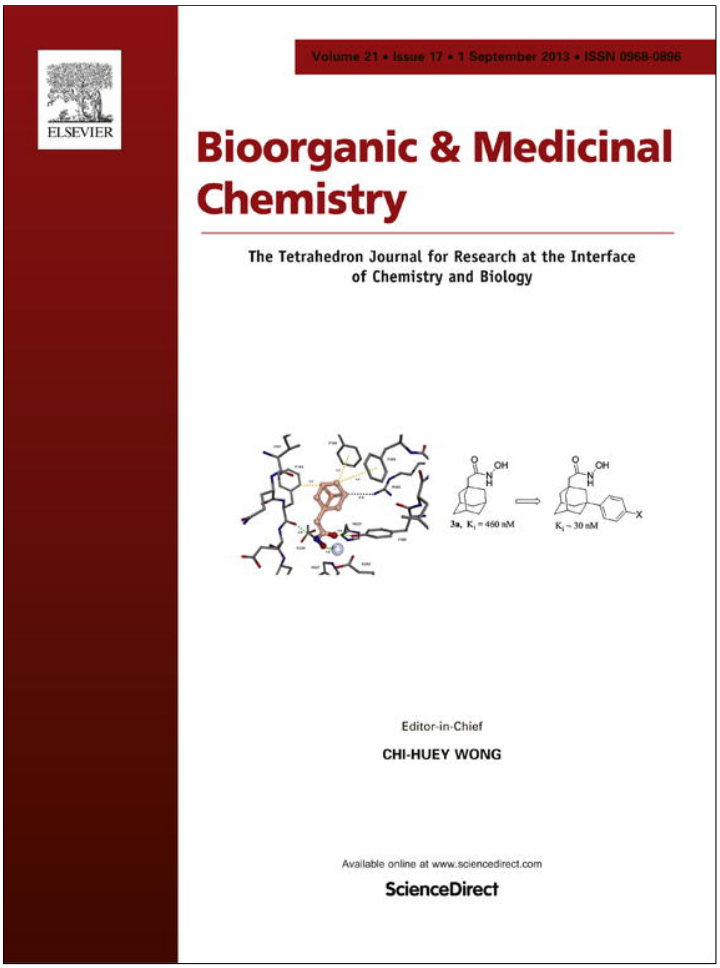

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Lipophilic derivatives of natural chlorins: Synthesis, mixed micelles with phospholipids, and uptake by cultured cells

Gelii V. Ponomarev [a,*], Maria N. Solovieva [a], Nikita O. Dugin [a], Maria G. Zavialova [a], Arif R. Mehtiev [a], Alexander Yu. Misharin [a], Roman A. Novikov [b], Yaroslav V. Tkachev [b], Vladimir I. Popenko [b], Vladimir P. Timofeev [b]

[a] Orekhovich Institute of Biomedical Chemistry RAMS, 10, Pogodinskaya Street, 119121 Moscow, Russia
[b] Engelhardt Institute of Molecular Biology RAS, Moscow, Russia

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ABSTRACT

The chemical synthesis of six lipophilic conjugates of chlorins was carried out, in which lipophilic fragment (either hexadecyl- or cholest-5-en-3β-yloxyethyl-) bound to 13^1-, 15^2-, 17^3-positions of macrocycle by formation of related carboxamides. Structure of synthesized conjugates was studied by spectral methods and molecular modeling. Lipophilic conjugates of chlorins, being mixed with egg yolk phosphatidyl choline, formed mixed micelles stable in aqueous media under physiological conditions. Mixed micelles of conjugates with phosphatidyl choline differing in stoichiometric compositions were prepared and characterized by absorption spectra, electron microscopy and laser scattering. These micelles were found to bind and internalized by human breast carcinoma MCF-7 cells. The presented data reveal that modification of macrocycle with lipophilic substituents, solubilization of obtained conjugates in aqueous medium as mixed micelles with phospholipids, and transfer of mixed micelles to cells is simple approach for targeting of chlorin derivatives, which apparently may be used in photodynamic therapy.

1. Introduction

Naturally occurring tetrapyrrolic macrocyles such as chlorins and porphyrins, owing to their unique photochemical properties are very attractive as photosensitizers for tumor photodynamic therapy (PDT).[1–6] A key challenge to the implementation of chlorins and porphyrins for PDT entails chemical modification of macrocycle to improve its delivery, specific targeting, and photodynamic properties. Conjugation of macrocycle with polyamines, amino acids, peptides, sugars have a significant impact on PDT efficacy, since it increases the solubility of sensitizer molecule in aqueous medium, facilitates transport through receptor or drug-mediated endocytosis, affects delivery of sensitizer to a specific location within the cells, and improves the biological effects of macrocycle-based compounds.[7–15] Aiming to improve delivery and distribution of sensitizers within targets several approaches were developed, including preparation of chlorin containing liposomes, dendrimer-like nanoparticles, and reconstructed low density lipoproteins.[16–19]

Our preliminary results showed that chlorin macrocycle, modified with cholesterol moiety acquires affinity to phospholipids and may be simply incorporated in phosphatidyl choline vesicles.[20,21] Chlorin derivatives modified with lipophilic substituents (in the contrast to chlorin polyamine derivatives) are insoluble in water and thereby could not penetrate the cells. However, these lipohilic chlorin derivatives may be solubilized in aqueous medium as mixed micelles with phosphatidyl choline, and then transferred to cells. We hope that this approach in future may be used for PDT.

Herein we present (i) facile synthesis of six chlorin amides containing either hexadecyl-, or cholest-5-en-3β-yloxyethyl-fragments in 13^1, 15^2, 17^3 positions (compounds **1–6**, Scheme 1), investigation of their structure by spectroscopic methods and molecular modeling; (ii) preparation and characterization of mixed micelles of conjugates **1–6** with phosphatidyl choline; (iii) uptake of conjugates by human breast carcinoma MCF-7 cells. The data presented below indicate that solubilized chlorin conjugates **1–6** are capable to penetrate within MCF-7 cells, the properties and characteristics of conjugate containing micelles, as well and as efficiency of cell labeling being dependent on the position of amide group and the structure of lipophilic substituent.

