Med Chem Res (2011) 20:1102–1110
DOI 10.1007/s00044-010-9442-y

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Evaluation of biological activity of new hemiesters of 17-hydroxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile

Suzana S. Jovanović-Šanta · Silvana Andrić · Nebojša Andrić · Gordana Bogdanović · Julijana A. Petrović

Received: 21 May 2010 / Accepted: 16 September 2010 / Published online: 6 October 2010

Abstract In uterotrophic assay newly synthesized compounds **2**–**5** showed a complete loss of estrogenic activity, whereas derivatives **2**–**4** exhibited slight, and compound **5** higher antiestrogenic effects. On the other hand, anti-aromatase assay showed that compounds **2**, **3**, and **4** possess inhibition potency, although lower than standard aromatase inhibitor aminoglutethimide. Cytotoxicity of compounds **2**–**5**, estradiol and tamoxifen against several human tumor or healthy cell lines (MCF-7, MDA-MB-231, HT-29, and MRC-5) was evaluated after short-time treatment.

Keywords 16,17-Secoestratriene derivatives · Synthesis · Hemiesters · Antiestrogens · Aromatase inhibitors · Cytotoxicity

Introduction

Estrogen hormones are female hormones—they promote primary, as well as secondary female characteristics. Further, they play important role in the function of brain, bones, liver, and cardiovascular system (Sarrel *et al*., 1994; Kneifel and Katzenellenbogen, 1981; Toney and Katzenellenbogen, 1986). Estrogen can elicit many other rapid changes in cells, including changes in ion fluxes across membranes and stimulation of kinases and phosphatases (Razandi *et al*., 1999; Stirone *et al*., 2005; Purves-Tyson and Keast, 2004; Warner and Gustafsson, 2005). They also inhibit apoptosis (Wang and Fang, 1995; Razandi *et al*., 2000; Hill *et al*., 2004). Unfortunately, except function in normal physiological processes, they play significant role in carcinogenesis of female reproductive tissues—they can cause DNA damage through free radicals (Han and Liehr, 1994) or show mitogen activity (Weinstain, 1991). Knowledge of the exact mechanism of estrogen hormones acting makes possible treating of patients with estrogen-dependent disorders or diseases. Interfering with the steroid hormone action could broadly be achieved by several fundamental mechanisms: by inhibiting their production (e.g., by inhibiting enzyme aromatase; Brodie and Long, 2001), transport and/or action (e.g., by competing for the estrogen receptors; Katzenellenbogen *et al*., 1995) or others. Some steroidal or non-steroidal compounds which show partial or complete loss of estrogenic potency and certain antiestrogenic activity (like tamoxifen or clomiphene) are therapeutics of choice in case of breast cancer or other estrogen-dependent diseases (Clarke *et al*., 2001). Furthermore, new generation of aromatase inhibitors, steroidal (exemestane), or non-steroidal (anastrozole) is very common class of therapeutics in case of infertility, breast cancer, or other estrogen-dependent diseases. In many cases antiestrogens are combined with aromatase inhibitors (Ziegler *et al*., 2005; Glück, 2005; Howell and Buzdar, 2005).

Previously we reported the synthesis of a series of 16,17-seco-estratriene derivatives (Jovanović-Šanta *et al*., 2000, 2003). Most of them, according to in vivo

S. S. Jovanović-Šanta (✉) · J. A. Petrović
Department of Chemistry, Faculty of Science, University of Novi Sad, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia
e-mail: suzana.jovanovic-santa@dh.uns.ac.rs

S. Andrić · N. Andrić
Department of Biology and Ecology, Faculty of Science, University of Novi Sad, Trg Dositeja Obradovića 2, 21000 Novi Sad, Serbia

G. Bogdanović
Oncology Institute of Vojvodina, Institutski put 4, 21204 Sremska Kamenica, Serbia

experiments, showed practically a total loss of estrogenic activity, whereby some expressed moderate antiestrogenic action. Several of these compounds compete for estrogen receptors (ER), other reduce nuclear translocation of E_2–ER complex, while other decrease complex binding to the DNA (Jovanović-Šanta *et al*., 2006). On the other hand, little changes in the molecule structure-substituent length or polarity of the moiety could be the reason for quite different biological properties of compounds (Zhang *et al*., 2004; Sakač *et al*., 2005). All of these facts prompted us to further syntheses, toward obtaining more potent antiestrogens. Accordingly, the aim of this article was the synthesis of 16,17-secoestratriene 17-hemiesters of glutaric and succinic acid with benzylated or free C-3 hydroxy function. We also tested biological activity of these compounds on experimental animals (estrogen vs. antiestrogen activity and inhibition of aromatase), as well as on tumor cells and studied correlation between their structure and biological activity, in order to present these substances as potential prodrugs.

Experimental

Synthesis and characterization of the newly synthesized compounds

General

Melting points were determined in open capillary tubes on a Büchi SMP apparatus and the values are uncorrected. Infrared spectra (ν in cm^{-1}) were recorded in KBr pellets on a Perkin-Elmer M457 or Carl Zeiss Specord 75 spectrophotometer. NMR-spectra were taken on a Bruker AC 250E spectrometer operating at 250 Hz (proton) and 62.9 Hz (carbon), using standard Bruker software. The signals are reported in ppm downfield from a tetramethylsilane internal standard (δ 0.00); symbols s, d, dd, and m denote singlet, doublet, double doublet, and multiplet, respectively. Mass spectra were recorded on a Finnigan-Math 8230 instrument using chemical ionization (*iso*-butane) techniques; the first number denotes *m/z* value, and the ion abundances are given in parentheses.

