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Synthesis of cyclic norspermidine derivative of protohemin IX

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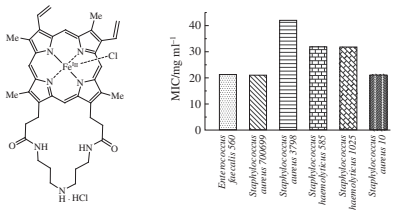
A simple synthesis of cyclic norspermidine derivative of protohemin IX without the use of selective protecting groups has been developed. The obtained water-soluble protohemin IX derivative has antibacterial activity and is non-toxic at a concentration of 250 μg ml^{-1}.



The resistance of microorganisms to antibiotics now becomes one of the global problems of medicine. The situation is complicated by the emergence of new multidrug-resistant bacteria and their ability to distribute resistance to other bacteria through horizontal gene transfer.[1] Therefore, the search for new effective antiseptic and therapeutic agents with antimicrobial properties, as well as the development of methods for their synthesis, becomes an urgent task for biologists and chemists. Currently, the search for such agents is carried out starting from various types of chemical compounds including natural ones. For this purpose, *e.g.*, cyclic antimicrobial peptides[2] and porphyrins modified by various substituents for applications in photodynamic therapy were investigated.[3,4]

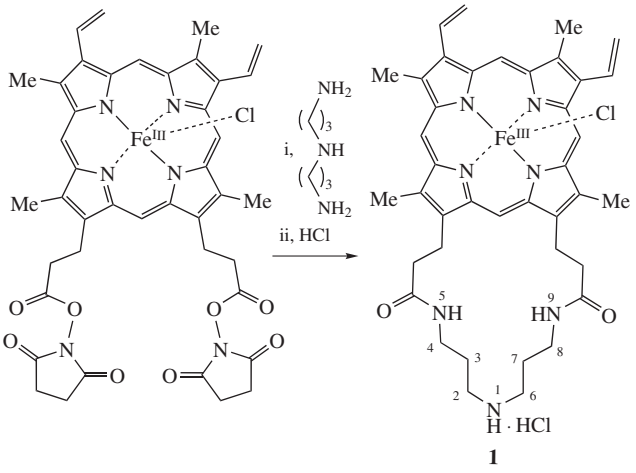
As an example of the latter case, protohemin IX as endogenous porphyrin-metal complex attracts attention in different areas including protohemin IX-based electrochemical biosensors with graphene or G-quadruplex moieties,[5,6] as well as its complexes and derivatives with proteins, peptides and G-quadruplexes used as peroxidase functional models.[5,7–9]

Protohemin IX exhibits certain antibacterial activity,[10] but its application as a pharmaceutical is hampered by hemolytic effect, insolubility in water, lack of efficiency and short duration of action. To overcome these drawbacks, we conducted the synthesis of a number of protohemin IX derivatives with amino acids and peptides. It has been shown that some of these compounds have pronounced antibacterial activity.[11]

In a search for protohemin IX derivatives with improved properties, we have developed a synthesis of its derivative with norspermidine [bis(3-aminopropyl)amine]. This biogenic polyamine is formed as a result of bacterial activity and participates in the process of biofilm spontaneous destruction.[12] The original purpose of this synthesis was to obtain protohemin IX disubstituted at propionic acid chains by two norspermidine residues.

Previously the similar synthetic procedures were carried out using polyamines with amino/imino functions selectively protected with trifluoroacetyl and benzyloxycarbonyl groups.[13,14] Here we used unprotected norspermidine. Our initial attempts employed the method described for protohemin IX disubstituted with amino acid residues and peptides,[11] namely simple mixing the amine component in double excess with protohemin IX bis(*N*-succinimidyl) ester [Hem(NSu)$_2$] in reasonably concentrated solutions. However, we did not obtain two residues of norspermidine bound by two carboxyl groups under these conditions, but had instead a mixture of different products.

