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Bacteriochlorophyll *a* Derivatives with Sulfur–Containing Amino Acids as Promising Photosensitizers for Cancer PDT

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Dedicated to Professor Oleg A. Golubchikov on the occasion of his 70-th birthday

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In present work sulfur amino acid-containing bacteriochlorophyll a residues were obtained. It has been found that the oxidation of free thiol groups in cysteine-containing bacteriopurpurinimide occurs under the action of atmospheric oxygen with the disulfide form formation. In cells this disulfide derivative undergoes monomerization with the participation of reduced form of glutathione. Oxidation of the methionine residue in the conjugate with dipropoxybacteriopurpurinimide results in the stable sulfoxide form. Mechanism for suppressing the antioxidant system of a cell under the action of sulfur-containing bacteriochlorophyll a derivatives is proposed.

Keywords: Sulfur-containing amino acids, glutathione, bacteriopurpurinimide derivatives, photosensitizers, photodynamic therapy of cancer.

Производные бактериохлорофилла *a* с серосодержащими аминокислотами в качестве перспективных фотосенсибилизаторов для ФДТ рака

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В настоящей работе впервые получены производные бактериохлорофилла а с остатками серосодержащих аминокислот. Установлено, что под действием кислорода воздуха происходит окисление свободных тиольных групп в цистеинсодержащем бактериопурпуринимиде, и образуется дисульфид, который в клетке претерпевает мономеризацию при участии восстановленной формы глутатиона. В случае окисления остатка метионина в составе конъюгата с дипропоксибактериопурпуринимидом образуется стабильный сульфоксид. В работе предложен механизм подавления антиоксидантной системы в опухолевых клетках под действием серосодержащих производных бактериохлорофилла а.

Ключевые слова: Серосодержащие аминокислоты, глутатион, производные бактериопурпуринимида, фотосенсибилизаторы, фотодинамическая терапия рака.

Introduction

Plenty of sulfur-containing compounds, including sulfur-containing amino acids, are abundantly used in medical chemistry in the development of new pharmaceuticals.[1-3] Cysteine, cystine and methionine are potent antioxidants that react with reactive forms of oxygen (ROS) and nitrogen (RNS) in cells, which present a hazard to the organism for their ability to damage proteins containing iron-sulfur clusters, such as aconitase, succinate dehydrogenase, NADH-ubiquinone oxidoreductase, *etc.*

The balance between the generation and elimination of the above-mentioned highly reactive units is important for the normal functioning of the organism. The effect of ROS/RNS on cysteine was previously thought to be a negative effect of oxidative stress leading to the destruction of proteins. However, it has now been found that oxidation of cysteine is a reversible and biochemically important process.[4]

In addition, oxidative stress forms the basis of some methods of antitumor therapy. One of such methods is photodynamic therapy (PDT), which consists in activation of intracellular oxygen under the action of a photosensitizer administered into the body and laser irradiation of the corresponding wavelength. The result of photochemical reactions is the generation of ROS acting on tumor cells and causing oxidative stress and death.

Recently, as a drug for PDT, special attention is attracted to photosensitizers (PS) based on natural chlorins, bacteriochlorins and their derivatives, which have low dark toxicity, are subjected to biodegradation and are rapidly eliminated from the body. All of them are characterized by intense absorption in the red and near infrared (IR) area of the spectrum (650–830 nm), where the tissue permeability for light is maximal, which allows to influence deep-lying tumors and pigmented tumors.[5-7]

Introduction of amino acids to photosensitizers allows, on the one hand, to increase the hydrophilicity of the pigment, and on the other hand, to improve the selectivity of accumulation by tumor tissues due to the involvement of amino acids in numerous intracellular processes, especially in rapidly proliferating cancer cells. In clinical practice, an officinal preparation is applied as a chlorin e_6 conjugate with aspartic acid (mono *L*-aspartylchlorin e_6, Talaporfin, NPe_6, MACE).[8,9]

This drug is PS of the second generation and used in the treatment of various forms of cancer. Due to its pharmacodynamic parameters, tumor growth inhibition (TGI), increase in life expectancy and elimination rate from the body, this drug exceeds Photofrin® which is widely used in clinical practice.[10-13]