*3-Benzyloxy-17-glutaroyloxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile (**2**) and 3-benzyloxy-17-succinoyloxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile (**3**)*

Secocyanoalcohol **1** (1 g, 2.67 mmol) and glutaric (0.78 g, 6.8 mmol) or succinic acid anhydride (0.8 g, 8.00 mmol) were solved in dry pyridine (20 ml) and the reaction mixtures were refluxed for 5 h. Cooled dark brown mixtures were poured into ice-cold water and acidified with HCl (1:1) until pH 3. Formed suspensions were extracted with methylene chloride (3 × 30 ml). The extracts were dried over anhydrous sodium sulphate and evaporated to dryness. Crude 3-benzyloxy-17-glutaroyloxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile (**2**) and 3-benzyloxy-17-succinoyloxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile (**3**) were purified by column chromatography on silica gel (toluene–ethyl acetate, /2:1/) giving 1.21 g (93.08%) and 0.86 g (67.79%) of pure compounds **2** and **3** (mp 112–113 and 127–128°C), respectively.

*Compound **2*** IR spectrum: 3600–3100, 2920, 2220, 1750, 1710, 1610, 1510, 1240, 1040, 890, 750, and 710.

^{1}H-NMR spectrum ($CDCl_3$): 1.00 (s, 3H, CH_3, H-18); 2.39 (m, 6H, $3CH_2$ from glutaroyloxy group); 2.50 (dd, 2H, H-15a, $J_{15a,14} = 16.92$ Hz, $J_{gem} = 4.53$ Hz); 3.83 (d, 1H, H-17a, $J_{gem} = 11.59$ Hz); 4.09 (d, 1H, H-17b); 5.06 (s, 2H, O–C**H**$_2$–C_6H_5); 6.75–7.47 (group of signals, 8H, aromatic protons).

^{13}C-NMR-spectrum ($CDCl_3$): 15.78 (CH_2, C-15); 16.08 (CH_3, C-18); 19.71 (C-3′ from the side chain); 25.89; 27.01; 29.91; 32.85 (C-2′ from the side chain); 33.02 (C-4′ from the side chain); 36.03; 37.58; 39.49; 42.16; 42.79; 69.91 (O–**C**H_2–C_6H_5); 71.32 (C-17); 112.68; 114.38; 119.31 (C≡N); 126.37; 127.39; 127.84; 128.51; 131.72; 137.14; 137.42; 156.92 (C-3); 172.69 (COOR); 178.57 (COOH).

Mass spectrum: 546 (10; $(M + i\text{-Bu-}1)^+$); 490 (100; $(M + 1)^+$); 472 (74; $(M\text{-OH})^+$); 358 (17; (M-glutaroyloxy + $1)^+$).

Anal. Calcd. for $C_{30}H_{35}NO_5$: C, 73.59; H, 7.21; N, 2.86. Found: C, 73.25; H, 7.47; N, 3.21.

*Compound **3*** IR spectrum: 3600–3100, 2930, 2220, 1730, 1710, 1610, 1510, 1230, 1150, 1040, 1000, 950, 750, and 700.

^{1}H-NMR spectrum ($CDCl_3$): 0.98 (s, 3H, CH_3, H-18); 2.50 (2dd, 2H, H-15, $J_{gem} = 26.03$ Hz, $J_{15a,14} = 4.62$ Hz); 2.66 (s, 4H, $2CH_2$ from succinoyloxy group); 3.81 (d, 1H, H-17a, $J_{gem} = 11.58$ Hz); 4.13 (d, 1H, H-17b); 5.05 (s, 2H, O–C**H**$_2$–C_6H_5); 6.74–7.47 (group of signals, 8H, aromatic protons).

^{13}C-NMR-spectrum ($CDCl_3$): 15.65 (CH_2, C-15); 16.06 (CH_3, C-18); 25.88; 26.93; 28.77 ($2CH_2$ from succinoyloxy group); 29.89; 35.96; 39.33; 41.73; 42.74; 69.87 (O–**C**H_2–C_6H_5); 71.33 (C-17); 112.44; 114.36; 119.28 (C≡N); 126.37; 127.37; 127.81; 128.48; 131.71; 137.11; 137.42; 156.87 (C-3); 171.83 (COOR); 178.02 (COOH).

Mass spectrum: 532 (12; $(M + i\text{-Bu-}1)^+$); 476 (100; $(M + 1)^+$); 458 (20; $(M\text{-OH})^+$); 358 (17; (M-succinoyloxy + $1)^+$).

Anal. Calcd. for $C_{29}H_{33}NO_5$: C, 73.24; H, 6.99; N, 2.94. Found: C, 73.38; H, 7.25; N, 3.23.

*3-Hydroxy-17-glutaroyloxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile (**4**) and 3-Hydroxy-17-succinoyloxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile (**5**)*

To the solution of the corresponding benzyl ether **2** or **3** (2 mmol) in methylene chloride–methanol mixture (2:1, 10 ml), 10% Pd/C (0.38 g) was added. The suspensions were stirred at room temperature for 1.5 and 18 h under an atmosphere of hydrogen. After the removal of the catalyst, the solvent was evaporated to dryness and the products were purified by crystallization from toluene, i.e., methylene chloride. Analytically pure 3-hydroxy-17-glutaroyloxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (**4**) and 3-hydroxy-17-succinoyloxy-16,17-secoestra-1,3,5(10)-trien-16-nitrile (**5**) were obtained in yields 50.33 and 60.46% (mp 113–115 and 129–131°C), respectively.

*Compound **4*** IR spectrum: 3450, 2930, 2220, 1750, 1720, 1630, 1510, 1190, 950, 890, and 810.

