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Microwave assisted synthesis and biomedical potency of salicyloyloxy and 2-methoxybenzoyloxy androstane and stigmastane derivatives

Katarina M. Penov Gaši [a], Evgenija A. Djurendić [a], Mihály Szécsi [b], János Gardi [b], János J. Csanádi [a], Olivera R. Klisurić [c], Sanja V. Dojčinović-Vujašković [a], Andrea R. Nikolić [a], Marina P. Savić [a], Jovana J. Ajduković [a], Aleksandar M. Oklješa [a], Vesna V. Kojić [d], Marija N. Sakač [a], Suzana S. Jovanović-Šanta [a,*]

[a] Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia
[b] First Department of Medicine, University of Szeged, Korányi fasor 8-10, H-6720 Szeged, Hungary
[c] Department of Physics, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 4, 21000 Novi Sad, Serbia
[d] Oncology Institute of Vojvodina, Put Dr Goldmana 4, 21204 Sremska Kamenica, Serbia

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ABSTRACT

A convenient microwave assisted solvent free synthesis as well as conventional synthesis of salicyloyloxy and 2-methoxybenzoyloxy androstane and stigmastane derivatives 7–19 from appropriate steroidal precursors 1–6 and methyl salicylate is reported. The microwave assisted synthesis in most cases was more successful regarding reaction time and product yields. It was more environmentally friendly too, compared to the conventional method. The antioxidant activity and cytotoxicity of the synthesized derivatives were evaluated in a series of in vitro tests, as well as their inhibition potency exerted on hydroxysteroid dehydrogenase enzymes (Δ^5-3βHSD, 17βHSD2 and 17βHSD3). All of the tested compounds were effective in OH radical neutralization, particularly compounds **9**, **11** and **14**, which exhibited about 100-fold stronger activity than commercial antioxidants BHT and BHA. In DPPH radical scavenging new compounds were effective, but less than reference compounds.

2-Methoxybenzoyl ester **10** exhibited strong cytotoxicity against MDA-MB-231 cells. Most compounds inhibited growth of PC-3 cells, where salicyloyloxy stigmastane derivative **15** showed the best inhibition potency. Compounds **9**, **10** and **11** were the best inhibitors of 17βHSD2 enzyme. X-ray structure analysis and molecular mechanics calculations (MMC) were performed for the best cytotoxic agents, compounds **10** and **15**. A comparison of crystal and MMC structures of compounds **10** and **15** revealed that their molecules conformations are stable even after releasing of the influence of crystalline field and that the influence of crystal packing on molecular conformation is not predominant.

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1. Introduction

It is well established that oxidative stress and oxidative damage are closely associated with carcinogenesis [1]. Thus, the antioxidant properties of androstane steroids which were proven in different cells and tissues [2–4] could potentially reduce cancer cells formation and proliferation. β-Sitosterol and stigmasterol expressed antioxidant activity, as well as cytotoxic activity against human carcinoma cell lines and are used in the prevention of certain cancers [5–7]. Further, prednisolone and its derivatives, besides their anti inflammatory effect, exhibited also antioxidant and cytotoxic activity [8,9].

Many phenolic substances of plant and synthetic origin possess powerful anti-oxidant and cytotoxic properties. Thus, for example, salicylic acid derivatives are phenolic compounds exhibiting such activities [10–17].

In addition, some substances could influence on the level of circulating steroids by affecting the steroidogenic enzymes, which is of high significance for the treatment of different endocrine disorders [18]. For example, some steroid substances expressed themselves as good inhibitors of 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2), so they can be used in the treatment of endocrine diseases, i.e., osteoporosis [19–21].

In this study, inspired by recent trends in using microwave (MW) irradiation methods for preparing steroids [22–24] we present efficient method for the synthesis of a series of steroidal salicyloyl and 2-methoxybenzoyl esters by reaction of the

corresponding steroidal compounds with methyl salicylate under solvent-free MW irradiation conditions. Synthesized compounds combine the structural characteristics of parent compounds, both steroidal (testosterone, sitosterol, stigmasterol, 3β-hydroxy-, 11β-hydroxy- and 3-oxo-steroids) and non-steroidal molecules (salicylates). For comparison purpose, we carried out similar reactions, using conventional heating in solution (toluene). Analytically pure steroidal esters were tested in vitro to assess their abilities as free radical scavengers (anti-oxidants), chemotherapeutic agents, and inhibitors of three hydroxysteroid dehydrogenases (Δ^5-3βHSD, 17βHSD2 and 17βHSD3), related to the development of endocrine diseases. Further, comparison of the X-ray crystallographic parameters obtained for two products (**10** and **15**) with the data obtained by molecular mechanic calculations was carried out to study the influence of crystalline field.

2. Experimental

2.1. Chemical synthesis

2.1.1. General

The infrared spectra (wave numbers in cm^{-1}) were taken on a Nexus 670 FT-IR spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC 250 apparatus operating at 250 MHz (proton) and 62.9 MHz (carbon), using standard Bruker software, with tetramethylsilane as the internal standard. Chemical shifts are given in ppm (δ-scale); coupling constants (J) are given in Hz. High resolution mass spectra (TOF) were recorded on a 6210 Time-of-Flight LC/MS Agilent Technologies (ESI+) instrument. GC/MS analyses were performed on an Agilent Technologies GC 7890A instrument with Mass Selective Detector 5975C. Absorbances of the reaction mixtures in free radical scavenging tests were recorded on a CECIL CE2021 spectrophotometer. The microwave reactor was a monomode system (Microwave Synthesis System – Discover Bench Mate from CEM) with focused waves. Melting points were determined using a Büchi SMP 20 apparatus and are uncorrected. Organic solutions were dried over Na_2SO_4 and evaporated on a rotary evaporator under reduced pressure. Column chromatography was performed on Merck grade 60 silica gel (0.063–0.2 mm).

2.1.2. General procedure for the preparation of compounds 7–19

2.1.2.1. Microwave irradiation. A mixture consisting of methyl salicylate (18 mmol), the corresponding steroidal compound (1 mmol) and sodium (3 mmol) was heated to 110 °C. When reaction with sodium was completed (5–10 min), the mixture was irradiated for 30 min at temperature 160–200 °C, using a 200 W MW source. After cooling the reaction mixture to room temperature, water (100 mL) and HCl (1:1, to pH 7) were added and crude product was extracted with dichloromethane (3 × 50 mL). The organic phase was dried and evaporated, resulted in an oily product. Chromatographic separation of crude product on silica gel column (toluene–EtOAc, 10:1 for **7** and **8**; toluene–EtOAc, 20:1 for **9** and **10**; n-hexane–acetone, 12:1 for **11**, **12**, **13**; petroleum ether for **14**; petroleum ether–acetone, 20:1 for **15** and **16**; petroleum ether–acetone, 3.5:1 for **18** and **19**) gave the pure products **7–16**, **18** and **19**.

2.1.2.2. Conventional heating. A mixture consisting of methyl salicylate (18 mmol), the corresponding steroidal compound (1 mmol), sodium (3 mmol) and toluene (3 mL) was refluxed during 1–50 h, depending of substrate. After the reaction completion, the reaction mixture was cooled to room temperature, diluted with water (100 mL), then neutralized with HCl (1:1) and extracted with dichloromethane (3 × 50 mL). The extract was dried and solvent was evaporated. The pure compounds **7**, **9** and **11–18** were obtained from crude mixture after chromatography on a silica gel column (toluene–EtOAc, 10:1 for **7**; n-hexane–acetone, 12:1 for **9**, **11**, **12** and **13**; petroleum ether for **14**; toluene for **15** and **16**; n-hexane–acetone, 7:1 for **17** and 8:1 for **18**).

