

Eremomycin pyrrolidide: a novel semisynthetic glycopeptide with improved chemotherapeutic properties

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Purpose: Development of new semisynthetic glycopeptides with improved antibacterial efficacy and reduced pseudoallergic reactions.

Methods: Semisynthetic glycopeptides 3–6 were synthesized from vancomycin (1) or eremomycin (2) by the condensation with pyrrolidine or piperidine. The minimum inhibitory concentration (MIC) for the new derivatives was measured by the broth micro-dilution method on a panel of clinical isolates of *Staphylococcus* and *Enterococcus*. Acute toxicity (50% lethal dose, maximum tolerated doses), antibacterial efficacy on model of systemic bacterial infection with *S. aureus* and pseudoallergic inflammatory reaction (on concanavalin A) of eremomycin pyrrolidide (5) were evaluated in mice according to standard procedures.

Results: The eremomycin pyrrolidide (5) was the most active compound and showed a high activity against Gram-positive bacteria: vancomycin-susceptible staphylococci and enterococci (minimum inhibitory concentrations [MICs] 0.13–0.25 mg/L), as well as vancomycin-intermediate resistant *Staphylococcus aureus* (MICs 1 mg/L). Antimicrobial susceptibility tested on a panel of 676 isolates showed that 5 had similar activity for the genera *Staphylococcus* and *Enterococcus* with MIC₉₀=0.5 mg/L, while vancomycin had MIC₉₀=1–2 mg/L. The number of resistant strains of *Enterococcus faecium* (vancomycin-resistant enterococci) (MIC =64 mg/L) with this value was 7 (8%) for vancomycin (1) and 0 for the compound 5. In vivo comparative studies in a mouse model of systemic bacterial infection with *S. aureus* demonstrated that the efficacy of 5 was notably higher than that of the original antibiotics 1 and 2. In contrast to 1, compound 5 did not induce pseudoallergic inflammatory reaction (on concanavalin A).

Conclusion: The new semisynthetic derivative eremomycin pyrrolidide (5) has high activity against staphylococci and enterococci including vancomycin-resistant strains. Compound 5 has a higher efficacy in a model of staphylococcal sepsis than vancomycin (1) or eremomycin (2). In striking contrast to natural antibiotics, the novel derivative 5 does not induce a pseudoallergic inflammatory reaction to concanavalin A and therefore has no histamine release activity. These results indicate the advantages of a new semisynthetic glycopeptide antibiotic eremomycin pyrrolidide (5) which may be a prospective antimicrobial agent for further pre-clinical and clinical evaluations.

Keywords: semisynthetic glycopeptides, antibacterial activity, pseudoallergic reaction, antibacterial efficacy

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Introduction

Polycyclic glycopeptide antibiotics are highly active against Gram-positive bacteria of *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium* genera, including strains resistant to β -lactams, fluoroquinolones, and tetracyclines. Vancomycin (1) (Figure 1) and teicoplanin are the drugs of last resort for treatment of life-threatening