The addition of sulfur-containing units to the chlorin macrocycle changes pharmacokinetics, biodistribution in organs and tissues and increases the photodynamic efficiency of PS. For chlorin e_6 and pheophorbide *a*, being the chlorophyll *a* derivatives, it was shown that introduction of cysteine and glutathione residues on the periphery of the macrocycle increases photoinduced cytotoxicity of PS with respect to HepG2 tumor cells, which in turn is associated with an increase in the quantum yield of singlet oxygen.[14]

Recently, we synthesized a variety of bacteriochlorophyll *a* derivatives with sulfur-containing compounds, including cysteamine, cystamine, lipoic acid. The presence of thiol (-SH) and disulfide groups (-S-S-) allows the incorporation of modified bacteriochlorins into various multifunctional platforms containing chemotherapeutic, diagnostic modules, as well as vector molecules for PS targeted transport. Lipoic acid contains a disulfide fragment in its structure, through which bacteriopurpurinimide has been immobilized on the gold nanoparticles (NP) surface. Such nanostructuring of the PS provides it's non-specific targeting in the tumor tissue, increases the selectivity of accumulation and, as a result, the photodynamic efficiency.[15]

In another work, we used aminoethanethiol (cysteamine) as a sulfur-containing agent, which is applied as a radioprotector and as a drug for the cystinosis treatment.[16] A conjugate with 2-mercaptoethylamine (cysteamine) was synthesized on the basis of the bacteriochlorophyll *a*-*O*-propyloxime-*N*-propoxybacteriourpurinimide derivative (DPBPI), which, being oxidized in air, was converted to the dimer with a disulfide bond (disulfo-BPI). It has been shown that in the presence of glutathione (GSH), a rapid reduction of the dimer's disulfide bond takes place, which suggested a mechanism for the cytotoxic effect of the latter, including a reduction of the reduced form of GSH in the tumor cells and a decrease of their resistance to oxidative stress. High levels of intracellular accumulation and singlet oxygen generation in experiments on S37 sarcoma culture cells provided photodynamic efficacy of disulfo-BPI at nanomolar pigment concentrations (IC50 = 0.05±0.005 μM). The kinetics of accumulation of disulfo-BPI in the tumor showed that the PS quickly enters the tumor tissue of animals and retains at a sufficiently high level for two hours, and then is eliminated from the body in 24 hours. Experiments to study the photodynamic efficiency of disulfo-BPI performed in mice with sarcoma 37 and rats with M-1 sarcoma showed a 100 % regression of the tumor at a PS dose of 5 mg/kg and light doses of 150–300 J/cm^2.[17]

The morphological studies of sarcoma M-1 in rats after PDT exposure with this pigment showed that the photoinduced antitumor effect is due to the destruction of M-1 sarcoma vasculature, rapid inhibition of proliferative activity and devitalization of tumor cells by apoptosis and necrosis.[18]

One of the problems in cancer treatment is tumor resistance caused by the establishment of reductive-oxidative equilibrium in response to the prooxidant action, which forms the basis of antitumor chemotherapy, radiation therapy and PDT.[19] For example, in case of PDT, an increase in the antioxidant response of the tumor is possible leading to a decrease in the ROS level in the cells of the neoplasms and, as a consequence, to a decrease in PDT efficiency.

Sulfur-containing amino acids in the composition of PS can increase the yield of ROS by inactivation of intracellular glutathione, which leads to an increase in PS cytotoxicity.

In the second half of the 20th century, changes in methionine metabolism in tumor cells of certain lines were found, which makes them more sensitive to the presence of this amino acid in the growth medium.[20,21]

It is believed that the dependence of tumor cells on methionine can be associated with a damage of one or more genes that encode enzymes involved in numerous metabolic pathways of methionine. Cancer cells with similar

defects are unable to regenerate methionine, and also use it to synthesize some necessary metabolites. At the same time, normal cells don't exhibit methionine deficiency under similar conditions.[22]

In this study, we proposed the synthesis of bacteriopurpurinimide with methyl esters of cysteine, cystine, methionine, and their chemical stability, as well as stability under physiological conditions in the presence of glutathione, to model the conditions for the functioning of these PSs within tumor cells.

Experimental

NMR spectra were registered using Bruker Avance 300 (Germany) with a frequency of 300 MHz. The calibration of the scale was carried out by the signals of the residual ^{1}H nuclei. MALDI mass spectra were registered on a Bruker Ultraflex mass spectrometer with DHB matrix (Germany). UV-Vis absorption spectra were obtained on a Ultrospec 2100 Pro UV-Vis spectrophotometer (GE Healthcare, USA) in CH_2Cl_2 using a standard 10 mm quartz cell.

