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Conjugates of Pyropheophorbide *a* with Androgen Receptor Ligands

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*Two new conjugates of pyropheophorbide a with testosterone and dihydrotestosterone: 17³[2-(17β-hydroxy-3-oxopregn-4-en-21-oylamido)ethylamido]pyropheophorbide a (**10**) and 17³[2-(17β-hydroxy-3-oxopregnan-21-oylamido)ethylamido]pyropheophorbide a (**11**) were synthesized. IC_{50} for conjugates **10** and **11** at 96 h incubation in LNCaP and PC-3 prostate carcinoma cells were 1.4 μM and 3.3 μM for compound **10**, and 4.5 μM and 6.1 μM for compound **11**, respectively. Irradiation with light at wavelength of 660 nm increased toxicity of the conjugates.*

Keywords: Conjugates, pyropheophorbide *a*, testosterone, dihydrotestosterone, prostate carcinoma cells, cytotoxicity.

Конъюгаты пирофеофорбида *a* с лигандами андрогенового рецептора

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*Синтезированы два новых конъюгата пирофеофорбида а с тестостероном и дигидротестостероном: 17³[2-(17β-гидрокси-3-оксопреген-4-ен-21-оиламидо)этиламидо]пирофеофорбид а (**10**) и 17³[2-(17β-гидрокси-3-оксопрегнан-21-оиламидо)этиламидо]пирофеофорбид а (**11**). IC_{50} для конъюгатов **10** и **11** при 96-часовой инкубации в клетках карциномы простаты LNCaP и PC-3 составляет 1.4 мкМ и 3.3 мкМ для соединения **10** и 4.5 мкМ и 6.1 мкМ для соединения **11**, соответственно. Облучение светом длиной волны 660 нм приводило к многократному повышению токсичности конъюгатов.*

Ключевые слова: Конъюгаты, пирофеофорбид *а*, тестостерон, дигидротестостерон, клетки карциномы простаты, цитотоксичность.

Tetrapyrrolic macrocycles, porphyrins and chlorins, owing to their unique photochemical and photophysical properties have wide range of biomedical applications such as optical imaging, fluorescent labeling, photodynamic inactivation of microbial infections, and photodynamic therapy of solid tumors. Coupling of macrocycles with fragments of biological active molecules improves delivery and distribution of macrocycle-based compounds to a specific location within the cells, facilitates its transport through receptor or drug mediated endocytosis, and affects its biological activity.[1-3] Synthesized earlier conjugates of macrocycles with polyamines, amino acids, peptides, peptidomimetics, antibiotics, nucleotides, carbohydrates, bile acids, lipids, steroids, *etc*., revealed prospective implications in biomedical studies and photodynamic therapy.[4-19]

