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# Chlorin e6–cholesterol conjugate and its copper complex. Simple synthesis and entrapping in phospholipid vesicles

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ABSTRACT

Synthesis of 13′[(cholest-5-en)-3β-yloxyethoxycarbamoyl]-chlorin e6 starting from methylpheophorbide and 3β(2-hydroxy)-ethoxycholest-5-ene is presented, as well as the preparation of related copper complex. Both conjugates obtained may be simply incorporated in phosphatidyl choline vesicles.



Tetrapyrrolic macrocyles, that is, porphyrins and chlorins, owing to their unique spectral, photochemical, photophysical, and metal chelating properties, have wide range of biomedical applications, such as optical imaging, fluorescent labeling, photodynamic inactivation of microbial infections, and photodynamic therapy of solid tumors.[1–6] A key challenge to the implementation of tetrapyrrolic macrocyles for biomedicine entails tailoring the molecules either with hydrophilic substituents to achieve its water solubility,[7,8] or with lipophilic substituents for the incorporation into liposomes and lipid micelles.[9,10] Coupling of phthalocyanine and pyropheophorbide macrocycles with estradiol and 3β-oleoyloxyanrost-5-en-17-amine was shown to be an efficient approach for the receptor-dependent targeting of macrocycles to cells.[11,12]

Modification of tetrapyrrolic macrocycle with lipophilic cholesterol moiety may be of interest, since cholesterol is essential component of mammalian membranes. The resulting conjugates are supposed to have affinity to membranes, and may be used as photosensitizers, being entrapped in liposomes. Insertion of paramagnetic, for example, copper ion, into the coordination sphere of macrocycle may convert them to spin probes suitable for membrane studies. Herein, the simple synthesis of chlorin e6–cholesterol conjugate from easily available methylpheophorbide,[13] preparation of related copper complex, and entrapping both these conjugates into phosphatidyl choline vesicles, are presented.

The synthetic pathway is shown in Scheme 1. 3β(2-Hydroxy)-ethoxycholest-5-ene **2** was prepared from cholesterol **1** according to published method;[14] substitution of hydroxyl group for amino group was carried out by three step procedure, the resulting 3β(2-amino)-ethoxycholest-5-ene[15] **3** was obtained in 62% overall yield (based on cholesterol **1**). For the coupling of sterol and macrocycle fragments the known reaction of nucleophilic opening of exocycle E in methylpheophorbide by amines was used:[16] incubation of methylpheophorbide with 1.5 equiv of aminosterol **3** in THF at 40 °C for 48 h led to target amide conjugate[17] **4** isolated in 90% yield. The copper complex of conjugate **5** was prepared in quantitative yield by heating of conjugate **4** with $Cu(CH_3COO)_2$ excess in $CH_2Cl_2$–MeOH mixture (1:3) at 45 °C for 1 h with subsequent isolation of product[18] **5** by silica gel flash chromatography. The formation of copper complex was confirmed by HRMS peak corresponding to molecular ion, hypsochromic shift of long wave maximum in absorption spectrum, and characteristic EPR spectrum.

Chlorin e6–cholesterol conjugates **4** and **5** may be simply incorporated in phospholipids bilayers. Mixed vesicles consisted of egg yolk phosphatidyl choline (PC) and compounds **4** and **5** were prepared according to known procedure[19] developed earlier for the preparation of unilamellar vesicles from pure PC, and PC–cholesterol mixtures. Incorporation of conjugates **4** and **5** in PC vesicles[20] provides their solubilization in aqueous medium, and leads to notable changes in absorption spectra when compared