For analytical TLC, aluminum plates ALUGRAM Xtra SIL G/UV254 (Macherey-Nagel, Germany), coated with silica gel 60 (0.2 mm) were used. For preparative chromatography in a thin layer, glass plates 20×20 cm, coated with silica gel 60 F254 (Merck, Germany) were utilized.

The following reagents were used: cysteine methyl ester (*L*-Cysteine methyl ester hydrochloride, "Sigma-Aldrich", Japan), cystine methyl ester (*L*-Cystine dimethyl ester dihydrochloride, "Sigma-Aldrich", Japan), methionine methyl ester *L*-Methionine methyl ester hydrochloride (Abcr GmbH, Germany), glutathione (reduced) (*L*-Glutathione reduced, "Sigma-Aldrich"), as well as reagents and solvents of domestic production. The solvents were purified and prepared according to standard procedures.

*Synthesis of Cys-DPBPI (**4**).* DPBPI (**3**) (15 mg, 21.5 μmol) and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (8 mg, 32.3 μmol) were dissolved in CH_2Cl_2 (1 ml) with stirring. After 15 minutes, cysteine methyl ester hydrochloride (7.38 mg, 43.0 μmol) was added to the reaction mass. The reaction was carried out for 24 hours under argon atmosphere, in the dark with stirring. The product (**4**) was isolated by extraction with CH_2Cl_2 (350 ml) and with water (500 ml), dried with Na_2SO_4 and purified using preparative TLC (CH_2Cl_2/CH_3OH, 45/1, v/v). The yield of the desired compound (**4**) was 5.7 mg (38 %). UV-Vis λ_{max} nm (relative intensities of peaks): 368, 420, 544, 798 (1:0.51:0.39:0.43). *m/z* (MALDI MS) calculated for $C_{43}H_{56}N_7O_7S$ [M+H]: 814.01, found.: 814.25 [M+H]. ^{1}H NMR (300 MHz, DMSO-d_6) δ_H ppm: 8.77 (H, s, 5-H), 8.68 (H, s, 10-H), 8.64 (H, s, 20-H), 8.15 (H, *J*=8.20 d, 17^3-NH), 7.13 (H, m, SH), 5.30 (H, m, 17-H), 4.33 (4H, m, -O*CH*$_2$CH$_2$CH$_3$), 4.20 (2H, m, 7-H, 18-H), 4.09 (H, m, 8-H), 3.98 (3H, s, 12-CH_3), 3.56 (4H, m, CHCH$_2$SH), 3.34 (3H, s, 2-CH_3), 2.70 (3H, s, 3^2-CH_3), 2.56 (H, m, 17^2-CH_2), 2.45 (3H, m, 8^1-CH_2, 17^1-CH_2, 17^2-CH_2), 2.15 (2H, m, 8^1-CH_2, 17^1-CH_2), 1.91 (3H, *J*=7.24 Hz d, 7-CH_3), 1.62 (9H, m, 18-CH_3, -OCH$_2$CH$_2$*CH*$_3$), 1.25 (4H, m, -OCH$_2$*CH*$_2$CH$_3$), 1.24 (3H, m, 8^2-CH_3), 0.11 (s, NH), -0.22 (s, NH).

