EXPERIMENTAL RESEARCH

CONJUGATES OF PYROPHEOPHORBIDE *A* WITH 17-SUBSTITU TED STEROIDAL ANDROGENS. SYNTHESIS, MOLECULAR MODELING, INTERACTION WITH SOME CANCER CELLS

In memory of Professor Gelii V. Ponomarev (13.05.1940 – 13.10.2021)

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Five new bifunctional conjugates of pyropheophorbide *a* with 17-substituted testosterone, dihydrotestosterone and epitestosterone differing in the length of linker (**1**–**5**) and two new complex conjugates **6** and **7** (containing three functional units: pyropheophorbide *a*, 17α-substituted testosterone, and lipophylic hexadecyl chain, connected with L-lysine joining block) were synthesized. Mutual influence of steroidal and macrocyclic fragments in conjugates (**1**–**7**) was established by analysis of ^{1}H NMR spectra and molecular models of conjugates. Studies of interaction of conjugates **1**–**5** with prostate carcinoma cells revealed that their uptake and internalization were dependent on the structure of conjugates, particularly on the stereochemical configuration of 17-hydroxyl group in steroidal moiety, and the length of linker connecting pyropheophorbide *a* with steroid fragments. Conjugates **1**–**5** significantly decreased the growth and proliferation of LNCaP and PC-3 cells. The highest antiproliferative activity demonstrated by epitestosterone derivative **3**, comprising short linker. Irradiation of labeled cells with light (λ=660 nm) significantly increased cytotoxicity. Trifunctional conjugates **6** and **7** easily formed mixed micells with phosphatidyl choline and pluronic F68; these mixed micelles efficiently internalized by human hepatocarcinoma Hep G2 cells. The binding of conjugates **6** and **7** in the form of mixed micelles to Hep G2 cells depended on the conjugate structure, rather than on the method of solubilization.

Key words: steroid conjugates; chemical synthesis; tetrapyrrolic macrocycles; molecular models; prostate carcinoma cells; phospholipid micelles

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INTRODUCTION

Tetrapyrrolic macrocycles porphyrins and chlorins have a wide range of biomedical applications owing to their unique photochemical and photophysical properties. They are used in optical imaging, fluorescent labeling, photodynamic inactivation of microbial infections, and photodynamic therapy of solid tumors. Coupling of macrocycles with drugs or fragments of biological active molecules significantly improves delivery and distribution of macrocycle-based compounds to a specific location within the cells, facilitates their transport via receptor or drug mediated endocytosis, increased their specificity and selectivity, affects their photo chemical properties and biological activity [1-7].

Conjugation of pheophorbide *a* or pyropheophorbide *a* with steroids is considered to be a promising approach for development of new bifunctional constructs possessing enhanced delivery to specific targets [8-11]. Specifically, such conjugates with estradiol were efficiently internalized by estrogen receptor positive cells, accumulated in nuclei, and revealed a potency of application as sensitizer for photodynamic therapy of breast cancer [8-11].

In this study we have synthesized and investigated seven new conjugates of pyropheophorbide *a* (**Pyro**) with androgen receptor ligands – testosterone, dihydrotestosterone and epitestosterone **1**–**7** (Figure 1). In bifunctional conjugates **1**–**5** macrocyclic and steroidal moieties are connected with ethylene diamine or 1,5-diamino pentane linkers; in conjugates **6** and **7** (L)-lysine residue was used as a linker. The latter allowed to introduce an additional functional fragment, the lipophylic hexadecyl chain, permitting simple solubilization of conjugates in an aqueous medium in the form of mixed micelles with phosphatidyl choline [12].

Presented here results of studies of spectral properties and molecular models of conjugates **1**–**7** revealed a significant influence of the structure on the conformation of conjugates. The study of interaction of conjugates with prostate carcinoma LNCaP and PC-3 cells indicated that conjugates **1**–**5** were efficiently uptaken and internalized by cells and potently inhibited their growth and proliferation. Antiproliferative activity of conjugates

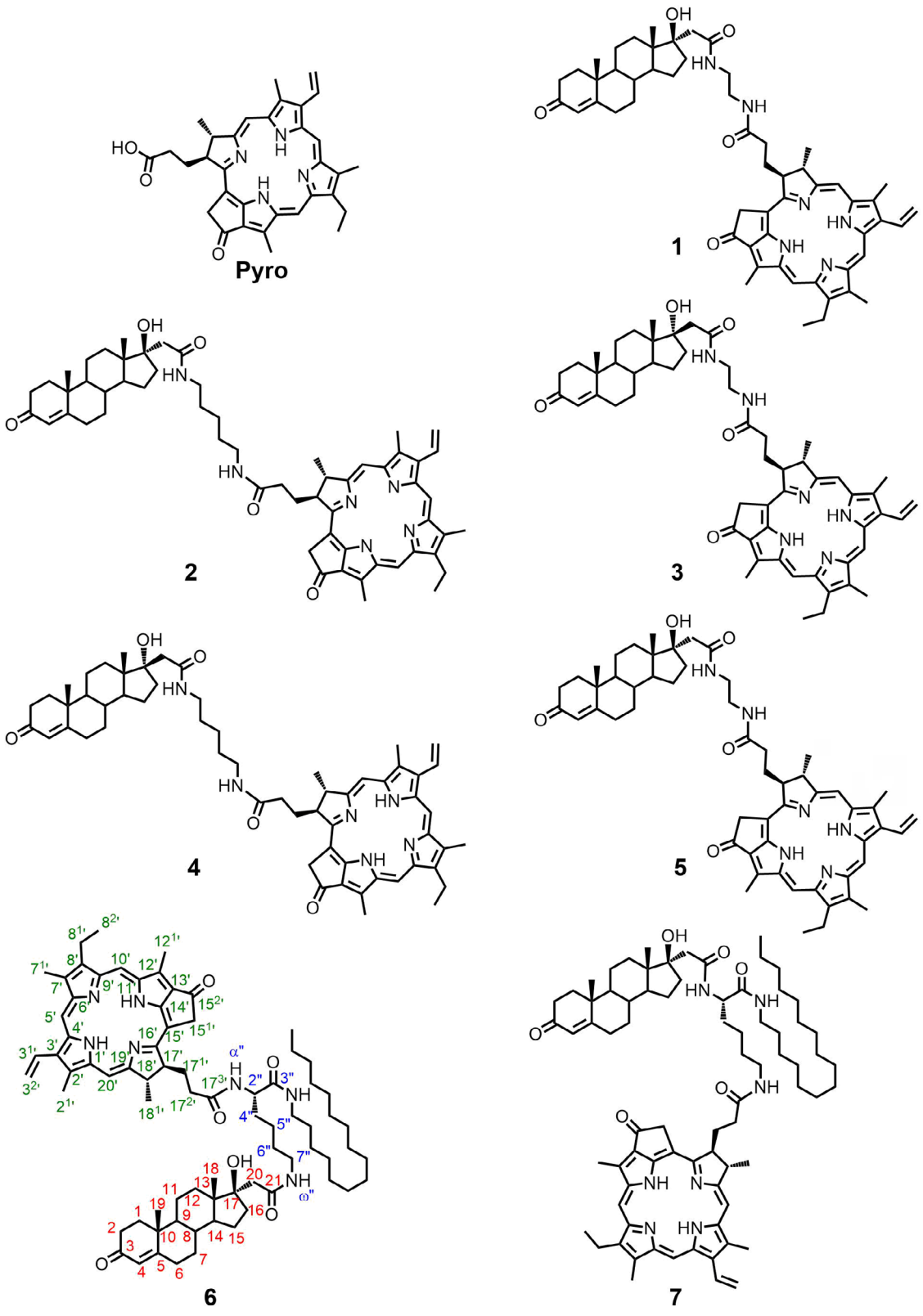


Figure 1. Structures of conjugates **1**–**7** (atom numbering is shown in **6**)

1–**5**, as well as their photo induced toxicity in prostate carcinoma cells depended on the length of a linker and the stereochemical configuration of C17 atom in steroidal part. On the other hand, conjugates **6** and **7** were insoluble in aqueous medium, they did not bind to prostate carcinoma LNCaP and PC-3 cells and did not affect the growth and proliferation of these cells. Nevertheless, they may be uptaken by human hepatocarcinoma Hep G2 cells in the form of mixed micelles with phosphatidyl choline or with biocompatible detergent.

The presented data show that some of new synthesized conjugates of pyropheophorbide *a* with steroids could be considered as prospective agents for various biomedical experiments in cultured cells.

MATERIALS AND METHODS

Materials and General Methods

HRMS were registered using Apex Ultra FT ICR MS ("Bruker") and Daltonics micrOTOF-Q II ("Bruker") instruments at the ion positive electrospray ionization mode; ^{1}H NMR and ^{13}C NMR spectra – on an AMX-III instrument ("Bruker", 400 MHz) in $CDCl_3$ (signals of ^{1}H in $CHCl_3$ was 7.28 ppm, and ^{13}C in $CDCl_3$ was 77.16 ppm). Assignment of ambiguous proton resonances in target compounds was performed by analyzing the set of 2D NMR spectra (data not shown). Absorption spectra were measured with a "Cary Spectra 100" spectro-photometer in $CHCl_3$ and CH_2Cl_2 using a quartz cell with the 1 mm optical path length; particle size distribution was measured using "DelsaNano Beckman Coulter" instrument.

Flash chromatography was performed on silica gel (0.035-0.070 mm) from "Acros", TLC – on HPTLC Silica gel F254 GLP 105564 glass plates from "Merck"; compounds on the plates were visualized by UV light (Filter 254 nm); and/or by spraying the dried developed plates with 5% $(NH_4)_2MoO_4$ in 10% sulfuric acid, followed by heating; pyropheophorbide *a* derivatives were visible on the plates without any treatments.

Dihydrotestosterone, testosterone, N-hydroxysuccinimide, dicyclohexyl carbodiimide, 4-dimethylaminopyridine, N(α)-Fmoc-N(ε)-Boc-Lys, ethylene diamine, 1,5-diamino pentane were obtained from "Acros"; soya bean PC "Lipoid S-100" was purchased from "Lipoids" (Germany), pluronic F68 – from "BASF" (Germany). **Pyro** was prepared from methyl pheophorbide *a* according to the procedure [13]; methyl [17(20) *E*]-6β-methoxy-3α,5α-cyclopregn-17(20)-en-21-oate **18** was synthesized according to procedure [14]; Dess-Martin periodinane – according to procedure [15], other reagents and solvents were purchased from "Aldrich" (USA), "Merck" (Germany), "Acros", "Fluka" (Switzerland) and "Spectra Chem" (Russia).

Chemical Synthesis

Methyl 6β-methoxy-17α,20(R,S)-epoxy-3α,5α-cyclopregn-17(20)-en-21-oate (19)

m-Chloroperbenzoic acid (2.06 g, 8.37 mmol) was added to the solution of compound **18** (2.00 g, 5.58 mmol) in CH_2Cl_2 (60 mL) and the mixture was stirred and heated under re-flux for 8 h, disappearance of starting compound was monitored by TLC. The saturated solutions of $NaHCO_3$ (70 mL) and $NaHSO_3$ (70 mL) were added; the resultant mixture was vigorously stirred for 30 min, then the layers were separated. The aqueous layer was then extracted with dichloromethane (2×30 mL). The combined extract was washed with brine (40 mL), dried over Na_2SO_4, and evaporated. The residue was purified by silica gel flash chromatography in hexane – EtOAc (8:1) followed by evaporation to obtain epoxide **19** (the mixture of two isomers in a ratio of 3:1, 1.34 g, 3.57 mmol, 64%) as colorless glass. HRMS, calculated for $[C_{23}H_{35}O_4]^+$: 375.2530; found: 375.2529. ^{1}H NMR for major isomer: 0.44 and 0.65 (each 1H, m, H-4); 0.93 (3H, s, H-18); 1.00 (3H, s, H-19); 2.78 (1H, t, J=2.7 Hz, H-6); 3.32 (3H, s, CH_3OC6); 3.45 (1H, s, H-20); 3.75 (3H, s, CH_3OC21); ^{1}H NMR for minor isomer: 0.44 and 0.65 (each 1H, m, H-4); 0.89 (3H, s, H-18); 1.02 (3H, s, H-19); 2.78 (1H, t, J=2.7 Hz, H-6); 3.32 (3H, s, CH_3OC6); 3.36 (1H, s, H-20); 3.75 (3H, s, CH_3OC21).

6β-Methoxy-17α,21-dihydroxy-3α,5α-cyclopregnane (20)

The solution of compound **19** (780 mg, 2.1 mmol) in abs. THF (20 mL) was added by drops to the stirred suspension of $LiAlH_4$ (175 mg, 4.6 mmol) in abs. THF (40 mL), and the mixture was stirred and heated under reflux for 2 h. After cooling, excess of $LiAlH_4$ was decomposed by adding of ice water. The mixture was filtered, the residue was washed with Et_2O (2×30 mL). The combined extract was dried over Na_2SO_4, and evaporated to obtain diol **20** (680 mg, 1.9 mmol, 90%) as colorless glass. HRMS, calculated for $[C_{22}H_{37}O_3]^+$: 349.2737; found: 349.2732; ^{1}H NMR: 0.43 and 0.64 (each 1H, m, H-4); 0.74 (3H, s, H-18); 1.03 (3H, s, H-19); 2.76 (1H, m, H-6); 3.32 (3H, s, CH_3O); 3.90 (2H, m, H-21); ^{13}C NMR: 13.2, 16.0, 19.4, 21.6, 22.4, 23.7, 25.0, 29.8, 30.8, 30.9, 33.5, 35.3, 36.8(×2), 43.6, 47.5, 48.1, 49.9, 56.7, 61.1, 82.4, 85.5.

6β-Methoxy-17α-hydroxy-3α,5α-cyclopregnan-21-oic Acid (***21***)

Diol **20** (2.23 g, 6.4 mmol) was dissolved in acetone (120 mL), then $KBrO_3$ (6.4 g, 38.5 mmol), water (80 mL), and RuO_2xH_2O (10 mg) were added, and the mixture was heated under reflux for 20 min, followed by cooling to room temperature. Thereafter EtOH (25 mL) was added by drops, the mixture was filtered, the residue was washed with acetone. The combined filtrate was evaporated, the residue was treated with $CHCl_3$ (150 mL) and water (50 mL). The chloroform extract was washed with brine (50 mL), dried over Na_2SO_4, and evaporated. The residue was applied on the top a silica gel column; the column was initially washed with hexane – EtOAc (2:1) to remove byproducts, then the target compound was eluted with hexane – EtOAc – CH_3COOH (50:49:1) to obtain acid **21** (1.55 g, 4.3 mmol, 67%) as white foam. HRMS, calculated for $[C_{22}H_{35}O_4]^+$: 363.2530; found: 363.2532; ^{1}H NMR: 0.43 and 0.64 (each 1H, m, H-4); 0.76 (3H, s, H-18); 1.01 (3H, s, H-19); 2.58 (2H, AB system, H-20); 2.78 (1H, m, H-6); 3.32 (3H, s, CH_3O); ^{13}C NMR: 13.2, 16.0, 19.7, 21.6, 22.2, 23.7, 25.0, 29.8, 30.8, 33.5, 35.2, 35.3, 37.5, 39.8, 43.5, 47.5, 47.8, 49.6, 56.6, 81.8, 82.5, 178.1.

