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Natural chlorins as a promising platform for creating targeted theranostics in oncology

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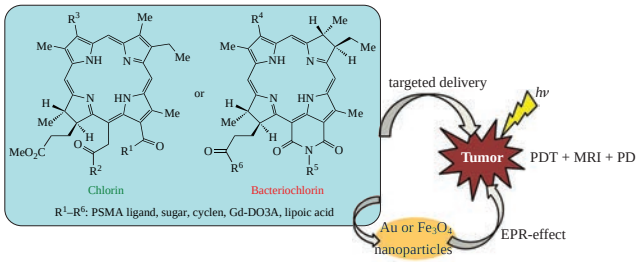
This review deals with the progress in the chemistry of natural chlorins and the prospects of using their derivatives as theranostics. Particular attention is paid to the creation of targeted photosensitizers by addition of vector molecules to chlorins and bacteriochlorins, or by immobilization onto nanoparticles of various nature. The results of biological tests that establish relationship between the structure, antitumor activity, and diagnostic potential of the resulting photosensitizers are presented.



Keywords: chlorins, bacteriochlorins, photodynamic therapy, targeted photosensitizers, nanoparticles, fluorescence diagnostics.

The successful development of photodynamic therapy (PDT) of cancer is closely related to the development of highly efficient drugs, *i.e.* photosensitizers (PSs) that lack their own bioactivity but, upon irradiation with light of a certain wavelength, pass into excited state and react with oxygen to generate active oxygen forms, including singlet oxygen and radical anion. The latter possess a cytotoxic effect but do not damage the surrounding normal tissues due to the limited lifetime and free path.[1–5] An optimal PS should absorb light in the red or near infrared spectral range, since in this case the light penetrates more deeply into a tissue. PSs should have high quantum yields for singlet oxygen and fluorescence in order to work as theranostics, and should also be amphiphilic compounds with high tropism toward tumors of various genesis.[6,7]

At the initial stage of PDT development, main attention in the creation of PSs was given to the search for compounds with optimum spectral and photophysical properties, and considerable success was achieved[8,9] among first-generation drugs based on natural hematoporphyrin such as Photofrin II (USA–Canada), Photosan (Germany) and Photogem (Russia).[10–14] In the creation of second-generation PSs with improved spectral characteristics, chlorophyll *a* derivatives were used, such as Talaporfin (Japan),

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Nikita V. Suvorov was born in 1993 in Alexandrov, Russia. In 2015, he received an engineer degree at the M. V. Lomonosov State University of Fine Chemical Technologies in Moscow, and in 2019 his PhD degree (thesis 'Modified natural chlorins of targeted action to tumor cells of various origins'). Currently he works at the Department of Chemistry and Technology of Biologically Active Compounds, Medical and Organic Chemistry of the Russian Technological University. His scientific interests involve synthesis and chemical modification of natural chlorins and bacteriochlorins, as well as the creation of targeted conjugates based on them, development of finished dosage forms of highly effective photosensitizers.

Andrey F. Mironov (born 1935) is currently the Professor at the Department of Chemistry and Technology of Biologically Active Compounds, Medical and Organic Chemistry of the Russian Technological University (Moscow). His scientific interests comprise tetrapyrrole macrocycles, synthesis and study of porphyrins, chlorins and bacteriochlorins, their conjugates with peptides, carbohydrates, fullerenes, *etc*., as well as their metal complexes. Application of such compounds as pharmaceuticals, sensor and light transforming devices is within his interests. Professor Mironov is the active promoter of the photodynamic cancer therapy in Russia. He is the Head of the scientific group responsible for the development of the first national photosensitizer 'Photogem'. Professor Mironov bears titles of the Master of Sports of the USSR, the Honoured Scientist of the Russian Federation, the Russian Government Prize Winner (in 2003 and 2007).

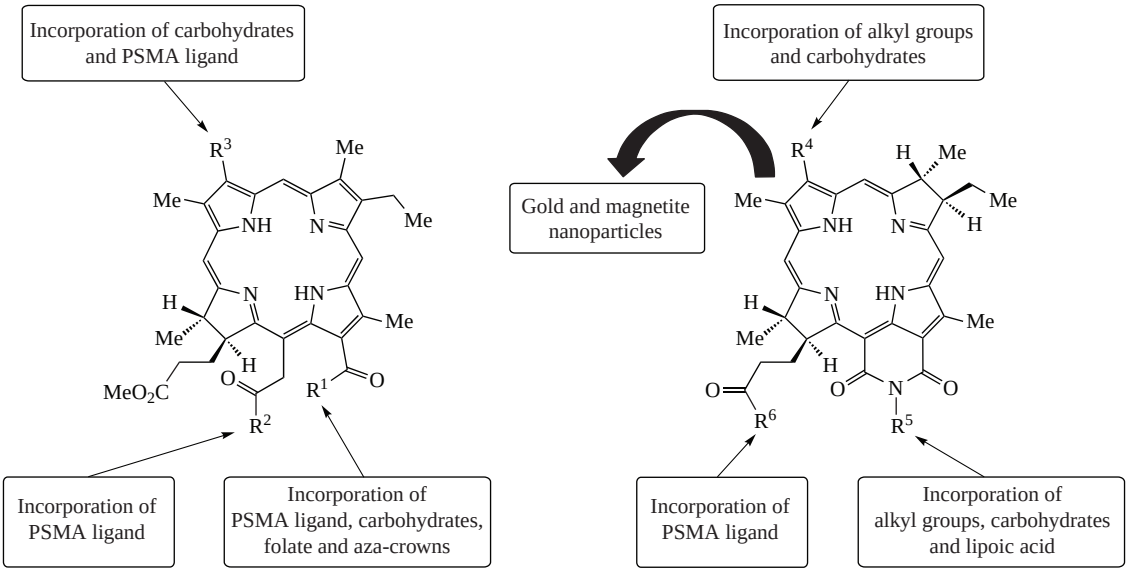


Figure 1 The strategy of synthesizing targeted photosensitizers.

Visudine (USA), Photoditazine and Radachlorin (Russia), Photolon (Belarus),[15–17] as well as synthetic chlorins such as Foscan (England),[18] and phthalocyanine Photosense (Russia).[19] In recent years, a PS with absorption in the near infrared region, Tookad (Israel), appeared.[20–22] The availability of these drugs made it possible to attain serious progress in fluorescence diagnostics and in the PDT of malignant neoplasms.

The PDT method belongs to high-tech medical care for cancer patients. Its main advantages include non-invasiveness, the absence of complications after surgical interventions, and its general toxicity characteristic in chemical and radiotherapy. The use of PDT does not require hospital treatment and long-term use of expensive drugs, which makes it an economically attractive method. However, other requirements for the development of drugs for the PDT of cancer have been coming forward in recent years. They are primarily related to a significant increase in the tropism for specific types of malignant neoplasms, since the selectivity of accumulation in the tumor compared to the surrounding tissues of the currently used PSs remains quite low and does not exceed 2.5–3. Therefore, along with the search for new PSs, special attention is now paid to the development of targeted means of their delivery to a tumor and to the improvement of the selectivity of accumulation in cancer cells.

Targeted molecular therapy is one of the new areas in the drug treatment (pharmacotherapy) of cancer. As a type of molecular medicine, targeted therapy blocks the growth of cancer cells by interfering with the mechanism of action of specific key molecules necessary for the carcinogenesis and tumor growth, rather than simply prevents the reproduction of all rapidly dividing cells. The targeted action solely on the tumor does not harm the healthy tissues around it and the overall health of the patient, thus eliminating the adverse effects of chemotherapy or radiation exposure. Currently, there are two approaches for increasing the tumorotropicity of chemotherapy drugs, and PSs in particular. The creation of conjugates of the latter with vector molecules allows active targeting of tumor cells to be implemented. Targeted peptides, steroids, carbohydrates, as well as folic acid derivatives can serve as such molecules.[23] Creating conjugates of antitumor agents with small targeted molecules is a promising approach for the targeted drug delivery to tumors.[24,25] This approach has a number of advantages, such as controlled synthesis and non-immunogenic nature.[26,27] The molecular mass of such conjugates is potentially much lower, which results in better internalization into tumor cells and better *in vitro* and *in vivo* stability.[28,29] The over-expression of a certain type of receptor on the surface of tumors of various genesis makes it possible to increase the accumulation of drugs due to receptor-mediated endocytosis.

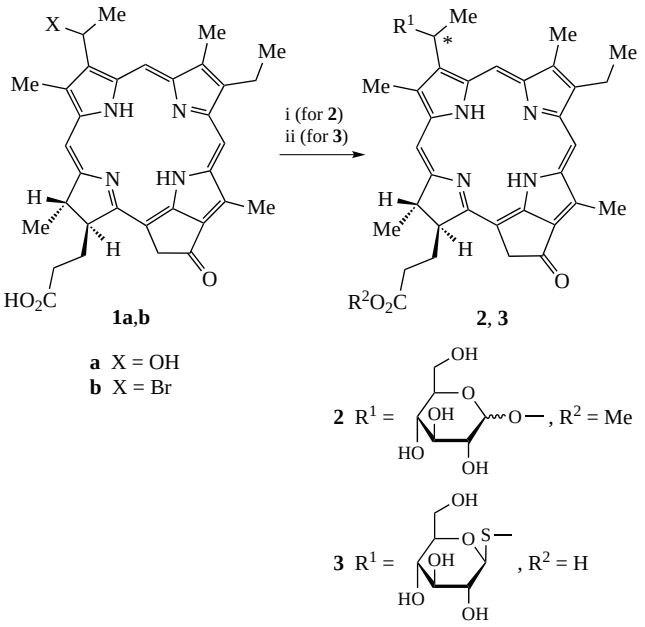
Yet another approach for increasing the accumulation selectivity involves the immobilization of active pharmaceutical compounds on nanoparticles (NPs) of various nature, which provides passive targeting, *i.e.*, the extravasation of loaded NPs from defective tumor vessels and their retention in the interstitium due to impaired tumor lymphatic drainage system (the EPR effect).

