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Synthesis and biotesting of new carrier prodrugs of 2-methoxyestradiol

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Molecular modeling

Computer molecular modeling was performed using a 3D model of the colchicine binding site (PDB ID: 4O2B). All water molecules and compounds used for X-ray of the protein were previously excluded from the model, all other molecules and ions at the interface of α- and β-subunits of the protein were maintained. Atomic charges of protein amino acids were assigned by standard Kollman method using AutoDock Tools 1.5.6.[1] Three-dimensional structures of the compounds were submitted to a conformational MMFF Amber ff14SB optimization using Gasteiger charges in USCF Chimera 1.13.1 program.[2] Molecular docking was carried out with AutoDock Vina 1.1.2[1] (grid box 15.75Å×18.0Å ×17.25Å, grid center size x = 19.003 Å, y = 67.056 Å, z = 42.513 Å, exhaustiveness = 16). Complexes with the best values of scoring functions were chosen and visualized using CLC Drug Discovery Workbench (Limited mode, Version 4).

Chemistry

All reaction temperatures correspond to internal temperatures unless otherwise noted. Solvents for extraction and chromatography were purified by standard procedures prior to use. Reactions were monitored by thin layer chromatography (TLC) carried out on Merck TLC silica gel plates (60 F_{254}), using UV light for visualization. Column chromatography purification was performed using Merck silica gel 60 (particle size 0.040–0.060 mm). Elemental analysis of synthesized compounds was performed on a Carlo-Erba ER-20 CNH analyser. ^{1}H and ^{13}C NMR spectra were recorded using an Agilent 400-MR spectrometer (400.13 MHz for ^{1}H; 100.6 MHz for ^{13}C) at room temperature; chemical shifts were measured with reference to the solvent ($CDCl_3$, δ_H = 7.24 ppm, δ_C = 77.0 ppm). Chemical shifts (δ) are given in ppm, spin-spin coupling constants (J) are reported in Hz; multiplicities are indicated by s (singlet), d (doublet), t (triplet) and m (multiplet). The melting points were measured in open capillaries and are given without correction. High resolution mass spectra (HRMS) were measured on a Thermo Scientific LTQ Orbitrap instrument using nanoelectrospray ionization (nano-ESI).

Signals of atoms for the 2-methoxyestradiol moiety in NMR spectra were assigned according to reference.[3]

1. General procedure for steroid esterification

To the solution of steroid and 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2, dichloroacetyl or chloroacetyl chloride was added dropwise and then the reaction mixture was stirred for 24 h at room temperature. The mixture was evaporated to dryness and the crude residue was dissolved in diethyl ether. The insoluble impurities were filtered off, the solvent was evaporated and the residue was washed with light petroleum or recrystallized from an appropriate solvent. The following compounds were obtained according to this protocol:

1.1 (17β)-3-hydroxy-2-methoxyestra-1(10),2,4-trien-17-yl dichloroacetate **1c** and *(17β)-2-methoxyestra-1(10),2,4-triene-3,17-diyl bis(dichloroacetate)* **1d**

The reaction of 50 mg (0.165 mmol) (17β)-2-methoxyestra-1(10),2,4-triene-3,17-diol, 10 mg (0.082 mmol) DMAP and 16 µl of dichloroacetyl chloride (0.165 mmol) in 3 ml of CH_2Cl_2 was carried out for 24 h, then additional 6 µl of dichloroacetyl chloride were added and reaction was stirred at room temperature for 48 h. After recrystallization from diethyl ether the mixture of compounds **1c** and **1d** was obtained as light yellow solid at ratio 1 : 4, respectively. After recrystallization from light petroleum the white solid **1c** was obtained, yield 15% (10 mg). The petroleum ether extract containing mixture of compounds **1c** and **1d** was evaporated to dryness and subjected to additional acylation in presence of 10 mg of DMAP in 3 ml of CH_2Cl_2 using 18 µl (0.19 mmol) of dichloroacetyl chloride. After 24 h at room temperature, the reaction mixture was evaporated to dryness and washed with diethyl ether. The pure compound **1d** was obtained as thick light yellow oil, yield 81% (70 mg).

For *(17β)-3-hydroxy-2-methoxyestra-1(10),2,4-trien-17-yl dichloroacetate* **1c**
^{1}H NMR ($CDCl_3$): 6.76 (s, 1H, Ar), 6.63 (s, 1H, Ar), 5.95 (s, 1H, -C<u>H</u>Cl_2), 4.82 (dd, J_1 = 7.8, J_2 = 8.9, 1H, -^{17}C<u>H</u>-O), 3.84 (s, 3H, C<u>H</u>$_3$O), 2.73-2.79 (m, 2H), 2.16-2.27 (m, 3H), 1.62-1.92 (m, 4H), 1.37-1.50 (m, 3H), 1.22-1.35 (m, 3H), 0.88 (s, 3H, C<u>H</u>$_3$). M.P. 132-135 °C
^{13}C NMR ($CDCl_3$, δ): 164.22, 144.19, 143.11, 130.93, 128.96, 114.18, 107.61, 85.45, 64.17, 55.65, 49.27, 43.58, 43.09, 38.06, 36.38, 28.49, 26.82, 26.67, 26.01, 22.76, 11.54.
HRMS (ESI) for $C_{21}H_{26}Cl_2O_4$ (M+nH): found: 412.1387, calculated: 412.1208.