2. Results and discussion

2.1. Synthesis and spectral properties of conjugates (1–6)

Synthesis of lipophylic conjugates (**1–6**) is shown in the Scheme 1.

* Corresponding author. Tel.: +7 4992465820.
E-mail address: gelii@yandex.ru (G.V. Ponomarev).

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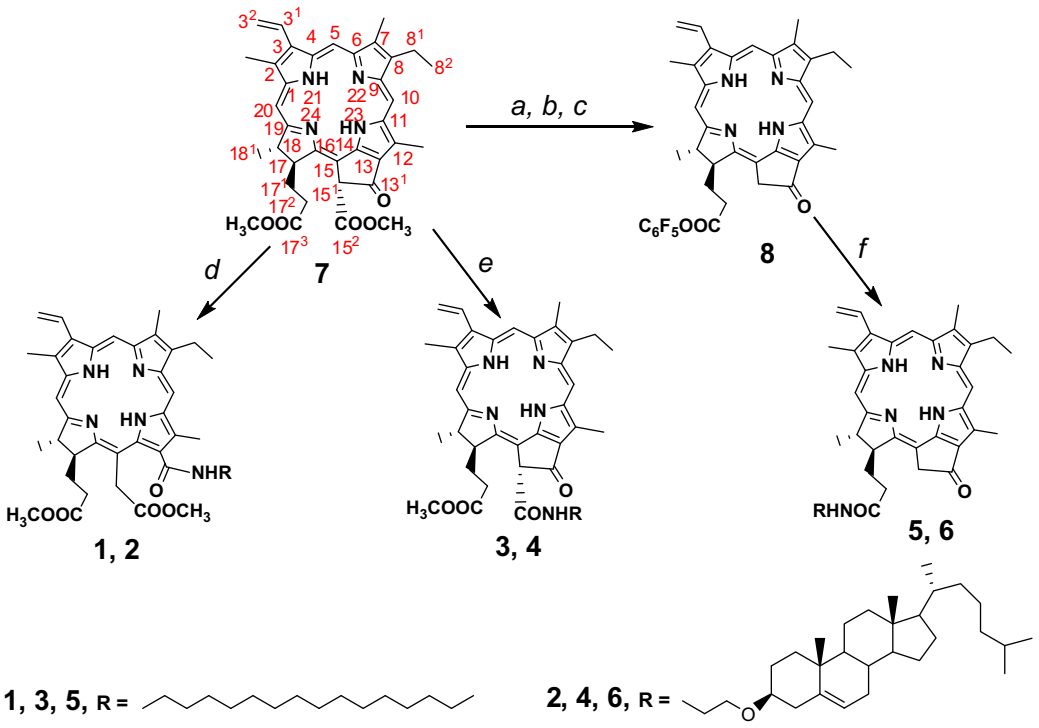

Scheme 1. Reagents and conditions: (a) Py, reflux, 8 h, (90%); (b) 50% aq H_2SO_4, rt, 2 h, (96%); (c) $CF_3COOC_6F_5$, Et_3N/CH_2Cl_2, rt, 15 min, (near quant); (d) NH_2R, THF, 40 °C, 24–72 h, (86–90%); (e) NH_2R, dioxane, reflux, 8 h, (80%); (f) NH_2R, Et_3N/THF, 40 °C, 1 h, (80–90%).

The nucleophilic opening of isocyclic ring in methyl pheophorbide a **7**[22] (method A) was used for the preparation of 13^1-carboxamides **1** and **2**. Earlier this reaction has been successfully used in synthesis of chlorin e6 13^1-carboxamides with various substituents at the periphery of macrocycle.[14,23–25] Reaction of methyl pheophorbide a **7** with excess of 3β(2-amino)ethoxycholest-5-ene proceeded slower in comparison with that of hexadecyl amine (and other primary aliphatic amines, ethylene diamine, benzylamine, piperidine[26]). 13^1-Carboxamides **1** and **2** were obtained in 86% and 90% yields by incubation of compound **7** with hexadecyl amine and 3β(2-amino)ethoxycholest-5-ene in THF at 40 °C for 24 h and 72 h, respectively.

We have found that interaction of methyl pheophorbide a **7** with the same amines in boiling dioxane for 6–8 h led to another products—15^2 carboxamides **3** and **4**, respectively (method B). The presence of isocyclic ring in compounds **3** and **4** was unequivocally established by absorption and ^{13}C NMR spectra. The yield of target products **3** and **4** in this reaction was near 80%; besides in the reaction mixture we have found 13^1-carboxamides **1** and **2** (11% and 8%, respectively). We did not find 13^1,15^2-dicarboxamides among the products of this reaction. Apparently, the exocyclic ring in 15^2-carboxamides **3** and **4** was more resistant toward nucleophilic opening, compared with that in methyl pheophorbide a **7**; this may be explained by different electron withdrawing effects of ester and amide groups.

These data showed that formation of either 13^1-carboxamide, or 15^2-carboxamide is dependent on reaction conditions. The use of nonvolatile amine, high temperature of reaction, and effect of β-carbonyl group in methyl pheophorbide a **7** promote formation of 15^2-carboxamide, which proceeds without exocyclic ring cleavage.[27] The prolong reaction in THF at a relatively low temperature (35–40 °C) is in favor to nucleophilic opening of exocyclic ring and formation of 13^1-carboxamide.[22–26]

Lipophilic conjugates of pyrophorbide **5** and **6** comprising 17^3-carboxamide function were obtained in high yield by substitution of pentafluorophenyl group in compound **8** for 3β (2-amino)ethoxycholest-5-ene and hexadecyl amine in warm THF in the presence of Et_3N (method C).[21] Both conjugates **1–6** were isolated as individual compounds according to TLC, their structures were completely characterized by HRMS, ^1H NMR, ^{13}C NMR and absorption spectra.