The majority of PSs currently used for the photodynamic treatment of cancer are natural or synthetic macroheterocyclic compounds, including porphyrins, chlorins, phthalocyanines and their metal complexes. Although the nature of these molecules favors their retention in a tumor node and targeted irradiation with light of a certain wavelength minimizes the risks of damage to surrounding tissues, nevertheless, the problem of improving the selectivity of PS accumulation in a zone of interest remains quite relevant. With this in mind, the creation of conjugates of PSs with small targeted molecules or with NPs is a promising approach for improving the efficiency of the photodynamic therapy method and expanding the scope of its clinical applications (Figure 1).

Glycoconjugates of natural chlorins and bacteriochlorins

Carbohydrates can act as ligands capable of specific binding to receptors on the surfaces of tumor cells. Many types of cancer are characterized by a higher expression level of galectins, *i.e.*, proteins with high affinity to β-galactosides. Conjugation of galactose and lactose with porphyrins and their analogs provides specific binding of conjugates to cell galectins. Furthermore, addition of carbohydrate moieties to hydrophobic tetrapyrrole macrocycle improves the pigment solubility in water and makes a molecule amphiphilic, which affects the accumulation and localization of a PS in the tumor cell.[30]

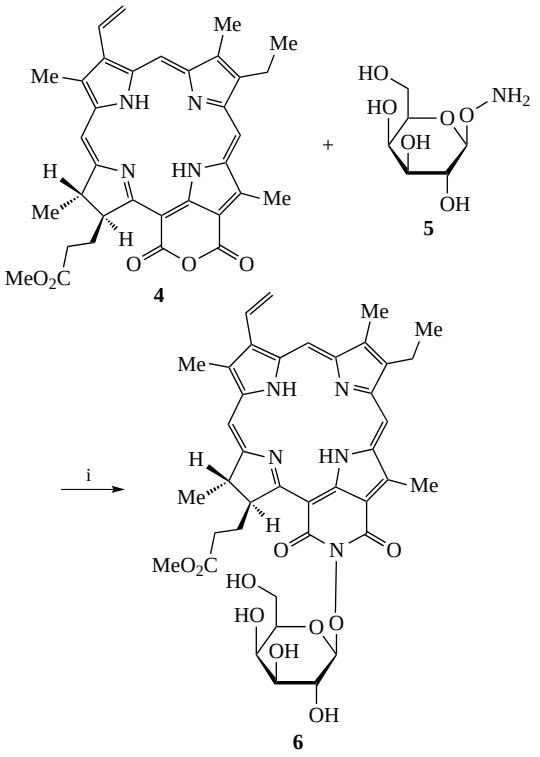
We performed new efficient syntheses of carbohydrate-containing conjugates of natural chlorins.[31,32] For example, starting from pyropheophorbide *a* **1** being a PS we obtained its *O*- and *S*-carbohydrate derivatives **2** and **3** (Scheme 1). To obtain *O*-glycoside **2**, 3-(α-hydroxyethyl)pyropheophorbide **1a** was condensed with 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose in the presence of boron trifluoride etherate. The yield of conjugate **2** was 36% with an anomer content of 83%. Thiogalactoside **3**



Scheme 1 *Reagents and conditions*: i, 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose, $Et_2O \cdot BF_3$, then MeONa/MeOH; ii, sodium β-D-galactopyranosylmercaptide, DMF.

was obtained in 54% yield by condensation of 3-(α-bromoethyl) pyropheophorbide **1b** with sodium salt of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside. Deacetylated conjugates **2** and **3** were well soluble in water–alcohol solutions but underwent aggregation with an increase in water content, which caused a broadening of the main absorption band around 665 nm.

A different approach was used in a synthesis of galactosyl-containing conjugate **6** (Scheme 2)[33] based on the reaction of purpurin 18 **4** with hydroxylamine.[34] The starting compound **4** was condensed with *O*-β-D-galactopyranosylhydroxylamine **5** obtained by treatment of D-galactose tetraacetate *N*-hydroxy-succinimide with hydrazine hydrate. The amphiphilic nature of the resulting conjugate **6** and the existence of an intense band at

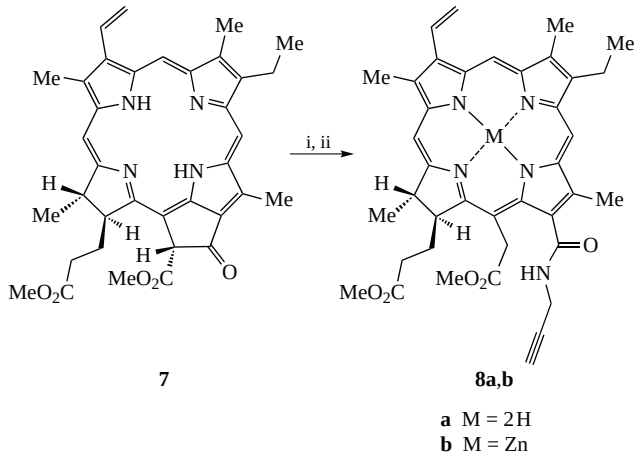


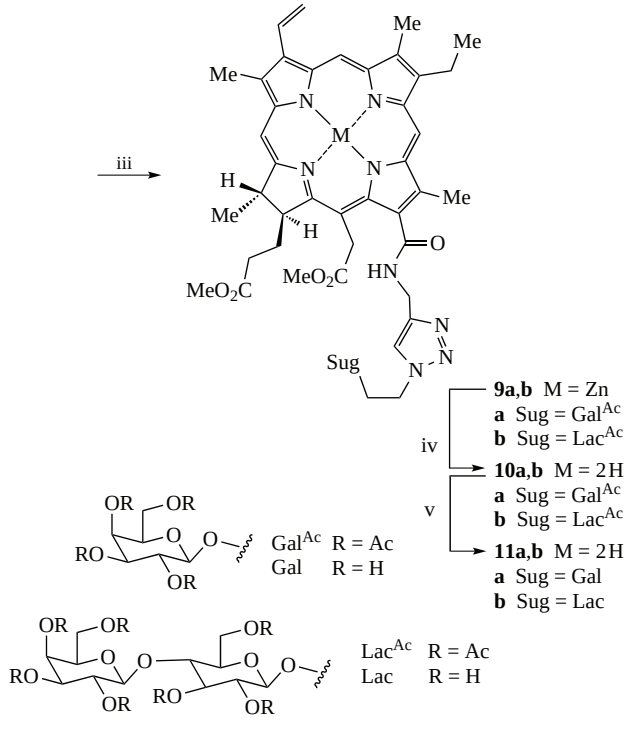
Scheme 2 *Reagents and conditions*: i, CH_2Cl_2, HCl.

714 nm allow it to be considered as a promising compound for the creation of targeted PSs by addition of vector molecules to pyrroles A and D.

We obtained a series of carbohydrate derivatives of chlorins and bacteriochlorins by Cu-catalyzed 1,3-dipolar cycloaddition of terminal alkynes and azides ('click chemistry').[35–37] To perform this reaction, we obtained new derivatives of chlorophyll *a* and bacteriochlorophyll *a* with a terminal triple bond at various positions of the macrocycle. Pheophorbide *a* methyl ester **7** was the key compound in the synthesis. Its reaction with propargylamine gave amide **8a** in 86% yield (Scheme 3). The latter was reacted with 2-azidoethyl β-D-galactopyranoside peracetate. It was found that copper was easily incorporated into the chlorin macrocycle in the course of the reaction. In view of this, the reaction was subsequently carried out with zinc complex **8b** that turned to be resistant to transmetallation. Removal of Zn from conjugate **9a** in a slightly acidic medium followed by treatment with MeOH/MeONa resulted in galactosylchlorin **11a** in a high yield. Lactosylchlorin **11b** was obtained similarly.