For *(17β)-2-methoxyestra-1(10),2,4-triene-3,17-diyl bis(dichloroacetate)* **1d**

^{1}H NMR ($CDCl_3$): 6.91 (s, 1H, Ar), 6.81 (s, 1H, Ar), 6.19 (s, 1H, C<u>H</u>Cl_2), 5.97 (s, 1H, C<u>H</u>Cl_2), 4.84 (dd, J_1=7.9, J_2=8.9, 1H, ^{17}C<u>H</u>-O), 3.81 (s, 3H, CH_3O-), 2.79-2.82 (m, 2H), 2.21-2.32 (m, 3H), 1.87-1.96 (m, 2H), 1.65-1.84 (m, 2H), 1.41-1.60 (m, 4H), 1.27-1.39 (m, 2H), 0.89 (s, 3H, C<u>H</u>$_3$).
^{13}C NMR ($CDCl_3$, δ): 164.81, 163.03, 148.33, 139.50, 136.92, 129.38, 121.85, 110.24, 89.96, 64.58, 64.03, 56.22, 49.72, 44.20, 43.45, 38.01, 36.72, 28.59, 27.08, 27.00, 26.16, 23.19, 11.94.
HRMS (ESI) for $C_{23}H_{26}Cl_4O_5$ (M+nH): found: 522.5967, calculated: 522.0531.

1.2 *(17β)-2-methoxyestra-1(10),2,4-triene-3,17-diyl bis(dichloroacetate)* **1d**

The reaction of 31 mg (0.103 mmol) (17β)-2-methoxyestra-1(10),2,4-triene-3,17-diol, 17 mg (0.082 mmol) DMAP and 40 µl of dichloroacetyl chloride (0.41 mmol) in 3 ml of CH_2Cl_2 was carried out for 24 h. Then 40 µl of dichloroacetyl chloride and 5 mg of DMAP were added and reaction mixture was stirred for 48 h. After evaporation of reaction mixture and extracting of dry residue with diethyl ether, the pure compound **1d** was obtained as thick light yellow oil, yield 88% (54 mg). The spectral and analytical data of obtained compound were the same as mentioned above for **1d**.

1.3 *(17β)-2-methoxyestra-1(10),2,4-triene-3,17-diyl bis(chloroacetate)* **1e**

The reaction of 30 mg (0.099 mmol) (17β)-2-methoxyestra-1(10),2,4-triene-3,17-diol, 12 mg (0.057 mmol) DMAP and 8 µl of chloroacetyl chloride (0.099 mmol) in 3 ml of CH_2Cl_2 was carried out for 24 h at room temperature. Then the 4 µl of chloroacetyl chloride were added, and reaction mixture was stirred at room temperature for 24 h. After evaporation of reaction mixture and extracting of dry residue with diethyl ether, the pure compound **1e** was obtained after ether evaporation as thick light yellow oil, yield 91% (41 mg).
^{1}H NMR ($CDCl_3$): 6.87 (s, 1H, Ar), 6.75 (s, 1H, Ar), 4.77 (t, J=9.0, 1H, ^{17}C<u>H</u>-O), 4.30 (s, 2H, C<u>H</u>$_2$Cl), 4.06 (s, 2H, C<u>H</u>$_2$Cl), 3.78 (s, 3H, C<u>H</u>$_3$O-), 2.75-2.79 (m, 2H), 2.19-2.29 (m, 3H), 1.83-1.91 (m, 2H), 1.71-1.78 (m, 1H), 1.53-1.64 (m, 2H), 1.40-1.50 (m, 4H), 1.28-1.37 (m, 2H), 0.84 (s, 3H, C<u>H</u>$_3$).
^{13}C NMR ($CDCl_3$): 167.37, 165.75, 148.37, 139.03, 137.07, 129.18, 122.26, 109.87, 84.49, 55.96, 49.69, 44.23, 43.07, 41.10, 40.65, 38.04, 36.75, 29.67, 27.39, 27.01, 26.16, 23.18, 11.99.
HRMS (ESI) for $C_{23}H_{28}Cl_2O_5$ (M+nH): found: 454.1519, calculated: 454.1314.

1.4 17-oxoestra-1(10),2,4-trien-3-yl dichloroacetate **2b**

The reaction of 100 mg (0.370 mmol) 17-oxoestra-1(10),2,4-trien-3-ol, 50 mg (0.41 mmol) DMAP and 90 µl of dichloroacetyl chloride (0.928 mmol) in 5 ml of CH_2Cl_2 was carried out for 24 h, then additional 9 µl of dichloroacetyl chloride were added and the reaction mixture was stirred at room temperature for 48 h. After evaporation of the reaction mixture and extraction of dry residue with diethyl ether, the pure compound **2b** was obtained after ether evaporation as dark yellow oil, yield 93% (119 mg).