3-Oxo-androst-4-en-17β-yl salicylate (**7**). White crystals, mp 265 °C from n-hexane–dichloromethane. IR (KBr): 3110, 2977, 2939, 1671, 1614, 1485, 1324, 1305, 1250, 1230, 1217, 1164, 767. ^{1}H NMR (CDCl$_3$): 0.99 (s, 3H, H-18); 1.21 (s, 3H, H-19); 4.87 (dd, 1H, J_1 = 7.7 Hz, J_2 = 9.0 Hz, H-17); 5.75 (s, 1H, H-4); 6.86–7.86 (group of signals, 4H, H–Ar); 10.87 (s, 1H, OH from salicyloyl group). ^{13}C NMR (CDCl$_3$): 12.2 (C-18); 17.4 (C-19); 20.5; 23.5; 27.6; 31.4; 32.7; 33.9; 35.4; 35.6; 36.6; 38.6; 42.9; 50.2; 53.6; 83.4 (C-17); 112.7; 117.6; 119.0; 123.9 (C-4); 129.7; 135.5; 161.6; 170.0; 170.8; 199.4 (C-3). HRMS (TOF) m/z: $C_{26}H_{32}O_4$ [M+H]$^+$ calculated 409.23734, found 409.23684; [M+Na]$^+$ calculated: 431.21928, found: 431.21910.

(3-Oxo-androst-4-en-17β-yl)-2-methoxybenzoate (**8**). White crystals, mp 158 °C from dichloromethane [25].

3-Oxo-5α-androstan-17β-yl salicylate (**9**). White crystals, mp 219 °C from n-hexane–dichloromethane. IR (KBr): 3110, 2937, 2853, 1710, 1671, 1614, 1485, 1303, 1250, 1214, 1158, 1091, 765, 702. ^{1}H NMR (CDCl$_3$): 0.95 (s, 3H, H-18); 1.03 (s, 3H, H-19), 4.85 (t, 1H, J = 8.2 Hz, H-17); 6.85–7.86 (group of signals, 4H, H–Ar); 10.88 (s, 1H, OH from salicyloyl group). ^{13}C NMR (CDCl$_3$): 11.4; 12.3; 20.8; 23.6; 27.6; 28.7; 31.2; 35.1; 35.7; 36.9; 38.0; 38.4; 43.0; 44.6; 46.5; 50.5; 53.6; 83.7 (C-17); 112.8 (C-1′); 117.5 (C-3′); 119.0 (C-5′); 129.7 (C-6′); 135.5 (C-4′); 161.6 (C-2′); 170.1 (C=O from salicyloyl group); 211.74 (C-3). HRMS (TOF) m/z: $C_{26}H_{34}O_4$ [M+H]$^+$ calculated 411.25299, found 411.25272; [M+Na]$^+$ calculated: 433.23493, found: 433.23503.

(3-Oxo-5α-androstan-17β-yl)-2-methoxybenzoate (**10**). White crystals, mp 165–167 °C from n-hexane–dichloromethane. IR (KBr): 3014, 2942, 2851, 1712, 1601, 1582, 1490, 1307, 1253, 1133, 1083, 1025, 756. ^{1}H NMR (CDCl$_3$): 0.91 (s, 3H, H-18); 1.01 (s, 3H, H-19), 3.88 (s, 3H, OCH$_3$); 4.81 (t, 1H, J = 8.7 Hz, H-17); 6.93–7.82 (group of signals, 4H, H–Ar). ^{13}C NMR (CDCl$_3$): 11.4; 12.3; 20.8; 23.6; 27.6; 28.7; 31.1; 35.1; 35.6; 36.8; 38.0; 38.4; 42.8; 44.5; 46.5; 50.5; 53.6; 55.8; 83.0 (C-17); 111.9; 119.9; 120.3; 131.5; 133.3; 159.2; 165.9 (C=O from 2-methoxybenzoyl group); 211.8 (C-3). HRMS (TOF) m/z: $C_{27}H_{36}O_4$ [M+H]$^+$ calculated 425.26864, found 425.26794; [M+Na]$^+$ calculated: 447.25058, found: 447.24984.

17β-Hydroxyandrost-5-en-3β-yl salicylate (**11**). White crystals, mp 184 °C from n-hexane–acetone. IR (KBr): 3273, 2943, 2905, 1667, 1614, 1485, 1301, 1249, 1211, 1157, 1137, 1086, 755. ^{1}H NMR (CDCl$_3$): 0.78 (s, 3H, H-18); 1.09 (s, 3H, H-19); 3.67 (t, 1H, J = 8.4 Hz, H-17); 4.89 (m, 1H, H-3); 5.43 (d, 1H, J = 4.6 Hz, H-6); 6.85–7.87 (group of signals, 4H, H–Ar), 10.92 (s, 1H, OH from salicyloyl group). ^{13}C NMR (CDCl$_3$): 11.0; 19.4; 19.7; 20.6; 23.4; 27.7; 30.5; 31.5; 31.9; 36.5; 37.0; 38.1; 42.7; 50.1; 51.2; 75.2 (C-3); 81.9 (C-17); 112.8; 117.5; 119.0; 122.8 (C-5); 129.9; 135.5; 139.4 (C-4); 161.7; 169.7 (C=O). HRMS (TOF) m/z: $C_{26}H_{34}O_4$ [M−H]$^-$ calculated: 409.23843, found 409.23834.

3β-Hydroxyandrost-5-en-17β-yl salicylate (**12**). White crystals, mp 211–212 °C from EtOAc. IR (KBr): 3468, 3134, 2981, 2964, 2936, 2854, 1666, 1611, 1485, 1303, 1248, 1215, 1162, 1092, 760, 733. ^{1}H NMR (CDCl$_3$): 0.96 (s, 3H, H-18); 1.04 (s, 3H, H-19); 3.54 (m, 1H, H-3); 4.86 (dd, 1H, J_1 = 7.5, J_2 = 9.0 Hz, H-17); 5.38 (d, 1H, = 4.9 Hz, H-6); 6.86–7.87 (group of signals, 4H, H–Ar), 10.90 (s, 1H, OH from salicyloyl group). ^{13}C NMR (CDCl$_3$): 12.2 (C-18); 19.4 (C-19); 20.6; 23.7; 27.7; 31.4; 31.6; 31.7; 36.6; 36.8; 37.2; 42.2; 42.8; 50.0; 51.0; 71.7 (C-3); 83.8 (C-17); 112.9; 117.6; 119.1; 121.2 (C-6); 129.8; 135.5; 140.9 (C-5); 161.6; 170.2 (C=O). HRMS (TOF) m/z: $C_{26}H_{34}O_4$ [M−H]$^-$ calculated: 409.23843, found: 409.23758.

Androst-5-ene-3β, 17β-diyl disalicylate (13). White crystals, mp 212–213 °C from dichloromethane–acetone. IR (KBr): 3423, 3119, 2945, 1667, 1614, 1584, 1486, 1301, 1249, 1217, 1158, 1091, 995, 767, 703. ^{1}H NMR (CDCl$_3$): 0.98 (s, 3H, H-18); 1.11 (s, 3H, H-19); 4.88 (overlapping, 2H, H-3 and H-17); 5.46 (d, 1H, $J = 4.3$ Hz, H-6); 6.85–7.88 (group of signals, 8H, H–Ar); 10.90 and 10,92 (2s, 2H, OH phenolic groups from salicyloyl residues). ^{13}C NMR (CDCl$_3$): 12.2 (C-18); 19.4 (C-19); 20.5; 23.7; 27.6; 27.7; 31.4; 31.6; 36.7; 36.8; 36.9; 38.0; 42.8; 49.9; 50.9; 75.1 (C-3); 83.7 (C-17); 112.8; 117.5; 118.9; 119.0; 122.6 (C-6); 129.8; 129.8; 135.5; 139.4 (C-5); 161.6; 161.7; 169.6 and 170.1 (C=O from salicyloyl group). MS (*m/z*, rel.%): 392 (M$^+$–C$_6$H$_4$(2-OH)CO$_2$H, 26.25); 255(M$^+$–2xC$_6$H$_4$(2-OH)CO$_2$H, 25); 194(17.5); 145(28.75); 121(100); 91(60); 55(42.5). For C$_{33}$H$_{38}$O$_6$ (530.65) calculated: 74.69% C; 7.22% H; found: 74.72% C; 7.83% H.