3β,17α-Dihydroxypregn-5-en-21-oic Acid (22)

Compound **21** (350 mg, 0.97 mmol) was dissolved in THF (15 mL) and after addition of 15% aqueous H_2SO_4 (4 mL) the mixture was heated under reflux for 20 min. After cooling water (50 mL) was added and the mixture was extracted with $CHCl_3$ (3×50 mL). The combined chloroform extract was washed with brine (30 mL), dried over Na_2SO_4, and evaporated to obtain acid **22** (300, mg, 0.86 mmol, 89%) as white powder which was used without purification. The analytical sample was obtained after silica gel flash chromatography in hexane – EtOAc – CH_3COOH (75:24:1). HRMS, calculated for $[C_{21}H_{33}O_4]^+$: 349.2373; found: 349.2370; ^{1}H NMR: 0.56 (3H, s, H-18); 0.84 (3H, s, H-19); 2.42 (2H, AB system, H-20); 3.38 (1H, m, H-3); 5.176 (1H, m, H-6); ^{13}C NMR: 14.91, 18.65, 20.37, 23.51, 29.90; 30.49, 31.68, 32.05, 36.28, 36.57, 36.97, 39.42; 41.12, 46.90, 49.45, 49.74; 71.55; 82.16; 121.36; 140.35; 178.21.

3-Oxo-17α-hydroxypregn-4-en-21-oic Acid (23)

Dess-Martin periodinane (902 mg, 2.12 mmol) was added to the stirred suspension of compound **22** (300 mg, 0.86 mmol) in dichloromethane (20 mL) and after addition of

water (10 mL, 0.56 mmol) the mixture was stirred for 30 min more; disappearance of compound **22** during the reaction was controlled by TLC. The mixture was cooled to 4°C, and after dropwise addition of EtOH (20 mL) it was poured into water (50 mL) and extracted with $CHCl_3$ (3×20 mL). The extract was washed with brine (30 mL), dried over Na_2SO_4, and evaporated. The residue dissolved in abs. EtOH (8 mL) was mixed with oxalic acid (36 mg, 0.4 mmol). The mixture was stirred and heating under reflux for 10 min and poured into water (50 mL). The suspension obtained was extracted with $CHCl_3$ (3×20 mL). The extract was washed with brine (30 mL), dried over Na_2SO_4 and evaporated. The residue was purified by silica gel flash chromatography in hexane – acetone – CH_3COOH (64:35:1) and evaporated to obtain 3-oxo-17α-hydroxypregn-4-en-21-oic acid **23** (193 mg, 0.56 mmol, 65%) as white solid. HRMS, calculated for $[C_{21}H_{31}O_4]^+$: 347.2217; found: 347.2219; ^{1}H NMR: 0.76 (3H, s, H-18); 1.18 (3H, s, H-19); 2.59 (2H, AB system, H-20); 3.38 (1H, br. s, 17-OH); 5.73 (1H, s, H-4); ^{13}C NMR: 15.5, 17.4, 20.6, 23.7; 30.2, 32.0, 32.9, 33.9, 35.7, 35.9, 37.4, 38.7, 39.6, 47.1, 49.0, 53.5, 81.5, 123.8, 171.8, 177.1, 200.0.

Pentafluorophenyl Pyropheophorbide a (24)

Pyropheophorbide *a* (**Pyro**) (150 mg, 0.28 mmol) was dissolved in 15 mL of CH_2Cl_2, then pentafluorophenyl trifluoroacetate (0.096 mL, 0.56 mmol) was added, thereafter Et_3N (0.039 mL, 2.8 mmol) was added dropvise to stirred solution during 10 min. The formation of pentafluorophenyl ester was controlled by TLC. After the reaction was completed, the solvent was evaporated in vacuo, the residue was twice evaporated with toluene, and purified by chromatography on silica gel in hexane – acetone (4:1) to give ester **24** (186 mg, 2.7 mmol, 95 %). HRMS, calculated for $[C_{39}H_{34}F_5N_4O_3]^+$: 701.2551; found: 701.2554. ^{1}H NMR: -1.45 (1H br.s, N-H); 1.68 (1H, t, J = 7.6 Hz, 82-H); 1.84 (3H, d, J = 7.3 Hz, 18-CH_3); 3.22, 3.41, 3.65 (each 3H, s, 2-, 7-, 12-CH_3); 4.37, 4.52 (each 1H, m, 17^1-H and 8^1-H); 5.18, 5.24 (each 1H, d, J = 19.7 Hz, 17^2-H), 6.17 (1H, dd, J = 11.5 Hz and J = 1.4 Hz, 8^2-H, *cis*), 6.22 (1H, dd, J = 17.9 Hz and J = 1.4 Hz, 8^2-H, *trans*), 7.98 (1H, dd, J = 11.5 Hz and J = 17.9 Hz, 8^1-H), 8.57, 9.37, 9.48 (each 1H, s, 5-, 10-, 20-H). ^{13}C NMR: 11.28; 12.12; 17.44; 19.53; 23.14; 29.72; 30.32; 47.99; 50.01; 51.41; 93.05; 97.41; 104.40; 105.98; 122.76; 128.57; 129.23; 130.37; 131.83; 136.17; 136.27; 136.54; 136.71; 136.83; 137.99; 138.38; 139.25; 139.92; 140.90; 141.92; 142.47; 145.27; 149.23; 151.02; 155.65; 159.57; 169.17; 177.30; 196.55; Absorption spectra in CH_2Cl_2, λ_{max}, nm: 398, 498, 660.

Procedure for Preparation of Amides 25 and 26

The mixture of C_6F_5-Pyro **25** (210 mg, 0.3 mmol), diamine (ethylene diamine or 1,5-diaminopentane, 6.0 mmol) and abs. CH_2Cl_2 (10 mL) was stirred for 2 h, then the mixture was poured into 0.1 M CH_3COONa buffer, pH 5 (20 mL), extracted with CH_2Cl_2 (2×20 mL), the combined extract was washed with brine (20 mL), dried over Na_2SO_4, and evaporated. Then the residue was dissolved in tetrahydrofuran (30 mL), the solution was dried over granulated KOH, followed by evaporation to dryness.

17^3[(2-Aminoethyl)-amido]-pyropheophorbide a (25)

Compound **25** was purified by flash chromatography in $CHCl_3$ – MeOH – NH_4OH (90:9:1) mixture, was obtained as a black amorphous powder (135 mg, 0.22 μmol, 73%). HRMS, calculated for $[C_{35}H_{41}N_6O_2]^+$: 577.3291, found: 577.3292; ^{1}H-NMR: -1.70, 0.33 (each 1H, br.s, N-H); 1.62 (3H, t, J = 7.6 Hz, H-8$^{2'}$); 1.75 (3H, d, J = 7.3 Hz, 18-CH_3); 3.18, 3.37, 3.41 (each 3H, s, H-2', H-7', H-12'); 4.23, 4.45 (each 1H, m, H-17$^{1'}$ and H-8$^{1'}$); 4.98, 5.19 (each 1H, d, J = 19.7 Hz, H-17$^{2'}$); 6.13 (1H, dd, J = 11.5 Hz and J = 1.4 Hz, H-3$^{2'}$, *trans*); 6.24 (1H, dd, J = 17.9 Hz and J = 1.4 Hz, H-3$^{2'}$, *cis*); 7.95 (1H, dd, J = 11.5 Hz and J = 17.9 Hz, H-3$^{1'}$); 8.50, 9.24, 9.30 (each 1H, s, H-5', H-10', H-20'); ^{13}C-NMR: 11.2; 11.8; 12.1; 17.4; 19.4; 23.0; 28.3; 30.2; 30.9; 32.8; 40.9; 41.7; 48.0; 47.0; 51.7; 92.9; 97.1; 103.9; 106.0; 122.7; 128.1; 129.2; 131.5; 135.8; 136.0; 136.1; 137.7; 144.9; 148.9; 150.7; 155.1; 160.4; 171.7; 172.4; 196.1. Absorption spectra in CH_2Cl_2, λ_{max}, nm (ε): 413 (85 000); 507 (8 900); 538 (8 000); 609 (7 000); 665 (35 200).

17^3[(2-Aminoethyl)-amido]-pyropheophorbide a (26)

Compound **26** was purified by flash chromatography in $CHCl_3$ – MeOH – NH_4OH (90:9:1) mixture, was obtained as black amorphous powder (130 mg, 0.21 μmol, 70%). HRMS, calculated for $[C_{38}H_{47}N_6O_2]^+$: 619.3760; found: 619.3749; ^{1}H NMR: −1.70, 0.40 (each 1H, br.s, N–H); 1.64 (3H, t, J=7.6 Hz, H-8$^{2'}$); 1.78 (3H, d, J=7.3 Hz, H-18$^{1'}$); 3.20, 3.38, 3.46 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$); 4.30, 4.48 (each 1H, m, H-17$^{1'}$ and H-8$^{1'}$); 5.05 (1H, br. s, NH-CO); 5.05, 5.21 (each 1H, d, J=19.7 Hz, H-15$^{1'}$); 6.15 (1H, dd, J=11.5 Hz and J=1.4 Hz, H-3$^{2'}$, *trans*); 6.26 (1H, dd, J=17.9 Hz and J=1.4 Hz, H-3$^{2'}$, *cis*); 7.96 (1H, dd, J=11.5 Hz and J=17.9 Hz, H-3$^{1'}$); 8.53, 9.31, 9.34 (each 1H, s, H-5', H-10', H-20'); ^{13}C NMR: 11.3, 11.9, 12.2, 17.5, 19.5, 23.2, 24.0, 29.1, 30.3, 32.9, 33.0, 39.3, 41.8, 48.1, 50.1, 51.8, 93.0, 97.2, 104.1, 106.2, 122.6, 128.3, 129.3, 130.5, 131.6, 135.9, 136.1, 136.2, 137.8, 141.6, 145.1, 149.0, 150.8, 155.3, 160.5, 171.9, 172.1 (C1', C6', C9', C17$^{3'}$, C19'); 196.2 (C13$^{1'}$). Absorption spectra in CH_2Cl_2, λ_{max}, nm (ε): 413 (85 000); 507 (8 900); 538 (8 000); 609 (7 000); 665 (35 200).

Procedure for Preparation of Conjugates 1–5

The mixture of carboxylic acid (**12**, **17**, or **23**, 0.1 mmol), amino containing amide (**25**, or **26**, 0.1 mmol), and DCC (23 mg, 0.11 mmol) in CH_2Cl_2 (5 mL) was stirred at room tem-perature for 2 h, evaporated to dryness, and the residue was then applied on the top of a silica gel column. The column was initially washed with $CHCl_3$ – acetone – CH_3COOH (75:24:1) to remove byproducts, washed with $CHCl_3$ (5 mL), and finally the target product was eluted with $CHCl_3$ – MeOH – 7 M solution of NH_3 in MeOH (93:5:2, v/v/v). After evaporation the conjugates were dried *in vacuo*.

17$^{3'}$[2"-(17β-Hydroxy-3-oxopregn-4-en-21-oylamidoethyl) amido]-pyropheophorbide a (Conjugate 1)

Conjugate **1** (48 mg, 51 μmol, 51%) was obtained as a black powder. HRMS, calculated for $[C_{56}H_{69}N_6O_5]^+$: 905.5329, found: 905.5327; ^{1}H-NMR: -1.65 (1H, br. s, N-H); 0.25, 0.75 (each 3H, s, H-18 and H-19); 1.58 (3H, t, J = 7.6 Hz, H-8$^{2'}$); 1.79 (3H, d, J = 7.3 Hz, H-18$^{1'}$); 3.16, 3.17, 3.39 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$); 4.24, 4.48 (each 1H, m, H-17$^{1'}$ and H-8$^{1'}$); 4.95, 5.19 (each 1H, d, J = 19.7 Hz, H-17$^{2'}$); 5.56 (1H, s, H-4); 6.14 (1H, dd, J = 11.5 Hz and J = 1.4 Hz, H-3$^{2'}$, *trans*); 6.29 (1H, dd, J = 17.9

Hz and J = 1.4 Hz, H-3$^{2'}$, *cis*); 6.30 (1H, br. t, J = 5.2 Hz, NH-CO); 6.61 (1H, br. t, J = 5.2 Hz, NH-CO); 7.91 (1H, dd, J = 11.5 Hz and J = 17.9 Hz, H-3$^{1'}$), 8.52, 9.04, 9.26 (each 1H, s, H-5', H-10', H-20'); ^{13}C-NMR: 11.1; 11.6; 12.0; 15.1; 16.9; 17.3; 19.2; 20.1; 23.0; 23.3; 29.9; 30.0, 30.4, 31.6; 32.6; 32.9; 33.7; 35.2; 35.5; 37.0; 38.2; 39.6; 40.4; 46.5; 48.0; 48.5; 50.1; 51.6; 52.9; 81.6; 94.0; 97.1; 103.9; 105.8; 122.6; 123.6; 127.8; 129.0; 129.9; 131.7; 135.9; 136.0; 136.3; 137.5; 141.7; 145.1; 148.8; 150.7, 155.4; 160.1, 171.2; 171.8; 173.6; 173.7; 196.3; 199.3. Absorption spectrum is presented in Fig. 2.