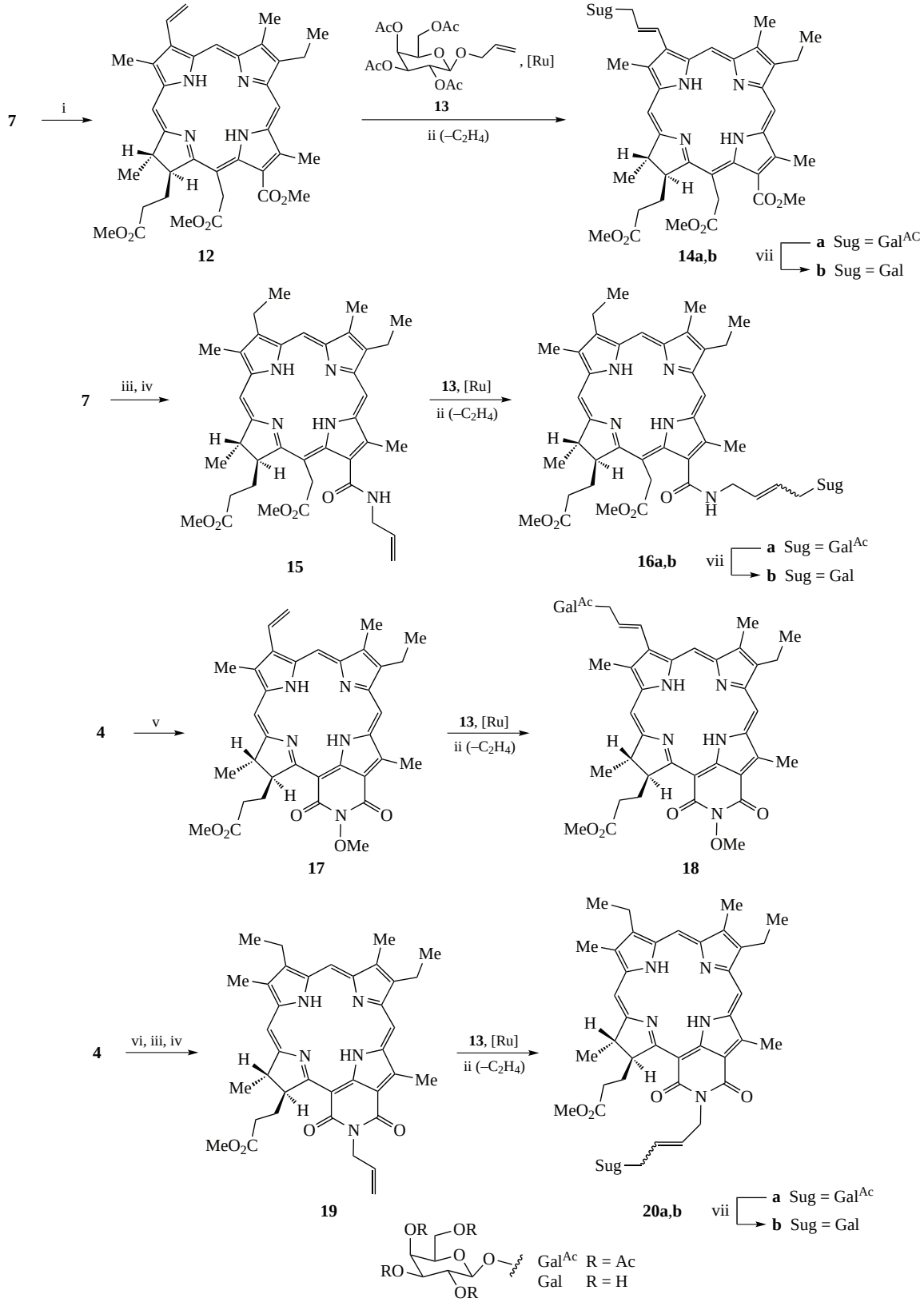
It was previously shown that incorporation of the same substituents at different positions of the chlorin macrocycle produced different effects on the amphiphilicity of the molecule





Scheme 3 *Reagents and conditions*: i, $HC{\equiv}CCH_2NH_2$, CH_2Cl_2, 20 °C; ii, $(AcO)_2Zn$, CH_2Cl_2/MeOH; iii, $Sug^{Ac}(CH_2)_2N_3$, CuI, MeCN, DIPEA, 20 °C; iv, HCl; v, MeOH/MeONa.

and, as a consequence, on the biological properties of the pigment. In particular, hydrophilic substituents at pyrrole ring A significantly increased the photodynamic efficiency of a PS.[38] The presence of a vinyl group in chlorophyll *a* derivatives allowed us to use the cross-metathesis of alkenes for incorporating sugars into pyrroles A and C and exocycle E (Scheme 4).[39] Chlorin e_6 trimethyl ether **12** obtained by opening the pentanone ring in methylpheophorbide *a* **7** on treatment with sodium methoxide was the key compound in the synthesis. Coupling of chlorin **12** with allyl β-D-galactopyranoside tetraacetate **13** was performed. Cross metathesis was carried out in the presence of the Grubbs catalyst II. A study of this reaction showed that the optimal reaction conditions involved refluxing of chlorin **12** with a four-fold excess of allyl galactoside **13** in the presence of 25 mol% of the catalyst providing the 40% yield of glycoconjugate **14a**. Increase in the concentration of the catalyst



Scheme 4 *Reagents and conditions*: i, MeONa, MeOH, CH_2Cl_2, then CH_2N_2; ii, allyl glycoside **13**, $PhCH{=}RuCl_2(PCy_3)(SIMes)$, CH_2Cl_2; iii, H_2, 10% Pd/C, then HCl; iv, $CH_2{=}CHCH_2NH_2$; v, $NH_2OH{\cdot}HCl/Py$, then CH_2N_2; vi, $Zn(OAc)_2$; vii, MeOH/MeONa.

and/or allyl galactoside **13** did not affect the conversion of the initial chlorin **12**. The reaction was stereoselective to provide *E*-configuration of the forming double bond.

In the synthesis of conjugates where galactose was located in the lower part of the macrocycle (see Scheme 4), the vinyl group in pheophorbide *a* was preliminarily hydrogenated, and the subsequent reaction of mesopheophorbide with allylamine afforded mesochlorin e_6 allylamide **15** in a high yield. The reaction of the latter with allyl β-D-galactopyranoside tetraacetate **13** in 1 : 5 ratio in the presence of 5 mol% of the Grubbs catalyst gave conjugate **16**. In this case the reaction occurred with lower stereoselectivity giving the *E*/*Z*-**16** conjugates in 5 : 1 ratio.

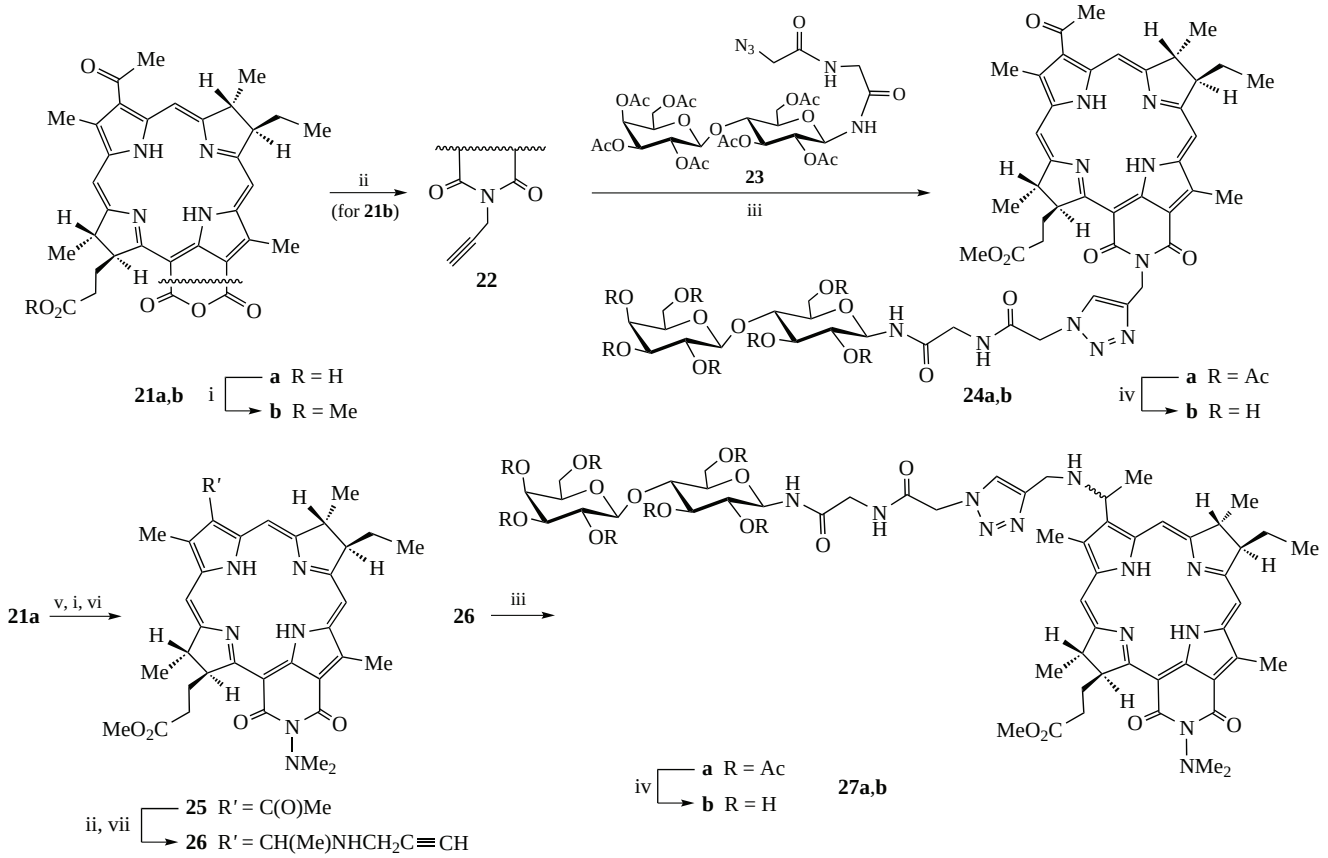
In contrast to the above reactions with chlorin e_6, the cross metathesis of allyl galactoside **13** at the vinyl group of purpurinimide **17** occurred with great difficulty (see Scheme 4), and even prolonged refluxing with a large excess of sugar **13** and with a higher amount of the Grubbs catalyst (up to 1 equiv.) did not give conjugate **18** in yields higher than 5%. This fact can be explained by a deactivating effect of the imide exocycle on the vinyl group in purpurinimide.