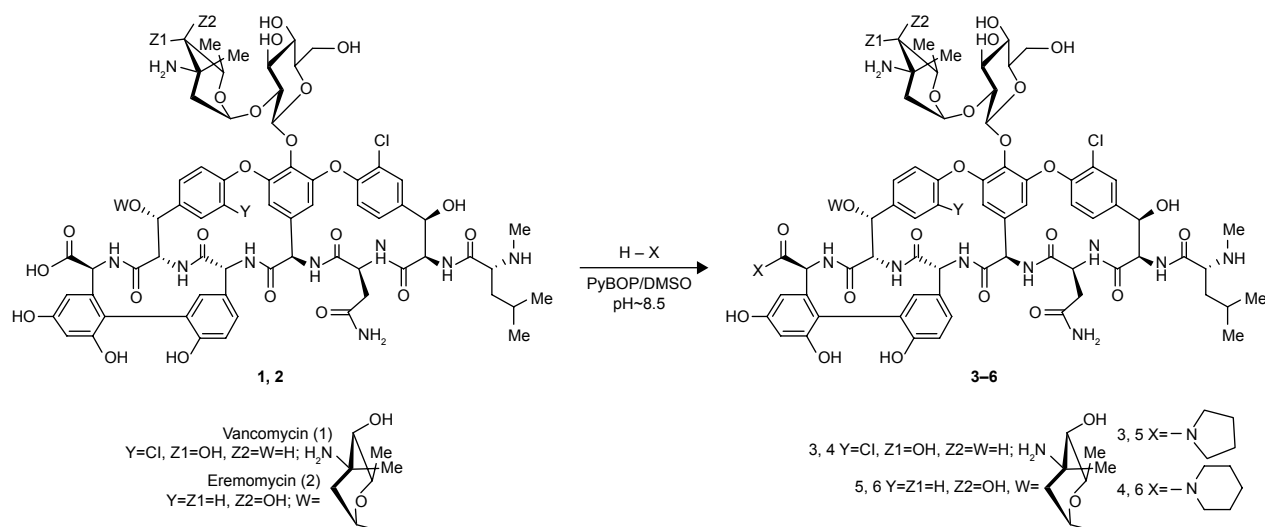


Figure 1 Synthesis of carboxamides of vancomycin and eremomycin 3–6.

infections caused by Gram-positive human pathogens.¹ These antibiotics are especially effective for treatment of staphylococci infections caused by multi-resistant strains, especially methicillin-resistant *Staphylococcus aureus* (MRSA).^{2,3} The antibiotics of this class do not have any alternative thus far, but their application is restricted by the emergence of resistance to glycopeptides, and by severe side effects.⁴ Due to its short half-life, rapid excretion, and a low accumulation in tissues, vancomycin is inefficient for treatment of pneumonia and some infections caused by Gram-positive bacteria.^{5–8}

Glycopeptide antibiotics selectively interfere with the formation of bacterial cell walls without significant effects on eukaryotic cells. These antibiotics bind to D-Ala-D-Ala dipeptide terminus of growing bacterial peptidoglycan, sequestering the substrate from transpeptidation and transglycosylation reactions of peptidoglycan synthesis.⁹ Due to the unique mechanism of action of glycopeptides, the resistance of microorganisms to vancomycin did not emerge for a long time.

However, the use of vancomycin (1) can be associated with adverse effects, largely nephro- and ear toxicity. Furthermore, vancomycin causes several types of hypersensitivity reactions, such as “red man syndrome” (RMS). RMS is a form of pseudoallergic drug reaction with symptoms that mimic immunological drug allergies but lack immunological mechanisms.^{5,10} Vancomycin rarely causes anaphylactic reactions; however, >50% of patients exhibit RMS as a result of histamine release from mast cells and basophils directly activated by vancomycin.¹¹

The wide usage of vancomycin (1) in the clinic and avoparcin in veterinary practice has resulted in the emergence of strains resistant to glycopeptides, such as vancomycin-resistant

enterococci (VRE)¹² and vancomycin-intermediate resistant *S. aureus* (VISA) strains.¹³ The isolates showed a modestly increased minimum inhibitory concentration (MIC) value for vancomycin (1) (MIC \geq 8 mg/L). Nevertheless, vancomycin treatment of infections caused by VISA isolates often led to therapeutic failure. The spread of resistance to glycopeptides in enterococci and *S. aureus* became a serious reason for the decreased efficacy of vancomycin against Gram-positive bacterial infections.^{3,14,15}

Thus, the resistance, adverse effects, and limitations in the application of vancomycin have stimulated the efforts to develop glycopeptides with improved pharmacological properties. Some second-generation glycopeptides have been identified in an extensive search for new agents active against poly-resistant strains of Gram-positive pathogens.^{3,16,17} Three most promising semisynthetic glycopeptides have been obtained by the modification of natural antibiotics: oritavancin (LY 333328; Eli Lilly, Indianapolis, IN, USA)¹⁸ was prepared by modifying chloroeremomycin, telavancin (TD-6424; Theravance Biopharma US, Inc., South San Francisco, CA, USA)¹⁹ was obtained from vancomycin, and dalbavancin (BI-397; Actavis, Edison, NJ, USA)²⁰ from A-40926. They were approved in the USA and European Union (EU) for treatment of skin infections and hospital-acquired bacterial pneumonia caused by resistant Gram-positive bacteria.^{16,21} According to preliminary data, the adverse-effect profile of these glycopeptides was similar to that of drugs currently used to treat severe Gram-positive infections.²² Further clinical evaluation revealed some safety problems, such as an increased risk of developing osteomyelitis with oritavancin; nephrotoxicity, a risk of QT interval prolongation with telavancin, and an elevation of hepatic enzymes after dalbavancin.⁴