Stigmast-5-en-3β-yl salicylate (14). White crystals, mp 168 °C from EtOAc. IR (KBr): 3141, 2951, 2868, 1670, 1612, 1300, 1249, 1213, 1157, 756, 701. ^{1}H NMR (CDCl$_3$): 0.69 (s, 3H, H-18); 1.07 (s, 3H, H-19); 4.88 (m, 1H, H-3); 5.43 (d, 1H, $J = 4.3$ Hz; H-6); 6.83–7.87 (group of signals, 4H, H–Ar), 10.93 (s, 1H, OH). ^{13}C NMR (CDCl$_3$): 11.8 (C-18); 12.0; 18.8; 19.0; 19.3; 19.8; 21.0; 23.0; 24.3; 26.0; 27.8; 28.2; 29.1; 30.3; 31.9; 33.7; 36.1; 36.6; 36.9; 38.1; 39.7; 42.3; 45.8; 50.0; 56.0; 56.7; 75.3 (C-3); 112.9; 117.5; 118.9; 123.1; 129.9; 135.4; 139.3 (C-5); 161.7; 169.6 (C=O). HRMS (TOF) *m/z*: C$_{36}$H$_{53}$O$_3$ M$^-$ calculated: 533.40002, found: 533.40035.

Stigmasta-5,22-dien-3β-yl salicylate (15). White crystals, mp 180–183 °C from dichloromethane. IR (KBr): 3132, 2950, 2867, 1669, 1612, 1300, 1213, 1157, 1087, 973, 756. ^{1}H NMR (CDCl$_3$): 0.73 (s, 3H, H-18); 1.06 (s, 3H, H-19); 5.05–5.17 (overlapping, 3H, H-3 and H-22 and H-23); 5.45 (d, 1H, $J = 4.3$ Hz, H-6); 6.85–7.88 (group of signals, 4H, H–Ar); 10.94 (s, 1H, OH). ^{13}C NMR (CDCl$_3$): 12.0; 12.3; 19.0; 19.3; 21.0; 21.1; 21.2; 22.1; 24.3; 25.4; 27.8; 28.9; 31.8; 31.9; 36.6; 36.9; 38.1; 39.6; 40.5; 42.2; 50.0; 51.2; 55.9; 56.8; 75.2 (C-3); 112.8; 117.5; 118.9; 123.1; 129.3; 129.9; 135.4; 138.3; 139.3 (C-5); 161.7; 169.6 (C=O). MS (*m/z*, rel.%): 394 (M$^+$–C$_6$H$_4$(2-OH)CO$_2$H, 46.15; 368 (5.76); 351 (8.46); 281 (11.53); 255 (30.76); 207 (16.15); 159 (22.31); 135 (36.15); 105 (51.92); 83 (59.23); 55 (100). For C$_{36}$H$_{52}$O$_3$ (532.80) calculated: 81.15% C; 9.84% H; found: 80.93% C; 9.71% H.

(Stigmasta-5,22-dien-3β-yl)-2-methoxybenzoate (16). White crystals, mp 155–157 °C from EtOAc. IR (KBr): 2954, 2868, 1724, 1601, 1583, 1490, 1463, 1299, 1252, 1132, 1081, 755. ^{1}H NMR (CDCl$_3$): 0.72 (s, 3H, H-18); 1.06 (s, 3H, H-19); 3.90 (s, 3H, OCH$_3$); 4.87 (m, 1H, H-3); 5.01–5.22 (overlapping, 2H, H-22 and H-23); 5.43 (d, 1H, $J = 4.7$ Hz, H-6); 6.95–7.79 (group of signals, 4H, H-Ar). ^{13}C NMR (CDCl$_3$): 12.0; 12.3; 19.0; 19.4; 21.0; 21.1; 21.2; 24.4; 25.4; 27.8; 28.9; 31.9; 31.9; 36.6; 36.9; 37.0; 38.0; 38.2; 39.6; 40.5; 42.2; 50.0; 51.2; 55.9; 55.9; 56.8; 74.3 (C-3); 112.0; 120.0; 120.8; 122.6; 129.2; 131.3; 133.2; 138.3; 139.8; 159.1; 165.5 (C=O). HRMS (TOF) *m/z*: C$_{37}$H$_{54}$O$_3$; [M+Na]$^+$ calculated: 569.39652, found: 569.39639. For C$_{37}$H$_{54}$O$_3$ (546,41) calculated: 81.27% C; 9.95% H; found: 80.93% C; 9.65% H.

11β-Hydroxyandrosta-1,4-diene-3,17-dione (17). White crystals, mp 185–186 °C from *n*-hexane–acetone (lit. [26] mp 186.5–189 °C; lit. [27] mp184–186 °C).

3,17-Dioxo-androsta-1,4-dien-11β-yl salicylate (18) Yellow oil. IR (film): 3185, 3052, 2940, 2855, 1739, 1665, 1612, 1485, 1299, 1249, 1214, 1157, 1082, 761, 735, 702. ^{1}H NMR (CDCl$_3$): 1.08 (s, 3H, H-18); 1.31 (s, 3H, H-19); 5.84 (d, 1H, $J = 2.9$ Hz, H-11); 6.05 (s, 1H, H-4); 6.28 (dd,1H, H-2, $J_1 = 1.8$ Hz, $J_2 = 10.1$ Hz); 6.88–7.78 (group of signals, 5H, H–Ar and H-1); 10.85 (s, 1H, OH). ^{13}C NMR (CDCl$_3$): 15.7 (C-18); 21.2 (C-19); 21.7; 31.3; 31.6; 32.3; 34.9; 36.7; 42.8; 46.2; 51.4; 54.6; 71.8 (C-11); 112.0 (C-1′); 118.1 (C-3′); 119.4 (C-5′); 123.0 (C-1); 128.6 (C-2); 129.2 (C-6′); 136.2 (C-4′); 153.8 (C-4); 162.2 (C-2′); 167.8 (O–C=O); 168.7 (C-5); 185.7

(C-3, C=O); 217.1 (C-17, C=O). HRMS (TOF) *m/z*: C$_{26}$H$_{28}$O$_5$ [M+H]$^+$ calculated: 421.20095, found: 421.20111, [M+Na]$^+$ calculated: 443.18290, found: 443.18378.