^{1}H-NMR spectrum (DMSO): 0.86 (s, 3H, CH_3, H-18); 2.35 (m, 6H, $3CH_2$ from glutaroyloxy group); 3.81 (d, 1H, H-17a, $J_{gem} = 11.59$ Hz); 4.03 (d, 1H, H-17b, $J_{gem} = 11.59$ Hz); 6.45 (d, 1H, H-4, $J_{4,2} = 2.26$ Hz); 6.53 (dd, 1H, H-2, $J_{2,1} = 8.45$ Hz, $J_{2,4} = 2.23$ Hz); 7.08 (d, 1H, H-1, $J_{1,2} = 8.50$ Hz); 9.06 (s, 1H, OH, C-3); 12.16 (s, 1H, COOH, C-17).

^{13}C-NMR-spectrum (DMSO-d_6): 15.05 (CH_2, C-15); 15.59 (CH_3, C-18); 19.96 (C-3′ from the side chain); 25.64; 26.62; 29.44; 32.67 (C-2′ from the side chain); 32.73 (C-4′ from the side chain); 37.29; 40.16; 40.50; 41.31; 42.20; 70.51 (C-17); 112.95; 114.61; 120.57 (C≡N); 126.27; 129.84; 136.88; 155.09 (C-3); 172.44 (COOR); 174.00 (COOH).

Mass spectrum: 400 (100; $(M + 1)^+$); 382 (81; $(M\text{-}OH)^+$); 2.68 (18; $(M\text{-glutaroyloxy} + 1)^+$).

Anal. Calcd. for $C_{23}H_{29}NO_5$: C, 69.15; H, 7.32; N, 3.50. Found: C, 69.43; H, 7.35; N, 3.37.

*Compound **5*** IR spectrum: 3450, 2930, 2220, 1730, 1710, 1610, 1510, 1230, 1160, 1040, 1000, 950,750, and 700.

^{1}H-NMR spectrum (DMSO-d_6): 0.86 (s, 3H, CH_3, H-18); 2.50 (2dd, 2H, H-15, $J_{gem} = 26.03$ Hz, $J_{15a,14} = 4.62$ Hz); 2.68 (s, 4H, $2CH_2$ from succinyloxy group); 3.81 (d, 1H, H-17a, $J_{gem} = 11.59$ Hz); 4.13 (d, 1H, H-17b); 6.45 (d, 1H, H-4, $J_{4,2} = 2.45$ Hz); 6.53 (dd, 1H, H-2, $J_{2,1} = 8.38$ Hz, $J_{2,4} = 2.50$ Hz); 7.07 (d, 1H, H-1, $J_{1,2} = 8.53$ Hz); 9.06 (s, 1H, OH, C-3); 12.24 (s,1H, COOH).

^{13}C-NMR-spectrum (DMSO): 15.03 (CH_2, C-15); 15.60 (CH_3, C-18); 25.67; 26.60; 28.77 (CH_2 from succinyloxy group); 28.95 (CH_2 from succinyloxy group); 29.43; 35.27; 37.39; 40.51; 41.00; 42.09; 70.52 (C-17); 112.95; 114.62; 120.49 (C≡N); 126.25; 129.90; 136.89; 155.08 (C-3); 171.88 (COOR); 173.44 (COOH).

Mass spectrum: 442 (4; $(M + i\text{-Bu-}1)^+$); 386 (100; $(M + 1)^+$); 368 (17; $(M\text{-OH})^+$); 342 (19); 269 (21; (M-succinoyloxy + 1)$^+$).

Anal. Calcd. for $C_{22}H_{27}NO_5$: C, 68.37; H, 7.30; N, 3.62. Found: C, 68.66; H, 7.32; N, 3.56.

Biological tests

All experiments were approved by the Local Ethical Committee of the University of Novi Sad and were performed and conducted in accordance with the principles and procedures of the NIH Guide for Care and Use of Laboratory Animals.

Uterotrophic and antiuterotrophic assay

The estrogenic and antiestrogenic effects of compounds **2**, **3**, **4**, and **5** were tested on experimental animals using the uterotrophic and antiuterotrophic methods (Emmens, 1950).

Immature Wistar strain female rats (21–23 days old, raised under controlled environmental conditions—temperature 22 ± 2°C and 14 h light/10 h dark, with food and water ad libitum) were randomly divided into groups of six to eight animals each. The animals were treated by subcutaneous injection once a day for 3 days with 0.1 ml of a solution of the test compound in olive oil, either solely or in combination with estradiol benzoate (EB). The control group obtained the vehicle only. The total administered amount of tested compounds was 5 mg/kg of body weight (b.w.), except in case of tamoxifen, used as a comparator, and compound **5**, where doses were 5 mg/kg b.w., i.e., 25 mg/kg b.w., whereas the EB dose was 30 μg/kg b.w. The animals were sacrificed on the fourth day. The uteri were removed, dissected free of adhering fat and blotted dry after expulsion of uterine fluid and the wet weights were recorded.

The differences of uteri weights of treated and control animals served for the calculation of the agonistic and antagonistic effects (Wakeling *et al*., 1984). Percentage of agonistic and antagonistic activity in immature rat uterine weight assays were calculated from the ratio of values recorded in the treated and control animals, thus:

$$\%\ \text{agonism} = (C - A) \times 100/(B - A)$$

$$\%\ \text{antagonism} = (B - D) \times 100/(B - A)$$

In these equations A, B, C, and D are uterine wet weights, corrected for differences in body weights (mg/100 g body weight) for vehicle alone, EB, test compound alone, or test compound plus EB, respectively.