Alteration of these reaction conditions, namely, the use of equimolar amounts of norspermidine and Hem(NSu)$_2$, dilution of the reaction mixture and slow addition of the amino component, resulted in a new compound **1** (Scheme 1).†



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Scheme 1

† *Protohemin IX* N,N′*-[azanediyldi(propane-3,1-diyl)diamide] hydrochloride* **1**. A solution of norspermidine (0.24 mmol, 34 μl) in DMF (3 ml) was added gradually (300 μl every 10 min) to Hem(NSu)$_2$ (200 mg, 0.24 mmol, prepared as previously described[15]) in DMF (20 ml), mixed thoroughly and kept for 16 h. The solvent was removed *in vacuo*, the residue was dissolved in MeOH (4 ml) and acidified with HCl (400 μl, 0.48 mmol) in MeOH. Again the solvent was removed *in vacuo* and the residue was purified by column chromatography on silica gel (2.2×40.0 cm column, 150 ml of chloroform–methanol–acetic acid–water, 5 : 4 : 1 : 1). The solvent was removed *in vacuo* from the fraction containing the

Table 1 Antibacterial activity of compound **1** (MIC, MBC/μg ml^{-1}).

Compound	*Enterococcus faecalis* 560		*Staphylococcus aureus* 700699		*Staphylococcus aureus* 3798		*Staphylococcus haemolyticus* 585		*Staphylococcus haemolyticus* 1025		*Staphylococcus aureus* 10	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	21.3	64.0	21.3	42.7	42.7	64.0	32.0	64.0	32.0	64.0	21.3	64.0
Ampicillin			>128	>128	>128	>128					1	5
Ceftazidime							>128	>128				
Azithromycin					>128	>128			>128	>128		
Vancomycin	>128	>128										

The reaction mixture contained a significant amount of unreacted Hem(NSu)$_2$, and the yield of the product after purification was as low as 21%. According to the NMR data, including modern techniques[16] (Figures S1, S2, S4 and S5, Online Supplementary Materials), the new compound is a cyclic derivative of protohemin IX (see Scheme 1)[‡] where the norspermidine residue is bound with both activated carboxyl groups forming a spacer between them.

The molecular ion of compound **1** corresponds to the structure with the norspermidine moiety attached to protohemin IX propionic acid residues by amide bonds. The bands typical of amide (amide I and amide II) are observed in the IR spectrum. The purity of the obtained product was confirmed by HPLC.

In the two-dimensional ^{1}H/^{15}N-HSQC spectrum of **1**, only one signal is observed (6.96, 121.9 ppm) (Figure S4). The values of its chemical shift correspond to the NH group of norspermidine located next to the carbonyl group.

The two-dimensional TOCSY spectrum of **1** reveals cross-peaks from the signal 6.95 pmm corresponding to proton at nitrogen atom to protons with chemical shifts 1.38 and 2.66 ppm [Figure S5(*a*)]. This means that protons with the chemical shift 2.66 ppm are located on the carbon atoms adjacent to the nitrogen atom, and a proton with the chemical shift 1.38 ppm is located on the adjacent carbon atom. The observed cross-peaks with the chemical shift 2.66 and 1.38 ppm in the DQF-COSY spectrum [Figure S5(*b*)] further confirm this arrangement. Thus, we can conclude that the signal with the chemical shift 2.66 ppm refers to protons at C^4 and/or C^2 atoms, and 1.38 ppm – to protons at C^3 atom of norspermidine moiety (see Scheme 1).

Moreover, the appearance of only one set of signals from the norspermidine moiety means that it is attached by the 5- and 9-positioned amino groups, or by the 1-positioned imino group (see Scheme 1). However, the latter is not confirmed by the mass spectrometry data. If the norspermidine moiety was attached to protohemin IX with participation of amino groups in 5-position and imino group in 1-position, the protons of CH_2 groups in 4-, 3-, 2-positions and 8-, 7-, 6-positions would have different chemical shifts. The proposed attachment is also confirmed by the peak area integrating in one-dimensional ^{1}H NMR spectrum of **1**.

Despite the presence of a single protonated NH group, compound **1** was soluble in water and possessed antimicrobial activity against a number of strains resistant to modern antibiotics such as ampicillin, ceftazidime, azithromycin and vancomycin (Table 1).[§] Unlike antimicrobial photosensitizers that are used for photodymamic therapy, the derivatives of protohemin IX such as product **1** do not require light exposure to achieve the antimicrobial effect. According to the values of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) for different strains (see Table 1), compound **1** exhibits non-selective antibacterial activity, including bactericidal activity.

