and dried over $MgSO_4$. Purification by flash chromatography (EtOAc/hexane 1:4) afforded the desired compound as a yellowish solid (0.165 g, 90%). Mp 151–152°C; ^{1}H NMR ($CDCl_3$) δ, 0.89 (t, J = 7.1 Hz, 3H, H-4), 2.44 (q, J = 7.2 Hz, 2H, H-3), 3.71 (s, 3H, $OC\underline{H}_3$), 3.81 (s, 3H, $OC\underline{H}_3$), 4.31 (s, 2H, $C\underline{H}_2$-Cl), 6.53–7.38 (m, 12H, H_{ar}); ^{13}C NMR ($CDCl_3$) δ, 13.56 (C-4), 28.92 (C-3), 40.87 ($\underline{C}H_2$-Cl), 112.79, 113.49 (C-3_{ar}, C-5_{ar}), 119.81, 120.62 (C-3_{ar}, C-5_{ar}), 130.62 (C-2_{ar}, C-6_{ar}), 131.87 (C-2_{ar}, C-6_{ar}), 134.18 (C-1_{ar}), 135.73 (C-$1'_{ar}$), 136.68 (C-$1''_{ar}$), 141.39 (C-1), 142.16 (C-2), 148.77 (C-4_{ar}), 158.29 (C-$4'_{ar}$, C-$4''_{ar}$), 165.75 ($\underline{C}OCH_2Cl$). Anal. ($C_{26}H_{25}O_4Cl$) C, H.

2.8. Daunorubicin-3′-NH-(E)-1-(p-acetylphenyl)-1,2-bis-(p-methoxyphenyl)-1-butene (4)

To an ice-cold stirred solution of daunorubicinhydrochloride (0.03 g, 0.06 mmol) and triethylamine (0.3 ml, 2.15 mmol) in anhydrous DMF (0.5 ml), a solution of **8** (0.08 g, 0.18 mmol) in DMF (1.5 ml) was added dropwise. The reaction mixture was allowed to reach the room temperature and stirred for additional 5 h. The solvent was evaporated under reduced pressure and the remaining slurry was purified by flash chromatography (CH_2Cl_2/Acetone 9:1) to produce the desired conjugate **4** (0.004 g, 7%) as a red amorphous powder. Furthermore, 78% of the unreacted starting material was recovered. ^{1}H NMR ($CDCl_3$) δ, *anthracycline* 2.11 (dd, J = 15, 4 Hz, 1H, H-8ax), 2.30 (dd, J = 15, 1 Hz, 1H, H-8eq), 2.39 (s, 3H, H-14), 2.95 (d, J = 18 Hz, 1H, H-10ax), 3.24 (d, J = 18 Hz, 1H, H-10eq), 4.07 (s, 3H, OCH_3), 5.27 (dd, J = 4, 1 Hz, 1H, H-7), 7.38 (d, J = 8 Hz, 1H, H-3), 7.78 (t, J = 8 Hz, 1H, H-2), 8.02 (d, J = 8 Hz, 1H, H-1); *sugar* 1.38 (s, 3H, CH_3), 1.80 (2H, H-2′), 3.30 (m, 1H, H-3′), 3.70 (br s, 1H, H-4′), 4.25 (m, 1H, H-5′), 5.56 (br s, 1H, H-1′); *ligand* 0.87 (t, J = 7 Hz, 3H, H-4″), 2.42 (q, J = 7 Hz, 2H, H-3″), 3.41 (d, J = 15 Hz, $\underline{CH}_2N$), 3.77 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 6.51–7.22 (m, 12H, H_{ar}); ^{13}C ($CDCl_3$) δ, *anthracycline* 24.71 (C-14), 33.30 (C-10), 34.97 (C-8), 56.71 (OCH_3), 70.46 (C-7), 77.20 (C-9), 112.89 (C-6a), 113.12 (C-10a), 118.42 (C-3), 119.80 (C-1), 122.00 (C-4a), 134.34 (C-12a), 135.08 (C-11a), 135.30 (C-5a), 135.81 (C-2), 155.98 (C-11), 156.57 (C-6), 160.09 (C-4), 187.50 (C-12 and C-5), 213.40 (C-13); *sugar* 16.25 (CH_3), 26.79 (C-2′), 50.03 (C-3′), 65.75 (C-5′), 68.50 (C-4′), 101.00 (C-1′); *ligand* 13.60 (C-4″), 28.98 (C-3″), 49.18 ($O\underline{C}H_2N$), 112.8, 113.3, 120.8, 199.9 (C_{ar}-3,5), 130.6, 131.9 (C_{ar}-2,6), 133.9, 134.2 (C_{ar}-1), 155.8, 156.5, 157.9 (C_{ar}-4), 141.20 (C-1″), 142.05 (C-2″), 168.8 ($O\underline{C}OCH_2$).

