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Guanidine and Biguanidine Derivatives of Natural Chlorins: Synthesis and Biological Assessment

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Dedicated to the memory of Prof. A. F. Mironov, and Prof. G. V. Ponomarev

Targeted molecular therapy is one of the approaches in the pharmacotherapy of cancer. The targeted action on the tumor alone does not harm the healthy tissues around the tumor and the overall patient's health, thus eliminating the adverse effects that arise upon chemotherapy or radiation treatment. The targeted delivery of drugs to specific cellular targets to increase the efficiency of drugs is an urgent goal of modern medicinal chemistry. In this work, guanidine and biguanidine groups were incorporated into chlorin e_6 aminoamide in order to create two targeting photosensitizers with high photodynamic efficiency that was proved in in vivo experiments in animals with tumors of various origins (mice Ehrlich carcinoma and rat sarcoma M-1). Optimal methods were suggested for the synthesis of the desired chlorins, which provide high reaction yields under relatively mild conditions. Taking into account the broad capabilities of guanidine and biguanidine derivatives, including heterocyclization, metal chelation, etc., the pigments suggested in this article may be considered as a platform for creating multifunctional photosensitizers of chlorin series.

Keywords: Guanidines, biguanidines, chlorins, photodynamic therapy, photosensitizers, targeted molecular therapy.

Гуанидиновые и бигуанидиновые производные природных хлоринов: синтез и оценка биологических свойств

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Светлой памяти наших Учителей,
проф. Андрея Федоровича Миронова и проф. Гелия Васильевича Пономарева

Молекулярно-таргетная терапия является одним из направлений фармакотерапии рака. Направленное действие только на опухоль не наносит вреда здоровым тканям вокруг нее и здоровью больного в целом, что исключает негативные последствия, которые возникают при химиотерапии или лучевом воздействии. Целевая доставка препаратов в конкретные клеточные мишени для увеличения эффективности препаратов является актуальной задачей современной медицинской химии. В настоящей работе гуанидиновая и бигуанидиновая

группы введены в аминоамид хлорина e_6 с целью создания двух таргетных фотосенсибилизаторов, имеющих высокую фотодинамическую эффективность, доказанную в экспериментах in vivo на животных с опухолями различного генеза (карцинома Эрлиха мышей и саркома крыс М-1). Для наработки целевых хлоринов предложены оптимальные способы их получения, обеспечивающие высокие выходы реакций при сравнительно мягких условиях их проведения. Учитывая широкие возможности гуанидиновых и бигуанидиновых производных, включая гетероциклизацию, хелатирование металлов и т.д., предложенные в данной статье пигменты можно рассматривать как платформу для создания многофункциональных фотосенсибилизаторов хлоринового ряда.

Ключевые слова: Гуанидины, бигуанидины, хлорины, фотодинамическая терапия, фотосенсибилизаторы, таргетная молекулярная терапия.

Introduction

It is known that derivatives of natural chlorins are widely used as photosensitizers (PS) for photodynamic therapy (PDT) in oncology and in other fields of medicine.[1,2] The antitumor effect of PS comprises direct cytotoxic and antiangiogenic effects. The accumulation of chlorins in tumor vessels or internalization into cancer cells largely depends on the structure of pigments. For example, bacteriochlorins tend to be accumulated in the endothelium of tumor vessels,[3] whereas chlorophyll *a* derivatives may be accumulated in certain compartments of tumor cells without penetration into their nuclei, depending on the nature and charge of substituents on the macrocycle periphery.[4–6]

Compounds containing guanidine and biguanidine groups are widespread in nature and participate in many biochemical processes in cells.[7] Owing to the biogenic nature of the above groups, they are contained in the structure of many anticancer, antiviral, antimicrobial, and other drugs.[8–10] The ability of guanidine (biguanidine) groups to be protonated under physiological conditions results in generation of a positive charge and high basicity in compounds that contain these groups. The biological activity profile of such compounds is very broad, including arginine amino acid, aminoglycoside antibiotics, *e.g.*, streptomycin, sulfanilamide antimicrobial drug sulgin, biguanides – hypoglycemic drugs used in diabetes mellitus, *etc*.

Studies by Matti *et al.* demonstrated the ability of delivery systems based on inositol and sorbitol modified with guanidine groups to overcome the blood-brain barrier and to be accumulated in mitochondria. These capabilities are promising for the intracellular targeted delivery of anticancer drugs.[11] The ability of positively charged groups to be bound to heterocyclic bases in DNA and RNA is well known and is used in the development of intercalating anticancer drugs based on anthracycline antibiotics.[12,13] In those works, the role of the latter in increasing the binding efficiency has been shown by comparison of DNA intercalator agents containing amino or guanidine groups.

Biguanides used for the treatment of type 2 diabetes as antihyperglycemic drugs, including metformin and its analogs, showed anti-tumor activity.[14] Two mechanisms of their impact on carcinogenesis have been suggested. The direct mechanism involves inhibition of important enzymes that regulate the processes of glycogenesis and lipogenesis, while the indirect one is due to the effect of biguanides on hepatocytes.[15]

Thus, incorporation of guanidine and biguanidine groups into the structure of pigment molecules is one of the ways to increase the selectivity of PS accumulation, both in intracellular compartments and in the entire tumor focus.

