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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and antifungal properties of arginine-containing hemin derivatives

G. A. Zheltukhina · S. A. Okorochenkov · N. V. Groza · V. G. Arzumanyan · V. E. Nebolsin

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Abstract Due to a steadily increasing number of clinical isolates of microscopic fungi that show resistance to conventionally administered antimycotics, the specialists involved have constantly been searching for novel fungicidal agents among various classes of chemical compounds. The present study was aimed at synthesizing and assessing the activity of arginine-containing hemin derivatives against strains of *Candida albicans* and *Cryptococcus neoformans* insensitive to commonly used antifungal agents. A hemin conjugate with a branched arginine-containing peptide was shown to exhibit in vitro fungicidal activity at a concentration of 25 μM and inhibit the growth of the yeast-like fungus *Candida albicans* at concentrations as low as 12.5 μM.

Keywords Hemin · Arginine-containing peptides · Antifungal agents · *Candida albicans* · *Cryptococcus neoformans* · 3-HETE

Introduction

Mycoses appear to have been accompanying the human civilization from time immemorial. They are induced by microscopic fungi, which are single- or multi-cellular hemoorganoheterotrophic eukaryotes. The prevalence of mycoses is currently growing at a rapid pace due to the migration of populations and alterations in the way of life in industrially developed countries. Mycosis-related damage is particularly perceived in transplantology, oncohematology, and neonatology; the most commonly encountered pathogens of mycoses, such as yeasts of the *Candida* spp., have been steadily ousting nosocomial bacterial infections from their leading positions in clinical medicine. Mycoses prevail in the structure of AIDS-associated pathologies and in individuals with impaired host defenses. Among the well-known pathogens of mycotic infections, the most dangerous are the yeasts of the *Candida* genus, inducing vulvovaginal candidiasis, bronchomycosis, deep invasive, and disseminated candidiases. In recent years, frequently encountered invasive fungal infections have also been associated with *Aspergillus fumigatis* and *Cryptococcus neoformans* (Clement *et al*., 2008). Cryptococcosis is typically treated with combinations of agents known to possess various mechanisms of action, which is intended to both attain a synergetic effect and due to increasingly frequent cases involving drug-resistant strains. Those few currently existing antifungal agents to which resistance does not develop at all or develops rarely (e.g., amphotericin B) are typically known to exhibit considerable human toxicity (Holeman and Einstein 1963). Taking the abovementioned facts into consideration, one cannot deny the vital importance of uninterruptedly searching for novel fungicidal agents among naturally occurring compounds and structural analogs thereof possessing wide-spectrum activity, especially against drug-resistant fungal strains. We have therefore attempted to study hemin conjugates (hereinafter referred to as HC) with synthetic peptides, including antimicrobial peptides.

G. A. Zheltukhina · S. A. Okorochenkov (✉) · N. V. Groza
Moscow State Academy of Fine Chemical Technology Named After M. V. Lomonosov, Moscow, Russia
e-mail: laboratory211@yandex.ru

V. G. Arzumanyan
Scientific Research Institute for Vaccines and Sera Named After I. I. Mechnikov Under the Russian Academy of Medical Sciences, Moscow, Russia

V. E. Nebolsin
Limited Liability Company "Pharmenterprises" Moscow, Moscow, Russia

The choice of hemin, a naturally occurring metalloporphyrin, as the basis for creating new fungicidal agents was largely predetermined by its known prooxidant properties, which probably also contribute to the antimicrobial activity of hemin, especially in the presence of hydrogen peroxide (Everse and Hsia, 1997; Malik *et al*., 1988). Production of reactive oxygen species (ROS) lies at the basis of the antifungal action of myeloperoxidase present in the specialized antimicrobial organelles of neutrophils, peroxisomes (Rogovin *et al*., 1977). However, the use of hemin in clinical practice is complicated due to its toxicity for red blood cells, water insolubility, and a short-term biocidal effect (Nitzan *et al*., 1987). Other promising antifungal agents are fungicidal peptides possessing high activity toward pathogenic fungi (Ajesh and Sreejith, 2009). However, these compounds appear to frequently render a considerable hemolytic effect. Besides, they are unstable in physiological media as a result of degradation when acted upon by proteinases. At the same time, hemin has been shown to inhibit proteinases, including chymotrypsin and HIV proteinase (Patent RU No. 2238959, 2004). Therefore, it might be expected that hemin conjugates with peptides, including antifungal peptides, could have increased resistance to proteinases, while at the same time remain active toward pathogenic fungi. Since the majority of antifungal peptides do have sequences enriched with residues of amino acids with positively charged lateral functions (Lys, Arg, or His), we have synthesized new hemin conjugates with the following arginine-containing peptides: HC with an analog of a combi-1-*N*-α-acetylated synthetic peptide possessing high fungicidal activity against *Candida albicans* (Blondlle and Houghten, 1996), HC with an RGD peptide from the sequence of a cell adhesion factor encountered in the sequences of antimicrobial peptides, and HC with the simplest branched arginine-containing peptide Glu(ArgOMe)ArgOMe. We investigated the antifungal activity of these novel HCs, the peptide Ac-RRWWRF-OH, and a previously synthesized hemin conjugate with an RGD peptide (Okorochenkov *et al*., 2010).