2.9. Determination of the estrogen receptor binding affinity (RBA)

Relative binding measurements were performed as previously reported [22], using lamb uterine cytosol, diluted to ~1.5 nM receptor. The protein solution was incubated with buffer or several concentrations of unlabeled competitor together with 10 nM [^{3}H]estradiol at 0°C for 18–24 h. The free ligand was removed by adsorption to dextran-coated charcoal. Unlabeled competitors were prepared and serially diluted in 1:1(v/v) dimethylformamide/TEA buffer (10 mM Tris, 1.5 mM EDTA, 3 mM sodium azide, pH 7.4 at 25°C) to ensure solubility. All data are reported relative to estradiol = 100%.

2.10. *Antitumor activity* assays

The following tumour cell lines were used for the assessment of the cytotoxic activity of the two conjugates:

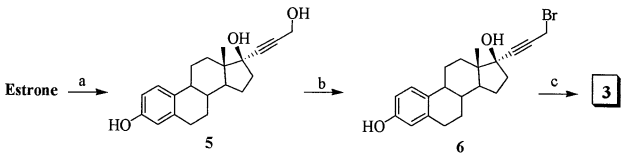
1. **MCF-7**, derived from a mammary adenocarcinoma of a 69-year old caucasian (ATCC: American Type Culture Collection, Rockville, USA). The cells are expressing estrogen receptors.
2. **MDA-MB-231**, derived from a mammary adenocarcinoma of a 51-year old caucasian (ATCC). The cells are estrogen-receptor negative.
3. **LNCaP** clone FGC, derived from a prostate carcinoma of a 50-year old caucasian (ATCC). The cells are expressing androgen and estrogen receptors.
4. **PC-3**, derived from a prostate adenocarcinoma of a 62-year old caucasian (ATCC). The cells are androgen insensitive.
5. **MES-SA**, derived from an uterine sarcoma of a 56-year old female (ECACC: European Collection of Cell Cultures, Salisbury, UK).
6. **MES-SA/Dx5**, derived from the above cell line after selection in the presence of doxorubicin (ECACC). The cells are multi drug-resistant, exhibiting a 100-fold resistance to doxorubicin.
7. **BC3c**, established in our laboratory [23] from a transitional cell carcinoma of the bladder of an 82-year old caucasian female.
8. **A549**, derived from a lung carcinoma of a 58-year old caucasian male (ECACC).
9. **L1210**, a lymphoblastic leukemia cell line derived from an 8-month old female mouse—strain: DBA subline 212 (ATCC).

All cell lines were routinely cultured in Dulbecco's Minimal Essential Medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% Fetal Bovine Serum (media and antibiotics from Biochrom KG, Berlin, Germany) in an environment of 5% CO_2, 85% humidity, 37°C. Adherent cells were subcultured using a trypsin 0.25%-EDTA 0.02% solution. Cytotoxicity was estimated by a modification of the MTT assay [24]. Briefly, cells were plated in 96-well flat-bottomed microplates at a density of 10 000 cells/well. Twenty four h after the plating, the test compounds were added, appropriately diluted in DMSO. After a 48 h incubation, the medium was replaced with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO, USA) dissolved at a final concentration of 1 mg/ml in serum-free, phenol-red-free RPMI (Biochrom KG), for a further 4 h incubation. Then, the MTT-formazan was solubilized in isopropanol and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm. In every experiment daunorubicin HCl was included as a positive control.