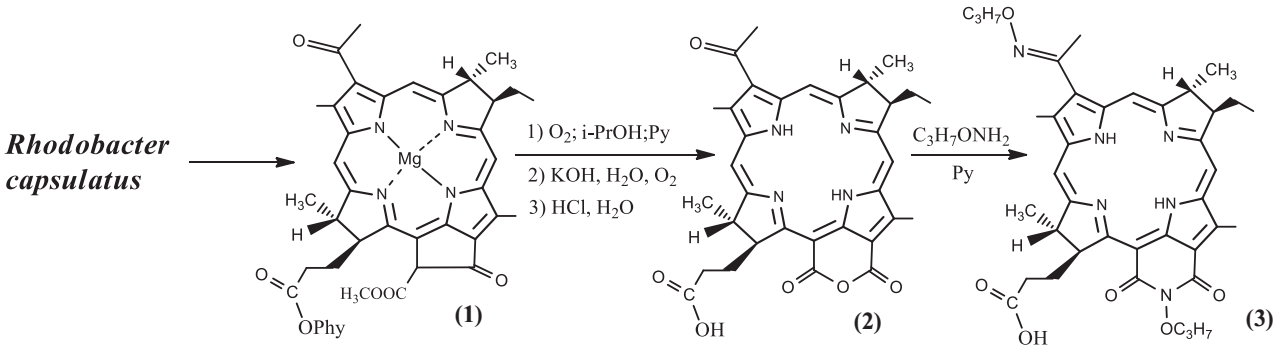
*Synthesis of Cys-Cys-DPBPI (**5**).* DPBPI (**3**) (15 mg, 21.5 μmol) and EEDQ (8 mg, 32.3 μmol) were dissolved in CH_2Cl_2 (1 ml) with stirring. After 15 minutes, cystine methyl ester hydrochloride (22.01 mg, 64.5 μmol) was added to the reaction mass. The reaction was carried out for 24 hours under argon atmosphere, in the dark with stirring. The product (**5**) was isolated by extraction with CH_2Cl_2 (350 ml) and with water (500 ml), dried with Na_2SO_4 and purified using preparative TLC (CH_2Cl_2/CH_3OH, 35/1, v/v). The yield of the desired compound (**5**) was 9.9 mg (66 %). UV-Vis λ_{max} nm (relative intensities of peaks): 368, 420, 544, 798 (1:0.50:0.37:0.44). *m/z* (MALDI MS) calculated for $C_{86}H_{108}N_{14}O_{14}S_2$ [M+H]: 1625.99, found: 1626.47 [M+H]. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: 8.63 (s, H, 10-H), 8.56 (s, H, 5-H), 8.38 (s, H, 20-H), 7.26 (d, *J*=8.15 H, 17^3-NH), 4.61 (m, O-CH$_2$-), 4.25 (m, 18-H, 7-H), 4.12 (m, 8-H), 3.68 (s, 12-CH_3), 3.53-3.46 (m, 4H, -CHCH$_2$S-), 3.50 (s, 17^5-CH_3), 3.53 (s, 2-CH_3), 3,27 (s, 3^2-CH_3), 2.77 (s, 17^7 -OMe), 2.75 (m, 8^1-CH$_2$), 2.73-2.66 (m, 17^7-CH$_2$S-), 2.40 (m, 17^1-H, 8^1-H), 1.83 (d, *J*=7.31, 7-CH_3), 1.70 (m, 18-CH_3), 1.12 (3H, m, 8^2–CH_3), 0.13 (s, NH), –0.18 (s, NH).

*Synthesis of Met-DPBPI (**6**).* DPBPI (**3**) (30 mg, 43 μmol) and EEDQ (16 mg, 64.6 μmol) were dissolved in CH_2Cl_2 (1 ml) with stirring. After 15 minutes, methionine methyl ester hydrochloride (17.2 mg, 86 μmol) was added to the reaction mass. The reaction was carried out for 24 hours under argon atmosphere, in the dark with stirring. The product (**6**) was isolated by extraction with CH_2Cl_2 (350 ml) and with water (500 ml), dried with Na_2SO_4 and purified using preparative TLC (CH_2Cl_2/CH_3OH, 45/1, v/v). The yield of the desired compound (**6**) was 16.8 mg (56 %). UV-Vis λ_{max} nm (relative intensities of peaks): 368, 420, 544, 798 (1:0.48:0.38:0.45). *m/z* MALDI MS calculated for $C_{45}H_{59}N_7O_7S$ [M+H]: 842.06, found: 842.38 [M+H]. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: 8.62 (s, H, 5-H), 8.55 (s, H, 10-H), 8.38 (s, H, 20-H), 7.05 (d, *J*=7.89, H, 17^3-NH), 5.14 (m, H, 17-H), 4.78 (m, H, -*CH*CH$_2$CH$_2$SCH$_3$), 4.56 (m, 4H, -OCH$_2$*CH*$_2$CH$_3$), 4.22 (m, 2H, 18-H, 7-H), 4.01 (m, H, 8-H), 3.70 (s, 3H, -OCH$_3$ Met), 3.63 (s, 3H, 12-CH_3), 3.29 (s, 3H, 2-CH_3), 2.74 (s, 3H, 3^2-CH_3), 2.67 (m, H, 17^2-CH_2), 2.58 (m, 2H, -CHCH$_2$*CH*$_2$SCH$_3$), 2.38 (m, 3H, 8^1-CH_2, 17^1-CH_2, 17^2-CH_2), 2.18 (m, 2H, -CH*CH*$_2$CH$_2$SCH$_3$), 2.10 (s, 3H, -S-CH_3), 2.04 (m, 2H, 8^1-CH_2, 17^1-CH_2), 1.68 (d, *J*=7.19 3H, 7-CH_3), 1.61 (m, 9H, -OCH$_2$CH$_2$*CH*$_3$, 18-CH_3), 1.12 (m, 3H, 8^2-CH_3), 0.15 (s, H, NH), –0.15 (s, H, NH).