Materials and methods

Experimental

General procedures and materials

The amino acids and L-series derivatives used in this study were manufactured by the Bachem Company (Germany). DIC, HOBt, DCC, 9-FmocONSu, Et_3N, and hemin (from a porcine source) were supplied by Sigma (USA). All solvents were anhydrous, except those used for extraction from aqueous solutions.

Oxylipin, 3-hydroxy-(5Z,8Z,11Z,14Z)-eicosatetraenoic acid (3-HETE), was obtained by means of complete chemical synthesis according to a technique we had worked out previously, with purity of 98–99% (Groza *et al*., 2002).

As a polymeric carrier, we used a styrene copolymer with 1% divinylbenzene and a 2-chlorotrityl chloride anchor group with the chlorine content amounting to 1.43 mmol/g (Bachem, Switzerland). Solid-phase synthesis of peptides was carried out according to the Fmoc strategy (Barlos *et al*., 1991). The degree of substitution of the carrier with the starting amino acid was determined spectrophotometrically (Dryland and Sheppard, 1986).

The identity of the obtained compounds was checked by means of TLC on Kieselgel 60 F254 plates (Merck, Germany) in the following systems: chloroform–methanol–25% ammonia (5:3:0.5) (1), chloroform–methanol 9:1 (2), chloroform–methanol–25% ammonia 6:1:0.2 (3). The chromatograms were developed with either the chlorine-benzidine reagent or UV light.

Analytical HPLC was performed on a Gilson 305 chromatograph (Gilson, France) under the following conditions: column (2 × 150 mm), reverse-phase C18, sorbent mean pore diameter 5 μm (Phenomenex, USA) in a gradient of 80% aqueous acetonitrile in a 0.05% TFA solution from 0 to 100%, and detection at 220 nm. Preparative HPLC was carried out under the following conditions: column (19 × 250 mm), reverse-phase C18, sorbent pores mean diameter 5 μm (Phenomenex, USA) at a gradient of 80% aqueous acetonitrile in a 0.15% TFA solution from 0 to 60%, and detection at 280 nm.

Column chromatography was performed on Sephadex LH-20 (Pharmacia, Sweden) or on Kieselgel 60 F 254 plates (Merck, Germany).

High-performance mass spectra were obtained on an Ultraflex time-of-flight spectrometer (Bruker, Germany) by means of matrix-assisted laser desorption ionization (MALDI-TOF), with 2,5-dihydroxybenzoic acid used as the matrix.

Electron spectra were registered on a UV–Visible spectrophotometer (Helios, UK).

Study of the antifungal action of hemin peptides and their combined effect with oxylipin 3-HETE. The objects acted upon were cells of museum strains of *Candida albicans* No. 927 and *Cryptococcus neoformans* No. 3465 grown on yeast–peptone–glucose (YPG) medium at 32°C for 24 h. The antimycotics pymafucin, amphotericin, and fluconazole were used as comparison compounds.

All experiments were carried out in duplicate using round-bottom 96-well culture plates (Lenmedpolimer, Russia). The first well was filled with 10 μl of the stock solution of water-soluble agents at a concentration of 10^{-2}–10^{-3} mol/l, the second one with 10 μl plus 10 μl of

the solvent corresponding to the given compound, followed by suspending and transferring 10 µl into the next well and so on for all eight wells of the row. The stock solutions of water-insoluble compounds were prepared in DMSO and diluted with DMSO and the medium. The DMSO concentration in the reaction well was ≤5% and did not influence the yeast growth (separate control).

The experiment involving combined incubation of the culture with a derivative of fatty acid (3-HETE) and HC was performed using the stock solution of oxylipin at a concentration of 550 µM in ethanol with serial dilutions to obtain the concentrations of 7 and 14 µM per well, with the ethanol concentration in the reactive well not exceeding 0.2%.

Each well was then supplemented with 190 µl of suspended cellular culture of *Candida albicans* or *Cryptococcus neoformans* in synthetic nutrient medium (a final concentration of approximately 10^3 CFU/ml), containing the pH indicator bromine-cresol blue (at a pH of 5.5 in the medium, the indicator turns blue). Once the pH value decreases to 4.5, the color of the indicator in the medium changes to yellow, as a sign of fungal growth. The synthetic medium consisted of the following components: salt, amino acids, trace elements, vitamins, an antibiotic, and glucose (with a total of 30 components) (Yarrow, 1998). After administering the suspension of *Candida albicans* cells, the plates were incubated for 1 h at 32°C with agitation, then transferred to the stationary thermostat and incubated for a further 24 h and 4 days at 27°C. After the addition of the suspension of *Cryptococcus neoformans*, the plates were incubated for 1 h at 32°C with agitation, then transferred to the stationary thermostat and incubated for 2 and 5 days at 27°C. In order to detect live cells, we performed inoculation from the wells containing HC after 4 days.

In the experiment aimed at clarifying the mechanism of action of **3**, a 24-h culture of. *Candida albicans* strain No. 927 was suspended in a potassium phosphate buffer of pH 4.6 to a cell density of approximately 10^6 CFU/ml. In a test tube, 100 µl of the given suspension and 100 µl of **3** at a concentration of 2×10^{-3} M were combined. In the control tube, 100 µl of the given suspension and 100 µl of the corresponding buffer were combined. Two series of experiments were performed: incubation of tubes for 2 h at 32°C or for 21 h at the same temperature. The samples were then centrifuged for 7 min at 16,000 rpm, discarding the supernatant, and the sediment was supplemented with 200 µl of bromocresol purple in the same buffer and incubated for 45 min at 32°C. The samples were then again centrifuged in the same way, and the obtained precipitate was examined by means of an MBI-6 light microscope (total magnification 1750×) and Sony Cybershot 7.2 camera.