Anti-aromatase assay

Chemicals

Antiestradiol serum no. 244, was kindly supplied by Dr. G. D. Niswender (Colorado State University, CO, USA). Pregnant Mares' Serum gonadotrophin (PMSG) was obtained from the Veterinary Institute Subotica (Serbia), [1,2,6,7-^{3}H(N)] estradiol was obtained from New England Nuclear (Belgium), NADPH, testosterone, and aminoglutethimide (AG) were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Animals, treatment, and assay

Anti-aromatase activity of the synthesized compounds was tested according to the procedure described elsewhere (Penov Gaši *et al*., 2001). In brief, in the final volume of 0.25 ml, the incubation mixture contained testosterone as substrate, 1 mM NADPH, 0.1 M phosphate buffer (pH 7.4), tested compound and 0.125 ml of denucleated ovarian fraction (0.33 mg of protein, prepared by known procedure; Brodie *et al*., 1976). Parallel incubations without tested compounds were also run as control samples. The desired concentration of tested compounds was prepared by evaporating the necessary amount of stock solution (in organic solvent) and dissolving it in 0.1 M phosphate buffer. Mixtures were incubated for 15 min at 37°C in a shaking water bath in 95% O_2–5% CO_2 atmosphere. The enzyme reaction was initiated by adding denucleated ovarian fraction and terminated by placing the tubes in an ice-cold bath. The samples were stored at −20°C until assayed for estradiol by RIA. Estradiol production in control and experimental samples were calculated after subtraction of the corresponding blanks. Namely, two sets of blanks were run in parallel with control and experimental samples: in the first set, testosterone and tested compounds were omitted in order to determine residual levels of estradiol and possible endogenous estradiol production in denucleated ovarian fractions used in each experiment, while in the second set, homogenate was omitted in order to determine cross-reactivity of testosterone and tested compounds.

For an assessment of anti-aromatase activity of the synthesized compounds, the given compounds were added in two concentrations (10 and 50 μM) to the incubation mixture containing 500 nM of testosterone as substrate (saturated concentration; the estimated K_m for testosterone was 49.17 nM, and V_{max} 5.76 pmol/min·mg protein). Standard anti-aromatase inhibitor, Ag, was tested in concentration 0.1 μM.

Statistics

The statistical significance was evaluated by two-tailed non-parametric Mann–Whitney test.

Tumor cell proliferation

Cell lines

The cell lines used in this study were: MCF-7 (human breast adenocarcinoma, estrogen receptor positive, ER+), MDA-MB-231 (human breast adenocarcinoma, estrogen receptor negative, ER−), HT-29 (human colon adenocarcinoma, estrogen receptor positive, ER+), and MRC-5 (human lung fibroblasts) as control. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose, supplemented with 10% of fetal calf serum (FCS, Veterinary Institute Novi Sad), and antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin (ICN Galenika). Only HT-29 cell line was grown in the presence of 15% of FCS. The cells were sub-cultured twice a week and a single cell suspension was obtained using 0.25% trypsin in EDTA (Serva). All cell lines were cultured in flasks (Costar, 25 cm^2) at 37°C in the 100% humidity atmosphere and 5% of CO_2. Only viable cells were used in the assays. The cell density (number of cells per unit volume) and percentage of viable cells were performed as described elsewhere (Bogdanović *et al*., 1994).

Tested substances

Newly synthesized compounds **2**–**5**, estradiol (E_2) and tamoxifen citrate (TC) were dissolved in DMSO (stock solution) and solutions were made, which, added 10 μl/well, in final volume of 100 μl gave concentrations 10^{-12}–10^{-6} M.

MTT assay

Growth inhibition was evaluated by colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) assay (Mosmann, 1983). Cells were harvested, counted by trypan blue exclusion and plated into 96-well microtiter plates (Costar, flat bottom) at optimal seeding density of 5×10^3 cells per well. Cells were plated in a volume of 90 μl per well and preincubated in complete medium at 37°C and 5% CO_2 for 24 h. Tested substances dissolved in growth medium were added to all wells (10 μl/well), except to the control ones, and micro plates were incubated for 48 h. After incubation period, 10 μl of MTT solution (5 mg/ml) was added to all wells and plates were incubated at 37°C for 3 h. After that time acid–isopropanol

(10 µl) was added to all probes, and absorbance (A) was measured on a micro plate reader (Multiscan MCC340, Labsystems) at 492/690 nm. The wells containing cells without tested substances were used as controls, while the wells without cells containing complete medium and MTT only acted as blank. Cells growth was expressed as a percent of control, according to the formula:

$$\text{Growth}(\%\ \text{of control}) = (A_{\text{test}}/A_{\text{control}}) \times 100$$

A_{test}—absorbance of cells treated with tested substances; A_{control}—absorbance of untreated cells.

Results and discussion

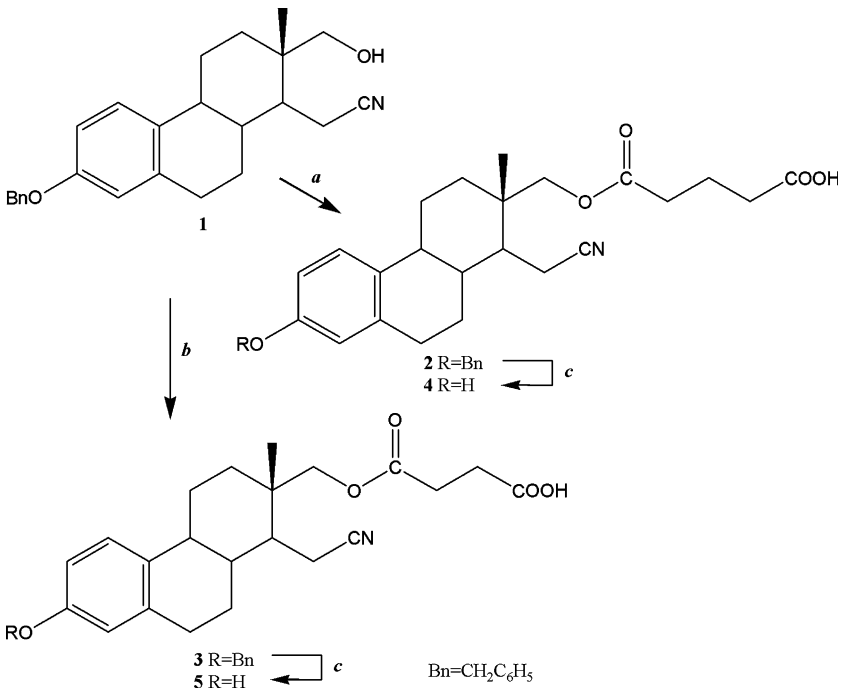
The syntheses of new compounds were performed according to Scheme 1, where starting compound, 3-benzyloxy-17-hydroxy-16,17-secoestra-1,3,5(10)-triene-16-nitrile (**1**), was synthesized in several synthetic steps, starting from estrone (Jovanović-Šanta *et al*., 2000).

