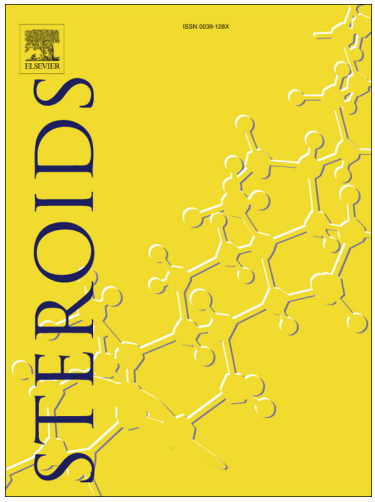

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Syntheses and Antiproliferative Effects of D-homo- and D-secoestrones

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Abstract

Substituted and/or heterocyclic D-homoestrone derivatives were synthetized via the intramolecular cyclization of a δ-alkenyl-D-secoaldehyde, -D-secoalcohol or -D-secocarboxylic acid of estrone 3-benzyl ether. The D-secoalcohol was modified at three sites in the molecule. The *in vitro* antiproliferative activities of the new D-homo- and D-secoestrone derivatives were determined on HeLa, MCF-7, A431 and A2780 cells through use of MTT assay. D-Homoalcohols **3** and **5** displayed cell line-selective cytostatic effects against ovarian and cervical cell lines, respectively. Two D-secoestrones (**6** and **12c**) proved to be effective, with IC_{50} values comparable with those of the reference agent cisplatin. A selected compound (**6**) was tested by tubulin polymerization assay and its cancer specificity was additionally determined by using noncancerous human fibroblast cells.

Keywords
Homoestrone, Secoestrone, Antiproliferative effect, MTT assay, Tubulin polymerization.

1. Introduction

Structural modifications of 17β-estradiol, a proliferation-inducing compound [1], may lead to antiproliferative estrone derivatives [2,3]. Substitution at position 2 and/or D-ring expansion usually reduces the estrogeneity, but enhances the antitumor behavior. Hillisch *et al.* recently patented antimitotic 2-substituted D-homoestrone-3-sulfamates [4]. We have found that unsubstituted D-homoestrone exerts a selective cytostatic effect on HeLa cells (IC_{50} = 5.5 μM), causing cell cycle arrest in the G2/M phase and inducing apoptosis, while its 3-methoxy counterpart has no impact on the proliferation of human reproductive cancer cell lines (HeLa, MCF-7 and A2780) [5]. We earlier reported an efficient route for the production of D-homoestrone from natural estrone, via the key δ-alkenyl-D-secoestrone-3-benzyl ether 17-carbaldehyde (**1**) [6]. The latter derivative readily takes part in ring-closure reactions due to the favorable position of its aldehyde and alkenyl moieties. In a preliminary publication, we described that reduction or oxidation of the aldehyde function of the 3-methoxy counterpart of the secoaldehyde leads to a secoalcohol or a secocarboxylic acid, which furnish 16-halomethyl-17-oxa derivatives in haloetherification or halolactonization processes [7]. In the 3-benzyl ether series only iodoetherification of the 3-benzyl-secoalcohol was carried out in order to synthetize cytotoxic estrone-talaromycin hybrids [8]. The resulting diastereomeric mixture of iodomethyl tetrahydropyrans was not separated as the new stereogenic center was lost in the following reaction step. In *in vitro* cytotoxicity tests of the natural product hybrids on A549 human lung cancer cells, the effective dosage for the most potent derivative proved comparable with that of the well-known anticancer agent cyclophosphamide. In a continuation of our earlier work, we now set out to produce 3-benzyloxy-D-homoestrone derivatives by the elaborated synthetic routes [6,7], extending the haloetherification and halolactonization with selenoetherification and selenolactonization. The 3-benzyl ether protecting group was preferred because of its easy removal. We planned to examine whether the synthetized D-homoestrones themselves have antiproliferative properties, without the formation of steroid-toxin hybrids. The potential antitumor activities of the newly synthetized D-homo derivatives were tested *in vitro* on four human reproductive cancer cell lines (HeLa, MCF-7, A2780 and Ishikawa) and one epidermoid tumor cell line (A431). Examination of the influence of the protecting group on C-3 and the substituent on C-16 on the antiproliferative properties was also projected. The tumor specificity of the potent compounds was investigated on noncancerous human foreskin fibroblast cells.

Besides the synthesis and pharmacological testing of the D-homoestrones, we planned to investigate the precursors of the cyclization reactions and their derivatives. The 3-benzyloxy secoalcohol was modified at three sites in the molecule: the alcoholic and/or phenolic hydroxy moiety and the alkenyl side-chain. The hydroxy groups were esterified under solvent-free microwave irradiation. The unsaturated side-chain was saturated under the conditions used for hydrogenolysis of the benzyl ether function.

2. Experimental

2.1. Chemistry

Melting points (mp) were determined with a Kofler hot-stage apparatus and are uncorrected. Elemental analyses were performed with a Perkin-Elmer CHN analyzer model 2400. Thin-layer chromatography: silica gel 60 F_{254}; layer thickness 0.2 mm (Merck); eluents: (A) diisopropyl ether, (B) dichloromethane, (C) 70% dichloromethane/30% hexane, (D) 2% ethyl acetate/98% dichloromethane; detection with iodine or UV (365 nm) after spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid and heating at 100–120 °C for 10 min. Flash chromatography: silica gel 60, 40–63 μm (Merck). The reactions under microwave irradiation were carried out with a CEM Corporation focused microwave system, Model Discover SP. ^{1}H NMR spectra were recorded in $CDCl_3$ solution (if not otherwise stated) with a Bruker DRX-500 instrument at 500 MHz, with Me_4Si as internal standard. ^{13}C NMR spectra were recorded with the same instrument at 125 MHz under the same conditions. Full scan mass spectra of the compounds were acquired in the range 50 to 800 *m/z* with an Agilent 500MS Ion trap mass spectrometer equipped with an electrospray ionization source. Analyses were performed in positive ion mode. The spectra were collected by continuous infusion of the steroid solution at 10 ng/μl in 1:1 acetonitrile/5 mM ammonium formate (v/v %) at a flow rate of 15 μl/min.

2.1.1. General procedure for the preparation of 17a-hydroxy-D-homoestrone derivatives **3** and **5**

Compound **2** [6] (372 mg, 1.00 mmol) or **4** [6] (284 mg, 1.00 mmol) was dissolved in methanol (20 ml), and potassium borohydride (270 mg, 5.00 mmol) was added in small portions in an ice-water bath. The mixture was allowed to stand at room temperature for 3 h, then poured onto ice (20 g) and acidified with dilute hydrochloric acid to pH 3. The precipitate that separated out was filtered off, washed until free from acid and dried. The crude product was subjected to column chromatography.

2.1.1.1. 3-Benzyloxy-17aβ-hydroxy-D-homoestra-1,3,5(10),16-tetraene (**3**)

According to section 2.1.1, compound **2** (372 mg, 1.00 mmol) was reacted with potassium borohydride (270 mg, 5.00 mmol). After work-up, the crude product was subjected to column chromatography with dichloromethane as eluent. **3** was obtained as a white solid (355 mg, 95%). Mp 202-205 °C, R_f = 0.40[A]. Anal. Calcd. for $C_{26}H_{30}O_2$: C, 83.38; H, 8.07. Found: C, 83.52; H, 7.95.

^{1}H NMR δ ppm 0.83(s, 3H, 18-H_3), 2.83(m, 2H, 6-H_2), 3.94(s, 1H, 17a-H), 5.04(s, 2H, OCH_2), 5.52(m, 1H) and 5.74(m, 1H): 16-H and 17-H, 6.71(d, 1H, *J* = 2.3 Hz, 4-H), 6.78(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.23(d, 1H, *J* = 8.5 Hz, 1-H), 7.24(m, 1H, 4'-H), 7.34(m, 2H, 3'-H and 5'-H), 7.42(m, 2H, 2'-H and 6'-H). ^{13}C NMR δ ppm 11.0(C-18), 26.2(2C), 27.1, 30.1, 37.4(C-13), 37.5, 39.8, 43.0, 43.8, 70.0(OCH_2), 78.1(C-17a), 112.4(C-2), 114.5(C-4), 126.4(C-1), 127.4(2C: C-2',6'), 127.7, 127.8(C-4'), 128.5(2C: C-3',5'), 130.1, 133.0(C-10), 137.3(C-1'), 137.9(C-5), 156.8(C-3). MS m/z (%): 375 (100), 397 (54), 91 (42).

2.1.1.2. 3,17aβ-Dihydroxy-D-homoestra-1,3,5(10)-triene (**5**)

According to section 2.1.1, compound **4** (284 mg, 1.00 mmol) was reacted with potassium borohydride (270 mg, 5.00 mmol). After work-up, the crude product was subjected to column chromatography with dichloromethane as eluent. **5** was obtained as a white solid (243 mg, 85%). Melting point 208-209 ºC [9]: mp 209–210 ºC. Compound **5** is identical with the compound described in [9]. Anal. Calcd. for $C_{19}H_{26}O_2$: C, 79.68; H, 9.15. Found: C, 79.92; H, 9.05.