The terminal double bond at exocycle E proved to be more reactive. This approach involved the catalytic hydrogenation of purpurin 18 in the form of a Zn complex followed by demetallation in an acid medium and by the reaction of the resulting mesopurpurine with allylamine. Isomeric 13- and 15-amides were treated with diazomethane and a KOH solution in methanol to provide *N*-allylmesopurpurinimide **19** in 85% overall yield (see Scheme 4). The cross metathesis was carried out under conditions similar to the synthesis of conjugate **16**, while the reaction stereoselectivity was somewhat higher and the *E*/*Z* ratio of isomers **20** was 6 : 1.

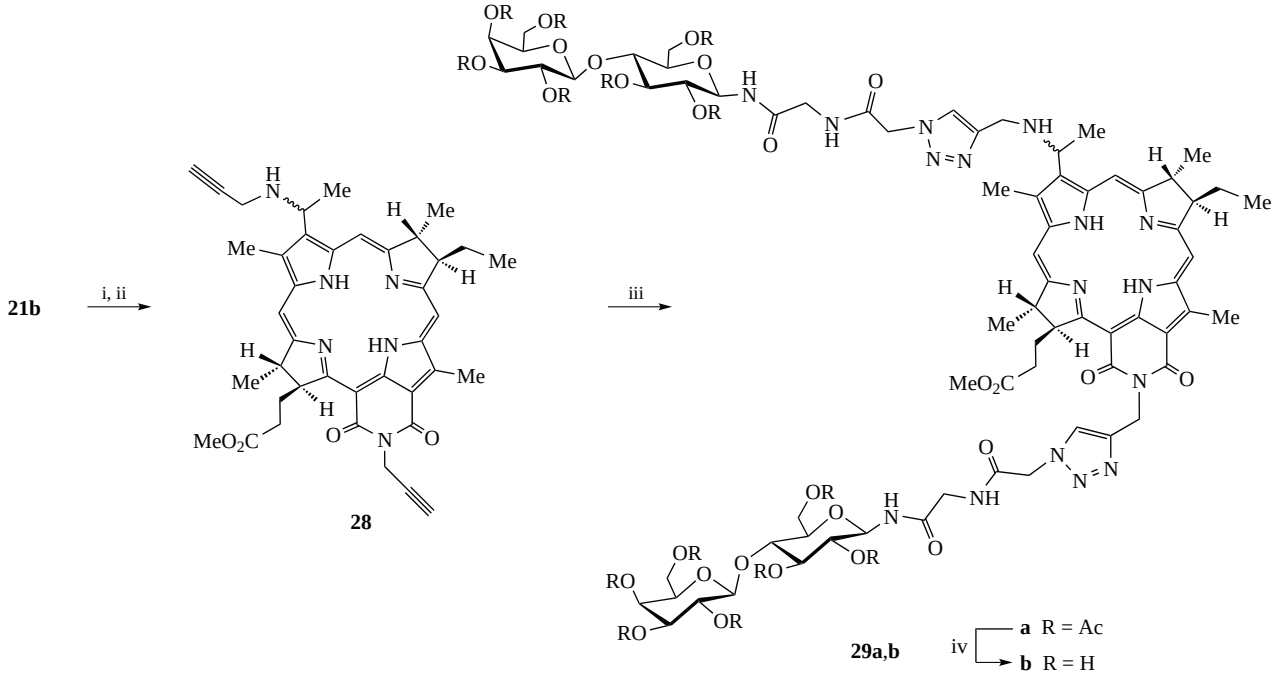
Biological tests of the resulting glycoconjugates on Hep2 cell line (epidermoid larynx pharynx carcinoma) allowed two main conclusions to be made. Galactosylchlorins obtained by the cross-metathesis and containing a double bond in the spacer exhibit higher photoinduced cytotoxicity than the triazole cycle. Of the glycosylated chlorins obtained, glycoconjugate **14b** with galactose in pyrrole A was the leader (IC_{50} = 0.02 μM). Its activity was 8 times higher than that of the initial chlorin e_6 trimethyl ester **12** (IC_{50} = 0.16 μM).[40]

Bacteriopurpurin **21a**[41] obtained from the biomass of purple bacteria *Rhodobacter capsulatus*[42] was used as the key compound in the syntheses of carbohydrate derivatives of bacteriochlorophyll *a* (Scheme 5). The reaction of bacteriopurpurin methyl ester **21b** with propargylamine occurred non-selectively and along with cycloimide **22** a Schiff base was also formed at the acetyl group of pyrrole A. The products were separated by chromatography. Further, cycloimide **22** was reacted with the azide derivative of lactoside **23** (obtained by sequential acylation of aminolactoside peracetate with glycine and azidoacetic acid). The choice of the carbohydrate was determined by the specificity of the carbohydrate-recognizing domain of galectins to the di- and oligosaccharide sequences with a β-galactose terminal residue. 1,3-Dipolar cycloaddition resulted in lactosyl bacteriopurpurinimide **24a** in 80% yield.

We also used *N*,*N*-dimethylaminobacteriopurpurinimide **25** obtained from bacteriopurpurin **21a** as the starting compound in the directed incorporation of a carbohydrate moiety into pyrrole ring A (see Scheme 5).[43] Refluxing cycloimide **25** with propargylamine in chloroform gave the corresponding Schiff base, which was then reduced with sodium borohydride. The resulting mixture of diastereomeric propargyl derivatives **26** was used in the coupling reaction with the azide derivative of lactose peracetate **23** to give conjugate **27a** in 83% yield. Removal of protective acetyl groups in compounds **24a** and **27a** by treatment with sodium methoxide in methanol afforded novel carbohydrate-containing conjugates **24b** and **27b** based on natural bacteriochlorins.



Scheme 5 *Reagents and conditions*: i, CH_2N_2, Et_2O; ii, $HC{\equiv}CCH_2NH_2$ (excess), $CHCl_3$, Δ; iii, **23**, CuI (10 mol%), DIPEA, CH_2Cl_2; iv, MeOH/MeONa; v, H_2NNH_2, then HCl; vi, MeI/DIPEA; vii, $NaBH_4$, MeOH.



Scheme 6 *Reagents and conditions*: i, $HC{\equiv}CCH_2NH_2$ (40 equiv.), $CHCl_3$, Δ, 40 h, ii, $NaBH_4$, MeOH; iii, **23**, CuI (20 mol%), DIPEA, CH_2Cl_2; iv, MeOH/MeONa.

It is known that an increase in the amount of carbohydrate residues in glycoconjugates increases the efficiency of binding to galectins. In view of this, a bivalent lactosyl-containing bacteriochlorin derivative based on purpurinimide was synthesized. For this end, bacteriopurpurin **21b** was reacted with excess propargylamine (Scheme 6). The product was reduced with sodium borohydride to give a mixture of diastereomeric cycloimides **28**, which were then entered into coupling with lactoside **23** (2 equiv.) to afford conjugate **29a** in 70% yield. After deprotection of the hydroxy groups, it was converted to pigment **29b** that had high hydrophilicity and solubility in water.

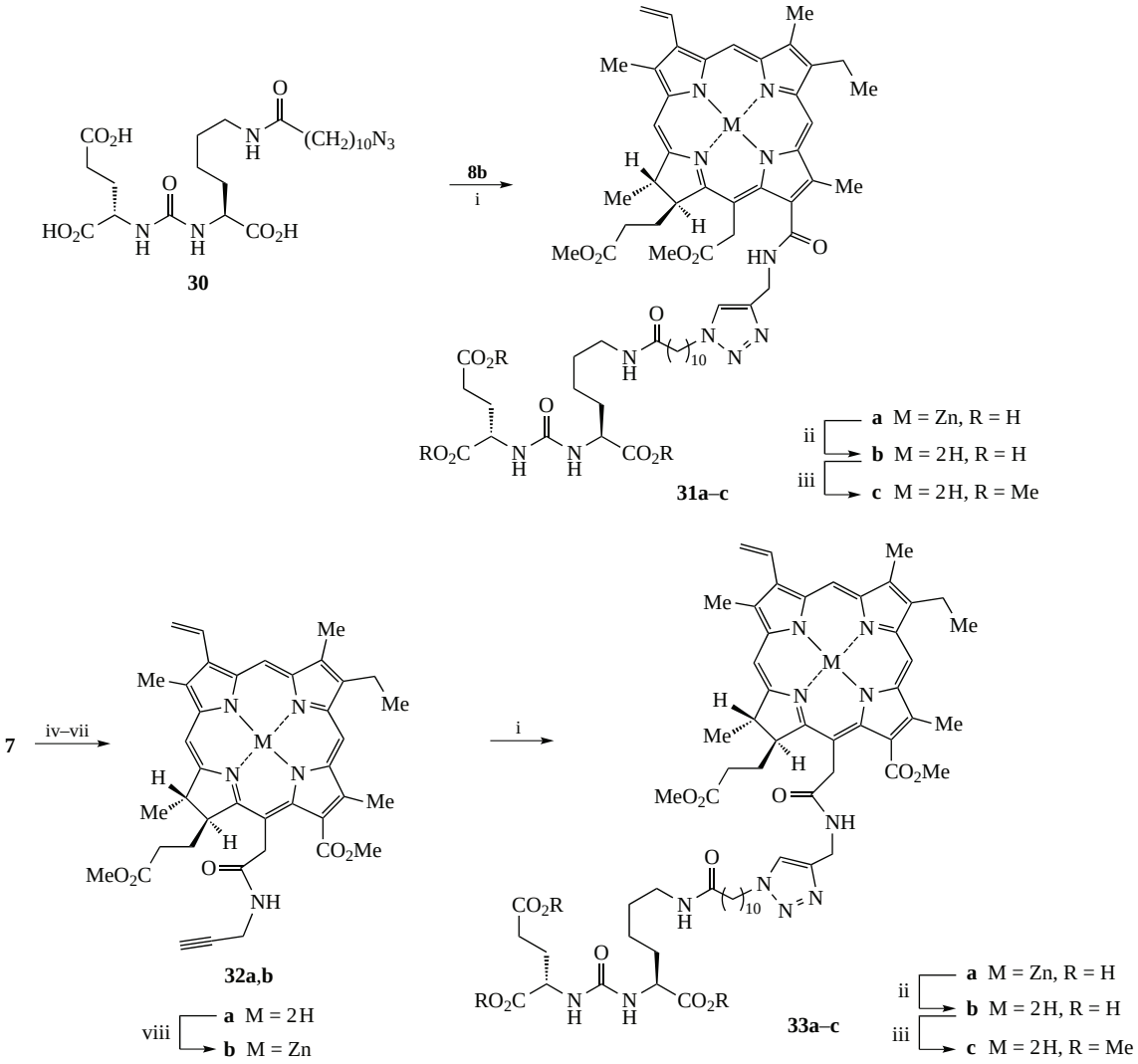
Natural chlorins with directed action against tumor cells of prostate cancer