^{1}H NMR ($CDCl_3$): 7.31 (d, J=8.6, 1H, Ar), 6.93 (dd, J_1 = 2.4, J_2 =8.6, 1H, Ar), 6.88 (d, J = 1.9, 1H, Ar), 6.13 (s, 1H, C<u>H</u>Cl$_2$), 2.91 (dd, J_1 = 3.9, J_2 =8.6, 2H), 2.52 (dd, J_1 = 8.6, J_2 =19.6, 1H), 2.38-2.43 (m, 1H), 2.25-2.31 (m, 1H), 2.17 (dd, J_1 = 9.0, J_2 =19.6, 1H), 1.94-2.09 (m, 3H), 1.39-1.66 (m, 6H), 0.91 (s, 3H, C<u>H</u>$_3$).
^{13}C NMR ($CDCl_3$): 222.27, 163.29, 148.03, 138.44, 138.40, 126.66, 120.66, 117.82, 64.19, 50.35, 48.10, 44.06, 37.86, 35.90, 31.40, 29.35, 26.22, 25.69, 21.58, 13.81.
HRMS (ESI) for $C_{20}H_{22}Cl_2O_3$ (M+nH): found: 380.1019, calculated: 380.0946.

1.5 17-oxoestra-1(10),2,4-trien-3-yl chloroacetate **2c**

The reaction of 50 mg (0.184 mmol) 17-oxoestra-1(10),2,4-trien-3-ol, 21 mg (0.20 mmol) DMAP and 37 µl of chloroacetyl chloride (0.518 mmol) in 5 ml of $CHCl_3$ was carried out for 48 h, then additional 37 µl of chloroacetyl chloride was added and the reaction mixture was stirred at room temperature for 24 h. After evaporation of reaction mixture and extraction of dry residue with diethyl ether, the pure compound **2c** was obtained after ether evaporation as light yellow oil, yield 97%, (62 mg).

^{1}H NMR ($CDCl_3$): 7.29 (d, J = 8.6, 1H, Ar), 6.88 (dd, J_1 = 8.2, J_2 = 2.0, 1H, Ar), 6.85 (d, J = 2.0, 1H, Ar), 4.28 (s, 2H, C<u>H</u>$_2$Cl), 2.90 (dd, J_1 = 7.8, J_2 = 3.1, 2H), 2.52 (dd, J_1 = 19.2, J_2 = 8.6, 1H), 2.37-2.42 (m, 1H), 2.24-2.29 (m, 1H), 2.15 (dd, J_1 = 19.2, J_2 = 9.0, 1H), 1.93-2.08 (m, 3H), 1.41-1.64 (m, 6H), 0.90 (s, 3H, C<u>H</u>$_3$).

^{13}C NMR ($CDCl_3$): 211.04, 166.24, 148.16, 138.26, 137.96, 126.55, 121.09, 118.24, 50.35, 48.06, 44.07, 40.90, 37.89, 35.89, 31.43, 29.36, 25.25, 25.69, 21.57, 13.80.
HRMS (ESI) for $C_{20}H_{23}ClO_3$ (M+nH): found: 346.1342; calculated 346.1336.

Biological tests

Cell cultures. A549 human lung epithelial carcinoma cells (CCL-185™) were cultured with Dulbecco´s Modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic penicillin/streptomycin at 37 °C under a 5% CO_2 humidified atmosphere.

MTT Cytotoxicity Assay. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] quantitative colorimetric assay was used to measure the cytotoxicity, viability and metabolic activity.[4] The A549 cells were seeded in 96-well plates at a density of 8000 cells per well. Experiments for all compounds were repeated 3–6 times and EC50 values were determined by sigmoid curve fitting using Excel based software.

Proliferation assay. A549 cells were incubated with 1, 10 and 100 μM of each tested compound for 24 and 48 h. 0.5% DMSO was used as a control. After culturing the cells were resuspended in PBS and counted directly by phase-contract microscopy using a hemocytometer.

Immunofluorescence staining of cellular microtubules and nuclei. A549 cells were cultured in 12-well plates on small glass coverslips (11 mm diameter) at a density of 20000 cells per a coverslip. Cells were incubated with tested compounds at concentrations of 1, 10 and 100 μM for 24 and 48 hours. 0.5 % DMSO served as a negative control. The cells were fixed and stained as described.[5] Fixed cells were labelled for tubulin with mouse monoclonal antibody against α-tubulin at a dilution of 1 : 300 (Sigma, St. Louis. USA), followed by incubation of Alexa Fluor488 labelled goat anti-mouse IgG at a dilution of 1 : 300 (Invitrogen, Germany). In order to analyze the compound effect on the apoptosis induction effect, the cell nuclei were stained with Hoechst No. 33258 (Sigma, St. Louis. USA) at concentration 5 μg ml^{-1}. Images of all samples were acquired using a Nikon Diaphot 300 inverted microscope (Nikon GmbH, Düsseldorf, Germany) equipped with a cooled charge-couple device camera system (SenSys Photometrics, Munich, Germany).