17$^{3'}$[5"-(17β-Hydroxy-3-oxopregn-4-en-21-oylamidopentyl) amido]-pyropheophorbide a (Conjugate 2)

Conjugate **2** (48 mg, 51 μmol, 51%) was obtained as a black powder. HRMS, calculated for $[C_{59}H_{75}N_6O_5]^+$: 947.5793; found: 947.5789; ^{1}H NMR: −1.66 (1H, br. s, N–H); 0.73, 1.00 (each 3H, s, H-18 and H-19); 1.64 (3H, t, J=7.6 Hz, H-8$^{2'}$); 1.78 (3H, d, J=7.3 Hz, H-18$^{1'}$); 3.19, 3.37, 3.46 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$); 4.27, 4.46 (each 1H, m, H-17$^{1'}$ and H-8$^{1'}$); 5.00, 5.16 (each 1H, d, J=19.7 Hz, H-15$^{1'}$); 5.28 (1H, br. t, J=5.2 Hz, NH-CO); 5.53 (1H, s, H-4); 6.15 (1H, dd, J=11.5 Hz and J=1.4 Hz, H-3$^{2'}$, *trans*); 6.25 (1H, dd, J=17.9 Hz and J=1.4 Hz, H-3$^{2'}$, *cis*); 6.33 (1H, br. t, J=5.2 Hz, NH-CO); 7.94 (1H, dd, J=11.5 Hz and J=17.9 Hz, H-3$^{1'}$); 8.51, 9.28, 9.31 (each 1H, s, H-5', H-10', H-20'); ^{13}C NMR: 11.3, 12.0, 12.2, 13.9, 17.3, 17.5, 19.5, 20.6, 23.1, 23.6, 23.9, 28.8, 29.0, 29.8, 30.5, 31.7, 32.7, 33.1, 33.9, 35.7, 36.2, 36.5, 38.6, 39.0, 39.1, 42.6, 46.2, 48.1, 50.1 (x2), 51.7, 53.8, 82.0, 93.1, 97.3, 104.1, 106.0, 122.7, 123.8, 128.2, 129.2, 130.3, 131.8, 136.0, 136.2, 136.4, 137.8, 141.8, 145.2, 149.1, 150.9, 155.5, 160.5, 171.0, 172.0, 172.5, 173.4, 196.3, 199.4. Absorption spectrum is presented in Fig. 2.

17$^{3'}$[2"-(17α-Hydroxy-3-oxopregn-4-en-21-oylamidoethyl) amido]-pyropheophorbide a (Conjugate 3)

Conjugate **3** (28 mg, 31 μmol, 31%) was obtained as a black powder. HRMS, calculated for $[C_{56}H_{69}N_6O_5]^+$: 905.5329, found: 905.5335; ^{1}H NMR: −1.67 (1H, br. s, N–H); 0.54, 0.88 (each 3H, s, H-18 and H-19); 1.55 (3H, t, J=7.7 Hz, H-8$^{2'}$); 1.75 (3H, d, J=7.3 Hz, H-18$^{1'}$); 3.13, 3.15, 3.35 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$); 4.20, 4.45 (each 1H, m, H-17$^{1'}$ and H-8$^{1'}$); 4.92, 5.15 (each 1H, d, J=19.7 Hz, H-15$^{1'}$); 5.53 (1H, s, H-4); 6.11 (1H, dd, J=11.6 Hz and J=1.4 Hz, H-3$^{2'}$, *trans*); 6.25 (1H, dd, J=17.8 Hz and J=1.4 Hz, H-3$^{2'}$, *cis*); 6.24 (1H, br. t, J=5.2 Hz, NH-CO); 6.78 (1H, br. t, J=5.2 Hz, NH-CO); 7.88 (1H, dd, J=11.6 Hz and J=17.8 Hz, H-3$^{1'}$); 8.58, 9.10, 9.24 (each 1H, s, H-5', H-10', H-20'); ^{13}C NMR: 11.2, 11.6, 12.1, 15.1, 16.9, 17.3, 19.3, 20.1, 23.0, 23.3, 29.9, 30.5, 31.6, 32.6, 33.0, 33.7, 35.2, 35.5, 37.0, 38.2, 39.6, 40.0, 40.5, 46.5, 48.1, 48.5, 50.1, 51.7, 53.0, 81.6, 93.0, 97.1, 103.9, 105.8, 122.7, 123.6, 127.8, 129.0, 129.9, 131.7, 135.9, 136.0, 136.3, 137.6, 141.7, 145.1, 148.9, 150.8, 155.4, 160.2, 171.2, 171.9, 173.6, 173.8, 196.3, 199.3. Absorption spectrum is presented in Fig. 2.

17$^{3'}$[5"-(17α-Hydroxy-3-oxopregn-4-en-21-oylamidopentyl) amido]-pyropheophorbide a (Conjugate 4)

Conjugate **4** (44 mg, 46 μmol, 46%) was obtained as a black powder. HRMS, calculated for $[C_{59}H_{75}N_6O_5]^+$: 947.5793; found: 947.5793; ^{1}H NMR: −1.67 (1H, br. s, N–H); 0.25, 0.81 (each 3H, s, H-18 and H-19); 1.61 (3H, t, J=7.6 Hz, H-8$^{2'}$), 1.78 (3H, d, J=7.3 Hz, H-18$^{1'}$), 3.17, 3.36, 3.37 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$); 4.26, 4.46 (each 1H, m, H-17$^{1'}$ and H-8$^{1'}$); 5.00, 5.15 (each 1H, d, J=19.7 Hz, H-15$^{1'}$); 5.53 (1H, br. t, J=5.2 Hz, NH-CO); 5.58 (1H, s, H-4); 6.14 (1H, dd, J=11.5 Hz and J=1.4 Hz, H-3$^{2'}$, *trans*); 6.22 (1H, br. t, J=5.2 Hz, NH-CO); 6.23 (1H, dd, J=17.9 Hz and J=1.4 Hz, H-3$^{2'}$, *cis*); 7.92 (1H, dd, J=11.5 Hz and J=17.9 Hz, H-3$^{1'}$); 8.50, 9.24, 9.32 (each 1H, s, H-5', H-10', H-20'); ^{13}C NMR: 11.3, 11.9, 12.1, 15.2, 17.1, 17.4, 19.4, 20.3, 23.1, 23.4, 23.7, 28.5, 28.9, 30.2, 30.6, 31.8, 32.0, 33.1, 33.9, 35.4, 35.7, 37.1, 38.4, 38.9, 39.0, 40.3, 46.6, 48.1, 48.7, 50.1, 51.7, 53.2, 81.8, 93.1, 97.2, 104.0, 105.9, 122.7, 123.7, 128.1, 129.1, 130.2, 131.8, 136.0, 136.2, 136.4, 137.8, 141.7, 145.2, 149.0, 150.8, 155.4, 160.5, 171.5, 172.0, 172.8, 173.1, 196.3, 199.6. Absorption spectrum is presented in Fig. 2.

17$^{3'}$[2"-(17β-Hydroxy-3-oxopregnan-21-oylamidoethyl) amido]-pyropheophorbide a (Conjugate 5)

Compound **5** (33 mg, 37 μmol, 69 %) was obtained as a black powder. HRMS, calculated for $[C_{56}H_{71}N_6O_5]^+$: 907.5486, found: 907.5490. ^{1}H NMR: –1.67, (1H, br.s, N–H); 0.56, 0.78 (each 3H, s, H–18 and H–19); 1.65 (3H, t, J=7.6 Hz, 8$^{2'}$); 1.77 (3H, d, J=7.3 Hz, 18$^{1'}$); 3.21, 3.37, 3.45 (each 3H, s, H-2$^{1'}$, H-7$^{2'}$, H-12$^{1'}$); 4.27, 4.46 (each 1H, m, H-17$^{1'}$, H-8$^{1'}$); 5.02, 5.21 (each 1H, d, J=19.7 H-17$^{2'}$); 5.86 (1H, br.t, J=5.2 Hz, NH–CO); 6.14 (1H, dd, J=11.5 Hz and J=1.4 Hz, H-32, *cis*), 6.20 (1H, dd, J=17.9 Hz and J=1.4 Hz, H-3$^{2'}$, *trans*), 6.58 (1H, br. t, J=5.2 Hz, NH–CO); 7.93 (1H, dd, J=11.5 Hz and J=17.9 Hz, H-3$^{1'}$); 8.52, 9.30, 9.33 (each 1H, s, H-5', H-10', H-20'); ^{13}C NMR: 11.4, 12.0, 13.6, 14.0, 17.3, 19.3, 20.6, 23.1, 23.4, 28.4, 28.7, 29.7, 31.5, 31.9, 33.0, 33.9, 35.3, 35.7, 36.2, 38.5, 39.7, 42.5, 46.0, 46.6, 48.0, 50.0 (x2), 51.7, 53.7, 81.9, 93.1, 7.1, 103.9, 105.9, 122.7, 124.2, 125.3, 128.2, 129.0, 130.0, 131.7, 136.0, 136.3, 137.6, 137.9, 141.7, 145.0, 148.7, 148.9, 160.5, 172.0, 173.6, 174.2, 174.5, 196.3, 211.5; 49. Absorption spectrum is presented in Fig. 2.

N(α)-Fmoc-N(ε)-Boc-Lys-hexadecyl Amide (28)

N(*α*)-Fmoc-N(*ε*)-Boc-Lys **27** (200 mg, 0.427 mmol) and DCC (97 mg, 0.47 mmol) were dissolved in dry CH_2Cl_2 (12 mL), after addition of hexadecyl amine (103 mg, 0.427 mmol), the mixture was stirred for 1 h, diluted with CH_2Cl_2, washed with $NaHCO_3$ saturated solution (20 mL), water (20 mL), brine (20 mL), dried over Na_2SO_4 and evaporated to obtain amide **28** (257 mg, 0.371 mmol, 87 %) as white powder. HRMS, calculated for $[C_{42}H_{66}N_3O_5]^+$: 692.4997, found: 692.4988. ^{1}H NMR: 0.87 (3H, t, J=6.7 Hz, CH_3-hexadecyl), 1.24 (28H, m, $(CH_2)_{14}$-hexadecyl), 1.42 (9H, s, CH_3-Boc), 3.09 (2H, q ,J=5.8 Hz, NCH_2(*ε*)-Lys), 3.21 (2H, q, J=5.4 Hz, NCH_2-hexadecyl), 4.07 (1H, m, CH(*α*)-Lys), 4.19 (1H, t, J=6.6 Hz, CH-Fmoc), 4.39 (2H, d, J=5.2 Hz, CH_2-Fmoc), 4.58 (1H, br.t, NH(*ε*)-Lys), 5.47 (1H, br.t, NH- hexadecyl), 6.09 (1H, br.d, NH(*α*)-Lys), 7.29 (3H, t, J=7.4 Hz, Ar-Fmoc), 7.38 (3H, t, J=7.3 Hz, Ar-Fmoc), 7.57 (2H, d, J=7.1 Hz, Ar-Fmoc), 7.75 (2H, d, J=7.4 Hz, Ar-Fmoc). ^{13}C NMR: 14.1, 22.5, 22.7, 24.9, 26.9 (×2), 28.4 (×3), 29.3, 29.4, 29.5, 29.6, 29.7 (×7), 31.9, 32.2, 34.0, 39.6, 47.2, 54.9, 67.0, 78.9, 120.0 (×2), 125.0 (×2), 127.1 (×2), 127.7 (×2), 141.3 (×2), 143.8 (×2), 156.2, 171.5.

N(ε)-Boc-Lys-hexadecyl Amide (29)

The mixture of amide **28** (1.512 g, 2.19 mmol), piperidine (220 μL, 3 mmol) and dry DMF (20 mL) was stirred for 1 h, then

poured into ice water (200 mL), stirred for 20 min, the resulted precipitate was filtered, washed with water and dried to obtain N(*ε*)-Boc-Lys-hexadecyl amide **29** (904 mg, 1.92 mmol, 88 %) as white solid. HRMS, calculated for $[C_{27}H_{56}N_3O_3]^+$: 470.4316, found: 470.4313. ^{1}H NMR: 0.86 (3H, t, J=6.7 Hz, CH_3-hexadecyl), 1.24 (26H, m, $(CH_2)_{13}$-hexadecyl), 1.42 (9H, s, CH_3-Boc), 3.10 (2H, q, J=5.31 Hz, NCH_2(*ε*)-Lys), 3.21 (2H, q, J=6.3 Hz, NCH_2-hexadecyl), 3.32 (1H, dd J_1=4.3 Hz, J_2=7.7 Hz, CH(*α*)-Lys), 4.17 (1H, br.t, NH-hexadecyl), 4.56 (1H, br.t, NH(*ε*)-Lys); ^{13}C NMR: 14.1, 22.7, 22.9, 24.9, 25.6, 27.0, 28.4 (×3), 29.3, 29.7 (×8), 29.9, 31.9, 34.6, 39.1, 40.2 (×2), 49.1, 55.1, 79.1, 156.1, 174.7.

N(α)-17$^{3'}$(Pyropheophorbide)carboxamido-Lys-hexadecyl Amide (30)

The solution of **Pyro** (300 mg, 0.56 mmol) and DCC (120 mg, 0.58 mmol) in dry CH_2Cl_2 (25 mL) was stirred for 30 min, then N(*ε*)-Boc-Lys-hexadecyl amide **29** (263 mg, 0,56 mmol) was added and the mixture was stirred for 40 min and evaporated to dryness. The residue was separated by silica gel flash chromatography in CH_2Cl_2 – acetone (9:1) mixture to obtain N(*α*)-Boc protected conjugate (329 mg, 0,33 mmol, 60 %) as black foam. HRMS, calculated for $[C_{60}H_{88}N_7O_5]^+$: 986.6841; found: 986.6844. ^{1}H NMR: -1.76 (1H, br.s, NH), 0.86 (3H, t, J=6.9 Hz, CH_3-hexadecyl), 1.18 (28H, m, $(CH_2)_{14}$-hexadecyl), 1.29 (9H, s, CH_3-Boc), 1.66 (3H, t, J=7.6 Hz, H-8$^{2'}$), 1.78 (3H, d, J=7.3 Hz, H-18$^{1'}$), 2.89 (2H, m, NCH_2-hexadecyl), 3.09 (2H, q, J=4.7 Hz, NCH_2(*ε*)-Lys), 3.22, 3.38, 3.58 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$), 4.11 (1H, m, H-17$^{1'}$), 4.28 (1H, m, H-17$^{1'}$), 4.47 (1H, m, H-8$^{1'}$), 4.54 (1H, br. t, NH($^{\varepsilon}$)-Lys), 5.04, 5.25 (each 1H, d, J=20.0 Hz, H-15$^{1'}$), 6.00 (1H, br. d, NH(*α*)-Lys), 6.15 (1H, dd J_1=11.7 Hz, J_2=1.4 Hz, H-3$^{2'}$, *trans*), 6.15 (br.t, 1H, NH-hexadecyl), 6.24 (1H, dd, J_1=17.9 Hz, J_2=1.4 Hz, H-3$^{2'}$, *cis*), 7.96 (1H, dd, J_1=11.6 Hz, J_2=17.8 Hz, H-3$^{1'}$), 8.55, 9.36, 9.42 (each 1H, s, H-5', H-10', H-20'). ^{13}C NMR: 11.2, 12.1, 14.1, 17.3, 19.5, 22.4, 22.6, 23.1, 26.8, 28.3 (×3), 29.6 (×14), 30.0, 31.6, 31.9, 32.4, 39.5, 39.8, 48.1, 50.0, 51.7, 52.9, 79.0, 93.3, 97.1, 103.9, 106.3, 122.6, 128.6, 129.1 (×2), 130.6, 131.74, 136.0, 136.3, 137.9, 141.6, 144.8, 149.1, 156.1, 160.6 (×2), 171.4, 171.7, 172.3, 196.1.