The prostate-specific membrane antigen (PSMA) is an oncomarker of prostate tumors. PSMA is a transmembrane glycoprotein, glutamate carboxypeptidase II (GCPII) that is a promising receptor for the targeted delivery of drugs and diagnostic agents.[44] The overexpression of these receptors is observed in prostate cancer cells and correlates with the level of histological differentiation of tumors, especially if metastases exist. In studying the structure of the PSMA receptor, several low molecular weight ligands were found, among which urea-based ones were most popular for the targeted delivery of therapeutic and diagnostic agents.[45,46]

We implemented a synthesis of targeted PSs based on chlorin e_6 and bacteriopurpurinimide containing the PSMA ligand at macrocycle positions 13, 15, 17 and 3 and studied their biological properties.[47,48] The strategy of synthesizing conjugates of chlorin e_6 with the PSMA ligand involved the Cu-catalyzed azide–alkyne cycloaddition (Scheme 7). In this way we performed ligand addition under mild conditions and in high yields. Zinc complexes of 13^1-(propargylamido)chlorin e_6 **8b** and 15^2-(propargylamido)chlorin e_6 **32b** were synthesized as the alkyne components. Compound **32a** was synthesized by forming an intermediate anhydride upon cyclization of the 13^1- and 15^2-positioned carboxy groups of chlorin e_6 in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) followed by anhydride ring opening with propargylamine and methylation of the resulting compound with diazomethane. The selective incorporation of the propargyl group to positions 13^1 and 15^2 of the macrocycle was confirmed by NMR spectroscopy. The signals for 17^3-Me, 15^2-Me and 13^1-propargyl groups were detected in the ^{1}H NMR spectrum of compound **8a**, which agrees with published data.[36] The regioselective incorporation of the propargyl group to position 15^2 was confirmed by NMR spectra of compound **32a**, in which the signals for 15^2-positioned methyl group were absent while the signals for the 13^1- and 17^3-positioned ones were present. Assignment of methyl group signals was carried out by comparison with the previously reported spectrum of chlorin e_6 trimethyl ester.[48] Moreover, the chemical shifts for the propargyl group in compounds **8a** and **32a** are markedly different. To prevent the possible metallization of the chlorin macrocycle in the course of the Cu-catalyzed reaction, zinc complexes **8b** and **32b** were preliminarily obtained.

Peptidomimetic **30** with a terminal azido group (see Scheme 7) was prepared by acylation of the terminal amino group of the PSMA ligand with 11-azidoundecanoic acid. The starting peptidomimetic was synthesized from glutamic acid di-*tert*-butyl ester and Cbz-protected lysine *tert*-butyl ester as described previously.[45,46] Coupling of precursor **30** with chlorins **8b** and **32b** resulted in derivatives **31b** and **33b**, respectively.

The phototoxicity of conjugates **31b** and **33b** was estimated on prostate cancer cell lines. The cells of one of them, 22Rv1, overexpress PSMA receptors, whereas the cells of the PC-3 line lack these receptors. Radachlorin (RadaPharma, Russia), which has been approved for clinical use in Russia, was used as the reference PS. The maximum photoinduced cytotoxicity of conjugate **31b** for 22Rv1 cells was 1.2 ± 0.1 μM, *i.e.*, 7 times higher than the photoactivity obtained on PC-3 cells, which is apparently due to the high expression of PSMA receptors in this cell culture. In contrast, the internalization of PS through receptor-mediated endocytosis is not possible for PC-3 cells that lack these receptors. It should be noted that Radachlorin was less efficient than conjugate **31b** for 22Rv1 cells (IC_{50} = 3.0 ± 0.2 μM). In the case of conjugate **33b**, the IC_{50} values (5.2–5.4 μM) were approximately the same for both cell lines. In general, incorporation of PSMA ligand into

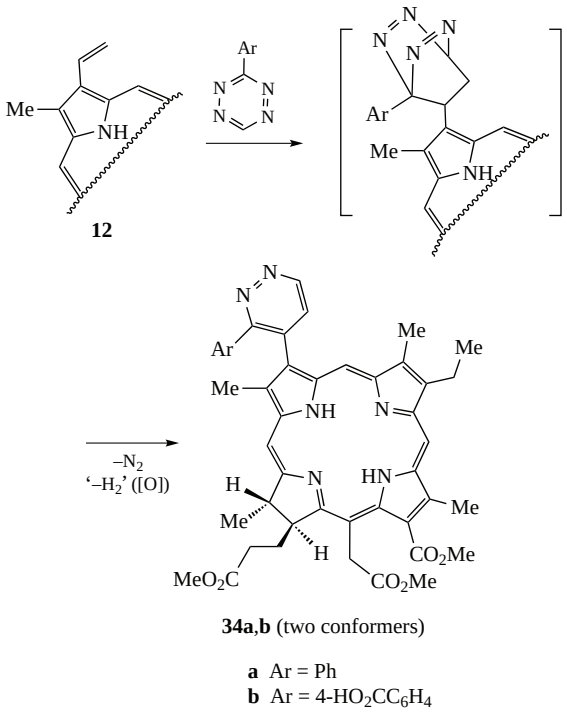


Scheme 7 *Reagents and conditions*: i, **30**, CuI, DIPEA, DMF, 20 °C, 16 h; ii, CF_3CO_2H (10%), DMSO, 20 °C, 2 h; iii, MeOH, $SOCl_2$, CH_2Cl_2; iv, NaOH, acetone, 50 °C; v, HCl; vi, $HC{\equiv}CCH_2NH_2$, EDC, DMAP, CH_2Cl_2; vii, CH_2N_2, CH_2Cl_2; viii, $Zn(OAc)_2$.

various positions of the macrocycle significantly affects the efficiency of PSs.[49]

In a continuation of studies on the structure–activity relationship for chlorins with the PSMA ligand, we implemented a regioselective incorporation of a peptidomimetic into pyrrole A of the chlorin macrocycle. The availability of a vinyl group in chlorophyll *a* derivatives makes it possible to use the catalyst-free tetrazine–alkene addition reaction (Inverse Electron Demand Diels–Alder reaction, IEDDA) for addition of a vector molecule.[50] In this reaction, a 1,2,4,5-tetrazine acts as a 'diene', while an alkene or alkyne act as a 'dienophile'. In the majority of cases, [4+2]-cycloaddition occurs with participation of the 3rd and 6th carbon atoms of the 1,2,4,5-tetrazine. Upon the following addition of a C,C-dienophile, a nitrogen molecule is released with formation of 1,2-dihydropyridazine ring which can then be oxidized to pyridazine.[51] In our studies, the reactions between chlorin e_6 trimethyl ester and aryltetrazines were carried out (Scheme 8). 3-Phenyl-1,2,4,5-tetrazine was reacted with chlorin **12** in non-polar (toluene, dichloromethane) and polar (methanol, DMF) solvents. High (94%) yield of the target product **34a** was achieved in polar DMF within 8 h.

The structure of the product was studied by NMR spectroscopy. The ^{1}H NMR spectrum contained doubled signals for the 20-positioned *meso*-proton of the chlorin macrocycle and for the pyridazine protons. Analysis of ^{1}H and ^{13}C spectra showed the presence of two conformers of compound **34a** in 1 : 1



Scheme 8 *Reagents and conditions*: DMF, 20 °C, 8 h.

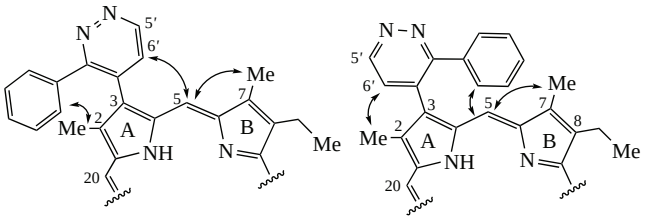


Figure 2 Fragments of the structures of compound **34a** conformers. The arrows indicate the NOE effects observed in the NOESY spectrum.

ratio that differed in the spatial position of the pyridazine ring (Figure 2). The assignment of signals in the NMR spectra of the latter was performed using COSY, NOESY and HSQC experiments.