In this work, we consider various methods for incorporating guanidine and biguanidine groups into the structure of chlorin e_6 13^1-*N*-(4-aminobutyl)amide that we have obtained and reported previously.[16]

Experimental

Materials and Methods

The reagents used in this work included 1*H*-pyrazole-1-carboxamidine (Sigma-Aldrich, USA), ethyldiisopropylamine (Sigma-Aldrich, USA), Cyanamide (Sigma-Aldrich, USA), Dicyandiamide (Sigma-Aldrich, USA), and Bis-Boc-thiourea (Sigma-Aldrich, USA). Solvents were purified and prepared by standard procedures. A Discover Proteomics microwave reactor (CEM Corporation) was used to synthesize compound **5**. Thin layer chromatography was performed on Kieselgel 60 F254 plates (Merck, Germany). The laboratory procedures for drying the compounds under reduced pressure were performed using a Rotavapor® R-300 rotary evaporator (Switzerland). The synthesis of 13^2-(5-biguanidylbutanamido)chlorin e_6 was carried out in a Biotage Initiator 2.0 microwave reactor (Biotage AB, Sweden). Absorption and fluorescence spectra were recorded on Shimadzu UV1800 UV/VIS spectrophotometer (Shimadzu, Duisburg, Germany). Absorption spectra were recorded in the range of 300–750 nm. ^{1}H and ^{13}C NMR spectra were recorded in deuterochloroform (chloroform-*d*, Sigma-Aldrich, USA) using a Bruker DPX-300 spectrometer (Germany) with a working frequency of 300 MHz. All spectral studies were performed at 25 °C. Analysis of the compounds obtained was carried out using a Vanquish ultra-high-performance liquid chromatograph (Thermo Scientific, USA) combined with a Q-exactive high-resolution hybrid mass spectrometer (Thermo Scientific, USA). The target compounds were isolated from the reaction mixtures using an ActaPure 25 preparative chromatographic system (Cytiva, Sweden) comprising a binary pump with a high pressure gradient of the mobile phase, an injector for sample injection with a 0.5 mL feeding loop, a monochromatic detector with detection of electromagnetic radiation absorption at a wavelength of 220 nm, and a fraction collector for automatic sampling. Preparative chromatographic separation was carried out in a Biotage Snap Discoveri C18 preparative column 120 mm long, 25 mm inner diameter, filled with a sorbent with a particle diameter of 10 μm. A 0.1 % formic acid solution in deionized water was used as component A of the mobile phase. A 0.1 % formic acid solution in acetonitrile was used as component B of the mobile phase.

Synthesis of 13²-(5-guanidylbutylmido)chlorin e_6 **2**

Methods using thiourea. Boc-protected thiourea (100 mg, 0.36 mmol) was added to a solution of chlorin e_6 13¹-*N*-(4-aminobutyl)amide **1** (70 mg, 0.1 mmol) in 3 mL of dichloromethane. The reaction was performed for 40 h with vigorous stirring in the presence of a catalyst, namely, mercury(II) chloride or copper(II) chloride (0.03 mmol). The reaction was monitored chromatographically. The intermediate product was isolated from the reaction mixture by extraction with a dichloromethane/water mixture. The bottom dark green organic layer was separated and washed with water. The top light green aqueous layer was extracted with dichloromethane until complete discoloration. The extracts were combined and dried with anhydrous sodium sulfate. After that, the conjugate was redissolved in 3 mL of dichloromethane, and 10 mL of 20 % trifluoroacetic acid solution in dichloromethane was added. The reaction was performed for 3 hours with stirring under an inert argon atmosphere. The product was isolated from the reaction mixture by extraction with a dichloromethane/water mixture. The bottom dark green organic layer was separated and washed with water. The top light green aqueous layer was extracted with dichloromethane until complete discoloration. The extracts were combined and dried with anhydrous sodium sulfate. Thereafter, the compound was purified by preparative TLC. The yield of **2** was 35 % in the presence of mercury(II) chloride, or 22 % in the presence of copper(II) chloride.

A method using cyanamide. Cyanamide (2.5 mmol) predissolved in 1.5 mL of methanol was added to a solution of chlorin e_6 13¹-*N*-(4-aminobutyl)amide **1** (70 mg, 0.1 mmol) in 4 mL of *N,N*-dimethylformamide. After that, 800 μL of 12M hydrochloric acid solution was added. The reaction was performed for 48 hours with vigorous stirring and with heating to 70 °C. The product was isolated from the reaction mixture by extraction with a dichloromethane/water mixture. The lower dark green organic layer was separated and washed with water. The upper light green aqueous layer was extracted with dichloromethane until complete discoloration. The extracts were combined and dried with anhydrous sodium sulfate. Thereafter, the compound was purified by preparative TLC. The yield of **2** was 43 %.

Methods using pyrazole-1-carboxyamidine. Pyrazol-1*H*-carboxyamidine (0.88 mmol) and ethyldiisopropylamine (0.09 mmol) were added to a solution of chlorin e_6 13¹-*N*-(4-aminobutyl)amide **1** (70 mg, 0.1 mmol) in 3 mL of dimethylsulfoxide. The reaction was performed for 8 hours with vigorous stirring and heating at 60 °C in an inert argon atmosphere. The product was isolated from the reaction mixture by repeated extraction in a dichloromethane/water mixture. The extracts were combined and dried with anhydrous sodium sulfate. Thereafter, the compound was purified by preparative TLC. The yield of **2** was 90 %.

In the other two methods used, the composition of solvents, temperature conditions and reaction times were changed (Table 1), while the amounts of the starting compound **1**, ethyldiisopropylamine and pyrazole-1*H*-carboxyamidine were the same.

^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: 9.68 (H, s, 10-H), 9.63 (H, s, 5-H), 9.41 (3H, br.s, 13⁹-NH_2, 13¹⁰-NH), 8.81 (H, s, 20-H), 8.07 (H, dd, *J* = 17.8 Hz, 11.5 Hz, 3¹ -H), 7.40 (H, m, 13⁷-NH) 6.98 (H, t, *J* = 5.2 Hz, 13²-NH), 6.33 (H, dd, *J* = 17.8 Hz,1.4 Hz, E-3²-H), 6.11 (H, dd, *J* = 11.5 Hz, 1.4 Hz, Z-3² -H), 5.55 (H, d, *J* = 18.9 Hz, 15-CH_2^a), 5.25 (H, d, *J* = 18.9 Hz, 15-CH_2^b), 4.47 (H, m, 18-H), 4.35 (H, m, 17-H), 3.80 (2H, m, 8¹-CH_2), 3.79 (3H, s, 15²-$COOCH_3$), 3.61 (3H, s, 12¹-CH_3), 3.54 (3H, s, 17³-$COOCH_3$), 3.49 (3H, s, 2¹-CH_3), 3.48 (2H, m, 13³-CH_2), 3.30 (3H, s, 7¹-CH_3), 2.78 (2H, m, 13⁶-CH_2), 2.53 (H, m, 17²-CH_2^a), 2.23 (H, m, 17¹-CH_2^a), 2.17 (H, m, 17²-CH_2^b), 1.81 (H, m, 17¹-CH_2^b), 1.70 (3H, d, *J* = 7.1 Hz, 18-CH_3), 1.62 (3H, t, *J* = 7.6 Hz, 8²-CH_3), 1.26 (4H, m, 13⁴ -13⁵ -CH_2), –1.63 (H, br.s, I-NH), –1.85 (H, br.s, III-NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C ppm: 173.8, 173.6, 168.9, 168.3, 167.1, 156.8, 153.7, 148.9, 144.6, 138.7, 135.9, 135.1, 134.3, 134.2, 129.9, 129.7, 128.5, 127.5, 121.2, 102.1, 101.4, 98.6, 93.8, 53.1, 51.9, 49.3, 38.9, 37.4, 32.1, 31.3, 29.6, 25.1, 23.8, 22.9, 19.4, 17.8, 11.9, 11.3, 10.9. MS *m/z* $[M+H]^+$ calculated for $C_{41}H_{52}N_8O_5$ + H 737.4133; found: 737.4118; $[M+2H]^{2+}$ calculated for $C_{41}H_{52}N_8O_5$ + 2H 369.2105; found: 369.2097.