The absorption spectra of conjugates **3–6** in CH_2Cl_2 display occurrence of cyclopentanone ring. The main long wavelength maxima revealed at 668 nm, and slightly split Soret bands—at 414 nm (with shoulder at 398 nm). Protonated forms of these compounds (in the presence of trifluoroacetic acid), had long wavelength maxima near 652 nm and considerably more intensive Soret bands shifted to 419 nm. The long wavelength maxima in absorption spectra of compounds **1** and **2** in CH_2Cl_2 (660 nm) were usual for chlorin e6 derivatives, they exhibited hypsochromic shift to 633 nm when related copper complex was derived.[20,21] Additionally, formation of copper complex led to the considerable bathochromic shift of Soret band, this band was slightly split and revealed maxima at 398, 399 and 410 nm.[21]

^1H NMR spectra of compounds **2**, **4** and **6** demonstrated significant differences in orientation of their steroid backbones regarding to macrocycle. We observed extraordinary low values for chemical shifts for exocyclic methyl protons occupying β-region of sterol moiety in compounds **4** and **6**. Namely, cholesterol H-18 protons in compounds **4** and **6** exhibited chemical shifts values of 0.18 and 0.45 ppm, respectively, while in compound **2** those were in common range for cholesterol derivatives. In compound **4** H-6 resonance was strongly shifted (4.67 ppm instead of 5.33 ppm in other cholesterol derivatives).

2.2. Molecular modeling

We performed molecular modeling of synthesized conjugates **1–6**. The lowest energy conformers of these compounds calculated by simulated annealing are shown on Figure 1.

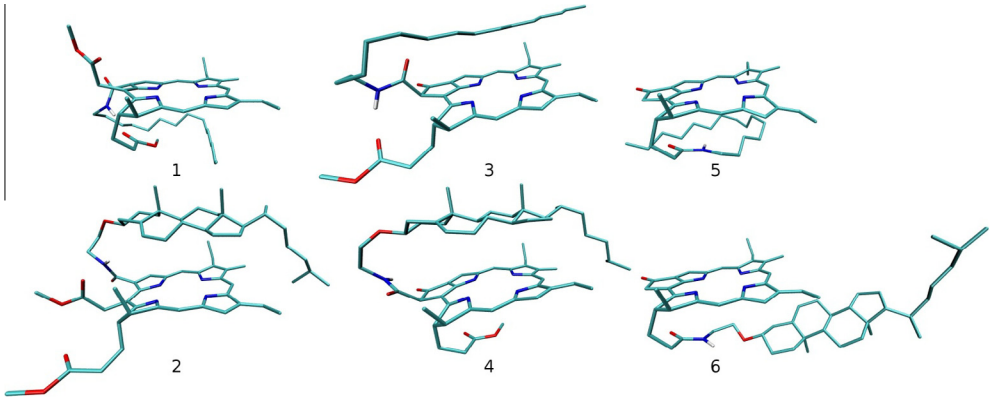

Figure 1. Calculated lowest energy conformers for 13^1(hexadecylcarbamoyl)chlorin (**1**); 13^1[(cholest-5-en)-3β-yloxyethoxycarbamoyl]chlorin (**2**); 15^2(hexadecylcarbamoyl)pheophorbide a (**3**); 15^2[(cholest-5-en)-3β-yloxyethoxycarbamoyl]pheophorbide a (**4**); 17^3(hexadecylcarbamoyl)pyropheophorbide a (**5**); 17^3[(cholest-5-en)-3β-yloxyethoxy-carbamoyl]pyropheophorbide a (**6**).

All these conjugates tend to fold lipophilic moiety over macrocylce; there also is a possibility of intermolecular hydrogen bond formation between amide group and ester group of second molecule of conjugate. Amide groups in conjugates **1–4** occupy off-plane position relative to plane of chloin ring. Abundance of folded conformation states and presence of out-of-plane ester groups must block ring-to-ring stacking of macrocycles in conjugates **1–4**. The steric interference of 17^3-substituents in conjugates **5** and **6** is much less compared with those in conjugates **1–4**. The absence of off-plane substituents in exocycle E in conjugates **5** and **6** renders at least one side of macrocycle open for possible ring-to-ring stacking. The intermolecular hydrogen bond also cannot be formed in these compounds making transition from folded to unfolded conformation easier, which exposes both sides of macrocyclic ring and making it freely available for stacking interaction.

Position of cholesterol 18- and 19-methyl groups in conjugates **2**, **4** and **6** also was evaluated from our conformational searches.

Among the low-energy conformers of compounds **4** and **6** (especially **4**) there are many states occupied with 18- and 19-methyls located close to ring plate, on the contrary, in conjugate **2** these upturned states were much less occupied (Fig. 2). This may explain the observed high field chemical shifts of corresponding resonances in ^{1}H NMR spectra of compounds **4** and **6**.

2.3. Preparation and properties of mixed micelles of PC with conjugates 1–6

Lipophilic conjugates (**1–6)** have affinity to phospholipids and may be solubilized in aqueous medium as mixed micelles with PC. We used the procedure which was developed earlier for preparation of stable micelles consisted of PC and cholesteryl esters or cholesteryl ethers.[28] Injection of mixed solution of PC and conjugate **1–6** in *iso*PrOH into aqueous buffer resulted in micelles, which size were dependent on the structure of conjugate and molar ratio conjugate/PC. For each of conjugate three types of micelles were prepared with the stoichiometric ratio conjugate/PC equal to 1:3; 1:5; 1:7 (mol/mol). Absorption spectra of obtained micelles are shown in Figure 3.

Spectra of all preparations displayed long wavelength maxima at 666–668 nm, that indicated more polar surrounding of chromophore in micelles compared with that in CH_2Cl_2 solution. The Soret band in spectra of conjugate/PC micelles exhibited hypsochromic shifts; the positions of related maxima being dependent on conjugate/PC ratio in micelles. Thus, the Soret band maximum in spectra of **3**/PC (1:3) revealed at 403 nm, while the spectrum of conjugate **3** in CH_2Cl_2 has related maximum at 411 nm (Fig. 3c).