To introduce an additional carboxy group into the chlorin macrocycle, chlorin e_6 trimethyl ester **12** was brought into a reaction with 4-(1,2,4,5-tetrazin-3-yl)benzoic acid to give product **34b** (see Scheme 8). Its NMR spectra also contained signals for two conformers, similarly to analogue **34a**.

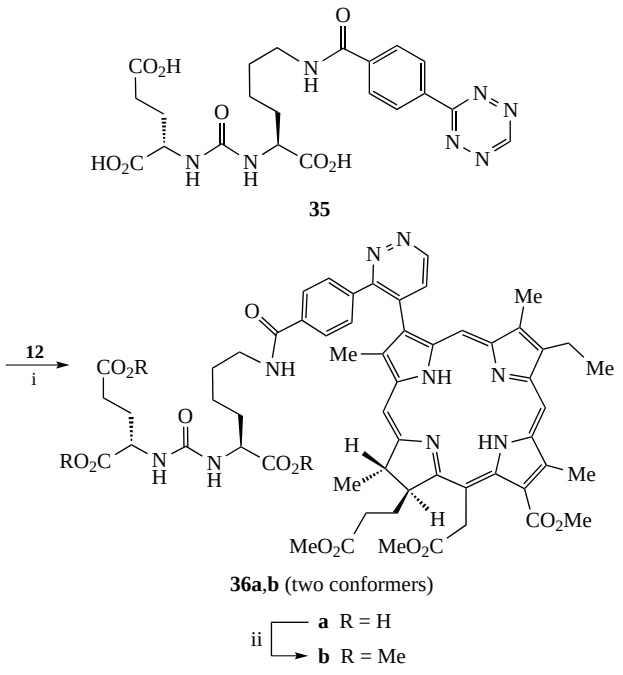
A study of the photophysical properties of the compounds thus obtained showed that incorporation of a pyridazine moiety into the molecule slightly affected the shift of the long-wavelength absorption maximum of the chlorine, namely, from 662 to 665 nm for compound **34a** and to 667 nm for compound **34b**. These chemical modifications did not result in a significant change in fluorescence properties compared to chlorin e_6: the fluorescence quantum yields φ were 0.16 and 0.11 for derivatives **34a** and **34b**, respectively, while φ was 0.16 for unsubstituted chlorin **12**.

Such chlorin functionalization allowed a vector molecule of a peptidomimetic to be incorporated at position 3 of the macrocycle (Scheme 9). The PSMA ligand was modified with 4-(1,2,4,5-tetrazin-3-yl)benzoic acid to give precursor **35** for the tetrazine–alkene addition to chlorin e_6 trimethyl ester **12**. To confirm the structure of the target conjugate **36a** by NMR, its free carboxy groups were esterified, and the NMR spectrum of the hexamethyl ester unambiguously confirmed the suggested structure **36b**. Moreover, like in the reaction described previously, the formation of two conformers that differed in the spatial arrangement of the vector peptidomimetic was observed.

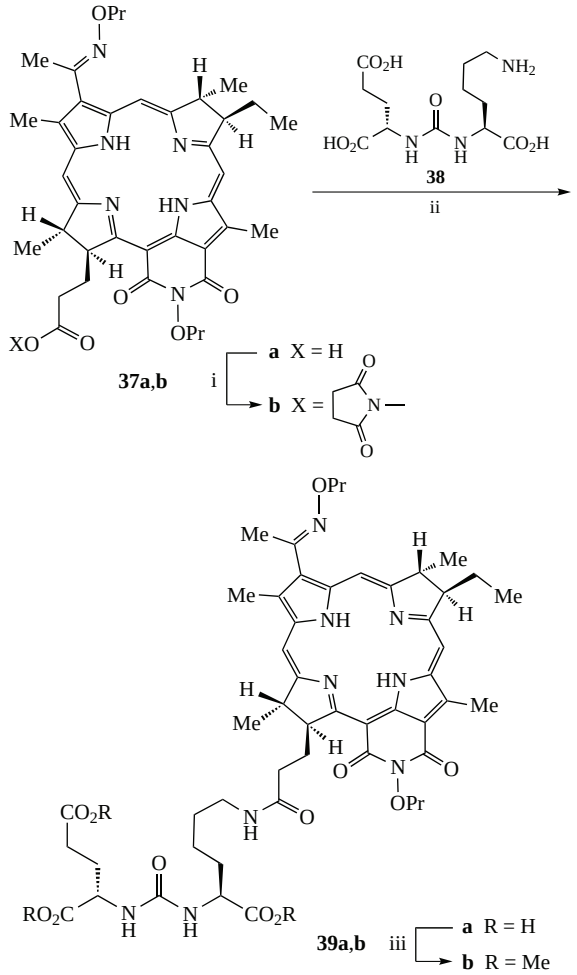
The phototoxicity of conjugate **36a** was estimated on 22Rv1 and PC-3 prostate cancer cell lines under conditions similar to those previously described for 13- and 15-substituted chlorins. Radachlorin (RadaPharma, Russia) was used as the reference PS. On both lines, a higher photodynamic activity of pigment **36a** compared to Radachlorin was observed (IC_{50} = 0.6 μM for **36a** *vs*. 2.7 μM for Radachlorin).

Previously, we obtained a bacteriopurpurinimide, namely *O*,*N*-dipropoxybacteriopurpurinimide (dipropoxy-BPI) **37a**, which was found to be a stable PS with high photoinduced cytotoxicity against tumor cells and *in vivo* photodynamic efficiency in tumors of various genesis.[52,53] To increase the efficiency of internalization of the aforementioned leader PS in prostate tumor cells, the PSMA ligand was added at the propionic residue of bacteriopurpurinimide (Scheme 10). The amidation of dipropoxy-BPI **37a** was carried out by the method of activated esters *via* an intermediate *N*-succinimide ester **37b**. The latter was brought into the reaction with peptidomimetic **38** containing unprotected carboxy groups. The structure of the resulting conjugate **39a** was confirmed by high resolution mass spectra in which a molecular ion peak was observed, as well as by NMR spectra of the corresponding trimethyl ester **39b**.

The presence of three carboxy groups in the structure of the leader PS pigment **39a** makes it soluble in sodium phosphate buffer (pH = 7.2), which is very convenient for biological studies. In addition, the high polarity of the conjugate decreases the efficiency of transmembrane transfer and reduces its photo-



Scheme 9 *Reagents and conditions*: i, **12**, DMF, 20 °C, 24 h; ii, MeOH, $SOCl_2$, CH_2Cl_2, 20 °C, 1.5 h.



Scheme 10 *Reagents and conditions*: i, *N*-chlorosuccinimide, EDC, CH_2Cl_2, 20 °C, 24 h; ii, **38**, CH_2Cl_2/Pr^iOH; iii, CH_2N_2, CH_2Cl_2.

induced cytotoxicity compared to an emulsion of the original dipropoxy-BPI **37a** in Kolliphor.

Thus, studies of the PS structure–activity relationship show that the direct cytotoxic effect of the PSs studied is a multifactor process that depends on the nature of the macrocycle (chlorin or bacteriochlorin), the presence or absence of a vector molecule in the pigment structure, its location on the periphery of the macrocycle, and the overall amphiphilicity of the PS, which determines the bioavailability at both cellular and organism levels.

Nanostructures based on magnetite and gold particles with derivatives of natural chlorins

The incorporation of various antitumor agents into biocompatible nanomaterials makes it possible to increase the selectivity of drug accumulation in tumors due to the EPR effect. Of various nanocarriers, researchers pay the most attention to magnetite and gold NPs[54–59] due to their low toxicity, possibility of functionalization, relative aggregative stability, as well as their own useful properties. We chose dipropoxy-BPI **37a** having an absorption maximum in the region of 800 nm and thus allowing the treatment of deep-lying and pigmented tumors[53] as the PS for magnetite NPs. Importantly, magnetite possesses superparamagnetic properties, therefore, its NPs can be used as contrast agents in magnetic resonance imaging (MRI). Immobilization of a PS on the surface of such NPs makes it possible to create theranostics for tumorfluorescence imaging, MRI diagnostics and PDT treatment.

Spherical magnetite NPs were prepared by a standard procedure from iron(III) oleate.[54] The mean particle size was 12 nm according to transmission electron microscopy (TEM). At the initial stage of the study, dipropoxy-BPI **37a** was immobilized on magnetite NPs coated with pluronic F127, an amphiphilic block copolymer of polyethylene and polypropylene glycols that is a solubilizer of hydrophobic dyes and drugs.[60] However, when this approach was used, PS aggregation took place, which was accompanied by a shift of the long-wavelength absorption band to the 900 nm region, fluorescence quenching, and a sharp decrease in the generation of singlet oxygen.

In view of this, we used a different approach involving the immobilization of a hydrophobic PS onto magnetite NPs coated with oleic acid, followed by treatment of the NPs with F127 pluronic (Figure 3).[61] In this case, the aggregation of the PS was really prevented and its spectral properties were preserved (Figure 4). After loading the PS and coating the NPs with Pluronic F127, the average hydrodynamic particle diameter determined by dynamic light scattering (DLS) was 125 nm, while the sizes of dipropoxy-BPI **37a** micelles in F127 pluronics were significantly smaller, in agreement with the reported data for similar complexes of NPs with PS.[55]

A study of intracellular accumulation conducted on two cell lines, LNCaP (PSMA+) and PC-3 (PSMA–), showed that

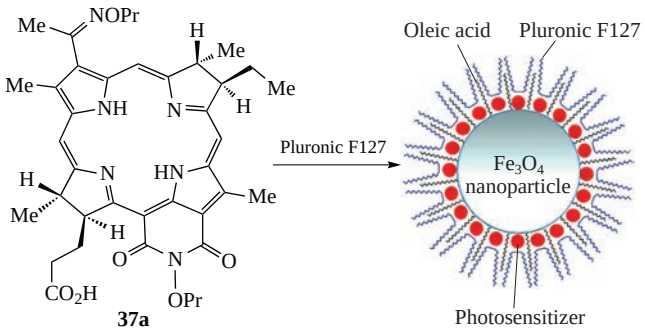


Figure 3 Preparation of magnetite nanoparticles loaded with dipropoxy-BPI **37a**.