References

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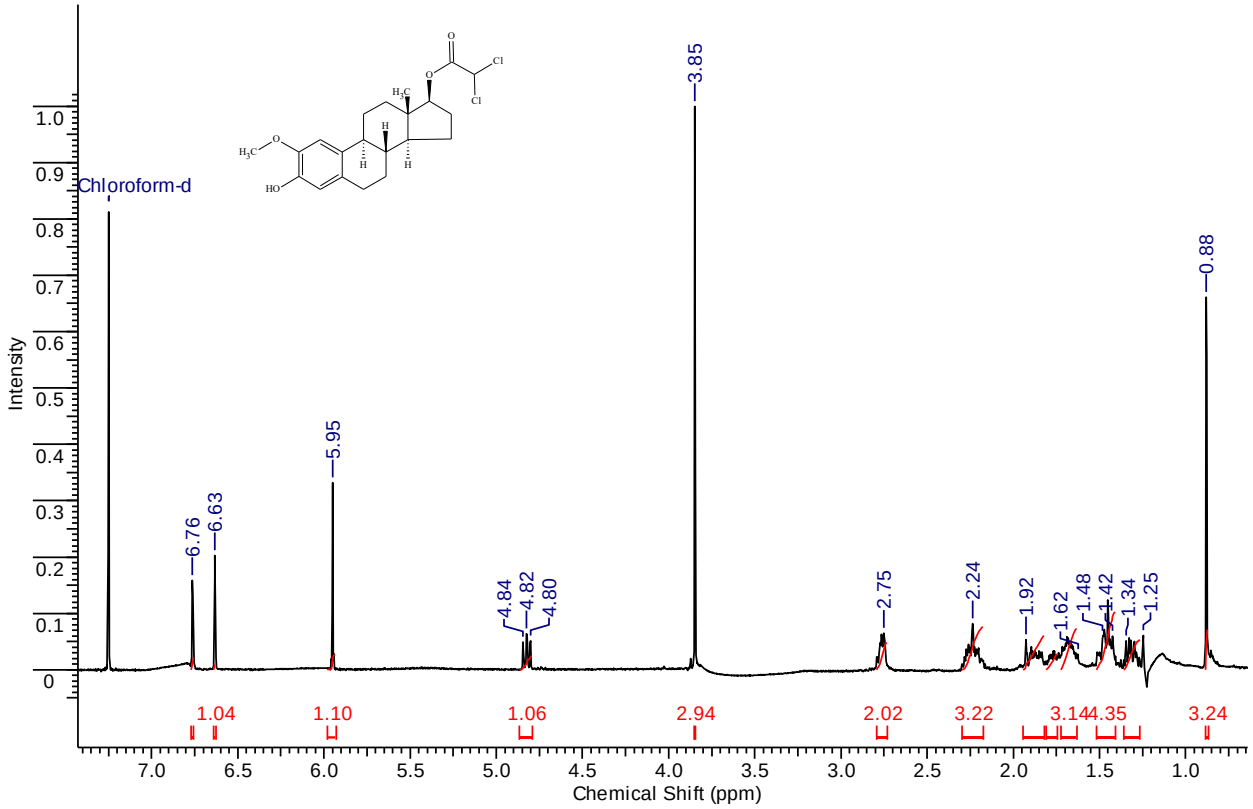
