Encapsulated Methionine γ-Lyase: Application in Enzyme Prodrug Therapy of *Pseudomonas aeruginosa* Infection

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**ABSTRACT:** Lung disease caused by *Pseudomonas aeruginosa* is the leading reason for death in cystic fibrosis patients. Therapeutic efficacy of the pharmacological pairs, the naked/encapsulated mutant form of *Citrobacter freundii* methionine γ-lyase and the substrates, sulfoxides of S-substituted L-cysteine, generating thiosulfates, was evaluated on the murine model of experimental sepsis caused by the multidrug-resistant *P. aeruginosa* 203-2 strain. The pairs containing the naked enzyme and substrates did not have antibacterial activity. The treatment of mice with the pair encapsulated enzyme and substrates did not have antibacterial activity. So, the substituents, attached to the thiosulfinate moiety, affect the antibacterial activity of thiosulfates against *P. aeruginosa*.

![Image](https://dx.doi.org/10.1021/acsomega.9b03555)

**INTRODUCTION**

The main cause of mortality in humans with cystic fibrosis is lung disease. Persistent bronchopulmonary infections caused by *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and other pathogens lead to chronic airway and systemic inflammation, tissue destruction, and respiratory insufficiency. Increasing the frequency of bacteria with multiple resistance to commercial antibiotics is a serious challenge for humanity. It requires studies of anti-infective agents with new mechanisms of action.

Helpful properties of garlic, such as antimicrobial, anti-inflammatory, anticancer, and so on, are known since ancient times. The main antibacterial compound of fresh garlic is allicin (diallyl thiosulfoxide), produced in the allicinase/alliin system, was demonstrated in vitro for the first time.2,3 In addition to bacteria, allicin is effective against fungi, protozoa, and viruses.4–6 However, the usage of pure allicin in medicine is restricted because of its instability as a result of high reactivity. The antimicrobial activity of diallyl thiosulfates, analogues of allicin (natural and synthetic), has been investigated as well.7–11 Diallyl thiosulfates are thermally and chemically more stable than allicin and differ in their antibacterial potential against specific organisms.12 Little is known about the in vivo investigation of the antibacterial and antifungal activity of allicin. It was shown that allicin can inhibit *Staphylococcus epidermidis* biofilm formation and enhance the bactericidal effect of vancomycin on the implant surface in vivo.13 Allicin significantly reduced mortality, prolonged survival, and reduced fungal load in mice infected with *Aspergillus fumigatus*.14 Antistaphylococcal efficacy of a polypropylene mesh pretreated with solutions of chlorhexidine and allicin for hernia repair was demonstrated on rabbit models.15 Site-directed generation of allicin by a binary system of conjugated monoclonal antibodies alliinase and allicin introduced the alliinase/alliin system in the enzyme prodrug therapy.16–18 Antibacterial and antifungal effects of the alliinase/alliin system were demonstrated in vitro.19 In our previous reports, we have found that pyridoxal 5′-phosphate-dependent *Citrobacter freundii* methionine γ-lyase (MGL, EC 4.4.1.11) catalyzes the nonphysiological, nonstereoselective β-elimination reaction of (±)-S-alk(en)yl-L-cysteine sulfoxides to yield allicin and other thiosulfates (Scheme 1).20–22 However, the enzyme was found to be inactivated in the course of the β-elimination reaction of alliin, as allicin oxidized three SH-groups of MGL.21 To avoid MGL inhibition caused by thiosulfimates, we obtained the C11SH mutant form of MGL.22 The antimicrobial effect of the binary system ("pharmacological pair") C11SH MGL/S-alk(en)yl-L-cysteine sulfoxides was demonstrated in vitro against a number of bacteria, including multidrug-resistant strains isolated from...
cystic fibrosis patients.\textsuperscript{22,23} In this report we tested the efficacy of naked/encapsulated C115H MGL/substrate binary systems on the murine model of experimental sepsis caused by \textit{P. aeruginosa} 203-2 strain isolated from cystic fibrosis patients.

## RESULTS AND DISCUSSION

The resistance of \textit{P. aeruginosa} 203-2 strain to antibiotics of different groups (β-lactams, macrolides, aminoglycosides, fluoroquinolones, etc) was assessed. It was found that the \textit{P. aeruginosa} 203-2 strain is resistant to imipenem, azlocillin, cefotaxime, cepofeme, ceftriaxone, gentamicin, tobramycin, and levomycetin (data are not shown). The results allowed us to classify the strain as multidrug-resistant. The virulence of the \textit{P. aeruginosa} 203-2 strain was assessed by determining LD\textsubscript{50} and LD\textsubscript{100} values. LD\textsubscript{50} and LD\textsubscript{100} were 5 \times 10\textsuperscript{8} cells/mouse and 5 \times 10\textsuperscript{9} cells/mouse.