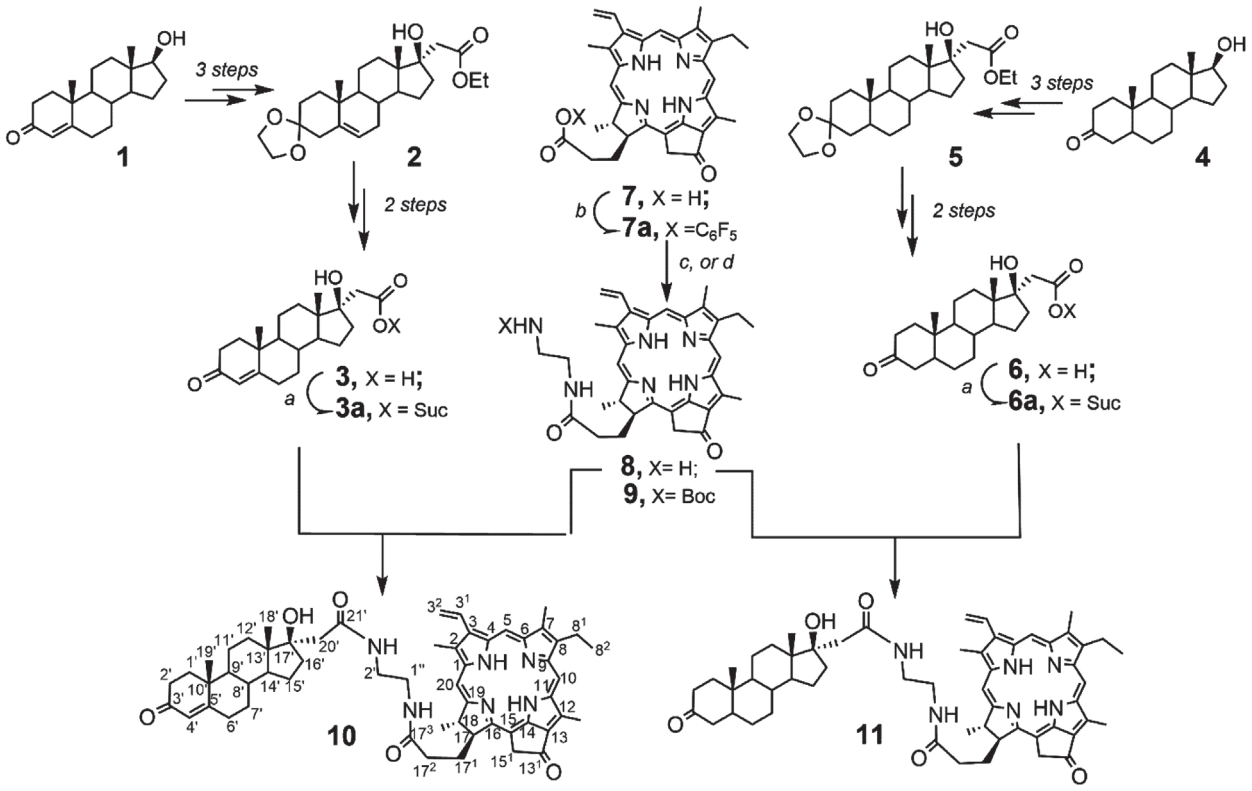
In this study we have synthesized conjugates of pyropheophorbide *a* with androgen receptor ligands – testosterone and dihydrotestosterone. Androgen receptor is known to be an important drug target for treatment of prostate cancer. Modern trends in preparation and application of various steroid conjugates targeting androgen receptor have been reviewed.[20, and the ref. therein] Until now conjugates of testosterone and dihydrotestosterone with tetrapyrrolic macrocycles have not been reported. Synthesis of new conjugates **10** and **11** is presented in the Scheme 1.

Testosterone **1** and dihydrotestosterone **4** were transformed to steroid blocks **2** and **5** by three steps including consecutive protection of carbonyl functions with formation of 1,3-dioxolanes, oxidation of 17β-hydroxyl groups, and Reformatsky reaction of obtained 17-ketones with Zn and ethyl bromoacetate.[21,22] The aforementioned reaction is known to pass stereoselectively and give appropriate 17β-OH isomer. Removal of ethylene ketal and ethyl ester protective groups in compounds **2** and **5** led to 21-carboxylic acids **3** and **6** in 49 % and 58 % overall yields (based on compounds **1** and **4**, respectively). Compounds **3** and **6** were transformed to related *N*-hydroxysuccinimide esters **3a** and **6a** by treatment with *N*-hydroxysuccinimide in the presence of *N,N*'-dicyclohexylcarbodiimide (DCC). HRMS, ^{1}H NMR, ^{13}C NMR data for compounds **2**, **3**, **3a**, **5**, **6** and **6a** are given in Supplementary section.