The product obtained (329 mg, 0.33 mmol), dioxane (10 mL) and 30% aqueous H_2SO_4 were stirred for 45 min, the removal of Boc-group was controlled by TLC. Thereafter the mixture was poured into the mixture of water (30 mL) and chopped ice (30 g), neutralized with NH_4OH, and extracted with dichloromethane (3×25 mL). The combined extract was washed with brine (30 mL), dried over Na_2SO_4 and evaporated to obtain compound **30** (284 mg, 0.32 mmol, 97 %) as black solid. HRMS, calculated for $[C_{55}H_{80}N_7O_3]^+$: 886.6317; found: 886.6318. ^{1}H NMR: -1.72 (1H, br.s, NH), 0.86 (3H, t, J=6.9 Hz, CH_3-hexadecyl), 1.19 (28H, m, $(CH_2)_{14}$-hexadecyl), 1.67 (3H, t, J=7.6 Hz, H-8$^{2'}$), 1.77 (3H, d, J=7.3 Hz, H-18$^{1'}$), 3.12 (2H, q, J=4.67 Hz, NCH_2(*ε*)-Lys), 3.21, 3.38, 3.59 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$), 4.20 (1H, br.t, NH-hexadecyl), 4.28 (1H, m, H-17$^{1'}$), 4.45 (1H, m, H-8$^{1'}$), 5.04, 5.23 (each 1H, d, J=19.9 Hz, H-15$^{1'}$), 5.92 (1H, br.d, NH(*α*)-Lys), 6.15 (1H, dd J_1=11.5 Hz, J_2=1.4 Hz, H-3$^{2'}$, *trans*), 6.26 (1H, dd, J_1=18.0 Hz, J_2=1.4 Hz, H-3$^{2'}$, *cis*), 7.97 (1H, dd, J_1=11.7 Hz, J_2=17.8 Hz, H-3$^{1'}$), 8.52, 9.34, 9.41 (each 1H, s, H-5', H-10', H-20'). ^{13}C NMR: 11.3, 12.1, 12.2, 14.2, 17.5, 19.5, 22.6, 22.8, 23.2, 23.8, 27.0 (×10), 32.0, 32.6, 32.8, 39.5, 39.6, 41.5, 48.1, 50.1, 50.9, 51.7, 53.2, 93.0, 97.2, 104.1, 106.2, 122.6, 128.2, 129.3 (×2), 131.6, 135.9, 136.1, 137.7, 137.9, 141.5, 141.6, 145.1, 148.9, 150.9, 155.4, 160.4, 171.6, 172.1, 196.2.

N(α)"-17$^{3'}$(Pyropheophorbide)carboxamido-N(ε)"-21(17β-hydroxy-3-oxo- pregn-4-ene-21-oyl)amido-Lys-hexadecyl amide (conjugate 6)

DCC (100 mg, 0.49 mmol) was added to the stirred solution of 17β-hydroxy-3-oxopregn-4-en-21-oic acid **12** in dry CH_2Cl_2 (15 mL); the mixture was stirred for 10 min; then compound **30** (217 mg, 0.25 mmol) was added, and the mixture was stirred for 12 h more. Thereafter the mixture was evaporated, the residue was separated by silica gel flash chromatography in CH_2Cl_2 – acetone – AcOH (84:15:1), evaporated, and dried to obtain conjugate **6** as black amorphous powder (141 mg, 0.12 mmol, 47 %). HRMS, calculated for $[C_{76}H_{108}N_7O_6]^+$: 1214.8356; found: 1214.8363. ^{1}H NMR: -1.67 (1H, br.s, NH), 0.71 (3H, s, H-18), 0.86 (3H, t, J=7.0 Hz, CH_3-hexadecyl), 0.95 (3H, s, H-19), 1.17 (28H, m, $(CH_2)_{14}$-hexadecyl), 1.60 (3H, t, J=7.6 Hz, H-8$^{2'}$), 1.74 (3H, d, J=7.2 Hz, H-18$^{1'}$), 2.34 (2H, AB system, H-20'), 3.11 (2H, q, J=6.5 Hz, NCH_2(*ε*)-Lys), 3.14, 3.33, 3.40 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$), 4.24 (1H, m, H-17$^{1'}$), 4.39 (1H, m, H-8$^{1'}$), 4.88, 5.13 (each 1H, d J=20.0 Hz, H-15$^{1'}$), 5.28 (1H, s, 17-OH), 5.48 (1H, s, H-4), 6.12 (1H, dd, J_1=11.6 Hz, J_2=1.4 Hz, H-3$^{2'}$, *trans*), 6.22 (1H, dd, J_1=17.9 Hz, J_2=1.4 Hz, H-3$^{2'}$, *cis*), 6.40 (1H, br.d, NH(*α*)-Lys), 6.46 (1H, br. t, NH-hexadecyl), 6.72 (1H, br.t, NH(*ε*)-Lys), 7.88 (1H, dd, J_1=14.7 Hz, J_2=17.8 Hz, H-3$^{1'}$), 8.46, 9.19, 9.23 (each 1H, s, H-5', H-10', H-20'). ^{13}C NMR: 11.1, 11.8, 12.0, 13.7, 14.1, 17.1, 17.6, 19.3, 20.4, 22.6, 22.7, 22.9, 23.4, 26.9, 28.6, 29.2, 29.6 (×10), 30.4, 31.4, 31.5, 31.8, 31.9, 32.5, 32.8, 33.8, 35.5, 36.1, 36.1, 38.4, 38.6, 39.6, 42.5, 46.0, 48.0, 49.8, 50.0, 51.5, 52.9, 53.5, 81.9, 92.9, 97.1, 103.9, 105.7, 122.6, 123.7, 128.2, 129.1, 130.0, 131.6, 135.9, 136.0, 136.3, 137.6, 141.7, 145.0, 148.9, 150.8, 155.4, 160.2, 170.9, 171.5, 171.7, 172.7, 173.4, 196.3, 199.2.

N(α)-Fmoc-Lys-hexadecyl Amide (31)

The mixture of amide **28** (911 mg, 1.32 mmol), CH_2Cl_2 (30 mL), and TFA (10 mL) was stirred for 30 min, evaporated to dryness, the residue was dissolved in CH_2Cl_2 (30 mL), the solution was washed with saturated $NaHCO_3$ solution (20 mL), brine (20 mL), dried over Na_2SO_4, and evaporated to obtain N(*α*)-Fmoc-Lys-hexadecyl amide **31** (766 mg, 1.29 mmol, 98 %) as a black film. HRMS, calculated for $[C_{37}H_{58}N_3O_3]^+$: 592.4473; found: 592.4477. ^{1}H NMR: 0.87 (3H, t, J=6.3 Hz, CH_3-hexadecyl), 1.36 (28H, m, $(CH_2)_{14}$), 2.69 (2H, m, CH_2(*ε*)-Lys), 3.22 (2H, m, NCH_2-hexadecyl), 4.07 (1H, m, CH(*α*)-Lys), 4.20 (1H, t, J=6.6 Hz, CH-Fmoc), 4.40 (2H, d, J=5.9 Hz, CH_2-Fmoc), 5.50 (1H, br.t, NH-hexadecyl), 6.16 (1H, br. d, NH(*α*)-Lys), 7.30 (3H, t, J=7.4 Hz, Ar-Fmoc), 7.39 (3H, t, J=7.4 Hz, Ar-Fmoc), 7.57 (2H, d, J=7.4 Hz, Ar-Fmoc), 7.75 (2H, d, J=7.6 Hz, Ar-Fmoc). ^{13}C NMR: 14.2, 22.7, 22.8, 27.0, 29.4, 29.4, 29.8 (×11), 32.0, 32.6, 39.7, 41.7, 47.3, 55.1, 67.1, 120.1 (×2), 125.1 (×2), 127.2 (×2), 127.8 (×2), 141.4 (×2), 143.9 (×2), 171.5.

N(ε)-17$^{3'}$(Pyropheophorbide)carboxamido-Lys-hexadecyl Amide (32)

The solution of **Pyro** (250 mg, 0,47 mmol) and DCC (97 mg, 0.47 mmol) in dry CH_2Cl_2 (25 mL) was stirred for 30 min, after addition of N(*α*)-Fmoc-Lys-hexadecyl amide **31** (227 mg, 470 μmol) the mixture was stirred for 45 min, and evaporated. TLC analysis revealed partial deletion of Fmoc-group in the resultant product. The residue dissolved in DMF (5 mL) was mixed

with piperidine (37 μL, 0.5 mmol). The resultant mixture was stirred for 1 h, diluted with CH_2Cl_2 (30 mL), washed with water (2×10 mL), dried over Na_2SO_4, and evaporated. The residue was separated by silica gel flash chromatography in CH_2Cl_2 – acetone (93:7) mixture to obtain compound **32** (146 mg, 170 μmol, 35 %). HRMS, calculated for $[C_{55}H_{80}N_7O_3]^+$: 886.6317; found: 886.6307. ^{1}H NMR: -1.69 (1H, br.s, NH), 0.86 (3H, t, J=7.5 Hz, CH_3-hexadecyl), 1.23 (28H, m, $(CH_2)_{14}$), 1.65 (3H, t, J=7.6 Hz, H-8$^{2'}$), 1.78 (3H, d, J=7.3 Hz, H-18$^{1'}$), 2.98 (2H, q, J=6.2 Hz, NCH_2(*ε*)-Lys), 3.20, 3.38, 3.51 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$), 4.21 (1H, m, H-17$^{1'}$), 4.50 (1H, m, H-8$^{1'}$), 5.05, 5.23 (each, 1H, d, J=19.9 Hz, H-15$^{1'}$), 5.42 (1H, br.t, NH-hexadecyl), 6.15 (1H, dd, J_1=11.6 Hz, J_2=1.4 Hz, H-3$^{2'}$, *trans*), 6.27 (1H, dd, J_1=17.8 Hz, J_2=1.4 Hz, H-3$^{2'}$, *cis*), 7.14 (1H, br.t, NH(*ε*)-Lys), 7.97 (1H, dd, J_1=11.5 Hz, J_2=17.8 Hz, H-3$^{1'}$), 8.52, 9.34, 9.35 (each 1H, s, H-5', H-10', H-20'). ^{13}C NMR: 11.3, 12.0, 12.2, 14.2, 17.5, 19.5, 22.8, 23.1, 23.2, 23.9, 27.0, 28.9, 29.0, 29.8 (×8), 30.4, 30.5, 32.0, 33.0, 34.3, 38.9, 39.1, 48.2, 50.1, 51.9, 54.8, 93.1, 97.2, 104.1, 106.2, 122.6, 128.3, 128.9, 129.3, 130.9, 131.7, 136.0, 136.1, 136.3, 141.6, 145.1, 149.1, 150.8, 155.3, 160.6, 172.0, 172.4, 174.5, 196.4.

N(α)"-21(17β-Hydroxy-3-oxopregn-4-ene-21-oyl)amido-N(ε)"-17$^{3'}$(pyropheophorbide)carboxamido-Lys-hexadecyl AQmide (Conjugate 7)

The mixture of compounds **32** (65 mg, 73 μmol), **12** (26 mg, 73 μmol), and DCC (17 mg, 80 μmol) was stirred for 25 min, the reaction was controlled by TLC. After mixture evaporation, the residue was applied on the top of silica gel column, the column was washed with $CHCl_3$ –acetone – AcOH (85:14:1) and the target product was then eluted with $CHCl_3$ – acetone – AcOH (79:20:1). The isolated crude conjugate was additionally purified by silica gel flash chromatography in $CHCl_3$ – MeOH – AcOH (93:6:1) to obtain conjugate **7** (47 mg, 39 μmol, 53 %) as black powder. HRMS, calculated for $[C_{76}H_{108}N_7O_6]^+$: 1214.8356; found: 1214.8362. ^{1}H NMR: -1.66 (1H, br.s, NH), 0.70 (3H, s, H-18), 0.86 (3H, t, J=7.0 Hz, CH_3-hexadecyl), 0.91 (3H, s, H-19), 1.21 (28H, m, $(CH^2)^{14}$), 1.61 (3H, t, J=7.6 Hz, H-8$^{2'}$), 1.76 (3H, d, J=7.1 Hz, H-18$^{1'}$), 2.29 (2H, AB system, H-20), 3.11 (2H, q, J=6.5 Hz, NCH_2(*ε*)-Lys), 3.16, 3.34, 3.38 (each 3H, s, H-2$^{1'}$, H-7$^{1'}$, H-12$^{1'}$), 4.30 (1H, m, H-17$^{1'}$), 4.44 (1H, m, H-8$^{1'}$), 4.97, 5.14 (each 1H, d, J=19.9 Hz, H-15$^{1'}$), 5.49 (1H, s, H-4), 5.80 (1H, br.t, NH-hexadecyl), 6.12 (1H, dd, J_1=11,5 Hz, J_2=1.4 Hz, H-3$^{2'}$, *trans*), 6.22 (1H, dd, J_1=18.0 Hz, J_2=1.4 Hz, H-3$^{2'}$, *cis*), 6.65 (1H, br. t, NH(*ε*)-Lys), 7.15 (1H, br.d, NH(*α*)-Lys), 7.89 (1H, dd, J_1=14.7 Hz, J_2=17.9 Hz, H-3$^{1'}$), 8.47, 9.22, 9.25 (each 1H, s, H-5', H-10', H-20'). ^{13}C NMR: 11.3, 11.9, 12.1, 13.9, 14.2, 17.1, 17.4, 19.4, 20.4, 22.8, 23.1, 23.5, 27.0, 29.0, 29.4, 29.4, 29.5, 29.6, 29.8 (×10), 30.8, 31.3, 31.5, 31.6, 32.0, 32.6, 33.4, 33.8, 35.5, 35.7, 36.2, 38.4, 38.8, 39.7, 42.9, 46.3, 48.1, 49.9, 50.1, 51.8, 53.1, 53.4, 81.1, 93.8, 97.2, 104.1, 105.7, 122.7, 123.8, 128.0, 129.1, 130.0, 131.7, 136.0, 136.2, 136.4, 137.7, 141.8, 145.1, 149.1, 150.9, 155.5, 160.5, 170.8, 171.6, 172.0, 172.8, 173.4, 196.4, 199.2.

Molecular Modeling

Conformation searches have been performed using molecular mechanics MMFF94 force field parameters *in vacuo*. OpenBabel package [16] was employed for initial structure preparation and energy minimizations. Simulated annealing molecular dynamics (MD) has been performed to sample low-energy conformation space of conjugates by means of NAMD [17] software. Parameters and topology files were generated with the aid of the SwissParam server [18] on the basis of the MMFF94 force field. The annealing protocol consisted of 4 ps high temperature runs at 500 K followed by 4 ps cooling phase bringing temperature down to 50 K, with total of 200 annealing cycles scheduled in 32 processes. This procedure yielded 6400 local energy minima for each compound. Resulting structures were then optimized by energy minimization with MMFF94 potential. VMD package [19] was used for MD trajectory post-processing, analysis, and visualization.