Very characteristic spectral changes (additional bands with maxima near 710–712 nm) were observed for micelles containing conjugates **5** and **6** (Fig. 3e and f). This red shift was known to appear if macrocycle chromophores associate to form stacked structures.[29,30] The intensity of maxima at 710–712 nm was elevated with increasing of conjugate/PC ratio, the intensity of maxima at 668 nm was simultaneously lowered. The spectral changes in Soret band (the broadering of band and appearance of shoulder near 450 nm) also confirmed self association of chromophores in micelles contained conjugates **5** and **6**. Apparently conjugates **5** and **6** comprising lipophilic substituents in 17^3-position are able to associate within mixed micelles, while 13^1- and 15^2-substituted conjugates—cannot. These observations are in good agreement with published results,[29–31] as well as with our molecular modeling calculations. The ratios of absorption at 710–712 nm versus 668 nm, and absorption at 450 nm versus 417 nm indicates relative contents of associated and non associated chromophores and proves that self association of chromophores is more abundant in case of conjugate **5** comprising 17^3-hexadedecyl substituent in comparison with that of conjugate **6** comprising 17^3-cholest-5-en-3β-yloxyethyl-fragment.

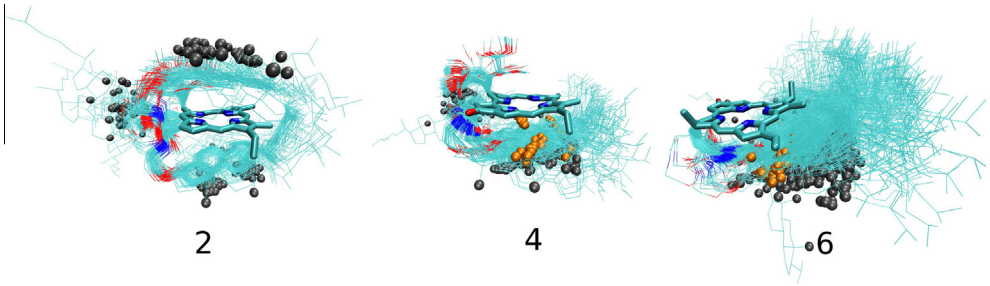

Figure 2. Calculated low energy conformers for conjugates **2**, **4** and **6** (cholesterol 18- and 19-methyl groups faced to macrocyle are shown as yellow balls; the same groups turned to the opposite site—as gray balls).

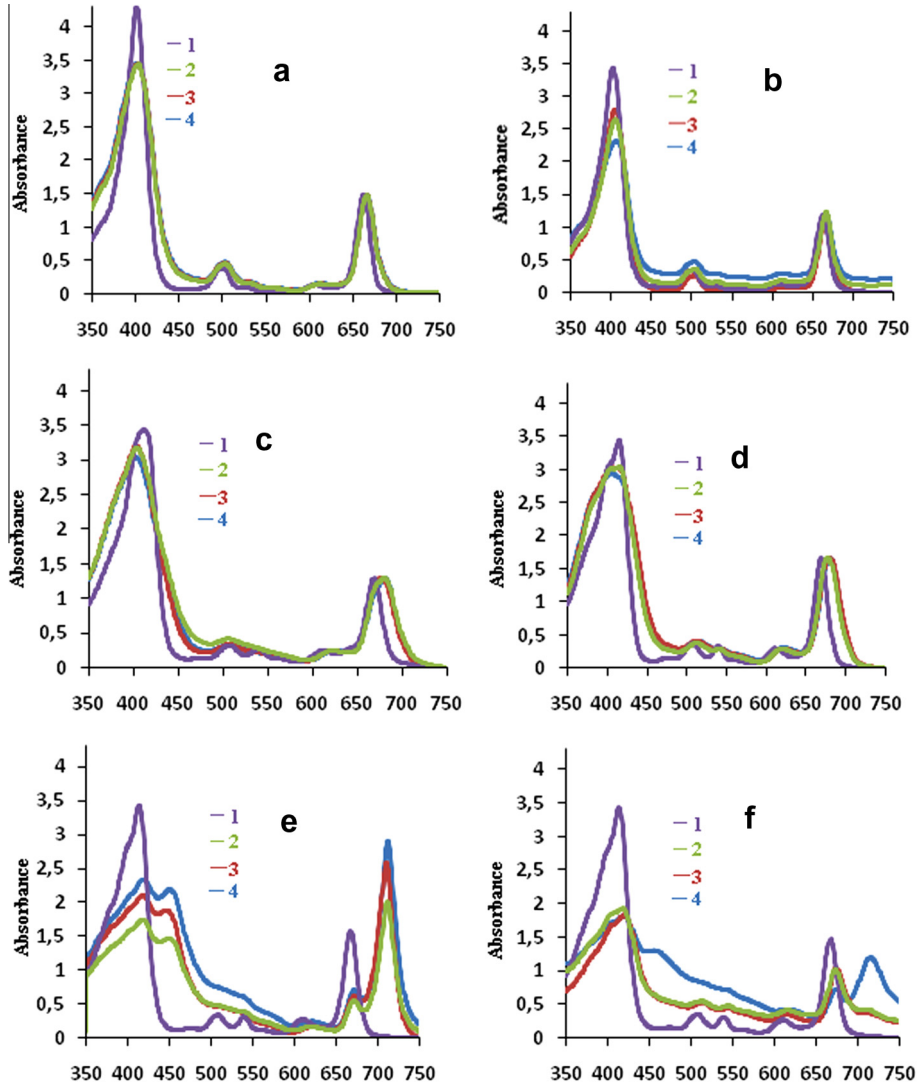

Figure 3. Normalized absorption spectra for mixed micelles of conjugates (**1–6**) with PC in PBS: (a) **1**:PC; (b) **2**:PC; (c) **3**:PC; (d) **4**:PC; (e) **5**:PC; (f) **6**:PC (for all patterns curve 1 corresponds to spectrum of pure conjugate in CH_2Cl_2; curves 2–4 correspond to mixed micelles with the ratio of conjugate to PC equal to 1:7 (mol/mol), 1:5 (mol/mol), and 1:3 (mol/mol), respectively.

These peculiarities may be explained in terms of different sterical effects of indicated substituents.

All obtained mixed micelles did not reveal any regular structures and were rather heterogeneous in sizes. Electron microphotography of mixed micelles conjugate **5**/PC (1:5) is shown in Figure 4; other preparations revealed close patterns.