Pyropheophorbide *a* derivative comprising primary amino group (compound **8**[23]) was prepared from pyropheophorbide *a* **7** through formation of pentafluorophenyl ester **7a**, followed by its treatment with excess of ethylene diamine. Compound **9**[24] comprising Boc-protected amino group was prepared from pentafluorophenyl ester **7a** by same reaction with mono-Boc ethylene diamine.[25]

Condensation of *N*-hydroxysuccinimide esters of steroid acids **3a** and **6a** with 17^{3}[(2-aminoethyl)amido] pyropheophorbide *a* (**8**) led to the target conjugates **10** and **11**,[26,27] respectively. These conjugates were isolated as individual compounds. Their structures were completely characterized by HRMS, ^{1}H NMR, ^{13}C NMR and electron absorption spectra.

Absorption spectra of conjugates **9**, **10** and **11** in CH_2Cl_2 were very close to those for pyropheophorbide *a* **7** and 17^{3}[(2-aminoethyl)amido]pyropheophorbide *a* (**8**). ^{1}H NMR spectra of conjugates **10** and **11** displayed strong high field shifts for H-18' and H-19' methyl protons in comparison with those in spectra of non conjugated steroids (s, 0.53 ppm and s, 0.91 ppm for compound **10** instead of s, 0.95 ppm and s, 1.19 ppm for compound **3**; s, 0.56 ppm and s, 0.78 ppm for compound **11** instead of s, 0.92 and s, 1.01 ppm for compound **6**). The H-4' resonance for compound **10** (s,



Scheme 1. (a) N-OSu, DCC/CH_2Cl_2; (b) $CF_3COOC_6F_5/CH_2Cl_2$; (c) $H_2N(CH_2)_2NH_2/CH_2Cl_2$; (d) $BocNH(CH_2)_2NH_2/CH_2Cl_2$.

5.46 ppm) was also shifted in high field compared with those for compound **3**(s, 5.73 ppm). These spectral peculiarities apparently were caused by influence of macrocycle on steroid moiety; close effects were reported earlier for conjugates of pyropheophorbide *a* with cholesterol.[28] Resonance of *tert*-butyl protons in conjugate **9** (s, 1.21 ppm) was also shifted in high field compared to those usually observed for Boc-amides (s, 1.4 ppm).

Speculating that steroid fragments may affect affinity conjugates **10** and **11** to prostate carcinoma cells, we investigated viability of androgen-sensitive LNCaP and androgen-insensitive PC-3 cells in the presence of these conjugates and 17^3[(2''-*tert*-butyloxycarbonylamidoethyl)-amido]pyropheophorbide *a* (**9**) (as reference compound). Two experiments were carried out: in the Experiment 1 we have measured LNCaP and PC-3 cells viability at 96 h incubation with compounds **9**, **10** and **11**; in the Experiment 2 we have compared dark toxicity and photo toxicity of conjugates in the same cells at short time incubation (labeling – 18 h; irradiation – 10 min; incubation without compounds – 24 h). Cell viability was measured with MTT method.[29] The protocol used is given in supplementary section. Student's *t*-test was used to estimate average values for all cases. All Student's *t*-tests were calculated by an online calculator (*http://www.graphpad.com/quickcalcs/ttest1.cfm*), confidence interval for each case did not exceed 6 % of the mean.

The results demonstrated that coupling of pyropheophorbide *a* with testosterone and dihydrotestosterone led to conjugates toxic in LNCaP and PC-3 cells. Figures 1a and 1b (see Supplementary section) showed that conjugates **10** and **11** were highly toxic in both prostate carcinoma cells at 96 h incubation; conjugate **10** being significantly more potent cytotoxic agent than conjugate **11,** steroid-free conjugate **9** exhibited rather low effect on cells viability.

Figures 1c and 1d showed that both conjugates **10** and **11** decreased LNCaP and PC-3 cells viability at short time incubation (dark toxicity), though less potently than at 96 h incubation; conjugate **9** at short time incubation stimulated proliferation of LNCaP, rather than PC-3 cells. Irradiation (LED AFS "Spectrum", Laser medical centrum Ltd, Moscow, Russia; wavelength of 660 nm, 10 min) potently increased toxicity of conjugates in all cases. However, at short time incubation (either with irradiation, or without irradiation) cells viability remained rather high (≈20 % for PC-3 cells, ≈40 % for LNCaP cells) even at 50 μM and 100 μM of conjugates **10** and **11**. IC_{50} for conjugates **10** and **11** at 96 h incubation in LNCaP and PC-3 prostate carcinoma cells were 1.4 μM and 3.3 μM for compound **10**, and 4.5 μM and 6.1 μM for compound **11**, respectively (Table 1, Supplementary section).