*Solubilization of Conjugates **6** and **7** in aqueous medium*

Solubilization of Conjugates with PC

To obtain solution conjugate/PC at ratio of 1:10 (mg/mg, 6.7 molar % of conjugate) calculated volumes of 10^{-2} M solutions of PC and conjugate (either **6**, or **7**) in $CHCl_3$ were mixed together. Mixed solutions were evaporated to dryness, and dissolved in *i*-PrOH at 40°C to obtain solutions with concentrations of conjugates equal to 10^{-3} M. Aliquots of heated isopropanolic solutions were injected during vortexing into 100-fold volume of PBS (for measuring of absorption spectra and particle size distributions) or in culture medium (for measuring of uptake and internalization of conjugates by cells).

Solubilization of Conjugates with Pluronic F68

Calculated volumes of 10^{-2} M solutions of pluronic F68 and conjugates (either **6**, or **7**) in $CHCl_3$ at ratios 1:10 and 1:50 (mg/mg) were mixed together to obtain solutions conjugate/pluronic. The Mixed solutions were evaporated to dryness, then calculated volumes of PBS, or culture medium were added to films, and the mixtures obtained were vortexed at 40 °C for 1 min.

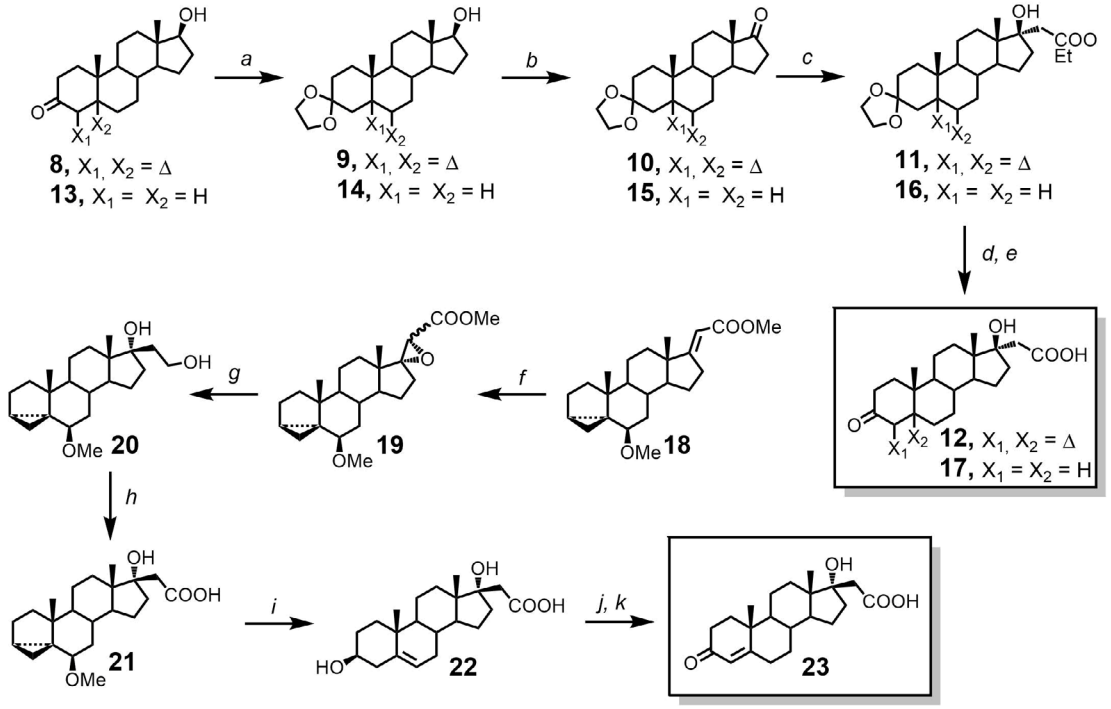
Biological evaluation

Cell Cultures

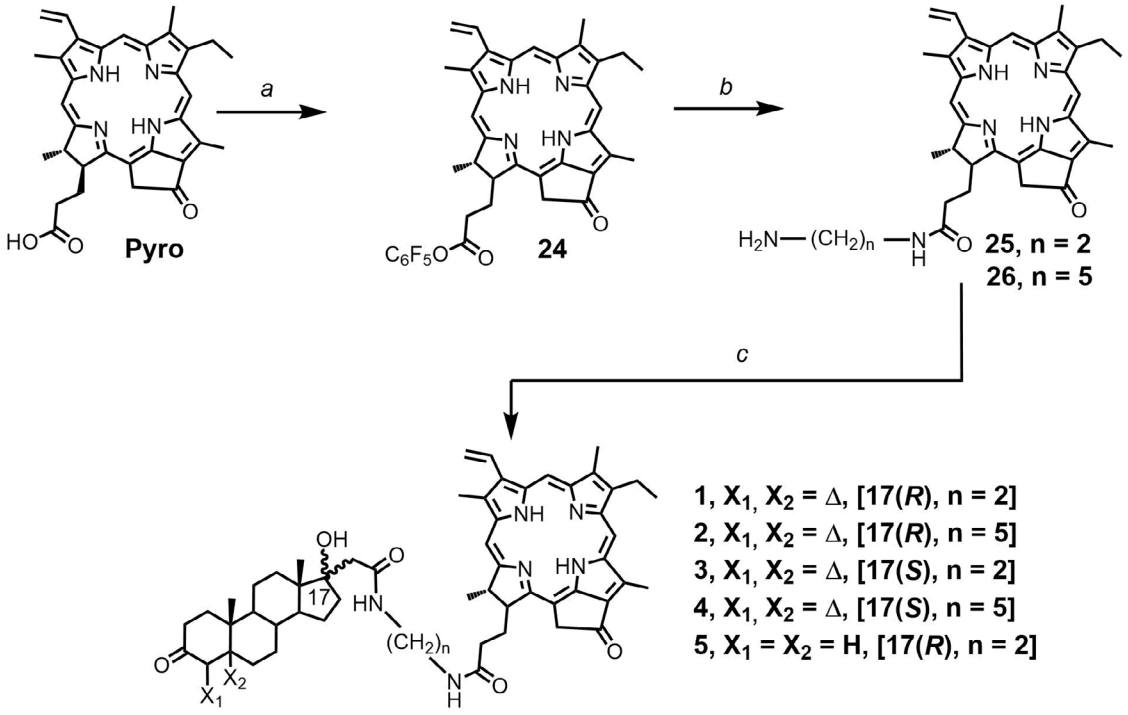
The human prostate carcinoma LNCaP and PC-3 cells, breast carcinoma MCF-7 cells, hepatocarcinoma Hep G2 cells were obtained from the American Type Culture Collection (USA). Cells were propagated in culture dishes at the desired densities in RPMI 1640 and DMEM medium supplemented with 10% fetal calf serum (FCS; "Gibco", USA) and 1% penicillin/streptomycin "Gibco" in a 5% CO_2 atmosphere at 37 °C for 24 h. Before experiments the cells were seeded either in 96-well plates at a density of 5·103 cells/well (for MTT assay), or in 6-well plates at a density of 106 cells/well (for investigation of uptake and internalization of conjugates) and incubated for 48 h.

Uptake and Internalization of Conjugates by Prostate Carcinoma Cells

LNCaP cells seeded in 6-well plates were incubated for 2 h, 6 h, 14 h and 20 h at 37 °C with conjugates **1**–**4** (25 μM in culture medium), then medium was aspirated, cells were washed with cold PBS at 4°C, and lipids were extracted from each well with hexane – *i*-PrOH mixture (3:2, 3×0.5 mL). Pellets were



Scheme 1. a - $(HOCH_2)_2$, H^+ / toluene; b – PCC / CH_2Cl_2; c - Zn, $BrCH_2COOEt$ / C_6H_6; d - MeOH – THF – 2N H_2SO_4; e – K_2CO_3 / H_2O – MeOH; f – mCPBA / CH_2Cl_2; g – $LiAlH_4$ / THF; h – RuO_4 – $KBrO_3$ / acetone – H_2O; i – THF – 2N H_2SO_4; j – DMP / CH_2Cl_2; k – $(COOH)_2$ / EtOH.

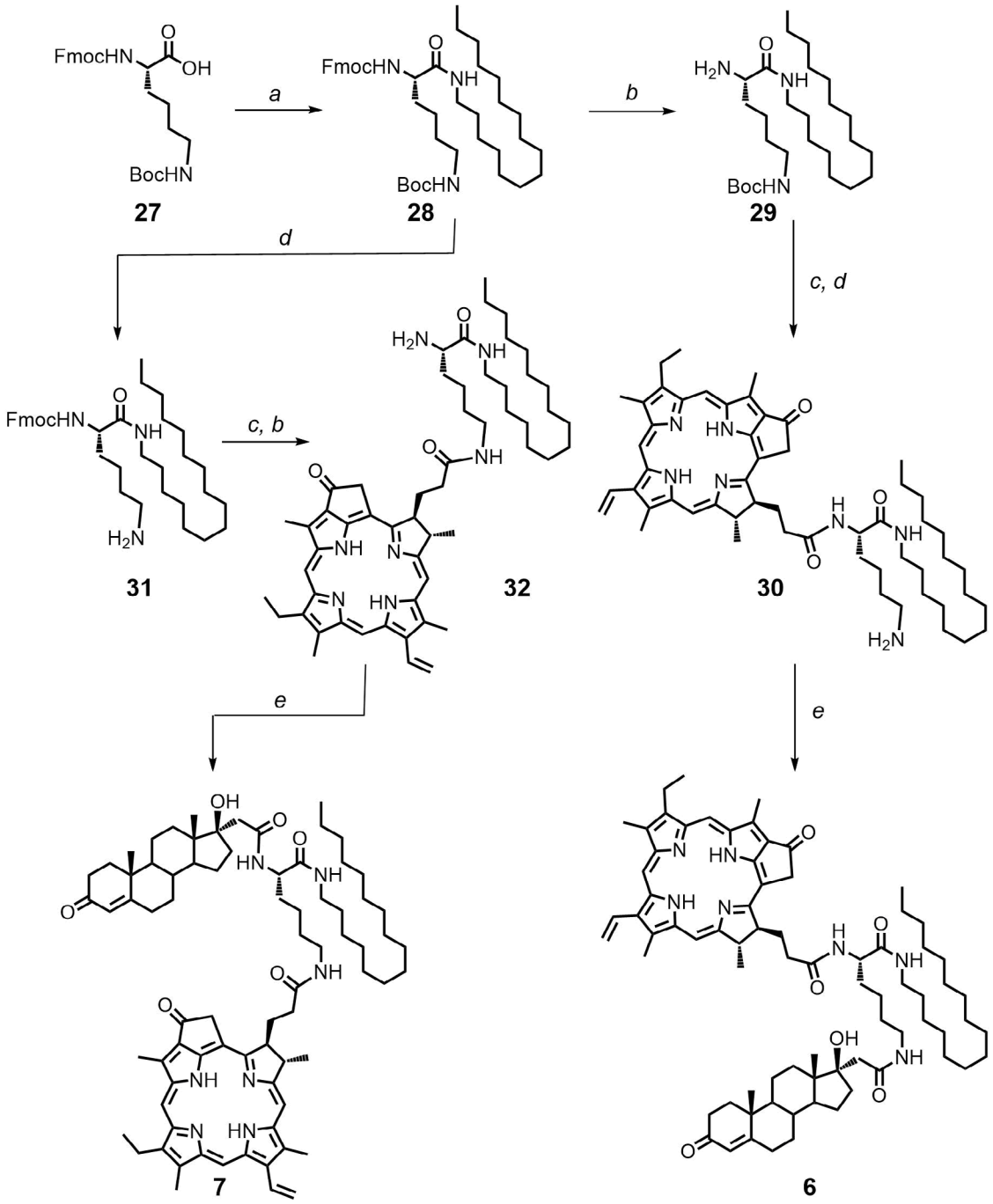


Scheme 2. a – Pentafluorophenyl trifluoroacetate, Et_3N / CH_2Cl_2; b - $H_2N(CH_2)_2NH_2$ or $H_2N(CH_2)_5NH_2$; c – 12 or 17, DCC / CH_2Cl_2.

used for cell protein concentration measurements. The lipid extracts were dried under nitrogen flow, residues were dissolved in CH_2Cl_2 (2 mL), and concentrations of conjugates were determined spectrophotometrically by means of a "Cary Spectra 100" spectrophotometer in $CHCl_3$ and CH_2Cl_2 using a quartz cell with the 1 mm optical path length. All measurements were carried out in triplicates. The efficiency of cell labeling was expressed in terms of ratios of internalized conjugates (nmol per mg of cell protein).

MTT Cell Viability Assay

LNCaP and PC-3 cells were treated with conjugates at the concentrations of 0.1 μM, 1 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM and incubated for 96 h in 96-well plates. Then, solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) was added and the cells were incubated for 4 h, followed by measuring absorbance at 570 nm,



Scheme 3. a – $CH_3(CH_2)_{15}NH_2$, DCC / CH_2Cl_2; b - piperidine; c – 25, DCC/ CH_2Cl_2; d – TFA; e – 12, DCC/ CH_2Cl_2.

with a "Techan Genius plus" microplate reader. The viability of treated cells was expressed as a percentage relative to that of control cells. Each experiment was performed in triplicate, and independently repeated at least four times.

Measurement of Photo Induced Toxicity

LNCaP cells were incubated with concentrations of conjugates **1** and **5** at ranged from 0.1 μM to 100 μM for 18 h in RPMI 1640 and DMEM medium supplemented with 10% fetal calf serum (FCS; Gibco, USA) and 1% penicillin/streptomycin "Gibco" in a 5% CO_2 atmosphere, then the cells were washed three times with PBS, thereafter fresh culture medium was added (100 μL/well), and cells were irradiated for 10 min with light (λ = 660 nm) using a LED AFS "Spectrum" instrument (p = 0,142 W; 85,2 J/cm^2). Then the cells were incubated in fresh medium for 24 h at 37°C. The viability of irradiated cells and control cells (incubated similarly except irradiation) was measured using the MTT assay. All experiments were carried out in triplicates.

RESULTS AND DISCUSSION

Chemical Synthesis

Preparation of new conjugates **1** – **7** (consisted of synthesis steroidal blocks **12**, **17** and **23**; synthesis of bifuntional conjugates **1** – **5** by coupling of **Pyro** with steroidal blocks **12**, **17** and **23** by means of diamino containing linkers; and synthesis of trifunctional conjugates **6** and **7**) is presented in the Schemes 1, 2, and 3, respectively.