Synthesis of 13²-(5-biguanidylbutylmido)chlorin e_6 **5**

A method using N-amidinopyrazole-1H-carboxyamidine. Pyrazole-1*H*-carboxyamidine **3** (152 mg, 1 mmol) was dissolved in 2 mL of dimethyl sulfoxide with addition of ethyldiisopropylamine (0.1 mmol). The reaction was performed for 24 hours with vigorous stirring and heating at 60 °C. The reaction mixture that remained once the solvent was distilled off was an oily substance. Therefore, product **4** was isolated by recrystallization from a mixture of methanol and diethyl ether. The yield of **4** was 94 %. *N*-Amidinopyrazole-1-carboxyamidine (0.88 mmol) was added to a solution of chlorin e_6 13¹-*N*-(4-aminobutyl)amide **1** (70 mg, 0.1 mmol) in 3 mL of dimethyl sulfoxide. Moreover, ethyldiisopropylamine (0.09 mmol) was added. The reaction was performed for 8 h with vigorous stirring and heating at 60 °C under an inert argon atmosphere. After the reaction products were isolated by extraction, product **5** was purified by preparative TLC. The yield of **5** was 74 %.

Methods using dicyandiamide. Method 1. Dicyandiamide (1.1 mmol) was added to a solution of chlorin e_6 13¹-*N*-(4-aminobutyl)amide **1** (70 mg, 0.1 mmol) in 5 mL of ethanol. The reaction was performed for 16 hours with heating at 70 °C. After the reaction products were isolated by extraction, product **5** was purified by preparative TLC. The yield of **5** was 37 %. *Method 2.* Dicyandiamide (1.1 mmol) was added to a solution of chlorin e_6 13¹-*N*-(4-aminobutyl)amide **1** (70 mg 0.1 mmol) in 4.5 mL of dimethylsulfoxide. Moreover, ethyldiisopropylamine (0.09 mmol) and copper(II) sulfate (0.05 mmol) were added. The reaction was performed for 48 hours at room temperature. After the reaction products were isolated by extraction, product **5** was purified by preparative TLC. The yield of **5** was 22 %. *Method 3.* Dicyandiamide (1.1 mmol) was added to a solution of chlorin e_6 13¹-*N*-(4-aminobutyl)amide **1** (70 mg, 0.1 mmol) in 4 mL of 1,4-dioxane. Moreover, 350 μL of 2M hydrochloric acid solution and 0.05 mmol of iron(III) chloride were added. The reaction was performed for 24 hours with heating at 70 °C. After the reaction products were isolated by extraction, product **5** was purified by preparative TLC. The yield of **5** was 23 %.

A method using dicyandiamide under microwave irradiation conditions. Dicyandiamide (0.08 mmol) was added to a solution of chlorin e_6 13¹-*N*-(4-aminobutyl)amide **1** (50 mg 0.072 mmol) in 2.5 mL of acetonitrile. Moreover, 0.08 mmol of trimethylchlorosilane and 0.24 mmol of isopropanol were added. The reaction was performed for 15 minutes with heating at 140 °C, with vigorous stirring and irradiation with adjustable power in the range of 0–400 W, at 2.45 GHz using a Biotage® Initiator 2.0 microwave reactor. After the reaction products were isolated by extrac-

Table 1. Conditions for synthesis of 13²-(5-guanidylbutylamido)chlorin e_6.

Solvent	Temperature, °C	Reaction time, h	Yield of compound **2**, %
N,N-Dimethylformamide	25	16	57
Acetonitrile	80	16	67

tion, product **5** was purified by preparative TLC. The yield of **5** was 72 %.