To avoid proteolysis, high immunogenicity and short-lasting biological activity of the enzyme component of the binary system C115H MGL was encapsulated in PIC spheres by mixing PEG-poly(α,β-aspartic acid)\textsubscript{70} and poly-(L-lysine)\textsubscript{70} with the enzyme, as described previously.\textsuperscript{24} The formation of enzyme-loaded PIC spheres was confirmed using the DLS technique. There were two types of particles—the prevailing fraction of about 52 ± 0.33 nm in size and a small number of particles of about 500 nm in size. The polydispersity index was 0.329. The zeta potential was 3.45 ± 0.36 mV. Exploitation of nanoreactors with encapsulated enzymes in enzyme prodrug therapy is very attractive because it avoids the problems associated with the use of the enzymes. There are few papers that describe an appliance of polymeric nanoreactors producing drugs \textit{in vivo}.\textsuperscript{25,26} The \textit{in vitro} potential of usage of \textit{Escherichia coli} penicillin acylase loaded into triblock copolymer vesicles to convert 7-aminodesacetoxycephalosporanic acid and phenylglycine methyl ester to cephalixin was demonstrated.\textsuperscript{27}

The antibacterial activity of two pharmacological pairs, C115H-loaded PIC spheres/alliin and C115H-loaded PIC spheres/S-methyl-L-cysteine sulfoxide (methiin), was studied on the murine model with a single administration of the infecting dose of the bacterial strain equal to LD\textsubscript{50} in the volume of 0.5 mL. The dose of administered sulfoxides corresponded to the MIC determined for the bacterium \textit{in vitro}.\textsuperscript{28} Pharmacokinetic data for the naked and C115H-loaded PIC spheres showed that the half-life of the naked enzyme proved to be 2 orders of magnitude less than that of the encapsulated enzyme.\textsuperscript{24} For this reason, we quadrupled the dosage of the naked C115H MGL compared to the dosage of C115H-loaded PIC spheres. The pair C115H-loaded PIC spheres/methiin showed the best antibacterial effect when using the infecting dose LD\textsubscript{50} (Figure 1). In the group that was treated with C115H-loaded PIC spheres/methiin, all animals survived (P ≤ 0.05); the average score of the general physical health of the animals was 0.2, which corresponds to completely healthy (0 points) animals. The weight loss in the group was less than 10%. A significant difference between the antibacterial action of C115H-loaded PIC spheres/methiin and C115H-loaded PIC spheres/alliin was found. The survival rate in the group treated with C115H-loaded PIC spheres/alliin was 67% (P = 0.5); the animals lost 14% of the weight, and the general physical health of the animals was 2 score. Allicin was shown to have a relatively low \textit{in vitro} antibacterial activity against \textit{P. aeruginosa}.\textsuperscript{23,28,29} Consistent with these data, our \textit{in vivo} results demonstrate low antibacterial activity of C115H-loaded PIC spheres/alliin against the strain as well. So, the substituents, attached to the thiosulfinate moiety, influence the antibacterial activity of thiosulfinates. This phenomenon was observed in the work.\textsuperscript{11} The inefficacy of the pairs naked C115H/sulfoxides (P > 0.99, 0.5) in comparison to the pairs C115H-loaded PIC spheres/sulfoxides was detected even when using simultaneous repeated administration of the enzyme and sulfoxides (Figure 1). Most probably, the quantity of the thiosulfinates, generated by the enzyme and the substrates, was not enough for a noticeable antibacterial effect because of the too short half-life of the naked enzyme in a bloodstream.

In addition, the effectiveness of the pharmacological pair C115H-loaded PIC spheres/methiin was studied on the murine model with an infecting dose of the bacterial strain equal to LD\textsubscript{100} (Figure 2). Three different protocols for administration of the pharmacological pair components and three different protocols of the pharmacological pair components administration were utilized. There was no difference in animal survival when single or fourfold methiin administrations were used (P ≤ 0.0095 in both cases). The survival rate was 50%. Weight loss in the groups was less than 10%. Reducing the dose of the enzyme component three times increased in animal mortality till 67% (P = 0.36). The optimal mode of administration of a pharmacological pair was intravenous separate single injections with an encapsulated enzyme and sulfoxide. The therapeutic doses of C115H-loaded PIC spheres and methiin were 75 U/kg and 100 mg/kg of an animal.