Synthesis of steroidal acids **12**, **17**, **23** was performed as follows. Testosterone **8** and dihydrotestosterone **13** were transformed to steroid blocks **12** and **17** by five steps including

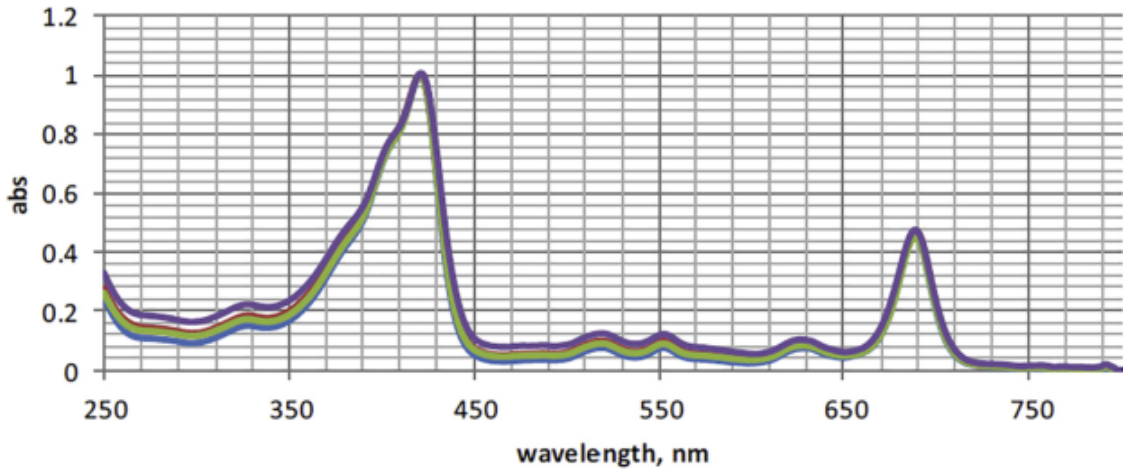


Figure 2. Normalized absorption spectra of conjugates **1** (olive); **2** (purple); **3** (blue); **4** (red) in $CHCl_3$.

Table 1. Chemical shifts (d, ppm) for selected characteristic resonances in the ^{1}H NMR spectra of conjugates **1** – **4**, **6** and **7**

Conjugate	H-4	H-18	H-19	H-5'	H-10'	H-20'	NHα"	NHω2"
1	5.56, s	0.25, s	0.75, s	9.26, s	9.04, s	8.52, s	6.61, br.t	6.30, br.t
2	5.53, s	0.73, s	1.00, s	9.31, s	9.28, s	8.51, s	6.35, br.t	5.30, br.t
3	5.53, s	0.54, s	0.88, s	9.24, s	9.10, s	8.48, s	6.78, br.t	6.24, br.t
4	5.54, s	0.25, s	0.81, s	9.32, s	9.24, s	8.50, s	6.22, br.t	5.53 br.t
6	5.48, s	0.71, s	0.95, s	9.23, s	9.19, s	8.46, s	6.40, br.d	6.75, br.t
7	5.49, s	0.70, s	0.91, s	9.25, s	9.22, s	8.47, s	7.15, br.d	6.56, br.t

consecutive protection of carbonyl functions with formation of 1,3-dioxolanes [20], oxidation of 17β-hydroxyl groups [15], and Reformatsky reaction of obtained 17-ketones **10** and **15** with Zn and ethyl bromoacetate [21,22]. The aforementioned reaction is known to pass stereoselectively and give an appropriate 17β-OH isomer. The consecutive removal of ethylene ketal and ethyl ester protective groups led to 21-carboxylic acids **12** and **17** in 49 % and 58 % overall yields (based on compounds **8** and **13**, respectively).

The steroidal block **23** was synthesized from cyclosteroid **18** [14] in five steps. The introdution of 17α-hydroxyl group was carried out by oxidation of 17(20)-double bond with *m*-chloroperbenzoic acid followed by the reduction of resulting of 17α,20-epoxide **19** [the mixture of related 17α,20(R)- and 17α,20(S)- isomers in the ratio of 3: 1] with $LiAlH_4$ in boiling THP to obtain 17α,21-diol **20**. Then diol **20** was transformed to hydroxy acid **21** by oxidation with ruthenate – potassium bromate reagent [23] in acetone – water (3:1) solution. Then acid **21** was subjected to acid hydrolysis to obtain 3β-hydroxy-5-ene acid **22**. Oxidation of compound **22** with Dess-Martin periodinane, followed by acid catalyzed isomerization of crude 3-oxo-5-ene resulted in target 17α-hydroxy-3-oxopregn-4-en-21-oic acid **23** in 22% overall yield (based on compound **18**).

Both 17β-hydroxy- and 17α-hydroxy acids **12**, **17** and **23** were obtained as pure compounds; the configuration of C17 was confirmed by ^{13}C NMR spectra. The differences in chemical shifts of C16, C17, and C18 resonances in compounds **12**, **17** and **23** were consistent with published data of ^{13}C NMR spectra for related 17α- and 17β-hydroxyestradiols [24].

Synthesis of bifunctional conjugates **1**–**5** is presented in Scheme 2. Initially, **Pyro** was transformed to related pentafluorophenyl ester **24**, which was then treated with excess of either ethylene diamine or 1,5-diaminopentane to obtain amides **25** and **26** comprising primary amino group. Condensation of compounds **25** and **26** with steroidal acids **12**, **17** and **23** in the presence of DCC led to the target conjugates **1**–**5**.

Synthesis of trifunctional conjugates **6** and **7** is presented in Scheme 3. Initially, N(α)-Fmoc-N(ε)-Boc-Lys **27** was condensed with hexadecyl amine to obtain protected lysyl amide **28**. To prepare conjugate **6**, amide **28** was consequently treated with piperidine to remove the Fmoc-protecting group; then the amino containing block **29** was coupled with **Pyro**. To remove the Boc-protecting group the obtained intermediate was treated in acidic conditions; and finally the resulting amine **30** was acylated with steroidal acid **12** to obtain conjugate **6**.

We observed sufficient racemization of C17 in 17-hydroxy-3-oxopregn-4-en-21-oyl amides in the conditions of the group removal. For this reason we changed the consequence of reactions as follows: initially we removed the Boc-protective group in amide **28** and coupled the obtained amine **31** with **Pyro** (wherein the partial removal of Fmoc-protecting group was observed); then, after complete Fmoc-group deletion, the amine **32** was condensed with steroidal acid **12** to obtain target conjugate **7**.

All synthesized conjugates **1-7** were prepared as pure compounds, their structures were completely characterized by HRMS, ^{1}H NMR, ^{13}C NMR and absorption spectra.

Spectral Properties and Molecular Models

The absorption spectra of conjugates **1–7** were similar to each other and were typical for **Pyro** and their derivatives. The normalized absorption spectra of conjugates **1–4** are presented in Figure 2.

The ^{1}H NMR spectra of conjugates **1–7** indicated a significant mutual influence of steroid and macrocyclic

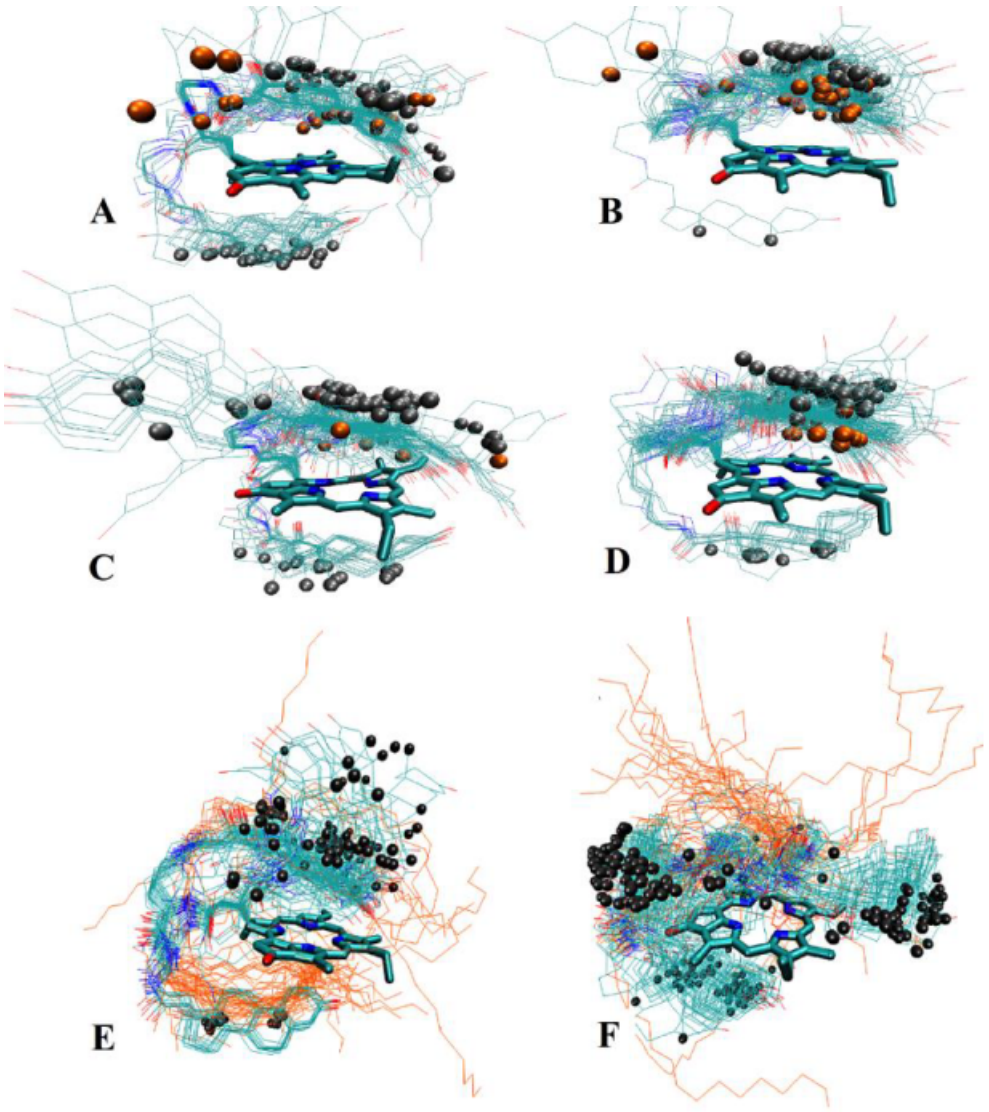


Figure 3. Ensembles of low energy conformers of compounds 1–4 , 6 and 7; 18- and 19-methyl groups of steroid core are depicted as balls (these faced to macrocyle are colored orange; away from macrocycle – gray).

fragments. The chemical shifts of selected protons in ^{1}H NMR spectra of conjugates **1–4**, **6** and **7** (comprising either testosterone or epitestosterone) are shown in Table 1. The strong high-field shifts for H-4, H-18, H-19 resonances were observed in the spectra of conjugates **1–4** in comparison with those in spectra of unconjugated steroidal acids **12** and **23**. The highest field shift for H-4 resonance was observed in spectra of conjugates **2** and **3**, while that for H-18 resonance – in spectra of conjugates **1** and **4**. The modest high field shifts for H-18 and H-19 resonances were observed in spectra of trifunctional conjugates **6** and **7**. Chemical shifts for amide NH" resonances strongly depended on the conjugate structure, while those for H-5', H-10' and H-20' resonances in pyropheophorbide *a* moieties differed insignificantly.

The data presented in the Table 1 are in agreement with results of molecular modeling (performed by simulated annealing) thus indicating differences in positional relationships of steroid and macrocycle moieties in the conjugates. The calculated ensembles of conformers, truncated at 10 kcal/mol above the lowest-energy conformer, are shown in the Figure 3; the structures for lowest energy conformers are presented in Figure 4.

Configuration of C17 affects the relative positioning of 17-hydroxy group, and in epitestosterone conjugates **3** and **4** this group was found to be oriented towards the macrocycle (Fig. 3C and 3D), and is capable of hydrogen bond formation with nearby amide proton (Fig. 4C and 4D). On the contrary, in testosterone derivatives **1** and **2** this hydroxyl group is directed outwards from the macrocycle, and completely exposed to the environment (Fig. 3A, 3B, 4A and 4B).

Due to the longer linker lengths in conjugates **2** and **4**, they exhibit higher conformational flexibility in comparison with conjugates **1** and **3**. This allows more conformers with NH proton to be axially positioned off the macrocycle plane, pushing corresponding NMR resonances into the higher field. Because position of the steroid moiety in the lowest-energy conformers was not significantly different between conjugates with different linker lengths, we have concluded that the main effect of linker lengthening is, indeed, enhanced conformational flexibility.

Structures with the steroid moiety hoisted over the surface of macrocycle were found energetically favorable for conjugates **1–4** (Fig. 3A–3D). This "folded" structure correlates well with observed high-field shifts of 18- and 19-methyl protons as compared to unconjugated steroids, because of shielding effect exerted by a large aromatic moiety of **Pyro** on atoms located above and below its surface. The presence of 'unfolded' conformers with relatively low energy in compounds **2** and **3**

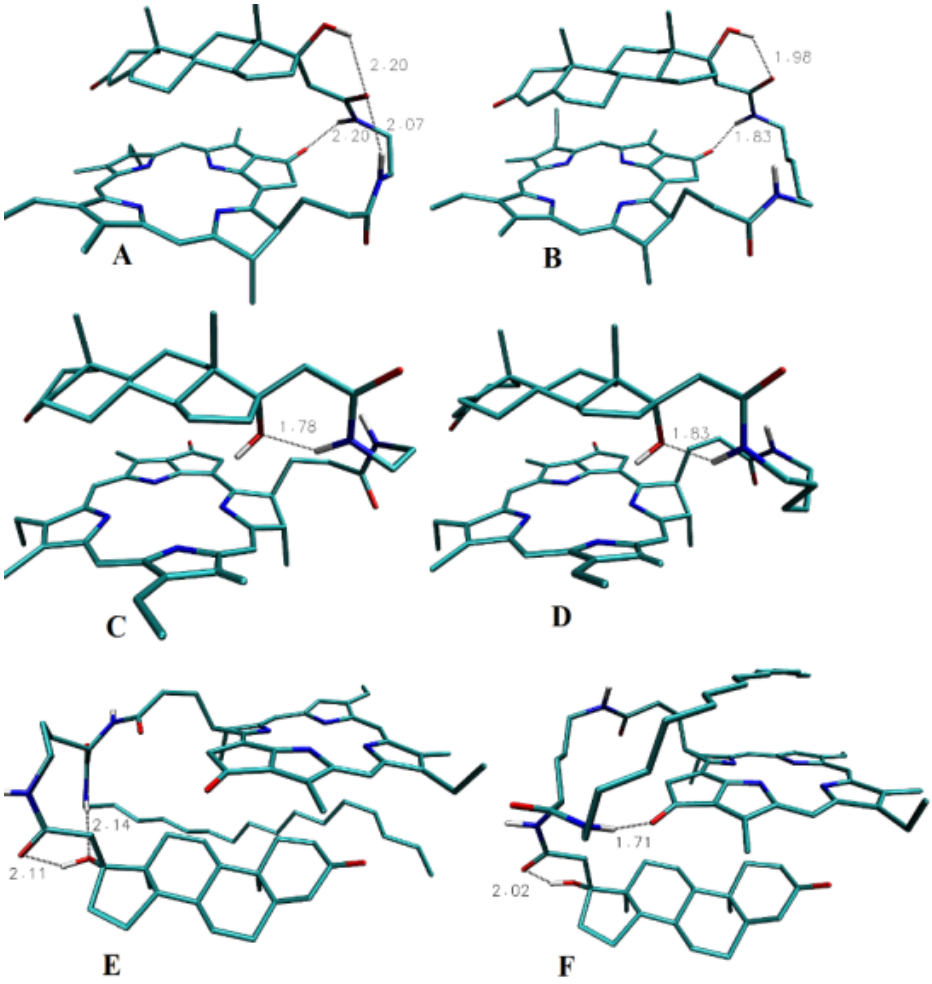


Figure 4. The lowest calculated energy conformers for conjugates **1–4, 6** and **7**. Short interatomic distances favorable for hydrogen bond formation are marked by hash lines; the numbers indicate distance in Angstroms.