We have synthesized and studied the biological properties of new semi-synthetic glycopeptides for selection of new drug candidates with improved chemotherapeutic properties.^{23,25} Special attention is given to the derivatives of an original antibiotic of this group, eremomycin (**2**) (Figure 1), discovered by Gause et al.²⁶ This glycopeptide antibiotic is highly active against Gram-positive bacteria, including the strains resistant to β -lactams, fluoroquinolones, and tetracyclines. It is 3–7 times more potent than one in vitro and in vivo toward many pathogenic strains, while the acute toxicity in animal tests is several times lower than that of vancomycin (**1**).^{27,28} Investigation of eremomycin (**2**) in immunological tests in vivo (reaction of generalized anaphylaxis [anaphylactic shock], active skin anaphylaxis, and delayed hypersensitivity reaction) showed that this antibiotic does not have allergenic properties but causes pseudoallergic reactions in rats.^{27,29,30}

Like vancomycin (**1**), eremomycin (**2**) is inactive against VRE but moderately potent against VISA (MIC =8 mg/L). Semisynthetic eremomycin carboxamides (C-terminus derivatives) showed a high activity against Gram-positive bacteria, including resistant strains for which the parent antibiotic was inert.^{23,24,30–32} Eremomycin *N*-adamantyl-2-amide had a promising activity against a range of clinical isolates, including methicillin-resistant staphylococci and VRE. This agent was equally active against ciprofloxacin-susceptible and ciprofloxacin-resistant *Bacillus anthracis* strains. The results of in vivo tests demonstrated that this compound efficiently prevented animal death caused by *S. aureus* or *B. anthracis*, showing better pharmacokinetic properties compared with **1** or **2**.²⁴

Another promising semisynthetic derivative of eremomycin amide was active against vancomycin-susceptible staphylococci and enterococci, as well as against VISA (MIC =0.5–0.25 mg/L).³³ In the rotational-echo double solid state nuclear magnetic resonance experiments with a labeled analog (Kim et al) was also shown that this compound has an additional binding site to the main site-D-Ala-D-Ala with fragments of the structure of cell-wall peptidoglycan of *S. aureus*.^{34,35} Like several semisynthetic eremomycin derivatives modified at the C-terminus of the peptide core, eremomycin amides exhibited less pronounced pseudoallergic reactions compared with the parent antibiotic **2** in animal tests.³⁰

Due to the increasing prevalence of diseases caused by resistant strains and some adverse effects of glycopeptides, the preparation and development of novel antibacterial antibiotics effective against poly-resistant bacteria (including glycopeptide-resistant strains) with diminishing of side effects remains a problem. The goal of this study was to synthesize and assess the antibacterial activity of new carboxamides of vancomycin and eremomycin (**3–6**). Among

this series, the derivative **5** demonstrated an improved activity against vancomycin-susceptible staphylococci and enterococci, including VISA and VRE strains. Thus, a new semisynthetic eremomycin pyrrolidide (**5**) showed advantages compared with natural antibiotics **1** and **2**.

Materials and methods

Reagents, instruments used and other general information; several properties of the compounds **3–6** and NMR data for eremomycin pyrrolidide **5** (Figures S1–S3) are presented in Supplementary materials.

Glycopeptide antibiotics

Vancomycin hydrochloride (**1**) was purchased from TEVA (Pharmaceutical Industries Ltd., Debrecen, Hungary). Sulfate of eremomycin (**2**) was produced at the pilot plant of the Gause Institute of New Antibiotics (Moscow, Russia).