*Solid-Phase Synthesis of Ac-RRWWRF-OH (**2**) on the Polymer with a 2-Chlorotrityl Chloride Anchor Group*

The peptide RRWWRF was synthesized on 2-chlorotrityl chloride resin using a modified standard Fmoc-SPPS protocol (Zheltukhina *et al.*, 2006). Since N^G-unprotected Fmoc-Arg-OH was used, after each Fmoc cleavage cycle, the resin was treated with HOBt (10 eq.) in 1 ml of DMF for arginine guanidine side chain protonation. Once synthesis and deblocking of the α-amino group was completed, 0.143 g of the peptidylpolymer was washed with DMF (1 ml × eight times, each for 2 min), washed with HOBt (3.6 eq.) in 1 ml of DMF, and acetylated with *p*-nitrophenyl acetate (3 eq.) in the presence of triethylamine (1 eq.) for 2 h. Completeness of amino-group replacement was controlled by means of the ninhydrin test. The acetylated peptidylpolymer was washed with DMF (1 ml, eight times, each for 2 min) and DCM (1 ml, twice, each for 2 min), dried in vacuo, and supplemented with 4 ml of a TFA:TFE:DCM mixture (1:1:8), stirring for 3 h under a nitrogen atmosphere. The polymer was separated and washed with 1:1:8 TFA:TFE:DCM (1 min × 2 ml); the solvents from the filter were removed in vacuo, the residue was triturated using a 10- to 12-fold excess volume of cooled anhydrous ether, and the deposit which formed was filtered, washed twice with ether, and vacuum-dried. The peptide was isolated with preparative HPLC. The yield amounted to 0.013 g (43%). R_f 0.8 (6). IR (KBr, cm^{-1}): 1724 (COOH), 1656 (C=O amide I), 1543 (C=O amide II); MALDI-MS (*m*/*z*): 1049.2 $[M]^+$ (calculated 1049.4); HPLC: individual peak, retention time 13.76 min.

*N-α-[6(7)-(Protohemin IX)-yl]-RRWWRF-OH · 3 CF_3COOH (**3**)*

A total amount of 0.29 g of the peptidylpolymer synthesized as mentioned above after Fmoc group cleavage was supplemented with 3 eq. of the 6(7)-mono-*N*-oxy-5-norboren-2,3-decarboxyimide ester of protohemin IX in 4.5 ml of DMF, agitated for 5 h, and allowed to stand for 24 h at room temperature. The hemin peptidylpolymer was separated and washed with DMF (3 ml, seven times, each for 3 min). The ninhydrin test was negative. The hemin peptidylpolymer was washed with DCM (2 ml, twice, each for 2 min), dried in vacuo, then supplemented with 4.5 ml of a TFA:TFE:DCM mixture (1:1:8), and stirred in a nitrogen-containing atmosphere for 3 h. The polymer was separated and washed with a 1:1:8 mixture of TFA:TFE:DCM (1 ml, four times, each for 1 min). The residue was triturated with cooled anhydrous diethyl ether (using a 10- to 12-fold excess in volume); the precipitate was filtered, washed twice with ether, and vacuum-dried. The product was purified on a Sephadex LH-20 column (150 × 20 mm), eluting the target

substance with a 20:1 ethanol–water mixture. The yield amounted to 0.027 g (25%). R_f 0.5 (1). Electron spectrum (methanol), λ_{max}, nm ($\varepsilon \times 10^{-3}$, M^{-1} cm^{-1}): 393.4. (65.8), 474.8 (8.7), 578.0 (3.61), 607.2 (3.19); IR (KBr, cm^{-1}): 1728 (COOH), 1659 (C=O amide I), 1546 (C=O amide II); MALDI-MS (*m/z*): 1605 $[M]^+$ (calculated 1603.5).

*Di-pentafluorophenyl ester of Boc-L-Glutamic Acid (**4**)*

A solution of 1.500 g (6.73 mmol) of Boc-L-glutamic acid in 9 ml of acetonitrile was supplemented with 2.235 g (12.145 mmol) of pentafluorophenol. The reaction mixture was cooled to −10°C and supplemented dropwise for 10 min with 2.505 g (12.145 mmol) of DCC in 4 ml of acetonitrile. The reaction mixture was agitated for 20 min at 0°C and kept for 24 h at 4–5°C. The reaction was monitored by means of TLC under conditions (10). The DCU sediment was filtered, and the solvent was removed by vacuum. The oil-like residue was twice crystallized from ethyl acetate with hexane. The solid residue was vacuum-dried over anhydrous $CaCl_2$. The yield was 3.410 g (84%), R_f 0.70 (10). m. p. 127–132°C. IR (KBr, cm^{-1}): 1737 (CO ester), 1697 (OCONH), 991, 1113 (C–F), 1521 (aromatic). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.18 (s, 1H, –N*H*), 4.37 (t, 1H, α-C*H* of Glu), 1.97–2.33 (m, 4H, –CH_2–CH_2–), 1.43 (s, 9H, (CH_3)$_3$C of Boc group); Anal. calcd. for $C_{22}H_{15}NO_6F_{10}$ C, 45.60; H, 2.59; N, 2.42 Found: C, 45.24; H, 2.60; N, 2.47. Ref. (Khrushchev *et al*., 2007): m. p. 128–132.