3. Results and discussion

3.1. Chemistry

The synthesis of estradiol-linker **6** and its attachment to daunorubicin is outlined in Fig. 2. Estrone was derivatizated



Reagents and conditions: (a) $HC{\equiv}CCH_2OH$, KOEt, THF, 0 °C (70%) ; (b) PPh_3, CBr_4, THF (65 %) ; (c) daunorubicinHCl, Et_3N, DMF, rt

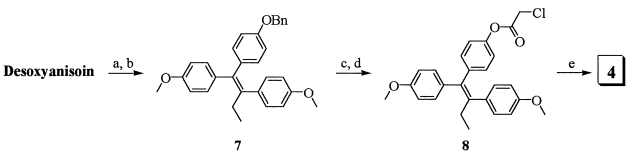
Fig. 2. Synthesis of steroidal ligand **6**.

at 17α position by reaction with propargyl alcohol in the presence of potassium ethoxide. Then the primary hydroxy group was selectively brominated by reaction with carbon tetrabromide and triphenylphosphine affording the estradiol-linker **6**. Subsequent reaction with daunorubicin in anhydrous DMF resulted in its attachment to the 3′-N position of daunorubicin producing the conjugate **3**. The yield of this conjugation reaction is low (10%), but during the purification procedure almost the entire amount of the unreacted steroid was recovered (71%).

Conjugate **4** was prepared in five steps from desoxyanisoine according to the synthetic sequence that is depicted in Fig. 3. More specifically, desoxyanisoin was ethylated by a known procedure [25] and subsequently treated with the benzyloxyphenyl magnesium bromide to furnish the corresponding Grignard product, which was dehydrated during the workup procedure to give the desired product **7**. The yield of the reaction is satisfactory, considering the competing enolization reaction of the activated, sterically hindered ketone [26]. Hydrogenolysis of the benzyl group and reaction with chloroacetylchloride produced in very good overall yield the non-steroidal-linker **8** which was reacted with daunorubicine furnishing the conjugate **4**. In this case the unreacted ligand was also recovered.

3.2. Estrogen receptor binding affinity (RBA)

The estrogen RBA's of the new compounds were determined by a competitive binding assay and are shown in Table 1. Ligands **6** and **8** displayed satisfactory binding affinities, while their corresponding daunorubicin conjugates **3** and **4** exhibit low but not negligible RBA's to the estrogen receptor. This expected reduction of RBA is attributed to the bulk of the cytotoxic agent indicating that a



Reagents and conditions: (a) NaH, EtI, DMF, THF (75 %) ; (b) i. $MgBrC_6H_4OBn$, Et_2O , 40 °C; ii. HCl ; (c) H_2, Pd/C, EtOAc, 1 bar (95 %) ; (d) $ClCH_2COCl$, Pyridine, Et_2O, 0-5 °C ; (e) daunorubicinHCl, Et_3N, DMF, 0-5 °C.

Fig. 3. Synthesis of non-steroidal ligand **8**.

Table 1
Estrogen binding affinity of ligands and daunorubicin conjugates

Compd.	RBA[a]
6	23 ± 1
3	0.079 ± 0.014
8	6.0 ± 0.6
4	0.85 ± 0.04

[a] Relative binding affinity for the estrogen receptor determined in a competitive radiometric assay relatively to [^{3}H] estradiol tracer (RBA = 100%), ±SD (n = 3).

further adjustment to the length of the linker chain is required in order to determine its optimum length.

3.3. Antitumor activity

The dose-response curves concerning the cytotoxicity of the conjugates **3** and **4** in comparison to that of daunorubicin are presented in Fig. 4, where the classical estrogen positive breast cancer cell line MCF-7 has been used as target. A slight stimulation above control at low concentrations could be the result of a combined hormonal and cytotoxic activity. The IC_{50} values obtained from similar experiments on all tumor-cell lines used in the present study are summarized in Table 2. Obviously, conjugation of daunorubicin resulted in a decrease of its cytotoxic activity, ranging from 1.4-fold to 350-fold and from 7.2-fold to 430-fold for conjugates **3** and **4**, respectively. This decrease could be possibly attributed to steric hindrance and/or to the use of the daunosamine nitro-