In cocnclusion, conjugates of pyropheophorbide *a* with androgen receptor ligands – testosterone and dihydrotestosterone – were synthesized. These conjugates were found to exhibit potent dark and photo toxicity in prostate carcinoma cells. We speculate that further investigation of uptake, distribution, subcellular localization, and possible participation in signaling and regulatory pathways of these compounds and related steroid conjugates may be helpful for development of new photo sensitizers possessing high specificity and activity.

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23. *17³[(2''-Aminoethyl)amido]pyropheophorbide a* (**8**). The mixture of pentafluorophenylpyropheophorbide *a* **7a** (202 mg, 0.29 mmol), ethylene diamine (580 μL, 520 mg, 8.65 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 (p*H* 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 THF (30 mL), the solution was dried over granulated KOH, followed by evaporation to dryness. The obtained black powder (139 mg, 0.24 mmol, 83 %) was used without further purification; the analytical sample was purified by TLC in $CHCl_3$:MeOH:NH_4OH (90:9:1) mixture. HRMS, calculated for $[C_{35}H_{41}N_6O_2]^+$: 577.3291, found: 577.3292. ^{1}H NMR δ ppm: –1.70, 0.33 (each 1H, br.s, N–H); 1.62 (3H, t, *J*=7.6 Hz, 8²–H); 1.75 (3H, d, *J*=7.3 Hz, 18–CH_3); 3.18, 3.37, 3.41 (each 3H, s, 2–, 7–, 12–CH_3); 4.23, 4.45 (each 1H, m, 17¹–H and 8¹–H); 4.98, 5.19 (each 1H, d, *J*=19.7 Hz, 17²–H); 6.13 (1H, dd, *J*=11.5 Hz and *J*=1.4 Hz, 3²–H, *cis*); 6.24 (1H, dd, *J*=17.9 Hz and *J*=1.4 Hz, 3²–H, *trans*); 7.95 (1H, dd, *J*=11.5 Hz and *J*=17.9 Hz, 3¹–H); 8.50, 9.24, 9.30 (each 1H, s, 5–, 10–, 20–H); ^{13}C NMR δ ppm: 11.18; 11.81; 12.05; 17.36; 19.37; 23.01; 28.30; 30.17; 30.88; 32.80; 40.92; 41.65; 48.01; 49.97; 51.70; 92.92; 97.08; 103.88; 106.03; 122.65; 128.10; 129.18; 131.50; 135.78; 135.96; 136.13; 137.68; 144.92; 148.86; 150.65; 155.11; 160.37; 171.68; 172.36; 196.14. UV-Vis (CH_2Cl_2) λ_{max} nm (ε): 413 (85,000); 507 (8,900); 538 (8,000); 609 (7,000); 665 (35,200).
24. *17³[(2''-tert-Butyloxycarbonylamidoethyl)amido]-pyropheophorbide a* (**9**). Compound **9** was synthesized from pentafluorophenylpyropheophorbide *a* **7a** (88 mg, 0.13 mmol) and mono-Boc-ethylene diamine (42 mg, 0.26 mmol) according the procedure described in ref.