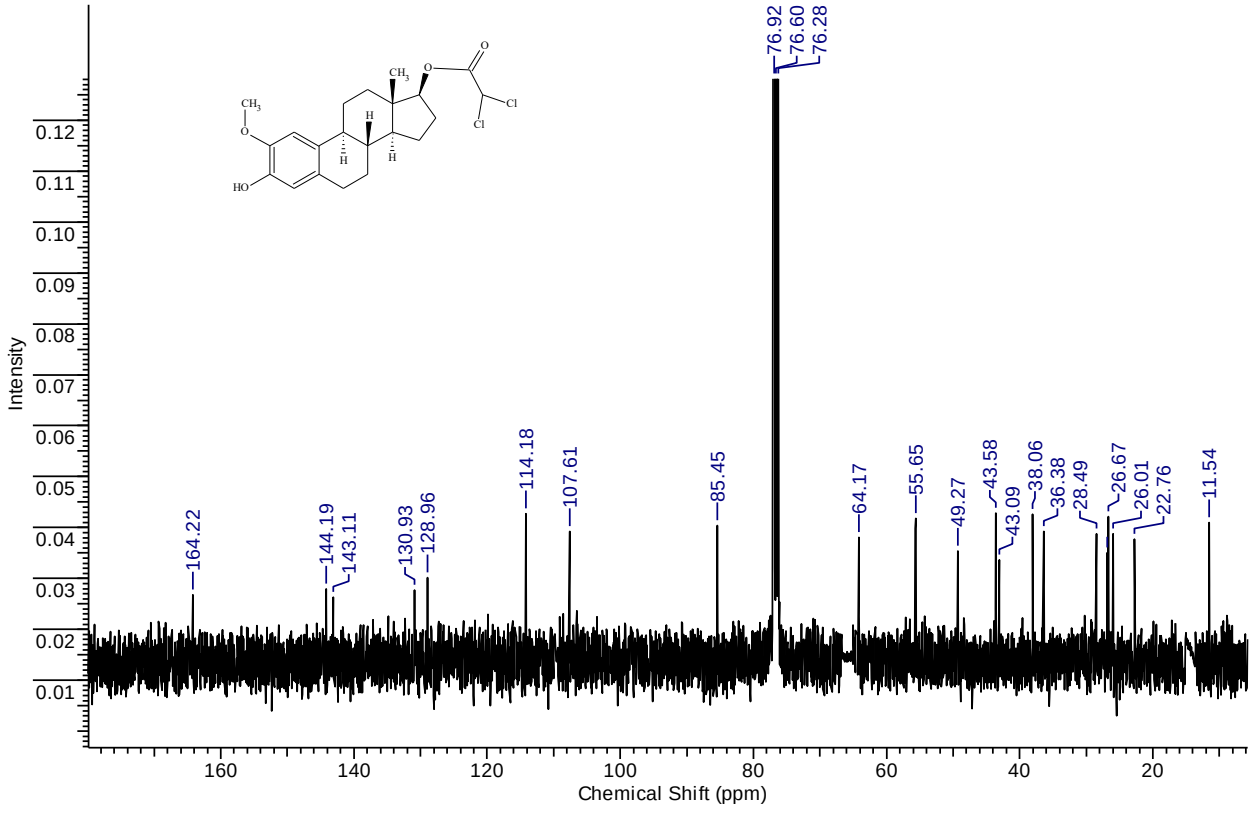
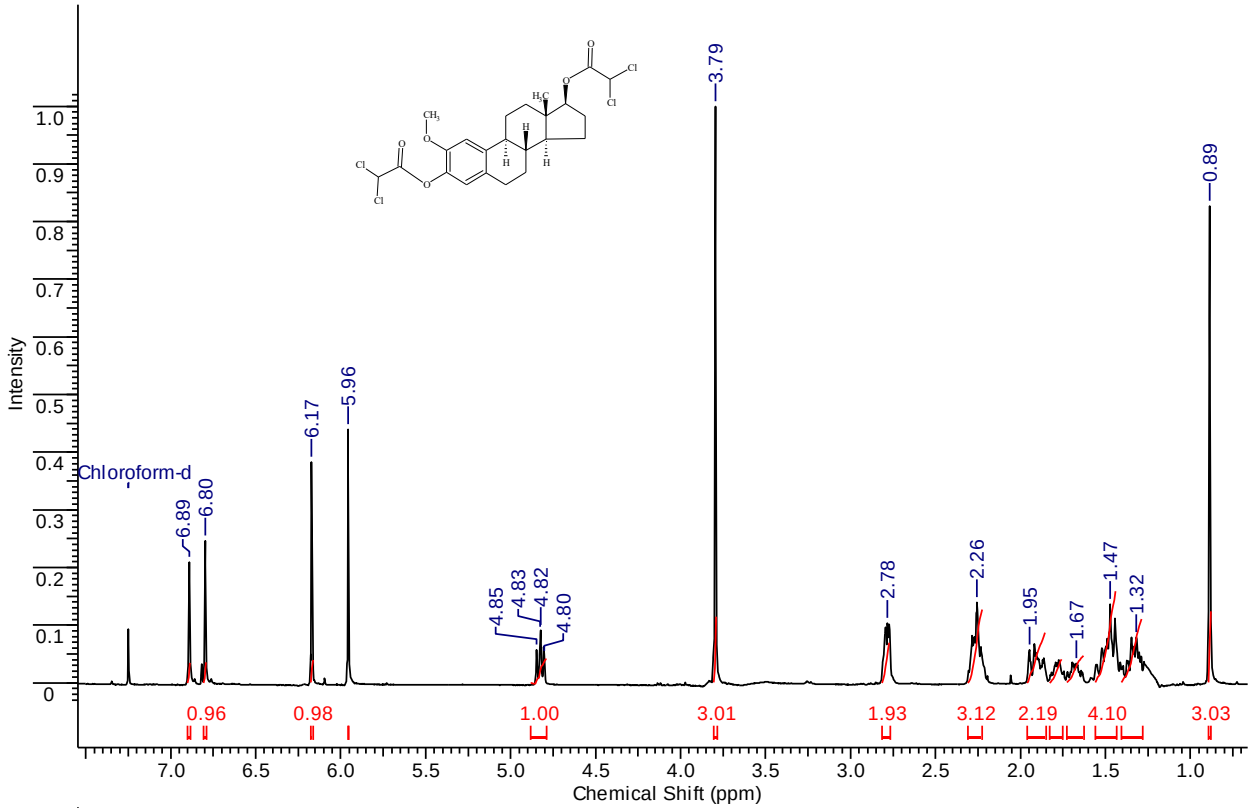
NMR spectral data for obtained compounds