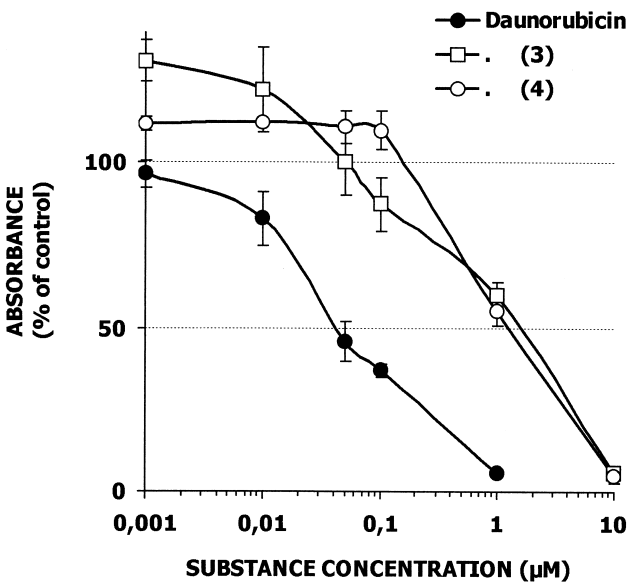


Fig. 4. Cytotoxicity of daunorubicin and of the conjugates **3** and **4**. After incubation of MCF-7 cells with the indicated concentrations of the three substances for 48 h, cytotoxicity was determined by the MTT assay. Details of the procedure are described in Experimental (see *2.10.*). Each point of the dose-response curves is the average of six wells. The error bars represent standard deviations. The results shown are representative of three experiments.

Table 2
In vitro cytotoxicity of daunorubicin conjugates against various cancer cell lines

Cell line	Type	IC_{50} (μM)		
		3	**4**	Daunorubicin
ER + : MCF-7	Human breast	1.2	1.6	0.22
ER − : MDA-MB-231	Human breast	0.4	7.0	0.22
AR + : LNCaP	Human prostate	7.0	7.0	0.02
AR − : PC-3	Human prostate	7.0	9.0	0.40
MES-SA	Human uterine sarcoma	0.1	30.0	0.07
MES-SA/Dx5	Human uterine sarcoma	7.0	>100.0	1.00
BC3c	Human bladder	11.0	22.0	0.55
A549	Human lung	2.0	10.0	0.50
L1210	Mouse leukemia	1.5	1.2	0.04

gen for the conjugation. Apart from that, the cytotoxicity of the two conjugates is clearly due to the daunorubicin moiety, since the doxorubicin-resistant MES-SA/Dx5 cell line exhibits a similar resistance to daunorubicin as well as to **3** and **4**, in comparison to its parental (doxorubicin-sensitive) cell line MES-SA. Furthermore, conjugate **3** was consistently more potent as cytostatic agent than conjugate **4** for most of the cell lines tested (exceptions are the murine leukemia L1210 and the human prostate cancer LNCaP). However, conjugate **3** showed no preferential inhibition of the proliferation of the estrogen receptor positive MCF-7 cells in comparison to the estrogen receptor negative MDA-MB-231. It is also noteworthy that the non-steroidal conjugate **4** inhibits the estrogen receptor positive MCF-7 cells more potently than the estrogen receptor negative MDA-MB-231 cells. These results are in agreement with the estrogen RBA's of the two conjugates presented above (see *3.2.*).

3.4. Conclusions

In summary, we have described the synthesis of two estrogen ligands and their conjugates with cytotoxic daunorubicine. Although this effort may be considered as another unsuccessful attempt to increase the selectivity of cytotoxic agents for cell rich in estrogen receptors, the reported results refer to chemistry that might be useful for the preparation of additional conjugates which may display the desired selectivity. Furthermore, conjugate **4** showed some selectivity to the estrogen receptor which was also reflected in its cytotoxic activity, since this compound was found to inhibit preferentially the estrogen receptor positive cells. Thus, further modification of linker chains by the introduction of functional groups or by changing their length in order to increase their binding affinities will be necessary. Additional work concerning the use of the other functional groups of daunorubicine for the attachment to the estrogen ligands may also be pursued.

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