General procedure for the preparation of carboxamides **3–6**

Semisynthetic derivatives **3–6** were synthesized from vancomycin (**1**) or eremomycin (**2**) by previously described methods^{23–25,31–33} for glycopeptide carboxamides (Figure 1). Compounds **3–6** were synthesized by condensation of **1** or **2** (1 eq.) with pyrrolidine or piperidine hydrochloride (5 eq.) using benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as a condensing reagent (1.5 eq.) in dimethyl sulfoxide (DMSO) in the presence of Et₃N (pH=8.5). After 1 hour of stirring at 22°C, Et₂O was added to the reaction mixture and shaken intensively to partly extract DMSO. The upper ethereal layer was separated, and the oil residue was dissolved in aqueous (0.1 n) H₂SO₄. The pH value was adjusted to 4.5, and the solution was poured into a stirred acetone to precipitate the product. The latter was filtered off, washed with acetone, and dried in vacuum to obtain a white powder of crude salts **3–6**. Purification of compounds was performed using column chromatography on silanized silica gel pre-equilibrated with water (1 g of solid for 70 cm³ of silica gel). The yields of **3–6** were ~48%–66%, and high-performance liquid chromatography (HPLC) purity was 96%–98%. Properties (yield, melting temperature, UV-, IR, HPLC, high-resolution mass spectrometry) and ¹H- and ¹³C-NMR-spectra of **3–6** are presented in Supplementary materials.

Microorganisms

The pre-screening of antibacterial activity of new derivatives **3–6** and starting glycopeptides **1** and **2** was performed on a panel of clinical isolates of Gram-positive microorganisms, including *Staphylococcus epidermidis* 533, *Staphylococcus*

Table 1 Antibacterial activities (MIC, mg/L) of derivatives 3–6 compared with the antibiotics 1 and 2

Compound	<i>S. epidermidis</i> 533	<i>S. haemolyticus</i> 602	<i>S. aureus</i> 3797 (VISA)	<i>S. aureus</i> 3798 (VISA)	<i>E. faecium</i> 568	<i>E. faecalis</i> 559
Vancomycin (1)	2	2	8	8	2	2
Eremomycin (2)	0.25	0.25	4	4	0.5	0.5
3	0.5	0.5	4	8	1	2
4	0.5	nt	1	1	0.5	nt
5	0.25	0.25	1	1	0.13	0.25
6	0.13	nt	0.5	2	0.5	nt

Abbreviations: *E.*, *Enterococcus*; MIC, minimum inhibitory concentration; nt, not tested; *S.*, *Staphylococcus*; VISA, vancomycin-intermediate resistant *S. aureus*.

haemolyticus 602, *S. aureus* 3797 (VISA HIP-5836; New Jersey), *S. aureus* 3798 (VISA HIP-5827; Michigan), *Enterococcus faecalis* 559 (vancomycin susceptible enterococci [VSE]), and *Enterococcus faecium* 568 (VSE). The strains were kindly provided by colleagues from the Lepetit Research Center (LePetit Group, Biosearch S.p.A., Varese, Italy). The results were usually identical and always within 2-fold of each other. Antimicrobial susceptibility testing of 5 and 1 was performed on the collection of clinical isolates from the Institute of Antimicrobial Chemotherapy (Smolensk, Russia). A total of 676 non-duplicate isolates were evaluated as follows: 255 strains of MRSA, 179 strains of methicillin-sensitive *S. aureus*, 45 strains of coagulase-negative *Staphylococcus*, 122 strains of *E. faecalis*, and 75 strains of *E. faecium*. We tested the material from hospitalized patients (adults and children) with clinical and laboratory symptoms of infection (out-of-hospital and nosocomial) of the skin and soft tissues, respiratory tract, bones, joints, urinary tract, and abdominal cavity. The in vitro study was performed according to the

recommendations of the European Committee on Antimicrobial Susceptibility testing. The MIC values were determined by the broth micro-dilution method using Mueller Hinton broth and National Committee for Clinical Laboratory Standards procedures.^{24,36} Data are presented in Tables 1 and 2.

Animals

Animals were obtained from the facility of the Russian Academy of Sciences (Moscow, Russia). The in vivo experiments were performed in accordance with the European Convention for the Protection of Vertebrate Animals, Directives 86/609/EEC,³⁷ the National Standard of the Russian Federation R 53434–2009 “Good Laboratory Practice”³⁸ and approved by Ethics of Animal Experimentation of Gause Institute of New Antibiotics.