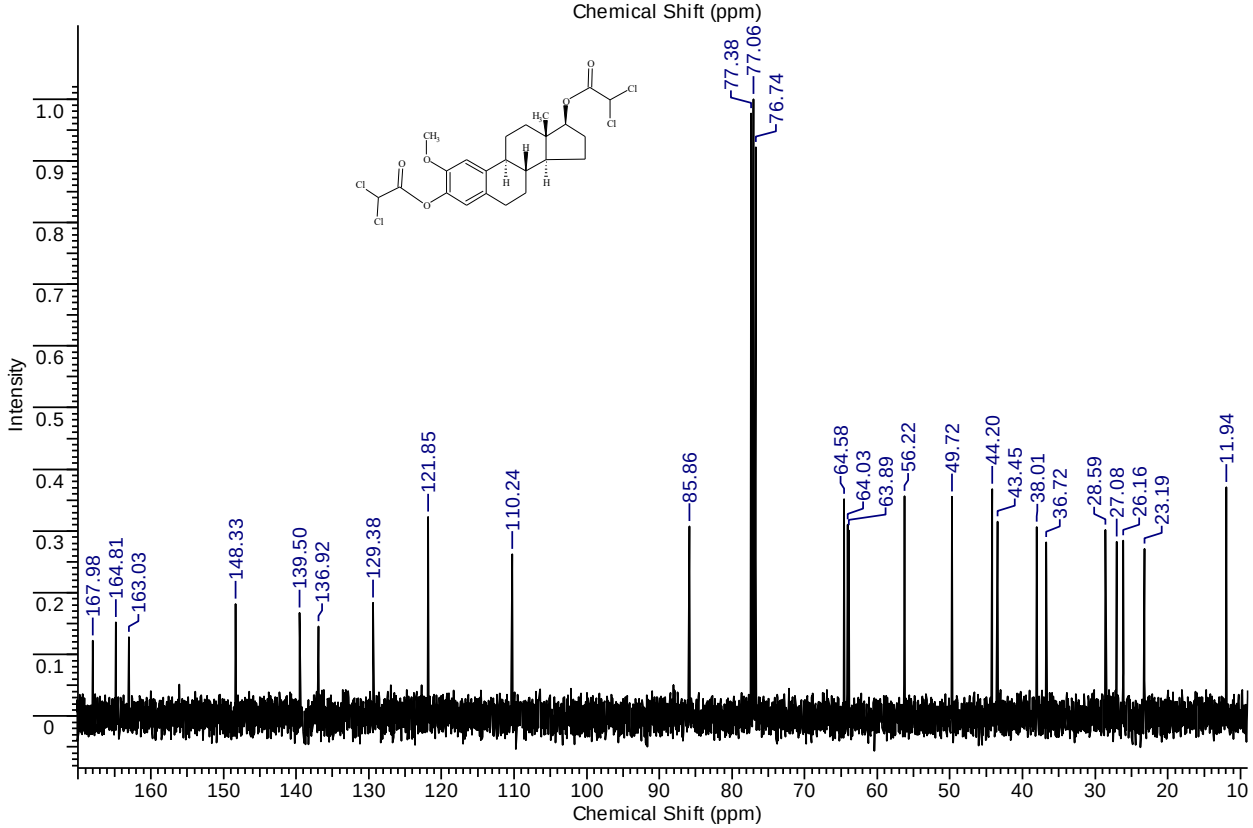
(17β)-3-hydroxy-2-methoxyestra-1(10),2,4-trien-17-yl dichloroacetate **1c**