The esterification of compound **1** with glutaric acid anhydride in dry pyridine afforded corresponding 3-benzyloxy-17-hemiglutarate **2**, in 93.08% yield, after purifying of the crude reaction mixture by column chromatography. The reaction of esterification of secocyanoalcohol **1** with succinic acid anhydride was carried out in similar reaction conditions and by similar mode of purifying. In this case, yield of corresponding 3-benzyloxy-hemisuccinate **3** was slightly lower (67.79%). The deprotection of the 3-hydroxy function of compounds **2** and **3** was performed by hydrogenolysis at room temperature in an atmosphere of hydrogen, using 10% Pd/C as catalyst, whereby 3-hydroxy-16,17-secoestrone derivatives **4** and **5** were obtained in 50.33%, i.e. 60.46% yields, respectively.

New compounds, synthesized in this study, were tested on estrogenic, antiestrogenic, and anti-aromatase activity. Influence on tumor cell proliferation was also studied.

The estrogenic and antiestrogenic effects of compounds **2**, **3**, **4**, and **5** were tested on experimental animals, using uterotrophic and antiuterotrophic methods (Emmens, 1950). The differences in uteri weights of treated and control animals served for the calculation of the agonistic and antagonistic effects (Wakeling *et al*., 1984). These results are presented in Table 1.

As can be seen from Table 1, all the compounds exhibited a total loss of estrogenic activity, whereas all new derivatives even prevented the action of endogenous estrogens. On the other hand, compounds **4** and **5** (compounds with free 3-hydroxy function) partially hindered the action of estradiol benzoate. Compound **4** behaved as moderate antagonist (13.78%), while compound **5** showed higher antagonistic activity (41.59%). Interestingly, this compound showed higher antihormonal properties in lower dose. Tamoxifen, the most used drug in breast cancer therapy showed higher antiestrogenic activity but it exhibited estrogenic potency also. Comparing the results of this experiment with results from earlier studies (Jovanović-Šanta *et al*., 2000; Table 1, compound **1** and its 3-hydroxy analog **1a**), we can see that compounds with free C-3 hydroxy function were more potent in in vivo tests, except in case of the starting compound, secocyanoalcohol **1** and its 3-hydroxy derivative **1a**.



Scheme 1 Reagents and rection conditions: *a* glutaric acid anhydride, abs. Py, reflux; *b* succinic acid anhydride, abs. Py, reflux; *c* H_2, 10% Pd/C, CH_2Cl_2, CH_3OH, and RT

Table 1 Agonistic and antagonistic effects of compounds **1–5**, **1a**, and tamoxifen

Compounds	Dose (mg/kg)	*n*	Estrogenic effect (%, mean ± SEM)	*n*	Antiestrogenic effect (%, mean ± SEM)
1	5	7	0.71 ± 0.90	8	31.47 ± 2.26
1a	5	6	−2.06 ± 1.06	8	21.13 ± 2.05
2	5	6	−3.92 ± 0.95	8	8.50 ± 6.22
3	5	6	−2.28 ± 1.16	8	2.82 ± 4.20
4	5	7	−9.63 ± 2.00	8	13.78 ± 9.88
5	5	7	−6.78 ± 1.52	8	41.59 ± 3.07
	25	7	6.42 ± 2.26	8	11.92 ± 5.44
Tamoxifen	5	7	39.78 ± 3.13	–	–
	25	8	39.36 ± 4.09	7	62.80 ± 2.13

Immature Wistar strain female rats (21–23 days old) were injected sc with 30 μg/kg b.w. (total dose) with the tested compounds **2–5** dissolved in olive oil (alone or in combination with EB) or with vehicle alone (control) for 3 days. Number of animals per group was 6–8 (*n*). Results are presented as percentage of agonistic and antagonistic activity. Numbers represent mean ± SEM

Anti-aromatase assay was carried out in purpose of screening potential inhibitory effect of the newly synthesized compounds. The assay was run in the denucleated ovarian fraction from PMSG-pretreated female rats (Penov Gaši *et al*., 2001; Brodie *et al*., 1976). The compounds were tested in two concentrations (10 and 50 μM). The results of the anti-aromatase assay are presented in Table 2.

The results, given in Table 2, showed that all tested compounds, except compound **5**, expressed inhibitory effect on aromatase activity in the presence of saturated concentration of testosterone. Compound **3** showed only minor inhibition property (21.22%), while its 3-hydroxy derivative **5** had no inhibition potency. On the other hand, compound **2** expressed the highest inhibitory effect (88.24%). Further, its 3-hydroxy analog **4** showed lower inhibition potency (67.33%). The structural difference between compounds **2** and **3** is that compound **2** has chain in position 17 longer than compound **3** for only one methylene group. That slight difference probably was the reason for different inhibition potency. It is known that small difference in the molecule structure causes significant difference in biological effect (Zhang *et al*., 2004). All compounds expressed higher effect when they were given in higher dose. Interestingly, compound **5**, which showed the highest antiestrogenic activity, expressed no anti-aromatase potency.