Mouse model of staphylococcal sepsis

A comparative study of the efficacy of sulfate of eremomycin pyrrolidide (5), vancomycin hydrochloride (1), and sulfate of eremomycin (2) was performed using a model of

Table 2 MIC distributions, MIC₅₀ and MIC₉₀ values for eremomycin pyrrolidide (5) and vancomycin (1) for isolates of Gram-positive pathogens

Organisms (n strains)/ antibiotics	The amount of strains of microorganisms with the values of MIC, mg/L										MIC, mg/L	
	0.06	0.125	0.25	0.5	1	2	4	8–32	64	50%	90%	
<i>S. aureus</i> (MRSA, n=255)												
5	0	23 (9%)	109 (43%)	107 (42%)	16 (6%)	0	0	0	0	0.25	0.5	
1	0	2 (1%)	2 (1%)	33 (13%)	172 (67%)	46 (18%)	0	0	0	1	2	
<i>S. aureus</i> (MSSA, n=179)												
5	2 (1%)	1 (0.5%)	82 (46%)	84 (47%)	10 (5.5%)	0	0	0	0	0.5	0.5	
1	0	0	4 (2%)	64 (36%)	106 (59%)	5 (3%)	0	0	0	1	1	
Coagulase-negative <i>Staphylococcus</i> (n=45)												
5	0	20 (44%)	17 (38%)	7 (16%)	1 (2%)	0	0	0	0	0.25	0.5	
1	0	1 (2%)	3 (7%)	3 (7%)	14 (31%)	24 (53%)	0	0	0	2	2	
<i>E. faecalis</i> (n=122)												
5	2 (2%)	21 (17%)	77 (63%)	20 (16%)	0	0	2 (2%)	0	0	0.25	0.5	
1	0	0	0	8 (6%)	106 (75%)	25 (18%)	1 (1%)	0	0	1	2	
<i>E. faecium</i> (n=75)												
5	35 (47%)	16 (22%)	13 (17%)	1 (1%)	1 (1%)	1 (1%)	8 (11%)	0	0	0.125	4	
1	0	0	0	24 (28%)	54 (62%)	2 (2%)	0	0	7 (8%)	1	2	

Abbreviations: *E.*, *Enterococcus*; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin susceptible *S. aureus*; *S.*, *Staphylococcus*.

staphylococcal sepsis in mice. We used female SHK colony mice weighing 22–25 g. After a 2-week quarantine, healthy animals were used in experiments. *S. aureus* (strain 10 sensitive to antibiotics, adapted to growth in mice) was used as an infectious agent. Initially, a 100% lethal dose (LD_{100}) of *S. aureus* was determined for a given strain of mice in the intravenous (IV) route of the infection. Death of mice was registered daily for 10 days. The LD_{100} was calculated as 3×10^8 CFU/mouse.

To determine the efficacy of tested drugs, the doses were determined at which 50% and 100% of animals survived (effective dose of compound required to cure 50% and 100% of animals [ED_{50}] and [ED_{100}]). Mice were housed in 10-head cages and infected IV with *S. aureus* at a lethal dose. Thirty minutes after infection, mice were injected with vancomycin hydrochloride (1), sulfate of eremomycin (2), and sulfate of eremomycin pyrrolidide (5) IV (6 doses in 5% glucose solution). As a control, a cohort of animals infected with *S. aureus* (at a lethal dose) but not treated with antibiotics was examined. Animals were monitored for 10 days, mortality was recorded daily.

To determine ED_{50} in the model of staphylococcal sepsis in mice, we used vancomycin hydrochloride (1) at 2.5–7.5 mg/kg; sulfate of eremomycin (2) at 0.5–5.0 mg/kg; and sulfate of eremomycin pyrrolidide (5) at 0.25–4.5 mg/kg. ED_{50} of the test preparations was determined by death of animals in Barents method (frequency accumulation).

Acute toxicity

The CBA mice (18–22 g) were randomized into groups ($n=6-10$) and received 1, 2, and 5 as single bolus IV injections. The substances were dissolved in 5% glucose solution (concentration of 1 and 5: 10 mg/mL; concentration of 2: 100 mg/mL) and administered at 200–700 mg/kg (1), 500–3,000 mg/kg (2), and 100–400 mg/kg (5). Acute toxicity was evaluated on the basis of mortality, survival time, and clinical manifestations of intoxication. The 50% lethal dose (LD_{50}) values and maximum tolerated doses (MTD = 10% lethal dose [LD_{10}]) were calculated by the method of Litchfield and Wilcoxon using StatPlus 2006 AnalystSoft StatPlus software.