^{1}H NMR (300 MHz, $CDCl_3$) δ_H ppm: 9.69 (H, s, 10-H), 9.61 (H, s, 5-H), 9.41 (3H, br.s, 13^9-NH_2, 13^{10}-NH), 8.83 (H, s, 20-H), 8.26 (3H, br.s, 13^{12}-NH, 13^{13}-NH_2) 8.07 (H, dd, J = 17.8 Hz, 11.5 Hz, 3^1 -H), 7.40 (H, m, 13^7-NH) 6.95 (H, t, J = 5.2 Hz, 13^2 -NH), 6.3 (H, dd, J = 17.8 Hz,1.4 Hz, E-3^2 -H), 6.11 (H, dd, J = 11.5 Hz, 1.4 Hz, Z-3^2 -H), 5.55 (H, d, J = 18.9 Hz, 15-CH_2^a), 5.25 (H, d, J = 18.9 Hz, 15-CH_2^b), 4.41 (H, m, 18-H), 4.35 (H, m, 17-H), 3.83 (2H, m, 8^1 -CH_2), 3.78 (3H, s, 15^2-$COOCH_3$), 3.6 (3H, s, 12^1-CH_3), 3.54 (3H, s, 17^3-$COOCH_3$), 3.49 (3H, s, 2^1-CH_3), 3.48 (2H, m, 13^3-CH_2), 3.30 (3H, s, 7^1-CH_3), 2.75 (2H, m, 13^6-CH_2), 2.53 (H, m, 17^2-CH_2^a), 2.23 (H, m, 17^1-CH_2^a), 2.17 (H, m, 17^2 -CH_2^b), 1.84 (H, m, 17^1-CH_2^b), 1.73 (3H, d, J = 7.1 Hz, 18-CH_3), 1.62 (3H, t, J = 7.6 Hz, 8^2-CH_3), 1.28 (4H, m, 13^4 -13^5 -CH_2), –1.61 (H, br.s, I-NH), –1.83 (H, br.s, III-NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C ppm: 173.9, 173.8, 169.2, 168.4, 167.2, 156.6, 155.7, 153.9, 148.7, 144.7, 138.3, 136, 135.4, 134.8, 134.4, 134.3, 129.9, 129.8, 128.7, 127.9, 121.2, 102.7, 101.4, 98.6, 93.7, 53.3, 52.3, 49.4, 39.6, 38.3, 32.1, 31.4, 29.7, 27.5, 23.0, 22.8, 19.3, 17.7, 11.9, 11.3, 10.9. MS *m/z*: $[M+2H]^{2+}$ calculated for $C_{42}H_{54}N_{10}O_5$ + 2H 390.2178; found: 390.2179.

*Isolation and identification of compounds **2** and **5** by chromatographic methods*

Table 2 lists the conditions used for the preparative isolation of the target compounds from the reaction mixtures.

The target fractions were collected into "Cellstar" polypropylene tubes of 50 mL capacity, cat. No. 210261. The collected fractions were combined into a round-bottom flask of 250 mL capacity, then concentrated *in vacuo* in a Rotavapor®R-300 rotary evaporator at a temperature of 40 °C and a rotation speed of 60 rpm. The fraction was concentrated to a volume of ~5–10 mL, then a 0.1 mL aliquot was taken from it and analyzed by ultra-high-performance liquid chromatography with tandem high-resolution mass spectrometric detection. The rest of the fraction was transferred into 15 mL dark glass tubes and evaporated to dryness in a nitrogen flow at room temperature.

Samples of purified fractions of the reaction mixture were analyzed in a Vanquish liquid chromatographic system coupled with a Q-Exactive HF-X high-resolution hybrid mass spectrometer.

The sample components were separated in a "Pyramid" reverse-phase column 75 mm long and with 2 mm inner diameter, with a sorbent particle diameter of 1.8 μm (Macherey-Nagel, Germany).

A solution of HPLC grade formic acid (Fluka, cat. No. 56302-1L), acetonitrile and Mili Q deionized water (18.2 S) in a volume ratio of 0.1/5/95 % was used as component A of the mobile phase. A solution of HPLC grade formic acid and acetonitrile (Panreac, USA, cat. No. 221881.1611) in a volume ratio of 0.1/95 % was used as component B of the mobile phase. The chromatographic separation parameters are presented in Table 3.

Compounds were analyzed in the positive ion detection mode with electrospray ionization at atmospheric pressure.

Table 2. Parameters of chromatographic separation in the Acta Pure preparative chromatographic system for the isolation of the target compounds from the reaction mixtures

Elution mode	Gradient		
Mobile phase flow rate, mL/min	25		
Gradient of mobile phase (MP) composition variation	Time, min	MP A content. %	MP B content, %
	0.00	90	10
	2.30	90	10
	8.00	0	100
	12.00	0	100
	12.01	90	10
Volume of a sample aliquot injected into the column, μL	500		
Target fraction collection interval, min.	8.9–9.5		
Separation time, min	14.5		

Table 3. Parameters of chromatographic separation of the samples being analyzed

Elution mode	Gradient		
Mobile phase (MP) flow rate, mL/min	0.500		
Gradient of variation in the mobile phase (MP) composition	Time, min	MP A content	MP B content, %
	0.00	95	5
	1.00	95	5
	9.00	5	95
	12.00	5	95
	12.01	95	5
Column thermostat temperature, °C	40		
Volume of the aliquot sample applied on the column, μL	3		
Analysis time, min	15		

Table 4. Working parameters of the mass spectrometer ionization source

No.	Parameter	Value, meas. unit
1.	Atomizing capillary voltage	4.0 kV
2.	Atomizing gas consumption	35 arb. units
3.	Auxiliary gas consumption	15 arb. units
4.	Drying gas consumption	5 arb. units
5.	Atomizing capillary temperature	200 °C
6.	Temperature of the mass spectrometer's inlet capillary	350 °C
7.	Auxiliary gas temperature	200 °C
8.	Ion optics input lens voltage	50 arb. units

Table 5. Working parameters of the mass spectrometer modes in the analysis of the target compounds

Mass spectrometer working mode	Adjustable working parameter	Parameter value
Full Scan MS	Resolution	70,000 rel. units
	Scanning range of parent ions' *m/z*	300–1800 Da
	Time of mode operation	100 ms
	Time of accumulation of precursor ions in the ion trap	100 ms
	Maximum allowable loading of precursor ions in the ion trap	5e 6, rel. units
PRM	Resolution	70,000 rel. units
	Maximum accumulation time of fragment ions in the ion trap	500 ms
	Maximum allowable loading of ions in the ion trap	5e 6, rel. units
	Scanning range of fragment ions' *m/z*	50–1000 Da
	Dissociation energy of precursor ions in the collision cell (NCE)	22 arb. units

Table 4 presents the parameters of the mass spectrometer's ionization source.

Compounds were detected in two mass spectrometer modes that switched sequentially:

1) Full Scan MS Positive/Negative (Full Scan) – scanning the full current of positive/negative precursor ions;

2) Parallel Reaction Monitoring (PRM) – scanning the total current of fragment ions formed upon dissociation of preselected precursor ions. The parameters of the mass spectrometer's working modes are presented in Table 5.