^{1}H NMR (DMSO-d_6) δ ppm 0.73(s, 3H, 18-H_3), 2.67(m, 2H, 6-H_2), 4.29(m, 1H, 17a-H),

6.42(m, 1H, 4-H), 6.50(m, 1H, 2-H), 7.05(m, 1H, 1-H), 8.96(s, 1H, 3-OH).

2.1.2. 3-Benzyloxy-13α-hydroxymethyl-14β-(prop-2-en-yl)-des-D-estra-1,3,5(10)-triene (**6**)
Compound **1** (374 mg, 1.00 mmol) was dissolved in a 1:1 mixture of dichloromethane and methanol, and potassium borohydride (270 mg, 5.00 mmol) was added in small portions in an ice-water bath. The mixture was allowed to stand at room temperature for 0.5 h, then diluted with water and extracted with dichloromethane. The combined organic phases were washed with water until neutral and dried over sodium sulfate, and the crude product was subjected to column chromatography with dichloromethane as eluent. **6** was obtained as a white solid (327 mg, 87%). Oil, R_f = 0.53[D]. Anal. Calcd. for $C_{26}H_{32}O_2$: C, 82.94; H, 8.57. Found: C, 83.05; H, 8.45. ^{1}H NMR δ ppm 0.80(s, 3H, 18-H_3), 2.85(m, 2H, 6-H_2), 3.30 and 3.61(2xm, 2x1H, 17-H_2), 5.03(m, 2H, 16a-H_2), 5.04(s, 2H, OCH_2), 5.93(m, 1H, 16-H), 6.73(d, 1H, *J* = 2.3 Hz, 4-H), 6.79(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.22(d, 1H, *J* =8.5 Hz, 1-H), 7.32(t, 1H, *J* = 7.3 Hz, 4'-H), 7.38(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.43(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 16.2(C-18), 26.2, 27.6, 30.4, 32.4, 35.9, 38.9(C-13), 41.2, 43.5, 44.3, 70.0(OCH_2), 71.0(C-17), 112.4(C-2), 114.3(C-16a), 114.4(C-4), 126.4(C-1), 127.4(2C: C-2',6'), 127.8(C-4'), 128.5(2C: C-3',5'), 133.1(C-10), 137.3(C-1'), 138.0(C-5), 140.5(C-16), 156.8(C-3). MS m/z (%): 431 (100).

2.1.3. 3-Benzyloxy-14β-(prop-2-en-yl)-des-D-estra-1,3,5(10)-trien-13α-carboxylic acid (**7**) and 3-benzyloxy-14β-(prop-2-en-yl)-des-D-estra-1,3,5(10)-trien-13α,9α-carbolactone (**8**)
Compound **1** (374 mg, 1.00 mmol) was dissolved in acetone (3 ml), then cooled in an ice-water bath and Jones reagent (0.4 ml, 8 N) was added in three portions. The reaction mixture was allowed to stand at room temperature for 4 h, then diluted with water and extracted with diethyl ether. The combined organic phases were washed with water until neutral and dried over sodium sulfate, and the crude product was subjected to column chromatography with dichloromethane as eluent. The first-eluted **8** was obtained as a white solid (144 mg, 37%). Mp 110-118 °C, R_f = 0.63[D]. Anal. Calcd. For $C_{26}H_{28}O_3$: C, 80.38; H, 7.26. Found: C, 80.45; H, 7.12. ^{1}H NMR δ ppm 1.22(s, 3H, 18-H_3), 2.73(m, 2H, 6-H_2), 5.05(s, 2H, OCH_2), 5.09–5.16(overlapping multiplets, 2H, 16a-H_2), 5.85(m, 1H, 16-H), 6.71(d, 1H, *J* = 2.3 Hz, 4-H), 6.85(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.26(d, 1H, *J* = 8.5 Hz, 1-H), 7.34(t, 1H, *J* = 7.3 Hz, 4'-H), 7.39(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.42(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 18.6(C-18), 25.3, 26.8, 30.1, 31.9, 34.5, 40.4(C-13), 44.7, 45.2, 69.9(OCH_2), 80.6(C-9), 113.5(C-2), 114.3(C-4), 117.0(C-16a), 127.4(2C: C-2',6'), 127.9(C-4'), 128.5(2C: C-3',5'), 129.1(C-1), 130.2 (C-10), 136.2(C-16), 136.9(C-1'), 139.3 (C-5), 158.7(C-3), 178.2(C-17). MS m/z (%): 446 (100), 411 (42).
Continued elution yielded **7**, which was obtained as a white solid (223 mg, 57%). Mp 120-122 °C. R_f = 0.32[D]. Anal. Calcd. for $C_{26}H_{30}O_3$: C, 79.97; H, 7.74. Found: C, 80.03; H, 7.68., ^{1}H NMR δ ppm 1.19(s, 3H, 18-H_3), 2.84(m, 2H, 6-H_2), 5.01(m, 2H, 16a-H_2), 5.05(s, 2H, OCH_2), 5.88(m, 1H, 16-H), 6.73(d, 1H, *J* = 2.3 Hz, 4-H), 6.80(dd, 1H, *J* = 8.5 Hz, *J*= 2.3 Hz, 2-H), 7.20(d, 1H, *J* = 8.5 Hz, 1-H), 7.32(t, 1H, *J* = 7.3 Hz, 4'-H), 7.39(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.43(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 15.0(C-18), 25.8, 27.1, 28.7, 30.2, 35.7, 37.4, 40.9, 43.0, 45.4, 47.4(C-13), 70.0(OCH_2), 112.5(C-2), 114.5(C-4), 115.3(C-16a), 126.4(C-1), 127.4(2C: C-2',6'), 127.9(C-4'), 128.5(2C: C-3',5'), 132.4(C-10), 137.2(C-1'), 137.9(C-5), 138.8(C-16), 156.8(C-3), 184.5(C-17). MS m/z (%): 435 (100).

2.1.4. 3-Benzyloxy-16β-bromomethyl-17-oxa-D-homoestra-1,3,5(10)-triene (**9a**)
Compound **6** (188 mg, 0.50 mmol) was dissolved in dichloromethane (5 ml), then cooled in an ice-salt bath to -15 ºC, and *N*-bromosuccinimide (89 mg, 0.50 mmol) was added in three

portions. The mixture was stirred for 30 min, the solvent was evaporated off and the residue was subjected to column chromatography with 70% dichloromethane/30% hexane, yielding **9a** as a white solid (155 mg, 68%). Mp 107–108 °C, $R_f = 0.51^C$. Anal. Calcd. for $C_{26}H_{31}BrO_2$: C, 68.57; H, 6.86. Found: C, 68.82; H, 6.75. ^{1}H NMR δ ppm 1.01(s, 3H, 18-H_3), 2.84(m, 2H, 6-H_2), 3.17(m, 1H) and 3.58(overlapping multiplets, 2H): 17-H_2 and 16-H, 3.46(m, 2H, 16a-H_2), 5.04(s, 2H, OCH_2), 6.73(d, 1H, *J* = 2.3 Hz, 4-H), 6.79(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.21(d, 1H, *J* = 8.5 Hz, 1-H), 7.30(t, 1H, *J* = 7.3 Hz, 4'-H), 7.37(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.42(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 16.5(C-18), 25.5, 25.7, 28.8, 29.9, 34.0(C-13), 35.1, 36.2, 38.5, 43.7, 47.2, 69.9(OCH_2), 77.6(C-16), 79.9(C-17), 112.4(C-2), 114.6(C-4), 126.1(C-1), 127.4(2C: C-2',6'), 127.8(C-4'), 128.5(2C: C-3',5'), 132.8(C-10), 137.3(C-1'), 137.8(C-5), 156.8(C-3). MS m/z (%): 457 (63), 455 (100), 453 (37).

2.1.5. 3-Benzyloxy-16β-iodomethyl-17-oxa-D-homoestra-1,3,5(10)-triene (**9b**) and 3-benzyloxy-16α-iodomethyl-17-oxa-D-homoestra-1,3,5(10)-triene (**10b**)

Compound **6** (188 mg, 0.50 mmol) was dissolved in diethyl ether (2 ml), and water (0.7 ml), sodium hydrogencarbonate (84 mg, 1.00 mmol) and iodine (127 mg, 0.50 mmol) were then added. The mixture was stirred for 1 h at room temperature, diluted with water and extracted with diethyl ether. The organic phase was separated and washed with a saturated solution of sodium thiosulfate and then with water. The organic phase was dried over sodium sulfate and evaporated, and the residue was subjected to column chromatography with 70% dichloromethane/30% hexane. **9b** eluted first as a white solid (127 mg, 50%). Mp 105–108 °C, $R_f = 0.51^C$. Anal. Calcd. for $C_{26}H_{31}IO_2$: C, 62.15; H, 6.22. Found: C, 62.02; H, 6.35. ^{1}H NMR δ ppm 1.01(s, 3H, 18-H_3), 2.85(m, 2H, 6-H_2), 3.18 and 3.58(2xm, 2x1H, 17-H_2), 3.26–3.33(overlapping multiplets, 3H, 16a-H_2 and 16-H), 5.04(s, 2H, OCH_2), 6.73(d, 1H, *J* = 2.3 Hz, 4-H), 6.79(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.21(d, 1H, *J* = 8.5 Hz, 1-H), 7.32(t, 1H, *J* = 7.3 Hz, 4'-H), 7.38(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.43(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 10.6(C-16a), 16.7(C-18), 25.4, 25.7, 29.9, 30.5, 33.9(C-13), 35.0, 38.5, 43.7, 47.2, 69.9(OCH_2), 77.4(C-16), 79.9(C-17), 112.4(C-2), 114.6(C-4), 126.1(C-1), 127.4(2C: C-2',6'), 127.8(C-4'), 128.5(2C: C-3',5'), 132.8(C-10), 137.3(C-1'), 137.8(C-5), 156.8(C-3). MS m/z (%): 504 (32), 503 (100), 214 (21), 113 (26).