Screening of cytotoxicity of the tested compounds **2–5** was evaluated after short-time treatment of the human malignant and nonmalignant cell lines. Growth inhibition of the cells treated by estradiol or TC was measured in parallel experiments in range of concentrations (10^{-12}–10^{-6} M) that included physiological concentration of estrogen hormones. Human breast adenocarcinoma cells MCF-7 and MDA-MB-231, colon adenocarcinoma cells

Table 2 Inhibitory effect of the tested compounds **2–5** and AG

Compounds	Concentration (μM)	Production (ng/min·mg P)	Aromatase inhibition (%)
Control	–	1.25 ± 0.11	–
2	10	1.02 ± 0.14	17.92 ± 11.42
	50	0.15 ± 0.06**	88.24 ± 4.79
3	10	1.16 ± 0.20	7.02 ± 16.06
	50	0.98 ± 0.09	21.22 ± 7.12
4	10	1.04 ± 0.14	16.55 ± 11.18
	50	0.41 ± 0.15**	67.33 ± 11.89
5	10	1.38 ± 0.138	−10.57 ± 10.62
	50	1.24 ± 0.23	0.10 ± 18.51
AG	0.1	0.73 ± 0.03**	41.33 ± 2.53

Estradiol production in the denucleated fraction of ovaries from PMSG-pretreated rats (animals): control group (ten probes), groups with tested compounds **2–5** and AG (four probes for each). Results are presented as estradiol production (ng/min·mgP) and the percentage of inhibition versus control. Numbers represent mean ± SEM of four replicates. Significance: ** $P < 0.005$ versus control (Mann–Whitney non-parametric test)

HT-29 and human lung fibroblasts MRC-5 (nonmalignant cells) were continuously treated for 48 h with the tested compounds. Growth inhibition was evaluated by MTT assay after approximately two cells doubling times (Bogdanović *et al*., 1994; Mosmann, 1983). Percentages of cells growth after the treatment were calculated according to the known formula. Figure 1a–f represents the growth of MCF-7, MDA-MB-231, HT-29, and MRC-5 cells treated for 48 h with tested compounds **2**–**5**, estradiol or TC, respectively.

From the data presented in Fig. 1 it can be obviously seen that dose-relationship is fundamentally hormetic, usually U-shaped (Calabrese *et al*., 2003; Calabrese, 2005) or biphasic.

Compound **2** (Fig. 1a) showed different growth inhibition activity depending on cell line. HT-29 and MCF-7, both ER+ cell lines, showed different response to compound **2** at lower concentrations (10^{-12}–10^{-9} M). Cytotoxicity of compound **2** was more expressed, although small (20–30%) in case of MCF-7 cells. The growth inhibition pattern of MDA-MB-31, ER- cells, was similar to that obtained with the same compound against MCF-7, ER+ cells. MRC-5 cells were the least sensitive to compound **2**. Obtained results indicated non-hormone