Gel filtration of mixed micelles on Sepharose CL 4B indicated that mixed micelles were eluted as single peaks with constant conjugate/PC ratios. However, the contents of conjugates and PC in eluted peaks was about 50% for micelles with initial molar ratio conjugate/PC equal to 1:3 and about 70% for micelles with initial molar ratio conjugate/PC equal to 1:5. Obviously, high content of conjugate in micelle promotes its aggregation and causes irreversible sorption of aggregated micelles on sepharose. In case of mixed micelles with initial molar ratio conjugate/PC equal to 1:7, the yield of components after gel filtration was not less than 92%. The results of size measuring by laser scattering is shown in Figure 5.

2.4. Uptake of conjugates by human breast carcinoma MCF-7 cells

Mixed micelles of conjugates with PC (1:7) were evaluated for their potency to deliver conjugates to cultured cells. Human breast carcinoma MCF-7 cells were used as an appropriate model. Preliminary experiments demonstrated that conjugates (**1–6**) did not affect cell viability: we did not observe any toxic effects (according

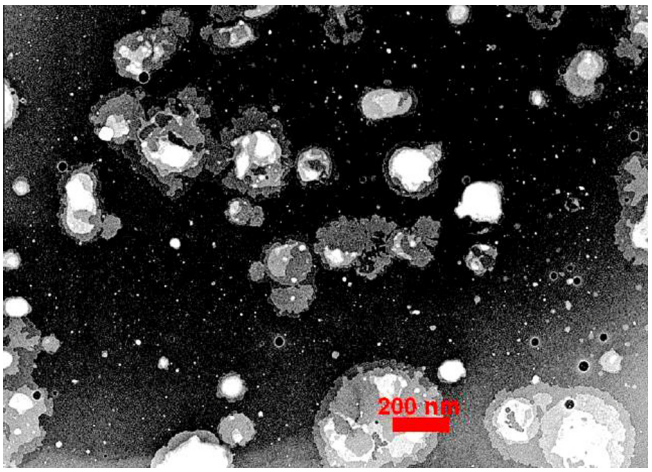

Figure 4. Electron microphotography (negative contrast) of mixed micelles of conjugate **5** with PC (components ratio was 1:5, mol/mol).

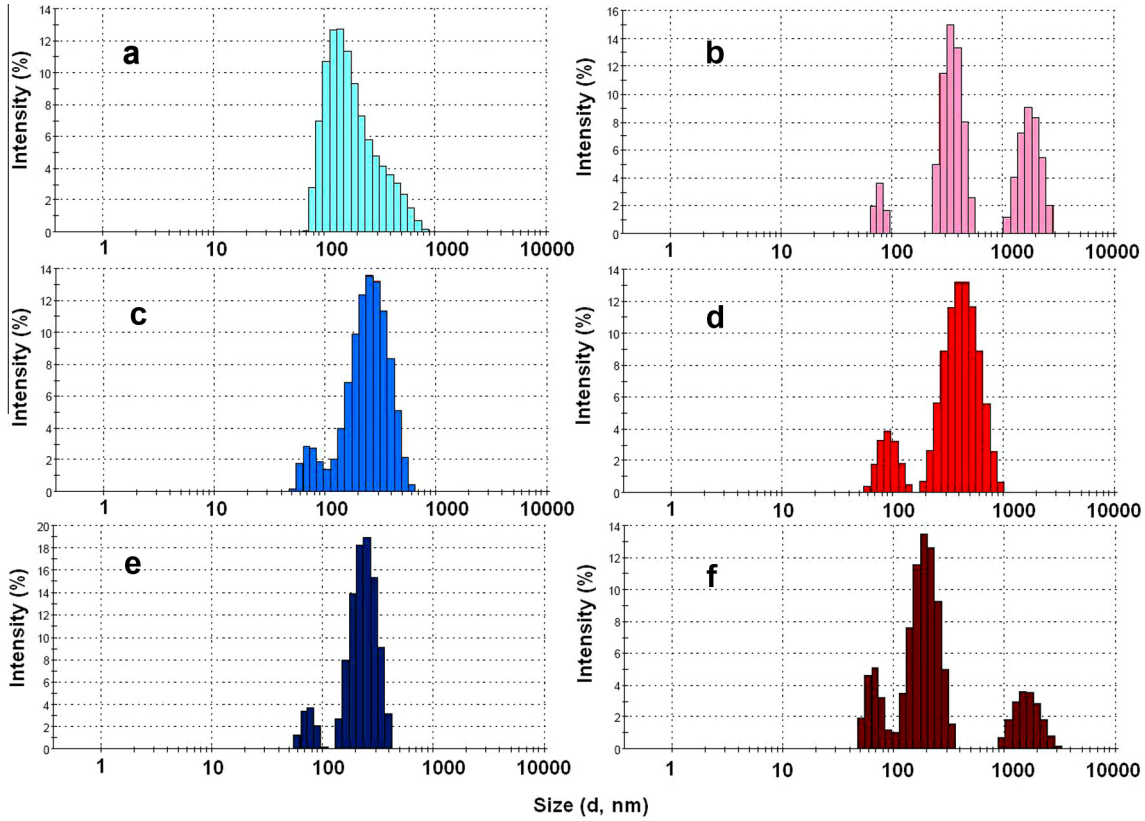

Figure 5. Particle size distribution for mixed micelles of conjugates (**1–6**) with PC measured by laser scattering (components ratio for all micelles were 1:7, mol/mol); (a) **1**:PC (average diameter—158,8 nm); (b) **2**:PC (average diameter—469.8 nm); (c) **3**:PC (average diameter—220.6 nm); (d) **4**:PC (average diameter—292.2 nm); (e) **5**:PC (average diameter—217.2 nm); (f) **6**:PC (average diameter—186.7 nm).

to MTT assay[32]) during prolong (48 h) incubation of MCF-7 cells with 30 μM conjugates incorporated in PC micelles.

The time course dependence of accumulation of conjugates by MCF-7 cells (at a concentration of conjugates **1–6** in culture medium equal to 10 μM) is shown in Figure 6.