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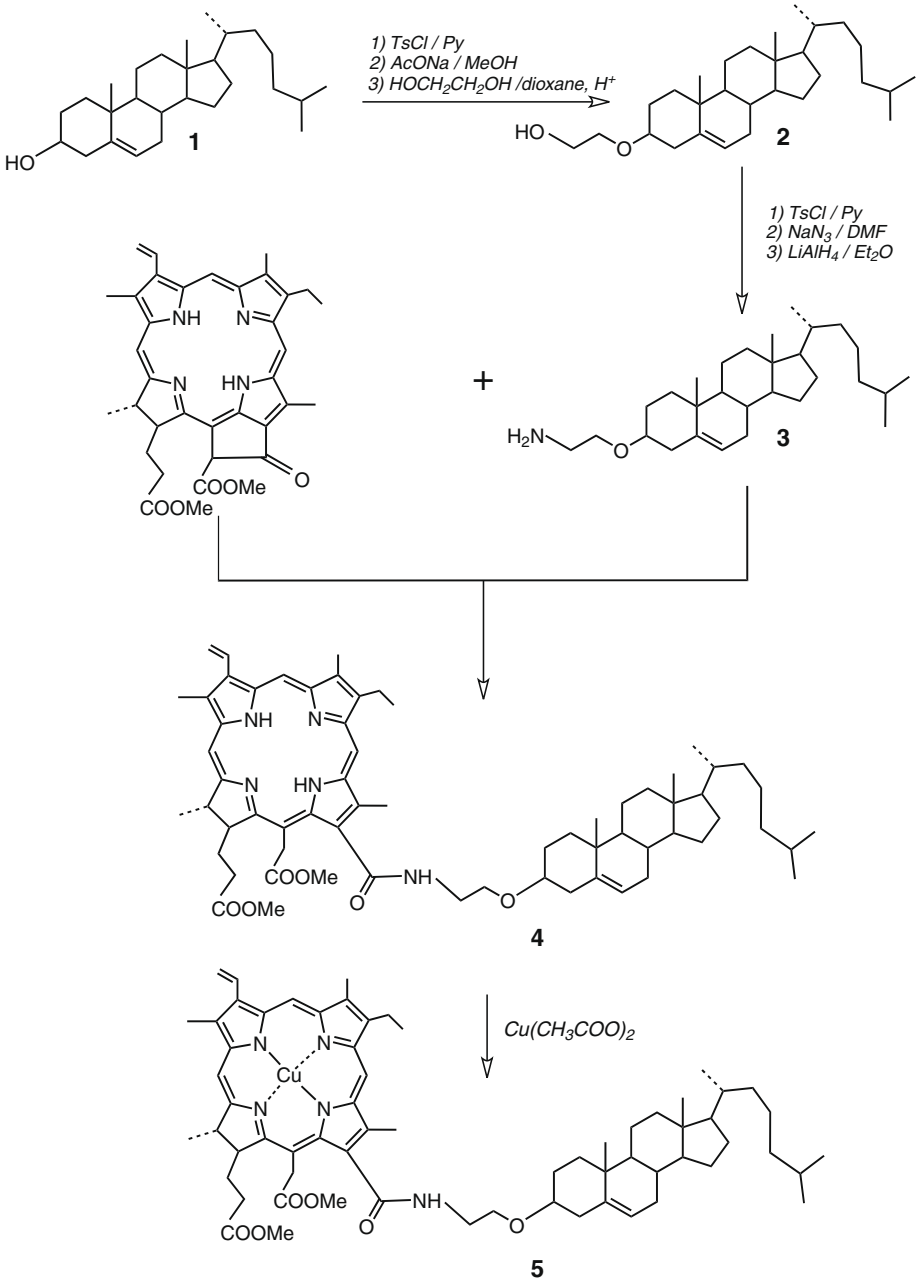


**Scheme 1.**

with these of $CH_2Cl_2$ solutions. Bathochromic shifts, apparently caused by increasing of the medium polarity, thus confirming the dye exposure on the bilayer surface, were observed both for Soret bands and for long wave maxima (Fig. 1).

EPR spectra[21] of conjugate **5** powder (1, Fig. 2A) and PC vesicles containing conjugate **5** in aqueous solution (2, Fig. 2A) look similar to well-known ones for porphin-like copper complexes.[22] High-field perpendicular manifold splits into two major components due to second-order effects ('angular anomalies'),[23] and this is typical for systems with highly anisotropic spin Hamiltonian. Angles about 70° (with respect to magnetic field) contribute mainly to the most high-field component,[23] thus providing some spatial resolution in perpendicular region. Splitting between low-field peaks (referenced as *A* parameter, Fig. 2A) provides a measure of motional spectrum narrowing. In rigid-limit state (no motion, with correlation time $\tau$ much larger than spin Hamiltonian anisotropy) it is equal to parallel component of $^{63}Cu$ nucleus hyperfine tensor (typically about 200G), while motions comparable to hyperfine tensor anisotropy leads to decreasing of *A* value.