Results and Discussion

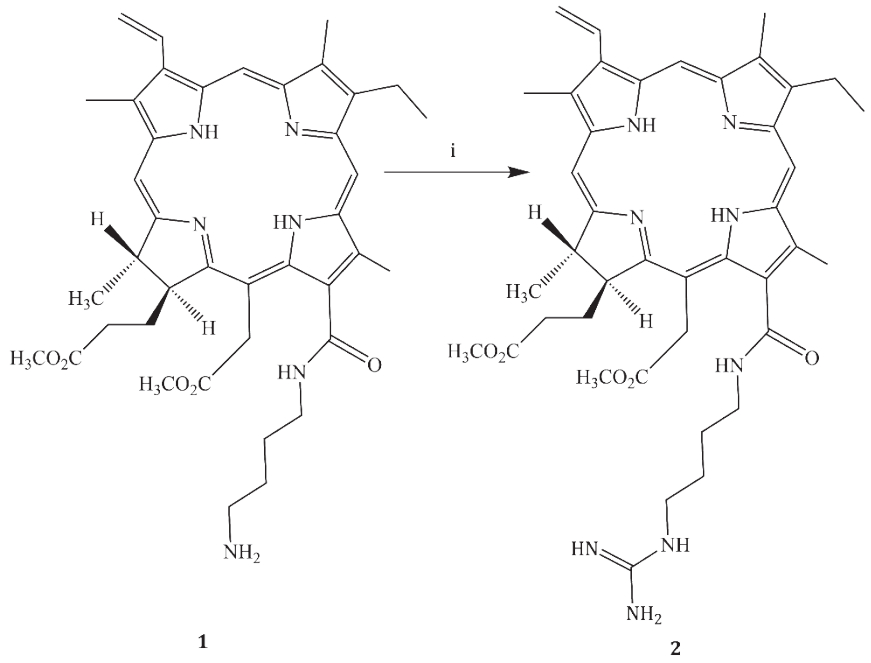
Chlorin e_6 aminoamide containing a terminal amino group **1** was the key compound for the synthesis of guanidine and biguanidine derivatives. Many methods for the incorporation of the above groups into organic amines are reported in literature, but not all of those proved to be sufficiently suitable in our case.

Based on the assessment of the guanylation and biguanylation yields and conditions of these reactions, the most optimal approaches for synthesizing the target compounds have been suggested.

The reported synthesis method using di-Boc-protected thiourea and copper or mercury chlorides as catalysts[17–18] did not give satisfactory yields of the target product **2** (Table 6, Scheme 2). Apparently, this method has low efficiency because it requires that the reaction mixture be treated with a trifluoroacetic acid solution, hence the nitrogen atoms of the macrocycle and guanidine group are protonated, which significantly complicates the chromatographic purification of product **2**. In addition, there is a high probability of chlorin metallation with metal salts, which leads to the formation of the corresponding metal complexes as side products.

Yet another group of methods that we used to incorporate a guanidine group into the chlorin structure involves the reaction of chlorin e_6 13^1-*N*-(4-aminobutyl)amide **1** with pyrazole-1-carboxyamidine.[19–21] The effect of the solvent and reaction temperature on the yield of the target product **2** was studied. It was shown that the highest degree of conversion was achieved if dimethylsulfoxide was used as the solvent and the temperature was no lower than 60 °C (Table 6, Scheme 1).

Moreover, a method for incorporating a guanidine moiety using cyanamide as the electrophilic agent is known from literature[22] (Table 6, Scheme 1). However, the low yield of the target product **2** did not allow this approach to be used for the preparative synthesis of a guanidine-containing chlorin in this case, either. Like in the scheme where pro-



1 **2**

Scheme 1. Scheme for the synthesis of 13^2-(5-guanidylbutylmido)chlorin e_6.
i = a. 1) *N,N*'-di-*tert*-butoxycarbonylthiourea, $HgCl_2$, CH_2Cl_2, 25 °C, 40 h; 2) CF_3COOH, CH_2Cl_2;
b. 1) *N,N*'- di-*tert*-butoxycarbonylthiourea, $CuCl_2$, CH_2Cl_2, 25 °C, 24 h; 2) CF_3COOH, CH_2Cl_2;
c. 1*H*-pyrazole-1-carboxyamidine, DIPEA, DMSO, 60 °C, 8 h;
d. 1*H*-pyrazole-1-carboxyamidine, DIPEA, DMF, 25 °C, 16 h;
e. 1*H*-pyrazole-1-carboxyamidine, DIPEA, CH_3CN, 80 °C, 16 h;
f. Cyanamide, 12M HCl, DMF, 70 °C, 48 h.

Table 6. Conditions and yields of reactions for incorporation of a guanidine group into chlorin e_6.

Reagent	Reaction conditions	Reaction time, hours	Temperature, °C	Catalyst	Yield, %
Thiourea	1) CH_2Cl_2, 2) CF_3COOH	40	25	$HgCl_2$	35
	1) CH_2Cl_2, Et_3N, 2) CF_3COOH	24	25	$CuCl_2$	22
Pyrazole-1-carboxyamidine	DMSO, DIPEA	8	60	–	90
	DMF, DIPEA	16	25	–	57
	CH_3CN, DIPEA	16	80	–	67
Cyanamide	DMF, CH_3OH, 12M HCl	48	70	–	43