[29] and isolated by silica gel flash chromatography in $CHCl_3$:MeOH:NH_4OH (90:9:1) mixture. After evaporation compound **9** (43 mg, 0.06 mmol, 43 %) was obtained as black powder. HRMS, calculated for $[C_{40}H_{49}N_6O_4]^+$: 677.3815, found: 677.3818. ^{1}H NMR δ ppm: –1.74, 0.36 (each 1H, br.s, N–H); 1.21 (9H, s, *t*-Bu); 1.59 (3H, t, *J*=7.6 Hz, 8³–H); 1.76 (3H, d, *J*=7.3 Hz,18–CH_3); 3.17, 3.27, 3.37 (each 3H, s, 2–, 7–, 12–CH_3); 4.25, 4.47 (each 1H, m, 17¹–H and 8¹–H); 5.01, 5.21 (each 1H, d, *J*=19.7 Hz,17²–H); 6.12 (1H, dd, *J*=11.5 Hz and *J*=1.4 Hz, 3²–H, *cis*); 6.23 (1H, dd, *J*=17.9 Hz and *J*=1.4 Hz, 3²–H, *trans*); 7.92 (each 1H, dd, *J*=11.5 Hz and *J*=17.9 Hz, 3¹–H); 8.51, 9.12, 9.28 (each 1H, s, 5–, 10–, 20–H). ^{13}C NMR δ ppm: 11.25; 11.76; 12.14; 17.41; 19.04; 23.15; 28.24; 28.46; 30.44; 33.02; 40.36; 40.63; 48.09; 50.08; 51.85; 79.56; 93.07; 97.14; 103.89; 106.08; 122.55; 128.06; 129.25; 130.28; 131.63; 135.86; 135.99; 136.23; 137.89; 141.61; 145.01; 148.95; 150.69; 155.19; 160.50; 171.80; 173.01; 196.26. UV-Vis (CH_2Cl_2) λ_{max} nm (ε): 413 (85,000); 507 (8,900); 538 (8,000); 609 (7,000); 665 (35,200).
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26. *17³[2''-(17'β-Hydroxy-3'-oxopregn-4'-en-21'-oylamidoethyl) amido]pyropheophorbide a* (**10**): The mixture of compounds **3a** (30 mg, 69 μmol), **8** (33 mg, 57 μmol), dry Py (3 mL), and dry THF (5 mL) was stirred at r. t. for 16 h, then evaporated to dryness with toluene, and the residue was applied on the top a silica gel column. The column initially was washed with $CHCl_3$:$(CH_3)_2CO$:AcOH (75:24:1) to remove byproducts, then washed with 5 mL $CHCl_3$, and finally the target product was eluted with $CHCl_3$:MeOH:7M NH_3 solution in MeOH (93:5:2, by vol). After evaporation the compound **10** (38 mg, 42 μmol, 73 %) was obtained as black powder. HRMS, calculated for $[C_{56}H_{69}N_6O_5]^+$: 905.5329, found: 905.5327. ^{1}H NMR δ ppm: –1.86 (1H, br.s, N–H); 0.53, 0.91 (each 3H, s, H–18' and H–19' in steroid moiety); 1.61 (3H, t, *J*=7.6 Hz, 8²–H in pyropheophorbide moiety), 1.74 (3H, d, *J*=7.3 Hz, 18–CH_3 in pyropheophorbide moiety), 3.19, 3.36, 3.39 (each 3H, s, 2–, 7–, 12–CH_3 in pyropheophorbide moiety), 4.23, 4.46 (each 1H, m, 17¹–H and 8¹–H in pyropheophorbide moiety), 4.98, 5.17 (each 1H, d, *J*=19.7 Hz, 17²–H in pyropheophorbide moiety), 5.46 (1H, s, H–4' in steroid moiety), 6.15 (1H, dd, *J*=11.5 Hz and *J*=1.4 Hz, 3²–H, *cis* in pyropheophorbide moiety), 6.17 (1H, br. t, *J*=5.2 Hz, NH–CO); 6.25 (1H, dd, *J*=17.9 Hz and *J*=1.4 Hz, 3²–H, *trans* in pyropheophorbide moiety), 6.71 (1H, br.t, *J*=5.2 