*Di-(methyl ester N-α-arginyl)-L-Glutamic Acid (**5**)*

A suspension containing 0.069 g (0.266 mmol) of H-Arg-OMe•2HCl in 2 ml of DMF was supplemented with 0.074 ml (0.531 mmol) of Et_3N and agitated at room temperature for 5 min. The obtained solution was supplemented with 0.077 g (0.133 mmol) of di-pentafluorophenyl ester of Boc-glutamic acid obtained previously and stirred for 4 h at ambient temperature. The reaction was monitored by means of TLC under the conditions (5). The solution was concentrated in vacuum to 1 ml and supplemented with 10 ml of diethyl ether. The oil-like sediment was separated from the solvent by decanting, and the residual solvent was removed under vacuum. The substance was purified by column chromatography on Kieselgel 60 F254 silica gel (Merck, Germany) (23 × 1 cm) and eluted with an ethanol–acetic acid–water mixture (4:0.5:0.5). The fractions containing the substance with R_f 0.67 (5) were collected, and then the solvent was removed under vacuum and the residue was dried. The yield was 0.073 g (77%), R_f 0.67 (3). IR (KBr, cm^{-1}): 1735 (CO ester), 1653 (amide I), 1542 (amide II). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.23–7.08 (m, 3H, α-N*H* of Glu and Arg), 4.37 (t, 1H, α-C*H* of Glu), 4.31–4.28 (m, 2H, α-*H* of Arg), 3.65 (s, 6H 2 × OCH_3), 3.17 (t, 4H, δ-CH_2 of Arg), 2.42–2.28 (m, 4H, –CH_2–CH_2– of Glu), 1.98–1.91 (m, 4H, β-CH_2 of Arg), 1.78–1.66 (m, 4H, γ-CH_2 of Arg), 1.36 (s, 9H, (CH_3)$_3$C of Boc group); MALDI-MS (*m/z*): $[M]^+$ 588.3 (calculated 588.6).

*Di-(methyl ester N-α-arginyl)-L-Glutamic Acid (**6**)*

To 0.073 g (0.0946 mmol) of compound **5**, 6 ml of approximately 3 N HCl/MeOH was poured. The obtained suspension was agitated for 2 h at room temperature. The process was monitored by TLC under the conditions (6).Once complete cleavage of the Boc protection of the compound was attained, the solvent was removed *in vacuo*. The oil-like residue was crystallized over anhydrous diethyl ether, with the solvent removed by decanting, and the residue was dried. The yield was 0.056 g (85 %), R_f 0.2 (2). IR (KBr, cm^{-1}): 1734 (CO ester), 1656 (amide I), 1532 (amide II); ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.23–7.08 (m, 4H, α-NH_2 of Glu, α-N*H* of Arg), 4.35 (t, 1H, α-C*H* of Glu), 4.28–4.20 (m, 2H, α-*H* of Arg), 3.65 (s, 6H 2 × OCH_3), 3.17 (t, 4H, δ-CH_2 of Arg), 2.38–2.22 (m, 4H, –CH_2–CH_2– of Glu), 1.96–1.89 (m, 4H, β-CH_2 of Arg), 1.76–1.66 (m, 4H, γ-CH_2 of Arg); MALDI-MS (*m/z*): $[M]^+$ 488.9 (calculated 488.3).

*6,7-Bis-[di-methyl ether of N-α-L-arginyl)-L-glutamyl]-Protohemin IX (**7**)*

To 0.082 g (0.167 mmol) of compound **6** in 1.5 ml of DMF, 0.047 ml (0.335 mmol) of Et_3N was added. Reaction mixture was stirred at room temperature for 2 min. The obtained suspension was supplemented with a solution of 0.047 g (0.0558 mmol) of 6,7,-bis-*N*-oxy succinimide ether of protohemin IX (I) in 2 ml of DMF and agitated for 22 h at room temperature. The reaction was monitored by means of TLC under the conditions (6). The solution was concentrated to 1 ml and supplemented with 10 ml of diethyl ether. In order to insert a counterion of Cl^-, the sediment was dissolved in 4 ml of MeOH and supplemented with 0.150 ml of ~3 N HCl/MeOH to adjust the pH to 4.0. The solvent was removed under vacuum at 30°C. The substance was purified by column chromatography on a Sephadex LH-20 column (13 × 1 cm), eluting with MeOH. The fractions containing the substance with R_f 0.1 (3) were combined. The solvent was removed under vacuum. The yield was 0.034 g (40 %), R_f 0.2 (2). Electron spectrum, λ_{max}, nm, chloroform:MeOH (8:2), ($\varepsilon \times 10^{-3}$): 400 (92.2), 508 (6.31), 636 (2.39); IR (KBr, cm^{-1}): 1737 (CO ester), 1649 (amide I), 1528 (amide II); MALDI-MS (*m/z*): $[M]^+$ 1554.9 (calculated 1555.2).