(17β)-2-methoxyestra-1(10),2,4-triene-3,17-diyl bis(dichloroacetate) **1d**





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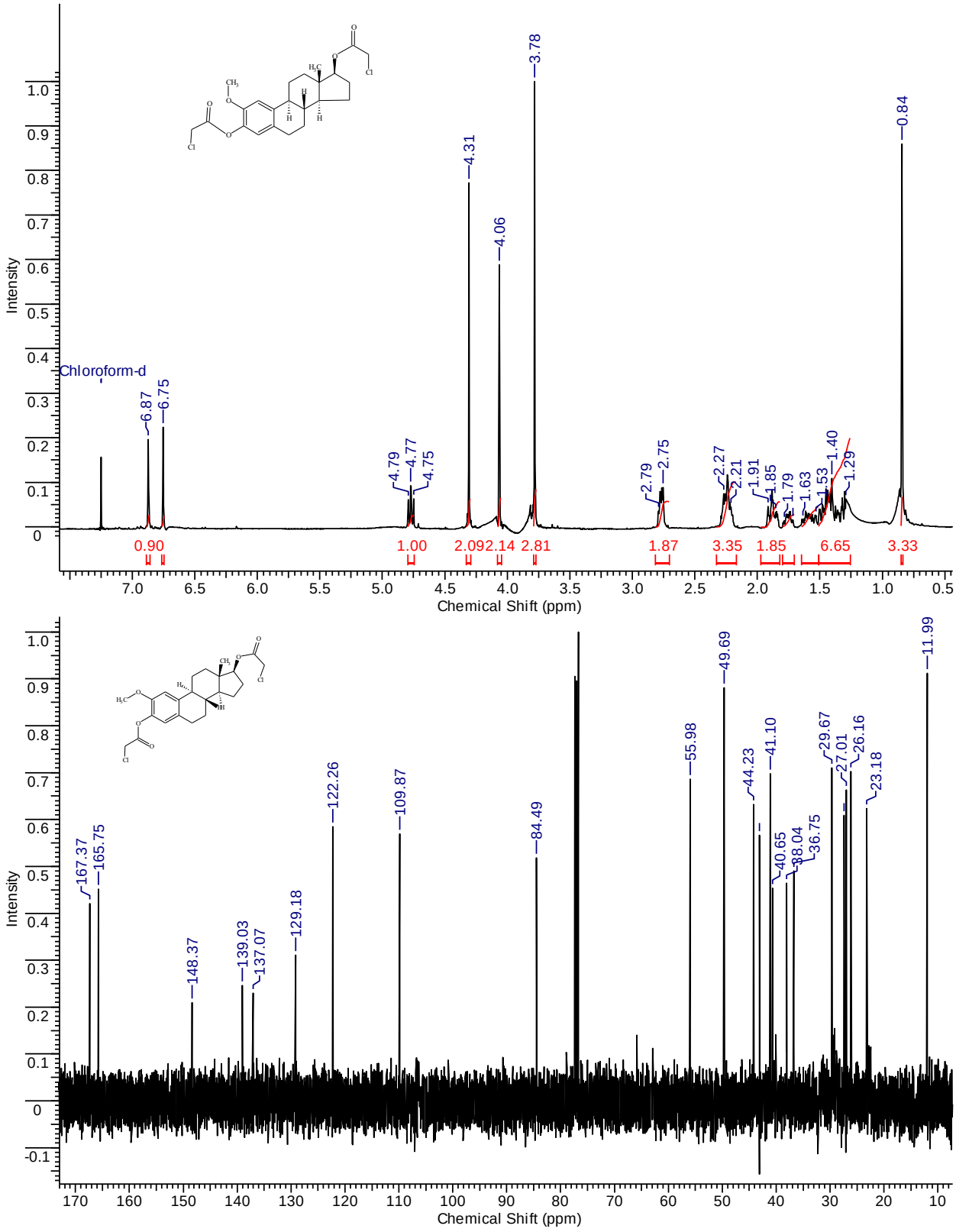