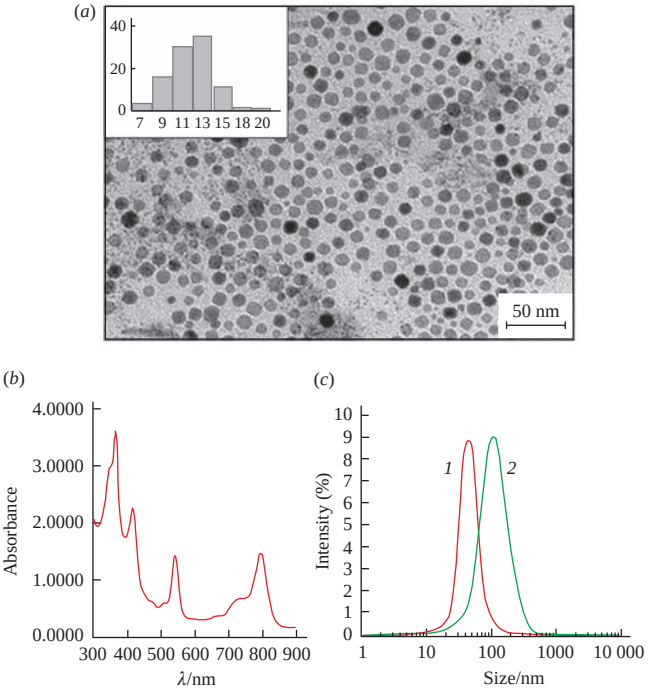
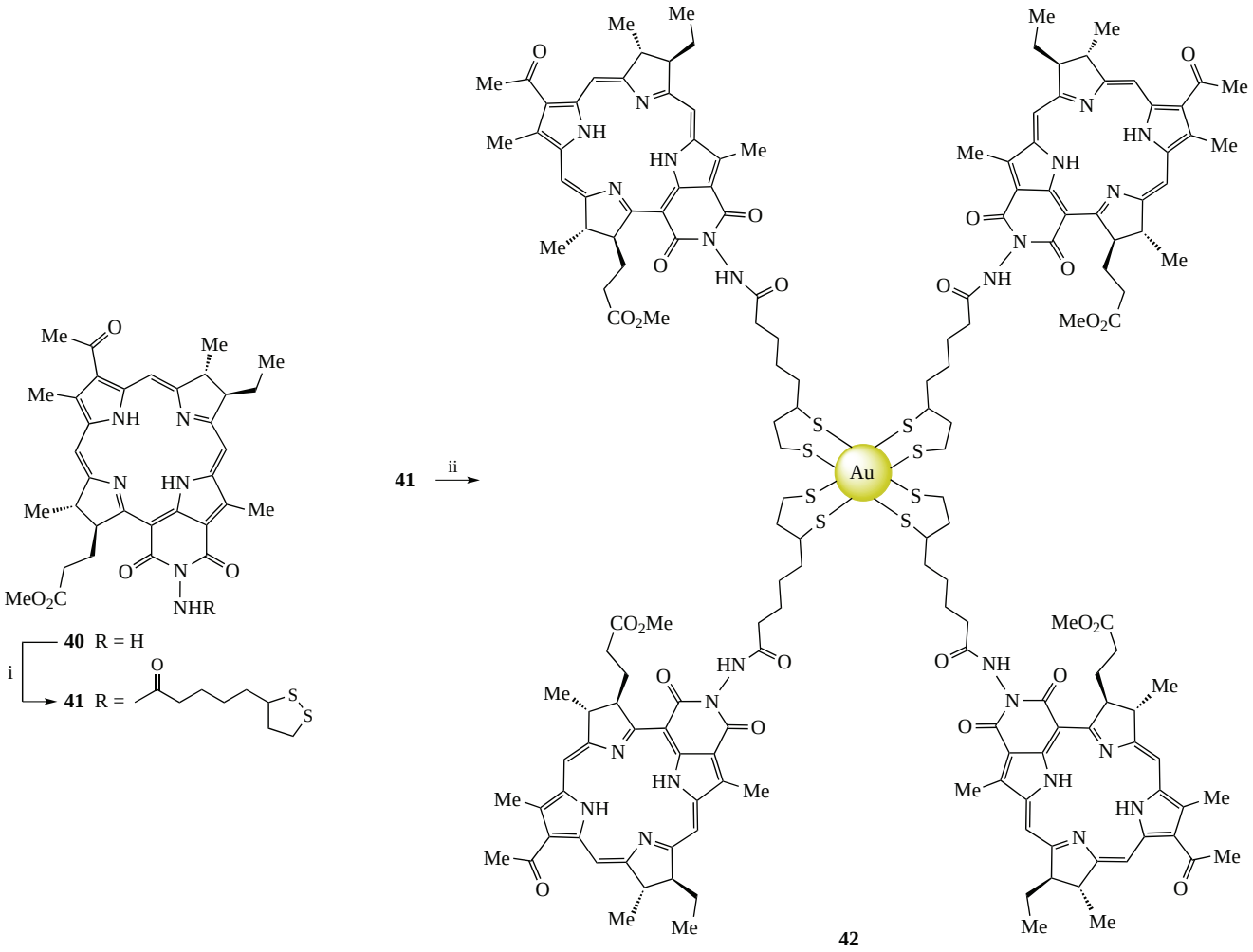


Figure 4 Characteristics of magnetite NPs modified with dipropoxy-BPI **37a**: (*a*) TEM micrograph and a histogram of the size distribution of NPs; (*b*) electronic absorption spectrum; (*c*) distribution diagram by the hydrodynamic diameter of (*1*) PS micelles in Pluronic F127 and (*2*) NPs modified with **37a**.

the internalization of nano-structured dipropoxy-BPI **37a** for PC-3 cells is two times higher compared to the micellar form of PS.

Gold NPs (NP-Au) have a number of unique characteristics, such as optical properties, strength, high surface area, chemical inertness and ability to withstand oxidation even in ultrafine (nanoscale) state. Depending on the shape, size and nature of surface functionalization, NP-Au can penetrate into cells by endocytosis or get attached to external structures of a cell membrane.[62] Gold NPs were used to successfully implement the delivery of antitumor agents both for chemotherapy and for PDT cancer PSs.[63,64]

We obtained a new sulfur-containing derivative of bacteriochlorophyll *a*, *N*-aminobacteriopurpurinimide **40** whose exocyclic amino group is acylated by the residue of lipoic acid **41**, a biogenic compound that serves as a cofactor of pyruvate dehydrogenase and ketoglutarate dehydrogenase complexes in the organism (Scheme 11).[65] Due to the presence of a disulfide moiety in the lipoic acid molecule, the pigment acquired aurophilic properties and could be immobilized on the surface of NP-Au due to formation of S–Au bonds. The shape and size of the resulting particles with immobilized (PS-Au) PS **42** were determined by DLS and TEM methods. The nano-structured PS consists of spheres with a hydrodynamic diameter of 100–110 nm. It absorbs light in the region of 824 nm and intensely fluoresces at 830 nm, which made it possible to study the kinetics of its distribution in healthy and tumor tissues in the organs of tumor-bearing animals. A comparison of the biological properties of the free PS and immobilized pigments (PS-Au) **42** in experiments on rats with M1 sarcoma showed that immobilization of bacteriopurpurinimide on NP-Au increases the circulation time of the nano-structured PS in the blood stream and increases its affinity to the tumor due to nonspecific targeting, including the extravasation of NPs loaded with the pigment from defective tumor vessels.[65]



Scheme 11 *Reagents and conditions*: i, EEDQ, CH_2Cl_2, 20 °C, 24 h; ii, NP-Au, sodium citrate, H_2O, 20 °C, 24 h.