Continued elution yielded **10b** as a white solid (64 mg, 25%). Mp 112–113 °C, $R_f = 0.47^C$. Anal. Calcd. for $C_{26}H_{31}IO_2$: C, 62.15; H, 6.22. Found: C, 62.28; H, 6.18. ^{1}H NMR δ ppm 1.02(s, 3H, 18-H_3), 2.84(m, 2H, 6-H_2), 3.20(m, 2H, 16a-H_2), 3.40 and 3.56(2xm, 2x1H, 17-H_2), 4.23 (m, 1H, 16-H), 5.04(s, 2H, OCH_2), 6.72(d, 1H, *J* = 2.3 Hz, 4-H), 6.79(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.24(d, 1H, *J* = 8.5 Hz, 1-H), 7.32(t, 1H, *J* = 7.3 Hz, 4'-H), 7.38(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.43(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 6.9(C-16a), 15.9(C-18), 25.3, 25.6, 29.8, 30.5, 34.2(C-13), 35.5, 38.3, 41.7, 43.7, 69.9(OCH_2), 72.5(C-17), 73.7(C-16), 112.4(C-2), 114.6(C-4), 126.0(C-1), 127.4(2C: C-2',6'), 127.8(C-4'), 128.5(2C: C-3',5'), 132.8(C-10), 137.3(C-1'), 137.8(C-5), 156.8(C-3). MS m/z (%): 504 (30), 503 (100).

2.1.6. 3-Benzyloxy-16β-phenylselenylmethyl-17-oxa-D-homoestra-1,3,5(10)-triene (**9c**) and 3-benzyloxy-16α-phenylselenylmethyl-17-oxa-D-homoestra-1,3,5(10)-triene (**10c**)

Compound **6** (188 mg, 0.50 mmol) was dissolved in acetonitrile (5 ml), and phenylselenyl bromide (118 mg, 0.50 mmol) was added in three portions. After stirring for 1 h at room temperature, potassium carbonate (70 mg, 0.50 mmol) was added and the stirring was continued for 1 h. The solvent was evaporated off and the residue was subjected to column chromatography with 70% dichloromethane/30% hexane. **9c** eluted first as a white solid (192 mg, 36%). Mp 135–137 °C, $R_f = 0.47^C$. Anal. Calcd. for $C_{32}H_{36}O_2Se$: C, 72.30; H, 6.83.

Found: C, 72.44; H, 6.95. ^{1}H NMR δ ppm 1.01(s, 3H, 18-H_3), 2.83(m, 2H, 6-H_2), 3.06 (m, 1H) and 3.14(overlapping multiplets, 2H) and 3.55(overlapping multiplets, 2H): 16-H and 16a-H_2 and 17-H_2, 5.04(s, 2H, OCH_2), 6.72(d, 1H, *J* = 2.3 Hz, 4-H), 6.79(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.21(d, 1H, *J* = 8.5 Hz, 1-H), 7.24–7.28 (overlapping multiplets, 3H) and 7.54 (m, 2H): 2",3",4",5",6"-H, 7.32(t, 1H, *J*= 7.3 Hz, 4'-H), 7.38(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.43(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 16.6(C-18), 25.5, 25.7, 29.9, 30.3, 33.8, 34.0(C-13), 35.2, 38.5(C-8), 43.7(C-9), 47.4(C-14), 69.9(OCH_2), 77.9(C-16), 80.0(C-17), 112.4(C-2), 114.6(C-4), 126.1(C-1), 126.7(2C: C-2',6'), 127.4(2C: C-3',5'), 127.8(C-4'), 128.5(2C: C-3",5"), 129.0(C-4"), 130.8(C-1"), 132.5(2C: C-2",6"), 132.9(C-10), 137.3(C-1'), 137.8(C-5), 156.8(C-3). MS m/z (%): 550 (34), 549 (100).
Continued elution yielded **10c** (192 mg, 36%). Mp 142–143 °C, R_f = 0.40[C]. Anal. Calcd. for $C_{32}H_{36}O_2Se$: C, 72.30; H, 6.83. Found: C, 72.38; H, 6.95. ^{1}H NMR δ ppm 1.00(s, 3H, 18-H_3), 2.80(m, 2H, 6-H_2), 3.11 and 3.40(2xm, 2x1H, 16a-H_2), 3.19 and 3.32(2xd, 2x1H, *J* = 11.2 Hz, 17-H_2), 4.24(m, 1H, 16-H), 5.03(d, 2H, OCH_2), 6.71(d, 1H, *J* = 2.3 Hz, 4-H), 6.78(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.19(d, 1H, *J* = 8.5 Hz, 1-H), 7.22–7.26 (overlapping multiplets. 3H): 3",4",5"-H, 7.32(t, 1H, *J* = 7.2 Hz, 4'-H), 7.38(t, 2H, *J* = 7.2 Hz, 3'-H and 5'-H), 7.43(d. 2H, *J* = 7.2 Hz, 2'-H and 6'-H), 7.54 (m, 2H, 2"-H and 6"-H), ^{13}C NMR δ ppm 16.1(C-18), 25.4, 25.5, 25.9, 29.4(C-16a), 29.8(C-6), 34.2, 35.6, 38.4(C-8), 41.8(C-9), 43.7(C-14), 69.9(OCH_2), 72.8(C-17), 73.1(C-16), 112.4(C-2), 114.6(C-4), 126.1(C-1), 126.9(2C: C-2',6'), 127.4(2C: C-3',5'), 127.8(C-4'), 128.5(2C: C-3",5"), 12.91(C-4"), 130.1(C-1"), 132.7(2C: C-2",6"), 132.9(C-10), 137.3(C-1'), 137.9(C-5), 156.8(C-3). MS m/z (%): 549 (100).

2.1.7. 3-Benzyloxy-16β-bromomethyl-17-oxa-D-homoestra-1,3,5(10)-trien-17a-one (**11a**)
Compound **7** (195 mg, 0.50 mmol) was dissolved in dichloromethane (5 ml), and *N*-bromosuccinimide (89 mg, 0.50 mmol) was added. The mixture was stirred at room temperature for 1 h. The solvent was evaporated off and the residue was subjected to column chromatography with dichloromethane as eluent. **11a** was obtained (193 mg, 82%). Mp 150–155 °C, R_f = 0.63[D]. Anal. Calcd. for $C_{26}H_{29}BrO_3$: C, 66.53; H, 6.23. Found: C, 66.72; H, 6.35. ^{1}H NMR δ ppm 1.26(s, 3H, 18-H_3), 2.88(m, 2H, 6-H_2), 3.56–3.62(overlapping multiplets, 2H, 16a-H_2), 4.54(m, 1H, 16-H), 5.04(s, 2H, OCH_2), 6.74(d, 1H, *J* = 2.3 Hz, 4-H), 6.80(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.22(d, 1H, *J* = 8.5 Hz, 1-H), 7.32(t, 1H, *J* = 7.3 Hz, 4'-H), 7.38(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.42(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 18.5(C-18), 25.4, 26.2, 26.3, 29.7, 34.2, 35.3, 37.7, 41.3(C-13), 42.6, 43.1, 70.0(OCH_2), 78.3(C-16), 112.6(C-2), 114.6(C-4), 126.2 and 127.4(2C) and 127.9 and 128.5(2C): C-1,2',3',4',5',6', 131.9(C-10), 137.2(C-1'), 137.4(C-5), 157.0(C-3), 176.3(C-17). MS m/z (%): 491 (63), 469 (100).