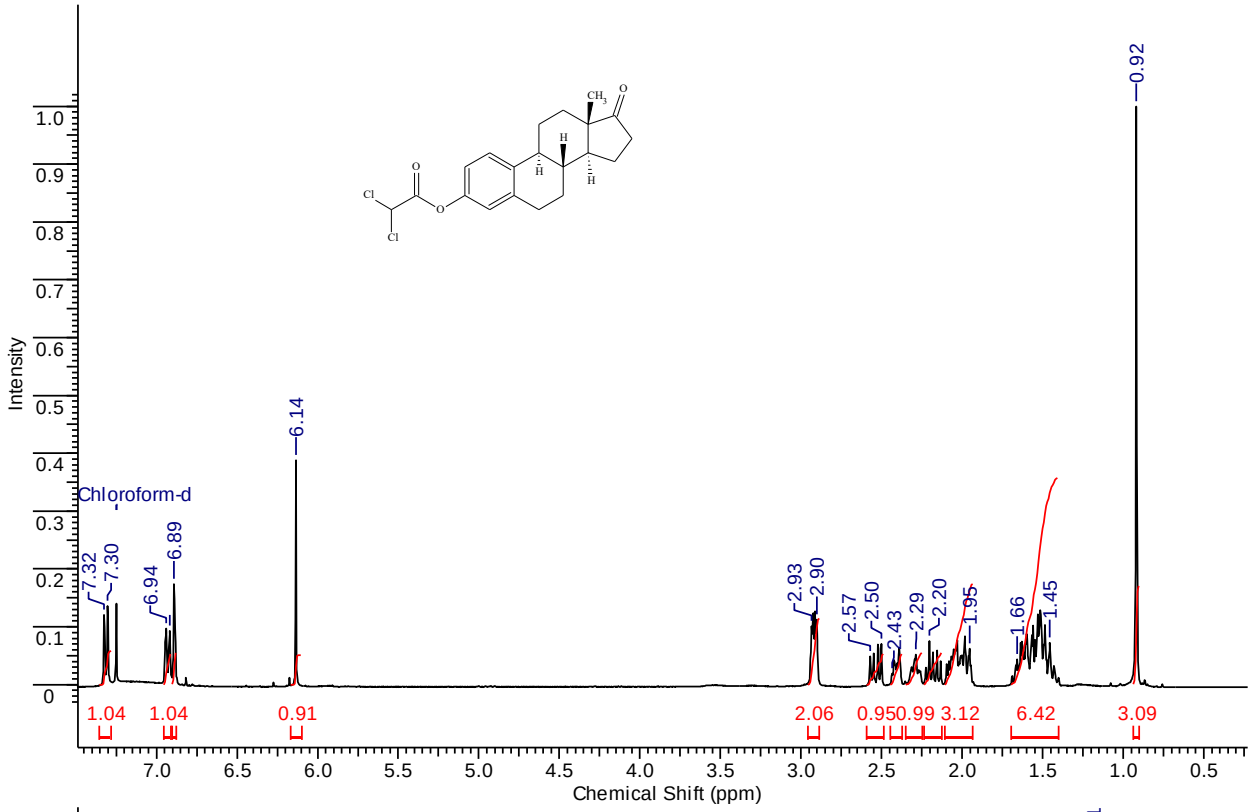
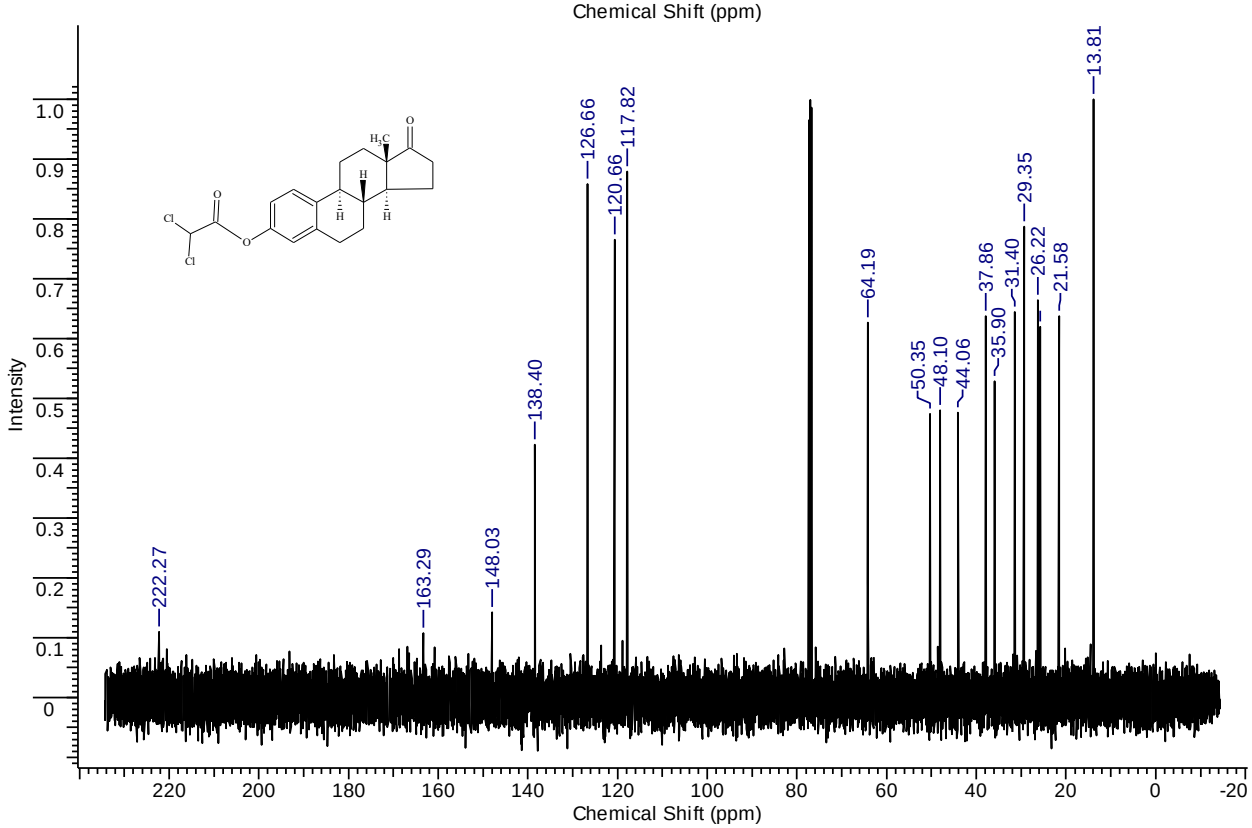


17-oxoestra-1(10),2,4-trien-3-yl dichloroacetate **2b**





17-oxoestra-1(10),2,4-trien-3-yl chloroacetate **2c**