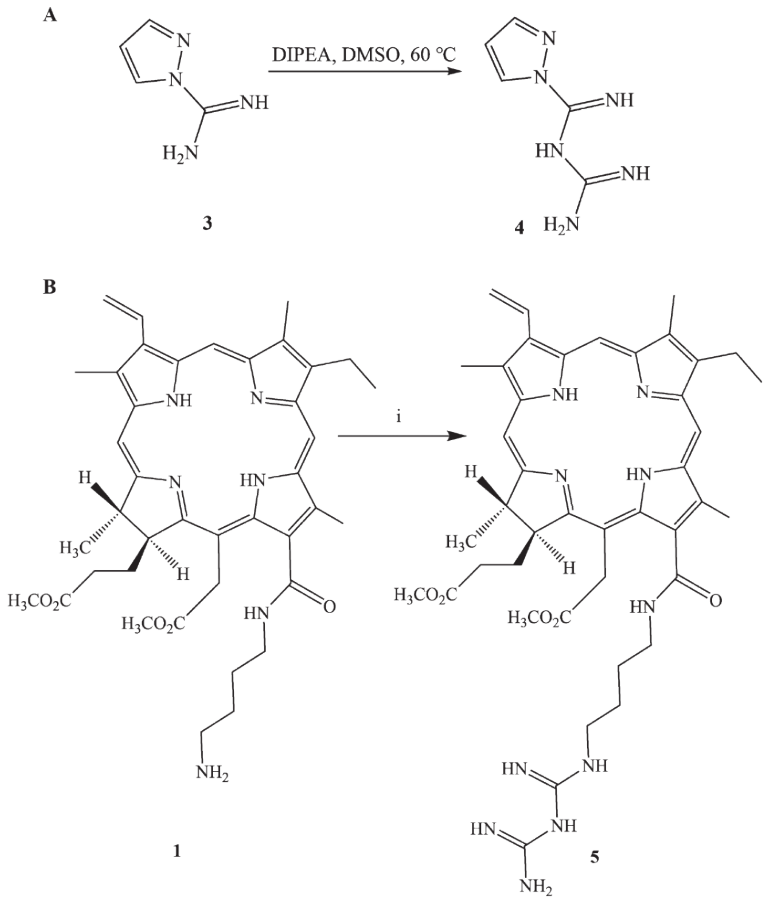
tected thiourea and treatment with trifluoroacetic acid are used, this reaction scheme uses hydrochloric acid required to activate cyanamide, which adversely affects the subsequent isolation and purification of chlorin **2**.

The conditions and yields of reactions for incorporation of a guanidine group into chlorin e_6 are presented in Table 6. Based on these data, the reaction using pyrazole-1-carboxyamidine with heating to 60 °C for 8 hours was found to be the optimal approach.

The conditions for the isolation and purification of target compound **2** are described in detail in Experimental. The structure of compound **2** was confirmed using ^{1}H and ^{13}C NMR spectroscopy and mass spectrometry methods. The spectra are presented in the Supplementary section.

As noted earlier, chlorin e_6 13^1-*N*-(4-aminobutyl) amide was chosen as the initial pigment for incorporating the biguanidine group. The reaction of the former with dicyandiamide should have led to biguanidine derivative **5**[23–28] (Table 7, Scheme 3).

Performing the reaction under various conditions, such as refluxing in ethanol and use of dimethylsulfoxide or dioxane as the solvents, did not allow us to achieve significant yields of the target product **5**. Therefore, a previously unreported scheme for incorporation of biguanidine into amines by the reaction with the pre-synthesized *N*-amidinopyrazole-1*H*-carboxyamidine **4** was developed. The latter was obtained by a well-known technique[29,30] involving pyrazole ring cleavage during heating



Scheme 4. A: Scheme for the synthesis of *N*-amidinopyrazole-1*H*-carboxyamidine; B: Scheme for the synthesis of 13^2-(5-biguanidylbutylmido)chlorin e_6.

i = a. *N*-amidinopyrazole-1*H*-carboxyamidine, DIPEA, DMSO, 60 °C, 8 h;
b. Dicyandiamide, C_2H_5OH, 70 °C, 16 h;
c. Dicyandiamide, DIPEA, $CuSO_4$, DMSO, 25 °C, 2 h;
d. Dicyandiamide, 2M HCl, $FeCl_3$, 1,4-dioxane, 70 °C, 24 h;
e. Dicyandiamide, TMSCl, CH_3CN, iPrOH, microwave, 140 °C, 15 min.

Table 7. Conditions and yields of reactions for incorporation of a guanidine group into chlorin e_6

Reagent	Reaction conditions	Reaction time, hours	Temperature, °C	Catalyst	Yield, %
N-amidinopyrazole-1*H*-carboxyamidine	DMSO, DIPEA	8	60	–	74
Dicyandiamide	C_2H_5OH, reflux	16	70	–	37
	DMSO, DIPEA	2	25	$CuSO_4$	22
	1,4-Dioxane, 2M HCl	24	70	$FeCl_3$	23
	AcN, iPrOH	0.25	140, microwave irradiation	TMSCl	72

in DMSO for 24 hours with high yield (94 %). The method for synthesizing **5** using dicyanamide, trimethylchlorosilane as the catalyst, and microwave irradiation proved to be rather efficient[28] (Table 7, Scheme 5).

As we expected, the spectral characteristics of the starting aminoamide **1** and its guanidine and biguanidine derivatives **2** and **5**, respectively, were found to be identical, since incorporation of these functional groups far on the macrocycle periphery does not affect the conjugated electronic system of the latter (Figure 1).

To study the biological activity, pigments **2** and **5** highly purified by means of HPLC were prepared and characterized by high-resolution mass spectrometry, ^1H and ^{13}C NMR spectroscopy (see Supplementary section).

Figure 2 demonstrates the chromatogram of the reaction mixture in the synthesis of conjugate **2**.

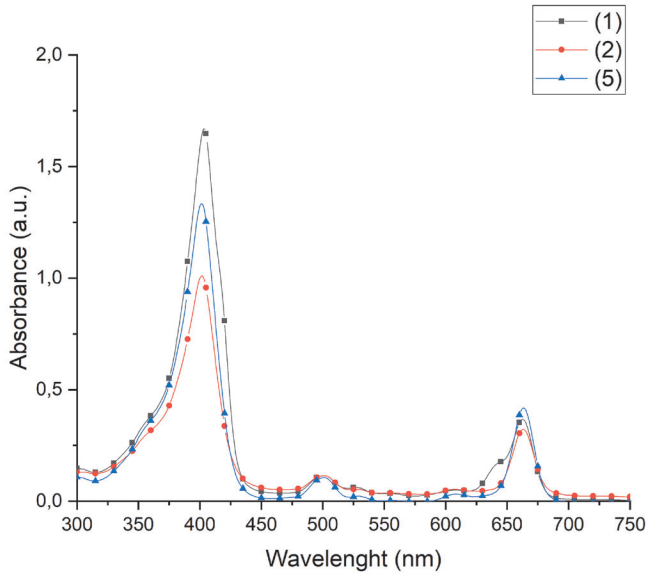


Figure 1. Absorption spectra of compounds **1**, **2** and **5**. The absorption spectra were obtained at the same solution concentrations and with dichloromethane as the solvent.