(3,17-Dioxo-androsta-1,4-dien-11β-yl)-2-methoxybenzoate (19). Colorless oil. IR (film): 3054, 2939, 2854, 1738, 1693, 1663, 1601, 1490, 1298, 1252, 1129, 1080, 1071. ^{1}H NMR (CDCl$_3$): 1.12 (s, 3H, H-18); 1.29 (s, 3H, H-19); 3.92 (s, 3H, OCH$_3$); 5.82 (d, 1H, $J = 2.9$ Hz, H-11); 6.03 (d, 1H, $J = 1.3$ Hz, H-4), 6.27 (dd, 1H, $J_1 = 1.3$ Hz, $J_2 = 10.1$ Hz, H-2); 6.98–7.87 (group of signals, 5H, H–Ar and H-1). ^{13}C NMR (CDCl$_3$): 15.4; 20.8; 21.8; 31.5; 31.6; 32.5; 35.0; 36.9; 43.1; 46.6; 51.6; 54.8; 55.6; 70.8; 112.0; 120.2; 122.9; 128.4; 131.5; 134.1; 154.6 (C-4); 159.4 (C-2′); 164.7 (–O–C=O); 168.2 (C-5); 186.0 (C-3, C=O); 217.7 (C-17, C=O). HRMS (TOF) *m/z*: C$_{27}$H$_{30}$O$_5$ [M+H]$^+$ calculated: 435.21660, found: 435.21633, [M+Na]$^+$ calculated: 457.19855, found: 457.19797.

2.2. Biological tests

2.2.1. Free radical scavenging assays

Free radical scavenging capacity (RSC) of the synthesized compounds was evaluated by measuring of their ability to neutralize 2,2-diphenyl-1-picrylhydrazyl (DPPH) or hydroxyl (OH) radicals. Final concentrations of the tested compounds were in the range of 0.01–8 mM.

2.2.1.1. DPPH assay. The DPPH-assay was performed as described previously [17]. Different aliquots (0.1–2.0 mL) of 0.01 M sample solution in dichloromethane were added to DPPH solution in methanol (90 μM, 1 mL; Sigma; St. Louis, MO) and filled with 95 vol.% of methanol to a final volume of 4 mL. The same reaction mixture with no tested compounds was used as control. Absorbencies of the reaction mixtures (A_{sample}) and control ($A_{control}$) were recorded at 515 nm (CECIL CE2021 spectrophotometer) after 1 h. Commercial synthetic antioxidants, 3,5-di-tert-butyl-4-hydroxytoluene (BHT) (Aldrich; Taufkirchen, Germany) and 3-tert-butyl-4-hydroxyanisole (BHA) (Fluka; Taufkirchen, Germany) were used as positive controls. For each sample, three replicates were recorded.

The percentage of DPPH radical scavenging capacity (DPPH RSC) was calculated using the following equation:

$$RSC\ (\%) = (A_{control} - A_{sample}/A_{control}) \times 100.$$

IC$_{50}$ values (the concentration of the tested compound in the reaction mixture which causes 50% of RSC) were determined by linear regression analysis from the obtained RSC values.

2.2.1.2. Hydroxyl-radical scavenging assay. Hydroxyl-radicals scavenging capacity (OH RSC) of the tested compounds was evaluated by measuring the degradation of 2-deoxy-D-ribose (Aldrich; Taufkirchen, Germany) in the reaction with OH radicals, generated *in situ* in Fenton's reaction [17]. These radicals attack 2-deoxy-D-ribose and degrade it into a series of fragments, some or all of which react with 2-thiobarbituric acid (TBA) (Sigma; St. Louis, MO) at low pH and high temperature to give a pink chromogen, which can be determined spectrophotometrically at 532 nm. Different aliquots (0.005–0.5 mL) of a sample solution in dichloromethane were added to test tubes (final concentration in the range of 0.01–8 mM, each containing 0.1 mL of 5 mM H$_2$O$_2$, 0.1 mL of 10 mM FeSO$_4$, 0.1 mL of 0.05 M 2-deoxy-D-ribose, and 0.067 M KH$_2$PO$_4$–K$_2$HPO$_4$ buffer of pH 7.4 to a final volume of 3 mL). The same reaction mixture without sample was used as control. After 1 h at 37 °C incubation, 2 mL of TBA reagent (10.4 mL of 60% (v/v) HClO$_4$, 3 g TBA and 120 g of trichloroacetic acid (TCA; Sigma; St. Louis, MO) and 0.2 mL of 0.1 M EDTA (Sigma; St. Louis, MO) were added to the reaction mixture, and the tubes were heated at 100 °C for 20 min. After cooling, absorbances of the reaction mixtures and of the control were recorded at

532 nm. Percentage of OH RSC was calculated using the same equation as for DPPH RSC.

Three replicates were recorded for each sample. BHT and BHA were used as reference compounds. IC_{50} values (the concentration at which 50% of OH radical is neutralized) were determined by linear regression analysis from the obtained RSC values.

2.2.2. Cytotoxicity assay

The cytotoxicity of newly synthesized compounds was evaluated using previously described method [28]. The chemotherapy drug doxorubicin (DOX), used as reference compound, was tested under the same experimental conditions.

2.2.2.1. Cell lines.
Three human tumor and one normal cell lines were used in this study: estrogen receptor positive human breast adenocarcinoma (ER+, MCF-7), estrogen receptor negative human breast adenocarcinoma (ER−, MDA-MB-231), human prostate cancer (PC-3) and normal fetal lung fibroblasts (MRC-5). These cells were grown in Dulbecco's modified Eagle's medium with 4.5% of glucose. Media were supplemented with 10% of fetal calf serum (FCS, Sigma) and antibiotics (100 IU/mL of penicillin and 100 μg/mL of streptomycin; ICN Galenika). All cell lines were cultured in flasks (Costar, 25 cm^2) at 37 °C in 100% humidity atmosphere and 5% of CO_2 incubator. Only viable cells were used in the assay. Viability was determined by dye exclusion assay with trypan blue.

2.2.2.2. SRB test.
Cytotoxicity was evaluated by colorimetric sulforhodamine B (SRB) assay [28]. In brief, single cell suspension (5×10^3 cells) was plated into 96-well microtiter plates (Costar, flat bottom). Plates were pre-incubated at 37 °C in a 5% CO_2 incubator during 24 h. Solutions of selected substances (at final concentrations ranging from 10^{-8} M to 10^{-4} M) were added to all wells except the control ones. After incubation period (48 h/37 °C/5% CO_2) cytotoxicity assay was carried out as follows: 50 μL of 80% TCA – was added to all wells; after 1 h the plates were washed with distilled water, and SRB (75 μL of 0.4%) was added to all wells; 30 min later the plates were washed with citric acid (1%) and dried at room temperature. Finally, 200 μL of 10 mM Tris basis (pH 10.5) was added to all wells. Wells containing cells without tested compounds were used as control. Wells without cells containing only complete medium were used as blank. Absorbances of samples (A_{sample}) and control ($A_{control}$) were measured on the microplate reader. Cytotoxicity was calculated according to the formula:

$$CI\,(\%) = (1 - A_{sample}/A_{control}) \times 100.$$

Two independent experiments were set out in quadruplicate for each concentration of the compound. Mean values and standard deviations (SD) were calculated for each concentration. The IC_{50} (value that defines the dose of compound that inhibits cell growth by 50%) for every compound was determined by Median effect analysis.