(Fig. 3B, 3C) is probably responsible for the observed weaker shielding of 18- and 19-methyls.

Figure 3E demonstrates that structures with the teroid moiety hoisted over the surface of macrocycle, and hexadecyl chain located in its opposite side, are energetically favorable for trifunctional conjugate **6**. On the contrary, Figure 3F reveals that three ensembles of low energy conformers differing in positions of steroid relatively to macrocycle, and random distribution of hexadecyl chain, are favored for trifunctional conjugate **7**. In both conjugates **6** and **7** 18- and 19-methyl groups were mainly turned away from macrocycle.

The lowest energy conformers of conjugates are stabilized by possibility of intramolecular hydrogen bonds formation (Fig. 4). In the lowest energy conformers of conjugates **1** and **2** Nα'' atom is located near carbonyl group of **Pyro**, thus making formation of corresponding hydrogen bond favorable. Additionally, in compound **1** the oxygen atom of the 21-amide group is located between 17β-hydroxyl group and Nω'' atom of ethylene diamine linker, and thus can serve as proton acceptor with either of these atoms being a donor (Fig. 4A, 4B). In the lowest energy conformers of conjugates **3** and **4** 17α-hydroxyl group is located close to Nα'' atom, and apparently may serve as proton acceptor to form the corresponding hydrogen bond (Fig. 4C, 4D).

In the lowest energy conformers of both conjugates **6** and **7**, the hydrogen atom of the steroid 17-hydroxyl group participates in hydrogen bond formation with oxygen of related 21-carboxamido group. Additionally, in the lowest energy conformer of conjugate **6**, the oxygen atom of the 17-hydroxyl group is located near the nitrogen atom of hexadecyl amide, and thus may serve as a proton acceptor to form the corresponding hydrogen bond (Figure 4E). In the lowest energy conformer of conjugate **7**, the nitrogen atom of hexadecyl amide is located near the carbonyl group of pyropheophorbide *a*, and may be involved in formation of the corresponding hydrogen bond (Fig. 4F).

Interaction of Bifunctional Conjugates with Cultured Cells

Conjugates **1–5** were efficiently internalized by prostate carcinoma LNCaP and PC-3 cells, breast carcinoma MCF-7 cells and hepatocarcinoma Hep G2 cells. The internalization was dependent on the structure of conjugates. Time course of conjugates **1–4** uptake by LNCaP cells is given in the Fig. 5A. Epitestosterone derivatives were internalyzed more efficiently than testosterone ones; in both pairs conjugates comprising shorter linkers were internalyzed more efficiently than those comprising long linkers (**3** > **4** ≥ **1** > **2**). According to our molecular models, this dependence must be correlated to diminished conformational flexibility of compounds **1** and **3**, combined with predominant 17-hydroxyl group exposure in testosterone derivatives.

The conjugate internalization by cells was confirmed as follows: LNCaP cells, initially labeled with conjugates **1–4** for 6 h, were incubated for 12 h in fresh medium, followed by determination of the conjugate content in it. The absence of

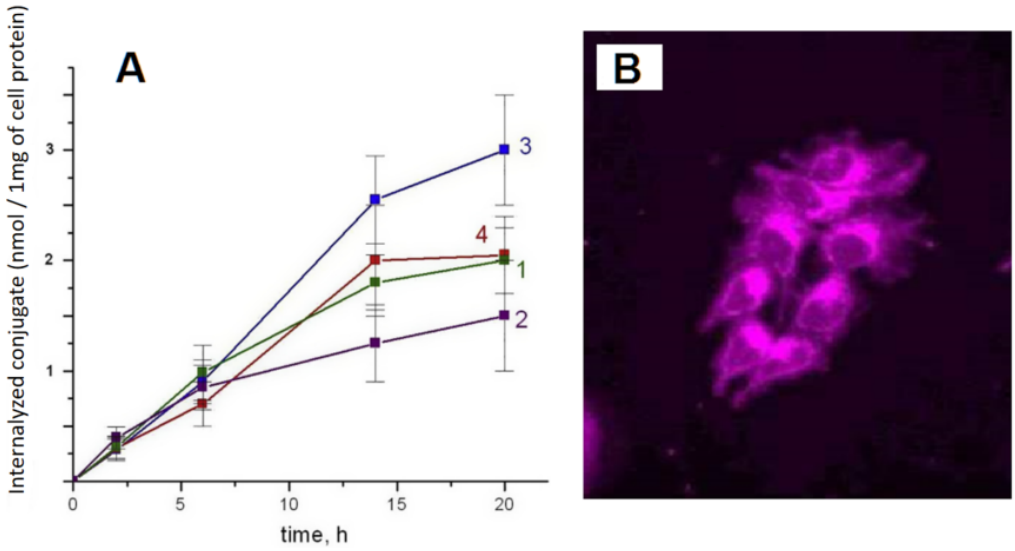


Figure 5. A - Internalization of conjugates **1–4** by LNCaP cells (concentration of each conjugate in media was 25 μM; the numbers of curves corresponded to numbers of conjugates). B – MCF-7 cells labeled with conjugate **1**.

Table 2. Effects of conjugates **1**–**5** on growth and proliferation of LNCaP and PC-3 cells during 96 h incubation

Conjugate	IC_{50}, μM	
	LNCaP	PC-3
1	4.8	6.1
2	12.1	21.4
3	1.3	2.6
4	6.4	14.2
5	18.3	24.1

Table 3. Dark and photo induced toxicity of conjugates **1** and **5** in LNCaP and PC-3 cells

Conjugate	LNCaP cells			PC-3 cells		
	Dark toxicity (IC_{50}, μM)	Photo induced toxicity (IC_{50}, μM)	Dark IC_{50} / Photo IC_{50} ratio	Dark toxicity (IC_{50}, μM)	Photo induced toxicity (IC_{50}, μM)	Dark IC_{50} / Photo IC_{50} ratio
1	24.2	5.4	4.5	16.0	1.4	11.4
5	24.0	2.6	9.2	17.4	1.6	10.9

detectable amounts of conjugates in the medium proves that they were completely internalized by cells. The photographs of MCF-7 cells labeled with conjugate **1** are presented in t Figure 5B.

Conjugates **1–5** moderately inhibited the growth and proliferation of LNCaP and PC-3 cells at 24 h incubation, however potently inhibited it at prolong of incubation. The MTT test [25] data demonstrating effects of conjugates **1–5** on the growth and viability of LNCaP and PC-3 cells at 96 h incubation are presented in the Table 2. Conjugates **1–5** inhibited growth of both LNCaP and PC-3 cells; however, the effect on LNCaP cells was more pronounced. Anti-proliferative activity of conjugates in LNCaP and PC-3 cells was dependent on the structure of steroid moiety and length of the linker and decreased in the following row: **3** > **1** > **4** > **2** > **5**.

Photo induced toxicity of conjugates **1** and **5** in LNCaP and PC-3 cells was also evaluated. For this purpose LNCaP and PC-3 cells were incubated with conjugates **1** and **5** for 18 h at concentration of 0.1 μM, 1 μM, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM, then the labeled cells were irradiated with light (λ=660 nm) for 10 min, thereafter the irradiated cells were incubated for 24 h in the fresh medium. The irradiated cells, as well as the control cells (treated by the same way, but without irradiation) were analyzed using the MTT test. The IC_{50} values for irradiated and non irradiated cells are presented in the Table 3. These data revealed that conjugates **1** and **5** exhibited significant photo induced toxicity in prostate carcinoma cells.

In contrast to bifunctional conjugates **1–5**, trifunctional conjugates **6** and **7** were insoluble in aqueous media, so we did not conduct experiments, as described above, with these conjugates.

Solubilization of Trifunctional Conjugates in Aqueous Medium

In order to solubilize trifunctional conjugates **6** and **7** in aqueous media we used two reported earler methods: (i) injection of mixed iso-propanolic solution of conjugate and phosphatidyl choline (PC) into aqueous buffer [12]; (ii) hydration of mixed films conjugate – pluronic F68 [26].

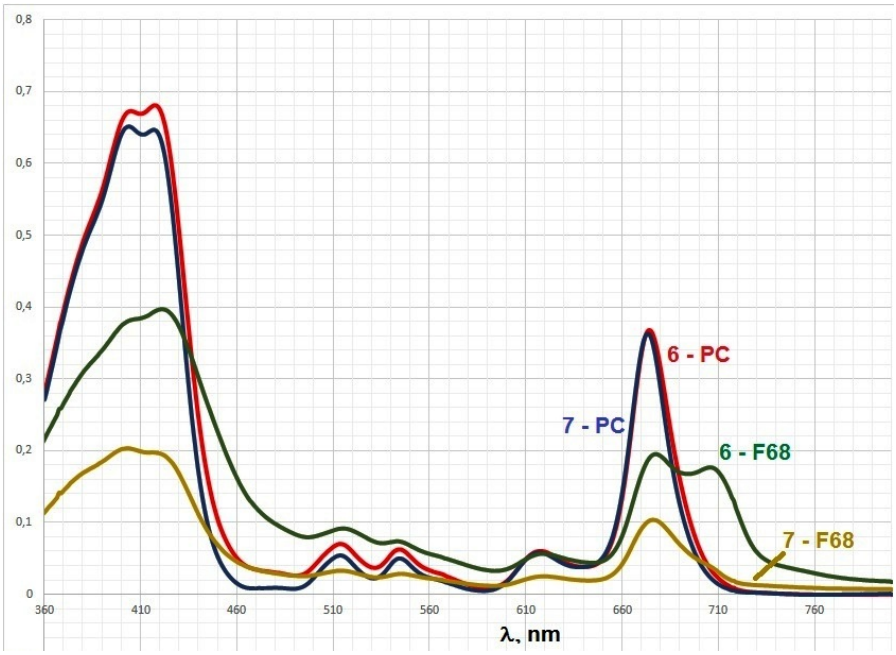


Figure 6. Absorbtion spectra of mixed micelles of conjugates **6** and **7** with PC or pluronic F68 in PBS.

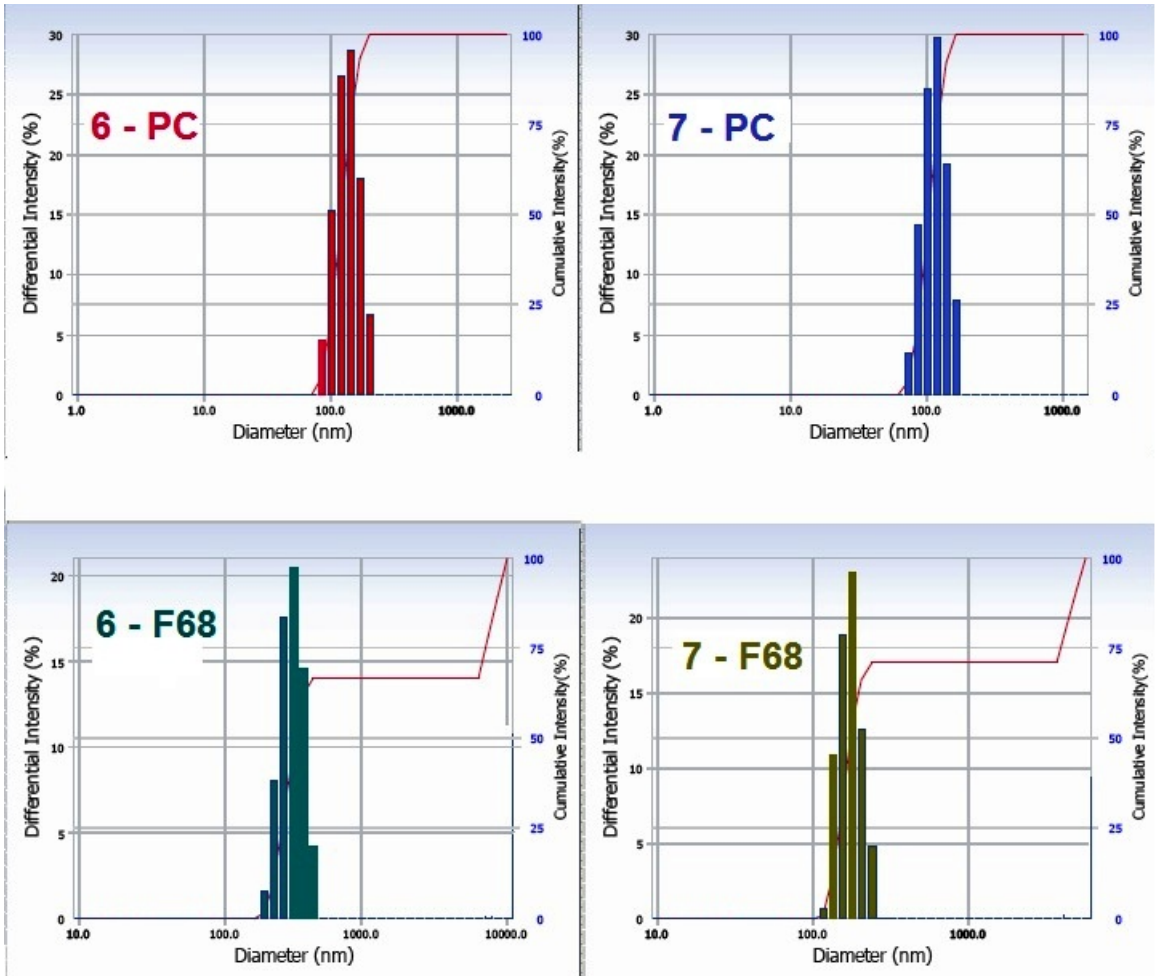


Figure 7. Particle size distribution for mixed micelles measured by laser scattering; **6 – PC** (average diameter – 123.3 nm); **7 – PC** (average diameter – 108.1 nm); **6 – F68** (average diameter – 621.3 nm); **7 – F68** (average diameter – 385.7 nm).