2.1.8. 3-Benzyloxy-16β-iodomethyl-17-oxa-D-homoestra-1,3,5(10)-trien-17a-one (**11b**)
Compound **7** (195 mg, 0.50 mmol) was dissolved in a 1:1 mixture (5 ml) of dichloromethane and tetrahydrofuran, and saturated sodium hydrogencarbonate solution (10 ml, pH = 9) and iodine (127 mg, 0.50 mmol) were added. The mixture was stirred for 1 h at room temperature, diluted with water and extracted with dichloromethane. The organic phase was separated out and washed with a saturated solution of sodium thiosulfate and with water. The organic phase was dried over sodium sulfate and evaporated, and the residue was subjected to column chromatography with dichloromethane as eluent. **11b** was obtained (217 mg, 84%). Mp 158–160 °C, R_f = 0.66[D]. Anal. Calcd. for $C_{26}H_{29}IO_3$: C, 60.47; H, 5.66. Found: C, 60.32; H, 5.75. ^{1}H NMR δ ppm 1.26(s, 3H, 18-H_3), 2.89(m, 2H, 6-H_2), 3.43(m, 2H, 16a-H_2), 4.22(m, 1H, 16-H), 5.04(s, 2H, OCH_2), 6.73(d, 1H, *J* = 2.3 Hz, 4-H), 6.80(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.21(d, 1H, *J* = 8.5 Hz, 1-H), 7.32(t, 1H, *J* = 7.3 Hz, 4'-H), 7.38(t, 2H, *J* = 7.3 Hz, 3'-H

and 5'-H), 7.42(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 9.3(C-16a), 18.7(C-18), 25.4, 26.3, 28.2, 29.7, 34.2, 37.7, 41.2(C-13), 42.7, 43.2, 69.9(OCH_2), 78.3(C-16), 112.6(C-2), 114.6(C-4), 126.2 and 127.4(2C) and 127.9 and 128.5(2C): C-1,2',3',4',5',6', 131.9(C-10), 137.2(C-1'), 137.3(C-5), 156.9(C-3), 176.4(C-17). MS m/z (%): 517 (58), 113 (100).

2.1.9. 3-Benzyloxy-16β-phenylselenylmethyl-17-oxa-D-homoestra-1,3,5(10)-trien-17a-one (**11c**)

Compound **7** (195 mg, 0.50 mmol) was dissolved in acetonitrile (5 ml), and phenylselenyl bromide (118 mg, 0.50 mmol) was added in three portions. After stirring for 1 h at room temperature, potassium carbonate (70 mg, 0.5 mmol) was added and the stirring was continued for 1 h. The solvent was evaporated off and the residue was subjected to column chromatography with dichloromethane as eluent. **11c** was obtained (216 mg, 79%). Mp 175–178 °C, $R_f = 0.66^D$. Anal. Calcd. for $C_{32}H_{34}O_3Se$: C, 70.45; H, 6.28. Found: C, 70.62; H, 6.14. ^{1}H NMR δ ppm 1.26(s, 3H, 18-H_3), 2.86(m, 2H, 6-H_2), 3.15 and 3.29(2xm, 2x1H, 16a-H_2), 4.52(m, 1H, 16-H), 5.04(s, 2H, OCH_2), 6.73(d, 1H, *J* = 2.3 Hz, 4-H), 6.80(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.21(d, 1H, *J* = 8.5 Hz, 1-H), 7.26–7.34(overlapping multiplets, 4H: 4',3",4",5"-H), 7.39(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.43(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), 7.56(m, 2H, 2"-H and 6"-H). ^{13}C NMR δ ppm 18.6(C-18), 25.4, 26.2, 27.4, 29.7, 33.3, 34.2, 37.8, 41.3(C-13), 42.7, 43.5, 69.9(OCH_2), 79.9(C-16), 112.2(C-2), 114.6(C-4), 126.2 and 127.4(3C) and 127.9 and 128.5(2C) and 129.3(2C): C-1,2',3',4',5',6',3",4",5", 129.6(C-1"), 132.0(C-10), 132.8(2C: C-2",6"), 137.2(C-1'), 137.4(C-5), 157.0(C-3), 176.8(C-17). MS m/z (%): 569 (100), 567 (53), 501 (47), 91 (42).

2.1.10. General procedure for the preparation of derivatives **12a** and **12d**A suspension of **6** (188 mg, 0.50 mmol) or **12c** (210 mg, 0.50 mmol) and Pd/C (0.30 g, 10%) in ethyl acetate (20 ml) was subjected to 20 bar of hydrogen pressure at room temperature for 3 h. The catalyst was then removed by filtration through a short pad of silica gel. After evaporation of the solvent *in vacuo*, the crude product was subjected to column chromatography with dichloromethane as eluent.

2.1.10.1. 3-Hydroxy-13α-hydroxymethyl-14β-propyl-des-D-estra-1,3,5(10)-triene (**12a**)

According to section 2.1.10, compound **6** (188 mg, 0.50 mmol) was subjected to hydrogenolysis. The chromatographic purification of the crude product yielded **12a** (118 mg, 87%). Mp 60–62 °C, $R_f = 0.17^D$. Anal. Calcd. for $C_{19}H_{28}O_2$: C, 79.12; H, 9.78. Found: C, 78.95; H, 9.86. ^{1}H NMR δ ppm 0.77(s, 3H, 18-H_3), 2.82(m, 2H, 6-H_2), 3.35 and 3.52(2xd, 2x1H, *J* = 10.9 Hz, 17-H_2), 6.56(d, 1H, *J* = 2.3 Hz, 4-H), 6.63(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.16(d, 1H, *J* = 8.5 Hz, 1-H), ^{13}C NMR δ ppm 14.7 and 15.9: C-18 and Ac-Me, 25.0, 26.4, 27.4, 30.5, 31.2, 35.6, 38.8(C-13), 41.8, 43.5, 45.3, 71.4(C-17), 112.8(C-2), 114.9(C-4), 126.7(C-1), 132.9(C-10), 138.3(C-5), 153.5(C-3). MS m/z (%): 289 (100).

2.1.10.2. 13α-acetoxymethyl-3-hydroxy-14β-propyl-des-D-estra-1,3,5(10)-triene (**12d**)

According to section 2.1.10, compound **12c** (210 mg, 0.5 mmol, described in section 2.1.11) was subjected to hydrogenolysis. The chromatographic purification of the crude product yielded **12d** (145 mg, 88%). Mp 84–90 °C, $R_f = 0.37^D$. Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.33; H, 9.15. Found: C, 76.45; H. 9.22. ^{1}H NMR δ ppm 0.83(s, 3H, 18-H_3), 2.09(s, 3H, Ac-Me), 2.82(m, 2H, 6-H_2), 3.86 and 3.92(2xd, 2x1H, *J* = 11.2 Hz, 17-H_2), 4.75(s, 1H, OH), 6.56(d, 1H, *J* = 2.3 Hz, 4-H), 6.63(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.16(d, 1H, *J* = 8.5 Hz, 1-H), ^{13}C NMR δ ppm 14.5 and 15.9: C-18 and Ac-Me, 24.8, 26.3, 27.3, 30.5, 31.3, 36.0, 37.4(C-13), 41.7, 43.4, 45.8, 72.3(C-17), 112.7(C-2), 114.9(C-4), 126.7(C-1), 132.7(C-10), 138.2(C-5), 153.4(C-3), 170.0(Ac-CO). MS m/z (%): 385 (100).

2.1.11. General procedure for the synthesis of derivatives **12c** and **12b** Compound **6** (188 mg,

0.50 mmol) or **12a** (145 mg, 0.50 mmol) and acetic anhydride (0.1 ml, 1 mmol) were mixed with silica gel (500 mg), placed into a pressure tube equipped with a stirrer bar and inserted into the cavity of a Discover SP microwave system apparatus. The mixture was heated at 160 ºC for 5 min, then poured onto water and extracted with diethyl ether. The organic phase was washed with water until neutral, dried over anhydrous sodium sulfate, filtered and evaporated. The crude product was subjected to column chromatography.

2.1.11.1. 3-acetoxy-13α-acetoxymethyl-14β-propyl-des-D-estra-1,3,5(10)-triene (**12b**)
According to section 2.1.11, compound **6** (188 mg, 0.50 mmol) was reacted with acetic anhydride (0.1 ml, 1 mmol) in a microwave reactor. The crude product was subjected to column chromatography with dichloromethane as eluent, yielding **12b** (160 mg, 86%). Oil, $R_f = 0.69^D$. Anal. Calcd. for $C_{23}H_{32}O_4$: C, 74.16; H, 8.66. Found: C, 74.25; H, 8.82. ^{1}H NMR δ ppm 0.83(s, 3H, 18-H_3), 2.09 and 2.28(2xs, 2x3H, 2xAc-Me), 2.86(m, 2H, 6-H_2), 3.86 and 3.92(2xd, 2x1H, *J* = 11.3 Hz, 17-H_2), 6.79(d, 1H, *J* = 2.3 Hz, 4-H), 6.83(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.27(d, 1H, *J* = 8.5 Hz, 1-H), ^{13}C NMR δ ppm 14.1 and 15.5 and 20.7: C-18 and 17-Ac-Me and 3-Ac-Me, 24.4, 25.8, 26.8, 30.0, 30.9, 35.6, 36.9(C-13), 40.9, 43.3, 45.4, 71.7(C-17), 118.2, 120.9, 126.2(C-4), 137.6 and 137.8: C-5 and C-10, 148.0(C-3), 169.4 and 170.9: C-17-Ac-CO and C-3-Ac-CO. MS m/z (%): 427 (100).