Assessment of pseudoallergic reaction

The potency of the semisynthetic derivative 5 to induce pseudoallergic reaction was assessed by non-immune activation of histamine release (inflammatory reaction to concanavalin A [Con A]) according to the “Guidelines on the

assessment of allergic properties of drugs.”³⁹ The experiment was carried out in male CBA mice ($n=40$) weighing 26–28 g. After a 2-week quarantine, healthy animals were caged in groups of 10 and fed ad libitum with standard laboratory food and tap water.

Compounds 1, 2, and 5 in 5% glucose solution were injected IV at single doses of MTD. Similarly, 10 mL/kg of 5% glucose solution was administered to the control group ($n=10$). One hour post injection, 0.5% Con A in isotonic sodium chloride solution (0.01 mL/10 g body mass) was injected into the pad of the hind legs, and the same volume of isotonic solution of sodium chloride was injected into the contralateral extremity of mice in the experimental and control groups. One hour later, the mice were decapitated, the extremities were amputated at the limit of the hock joint, and their mass was determined. The reaction index of inflammation (RI) was calculated using the formula $RI = (M_c - M_e) / M_e \times 100\%$, where M_e is the mass of the hind leg foot injected with Con A and M_c is the mass of the hind leg foot injected with isotonic sodium chloride solution.

Results and discussion

Chemistry

Modification of the C-terminal carboxyl group of the peptide core is an effective synthetic method of chemical transformation of glycopeptides.^{16,17,40} We synthesized new vancomycin and eremomycin carboxamides 3–6 (Figure 1). The reaction of 1 or 2 with pyrrolidine or piperidine in the presence of the coupling reagent resulted in pyrrolidides or piperidides of vancomycin 3 and 4 or eremomycin 5 and 6. Several appropriate reagents (PyBOP, O-(benzotriazol-1-yl)-N,N,N',N''-tetramethyluronium tetrafluoroborate, 1-[Bis(dimethylamino)methylene]-1 H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate, 1-propanephosphonic anhydride (), diphenylphosphoryl azide, or [1-cyano-2-ethoxy-2-oxoethylideneaminoxy]dimethylamino-morpholino-carbenium hexafluorophosphate) were tested for the amidation of glycopeptides.⁴⁰ The best reagent in this reaction was PyBOP, which gave the final carboxamides 3–6 in 48%–66% yields in optimal conditions. The purity of final derivatives 3–6 was >95% according to HPLC analysis. The analytical and spectroscopic data of new compounds were in full accordance with the assigned structures (Supplementary materials).

In vitro antibacterial testing

Screening of the antibacterial activity of new antibiotics was carried out using a panel of clinical isolates of

Gram-positive microorganisms using the micro-dilution method to determine MIC. Generally, the semisynthetic vancomycin derivatives 3 and 4 were less active against the tested strains (MICs ~0.5–8 mg/L) compared with the corresponding eremomycin derivatives 5 and 6 (Table 1). The activity of 3 and 4 or 5 and 6 were close to the potency of the corresponding starting antibiotics 1 or 2. However, it should be noted that carboxamides 4, 5, and 6 were more active against resistant strains VISA (MICs =0.5–2 mg/L) than the starting antibiotics 1 or 2 (MICs =4–8 mg/L).

The derivative 5 showed the highest activity against all tested strains and better MICs values for VSE (*S. epidermidis*, *S. haemolyticus*; MICs =0.13–0.25 mg/L) and vancomycin-resistant strains (VISA MICs =1 mg/L) than the carboxamides 3, 4, and 6. Based on results of pre-screening of antimicrobial properties, eremomycin pyrrolidide (5) was selected for a broad assessment of activity compared with vancomycin (1) as the gold standard for treatment Gram-positive human pathogens. The MIC distribution, MIC₅₀, MIC₉₀, and the percent susceptibility rates are summarized in Table 2.