Compared to EPR spectrum of conjugate **5** in $CH_3Cl$ solution (Fig. 2B), the spectrum of conjugate **5** in PC vesicles (2, Fig. 2A) displays much higher value of *A* (209.7G vs 110.6G). This clearly confirms an entrapping of conjugate **5** into PC vesicles, because of $\tau_{(\text{free conjugate})} \ll \tau_{(\text{vesicle})}$. Moreover, the value of *A* at 293 K for PC-entrapped complex is even larger than in powder spectrum of free one (209.7G vs 201.5G), approaching rigid-limit (211.9G, as measured at 77 K) indicating intramolecular fast oscillations being hindered by the bilayer. This allows relating membrane director tilt angles with hyperfine splitting.

Additionally, in spectrum of compound **5** powder (1, Fig. 2A), the super-hyperfine splitting in the region of perpendicular mani-

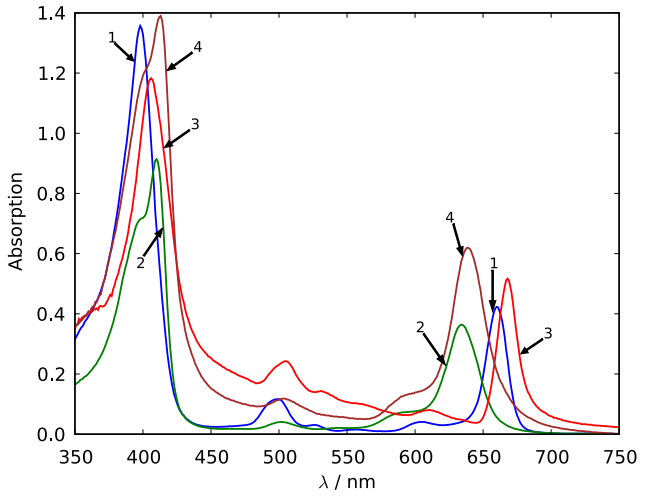


**Figure 1.** Absorption spectra of compounds **4** and **5** in $CH_2Cl_2$ (curves 1 and 2, respectively) and mixed vesicles of compounds **4** and **5** with PC in aqueous solution, pH 7.4 (curves 3 and 4, respectively).

fold (arising from four nitrogen nuclei of chlorin ring) is almost unresolved, while in presence of PC it is clearly emphasized. Therefore, entrapping of cholesterol moiety of conjugate **5** into PC vesicle, leads to exposure of its chlorin ring on the surface of bilayer, hindering its reorientational motion, and preventing copper centers from spin–spin interaction due to spatial separation, which otherwise leads to line broadening.

In conclusion, chlorin e6–cholesterol conjugates were synthesized and characterized, as well as the related mixed vesicles with PC. The affinity of cholesterol moiety of conjugates to phospholipids ensures efficient incorporation in lipid aggregates. Being used as spin probes, chlorin e6–cholesterol conjugates containing paramagnetics, may provide structural (distinguishing between parallel, perpendicular, and 70° orientations) and dynamical information (with maximum sensitivity at $\tau \approx 1$ ns and less). Being incorporated in PC vesicles, chlorin e6–cholesterol conjugates are efficiently taken up by the cultured cells, enabling them to be considered as potential sensitizers for photodynamic therapy.