2.1.11.2. 13α-acetoxymethyl-3-benzyloxy-14β-(prop-2-en-yl)-des-D-estra-1,3,5(10)-triene (**12c**)
According to section 2.1.11, compound **12a** (145 mg, 0.50 mmol) was reacted with acetic anhydride (0.1 ml, 1 mmol) in a microwave reactor. The crude product of **12c** was subjected to column chromatography with 2% ethyl acetate/98% dichloromethane as eluent, yielding **12c** (191 mg, 91%). Mp 90–92 °C, $R_f = 0.78^D$. Anal. Calcd. for $C_{28}H_{34}O_3$: C, 80.35; H, 8.19. Found: C, 80.12; H, 8.22. ^{1}H NMR δ ppm 0.87(s, 3H, 18-H_3), 2.09(s, 3H, Ac-Me), 2.85(m, 2H, 6-H_2), 3.84 and 4.00(2xd, 2x1H, *J* = 11.2 Hz, 17-H_2), 4.97(m, 2H, 16a-H_2), 5.04(s, 2H, OCH_2), 5.84(m, 1H, 16-H), 6.72(d, 1H, *J* = 2.3 Hz, 4-H), 6.79(dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.21(d, 1H, *J* = 8.5 Hz, 1-H), 7.30(t, 1H, *J* = 7.3 Hz, 4'-H), 7.38(t, 2H, *J* = 7.3 Hz, 3'-H and 5'-H), 7.43(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), ^{13}C NMR δ ppm 16.2 and 20.9: C-18 and Ac-Me, 26.2, 27.5, 30.4, 32.7, 36.3, 37.6(C-13), 41.3, 43.4, 44.8, 69.9(OCH_2), 71.9(C-17), 112.4(C-2), 114.4(C-16a), 114.5(C-4), 126.4(C-1), 127.4(2C: C-2',6'), 127.8(C-4'), 128.5(2C: C-3',5'), 132.9(C-10), 137.3(C-1'), 137.9(C-5), 139.7(C-16), 156.8(C-3), 171.2(C-17). MS m/z (%): 431 (100).

2.1.12. Synthesis of 3-benzyloxy-13α-(*p*-tolylsulfonyloxymethyl)-14β-(prop-2-en-yl)-des-D-estra-1,3,5(10)-triene (**12e**)
Compound **6** (188 mg, 0.50 mmol) was dissolved in pyridine (3 ml), then cooled in an ice-water bath and 286 mg (1.50 mmol) of *p*-tolylsulfonyl chloride was added in small portions. After stirring for 3 h at room temperature, the reaction mixture was diluted with water (50 ml) and extracted with dichloromethane. The organic phase was washed with water until neutral, dried over anhydrous sodium sulfate, filtered and evaporated. The crude product was subjected to flash chromatography with dichloromethane as eluent, yielding **12e** (252 mg, 95%). Mp 105–110 °C, $R_f = 0.82^D$. Anal. Calcd. for $C_{33}H_{38}O_4S$: C, 74.68; H, 7.22. Found: C, 74.83; H, 7.35. ^{1}H NMR δ ppm 0.81(s, 3H, 18-H_3), 2.47(s, 3H, Ts-Me), 2.82(m, 2H, 6-H_2), 3.74 and 3.90(2xd, 2x1H, *J* = 9.4 Hz, 17-H_2), 4.92(m, 2H, 16a-H_2), 5.03(s, 2H, OCH_2), 5.75(m, 1H, 16-H), 6.71(d, 1H, *J* = 2.3 Hz, 4-H), 6.78 (dd, 1H, *J* = 8.5 Hz, *J* = 2.3 Hz, 2-H), 7.18(d, 1H, *J* = 8.5 Hz, 1-H), 7.32(t, 1H, *J* = 7.3 Hz, 4'-H), 7.36–7.39(overlapping multiplets,

4H: 3',5',3",5"-H), 7.43(d, 2H, *J* = 7.3 Hz, 2'-H and 6'-H), 7.82(d, 2H, *J* = 8.2 Hz, 2"-H and 6"-H), ^{13}C NMR δ ppm 15.7 and 21.4: C-18 and Ts-Me, 25.7, 27.1, 30.1, 32.4, 35.6. 37.9(C-13), 40.7, 42.8, 43.8, 69.7(OCH_2), 76.5(C-17), 112.2(C-2), 114.2(C-4), 114.5(C-16a), 126.1 and 127.2(2C) and 127.6 and 127.7(2C) and 128.3(2C): C-1,2',3',4',5',6',2",6", 129.5(2C: C-3",5"), 132.4(C-10), 132.7(C-1"), 137.0(C-1'), 137.6(C-5), 138.9(C-16), 144.4(C-4"), 156.5(C-3). MS m/z (%): 530 (100), 552 (45).

2.2. Antiproliferative (MTT) assay

The growth-inhibitory effects of the test compounds were determined on HeLa, MCF-7, A2780, Ishikawa and A431 cells isolated from cervical, breast, ovarian, endometrial and skin cancer, respectively. These cell lines were obtained from the European Collection of Cell Cultures, Salisbury, UK. The cells were cultivated in minimal essential medium (Sigma-Aldrich, Budapest, Hungary) supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic-antimycotic mixture. All media and supplements were obtained from Life Technologies (Paisley, Scotland, UK). All cell types were seeded into 96-well plates at a density of 5000 cells/well and incubated with increasing concentrations (0.1–30 μM) of the compounds at 37 °C under cell culturing conditions. After a 72-h incubation, cells were treated with 5.0 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution for 4 h, the precipitated formazan crystals were dissolved in dimethyl sulfoxide, and the absorbance was read at 545 nm with a microplate reader; wells with untreated cells were utilized as controls [10]. Sigmoidal concentration–response curves were fitted to the measured points, and the IC_{50} values were calculated by means of GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Cisplatin was used as a positive control in the same concentration range as the test compounds.

To determine the selective cytotoxic effect, compound **6** was subjected to the MTT assay with intact, noncancerous, human foreskin cells under the same experimental conditions.

2.3. Tubulin polymerization assay

The effect of 3-benzyloxy-D-secoalcohol (**6**) on tubulin polymerization was tested with the Tubulin Polymerization Assay Kit (Tebu-bio, Le Perray-en-Yvelines, France) according to the manufacturer's recommendations. Briefly, 10 µl of a 0.1 or 0.2 mM solution of the test compound (**6**) was placed on a prewarmed (37 °C), UV-transparent microplate. 10 µl 10 µM paclitaxel and 10 µl General Tubulin Buffer were used as positive and negative control, respectively. 100 µl 3.0 mg/ml tubulin in 80 mM PIPES pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP, 10.2% glycerol was added to each sample, and the microplate was immediately placed into a prewarmed (37 °C) UV-spectrophotometer (SpectoStarNano, BMG Labtech, Ortenberg, Germany) to start the recording reaction. A 60-min kinetic measurement protocol was applied to determine the absorbance of the reaction solution per minute at 340 nm. For evaluation of the experimental data, a tubulin polymerization curve was used in which optical density was plotted against time, and the maximum reaction rate (V_{max}: Δabsorbance/min) was calculated. Differences between absorbances determined at two consecutive timepoints were calculated and the highest difference was taken as the V_{max} of the tested compound in the tubulin polymerization reaction. Each sample was prepared in two parallels. For statistical evaluation, V_{max} data were analyzed by the unpaired Student's t-test by using Prism 4 software. The V_{max} values of the tested compounds were compared with the rate of the basal tubulin polymerization (i.e. the untreated control).

2.4. Docking calculations

Docking calculations were carried out according to the DockingServer methodology [11]. The MMFF94 force field [12] was used for energy minimization of the ligand molecule by using DockingServer. PM6 semiempirical charges calculated by MOPAC2009 [13] were ascribed to the ligand atoms. Nonpolar hydrogen atoms were merged, and rotatable bonds were defined.

Docking calculations were carried out on the tubulin structure with the pdb code 1Z2B. Essential hydrogen atoms, Kollman united atom type charges and solvation parameters were added with the aid of AutoDock tools [14]. Affinity (grid) maps of 25×25×25 Å grid points and 0.375 Å spacing were generated by using the Autogrid program [14]. The simulation box center was set to the center of the crystallized ligand. AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively.

Docking simulations were performed by using the Lamarckian genetic algorithm and the Solis & Wets local search method [15]. The initial positions, orientations and torsions of the ligand molecules were set randomly. Each docking experiment ivolved 50 different runs that were set to terminate after a maximum of $2.5x10^6$ energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å and quaternion and torsion steps of 5 were applied.

3. Results and discussion

3.1. Synthesis of D-homo- and D-secoestrones

The literature reveals several synthetic approaches for the formation of D homologs of estrone. The total synthesis of D-homoestrone (**4**) was first reported by Ananchenko *et al.* [16]. Semi-synthetic methods, and particularly the ring expansion of five-membered ring D of estrone analogs, are complex, multistep reactions and therefore exhibit low efficiency [17]. We recently developed an efficient route for the synthesis of D-homoestrone (**4**) from a δ-alkenyl-D-secoestrone-3-benzyl ether (**1**), which is available in several steps from estrone 3-benzyl ether via a Grob fragmentation as key step [6,18]. In the present work we used the D-secoaldehyde (**1**) for the synthesis of D-homoestradiol (**5**), as the impact of **5** on the proliferation of the human cancer cell lines HeLa, MCF-7, A2780, Ishikawa and A430 does not appear to have been described in the literature. The intramolecular Prins cyclization of **1** with 1.1 equivalents of *p*-toluenesulfonic acid and the subsequent Jones oxidation (and simultaneous β-elimination of the tosyloxy group) led to the desired α,β-unsaturated ketone (**2**, Scheme 1). The hydride reduction of **2** selectively yielded its 17aβ-hydroxy derivative (**3**). During the hydrogenation of **2**, hydrogenolysis of the 3-benzyl group and saturation of the 16,17-double bond occurred, leading to D-homoestrone (**4**), hydride reduction of which selectively yielded 17aβ-hydroxy-D-homoestradiol (**5**).