Eremomycin pyrrolidide (5) revealed a high potency against *S. aureus*, coagulase-negative *Staphylococcus*, and *Enterococcus* strains, exceeding that of vancomycin (1) by 2–4 dilutions. Of note, eremomycin pyrrolidide (5) had similar activity for the genera *Staphylococcus* and *Enterococcus* (except for *E. faecium*) with MIC₉₀ =0.5 mg/L while vancomycin had MIC₉₀ =1–2 mg/L. The activity of both antibiotics against *Staphylococcus* and *Enterococcus* strains had no dependence on resistance to beta-lactams, fluoroquinolones, tetracyclines, and aminoglycosides. The distribution of MICs was monomodal for *Staphylococcus* and *E. faecalis*. Strains of vancomycin-resistant *E. faecium* (VRE, MIC >64 mg/L) were susceptible to 5, but an increased MIC value for these isolates led to the increase of MIC₉₀ to 4 mg/L, which was slightly higher than that of 1 (MIC₉₀ =2 mg/L).

The derivative 5 was significantly more active than vancomycin (1) for *E. faecium* (MIC₅₀ 0.125 mg/L vs 1 mg/L, respectively). It is important to mention that the number of resistant strains of *E. faecium* (MIC >64 mg/L) with this value was 7 (8%) for vancomycin (1) and zero for 5 (Table 2). Given that eremomycin pyrrolidide (5) demonstrated an obvious advantage over vancomycin (1), we compared their antibacterial efficacy in vivo and their acute toxicity in mice.

Staphylococcal sepsis in mice

Figure 2 indicates that the survival rate of mice infected with *S. aureus* increased after IV single-dose injections of 5 in a dose-dependent manner. Moreover, the efficacy of 5 in this model was notably superb compared with vancomycin (1)

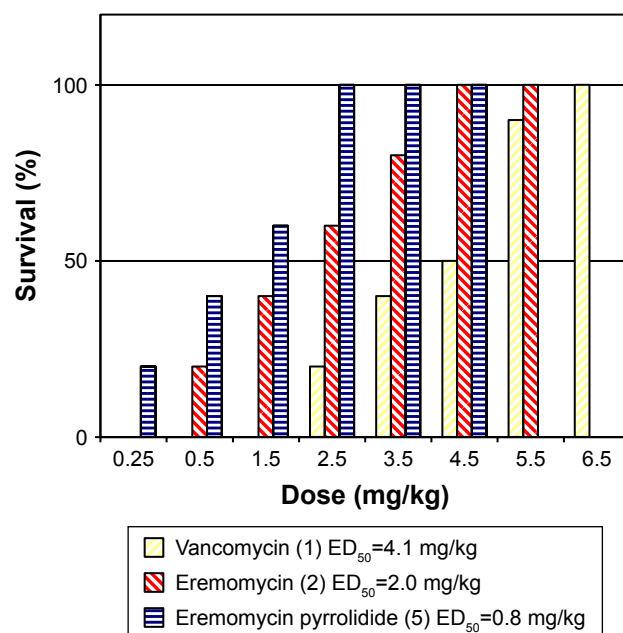


Figure 2 Efficacy of vancomycin (1), eremomycin (2), and eremomycin pyrrolidide (5) in the mouse model of staphylococcal sepsis.

Abbreviation: ED₅₀, effective dose of compound required to cure 50% of animals.

and eremomycin (2). Thus, the calculated ED₅₀ values for 5 (0.8 mg/kg) were 2.5 and 5 times lower than those for 2 or 1 (2.0 and 4.1 mg/kg, respectively). Survival of 100% animals was achieved with eremomycin pyrrolidide (5) at a noticeably lower dose (2.5 mg/kg) than after the administration of natural antibiotics 2 or 1 (4.5 and 6.5 mg/kg, respectively).