To obtain the most reliable data, compounds were detected in two operation modes of the mass spectrometer switched sequentially. Figure 3 shows the mass chromatogram of a chlorin **2** sample.

Figure 4 shows the mass chromatogram and the first order mass spectrum of biguanidyl chlorin **5**.

The mass spectral characteristics obtained make it possible to perform a reliable identification of the declared compounds **2** and **5**.

The *in vivo* studies conducted in animals with transplanted malignant tumors (mice Ehrlich carcinoma and rat sarcoma M-1) demonstrated a high antitumor efficiency of PDT with chlorin e_6 guanidine and biguanidine derivatives **2** and **5**, respectively, at the following laser irradiation parameters: E = 150 J/cm^2, Ps = 0.48 W/cm^2. For both PS, complete tumor regression of mice Ehrlich carcinoma was achieved at a dose of 1.25 mg/kg (100 % cure rate) and a considerable efficiency (60–80 % cure rate), at a dose of 0.70 mg/kg. In the case of rat sarcoma M-1, 100 % cure was observed in animals on day 90 after PDT at a dose of 2.5 mg/kg PS.

Conclusions

In this work, mono- and biguanidine derivatives of chorin e_6 were obtained for the first time and efficient methods for their preparation were suggested and optimized. Using the HPLC method, highly purified samples of pigments **1**, **2**, and **5** were obtained. Biological tests of these compounds showed a high photodynamic efficiency at much lower doses (1.25 and 0.75 mg/kg) than those generally used in experiments with animals. Moreover, functional-

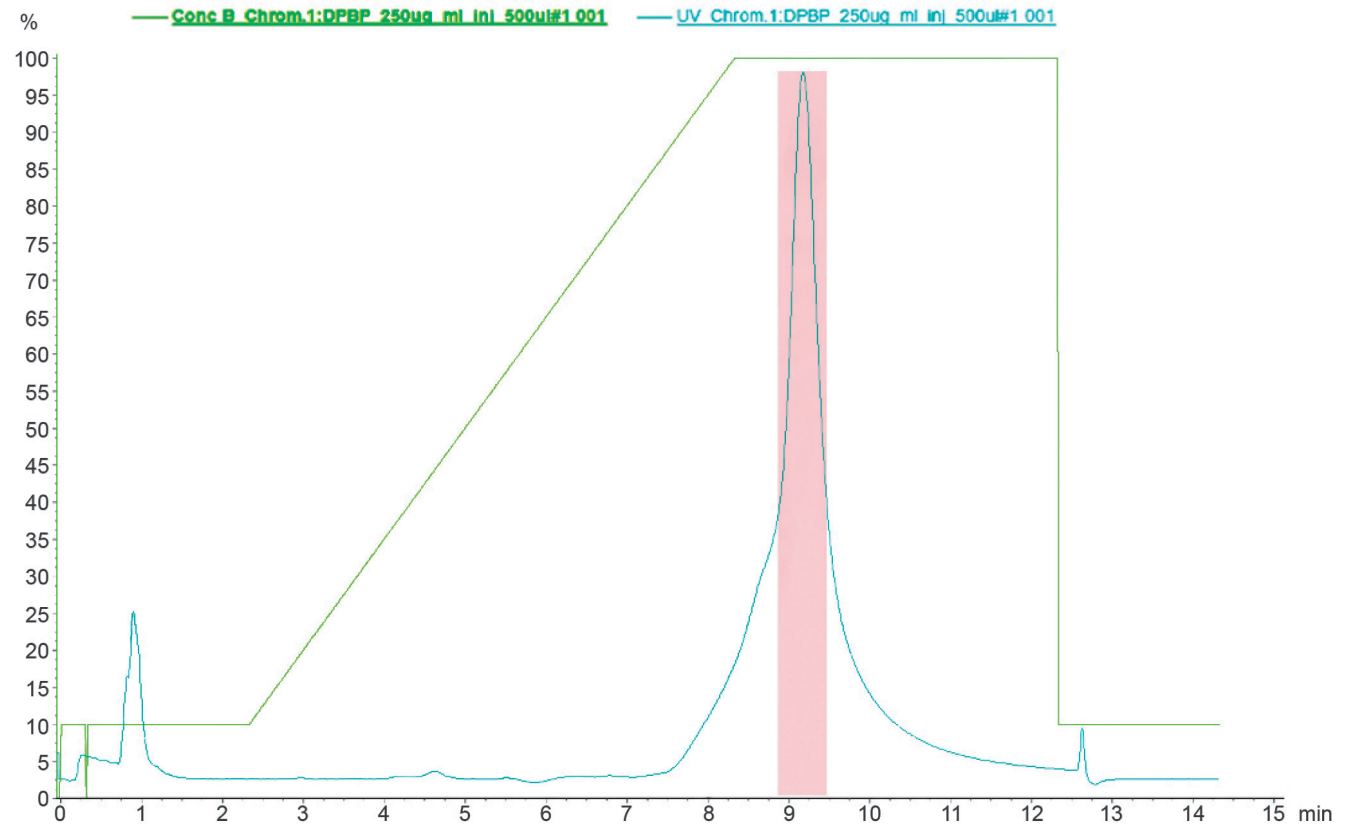


Figure 2. Chromatogram of preparative isolation of 13^2-(5-guanidylbutylamido)chlorin e_6 from the reaction mixture performed using the "Acta Pure" chromatographic system.