*Synthesis of Met(SO)-DPBPI (**7**).* To the DPBPI conjugate with methionine methyl ester (**6**) (10 mg, 11.87 μmol) 30 % aq. solution of H_2O_2 (0.5 ml) was added. The reaction proceeded in the dark for 30 minutes. The product (**7**) was isolated by extraction with CH_2Cl_2 (350 ml) with water (500 ml), dried on Na_2SO_4. Compound was purified by preparative TLC (CH_2Cl_2/CH_3OH, 30/1, v/v). The yield of the compound (**7**) was 8.5 mg (85 %). UV-Vis λ_{max} nm (relative intensities of peaks): 368, 420, 544, 798 (1:0.49:0.37:0.46). *m/z* (MALDI MS) calculated for $C_{45}H_{59}N_7O_8S$ [M+H]: 858.06, found: 858.72 [M+H]. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: 8.62 (s, 5-H), 8.55 (s, 10-H), 8.38 (s, H, 20-H), 7.23 (d, *J*=8.07 17^4-NH), 5.14 (m, 1H, 17-H), 4.78 (m, H, -*CH*CH$_2$CH$_2$SCH$_3$), 4.56 (m, 4H, -OCH$_2$CH$_2$CH$_3$), 4.22 (m, 2H, 18-H, 7-H), 4.01 (m, 1H, 8-H), 3.70 (s, 3H, -OCH$_3$ Met), 3.63 (s, 3H, 12-CH_3), 3.29 (s, 3H, 2-CH_3), 2.74 (s, 3H, 3^2-CH_3), 2.67 (m, 1H, 17^2-CH_2), 2.58 (m, 2H, -CHCH$_2$*CH*$_2$S(O)CH$_3$), 2.38 (m, 3H, 8^1-CH_2, 17^1-CH_2, 17^2-CH_2), 2.33 (m, 2H, -CH*CH*$_2$CH$_2$SCH$_3$), 2.18 (s, 3H, -S-CH_3), 2.04 (m, 2H, 8^1-CH_2, 17^1-CH_2), 1.68 (d, *J*=7.26 3H, 7-CH_3), 1.61 (m, 9H, -OCH$_2$CH$_2$CH$_3$, 18-CH_3), 1.12 (m, 3H, 8^2-CH_3), 0.12 (s, 1H, NH), –0.12 (s, 1H, NH).

Results and Discussion

As a starting compound, it was used natural bacteriochlorophyll *a* (Bcl *a*) (**1**) which was obtained from the Rhodobacter capsulatus bacteria biomass by extraction with isopropyl alcohol. The long-wavelength maximum of the pigment absorption is in the region of 770 nm. Synthesis of bacteriopurpurin (**2**) – a bacteriochlorophyll *a* derivative containing an anhydride exocycle – was carried out by oxidizing the latter with air oxygen under alkaline conditions. The reaction was monitored spectrophotometrically from the shift of the absorption band to the 812 nm region. The resulting bacteriopurpurin is a labile compound because of the presence of an anhydride cycle, ready to the open-



Scheme 1. Synthesis of *O*-propyloxime-*N*-propoxybacteriopurpurinimide (DPBPI).

ing in alkaline medium. Therefore, *O*-propyloxime-*N*-propoxybacteriopurpurinimide (DPBPI) (**3**), a promising photosensitizer of high stability, having an absorption band at 800 nm and showing significant photoinduced activity on tumor cells and on tumors of various genes in animals, was proposed as a leading compound in this research (Scheme 1).[23,24]

Based on DPBPI (**3**), its derivatives with methyl esters of cysteine (*Cys*-DPBPI, **4**), cystine (*Cys*-*Cys*-DPBPI, **5**), methionine (Met-DPBPI, **6**) and methionine sulfoxide (MetSO-DPBPI, **7**) were synthesized (Scheme 2).