![Scheme 1. Reaction of β-Elimination of (+)-S-alk(en)yL-cysteine Sulfoxides Catalyzed by C115H MGL](https://acseboss.elsevier.com/10.1021/acsomega.9b03555)
CONCLUSIONS
The present study has demonstrated that the pharmacological pair C115H-loaded PIC spheres/methi
inhibits the development of antibacterial drugs with a new mechanism of action against P. aerugi
acid, ampicillin, azithromycin, aztreonam, cefepime, ceftazi-dime, ceftriaxone, cephotaxime, cepoxitin, chloramphenicol, ciprofloxacine, colistin, doxycline, erythromycin, gentamicin, imipenem, levofloxacine, lincomycin, norfloxacin, ofloxacine, oxacilin, rifampicine, spiramycin, tobramycin, and vancomycin are promising for the development of antibacterial drugs.

MATERIALS AND METHODS
Materials. Reduced form of nicotinamide adenine dinucleotide (NADH), lactate dehydrogenase (LDH) from rabbit muscle, and (±)-L-allin were purchased from Sigma-Aldrich; pyridoxal S’-phosphate (PLP) and DL-dithiothreitol (DTT) were from Serva; kanamycin is a domestic product (OAO Biokhimik); DEAE-sepharose was from Amersham. 2-Nitro-5-thiobenzoate (NTB) was prepared according to ref31. N-(2-Hydroxyethyl)-2-aminoethanesulfonic acid, amikacin, amoxycillin/clavulanic acid, ampicillin, azithromycin, aztreonam, cefepime, cefactazidime, ceftriaxone, cephotaxime, cepoxitin, chloramphenicol, ciprofloxacine, colistin, doxycline, erythromycin, gentamicin, imipenem, levofloxacine, lincomycin, norfloxacin, ofloxacine, oxacilin, rifampicine, spiramycin, tobramycin, and vancomycin were from HiMedia Laboratories Pvt. Limited (India).

Purification of C115H MGL. The C115H mutant form of MGL from C. freundii was obtained and purified, as described by Morozova et al.22 To eliminate endotoxin, the enzyme was mixed with Endotoxin Extractor (Sileks, Russia) and incubated for 30 min at 0 °C; then, it was incubated for 15 min at 50 °C and centrifugated for 5 min at 5000 rpm. The transparent aqueous phase containing endotoxin-free enzyme was collected. An endotoxin level was measured by the Limulus Amebocyte Lysate (LAL) chromogenic endotoxin quantitation kit (Thermo Fisher Scientific, USA). The endotoxin level in the samples was 0.07 EU/mg.

Preparation and Characterization of C115H-Loaded PIC Spheres. PEG-P(Asp)70 and PLL70 were dissolved in 50 mM potassium phosphate buffer of pH 7.4 at 1 mg/mL and filtrated through a 0.22 μm membrane filter to remove dust. The solutions of the two polymers were mixed in an equal ratio of −COO− and −NH3+ units by vortex mixing for 2 min. The enzyme (6 mg/mL in 10 mM potassium phosphate buffer of pH 7.4) was added, and the mixture was stirred for 2 min. To remove the nonencapsulated enzyme, the mixture was centrifuged at 12,000 rpm for 5 min, and the supernatant was exchanged with 10 mM potassium phosphate buffer four times. The absence of the enzyme in a supernatant was checked by the disappearance of the characteristic holoenzyme band at 420 nm in the supernatant spectrum.22 The average particle size and zeta potential of C115H-loaded PIC spheres were determined via phase analysis light scattering with ZetaPALS at 25 °C (Brookhaven Instruments Corporation, USA).

Animals and Ethical Approval. A total of 84 outbred male mice weighing 14–16 g were obtained from Andreveka FSBI SCBT FMBA, Russia. To acclimatize, mice were housed in ventilated cages with 12/12 light–dark cycle and had free access to food and water for 14 days before the start of the experiments. All experiments were carried out in accordance with the “Rules of work with use of experimental animals” (Appendix to the order of the Ministry of Health of the USSR of 12.08 1977, no. 755).

Determination of Antibiotic Resistance of P. aerugi-nosa 203-2 Strain. The P. aeruginosa 203-2 strain isolated from cystic fibrosis patients was from the collection of the Laboratory of Molecular Epidemiology of Nosocomial Infections at the National Research Center for Epidemiology and Microbiology named after the honorary academician N. F. Gamaleya. The resistance of P. aeruginosa 203-2 to standard antibiotics prescribed to treat cystic fibrosis was determined by the serial dilution method, according to the clinical recommendations on the threshold MIC values for each antibiotic [Determination of the sensitivity of microorganisms to antimicrobial drugs (version 2015-02). Methodological guidelines 4.2.1890-04].