All conjugates besides compound **5** bound to cells in a saturable manner. Under used conditions the binding was dependent on the structure of conjugates; the maximal binding was observed for conjugates **5** and **6** bearing lipophilic substituents in 17^3-position. We speculate that number of polar groups in conjugate molecule affect binding and internalization. Indeed, conjugates **5** and **6** comprise one carboxamide group and one carbonyl group; conjugates **3** and **4**—one carboxamide group, one ester group and one carbonyl group; conjugates **1** and **2**—one carboxamide group and two ester groups.

Photographs of MCF-7 cells labeled with conjugate **5** are shown in Figure 7. The fluorescent photograph (Fig. 7b) display rather diffuse distribution of fluorophore around dark nucleus.

The fact of internalization of conjugates by cells was also confirmed by independent experiments: MCF-7 cells, labeled with conjugates **1–6** for 20 h, as indicated above, were then incubated for 24 h in fresh medium, followed by determination of conjugates in incubation medium. We did not found any detectable amounts of conjugates in medium, that shows the absence of non specific binding and proves the internalization of conjugates by cells.

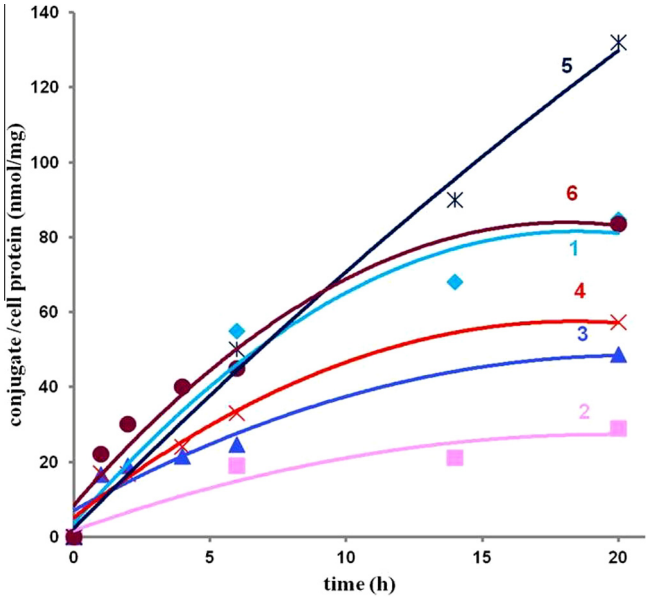

Figure 6. Time course uptake and internalization of conjugates (**1–6**) by MCF-7 cells at a concentration of each conjugate in culture medium equal to 10 μmol. Cells were incubated for indicated times with mixed micelles of conjugates (**1–6**)/PC (molar ratio was 1:7) followed by measuring of conjugates concentration within the cells as described in Section 3.

3. Experimental procedures

3.1. General methods and materials

Absorption spectra were registered with a 'Thermospectronic Helios α' spectrophotometer; ^{1}H NMR and ^{13}C NMR spectra—with an 'AMX-III' 400 MHz Bruker instrument in $CDCl_3$; high resolution mass spectra (HMRS)—with a Bruker 'Apex Ultra' FT ICR MS instrument at ion positive electro spray ionization mode; size distribution of mixed micelles was measured with Zetasizer Nano ZS

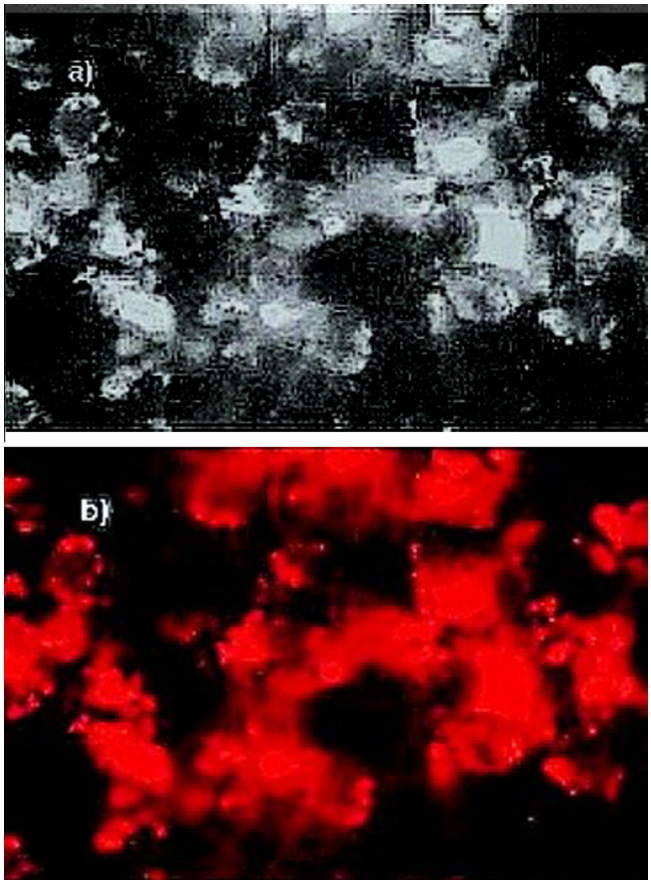

Figure 7. Subcellular localization of conjugate **5** in MCF-7 cells after 20 h incubation of cells with mixed micelles **5**:PC (1:7) at a concentration of conjugate in culture medium equal to 10 μmol: (a) phase contrast; (b) fluorescence.

(Malvern Instrument); electron microphotographs were obtained with JEOL 100 CX instrument; flash chromatography was performed on silica gel G (0.015–0.040 mm), analytical TLC—on 254-HPTLC silica gel plates, preparative TLC—on UV254-PTLC silica gel plates, both purchased from 'Merck'.