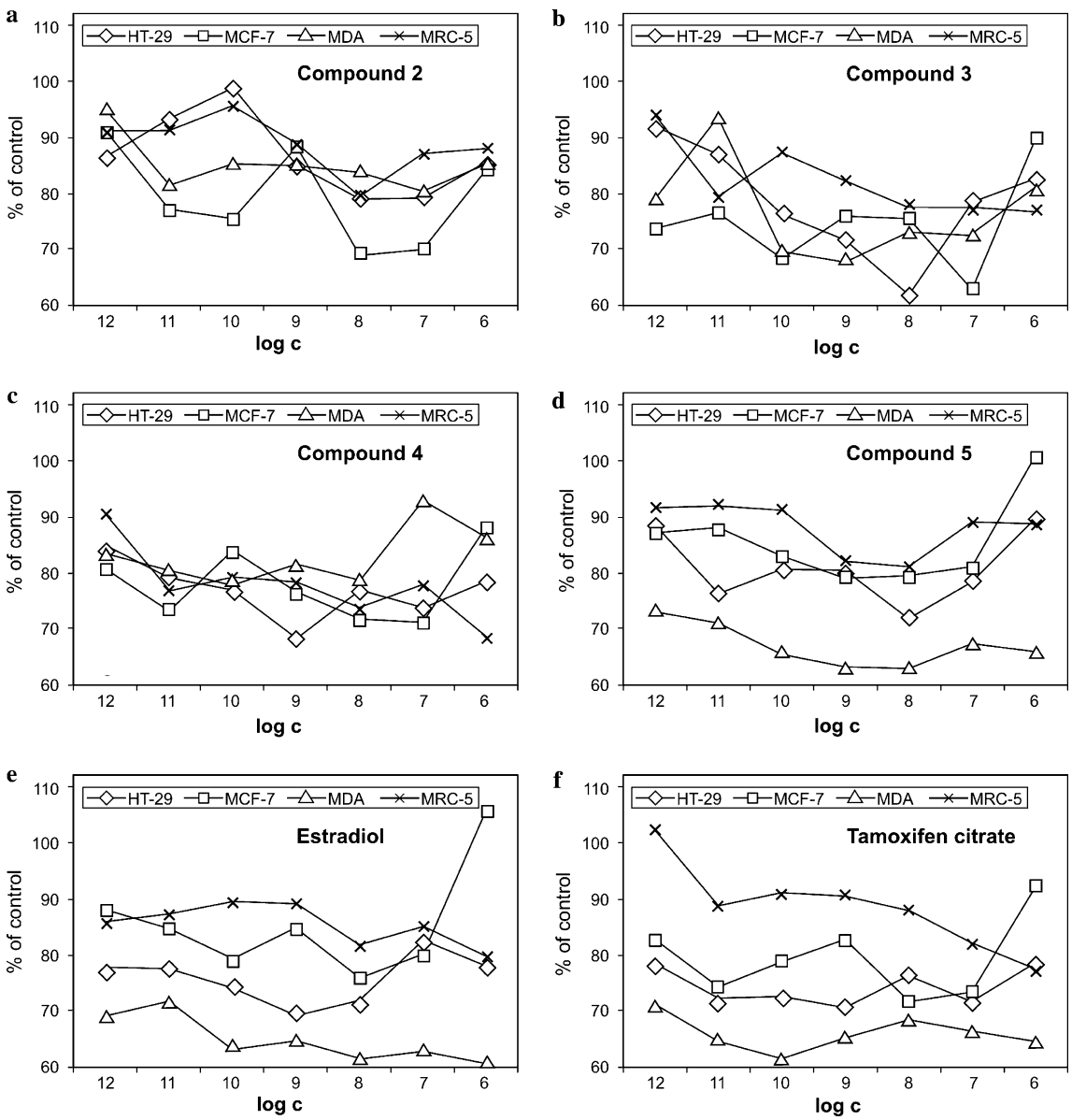


Fig. 1 Growth of MCF-7, MDA-MB-231, HT-29, and MRC-5 cells after 48 h treatment with compounds **2**–**5**, estradiol, or TC. Cells growth (percentage of control) for all cell lines was evaluated 48 h after the treatment of cells with compounds **2**–**5**, E_2 or TC. Data are the means of at least two experiments performed in quadruplicate (SEM values are not given in graphs because of clarity)

mechanism of action of the compound **2** on investigated cell lines.

All malignant and nonmalignant (MRC-5) cell lines showed very similar response to the treatment with compound **3** (Fig. 1b). The growth inhibition did not exceed 40% in the whole range of concentrations. Compounds **2** and **3** are similar in structure. They gave different biological response when administered at lower doses, while their biological properties at higher doses were very similar.

The compounds **4** and **5**, although similar in structure, expressed quite different effects on cell lines. Compound **4** (Fig. 1c) inhibited cell growth at the whole range of concentrations by 10–30%, regardless to the cell type. Compound **5** (Fig. 1d) slightly inhibited (up to 30%) the growth of MCF-7 and HT-29 cells. However, the same compound had the highest effect on the growth of MDA-MB-231 (ER− cells). Growth inhibition ranged from 30 to 40% for the whole concentration range.

Estradiol (Fig. 1e), estrogen hormone present in the human body in physiological concentration was used in our experiments as control. HT-29 cells showed almost the same response at all its concentrations (20–30%), as well as MCF-7 cells at dose range 10^{-12}–10^{-7} M (10–20%). There was no significant growth inhibition or stimulation of MCF-7 cells at concentration 10^{-6} M. MDA-MB-231 cells growth was inhibited by 30 to 40% at all doses.

Tamoxifen citrate (Fig. 1f), the most widely used therapeutic for breast cancer treatment, was the second control substance in our experiments. Growth inhibition pattern of TC was comparable to that obtained with estradiol against all cell lines including MRC-5 cell line. The highest growth inhibition effect was obtained on ER− MDA-MB-231 cells, which indicates possible non-hormonic mechanisms of action of this compound, similar to effects of estradiol and compound **5**.

Comparing the effect of hemiglutarate **2** and its 3-hydroxy analog (compound **4**) on cell lines, it can be seen that curve shape of MCF-7 cells is very similar for both compounds. It is interesting that the effect of **2** is quite different on HT-29 cells compared to the effect of **4** at concentration range 10^{-12}–10^{-9} M: compound **2** was less active than compound **4**, maybe because of time needed for compound **2** to release benzyl ether function in position 3 and achieve structure of compound **4**.

Further, comparing the influences of hemisuccinate **3** and its 3-hydroxy analog **5**, it is obvious that these structurally quite similar compounds have different effects on cells growth, though growth inhibition was always below 50%.

Interestingly, 3-hydroxy-17-hemisuccinate **5** showed the highest antiestrogenic action in in vivo experiments (even 42%), but did not express high growth inhibition effect on estrogen receptor positive cancer cells (HT-29 and MCF-7 cell lines, 10–30% only). Therefore, it could be presumed that growth inhibition effect of this compound did not include mechanism of estrogen action or the incubation period was too short.

On the other hand, 3-benzyloxy-17-hemiglutarate **2** and 3-hydroxy-17-hemiglutarate **4**, the best aromatase inhibitors of the tested derivatives, had influence less than 50% on the cell growth and thus it could be assumed that growth inhibition is not necessarily related to aromatase action in cell cultures.

Conclusion

In experiments in vivo 3-hydroxy-17-hemisuccinate **5** expressed the highest antiestrogenic effect. Its 3-benzyloxy analog **3** or 17-glutaroyloxy analog **4** showed much lower antihormonal properties. All new compounds had no agonistic activity.

Aromatase inhibition potency showed 17-hemiglutarates with free or benzylated 3-hydroxy function. Compound with the best inhibiting activity was 3-benzyloxy 17-hemiglutarate **2**, while its 3-hydroxy analog **4** showed something smaller effect on aromatase. 3-Benzyloxy or 3-hydroxy-17-hemisuccinate (**3** or **5**, respectively) showed practically no aromatase inhibition potency.

Antiproliferative activity of the tested substances against human tumor or healthy cells (lines: MCF-7, MDA-MB-231, HT-29, and MRC-5) after short-time treatment was quite low. According to this, we are planning to continue study in longer period of exposure of cell lines to the tested substances. Growth inhibition of the cancer cells, treated with known or new compounds in steroid-free medium, is planed to be examined also.

In frame of all these results, further derivatization of compound **1** as well as the synthesis of new estratriene derivatives are planed with an aim of obtaining compounds with higher antagonistic and anti-aromatase properties.

Acknowledgment This work was supported by a research grant from the Ministry of Science and Technology Development, Republic of Serbia (grant No. 142052B).