Mice Models of Experimental Sepsis. The P. aeruginosa 203-2 strain was used for the induction of experimental sepsis in animals. A single colony of pure 18–24 h culture of bacteria grown on 5% blood agar was grown overnight in LB at 37 °C. The bacteria were collected by centrifugation at 3000 rpm for 10 min, and the pellet was suspended in 0.9% NaCl. The concentration of the bacterial suspension was determined by the visual comparison of the suspension with the 0.5 MF turbidity standard. To clarify the content of viable cells in the infecting suspensions, seeding from a number of dilutions to a dense nutrient medium was carried out, followed by counting the colonies after 24 h of incubation.

To simulate the infection, 5 groups of animals (six mice each) were formed, four of which were administered different doses of P. aeruginosa 203-2 obtained by 10-fold serial dilution of the initial concentration of inoculums (5 × 104, 5 × 105, 5 × 106, 5 × 107 KFU/mice). The control group of animals was administered a sterile saline buffer. Sepsis was modeled by a single intraperitoneal injection of 0.5 mL inoculate of a strain. The duration of observation was 7 days, after which the survived animals were euthanized by the administration of
diethyl ether using ether-impregnated cotton. As the evaluation of virulence, the LD$_{50}$ and LD$_{100}$ were determined.

**Antibacterial Activity of Pharmacological Pairs on the Model of Experimental Infection Caused by *P. aeruginosa* 203-2.** The antibacterial activity of the two drug combinations, C115H-loaded PIC spheres/alliin and C115H-loaded PIC spheres/methiin, was studied on the murine model with a single administration of the infecting dose of the bacterial strain equal to LD$_{50}$ in a volume of 0.5 mL. In addition, the effectiveness of the pharmacological pair C115H-loaded PIC spheres/methiin was studied on the murine model with the infecting dose of the bacterial strain equal to LD$_{100}$.

For the model with the infecting dose of *P. aeruginosa* 203-2 equal to LD$_{50}$, mice were assigned to 5 groups (six mice each): C115H-loaded PIC spheres/methiin and C115H-loaded PIC spheres/alliin groups with a separate injection containing encapsulated enzyme alone at a dose of 75 U/kg 1 h after the infection and fourfold injection of methiin/alliin at a dose of 100 mg/kg at 1, 24, 48, and 72 h after the infection; naked infection and fourfold injection of methiin at a dose of 100 mg/kg at 1, 24, 48, and 72 h after the infection; control group with the separate injection containing the encapsulated enzyme alone at a dose of 300 U/kg and alliin/methiin at a dose of 100 mg/kg at 1, 24, 48, and 72 h after the infection; control group with an injection of 0.2 mL buffer at 1, 24, 48, and 72 h after the infection.

For the model with a LD$_{100}$ infecting dose of *P. aeruginosa* 203-2, mice were assigned to 4 groups (six mice each): C115H-loaded PIC spheres/methiin group 1 with the separate injections of the encapsulated enzyme alone at a dose of 75 U/kg and methiin at a single dose of 100 mg/kg 1 h after the infection; C115H-loaded PIC spheres/methiin group 2 with a separate injection containing the encapsulated enzyme alone at a dose of 75 U/kg 1 h after the infection and fourfold injection of methiin at a dose of 100 mg/kg at 1, 24, 48, and 72 h after the infection; C115H-loaded PIC spheres/methiin group 3 with separate injections containing the encapsulated enzyme alone at a dose of 25 U/kg 1 h after the infection and fourfold injection of methiin at a dose of 100 mg/kg at 1, 24, 48, and 72 h after the infection; control group with an injection of 0.2 mL of buffer at 1, 24, 48, and 72 h after the infection.

The preparations of the naked/encapsulated enzyme and sulfoxides were intravenously injected at a volume of 0.1 mL in 0.1 mM potassium phosphate buffer, pH 7.5, with 0.1 mM PLP. The same buffer was used as the control.

The body weight and discomfort, such as reduced locomotor activity, were daily observed. Animals that survived after 7 days of observation were euthanized.

**Statistical Analysis.** Statistical analysis of mice survival was performed by the log rank test using the Graphpad Prism 8 software package (Graphpad Software Inc., San Diego, CA, USA). P values of <0.05 were considered significant in the analyses.

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