Results and discussion

Synthesis of hemin peptides

Most fungicidal and antimicrobial peptides (AMP) appear to be amidated at the C-terminus; however, effective AMPs also include peptides with a C-terminal carboxylic group, for instance magainine-1 and magainine-2 (Kokryakov, 1999). Therefore, we synthesized a 2-chlorotrityl chloride polymer-based peptide Ac-RRWWRF-OH **2** amidated at the C-terminus whose analog (combi-1) exhibits high antifungal activity (Blondlle and Houghten, 1996), as well as the corresponding conjugate with hemin **2**. Synthesis of the latter is of special independent interest and associated with a series of certain difficulties, since the use of conventional polymers for solid-phase synthesis and standard acid-labile protective groupings is limited by the presence of the metalloporphyrin residue in the molecule. Analog **2** of the antifungal peptide combi-1 and the corresponding hemin peptide **3** were synthesized by a solid-phase method with previously developed methodology on a polymer with a 2-chlorotrityl chloride anchor group (Blondlle and Houghten, 1996). While doing so, the guanidine group of arginine was protected by protonation, leaving the indole nucleus of tryptophan unprotected, since detachment of the peptide from the chlorotrityl chloride polymer occurs under sufficiently mild conditions under which side reactions along the lateral functional group of tryptophan are excluded. It is known (Rubina *et al*., 2000) that at the stage of deblocking the α-alpha-amino group resulting from treatment with piperidine having a pKa 11.12, partial deprotonation of the guanyl group takes place which further on under the effect of an excess amount of an acylating agent may lead to the formation of by-products. In fact, the literature data strongly suggest the ability of the guanine group of arginine to participate in acetylating reactions and to engage in other side reactions (Rink *et al*., 1984). The introduction of an additional stage of protonation of the guanidine group after the amino group and prior to the subsequent stage of condensation makes it possible to entirely prevent the course of this side reaction (Rubina *et al*., 2000). Therefore, in order to avoid the formation of by-products, we additionally treated the peptidylpolymer with HOBt after each stage of deblocking with piperidine.

Since exposure to acetic anhydride conventionally used for the acetylation of amino groups of peptides on a solid phase might be associated with modifications of the protonation-protected guanidine group of arginine residues, acetylation of the N-terminus of peptide **5** was performed by the action of a mild acylating agent, *p*-nitrophenyl acetate, in the presence of triethylamine. Cleavage of peptide **2** and hemin peptide **3** from the polymer was carried out using a mixture of trifluoroacetic acid, trifluoroethanol, and dichloromethane (1:1:8) for 3 h (Scheme 1). The obtained Ac-RRWWRF-OH was purified by means of preparative HPLC.

The conjugate of hemin and peptide **3** was obtained on the solid phase by acylating the appropriate deblocked peptidylpolymer with a threefold excess of the ONb-ether of protohemin IX obtained according to (Okorochenkov *et al*., 2010) (Scheme 1). Compound **2** was detached from the respective hemin peptidylpolymer and purified by column chromatography on Sephadex LH-20 with methanol-mediated elution. The procedure resulted in obtaining compounds **2** and **3** with satisfactory yields (43 and 25%, respectively).

Synthesized peptide **2** was characterized by means of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (Fig. 1), as well as spectrometry, while hemin peptide **3** was assessed by means of TLC, IR and UV–Vis spectroscopy, and mass spectrometry.

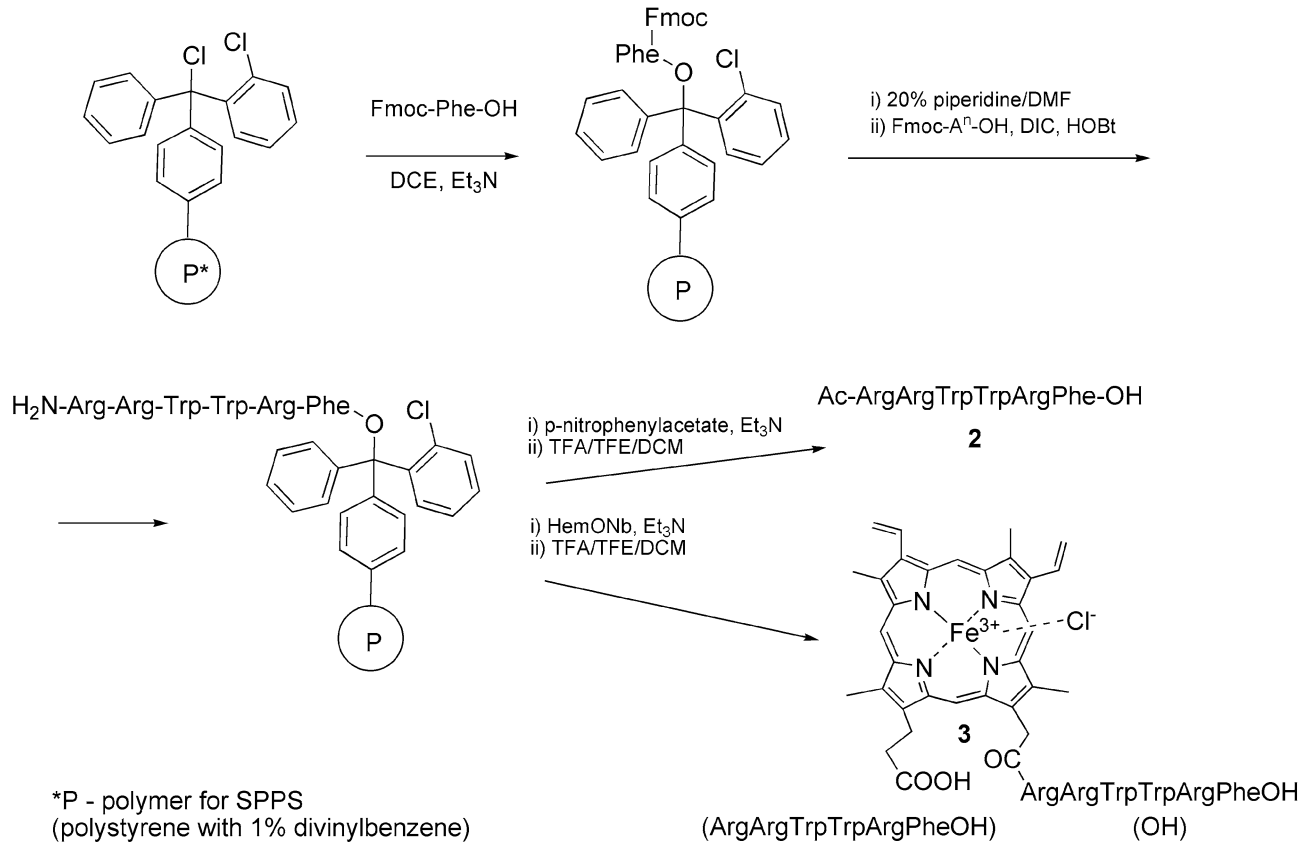
Branched positively charged peptides have been shown (Khrushchev *et al*., 2007; Bruschi *et al*., 2010) to possess high activity toward various pathogens; however, the available literature seems to contain no reports regarding the antifungal activity of conjugates of porphyrins and branched peptides. In order to investigate the promising nature of such compounds as antifungal agents, we have obtained a compound, i.e. a hemin conjugate with the simplest branched positively charged peptide **7**.