Scheme 1.

Hydride reduction of the D-secoaldehyde (**1**) with potassium borohydride or the oxidation of **1** with Jones reagent (8 N) was carried out in order to obtain the 3-benzyloxy-D-secoalcohol (**6**) and the 3-benzyloxy-D-secocarboxylic acid (**7**), which can be used as starting materials for electrophile-induced ring-closure reactions (Scheme 2). Jones oxidation of the aldehyde function in **1** was accompanied by the formation of a bridged lactone **8**, similarly as described earlier [7]. It was previously shown that the secoaldehyde in the 3-methoxy 13β-estrone and 13α-estrone series readily cyclizes and furnishes 17-oxa-D-homo-tetrahydropyrans or 17-oxa-D-homo-δ-lactones (NBS or I_2 were used as electrophile triggers). In the present work, we carried out halo- or selenoetherification and halo- or selenolactonization reactions to obtain 16-halomethyl- or 16-phenylselenylmethyl-substituted 17-oxa-D-homo-3-benzyl ethers. Bromoetherification of **6** with an equimolar amount of NBS in MeCN afforded **9a** (Scheme 1, Table 1, Entry 1). The reaction was carried out at -15 °C to avoid the formation of 2-bromo derivatives by electrophile substitution. A 2:1 diastereomeric ratio was observed in the iodoetherification with iodine, yielding a mixture of **9b** and **10b** (Table 1, Entry 2). The application of phenylselenyl bromide as electrophile trigger led to a different isomeric ratio (**9c** + **10c** = 1:1, Table 1, Entry 3). The configuration at C-16 in the tetrahydropyrans **10** was established from NOESY experiments, which demonstrated a crosspeak between the multiplet of 16-H and the singlet of the angular methyl group. The orientation of the 16a substituent in the minor diastereomers (**10**) is therefore the opposite (α) of that of the angular methyl group. The electrophile-induced cyclizations of the secocarboxylic acid **7** furnished one diastereomer **11** stereoselectively (Table 1, Entries 4–6). NOESY experiments revealed the β-position for the 16 substituent. These results are in good agreement with the earlier observations in the 3-methoxy series [7].

Scheme 2.

Table 1.

For functionalization of the alcoholic and phenolic hydroxy groups in the secoalcohol **6**

(Figure 1, Table 2), esterification was carried out by using acetic anhydride under microwave irradiation on a solid support, since microwave heating is a very effective and non-polluting method of activation. Irradiation under solvent-free conditions usually leads to higher conversions and shorter reaction times and has several environmental advantages (green chemistry) [19]. **6** was subjected to hydrogenolysis in order to remove the benzyl protecting group and to saturate the alkenyl side-chain (Table 2, Entry 1). The resulting diol **12a** was acetylated with acetic anhydride on a silica gel support in a microwave reactor, yielding **12b** (Table 2, Entry 2). The partially acetylated **12c** was synthetized from the secoalcohol **6** by microwave-induced acetylation (Table 2, Entry 3). Removal of the benzyl ether group from **12c** led to **12d** (Table 2, Entry 4). Tosylation of **6** furnished **12e** (Table 2, Entry 5).

Figure 1.

Table 2.

3.2. Determination of antiproliferative properties of the newly synthetized compounds (6–12) and the effect of secoalcohol 6 on tubulin polymerization

Microtubuli are polymers of tubulin, which in turn is a dimer involving an α unit and a β unit. They have a wide range of biological functions, including maintenance of the cytoskeleton and the organization of intracellular transport, but the formation of mitotic spindles is generally regarded as the most relevant and is the most intensively investigated aspect [20]. A variety of cytotoxic agents such as colchicine, vinca alkaloids, taxans, combrestatin and 2-methoxyestradiol (2ME) influence the equilibrium between the polymerization and degradation of the microtubuli, and are therefore able to influence cell division in a cell cycle phase-specific manner [21]. It has recently been established that some agents that interact with tubulin are also able to target the vascular system of tumors [22], causing vasculature recession or vasculature 'normalization' [23]. 2ME, a well-known nonpolar endogenous metabolite of 17β-estradiol, is a potent antiproliferative, apoptotic and antiangiogenic agent *in vitro* and *in vivo* at pharmacological concentrations [24]. Investigation of its antitumor activity on different malignancies in clinical studies revealed that 2ME can be tolerated up to very high doses (e.g. 1200 mg/day) [25,26]. The most important mechanisms of its action seem to be the inhibition of neoangiogenesis, microtubule disruption and the upregulation of factors regulating extrinsic and intrinsic apoptotic pathways [27]. Cushman *et al.* reported that 2-ethoxyestradiol is a more potent cytotoxic agent than 2ME in cancer cell cultures, and is also a tubulin polymerization inhibitor [28]. Hillisch *et al.* patented the finding that some D-homoestra-1,3,5(10)-trien-3-yl 2-substituted sulfamates are potential pharmaceuticals for the treatment of tumorous diseases [4] by acting as tubulin polymerization inhibitors. We recently characterized the antitumor activities of 13-epimers of D-homoestrone and their 3-methyl ether counterparts on HeLa, MCF-7 and Ishikawa cells, providing the first evidence that D-homoestrone selectively suppresses HeLa cell proliferation and modulates cell cycle progression, leading to the accumulation of cells in the G2/M phase. Moreover, no significant *in vivo* estrogenic activity was observed as concerns the weight gain of the uterine tissue of gonadectomized rats after a 7-day treatment with D-homoestrone. The 3-methyl ether and 13-*epi* counterparts did not display any effect on the proliferation of the tested cells [5].

In the present study, we tested D-homoestrone derivatives that are readily available via the D-homoestrone synthetic pathway [5]. Compounds **2**–**5** differ only in the functional groups on C-3 and C-17a, and possess no other substituents. I*n vitro* antiproliferative tests were carried out on a panel of human adherent cancer cell lines (Table 3). Compounds **3** and **5** proved to exert substantial cell-line selective antiproliferative activities against ovarian and cervical cancer cell lines, respectively. 17aβ-D-Homoestradiol (**5**) behaved similarly to D-homoestrone (**4**) [5]. The 3-benzyl ether of the unsaturated ketone **2** was not active. As concerns the results for **2**–**5** and D-homoestrone-3-methyl ether [5], three of the five tested 13β-methyl-D-homoestrone derivatives are potent selective cytotoxic agents, acting in the low micromolar range. The nature of the protecting group at position 3 has a great influence on the antiproliferative properties.

Möller *et al.* found that some 2- and/or 16-halogenated D-homoestrone derivatives are efficient inhibitors of 17β-hydroxysteroid dehydrogenase type 1, and their binding affinity to human estrogen receptor α is reduced by 100- to 1000-fold relative to that of estradiol [29]. Accordingly, such modified D-homoestrone derivatives may be used for the treatment of estradiol-dependent diseases. Those previous results led us to examine the antiproliferative properties of the synthetized halogen-containing D-homoestrones. The 16-halomethyl-17-oxa-D-homosteroids (**9**–**11**) and their starting compounds (**6**, **7**) were tested *in vitro*. None of the 16-halomethyl derivatives influenced the proliferation of the tested tumor cells, but the secoalcohol (**6**) proved to be effective against all the cell lines, and especially on the estrogen receptor-positive breast adenocarcinoma (MCF-7), skin epidermoid (A431) and endometrial

(Ishikawa) carcinoma cells (IC_{50} = 5-8 μM). Structural modifications of secoalcohol **6** at three sites in the molecule yielded diverse derivatives (**12**) which displayed different effects on the proliferation of the cell lines. Acetylation of the 17-OH group led to the 17-acetoxy-3-benzyl ether (**12c**) which exhibited similar antiproliferative action to that of its 17-hydroxy counterpart (**6**), whereas the 17-tosyl ester (**12d**) appeared to be inactive. Removal of the 3-benzyl protecting group and simultaneous saturation of the propenyl side-chain furnished **12a**, which demonstrated complete loss of the growth-inhibitory behavior, and the 16-acetoxy-3-ol (**12d**) and the 3,17-diacetate (**12b**) were also totally inactive. The results of the MTT assays for the secoestrones **6** and **12** indicate that the benzyl ether protecting group is necessary for the antiproliferative activity, which is greatly affected by the nature of the 17-functional group.

Table 3.