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14. Misharin, A. Y.; Malugin, A. V.; Steinschneider, A. Y.; Kosykh, V. A.; Novikov, D. K. *Med. Chem. Res.* **1993**, *3*, 451. Analytical data for *3β(2-hydroxy)-ethoxycholest-5-ene* **1**: white needles, mp 104 °C (from $CH_3CN$); $^1H$ NMR (here and below were obtained on a Bruker AMX-III instrument, 400 MHz, in $CDCl_3$): 0.67 (s, 3H, H-18); 0.85 and 0.86 (each d, $J$ = 6.6 Hz, 3H, H-26 and H-27); 0.91 (d, $J$ = 6.6 Hz, H-21); 1.00 (s, 3H, H-19); 3.19 (m, 1H, H-3); 3.58 (m, 2H, $COCH_2$); 3.70 (m, 2H, $HOCH_2$); 5.34 (m, 1H, H-6); $^{13}C$ NMR: 12.78; 19.61; 20.22; 21.95; 23.38; 23.60; 24.70; 25.12; 29.02; 28.78; 29.23; 32.70; 32.71; 36.53; 36.97; 37.60; 37.96; 39.86; 40.25; 40.55; 43.05; 50.89; 56.85; 57.40; 62.59; 69.55; 79.89; 121.71; 140.71.
15. *3β(2-Amino)-ethoxycholest-5-ene* **2** was isolated as white solid homogenous by TLC and indicated positive reaction with ninhydrin. $^1H$ NMR: 0.67 (s, 3H, H-18); 0.85 and 0.86 (each d, $J$ = 6.6 Hz, 3H, H-26 and H-27); 0.91 (d, $J$ = 6.6 Hz, 3H, H-21); 1.00 (s, 3H, H-19); 3.20 (m, 1H, H-3); 3.34 (m, 2H, $NCH_2$); 3.65 (m, 2H, $COCH_2$); 5.34 (m, 1H, H-6); $^{13}C$ NMR: 12.02; 18.89; 19.53; 21.25; 22.70; 22.95; 24.01; 24.45; 28.16; 28.38; 28.65; 32.08; 35.95; 36.38; 37.06; 37.42; 39.36; 39.69; 39.98; 42.41; 42.50; 50.40; 56.37; 56.96; 70.24; 79.43; 121.72; 141.09. Analytical data for intermediates: *3β(2-p-tolyolsulfonyloxy)-*

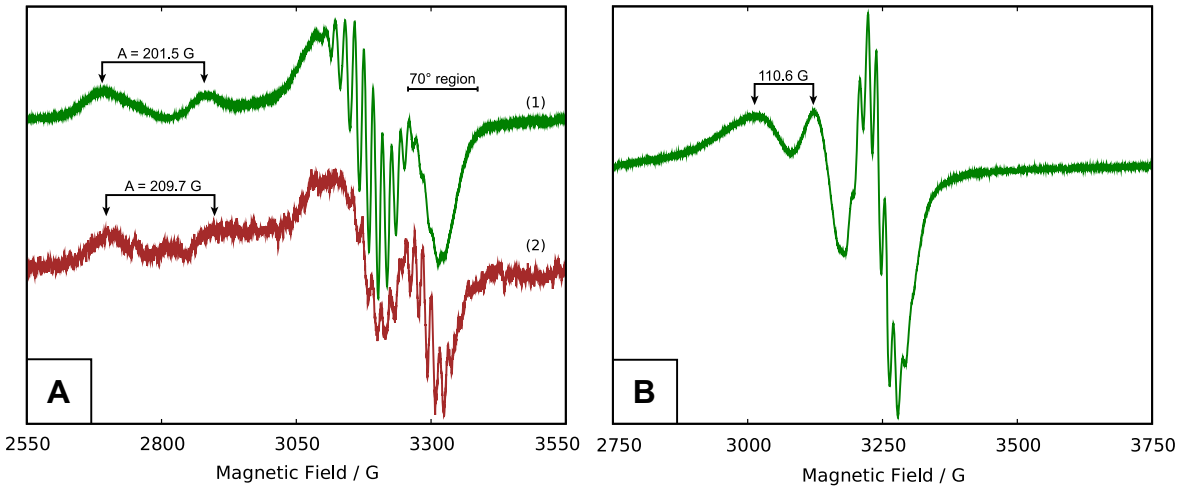


**Figure 2.** X-band EPR spectra of conjugate **5.** (A) Solid-state powder spectrum (1); spectrum of conjugate **5**—PC mixed vesicles in aqueous solution, pH 7.4 (2). (B) Spectrum in $CHCl_3$ solution.