An important feature of compound **1** is the lack of toxicity of its aqueous solutions at a high concentration of 250 μg ml^{-1} to normal blood cells – erythrocytes and leukocytes.[¶]

Thus, a simple access to the novel cyclic norspermidine derivative of protohemin **1** without the use of selective protection of primary and secondary amino groups of norspermidine has been elaborated. Despite the relatively low hydrophilicity of the norspermidine moiety, the product obtained is soluble in water. It demonstrates antibacterial activity against antibiotic-resistant bacteria and is non-toxic.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2019.03.032.

target substance. Additional purification was achieved by gel-permeation chromatography on Sephadex LH-20 column in methanol. Removal of methanol *in vacuo* gave the substance soluble in water and methanol. The residue was dried over $CaCl_2$ *in vacuo*, yield 35 mg (18.6%). Analytical HPLC: Knauer Pump 64 chromatograph, 4.6×150 mm column with reversed phase Luna C18(2), 100 Å, average particle size 5 μm, acetonitrile gradient from 5 to 100% in 0.1% aqueous TFA, flow rate 1 ml min^{-1}, detection at 400 nm, retention time 32.1 min. ^{1}H NMR (700 MHz, DMSO-d_6) δ: –1.6, 0.1, 1.57, 1.67 (s, 1H, *meso*-H), –0.42, –0.45 [2H, $CH_2CH_2C(O)N$], 1.38 (m, 4H, $NHCH_2CH_2CH_2NH$), 2.31, 2.59 (dd, 2H, vinyl H_β), 2.68 (8H, $NHCH_2CH_2CH_2NH$), 5.19, 5.58 [2H, $CH_2CH_2C(O)N$], 6.95 [2H, C(O)NH], 9.97, 10.51 (m, 1H, vinyl H_α), 10.46, 11.72, 14.73, 15.12 (s, 3H, Me). FT-IR (KBr, ν/cm^{-1}): 1647 (amide I), 1568 (amide II). UV (DMSO) [λ_{max}/nm (ε)]: 400 (74000), 503 (27900), 603 (15600) (Figure S3). MS (MALDI), *m*/*z*: 711.017 [M–Cl]$^+$.

‡ For the convenience of the NMR data interpretation the own author numeration for atoms in the norspermidine moiety is presented. The sample of compound **1** was dissolved in DMSO-d_6 at a concentration of 2 mg ml^{-1}. Low-spin state of ferrous ion was reached by addition of KCN up to 100 mmol dm^{-3} concentration.[17] One-dimensional NMR and two-dimensional ^{1}H/^{15}N-HSQC and ^{1}H/^{13}C-HSQC NMR spectra were recorded on a Bruker Avance 700 spectrometer operating at 700.22 MHz and equipped with a pulse gradient triple resonance sensor (H/C/N).

§ The bacterial strains were received from the collection of the Gause Institute of New Antibiotics. The minimal inhibitory concentration (MIC) was determined according to the described procedure.[11] Tests were carried out with the initial inoculum of 5×10^5 cfu ml^{-1}. Minimum bactericidal concentration (MBC) was determined applying a culture broth from the cells used in the previous MIC determination, where the inhibition of bacterial growth was clearly recognized, on plates with antibiotic-free medium. MBC was taken as the lowest concentration of the substance in a cell where inoculation showed no growth.

¶ To study the toxicity of compound **1** towards leukocytes and erythrocytes, fresh blood from healthy donors was used according to the described procedure.[11] The concentration of leukocytes and erythrocytes was determined in Goryaev's chamber. Dead and living leukocytes after incubation with compound **1** were determined using propidium iodide and Hoechst 33342, the analysis was performed using inverted and fluorescent microscopes.

The fraction of hemoglobin released from the erythrocytes into the external medium was used as hemolytic activity parameter. Hemoglobin was determined by the supernatant absorption at 414 nm. Since the investigated compound absorbed light at this wavelength as well, correction was made for its absorption.

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