Hz, NH–CO); 7.90 (1H, dd, *J*=11.5 Hz and *J*=17.9 Hz, 3¹–H in pyropheophorbide moiety), 8.58, 9.30, 9.35 (each 1H, s, 5–, 10–, 20–H in pyropheophorbide moiety). ^{13}C NMR δ ppm: 11.29; 12.00; 12.15; 13.60; 17.16; 17.32; 19.51; 20.36; 20.51; 23.17; 23.31; 30.35, 31.32; 32.59; 32.78; 33.83; 35.46; 36.00; 38.42; 39.59; 42.43; 45.97; 48.10; 49.62; 50.10; 51.92; 53.33; 81.77; 93.88; 97.10; 103.93; 106.51; 123.09; 123.67; 128.30; 129.04; 130.53; 132.19; 135.81; 136.35; 136.50; 137.83; 141.97; 144.94; 146.94; 149.36, 153.65; 155.65, 161.35; 171.17; 172.46; 173.59; 173.84; 174.03; 196.12; 199.41. UV-Vis (CH_2Cl_2) λ_{max} nm (ε): 413 (86,400); 507 (8,700); 538 (7,800); 609 (6,900); 667 (36,000).
27. *17³[2''-(17'β-Hydroxy-3'-oxopregnan-21'-oylamidoethyl) amido]pyropheophorbide a* (**11**). The synthesis of compound **11** was carried out from compounds **6a** (26 mg, 60 μmol) and **8** (30 mg, 52 μmol) using the procedure described in ref.[25] Compound **11** (33 mg, 37 μmol, 69 %) was obtained as black powder. HRMS, calculated for $[C_{56}H_{71}N_6O_5]^+$: 907.5486, found: 907.5490. ^{1}H NMR δ ppm: –1.67, (1H, br.s, N–H); 0.56, 0.78 (each 3H, s, H–18' and H–19' in steroid moiety); 1.65 (3H, t, *J*=7.6 Hz, 8²–H in pyropheophorbide moiety); 1.77 (3H, d, *J*=7.3 Hz, 18–CH_3 in pyropheophorbide moiety); 3.21, 3.37, 3.45 (each 3H, s, 2–, 7–, 12–CH_3 in pyropheophorbide moiety); 4.27, 4.46 (each 1H, m, 17¹–H, 8¹–H in pyropheophorbide moiety); 5.02, 5.21 (each 1H, d, *J*=19.7 17²–H in pyropheophorbide moiety); 5.86 (1H, br.t, *J*=5.2 Hz, NH–CO); 6.14 (1H, dd, *J*=11.5 Hz and *J*=1.4 Hz, 3²–H, *cis* in pyropheophorbide moiety), 6.20 (1H, dd, *J*=17.9 Hz and *J*=1.4 Hz, 3²–H, *trans* in pyropheophorbide moiety), 6.58 (1H, br. t, *J*=5.2 Hz, NH–CO); 7.93 (1H, dd, *J*=11.5 Hz and *J*=17.9 Hz, 3¹–H in pyropheophorbide moiety), 8.52, 9.30, 9.33 (each 1H, s, 5–, 10–, 20–H in pyropheophorbide moiety). ^{13}C NMR δ ppm: 11.39; 12.03; 13.62; 14.02; 17.34; 19.31; 20.61; 23.05; 23.36; 28.42; 28.74; 29.69; 31.46; 31.87; 32.98; 33.90; 35.25; 35.66; 36.16; 38.48; 39.72; 42.49; 46.00; 46.63; 48.04; 49.95; 50.03; 51.72; 53.67; 81.85; 93.11; 97.11; 103.85; 105.86; 122.65; 124.15; 125.29; 128.22; 129.02; 130.01; 131.73; 135.97; 136.32; 137.58; 137.86; 141.70; 144.98; 148.66; 148.93; 160.45; 171.97; 173.58; 174.20; 174.48; 196.31; 211.49. UV-Vis (CH_2Cl_2) λ_{max} nm (ε): 413 (85,900); 507 (8,500); 538 (8,000); 609 (6,500); 667 (35,800).
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