2.2.3. Determination of Δ^5-3βHSD, 17βHSD3 and 17βHSD2 activities and their inhibition

Inhibitory effects exerted by the newly synthesized compounds (**7**, **9–18**) on the rat androgen synthesis were investigated with *in vitro* radiosubstrate incubations. Our previously published method for Δ^5-3βHSD [29,30] was used with certain modifications and adapted also to the measurement of 17βHSD3 and 17βHSD2 [31] activities. Δ^5-3βHSD activity was investigated *via* dehydroepiandrosterone–androst-4-ene-3,17-dione conversion, whereas the 17βHSD3 was measured by the androst-4-ene-3,17-dione–testosterone conversion. Homogenate of testicular tissue obtained from adult Wistar rats was used as source of these enzymes. Activity of the 17βHSD2 was studied *via* conversion of testosterone to

androst-4-ene-3,17-dione and microsomes prepared from liver tissue of adult female rats served as enzyme source in this case. During procedures, tissue preparations suited as enzyme source were incubated with 1 μM [^{14}C]-labeled substrate steroid in the presence of 1 mM coenzyme NAD or NADPH in 0.1 M HEPES buffer (pH = 7.3) containing 1 mM EDTA and 1 mM dithiothreitol. The appropriate substrate was added to the incubate in 10 μL of a 25 v/v% propylene glycol in HEPES buffer solution, whereas test compounds were applied in 10 μL of dimethyl sulfoxide solution. These organic solvent contents of 200 μL final volume of the incubation medium did not reduce the enzyme activity substantially. Incubation was carried out at 37 °C for 20 min and the enzymatic reaction was stopped by the addition of ethyl acetate and freezing. After extraction, unlabeled carriers of the substrate and the product steroids were added to the samples. Substrates and products were separated by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick) with the solvent system diisopropyl ether/CH_2Cl_2 (50:50 v/v) and UV spots were used to trace the separated steroids. Spots were cut out and the radioactivity of the product formed and of the substrate remaining was measured by means of liquid scintillation counting. Test compounds were applied at 50 μM concentrations and control incubates without test substances were also prepared in every series. Two experiments were performed with each test compound and the standard deviations of the mean enzyme activity results were within ±10%. IC_{50} values were determined for more potent inhibitors. In this case, conversion was measured at five or six different concentrations of the test compound between 0.1 and 50 μM. IC_{50} results were calculated by linear regression analysis following a logit–log transformation of the data, and the standard deviations were determined from the fitted lines.

2.3. X-ray crystallographic analysis of compounds **10** and **15**

X-ray diffraction data for compounds **10** and **15** was collected at room temperature on an Oxford Diffraction (Agilent Technologies) Gemini S diffractometer with graphite-monochromated MoKα radiation (λ = 0.7107 Å). Data reduction for all compounds was performed with the program package CrysAlis RED [32]. Space group determinations were based on analysis of the Laue class and systematically absent reflections. Structures were determined by direct methods using SIR92 [33]. All structures were refined using full-matrix least-squares. Non-hydrogen atoms were refined anisotropically for all compounds, C–H hydrogen atoms were included on calculated positions riding on their attached atoms with fixed distances of 0.93 (CH) or 0.97 Å (CH$_2$), and O–H hydrogen atoms were identified on difference electron density maps and isotropically refined. All calculations were performed using SHELXL97 [34], PARST [35] and PLATON [36], as implemented in the WINGX [37] system of programs. Crystal data and refinement parameters are summarized in Table S1.
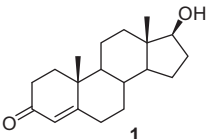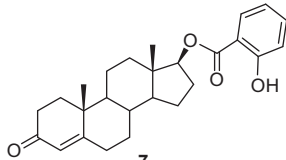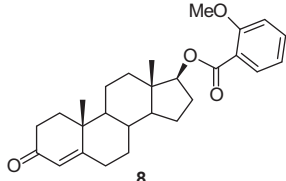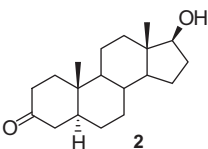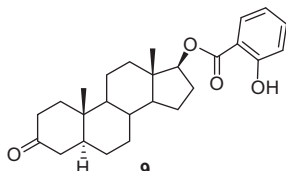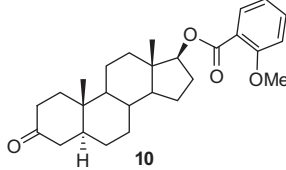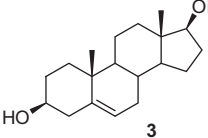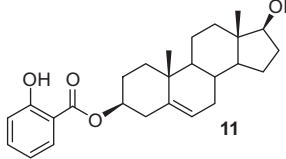
3. Results and discussion

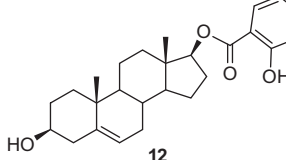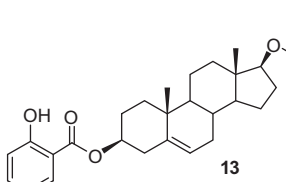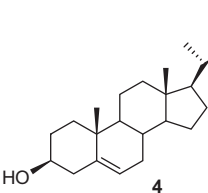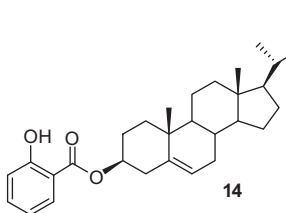### 3.1. Synthesis of new compounds

The primary objective of this study was the synthesis of salicyloyl esters of steroids **1–6** which was achieved by conventional heating of a mixture of steroidal compound (**1–6**), sodium and methyl salicylate in toluene. Otherwise, in solvent-free MW assisted transesterification reaction, in addition to esterification of steroidal alcohols, in most cases methylation of the phenol moiety occurred also, leading to the mixtures of salicyloyl and 2-methoxybenzoyl esters, except in the case of substrates **3** and **4**, when only salicyloyl esters were obtained as the reaction products. All mixtures of salicyloyl and 2-methoxybenzoyl esters were
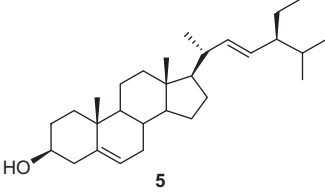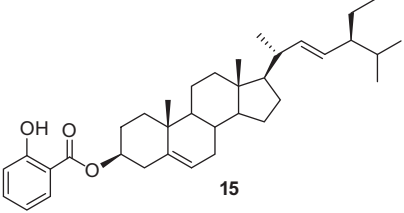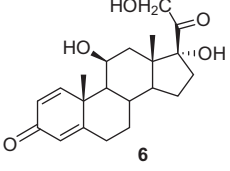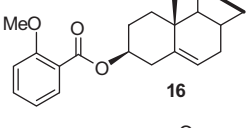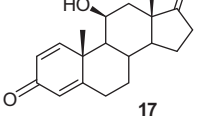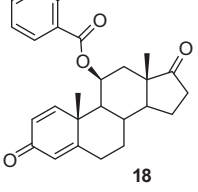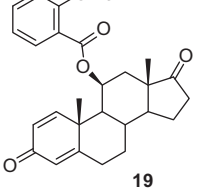
Table 1
The structures of starting and product compounds and reaction conditions for both MW assisted and conventional synthesis.

Substrates	Products	MW irradiation (200 W) [temp (°C)/time (min)/yield (%)]	Conventional heating [time (h)/yield (%)]
1	**7**	200/30/34.6	8/19.6
	8	200/30/43.4	–
2	**9**	180/30/66	13/33.4
	10	180/30/8.4	–
3	**11**	160/30/4.2	16/13.4
	12	160/30/6	16/9.6
	13	160/30/44.4	16/4
4	**14**	190/30/53	2/74

(*continued on next page*)

Table 1 (*continued*)

Substrates	Products	MW irradiation (200 W) [temp (°C)/time (min)/yield (%)]	Conventional heating [time (h)/yield (%)]
5	**15**	200/30/18	2/43.5
	16	200/30/26	2/15
6	**17**	–	1/32.8
	18	200/30/15.2	50/23.3
	19	200/30/10.6	–

chromatographically separated. We assume that there are two possible mechanisms of 2-methoxybenzoyl esters formation. First mechanism supposes that salicyloyl esters could be formed primarily and then methylated *in situ* under MW irradiation, giving 2-methoxybenzoyl esters. Another mechanism includes reaction of steroidal alcohols with methyl 2-methoxybenzoate, probably formed during heating of methyl salicylate at high temperatures with sodium. However, prednisolone (**6**) underwent fragmentation first, and then esterification, giving one fragmentation product and its salicyloyl ester, when reaction mixture was conventionally heated, while in the MW assisted reaction salicyloyl and 2-methoxybenzoyl esters were obtained.