A suitable syntone for obtaining dendrimers based on glutamic acid is a di-pentafluorophenyl-activated ether of its Boc derivative (Khrushchev *et al*., 2007). Glutamic acid dipentafluorphenyl ester was obtained by the action of DCC on Boc-protected glutamic acid in the presence of pentafluorophenol in acetonitrile. Compound **6** was synthesized by the coupling of HArgOMe·2HCl with dipentafluorphenyl ester **4** in the presence of Et_3N in DMF with further cleavage of Boc-protecting group from compound **5**. (Scheme 2).

After deblocking the amino group of the obtained N-Boc-protected peptide in acidic medium, branched peptide **6** was added to the reaction with 6,7-bis-*N*-oxysuccinidic ester of hemin ($Hem(ONSu)_2$), obtained according to (Okorochenkov *et al*., 2010), with the resulting formation of target HC **7**.

Antifungal activity of HCs

We then investigated the activity of the synthesized HCs toward human opportunistic yeast-like fungi of the. *Candida albicans* and *Cryptococcus neoformans* species. Compound **7** was found to inhibit the growth of. *Candida albicans*, despite exhibiting no fungicidal action even at high concentrations, with the dynamics of colony growth in the control inoculations



Scheme 1 Synthesis of the peptide on the 2-chlorotrityl chloride polymer

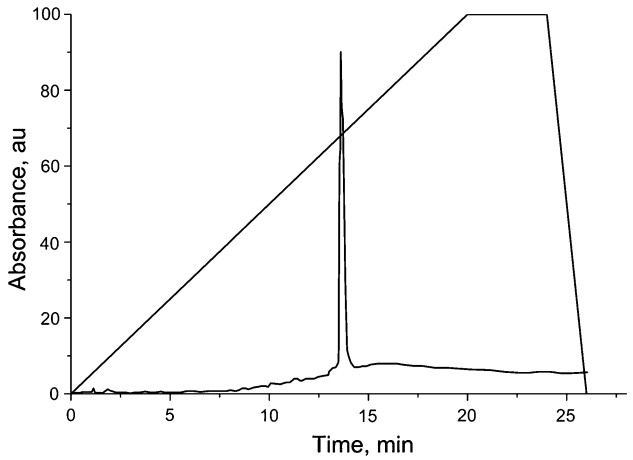


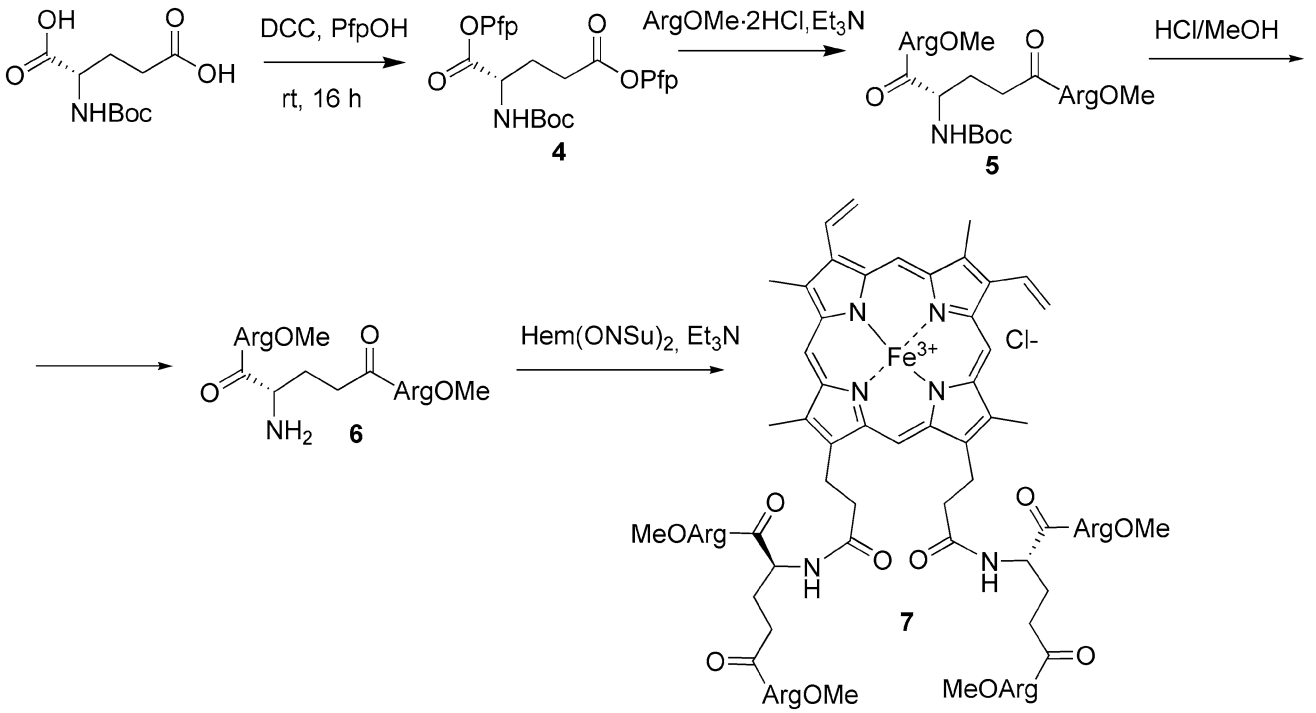
Fig. 1 Chromatogram of Ac-RRWWRF-OH (**2**). HPLC was performed on a Gilson 305 unit (Gilson, France) using a 2 × 150 mm column, reverse-phase C18, sorbent pores mean diameter 5 μm (Phenomenex, USA) with a gradient of acetonitrile in a 0.05% aqueous solution of TFA from 0 to 100%. Detection at 220 nm

following exposure to compound **3** not differing from that of the intact culture. The hemin conjugate with the RGD peptide (HemRGD) exhibited no activity against either of the fungi examined. As can be seen from Tables 1 and 2, the most efficient peptide proved to be compound 3, which suppressed the growth of both *Candida albicans* and *Cryptococcus neoformans* at concentrations as low as 12.5 μM. Using control inoculations, it was shown that compound **7** rendered a fungicidal effect on *Candida albicans* at concentrations down to 25 μM inclusive (the colonies in the control inoculations did not grow at all) and showed a pronounced activity against *Cryptococcus neoformans* (the colonies in the control inoculations did grow; however, their numbers were significantly lower compared with the control).