The absorption spectra of all the sulfur-containing compounds obtained at the same concentration had an absorption band of Q_2 of the same intensity, except for compound **5** (Figure 1).

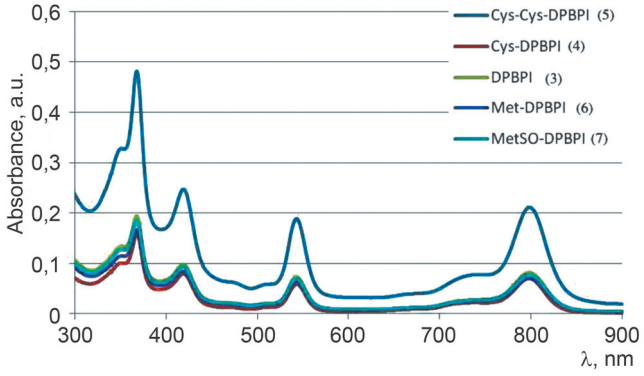
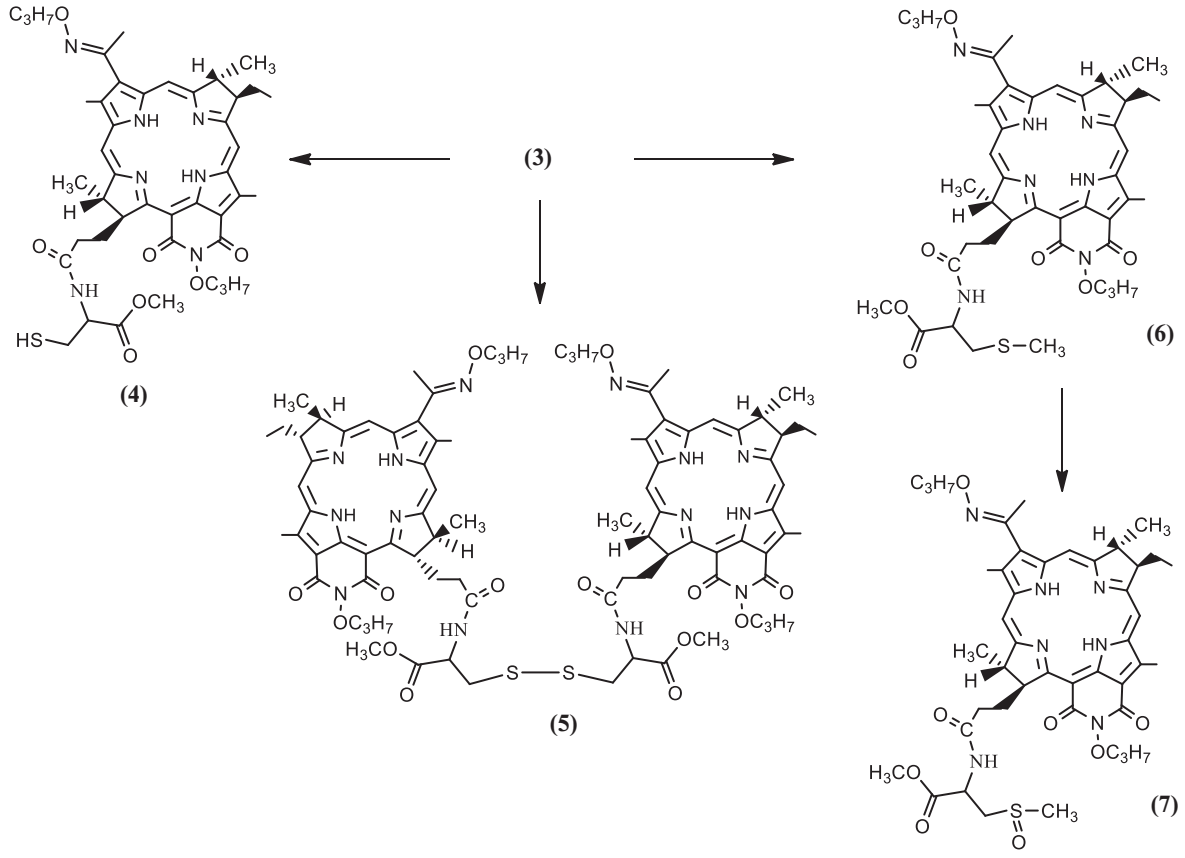
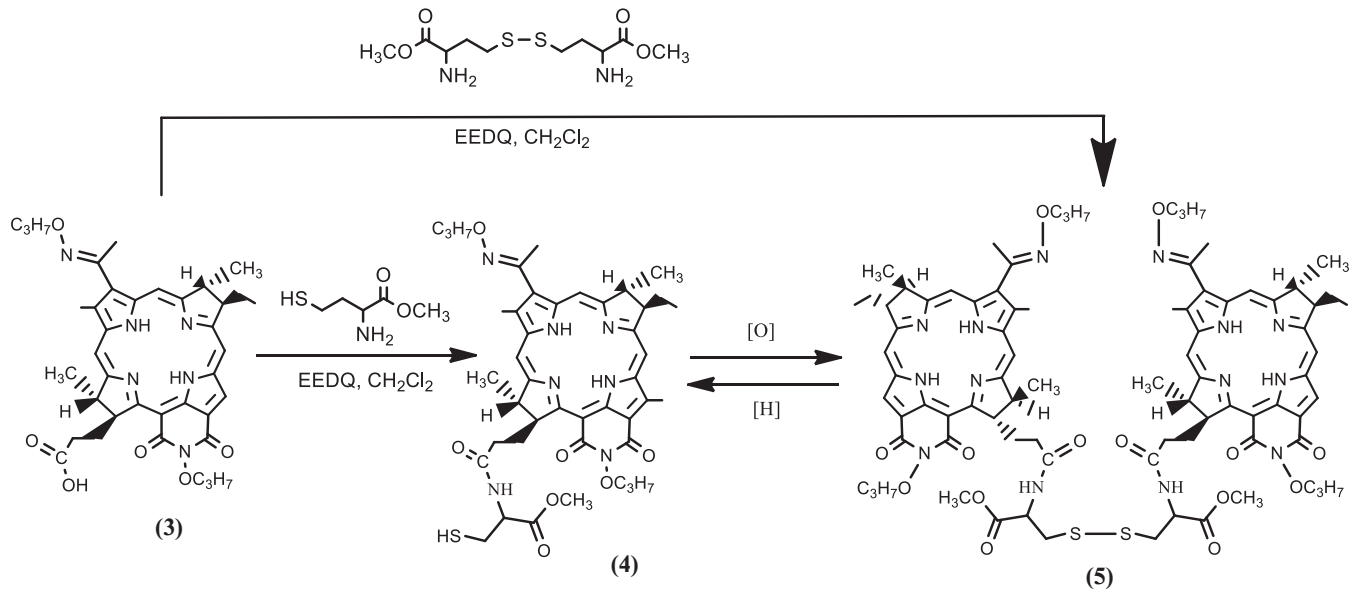