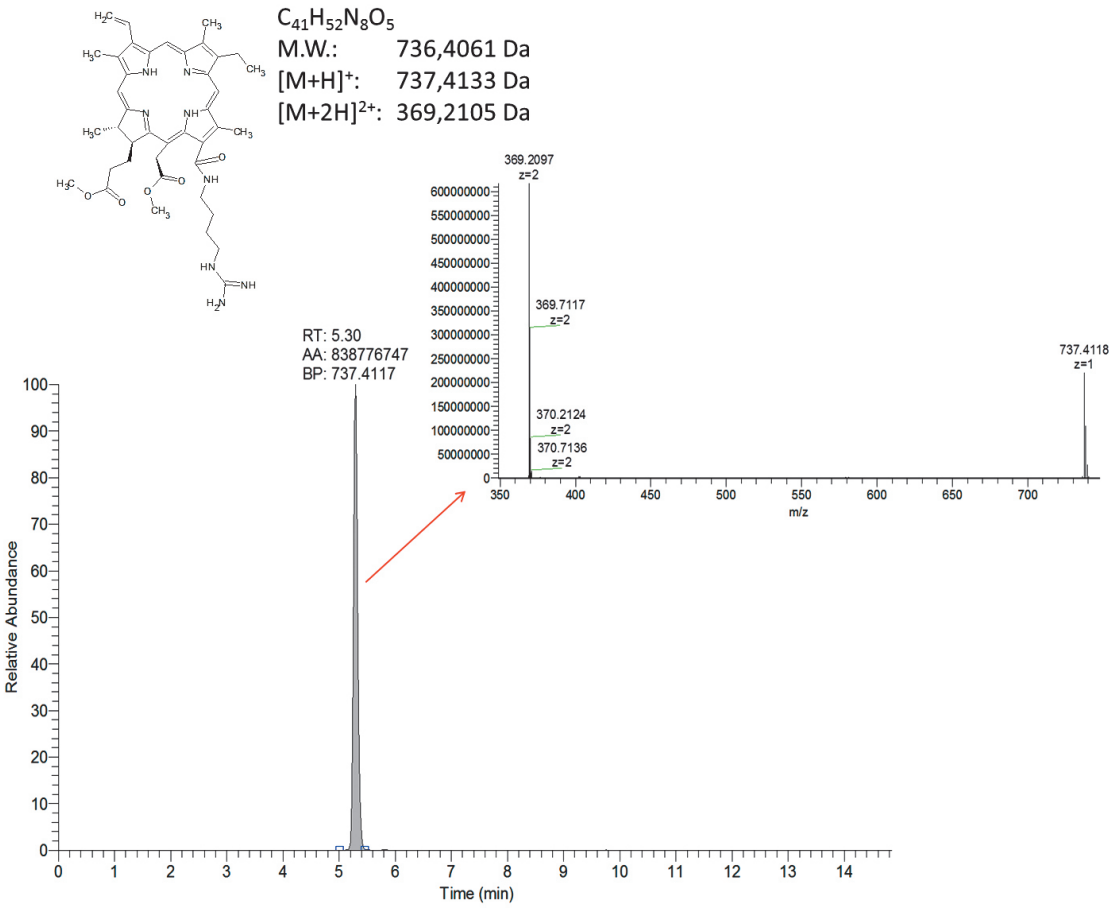


Figure 3. Mass chromatogram of a sample of guanidyl chlorin **2** from the reaction mixture. The retention time of the target compound is 5.30 min, *m/z* $[M+H]^+$ = 737.4118, $[M+H]^{2+}$ = 369.2097.

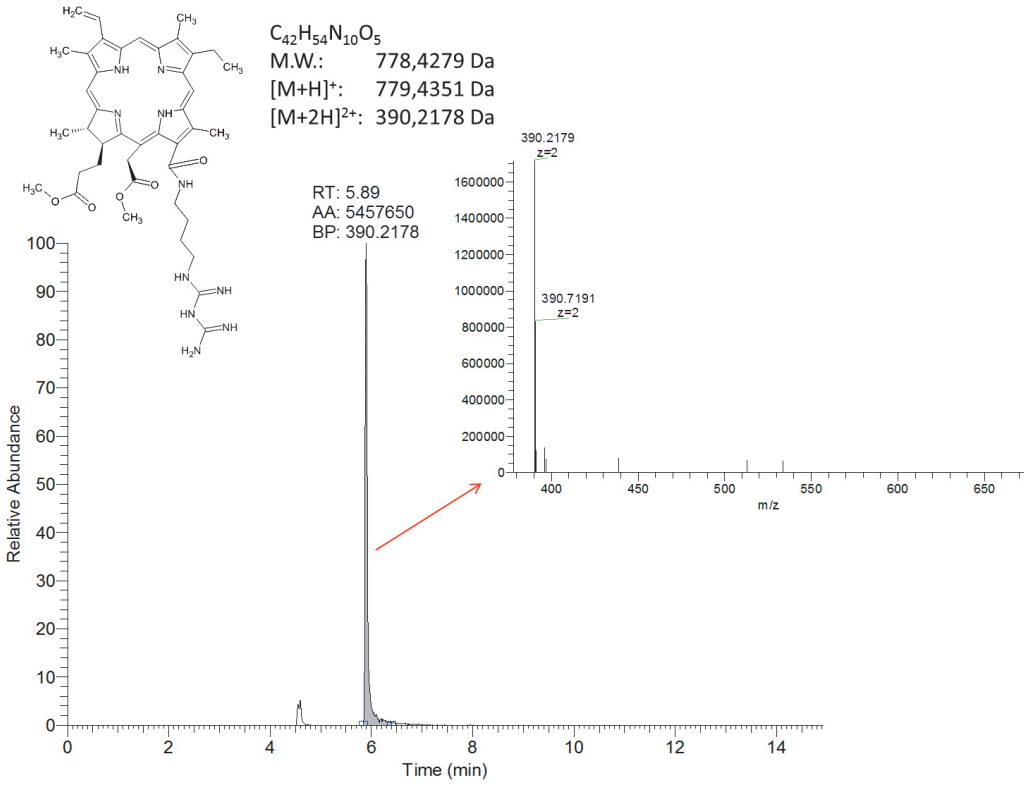


Figure 4. Mass chromatogram of a sample of the reaction mixture in the synthesis of 13^2-(5-biguanidylbutylamido)-chlorin e_6. The retention time of the target compound is 5.89 min, *m/z* $[M+H]^{2+}$ = 390.2178.

ized chlorins **2** and **5** were found to be more efficient than the starting chlorin e_6 aminoamide.

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