The obtained findings are of special value, given the known resistance of *Candida albicans* strain No. 927 to exoderil and low sensitivity to amphotericin, pymafucin, miramistin, nitrofungin, as well as the resistance of *Cryptococcus neoformans* strain No. 3465 to amphotericin and low sensitivity to pimafucin and nitrofungin.

Moreover, *Cryptococcus neoformans* is known for being insensitive to azole-series drugs (nizoral, clotrimazole, fluconazole, and itraconazole), which is typical of basidiomycetes class the given strain belongs to.

In order to investigate the mechanisms of the antifungal activity of compound **7,** a microscopic study was carried out on *Candida albicans* cells treated with compound **7** for 2 h (Fig. 2a) and for 21 h (Fig. 2b). Figure 2 shows that the control sample (Fig. 2c) contains intact cells separated



Scheme 2 Synthesis of 6,7-bis-[di-methyl ether *N*-α-L-arginyl)-L-glutamyl]-protohemin IX

Table 1 Growth of the *Candida albicans* culture in the presence of HC

Compounds	Growth of the test culture at various concentrations of the compounds (μM)							
	100	50	25	12.5	6.25	3.13	1.56	0.78
2	+	+	+	+	+	+	+	+
3	±	±	±	±	±	±	±	±
7	−	−	−	±	±	±	±	±
HemRGD	+	+	+	+	+	+	+	+
Pimaricin	−	−	−	−	+	+	+	+
Clotrimazole	+	+	+	+	+	+	+	+
Control	+	+	+	+	+	+	+	+

(−) No growth, blue indicator

(±) Weak growth, yellow indicator but the precipitate of the grown cells is in the form of a fuzzy spot

(+) Intensive growth, with yellow indicator and the cellular precipitate in the form of a compact spot

Table 2 Growth of the *Cryptococcus neoformans* culture in the presence of HC

Compounds	Growth of the test culture at various concentrations of the compounds (μM)							
	50	25	12.5	6.25	3.13	1.56	0.78	0.39
2	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+
7	−	−	−	+	+	+	+	+
HemRGD	+	+	+	+	+	+	+	+
Pimaricin	−	−	−	−	+	+	+	+
Clotrimazole	+	+	+	+	+	+	+	+
Control	+	+	+	+	+	+	+	+