Reagents and solvents were obtained from 'Aldrich', 'Acros Organics' and 'Ekos-1 Ltd'. Egg yolk posphatidyl cholne (PC) was purchared from 'Sigma'; methyl pheophorbide a **7** was isolated from *Spirulina platensis* according to;[33] pentafluorophenyl pyropheophorbide a **8** was prepared from methyl pheophorbide a **7** by subsequent decarbmetoxylation, hydrolysis of obtaining methyl pyrophorbide, and transformation of resulting carboxylate to pentafluorophenyl ester by reaction with pentafluorophenyl trifluoroacetate according to reported methods[21,34] with minor modifications; hexadecyl amine was purchased from 'Acros Organics', 3β(2-amino)ethoxycholest-5-ene was synthesized according to .[21]

3.1.1. Synthesis of 13^1(hexadecylcarbamoyl)chlorin 1 and 13^1[(cholest-5-en)-3β-yloxyethoxycarbamoyl]chlorin 2 (method [A])

The mixture of methyl pheophorbide a **7** (60 mg, 0.1 mmol) and hexadecyl amine (or 3β(2-amino)ethoxycholest-5-ene, 0.55 mmol) in abs. THF (4 mL) was incubated at 40 °C until the formation of target product was complete according to TLC. Thereafter the mixture was evaporated to dryness, the product was isolated by flash chromatography in CH_2Cl_2 containing MeOH (1% v/v), and finally was purified by preparative TLC in the same system. Compound **1** (72 mg, 0.085 mmol, 86%) HRMS, calculated for $[C_{52}H_{74}N_5O_5]^+$: 848.5690; found: 848.5645; data of ^{1}H NMR, ^{13}C NMR and absorption spectra (Supplemented materials) were the same as was reported.[27] Compound **2** (96 mg, 0.09 mmol, 90%). HRMS, calculated for $[C_{67}H_{98}N_5O_6]^+$: 1068.7517; found: 1068.7444; data of ^{1}H NMR, ^{13}C NMR and absorption spectra (Supplemented materials) were the same as was reported.[20]

3.1.2. Synthesis of 15^2(hexadecylcarbamoyl)pheophorbide a 3 and 15^2[(cholest-5-en)-3β-yloxyethoxy-carbamoyl]pheophorbide a 4, (method [B])

The mixture of methyl pheophorbide a **7** (60 mg, 0.1 mmol) and hexadecyl amine (or 3β(2-amino)ethoxycholest-5-ene, 0.33 mmol) in dioxane (10 mL) was stirred under reflux for 6 h, until the formation of target product was complete according to TLC. Thereafter the mixture was evaporated to dryness, the product was isolated by flash chromatography in CH_2Cl_2 containing MeOH (1% v/v), and finally purified by preparative TLC in the same system. 15^2-(Hexadecylcarbamoyl)pheophorbide a **3** (65 mg, 0.08 mmol, 80%); HRMS, calculated for $[C_{51}H_{70}N_5O_4]^+$: 816.5428, found: 816.5445; data of ^{1}H NMR, ^{13}C NMR and absorption spectra (Supplemented materials) were the same as was reported.[27] 15^2[(Cholest-5-en)-3β-yloxyethoxy-carbamoyl]pheophorbide a **4** (81 mg, 0.08 mmol, 81%); MS, calculated for $[C_{64}H_{86}N_5O_5]^+$: 1004.6629, found: 1004.6613. ^{1}H NMR ($CDCl_3$): −1.61 (2H s, NH); 0.18 and 0.58 (each 3H, s, H-18 and H-19 in cholesterol moiety); 0.87 and 0.88 (each 3H, d, J = 6.6 Hz, H-26 and H-27 in cholesterol moiety), 0.94 (3H, d, J = 6.6 Hz, H-21 in cholesterol moiety); 1.70 (3H, t, J = 7.2 Hz, 18^2-H), 1.87 (3H, d, J = 7.3 Hz, 8^2-H); 3.23, 3.40, 3.54, 3.68 (each 3H, s, 2-, 7-, 12- and OCH_3); 3.54 (1H, m, H-3 in cholesterol moiety); 3.72 (4H, m, NCH_2CH_2O and NCH_2CH_2O); 4.38–4.50 (2H, m, 8^1-H and 17^1-H); 4.67 (m, 1H, H-6 in cholesterol moiety); 5.33 (br 1H, NHCO); 6.17 (1H, dd, J = 11.6, 1.5 Hz, 3^2-H, *trans*), 6.29 (1H, dd, J = 17.8, 1.5 Hz, 3^2-H, *cis*); 7.98 (1H, dd, J = 11.6, 17.8 Hz, 3^1-H); 8.56, 9.38, 9.51 (each 1H, s, 5-, 10-, 20-H). ^{13}C NMR ($CDCl_3$): 11.34; 11.85; 12.18; 12.21; 17.49, 18.56, 18.85; 19.58; 20.73; 22.67; 22.91; 23.21; 23.96; 24.34; 27.95; 28.13; 28.32; 29.79; 30.08; 31.14; 31.65; 32.01; 35.90; 36.26; 36.48; 37.14; 38.59; 39.66; 39.45; 40.53; 42.32; 49.76; 50.29; 51.35; 51.72; 56.27; 56.65; 65.92; 66.66; 68.28; 79.22; 93.31; 104.38; 105.58; 121.22; 122.78; 128.74; 129.22; 130.97; 136.15; 136.28; 136.71; 138.08; 140.03; 142.15; 145.30; 149.70; 150.98; 155.82; 162.62; 167.74; 172.43; 173.59; 191.71. Absorption spectra in CH_2Cl_2, λ_{max}, nm (ε): 415 (74,500); 508 (7700); 540 (7000); 611 (5300); 669 (35,900).

3.1.3. Synthesis of 17^3(hexadecylcarbamoyl)pyropheophorbide a 5 and 17^3[(cholest-5-en)-3β-yloxyethoxy-carbamoyl]pyropheophorbide a 6 (method [C])