*ethoxycholest-5-ene* (white needles from hexane, mp 115 °C); $^1$H NMR: 0.67 (s, 3H, H-18); 0.85 and 0.86 (each d, $J$ = 6.6 Hz, 3H, H-26 and H-27); 0.91 (d, $J$ = 6.6 Hz, H-21); 0.96 (s, 3H, H-19); 2.43 (s, 3H, $CH_3$, tosyl); 3.09 (m, 1H, H-3); 3.64 (m, 2H, $COCH_2$); 4.14 (m, 2H, $SOCH_2$); 5.31 (m, 1H, H-6); 7.33 (d, $J$ = 8.0 Hz, tosyl); 7.80 (d, $J$ = 8.0 Hz, tosyl); $^{13}$C NMR: 12.02; 18.90; 19.49; 21.24; 21.78; 22.71; 22.96; 24.01; 24.45; 28.17; 32.08; 32.11; 28.17; 28.38; 32.11; 35.95; 36.38; 36.98; 37.27; 39.05; 39.70; 39.97; 42.51; 50.35; 56.37; 56.96; 66.56; 69.81; 79.79; 121.95; 128.17; 129.92; 133.50; 140.77; 144.82. *3β(2-Azido)-ethoxycholest-5-ene* (white cubes from EtOH, mp 60 °C); $^1$H NMR: 0.67 (s, 3H, H-18); 0.85 and 0.86 (each d, $J$ = 6.6 Hz, 3H, H-26 and H-27); 0.91 (d, $J$ = 6.6 Hz, H-21); 1.00 (s, 3H, H-19); 3.20 (m, 1H, H-3); 3.34 (m, 2H, $NCH_2$); 3.65 (m, 2H, $COCH_2$); 5.34 (m, 1H, H-6); $^{13}$C NMR: 12.02; 18.90; 19.53; 21.26; 22.71; 22.96; 24.01; 24.46; 28.17; 28.39; 28.49; 32.09; 32.12; 35.95; 36.39; 37.02; 37.36; 39.16; 39.70; 39.99; 42.52; 50.40; 51.22; 56.38; 56.98; 66.91; 79.88; 121.93; 140.90.