(−) No growth, blue indicator

(+) Intensive growth, with yellow indicator and the cellular precipitate in the form of a compact spot

from each other, whereas the experimental variants (Fig. 2a, b) are represented in the form of agglomerates consisting of intact whole cells and cellular debris. Apparently, the mechanism of action of compound **7** on fungal cells consists of complete destruction (lysis) of the major part of the cells. The interaction between compound **7** and the cells of *Candida albicans* probably occurs very rapidly; 2 h and 21 h after adding compound **7** to the culture, similar results were observed with a small portion of non-destroyed fungal cells and a large amount cellular debris, since the cellular organelles remained after cell lysis. When assessing these findings, it should be borne in mind that under the conditions of the experiment, the ratio of HC to fungal cells was 25 times lower compared with that observed under the conditions of the standard experiment (Table 1). This factor may have led to incomplete destruction of fungal cells (Fig. 2).

Therefore, compound **7** has fungicidal action upon cells of *Candida albicans*, which is probably mediated by complete lysis of both the cellular wall and membrane.

We then attempted to investigate a possibility of increasing the efficacy of the most promising HC **7** due to a

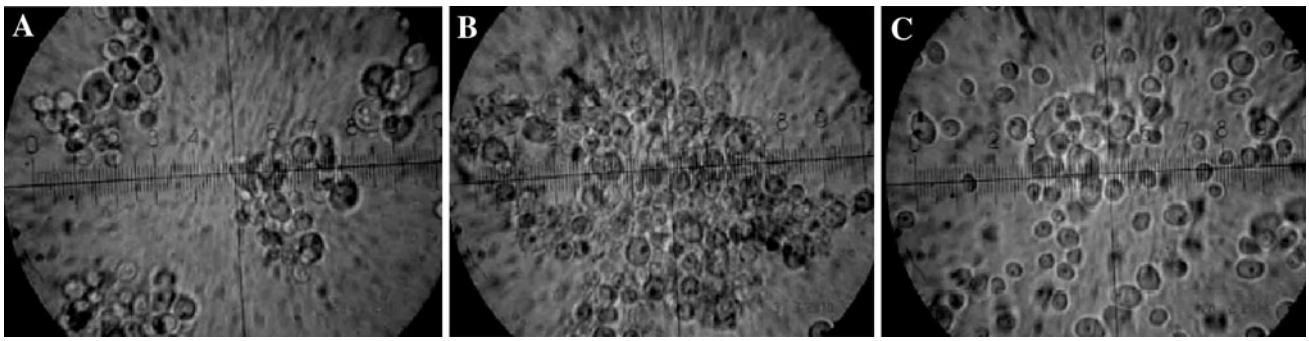


Fig. 2 Cells of *Candida albicans*, strain No. 927, 5×10^5 CFU/ml after exposure to compound **7** (2×10^{-3} M). **a** after 2 h; **b** after 21 h; **c** control

potential synergetic effect with another naturally occurring agent. As a novel potential cellular target of fungi, special attention has been drawn to their lipid metabolism. It has been shown that polyunsaturated fatty acids are capable of inhibiting *C. albicans* culture growth in vitro at concentrations varying from 4 to 20 μM (Clement *et al*., 2008). Moreover, they are capable of considerably altering the proprieties of cellular membranes, including fluidity, permeability, and the activity of membrane-bound proteins, thereby increasing the absorption and accumulation of therapeutic substances in the cell (Gleissman *et al*., 2010). Such compounds may at the same time induce oxidative stress in the cell, as they are good substrates for oxidation and generators of reactive oxygen species (Gleissman *et al*., 2010). Hydroxy derivatives of unsaturated fatty acids (oxylipins), in particular 3-HETE, play an important role in the processes of adhesion, growth, and reproduction of fungal cells, including *Candida albicans* (Groza *et al*., 2010).

In regard to the abovementioned results, the 3-HETE-oxidized derivative of arachidonic acid (C20n-6) was chosen to investigate its combined effect with HC **7** on the commonly occurring species of opportunistic fungi *Candida albicans* and *Cryptococcus neoformans*.

Based on the obtained findings, it was determined that oxylipin 3-HETE at concentrations equaling 7 and 14 μM had neither fungistatic nor fungicidal action on *Candida albicans* strain No. 927 under the standard experimental conditions. Taking into consideration that 3-HETE is a bioregulator of fungal vital functions, the choice of its optimal concentration for reliable inhibition thereof might be a separate task. Besides, the lack of an effect of 3-HETE might to some degree be determined by the resistance and low sensitivity of the given strain of *Candida albicans* to a series of antifungal agents and related mechanisms of action.

Combined exposure to 3-HETE (7 and 14 μM) and compound **7** (12.5 and 25 μM) resulted in the disappearance of the antifungal effect, which may have been the result of their opposite action on the same targets in the fungal cell. Determining the actual causes of this phenomenon requires additional study.

Conclusion

In this study, new hemin conjugates with arginine-containing peptides were synthesized, showing the antifungal activity of a hemin conjugate with a branched arginine-containing peptide against human opportunistic fungi resistant or poorly sensitive to known antifungal agents. Based on the obtained findings, a conclusion may be drawn that such structural peculiarities of HCs, such as the presence of several Arg residues, the modification of both propionic acid groups of hemin, the lack of unprotected carboxylic groups in the HC molecule, and the total positive charge of the substituent in the HC, may have contributed to the antifungal activity of this compound.

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