We are not aware of literature reports of effective antitumor D-secoestrone derivatives against the HeLa, MCF-7, A2780, A431 and Ishikawa cell lines. Saloranta *et al.* described 17-homoallylic D-secoestrones with low antitumor potentials on these cell lines (IC_{50} > 30 μM) [30]. D-Secoestrone nitriles were recently tested *in vivo* for estrogenic activity. Opening of ring D resulted in complete loss of the estrogenic activity, whereas some derivatives exerted moderate antiestrogenic effects [31–33]. As D-secoestrone derivative **6** suppresses the proliferation of MCF-7, A431 and Ishikawa cells, it was selected for additional *in vitro* experiments. Since tumor specificity is one of the greatest challenges facing new anticancer drug candidates, this feature was additionally investigated for **6**. When intact, noncancerous human foreskin fibroblast cells were incubated with 3.0 or 10 μM **6** for 72 h, a weak proliferation-inhibiting effect (8.3 and 17.7% inhibition, respectively) was detected (Table 4). Cisplatin, our positive control molecule, elicited similar inhibitory activity on fibroblast cell proliferation at these concentrations. Although the tumor specificity of secoestrone **6** does not differ significantly from that of cisplatin, a compound generally used in the therapy of cancerous diseases of female reproductive organs, it may be noted that to date there have been no reports of the tumor specificity of D-secoestrone derivatives with potent antitumor activity.

Table 4.

Various cytotoxic estrones behave as tubulin polymerization inhibitors [3, 29, 34, 35], and there is evidence relating to a B-homoestrone derivative which enhances tubulin assembly and stabilizes microtubules similarly to paclitaxel [36]. However, no information was previously available on the impact of D-secoestrones on tubulin polymerization. We present here data on the effect of a D-secoalcohol (**6**) on the kinetics of *in vitro* tubulin polymerization. Our test compound (**6**) significantly enhanced the maximum rate (V_{max}) of formation of microtubules (Figure 2).

Figure 2.

This significant increase in V_{max} might be due to the interaction of compound **6** with the colchicine binding site of tubulin, as previously reported for several estrone analogs [36]. To determine whether compound **6** interacts with tubulin, it was docked into both the colchicine and the vinblastine binding sites of tubulin. Molecular docking calculations were carried out using the available crystal structure of tubulin crystallized with both colchicine and vinblastine (PDB code: 1Z2B) [37]. The results revealed that both the interaction energy and

the frequency of docking suggest more favorable binding of the ligand at the colchicine binding site. The interactions of the ligand at the binding site are presented in Figure 3. Compound **6** is seen to be stabilized mainly by hydrophobic interactions between the hydrophobic amino acids at the binding site and the hydrophobic ring of the ligand: LEU-248, ALA-250, LEU-255, ALA-316, VAL-315 and MET-299 in the B chain and VAL-181 in the A chain are within interacting distance. The alcoholic oxygen of the ligand accepts a hydrogen-bond from the amide nitrogen of ASN101 in the A chain, whereas the oxygen is donated to GLN11 in the A chain.

Figure 3.

4. Acknowledgments

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Footnotes for tables

Table 1 Synthesis of D-homoestrone derivatives (**9**–**11**)

Table 2 Synthesis of D-secoestrone derivatives (**12**)

Table 3 Antiproliferative properties of the synthetized compounds
* Mean values from two independent determinations with five parallel wells; standard deviation <15%.

Table 4 Proliferation-inhibitory effects of compound **6** and cisplatin on noncancerous human foreskin fibroblast cells, determined in the 72-h antiproliferative assay described in section 2.2.
* Mean values from two independent determinations with five parallel wells.

Tables

Table 1.

Entry	Starting secosteroid	Electrophilic reagent	Product	Yield (%)
1	**6**	NBS	**9a**	68
2	**6**	NIS	**9b + 10b**	50 + 25
3	**6**	PhSeBr	**9c + 10c**	36 + 36
4	**7**	NBS	**11a**	82
5	**7**	NIS	**11b**	84
6	**7**	PhSeBr	**11c**	79

Table 2.

Entry	Substrate	Reagent	Reaction conditions	Product	Yield (%)
1	**6**	H_2, Pd/C	10 bar, rt	**12a**	87
2	**12a**	Ac_2O	MW, 5 min, 160 ºC	**12b**	86
3	**6**	Ac_2O	MW, 5 min, 160 ºC	**12c**	91
4	**12c**	H_2, Pd/C	10 bar, rt	**12d**	88
5	**6**	TsCl	3 h, rt	**12e**	95

Table 3.

	Calculated IC_{50} values [μM]				
	HeLa	MCF-7	A2780	A431	Ishikawa
2	>30	>30	>30	>30	>30
3	18.9	13.9	3.0	11.3	>30
5	3.5	>30	>30	>30	>30
6	13.2	6.4	12.0	5.4	7.3
7	>30	>30	>30	>30	>30
8	>30	>30	20.6	>30	>30
9a	>30	>30	>30	>30	>30
9b	>30	>30	>30	>30	>30
9c	>30	>30	>30	>30	>30
10b	>30	>30	>30	>30	>30
10c	>30	>30	>30	>30	>30
11a	>30	>30	19.2	>30	>30
11b	>30	24.1	>30	>30	>30
11c	>30	>30	>30	>30	>30
12a	>30	>30	>30	>30	>30
12b	>30	>30	>30	>30	>30
12c	14.9	7.9	14.4	7.3	9.5

12d	>30	23.3	>30	>30	>30
12e	>30	>30	>30	>30	>30
Cisplatin	5.3	7.3	0.7	8.9	3.5

Table 4.

Compound	Applied concentration [µM]	% Inhibition of proliferation ± SEM*
6	3.0	8.3 ± 1.2
	10.0	17.7 ± 2.1
Cisplatin	3.0	12.5 ± 1.7
	10.0	22.1 ± 4.6

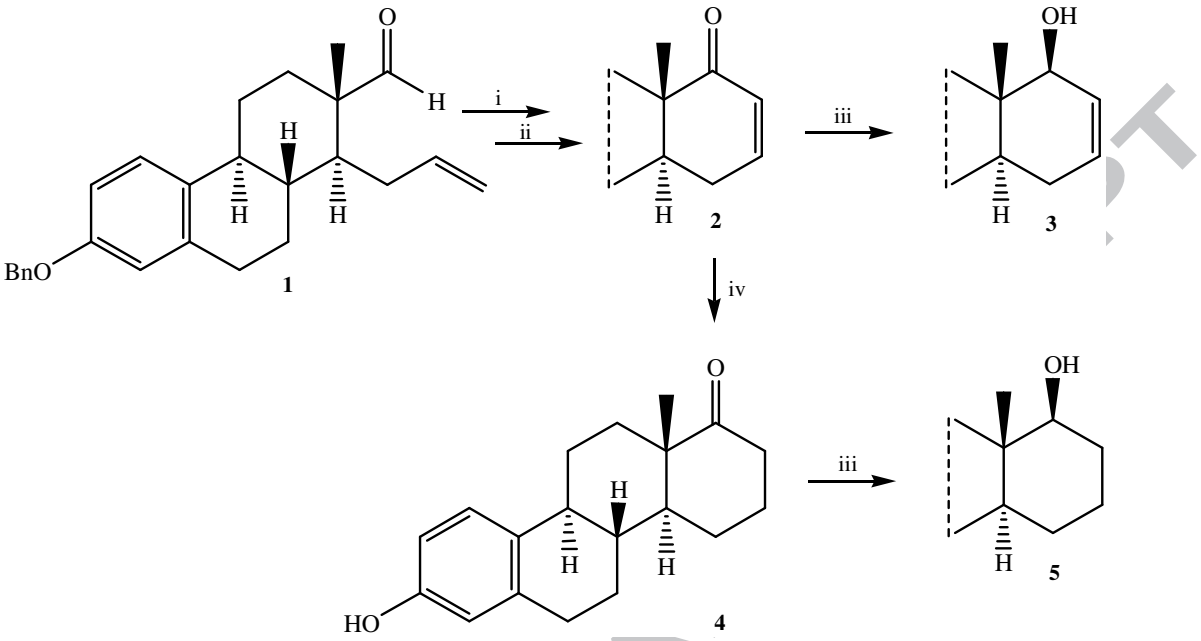
Scheme legends

Scheme 1. Reagents and conditions: (i) *p*-TsOH, CH_2Cl_2, 0 ºC – r.t., 24 h; (ii) Jones reagent (8 N), Me_2CO, 0 ºC, 1 h; (iii) KBH_4, MeOH, 0 ºC – r.t., 3 h; (iv) H_2 (20 bar), Pd/C, EtOAc, 24 h.

Scheme 2. Reagents and conditions: (i) 5 equiv. KBH_4, CH_2Cl_2:MeOH=1:1, 0 ºC – r.t., 0.5 h; (ii) Jones reagent (8 N), Me_2CO, 0 ºC – r.t., 4 h; (iii) NBS, MeCN, -15 ºC, 0.5 h; (iv) I_2, $NaHCO_3$, CH_2Cl_2/THF, r.t., 1 h; (v) PhSeBr, MeCN, r.t., 1 h.

Schemes

Scheme 1.



Scheme 2.