In order to compare the efficiency of both reaction conditions, we compared the reaction durations, as well as the yields of newly synthesized compounds (Table 1) for both MW assisted and conventional methods.

Compound **7** was prepared in reaction of testosterone (**1**) and methyl salicylate by MW assisted, as well as conventional heating (Table 1). MW assisted reaction lasted 16 times shorter than conventional one, and compound **7** was obtained in better yield (34.6% vs. 19.6%). In the MW assisted reaction 2-methoxybenzoyl ester **8** was obtained as well (43.4%; total yield of compounds **7** and **8** was 78%), which was not obtained by conventional heating. Compound **8** was prepared before from testosterone and 2-methoxybenzoyl chloride in the presence of 4-dimethylaminopyr-

idine and triethylamine for 48 h at room temperature [25], which is almost 100 times slower than in MW assisted reaction. The spectroscopic data of compound **8** are in good agreement with earlier data [25].

Starting from 17β-hydroxy-5α-androstan-3-one (**2**), MW assisted reaction resulted in formation of two products: salicyloyl ester **9** (66%) and 2-methoxybenzoyl ester **10** (8.4%). However, derivative **9** was the only product formed by conventional heating (33.4%). The yield of compound **9** was twice higher in the MW assisted reaction, while the reaction time was 26-fold shorter.

Both MW assisted and conventional heating of 3β,17β-dihydroxy-androst-5-ene (**3**) gave three products: 3β-salicyloyl derivative **11**, 17β-salicyloyl derivative **12** and 3β,17β-*bis*(salicyloyl) derivative **13**. MW assisted reaction conditions resulted in a shorter reaction time (32-fold) and 2-fold greater total yield for all products (**11**, **12** and **13**) (54.6%), with compound **13** (44.4%) constituting the major product, in comparing to the results of conventional heating where the total yield of all products was 27%.

Transesterification of methyl salicylate with β-sitosterol (**4**) in conventional reaction conditions resulted in salicyloyl derivative **14** in 74% yield, while the MW assisted reaction gave the same product in 53% yield. Otherwise, MW assisted reaction conditions resulted in a shorter reaction time (4-fold). Similarly, by conventional heating of stigmasterol (**5**) with methyl salicylate, salicyloyl derivative **15** and 2-methoxybenzoyl derivative **16** were obtained

Table 2
Scavenger activity of the selected steroidal compounds; standard deviations of the mean results were within ±10%.

Compound	DPPH (IC$_{50}$ mM)	OH (IC$_{50}$ mM)
9	1.00	0.02
10	1.00	0.60
11	0.65	0.03
13	0.85	0.18
14	0.03	0.02
15	0.15	0.09
16	1.60	0.06
17	0.52	0.09
18	1.64	0.29
β-Sitosterol (**4**)	0.09	0.18
Stigmasterol (**5**)	0.13	0.19
BHT	0.04	1.94
BHA	0.012	2.13

Table 3
In vitro cytotoxicity of the tested compounds: standard deviations of the mean results were within ±10%.

Compound	IC$_{50}$ (μM)			
	MCF-7	MDA-MB-231	PC-3	MRC-5
7	>100	>100	16.51	>100
9	>100	>100	34.79	>100
10	68.32	3.45	58.31	>100
11	>100	32.91	26.27	>100
12	>100	>100	29.31	>100
13	48.25	88.01	51.75	>100
14	61.02	>100	86.80	>100
15	45.11	19.10	10.89	>100
16	>100	42.02	42.44	>100
17	>100	>100	18.59	>100
18	>100	40.58	98.79	>100
DOX	0.75	0.12	95.61	0.12

in better total yield (58.5%) than in MW assisted reaction conditions (total yield 44%).

MW irradiation and conventional heating of prednisolone (**6**) with methyl salicylate and sodium gave androstane derivatives **17**, **18** and **19** as a result of primary cleavage of the C17-dihydroxyacetone side chain of prednisolone. Here we present degradation of prednisolone (**6**) in the highly functionalized 17-ketosteroid **17** (32.8%), achieved with methyl salicylate and sodium in toluene by conventional heating during 1 h (Table 1). However, the cleavage of the C17-dihydroxyacetone side chain of prednisolone under different reaction conditions was reported before [26,27]. Thus, the degradation of prednisolone using ethanolic KOH at room temperature during 1.5 h gave 17-ketosteroid **17** in 23% yield [26]. On the other hand, the reaction of prednisolone with Bi(OTf)$_3$·H$_2$O, in 1,4-dioxane, at 80 °C, for 14 h [27], afforded **17** in better yield (78%) but longer time than in our work.

On the other hand, conventional heating of prednisolone (**6**) with methyl salicylate for 50 h gave only 11β-salicyloyl ester **18** (23.3%). However, the MW assisted reaction resulted in formation of two products: compound **18** (15.2%) and 2-methoxybenzoyl ester **19** (10.6%). The yield of compound **18** was a little bit lower in the MW assisted reaction, compared to the conventional heating, but the reaction time was 100-fold shorter.

Based on a comparison of the transesterification reaction of methyl salicylate with steroidal alcohols **1–6** under solvent-free MW irradiation conditions versus conventional heating, MW irradiation considerably accelerated the reactions (4–100-fold). In the case of steroidal compounds **1**, **2**, **3** and **6**, MW assisted reaction resulted in higher total product yields. Although, in general, yields and reaction times were higher and shorter, respectively, in the

MW assisted reactions, the transesterification reaction gives better yields in solution (conventional heating) with sterols **4** and **5** possessing an additional hydrocarbon side chain.

It should be noted that at 160 °C and 180 °C under MW irradiation, salicyloyl esters were the main or only products, while at higher temperature (200 °C) 2-methoxybenzoyl esters are, in most cases, the main reaction products (Table 2). Namely, comparison between substrates **1** and **2**, or **4** and **5** (Table 1), and both pairs with **3** indicated an increasing of the yields of 2-methoxybenzoyl esters with increasing of temperature. However, the apparent temperature trend was broken if one compares the product profile of entry **2** (180 °C) and **4** (190 °C). When the reaction was carried out at 160 °C (substrate **3**), salicyloyl esters as the only reaction products were obtained. However, at 180 °C (substrate **2**) the salicyloyl ester was obtained as main product and corresponding 2-methoxybenzoyl ester was obtained as side product. By applying 200 °C (substrates **1**, **5** and **6**) the yields of the corresponding 2-methoxybenzoyl esters were higher or similar as yields of salicyloyl esters.

3.2. Biological properties

3.2.1. Scavenger activity of the tested compounds

The antioxidant activities of the synthesized salicylic acid steroidal derivatives were evaluated in a series of *in vitro* tests and compared with those of commercial antioxidants BHT and BHA. In the DPPH assay, the ability of the tested compounds to act as hydrogen or electron donors in transforming DPPH radical to its reduced form, DPPH-H, was measured spectrophotometrically [17]. The hydroxyl radical scavenging activity of the examined compounds was measured by the deoxyribose assay [17]. The protective effects of the tested compounds on 2-deoxy-D-ribose were assessed as their ability to remove hydroxyl radicals (formed in the Fenton reaction) from the test solution and prevent the sugar degradation. The OH radical scavenging activity of the tested compounds was determined indirectly, by measuring the absorbance of the pink colored solutions. Scavenger activity of the selected steroidal compounds is given in Table 2.