The 1 M solution of Et_3N in abs. THF (100 μL) was added to the solution of pentafluorophenyl pheophorbide a **8** (70 mg, 0.1 mmol) and hexadecyl amine (or 3β(2-amino)ethoxycholest-5-ene, 0.33 mmol) in 6 mL of abs. THF, and the mixture was stirred for 1 h at 40 °C, until the formation of target product was complete according to TLC and absorption spectrum. Thereafter the mixture was evaporated to dryness, the product was isolated by flash chromatography in CH_2Cl_2 containing MeOH (2% v/v), and finally was purified by preparative TLC in the same system. Compound **5** (60 mg, 0.08 mmol, 80%). HRMS, calculated for $[C_{49}H_{68}N_5O_2]^+$: 758.5373, found: 758.5399. ^{1}H NMR ($CDCl_3$): −1.60 (2H br, NH); 0.86 (3H, as t, CH_3 in hexadecyl moety), 1.25 (br, CH_2 in hexadecyl moety); 1.68 (3H, t, J = 7.2 Hz, 18-H), 1.79 (3H, d, J = 7.2 Hz, 8^2-H); 3.23, 3.40, 3.58 (each 3H, s, 2-, 7-, and 12-CH_3); 4.34 (1H, qd, J = 7.2, 2.0 Hz, 8^1-H); 4.51 (dt, J = 8.7, 2.0 Hz, 17^1-H); 6.16 (1H, dd, J = 11.6, 1.5 Hz, 3^2-H, *trans*); 6.28 (1H, dd, J = 17.8, 1.5 Hz, 3^2-H, *cis*); 7.99 (1H, dd, J = 11.6, 17.8 Hz, 3^1-H); 8.54, 9.38, 9.43 (each 1H, s, 5-, 10-, 20-H). ^{13}C NMR ($CDCl_3$): 11.34; 12.20; 14.18; 14.49; 17.38;

18.53; 19.62; 22.76; 23.09; 23.32; 23.88; 24.58; 24.90; 26.80; 27.12; 29.19; 29.44; 29.50; 29.73; 30.01; 30.49; 30.81; 30.97; 32.62; 32.87; 37.14; 48.21; 50.13; 51.91, 52.07; 53.48; 58.56; 93.51; 97.32; 104.07; 122.90; 129.25; 129.91; 130.94; 131.95; 136.04; 136.46; 138.02; 141.86; 144.87; 149.48; 154.96; 172.07; 196.02. Absorption spectra in CH_2Cl_2, λ_{max}, nm (ε): 414 (78,800); 508 (7500); 539 (5500); 611 (5100); 668 (36,000). Compound **6** (85 mg, 0.09 mmol, 90%). HRMS, calculated for $[C_{62}H_{84}N_5O_3]^+$: 946.6496; found: 946.6500; data of ^{1}H NMR, ^{13}C NMR and absorption spectra (Supplemented materials) were the same as was reported.[21]

3.2. Molecular modeling

The OpenBabel package has been employed for calculations with MMFF94 (Merck) force field parameters.[35–43] In order to find low-energy conformations of compounds, we performed molecular dynamics (MD) simulations, which were performed with NAMD software. Parameters and topology files were generated with the aid of SwissParam server which uses the same Merck force field. The annealing protocol has been implemented to find low energy conformes, with 4 ps high temperature run at 500 K followed by 4 ps cooling down to 50 K. Total of 200 annealing cycles were scheduled in 24 processes, yielding 4800 conformers for each compound. The resulting structures were optimized by energy minimization with MMFF94 potential. The VMD package was utilized for all the further post-processing, analysis, and visualization of these structures.

3.3. Preparation and characterization of mixed micelles PC with conjugates (1–6)

Calculated volumes of 10^{-2} M solutions of PC and conjugate (**1–6**) in chloroform were mixed together to obtain mixed solution conjugate/PC with ratios 1:3, 1:5, and 1:7 (mol/mol). Mixed solutions were evaporated to dryness, and dissolved in *iso*PrOH at 60 °C to obtain solutions with concentrations of conjugates (**1–6**) equal to 5×10^{-2} M. Aliquotes of heated isopropanolic solutions (100 μL) were injected into 5 ml of water at 60 °C during wortexing to obtain stock micellar solution of conjugates (**1–6**) with PC containing aforementioned conjugates in concentration of 10^{-3} M and PC in concentration of 3×10^{-3} M; 5×10^{-3} M, and 7×10^{-3} M, respectively. Stock solutions were diluted with 10 volumes of appropriate buffer (phosphate buffered saline for measuring absorption spectra, and particle sizes by laser scattering; 10^{-3} M Tris–HCl buffer (pH 7.4), containing 0.14 M NaCl for gel filtration experiments and stoichiometic compositions determination; 10^{-5} M NH_4HCO_3 solution for electron microscopy). Gel filtration was performed on a 1.6×100 cm (LKB) column with CL 4B Sepharose using 10^{-3} M Tris–HCl buffer (pH 7.4), containing 0.14 M NaCl for elution. Gel filtration was performed at a rate of 12 mL/h, 1 mL fractions were collected and used for quantitative determination of PC content according to method[44] and conjugates (**1–6**) from absorption spectra. All determinations were carried out in triplicates.

3.4. Uptake and internalization of conjugates (1–6) by MCF-7 cells

For the experiments mixed micelles of conjugates (**1–6**) and PC with molar ratio conjugate/PC equal to 1:7 (12.5 mol % of each conjugate) were used. MCF-7 cells in a 12-well plates were incubated for 1, 2, 4, 6, 14 and 20 h with mixed micelles, then medium was aspirated, cells were washed with cold PBS at 4 °C, and lipids from each well were extracted with mixture hexane-*iso*PrOH (3:2) (3×0.5 mL), the cell pellets were used for measuring of cell protein concentrations.[45] Lipid extracts were dried under nitrogen flow, residues were dissolved in CH_2Cl_2 (2 mL) and the concentration of conjugates (**1–6**) were determined from absorption spectra. All measurements were carried out in triplicates. The efficiency of cell labeling was expressed in terms of ratios of internalized conjugates (nmol/1 mg of cell protein).

4. Conclusions

The data presented above reveal that modification of chlorin macrocycle with lipophilic amines is very simple procedure allowing to obtain regioisomeric 13^1-, 15^2- 17^3-lipophilic conjugates in high yields. These lipophilic conjugates may be solubilized in aqueous medium as mixed micelles with phospholipids. Mixed micelles containing of lipophilic conjugates may be used for delivery of photosensitizers to cells. Investigation of photo induced toxicity of aforementioned conjugates in cultured cells are in progress in our team. We hope that this approach may be used also in vivo, since incorporation of drugs into amphiphilic lipid complexes is known to provide their preferential uptake by carcinoma cell line over normal cells.[46]

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Supplementary data

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