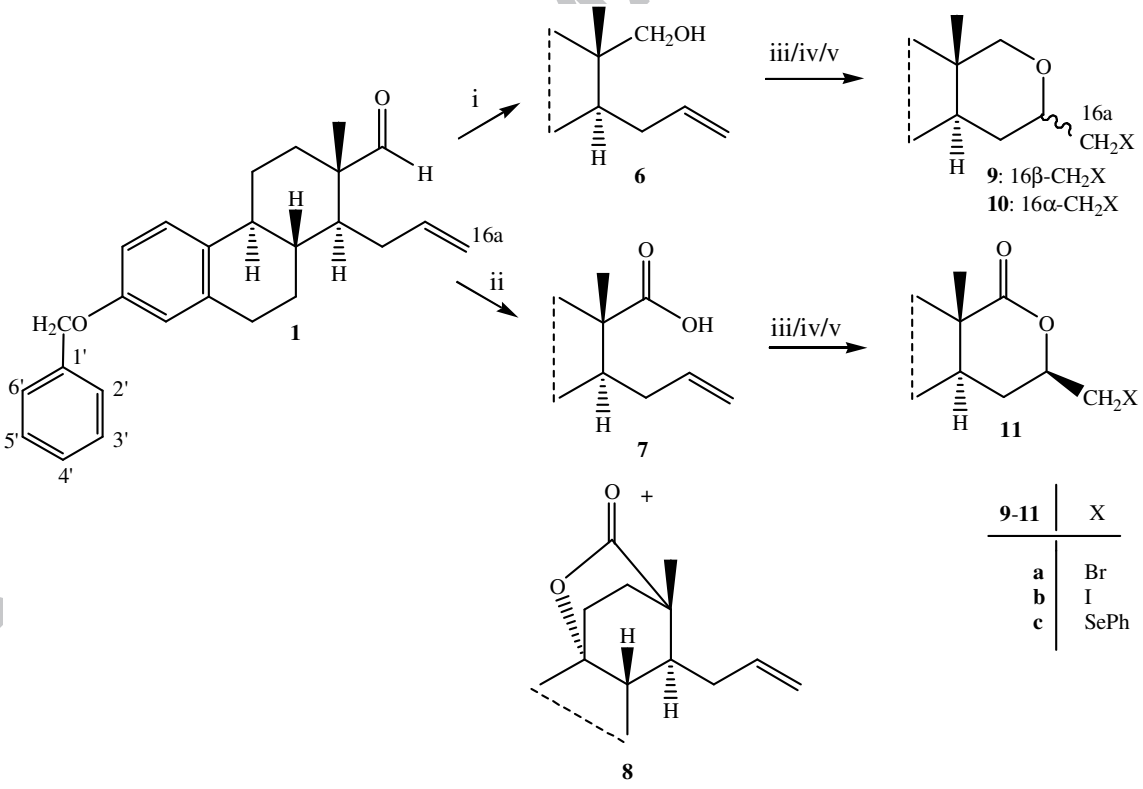


Figure legends

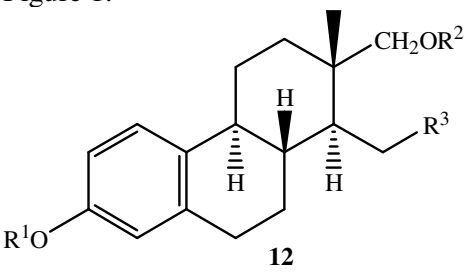
Figure 1. The synthetized D-secoestrone derivatives

Figure 2. Effects of compound **6** on the calculated maximum reaction rate (V_{max}) of *in vitro* microtubule formation. Kinetic curves of tubulin polymerization in the presence of 100 or 200 μM of **6** and 10 μM paclitaxel (Panel A). Calculated V_{max} values of tubulin polymerization (Panel B). Control: untreated samples, PAC: 10 μM paclitaxel-treated samples. The experiment was performed in two parallels. Each bar denotes the mean ± SEM, n = 4. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the control values.

Figure 3. Interactions of ligand **6** at the colchicine binding site of tubulin

Figures

Figure 1.



12

12	R^1	R^2	R^3
a	H	H	$-CH_2-CH_3$
b	Ac	Ac	$-CH_2-CH_3$
c	Bn	Ac	$-CH=CH_2$
d	H	Ac	$-CH_2-CH_3$
e	Bn	Ts	$-CH=CH_2$

Figure 2.

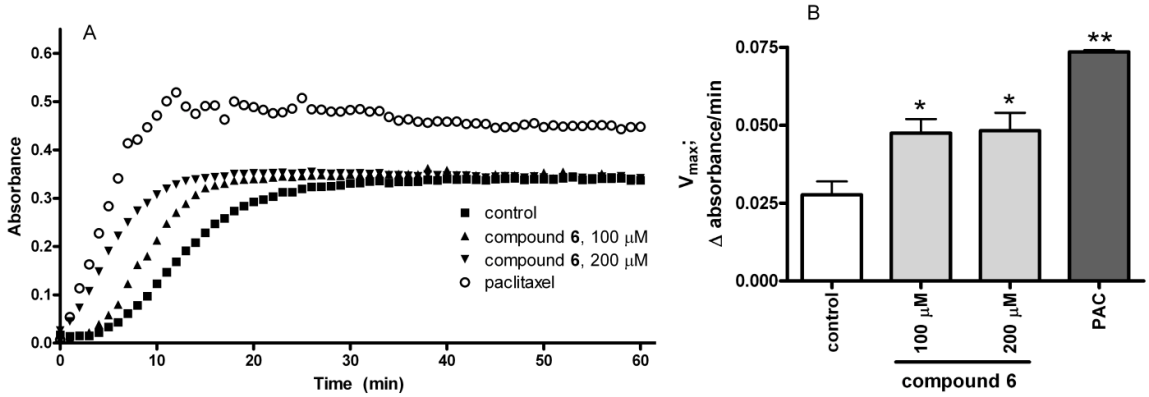
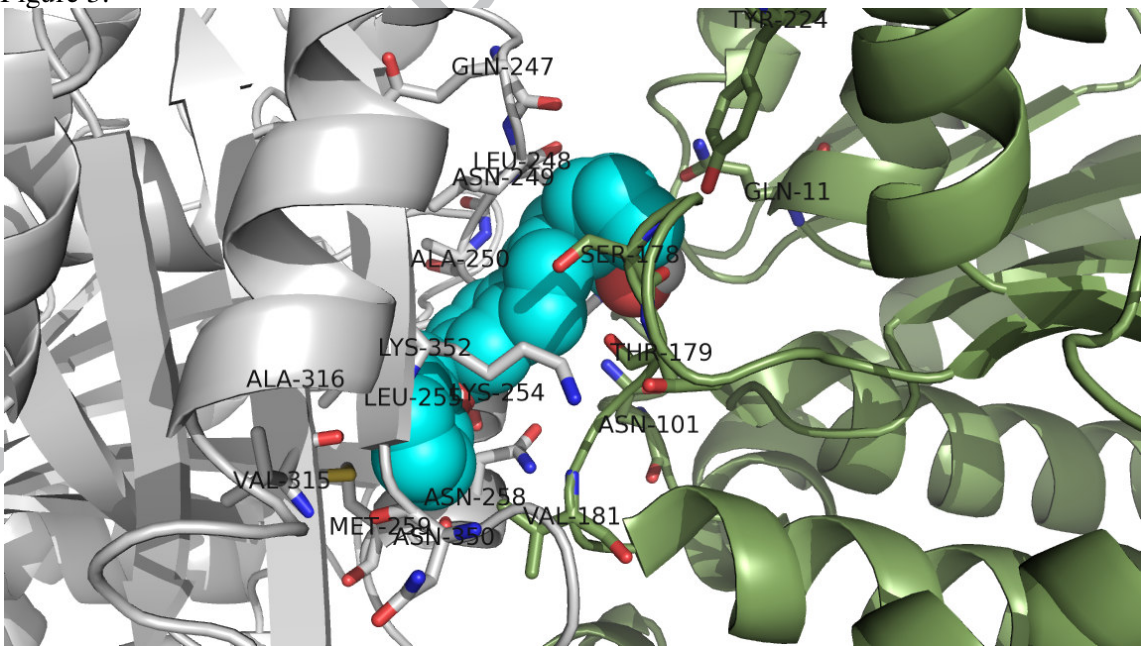
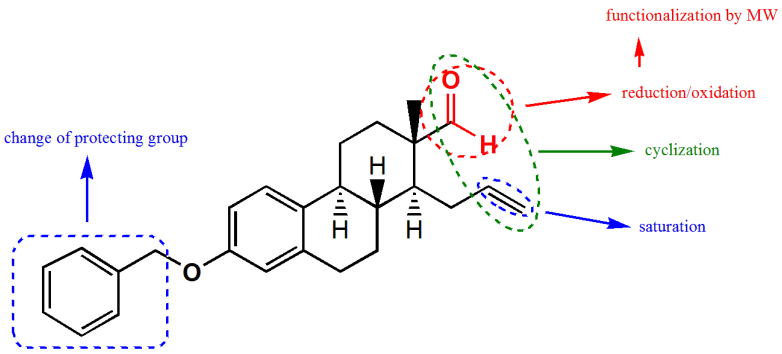


Figure 3.





Highlights

1. Synthesis of D-homoestrones via the intramolecular cyclization of D-secoestrones.
2. Significant *in vitro* antiproliferative effect of D-homo and D-secoestrones.
3. Enhancement of the maximum rate of *in vitro* formation of microtubules.