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17. *13'[(Cholest-5-en)-3β-yloxyethoxycarbamoyl]-chlorin e6* **4** was isolated by silica gel flash chromatography in $CH_2Cl_2$ containing MeOH (1% v/v). $^1$H NMR (assignment of signals was obtained from COSY spectra): −1.88 (broad s, 1H, NH in chlorin e6 moiety); −1.40–1.90 (broad, 1H, NH in chlorin e6 moiety); 0.64 (s, 3H, H-18 in cholesterol moiety); 0.84 and 0.85 (each d, $J$ = 6.6 Hz, 3H, H-26 and H-27 in cholesterol moiety); 0.90 (d, $J$ = 6.6 Hz, H-21 in cholesterol moiety); 0.93 (s, 3H, H-19 in cholesterol moiety); 1.71 (d, $J$ = 7.2 Hz, 3H, $CH_3CH$ in chlorin e6 moiety); 1.72 (t, $J$ = 7.2 Hz, 3H, $CH_3CH_2C$= in chlorin e6 moiety); 3.21 (m, 1H, H-3 in cholesterol moiety); 3.31, 3.48, 3.58, 3.61, and 3.71 (each s, 3H, $CH_3CC$= and $CH_3OO$ in chlorin e6 moiety); 3.80 (q, 2H, $J$ = 7.5 Hz, $CH_3CH_2C$= in chlorin e6 moiety); 3.82 (m, 2H, $NCH_2CH_2O$); 4.03 (m, 2H, $NCH_2CH_2O$); 4.40 (dd, $J$ = 9.7 Hz and $J$ = 1.9 Hz, 1H, $CH_3CHCH$ in chlorin e6 moiety); 4.47 (q, $J$ = 7.2 Hz, $CH_3CH_2C$= in chlorin e6 moiety); 5.29 (d, $J$ = 18.9 Hz, 1H, $CCH_2COO$ in chlorin e6 moiety); 5.32 (m, 1H, H-6 in cholesterol moiety); 5.56 (d, $J$ = 18.9 Hz, 1H, $CCH_2COO$ in chlorin e6 moiety); 6.13 (dd, $J$ = 11.5 Hz and $J$ = 1.4 Hz, 1H, $H_2C$=CH (trans) in chlorin e6 moiety); 6.35 (dd, $J$ = 17.7 Hz and $J$ = 1.4 Hz, 1H, $H_2C$=CH (cis) in chlorin e6 moiety); 6.72 (broad m, 1H, HNCO); 8.07 (dd, $J$ = 11.5 Hz and $J$ = 17.7 Hz, 1H, $H_2C$=CH in chlorin e6 moiety); 8.80, 9.64, and 9.71 (each s, 1H, β-H, CHC= in chlorin e6 moiety); $^{13}$C NMR: 11.42; 11.92; 12.04; 12.22; 17.75; 18.81; 19.42; 19.79; 21.14; 22.64; 22.89; 23.15; 23.94; 24.35; 28.10; 28.30; 28.54; 29.82; 31.22; 31.97; 31.99; 35.86; 36.30; 36.93; 37.25; 37.99; 39.18; 39.63; 39.86; 40.90; 42.41; 49.36; 50.26; 51.65; 52.25; 53.24; 56.28; 56.84; 66.49; 70.67; 71.72; 79.57; 93.82; 98.89; 101.46; 102.58; 121.58; 121.72; 121.87; 128.51; 129.60; 130.12; 130.27; 134.72; 134.92; 135.01; 135.30; 136.07; 139.05; 140.72; 144.73; 148.84; 153.92; 166.94; 169.02; 169.44; 173.56; 173.81; HRMS (here and below were obtained on a Bruker 'Apex Ultra' FT ICR MS instrument, at ion positive electro spray ionization mode) calculated for $[C_{67}H_{98}N_5O_6]^+$: 1068.7517; found: 1068.7444; $\lambda_{max}$(nm) (here and below: absorption spectrum were obtained on a 'Thermospectronic Helios α' spectrophotometer) in $CH_2Cl_2$: 398; 498; 660.
18. *13'[(Cholest-5-en)-3β-yloxyethoxycarbamoyl]-chlorin e6–Cu complex* **5** was isolated by silica gel flash chromatography in $CH_2Cl_2$ containing MeOH (1% v/v). HRMS calculated for $[C_{67}H_{97}CuN_5O_6]^+$: 1129.6657; found: 1129.6620; $\lambda_{max}$,(nm) in $CH_2Cl_2$: 398 (sh), 410, 634.
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20. Stoichiometric composition of PC vesicles containing conjugates **4** and **5** was determined as follows: 1 mL of obtained vesicles was extracted with $CHCl_3$/MeOH (2:1 by vol.) mixture (3 × 3 ml), followed by quantitative determination of PC concentration according to known method: Vaskovsky, V. E.; Kostetsky, E. V.; Vasendin, I. M. *J. Chromatogr.* **1975,** *114*, 129–141; and chlorin e6–cholesterol conjugates concentration from absorption spectra (suggesting $\varepsilon_{660}$ value for compound **4,** and $\varepsilon_{630}$ value for compound **5** to be equal to 36,000). The molar ratio of conjugate/PC was found to be 1:50 in both cases. Compound **4**—PC mixed vesicles: $\lambda_{max}$ (nm) in $H_2O$: 406, 505, 668. Compound **5**—PC mixed vesicles: $\lambda_{max}$ (nm) in $H_2O$: 401(sh), 413, 503, 592(sh), 639.
21. EPR spectra of 13'[(cholest-5-en)-3β-yloxyethoxycarbamoyl]-chlorin e6–Cu complex **5** (as a powder, mixed with solid silica gel, as solution in $CHCl_3$, and as mixed vesicles with PC in phosphate buffered saline, pH 7.4) were obtained on a Varian E-104A X-band (9.15 GHz) spectrometer at room temperature (293 K), with microwave irradiation level of 20 mW, modulation amplitude of 4G and sweep width of 1000G.
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