References

Bogdanović G, Raletić-Savić J, Marković N (1994) In vitro assays for antitumor-drug screening on human tumor cell lines: dye exclusion test and colorimetric cytotoxicity assay. Arch Oncol 2:181–184

Brodie A, Long B (2001) Aromatase inhibition and inactivation. Clin Cancer Res 7:4343s–4349s discussion 4411s–4412s

Brodie AMJ, Schwarzel WC, Brodie HJ (1976) Studies on the mechanism of estrogen biosynthesis in the rat ovary. J Steroid Biochem 7:787–793

Calabrese EJ (2005) Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. Environ Pollut 138(3):379–411

Calabrese EJ, Baldwin LA (2003) Hormesis: the dose-response revolution. Annu Rev Pharmacol Toxicol 43:175–197

Clarke R, Leonessa F, Welch JN, Skaar TC (2001) Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacol Rev 53:25–71

Emmens CW (1950) Hormone assay. Academic Press, New York

Glück S (2005) Changing the gold standard in adjuvant therapy for breast cancer: from tamoxifen to aromatase inhibition. Biomed Pharmacother 59:S321–S322

Han X, Liehr JG (1994) 8-Hydroxylation of guanine bases in kidney and liver DNA of hamsters treated with estradiol: role of free radicals in estrogen-induced carcinogenesis. Cancer Res 54(21):5515–5517

Hill RA, Pompolo S, Jones ME, Simpson ER, Boon WC (2004) Estrogen deficiency leads to apoptosis in dopaminergic neurons in the medial preoptic area and arcuate nucleus of male mice. Mol Cell Neurosci 27:466–476

Howell A, Buzdar A (2005) Are aromatase inhibitors superior to antiestrogens? J Steroid Biochem Mol Biol 93:237–247

Jovanović-Šanta S, Andrić S, Kovačević R, Pejanović V (2000) Synthesis and biological activity of new 16, 17-secoestrone derivatives. Collect Czech Chem Commun 65:77–82

Jovanović-Šanta S, Petrović J, Andrić S, Kovačević R, Đurendić E, Sakač M, Lazar D, Stanković S (2003) Synthesis, structure, and screening of estrogenic and antiestrogenic activity of new 3, 17-substituted-16, 17-seco-estratriene derivatives. Bioorg Chem 31:475–484

Jovanović-Šanta S, Petrović J, Sakač M, Žakula Z, Isenović E, Ribarac-Stepić N (2006) The influence of 17-oxo and 17-hydroxy-16, 17-secoestratriene derivatives on estrogen receptor. Collect Czech Chem Commun 71:532–542

Katzenellenbogen BS, Montano M, Le Goff P, Schodin DJ, Kraus WL, Bhardwaj B, Fujimoto N (1995) Antiestrogens: mechanisms and actions in target cells. J steroid Biochem Mol Biol 53:387–393

Kneifel MA, Katzenellenbogen BS (1981) Comparative effects of estrogen and antiestrogen on plasma renin substrate levels and hepatic estrogen receptors in the rat. Endocrinology 108(2):545–552

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Penov Gaši K, Stanković S, Csanadi J, Đurendić E, Sakač M, Medić-Mijačević Lj, Arcson O, Stojanović S, Andrić S, Molnar Gabor D, Kovačević R (2001) New D-modified androstane derivatives as aromatase inhibitors. Steroids 66(8):645–653

Purves-Tyson TD, Keast JR (2004) Rapid actions of estradiol on cyclic amp response-element binding protein phosphorylation in dorsal root ganglion neurons. Neuroscience 129(3):629–637

Razandi M, Pedram A, Greene GL, Levin ER (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERα and ERβ expressed in Chinese Hamster ovary cells. Mol Endocrinol 13(2):307–319

Razandi M, Pedram A, Levin ER (2000) Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. Mol Endocrinol 14(9):1434–1447

Sakač MN, Penov Gaši KM, Popsavin M, Djurendić EA, Andrić S, Kovačević RM (2005) Synthesis and estrogenic activity screening of some 6, 9-substituted estradiol derivatives. Collect Czech Chem Commun 70:479–486

Sarrel PM, Lufkin EG, Oursler MJ, Keefe D (1994) Estrogen actions in arteries, bone, and brain. Sci Am Sci Med 1(3):44–53

Stirone C, Boroujerdi A, Duckles SP, Krause DN (2005) Estrogen receptor activation of phosphoinositide-3 kinase, Akt, and nitric oxide signaling in cerebral blood vessels: rapid and long-term effects. Mol Pharmacol 67:105–113

Toney TW, Katzenellenbogen BS (1986) Antiestrogen action in the medial basal hypothalamus and pituitary of immature female rats: insights concerning relationships among estrogen, dopamine, and prolactin. Endocrinology 119:2661–2669

Wakeling AE, Valcaccia B, Newboult E, Green LR (1984) Non-steroidal antioestrogens-receptor binding and biological response in rat uterus, rat mammary carcinoma and human breast cancer cells. J Steroid Biochem 20(1):111–120

Wang TTY, Fang JM (1995) Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. Cancer Res 55:2487–2489

Warner M, Gustafsson JA (2005) Nongenomic effects of estrogen: why all the uncertainty? Steroids 71(2):91–95

Weinstain IB (1991) Mitogenesis is only one factor in carcinogenesis. Science 251:387–388

Zhang JX, Labaree DC, Mor G, Hochberg RB (2004) Estrogen to antiestrogen with a single methylene group resulting in an unusual steroidal selective estrogen receptor modulator. J Clin Endocrinol Metab 89:3527–3535

Ziegler D, Mattenberger C, Luyet C, Romonscanu I, Fournet Irion N, Bianchi-Demicheli F (2005) Clinical use of aromatase inhibitors (AI) in premenopausal women. J Steroid Biochem Mol Biol 95:121–127