Though all of the tested compounds neutralized DPPH radical, the highest scavenger activity was displayed by the salicyloyl ester of β-sitosterol **14**, which was slightly more effective than the commercial antioxidant BHT and 3 times more effective than its parent molecule, β-sitosterol.

All of the tested compounds were more effective in OH radical neutralization than commercial antioxidants BHT and BHA, particularly androstane 17β-salicyloyloxy derivative **9**, androstane 3β-salicyloyloxy derivative **11** and salicyloyl ester of β-sitosterol **14**, which exhibited the highest activity with respect to IC$_{50}$ values, about 100-fold lower than those observed for BHT and BHA. 3β,17β-*Bis*-salicyloyl ester **13** was less active than the corresponding mono-3β salicyloyl ester **11**. Compound **18** with salicyloyloxy group at C11 exhibited the lowest activity.

It can be seen that introduction of salicyloyl or 2-methoxybenzoyl group in the C3 position of β-sitosterol and stigmasterol led to an increasing of the OH radical neutralization potential. Namely, salicyloyl and 2-methoxybenzoyl esters of β-sitosterol and stigmasterol **14–16** expressed themselves as 2–9-fold better hydroxyl radical scavengers than their parent compounds, where for β-sitosterol antioxidant activity was established before [5]. Salicyloyl ester of β-sitosterol **14** was more active than salicyloyl ester of stigmasterol **15**.

3.2.2. Cytotoxicity

The synthesized androstane and stigmastane derivatives **7–18** were evaluated for their *in vitro* cytotoxicity against human breast adenocarcinoma (MCF-7, ER+ and MDA-MB-231, ER−) and
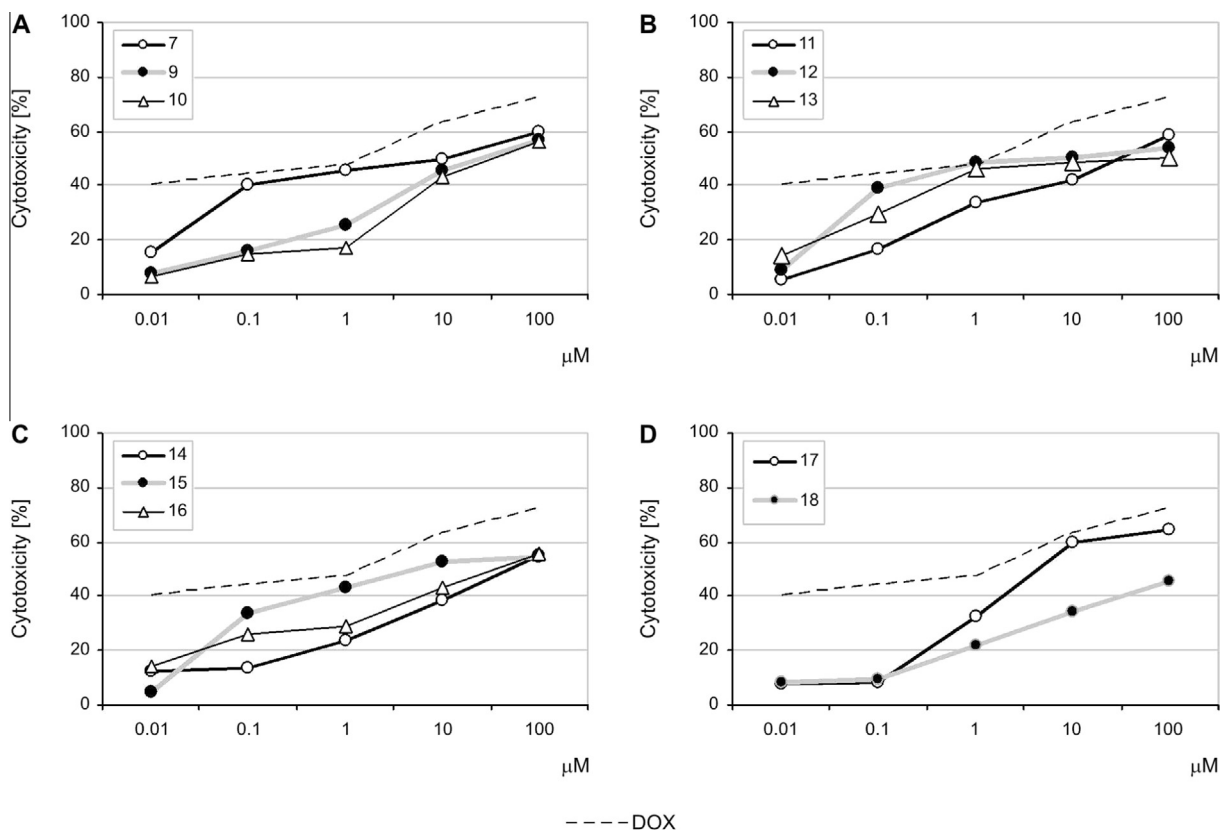
Fig. 1. Cytotoxicity trough the range of investigated concentrations of the tested steroidal compounds against the most affected tumor cell line PC-3 (A: compounds **7**, **9** and **10**; B: compounds **11**–**13**; C: compounds **14**–**16** and D: compounds **17** and **18**). The corresponding cytotoxicity curves of reference compound doxorubicin is also presented on each graph (dashed line). All derivatives expressed an expected dose-dependent toxicity.
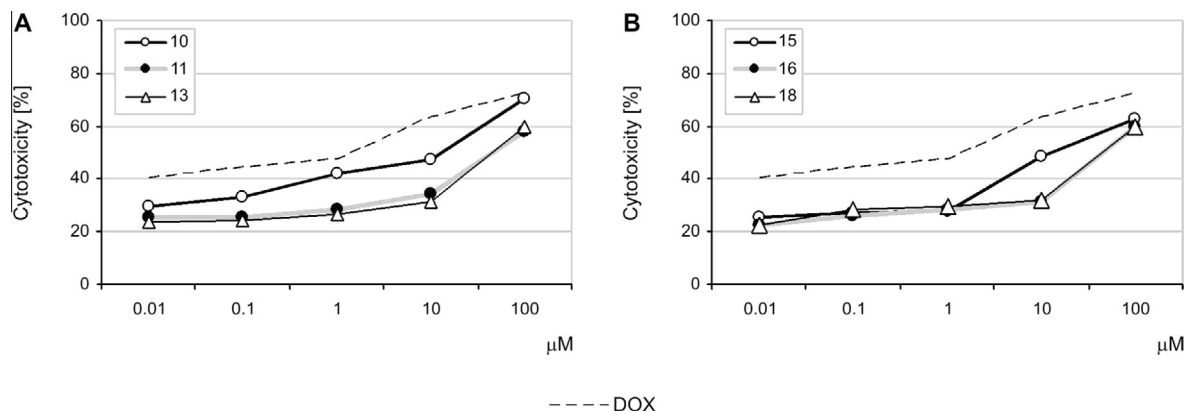

Fig. 2. Cytotoxicity trough the range of investigated concentrations of the most effective tested steroidal compounds against MDA-MB-231 tumor cell line (A: compounds **10**, **11** and **13**; B: compounds **15**, **16** and **18**). The corresponding cytotoxicity curves of reference compound doxorubicin is also presented on both graphs (dashed line). All derivatives expressed an expected dose-dependent toxicity.

prostate cancer cells (PC-3), as well as normal fetal lung fibroblasts (MRC-5). Cytotoxicity was determined using the standard SRB assay, after exposure of cells to the tested compounds for 48 h [28]. Doxorubicin served as reference compound, used as positive control for general toxicity. The results of cytotoxicity assay for the tested compounds are presented in Table 3.