Creation of transition metal chelators based on natural chlorins for application in cancer diagnostics

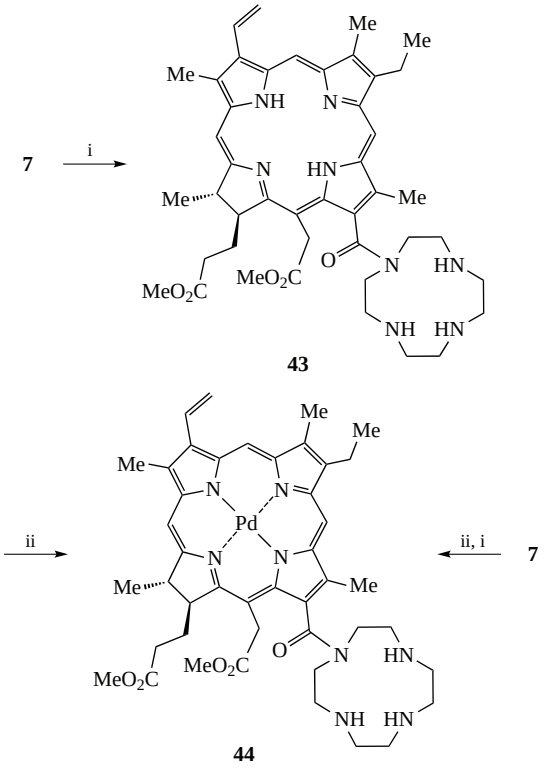
Along with the use of chlorophyll *a* and bacteriochlorophyll *a* derivatives as PSs for photodynamic therapy, great opportunities for reliable diagnostics and visualization of tumors appear if metal ions are included both directly in a tetrapyrrole macrocycle and on its periphery in external chelating substituents. An example of the first approach is the development of a palladium complex of bacteriochlorin (WST11) by scientists from Israel. Along with successful elimination of rather large and deep-lying tumors, it allows one to perform their fluorescence diagnostics.[66,67] Significantly more prospective is the preparation of bimetallic complexes based on conjugates of natural chlorins with external chelators, including *e.g.* tetraaza macrocycle widely used for these purposes.[68] The latter allows metal ions with large radii, such as lanthanides, to be included. In this case, illumination of the chlorin macrocycle results in generation of reactive oxygen species for tumor destruction, while lanthanide ions in the ring allow fluorescence diagnostics in the infrared region (Er, Yb, Eu) or visualization of the tumor by magnetic resonance imaging (Gd, Mn) to be performed.[69–72]

We obtained derivatives of chlorophylls with nucleophilic 1,4,7,10-tetraazacyclododecane by its straightforward reaction with methylpheophorbide **7** which underwent the pentanone exocycle opening to give conjugate **43** (Scheme 12).[73] Since binding of two subunits due to formation of an amide bond hinders the conformational mobility, and taking the steric factor into account, we expected that conversion of methylpheophorbide *a* would be low. Luckily, the yield of compound **43** was rather high (77%). The availability of two coordination cavities in conjugates of this kind makes it possible to synthesize homo- and heteronuclear metal complexes that may be in demand in fluorescence diagnostics, MRI and positron emission tomography.

In this case, taking into account the nonplanar structure of the tetraazacyclododecane, transition metal ions should preferably be incorporated into the chlorin macrocycle. To confirm this assumption, we synthesized a monopalladium complex of conjugate **44** by two pathways (Scheme 12). In the first case, palladium was initially incorporated into pheophoribide *a* **7**, followed by the reaction of the resulting metal complex with tetraazacyclododecane. In the second case, the pre-synthesized non-metallated conjugate **43** was treated with palladium acetate in dichloromethane. The compounds obtained by the two different schemes had the same chromatographic mobility and contained one palladium cation according to the MALDI mass spectrum manifesting characteristic isotope cluster for palladium. In the electronic spectrum, an intense absorption band Q_2 at 624 nm also confirmed the structure of the complex.

We found that when Pd^{2+} cation is incorporated into the chlorin macrocycle, the fluorescence of conjugate **44** is quenched dramatically, while its photosensitizing activity approaches 100% (Table 1) as estimated from the quantum yield of singlet oxygen generation (Φ_Δ) using 1,3-diphenylisobenzofuran as the chemical trap. It allows us to make the conclusion about the high photodynamic potential of this metal complex.

As a logical continuation of the work on the creation of a theranostic agent, another transition metal capable of implementing the diagnostic capabilities of the above conjugate was incorporated into the tetraazacyclododecane subunit. To ensure the chelating properties of tetraazacyclododecane,



Scheme 12 *Reagents and conditions*: i, 1,4,7,10-tetraazacyclododecane, CH_2Cl_2, DIPEA; ii, $Pd(OAc)_2$, CH_2Cl_2.

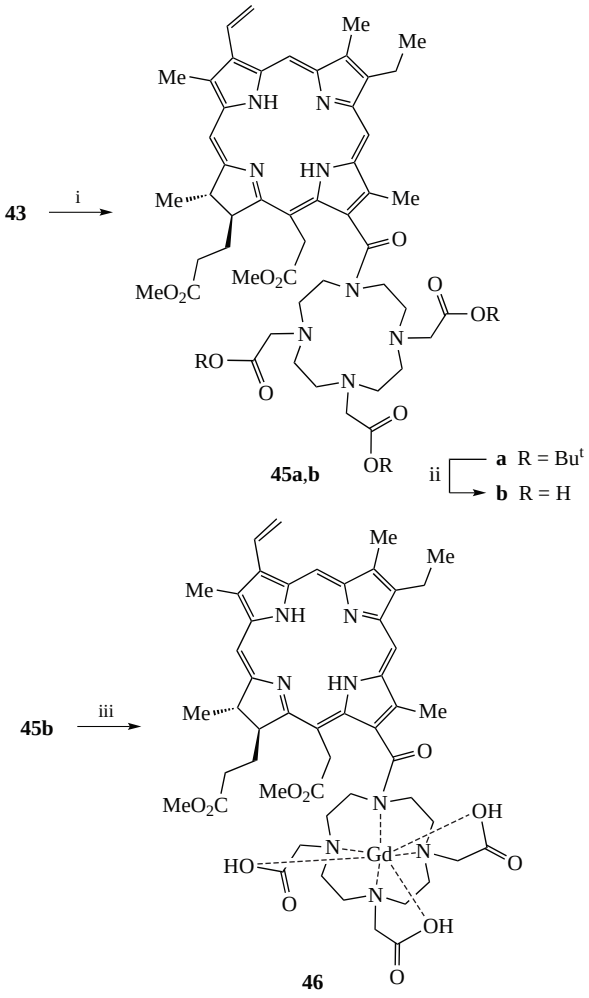
Table 1 Quantum yields of fluorescence and singlet oxygen generation for the chlorintetraazacyclododecane conjugate **43** and its Pd complex **44** in acetone.

Sample	Fluorescence[a]		Singlet oxygen[b]	
	λ_{ex}/nm	Φ_F	λ_{ex}/nm	Φ_Δ
43	400	0.266 ± 0.005	663	0.71 ± 0.05
44	395	0.0005	515	0.98 ± 0.05

[a] Fluorescence excitation wavelength (λ_{ex}), fluorescence quantum yield (Φ_F). [b] Excitation wavelength for the absorption band (λ_{ex}), quantum yield of singlet oxygen generation (Φ_Δ).

additional modification of its moiety was required, which comprised the incorporation of acetic acid residues at secondary nitrogen atoms. The described method of direct N-alkylation with chloro- or bromoacetic acids failed in our case because the mono-, di- and trisubstituted derivatives formed were inseparable due to their close chromatographic mobility. Therefore, for the alkylation of conjugate **43**, *tert*-butyl bromoacetate was applied (Scheme 13). To liberate the carboxy groups, acid hydrolysis of triester **45a** with 80% trifluoroacetic acid was carried out. To incorporate the Gd^{3+} cation, conjugate **45b** was brought into a reaction with $GdCl_3 \cdot 6H_2O$ in dichloromethane with addition of a small amount of a methanolic solution of NaOH (pH ~ 7.5) with heating to 40 °C. The presence of a Gd^{3+} cation in the tetraazacyclododecane moiety was confirmed by difference in the IR spectra of compounds **45b** and **46**, including the appearance of a band from COOGd and suppression of the COOH peak. The presence of Gd^{3+} cation in complex **46** was proved directly by atomic emission spectroscopy.

As concerns the fluorescent properties of conjugate **46**, the presence of the Gd^{3+} cation in immediate vicinity of the chlorin macrocycle extinguished the fluorescence, apparently due to the discharge of a fraction of the energy of the triplet state of chlorin to the metal. However, the existence of residual fluorescence makes it possible to predict that the resulting theranostic



Scheme 13 *Reagents and conditions*: i, $BrCH_2CO_2Bu^t$, CH_2Cl_2, DIPEA; ii, CF_3CO_2H (80% aq.); iii, $GdCl_3 \cdot 6H_2O$, CH_2Cl_2, NaOH/MeOH, 40 °C.

compound may find use in two diagnostic approaches, including magnetic resonance imaging and fluorescence diagnostics.

Thus, the spectral properties of tetraazacyclododecane–chlorin conjugates and their complexes with transition metals such as palladium and gadolinium have been studied and the prospects of their application as theranostics for non-invasive diagnostics and therapy in oncology have been demonstrated. Based on the results obtained, a large group of mono- and binuclear complexes with Zn, Cu, Mn, Ga, Pd and In in the tetrapyrrole macrocycle and with Ga, In, Gd in tetraazacyclododecane was synthesized.[74]

Conclusion

This review covers the studies in the area of chlorophyll *a* and bacteriochlorophyll *a* chemical derivatives carried out over the past 25 years at N. A. Preobrazhensky Department of Chemistry and Technology of Biologically Active Compounds, Medical and Organic Chemistry of the Russian University of Technology. Over these years, we have developed a new strategy for the functionalization of natural chlorins that allowed one to obtain highly reactive chlorophyll *a* and bacteriochlorophyll *a* derivatives for subsequent chemical modifications. Methods for synthesizing conjugates of natural chlorins with molecules of other classes based on modern organic chemistry reactions, including click chemistry (Cu-catalyzed 1,3-dipolar cycloaddition of terminal alkynes with azides), cross-coupling reactions (olefin metathesis), tetrazine–alkene addition (Inverse electron demand Diels–Alder reaction), *etc*. have been suggested. As a result of these large-scale studies, we have invented the

photosensitizer leaders that successfully passed preclinical trials and showed great potential as theranostic agents in oncology.

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