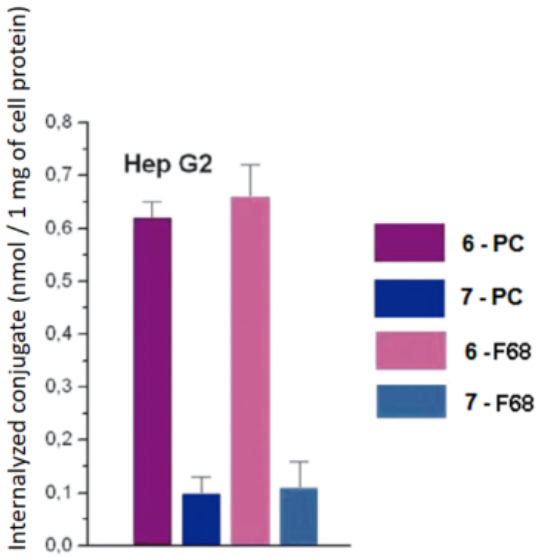


Figure 8. Internalization of conjugates **6** and **7** in form of mixed micelles by Hep G2 cells (concentration of each conjugate in media was 25 μM)

We have prepared mixed micelles **6 – PC** and **7 – PC** with mass ratio conjugate/PC equal to 1:10 (which corresponded to concentration of 6.7 molar % of conjugates); and micelles **6 – F68** and **7 – F68** with mass ratio conjugate/pluronic equal to 1:10 and 1:50. Absorption spectra and particle size distribution (measured by laser scattering) for these preparations are presented in Figures 6 and 7, respectively.

The spectra of micelles **6 – PC** and **7 – PC** were nearly identical and highly resolved; the Soret bands had two maxima at 402 nm and 417 nm (the last one is known to be characteristic for the aggregated form of conjugates); the long wave maxima had red shifts of about 6 nm (as compared to those for spectra of conjugates **6** and **7** in CH_2Cl_2) and were observed at 674 nm; the maxima at 516 nm, 544 nm, 618 nm were clearly visible (Fig. 6). The mean sizes of micelles **6–PC** and **7–PC** were 123.3 nm and 108.0 nm, respectively (Fig. 7). These mixed micelles possessed high stability: their absorption spectra and particle size distribution did not show any visible changes during the storage for 1 week.

The spectra of micelles **6–F68** and **7–F68** (at the conjugate/pluronic ratio of 1:50) were insufficiently resolved; the Soret bands were broad; the long wave maxima had additional shoulder near 710 mn, that indicated association of macrocycle chromophores with formation of stacked structures [27, 28]. The spectra of micelles **6–F68** and **7–F68** (at the conjugate/pluronic ratio of 1:10) were similar to those presented in Figure 6, but displayed certain turbidity and poor resolution. The mean sizes of conjugate-pluronic micelles **6–F68** and **7– F68** (with the mass ratio conjugate/pluronic 1:50) were 621.3 nm and 385.7 nm, respectively (Fig. 7). The absorption spectra of these micelles displayed significant changes after 24 h of storage, and after 1 week of storage the presence of mixed micelles was undetectable, that indicates low stability of mixed micelles conjugate-pluronic.

Mixed micelles of conjugates **6–PC**, **7–PC**, **6–F68**, and **7–F68** poorly interacted with prostate carcinoma LNCaP and PC-3 cells, however significantly internalized by hepatocarcinoma Hep G2 cells. The internalization of conjugate **6** was about 5-fold stronger than that of conjugate **7**. It depended on the structure of conjugate, rather than on the method of solubilization – the micelles conjugate – PC and conjugate – pluronic were internalized similarly (Fig. 8).

Apparently, binding of conjugates to Hep G2 cells may be explained by the existence of lipid binding sites (LBS) on the surface of these cells and affinity of hexadecyl moiety of conjugates to LBS [24].

COMPLIANCE WITH ETHICAL STANDARDS

This work does not contain any studies using humans and animals as research subjects.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Sharman W.M., van Lier J.E., Allen C.M. (2004) Targeted photodynamic therapy via receptor mediated delivery systems. Advanced Drug Delivery Reviews, **56**(1), 53–76. DOI: 10.1016/j.addr.2003.08.015

2. Giuntini F., Alonso C.M.A., Boyle R.W. (2011) Synthetic approaches for the conjugation of porphyrins and related macrocycles to peptides and proteins. Photochemical & Photobiological Sciences, **10**(5), 759–791. DOI: 10.1039/c0pp00366b

3. You H., Yoon H.-E., Yoon J.-H., Ko H., Kim Y.-C. (2011) Synthesis of pheophorbide-a conjugates with anticancer drugs as potential cancer diagnostic and therapeutic agents. Bioorganic & Medicinal Chemistry, **19**(18), 5383–5391. DOI: 10.1016/j.bmc.2011.07.058

4. Abrahamse H., Hamblin M.R. (2016) New photosensitizers for photodynamic therapy. The Biochemical Journal, **473**(4), 347–364. DOI:10.1042/BJ20150942

5. Baskaran R., Lee J., Yang S-G. (2018) Clinical development of photodynamic agents and therapeutic applications. Biomaterials Research, **22**, 25. DOI: 10.1186/s40824-018-0140-z

6. Tsolekile N., Nelana S., Oluwafemi O.S. (2019) Porphyrin as Diagnostic and Therapeutic Agent. Molecules, **24**(14), 2669. DOI:10.3390/molecules24142669

7. Pathak P., Zarandi M. A., Zhou X.· Jayawickramarajah J. (2021) Synthesis and Applications of Porphyrin-Biomacromolecule Conjugates. Frontiers in Chemistry, **9**, 764137. DOI: 10.3389/fchem.2021.764137

8. James D.A., Swamy N., Paz N., Hanson R.N., Ray R. (1999) Synthesis and estrogen receptor binding affinity of a porphyrin-estradiol conjugate for targeted photodynamic therapy of cancer. Bioorganic & Medicinal Chemistry Letters, **9**(16), 2379–2384. DOI: 10.1016/s0960-894x(99)00390-x

9. Swamy N., James D.A., Mohr S.C., Hanson R.N., Ray R. (2002) An estradiol-porphyrin conjugate selectively localizes into estrogen receptor-positive breast cancer cells. Bioorganic & Medicinal Chemistry Letters, **10**(10), 3237–3243. DOI: 10.1016/s0968-0896(02)00242-0

10. El-Akra N., Noirot A., Faye J.C., Souchard J.P. (2006) Synthesis of estradiol - pheophorbide conjugates: evidence of nuclear targeting, DNA damage and improved photodynamic activity in human breast cancer and vascular endothelial cells. Photochemical & Photobiological Sciences, **5**(11), 996–999. DOI: 10.1039/b606117f

11. Sadler S., Persons K.S., Jones G.B., Ray R. (2011) Internalization of a C17α-alkynylestradiol-porphyrin conjugate into estrogen receptor positive MCF-7 breast cancer cells. Bioorganic & Medicinal Chemistry Letters, **21**(15), 4638–4641. DOI: 10.1016/j.bmcl.2011.05.094

12. Ponomarev G.V., Solovieva M.N., Dugin N.O., Zavialova M.G., Mehtiev A.R., . Misharin A.Y, Novikov R.A., Tkachev Y.V., Popenko V.I., Timofeev V.P. (2013) Lipophilic derivatives of natural chlorins: synthesis, mixed micelles with phospholipids, and uptake by cultured cells. Bioorganic & Medicinal Chemistry, **21**(17), 5420–5427. DOI: 10.1016/j.bmc.2013.06.016

13. Nikolaeva I.A., Morozova J.V., Zavialova M. G., Novikov R.A.,. Tkachev Y.V, Timofeev V.P., Misharin A.Y., Ponomarev G.V. (2010) Natural Chlorins Modified with Cholesterol Moiety. Synthesis, Characteristics, Copper Complexes, and Entrapping in Phospholipid Vesicles. Macroheterocycles, **3**(2-3), 150–156. DOI: 10.6060/mhc2010.2-3.150

14. Zolottsev V.A., Tkachev Y.V., Latysheva A.S., Kostin V.A., Novikov R.A., Timofeev V.P., Morozevich G.E., Kuzikov A.V., Shumyantseva V.V., Misharin A.Y.

(2018) Comparison of [17(20)*E*]-21-Norpregnene oxazolinyl and benzoxazolyl derivatives as inhibitors of CYP17A1 activity and prostate carcinoma cells growth. Steroids, **129**, 24–34. DOI: 10.1016/j.steroids.2017.11.009
15. Tojo G., Fernandez M. (2006). Oxidation of Alcohols to Aldehydes and Ketones. Springer, NY, pp. 181–200 Chapter 3.
16. The Open Babel Package, version 2.0.1, http://openbabel.sourceforge.net/.
17. Phillips J.C., Braun R., Wang W., Gumbart J., Tajkhorshid E., Villa E., Chipot C., Skeel R.D., Kale L., Schulten K. (2005) Scalable molecular dynamics with NAMD, Journal of Computational Chemistry, **26**, 1781–1802. DOI: 10.1002/jcc.20289
18. Zoete V., Cuendet M.A., Grosdidier A., Michielin O. (2011) SwissParam, a fast force field generation tool for small organic molecules. Journal of Computational Chemistry, **32**, 2359–2368. DOI: 10.1002/jcc.21816
19. Humphrey W., Dalke A., Schulten K.J. (1996) VMD: visual molecular dynamics. Molecular Graphics, **14**, 33–38. DOI: 10.1016/0263-7855(96)00018-5
20. Liu A., Carlson K.E., Katzenellenbogen J.A. (1992) Synthesis of high affinity fluorine-substituted ligands for the androgen receptor. Potential agents for imaging prostatic cancer by positron emission tomography. Journal of Medicinal Chemistry, **35**(11), 2113–2129. DOI: 10.1021/jm00089a024
21. Oliveto E.P. (1972) In: Organic Reactions in Steroid Chemistry, Vol.II. (Fried J., Edwards J.A., Eds.), Van Nostrand Reinhold Co, 139 p.
22. Bittler, D., Laurent, H., Rach, P., Topert, *M*. (1991) US Patent 5010071
23. Boelrijk A.E.M., Reedijk J. (1994) Oxidation of methyl- and octyl-a-D-glucopyranoside, catalyzed by high-valent ruthenium species. Journal of Molecular Catalysis, **89**(1-2), 63 –76. DOI: 10.1016/1381-1169(95)00112-3
24. Dionne P., Ngatcha B.T., Poirier D. (1997) D-ring allyl derivatives of 17β- and 17α-estradiols: chemical synthesis and ^{13}C NMR data. Steroids, **62**(10), 674–681. DOI: 10.1016/s0039-128x(97)00067-6
25. Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods. Methods, **65**(1-2), 55–63. DOI: 10.1016/0022-1759(83)90303-4
26. Park H., Na K. (2013) Conjugation of the photosensitizer Chlorin e6 to pluronic F127 for enhanced cellular internalization for photodynamic therapy. Biomaterials, **34**(28), 6992–7000. DOI: 10.1016/j.biomaterials.2013.05.070
27. Tamiaki H., Michitsuji T., Shibata R. (2008) Synthesis of zinc bacteriochlorophyll-d analogues with various 17-substituents and their chlorosomal self-aggregates in non-polar organic solvents. Photochemical & Photobiological Sciences, **7**, 1225–1230. DOI: 10.1039/B802359J
28. Tamiaki H., Fukai K., Shimazu H., Nishide K., Shibata Y., Itoh S., Kunieda M. (2008) Covalently linked zinc chlorophyll dimers as a model of a chlorophyllous pair in photosynthetic reaction centers. Photochemical & Photobiological Sciences, **7**, 1231–1237. DOI: 10.1039/B802353K
29. Medvedeva N.V., Kisseleva A.F., Misharin A.Y. (1999**)** Preparation and properties of aqueous dispersions of phosphatidyl choline-cholesteryl esters or ethers. Russian Journal of Bioorganic Chemistry, **25**, 147–152.

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КОНЪЮГАТЫ ПИРОФЕОФОРБИДА *А* С 17-ЗАМЕЩЕННЫМИ СТЕРОИДНЫМИ АНДРОГЕНАМИ. СИНТЕЗ, МОЛЕКУЛЯРНОЕ МОДЕЛИРОВАНИЕ, ВЗАИМОДЕЙСТВИЕ С НЕКОТОРЫМИ ЛИНИЯМИ РАКОВЫХ КЛЕТОК

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Синтезированы пять новых бифункциональных конъюгатов пирофеофорбида *а* с 17-замещенными тестостероном, дигидротестостероном и эпитестостероном, различающихся длиной линкера (**1**–**5**), и два новых комплексных конъюгата **6** и **7** (содержащие три функциональные группы: пирофеофорбид *а*, 17α-замещенный тестостерон и липофильная гексадецильная цепь, связанные между собой L-лизиновым блоком). Анализом спектров 1H ЯМР и молекулярных моделей конъюгатов **1**–**7** установлено взаимное влияние стероидного и макроциклического фрагментов. Исследование взаимодействия конъюгатов **1**–**5** с культурами клеток карциномы предстательной железы показало, что поглощение и интернализация зависят от структуры конъюгата, в частности, от стереохимической конфигурации 17-гидроксильной группы в стероидной части и длины линкера, соединяющего пирофеофорбид а со стероидным фрагментом. Конъюгаты **1**–**5** значительно снижали рост и пролиферацию клеток LNCaP и PC-3 при 96-часовой инкубации; наиболее высокой антипролиферативной активностью обладало производное эпитестостерона с коротким линкером **3**. Облучение обработанных конъюгатами клеток светом (λ=660 нм) значительно повышало цитотоксичность. Трифункциональные конъюгаты **6** и **7** легко образовывали смешанные мицеллы с фосфатидилхолином и плюроником F68; данные смешанные мицеллы эффективно интернализовались клетками гепатокарциномы человека Hep G2. Связывание конъюгатов **6** и **7** в виде смешанных мицелл с клетками Hep G2 зависело от структуры конъюгата и не зависело от способа его солюбилизации.

Ключевые слова: стероидные конъюгаты; химический синтез; тетрапиррольные макроциклы; молекулярные модели; клетки карциномы простаты; фосфолипидные мицеллы

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