Figure 1. Absorption spectra of DPBPI and its sulfur-containing derivatives, with concentration 0.5 mg/ml in CH_2Cl_2.



Scheme 2. Derivatives of DPBPI with sulfur-containing amino acids.



Scheme 3. Synthesis of DPBPI derivatives with methyl esters of cysteine and cystine.

The preparation of the *O*-propyloxime-*N*-propoxybacteriopurpurinimide derivative with methyl ester of cysteine was carried out by creating an amide bond between the carboxyl group of the propionic residue at position 17 of the macrocycle and the α-amino group of cysteine in the presence of the condensing agent – *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). The reaction progress was monitored chromatographically by increasing the mobility of the reaction product (**4**). The latter turned out to be unstable in the air and oxidized to a dimer (**5**), which was obtained by the on-going synthesis from DPBPI and methyl ester of cystine. The synthesis was carried out under conditions similar to those described earlier (Scheme 3). The structure of compounds (**4**) and (**5**) was confirmed by the NMR spectroscopy.

Another sulfur-containing amino acid, attached to the DPBPI and not containing the thiol group, was methionine (**6**). The latter did not dimerize like cysteine and its oxidation proceeded differently (Scheme 4).

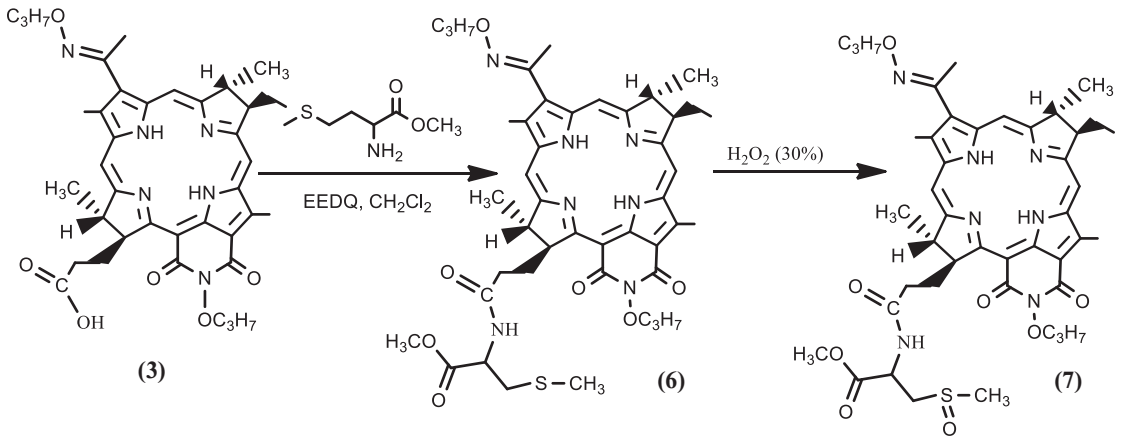
It was shown that under the influence of atmospheric oxygen, the sulfur atom is slowly oxidized to form methionine sulfoxide (**7**). More easily, a similar conversion occurs under the action of a 30 % solution of hydrogen peroxide.[25,26]

The structure of the conjugate (**6**) was confirmed by the NMR spectrum, in which a signal of the methyl group is observed at the sulfur atom in the region of 2.1 ppm. In the NMR spectrum of the oxidized form (**7**), the signals from the amino acid are shifted to the weak-field region.

Thus, the oxidized forms of the amino acid derivatives of DPBPI, including conjugates with cystine and methionine sulfoxide, are more stable comparing to the original pigments and can be considered as prodrugs in the development of pharmacologically active substances.

Stability of DPBPI derivatives with sulfur-containing amino acids with glutathione

It is known that intracellular glutathione (GSH) interacts with compounds containing a disulfide bond, and as a result of the disulfide exchange reaction it is converted to a dimeric form (GSSG).



Scheme 4. Obtaining of DPBPI derivatives with methionine and methioninesulfoxide methyl esters.

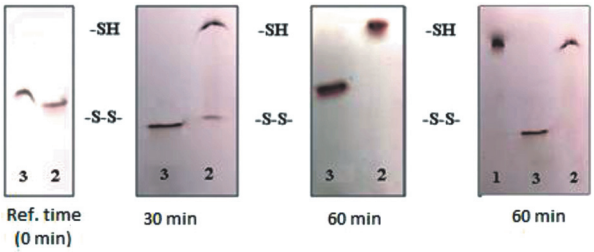


Figure 2. Interaction of dimer (**5**) with glutathione (1 – monomer standart, 2 – disulfide in the presence of glutathione, 3 – disulfide standart).

To study this reaction, which takes place in the intracellular environment, the interaction of the disulfide derivative of DPBPI with the reduced form of glutathione was realized.

Cystine-DPBPI (**5**) was dissolved simultaneously with the reduced form of glutathione in a 5 % aqueous solution of dimethylsulfoxide (DMSO). The course of the reaction was monitored by the change in the chromatographic mobility of the reaction product compared to the initial cystine-DPBPI (**4**) (Figure 2).

Analysis of the data obtained suggests that the dimer (**5**), when interacting with glutathione, converts to the monomer (**4**), which apparently occurs during internalization of substances with a disulfide.

Such interaction can lead to inactivation of the glutathione antioxidant system (due to the cystine-induced transition of the reduced form of glutathione to the oxidized one), potentially weakening of the antioxidant system of tumor cells, and, as a result, to an increase of the photodynamic effect.

Conclusions

In the present work, bacteriochlorophyll *a* derivatives with the residues of four sulfur-containing amino acids were obtained for the first time. It has been found that the oxidation of free thiol groups in cysteine-containing bacteriopurpurinimide occured under the action of atmospheric oxygen with disulfide formation, which undergoes monomerization with the participation of the reduced form of glutathione in the cell. In the case of oxidation of the methionine residue in the conjugate with dipropoxybacteriopurpurinimide, a stable sulfoxide is formed.

A possible mechanism of suppression of the antioxidant system of tumor cells under the action of sulfur-containing bacteriochlorophyll *a* derivatives is proposed.

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