Prostate cancer PC-3 cells were the most sensitive on the tested steroidal compounds (Table 3). All the tested compounds decreased this cell line proliferation, where stigmastane 3β-salicyloyloxy derivative **15**, testosterone salicyloyl ester **7** and compound **17** without salicyloyl group expressed the best cytotoxic

potential (IC$_{50}$ 10.89, 16.51 and 18.59 μM, respectively). Against MDA-MB-231 cell line the most active was 2-metoxybenzoyl ester **10** (IC$_{50}$ 3.45 μM), whereas moderate cytotoxicity showed salicyloyl ester **15** (IC$_{50}$ 19.10 μM).

For both PC-3 and MDA-MB-231 cell line all cytotoxic compounds expressed an expected dose-dependent toxicity (Figs. 1 and 2, respectively). Estrogen receptor positive breast cancer cell line (MCF-7) was less sensitive on the tested steroidal compounds.

Note that doxorubicin was extremely toxic to normal noncancerous MRC-5 cells, consistent with its nonspecific cellular cytotoxicity. In addition, doxorubicin controls showed low

Table 4
In vitro inhibition of Δ^5-3βHSD, 17βHSD3 and 17βHSD2 by selected steroidal compounds; standard deviations of the mean results were within ±10%. Non-inhibited control taken as 100%. S.D.: standard deviation. NI: no inhibition.

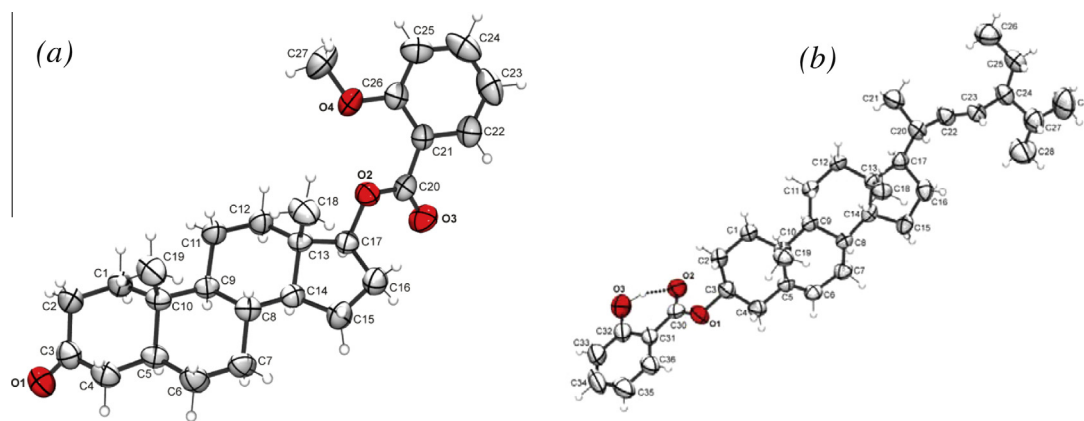| Compound	3βHSD inh.	17βHSD3 inh.	17βHSD2 inh.	
	Rel. conv. at 50 μM (%)	Rel. conv. at 50 μM (%)	Rel. conv. at 50 μM (%)	IC$_{50}$ (μM)
7	78	84	–	19.0
9	NI	80	–	5.8
10	67	77	–	3.7
11	79	NI	–	1.8
12	69	NI	74	–
13	NI	76	88	–
14	NI	85	60	–
15	NI	93	78	–
16	90	NI	NI	–
17	NI	95	NI	–
18	56	79	–	22.4

Fig. 3. ORTEP drawings of the molecular structures of compounds **10** (a) and **15** (b) with labeling of non-H atoms. Displacement ellipsoids are shown at the 50% probability level and H atoms are drawn as spheres of arbitrary radii. Intramolecular hydrogen bond is shown as dashed.

cytotoxicity against PC3-cells (IC$_{50}$ 95.61 μM), which was shown before [38]. On the contrary, none of the newly synthesized steroidal compounds were toxic against healthy cells (MRC-5).

3.2.3. Inhibition of Δ^5-3βHSD, 17βHSD3 and 17βHSD2 activities

Since hydroxysteroid dehydrogenase enzymes are involved in the biosynthesis of active steroids, their inhibition constitutes an interesting approach for treating steroid-dependent cancers. The inhibitory effects of newly synthesized steroidal compounds exerted on three enzymes from hydroxysteroid dehydrogenase family were tested, in order to study potential of these substances to influence the peripheral conversion of steroid precursors into biologically active forms.

Inhibitory effects exerted on the rat testicular Δ^5-3βHSD and 17βHSD3 activity by the newly synthesized compounds (**7**, **9**–**18**), investigated *via* measurement of steroidal substrate to corresponding product conversion by *in vitro* radiosubstrate incubation methods, showed that these compounds do not affect the activity of these two enzymes (Table 4). However, almost all of the tested substances expressed the inhibitory effects on the 17βHSD2 activity, whereas salicyloyl esters **9** and **11**, and 2-methoxybenzoyl ester **10** efficiently inhibited this enzyme (IC$_{50}$ = 5.8, 1.8 and 3.7 μM, respectively), while salicyloyl esters **7** and **18** modestly inhibited it.

Summarizing biological properties of the investigated compounds, it can be concluded that some of androstane and stigmastane salicyloyl and 2-methoxybenzoyl esters have promising biomedical potential. Namely, all of the tested compounds were effective in OH radical neutralization, even more than commercial antioxidants, particularly compounds **9**, **11** and **14** with

salicyloyloxy group in C-3 or C-17 position. Similarly, all of the tested compounds decreased proliferation of prostate cancer PC-3 cells, where 3- and 17-salicyloyl esters **15** and **7**, as well as compound **17** expressed the best cytotoxic effect. 17-(2-Methoxybenzoyl) ester **10** showed considerable antiproliferative activity against MDA-MB-231 cells. Newly synthesized compounds were no toxic against normal, non-cancerous cells (MRC-5), while doxorubicin was extremely toxic against these cells. Most of the tested substances effectively inhibited 17βHSD2 enzyme, whereas 17-salicyloyl esters **9** and **11**, as well as 17-(2-methoxybenzoyl) ester **10**, were the strongest inhibitors.

3.3. Crystallographic study

To complement our investigation, we defined three-dimensional structures in the crystalline state of compounds **10** and **15**, which exhibited the best cytotoxic activity against breast i.e. prostate cancer cells (Table 3). Representative views of molecules **10** and **15** are shown in Fig. 3 [39]. Since we investigated behavior of compounds **10** and **15** in biological (live) systems, we were interested in the conformation of molecules released from the influence of crystalline field (the conformations in which the compounds exhibit their biological activity). After determining three-dimensional structures of these compounds in crystalline state, next step in our study was defining the conformation of the molecules **10** and **15** in terms of energy minima. For this purpose, we performed the MMC using PCMODEL [40]. The conformation of molecules remains stable at their energy minima, which is confirmed by comparing selected torsion angles of molecules, obtained from crystal structure analyses and after MMC (Table S2). As can be seen in Fig. S1, very good

overlap was observed for the molecular structures of molecules **10** and **15** in the crystalline state and after MMC.

Figs. 3 and S1 illustrate that compound **15** contains intramolecular O–H···O hydrogen bond (hydrogen bond parameters are given in Table S3), which remains after MMC. Based on our analyses, the presence of O–H···O intramolecular hydrogen bonds appears to stabilize the conformations of the compound **15**. Otherwise, the absence of intermolecular hydrogen bonding in the crystal packing of both compounds **10** and **15** (illustrated in Fig. S2) could be one of the reasons for the stability of their molecular conformations. The influence of crystal packing molecular conformation is not predominant.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2014.12.008.

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