Acute toxicity

The signs of acute toxicity caused by a single IV administration of 1, 2, and 5 were generally similar. After the injection of the biggest doses, the animals in all groups died within 1–3 hour exhibiting neurotoxicity. Lower doses were lethal 1–3 days post treatment due to cardiovascular failure. Quantitative parameters of acute toxicity of 1, 2, and 5 are presented in Table 3. The LD₅₀ and MTD (LD₁₀) values for 1 correlated with the results reported by US Pharmacopeia.^{41,42} The acute toxicity of 2 (LD₅₀ ~1,500 mg/kg) was ~3-fold lower than that of 1 (LD₅₀ ~500 mg/kg), but the acute toxicity of 5 (LD₅₀ ~250 mg/kg)

Table 3 Acute toxicity LD₅₀ and MTD (LD₁₀) of vancomycin (1), eremomycin (2), and eremomycin pyrrolidide (5) in mice

Parameter	Vancomycin (1)	Eremomycin (2)	Eremomycin pyrrolidide (5)
LD ₅₀ (mg/kg)	525 (477.3÷577.5)*	1,530.3 (1,172.3÷1,888.2)*	248.4 (195.4÷301.4)*
MTD (LD ₁₀) (mg/kg)	360	877.9	119.7

Note: *Data shown as mean LD₅₀ (significant deviation of LD₅₀ for P≤0.05).

Abbreviations: LD₁₀, 10% lethal dose; LD₅₀, 50% lethal dose; MTD, maximum tolerated dose.

Table 4 Reaction index: pseudoallergic inflammatory reaction (RI) on concanavalin A in mice treated with eremomycin pyrrolidide (5), eremomycin (2), and vancomycin (1)

Compound	Relative increase M_e over M_c , %, mean \pm SD	RI, %
Vancomycin (1)	20.1 \pm 3.3	52.1
Eremomycin (2)	18.8 \pm 1.7	42.7
Eremomycin pyrrolidide (5)	13.9 \pm 2.1	5.5
Control	13.2 \pm 1.1	–

Notes: M_e is the mass of the hind leg foot injected with concanavalin A. M_c is the mass of the hind leg foot injected with isotonic sodium chloride solution.

was higher about 2-fold. Nevertheless, the ratio between therapeutic (ED_{50}) and toxic doses (MTD) for compound 5 was 1/150 compared with 1/90 for compound 1.

Pseudoallergic reaction

Finally, we compared the potency to induce a non-immune activation of histamine release for the selected derivative 5 and natural antibiotics 1 and 2. The inflammatory reaction to Con A is based on the capacity of this lectin to directly act on mast cell and basophil receptors, thereby liberating the inflammatory mediators. The results of this test demonstrated that the IV injection of vancomycin (1) and eremomycin (2) produced statistically significant elevations of M_e with respect to M_c (Table 4). The RI values in these groups were 52.1% and 42.7%, respectively. In contrast, a non-significant difference was found in M_e and M_c in mice treated with 5. The RI value in this group exceeded the respective value in the control cohort by only 5.5%.

Conclusion

Four new glycopeptides carboxamides of 3–6 were obtained by amidation of natural antibiotics vancomycin (1) or eremomycin (2) with pyrrolidine or piperidine in the presence of the coupling reagent PyBOP. Evaluation of biological properties of eremomycin pyrrolidide (5) demonstrated a pronounced antibacterial activity in vitro against a panel of clinical isolates of Gram-positive bacteria, including strains resistant to glycopeptides (VISA and VRE). The highest in vivo efficacy was also achieved in a murine model of staphylococcal sepsis. Eremomycin pyrrolidide (5) showed advantages over vancomycin (1) in all in vitro tests against Gram-positive bacteria. The response to Con A showed that 5 had no histamine release activity and, therefore, did not induce a pseudoallergic reaction. In addition, compound 5 demonstrated a favorable toxicology profile because its therapeutically efficient dose was 150-fold smaller than MTD. Thus, eremomycin pyrrolidide (5) is advantageous over the

natural antibiotics vancomycin (1) and eremomycin (2) and is perspective for further investigations.

Acknowledgments

We are grateful to Natalia M Malyutina for HPLC analysis, Alexandr M Korolev for electrospray ionization mass spectra, and Yurii N Luzikov for NMR-spectra (all of whom are from the Gause Institute of New Antibiotics, Moscow). †Professor Maria N Preobrazhenskaya passed away on December 25, 2014.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Reagents, instruments used, and other general information

All reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fluka (Munich, Germany), and Merck (Kenilworth, NJ, USA). The evaluation of the reactions, column eluates, and all final samples were analyzed by thin layer chromatography (TLC) using Merck Silica Gel 60F₂₅₄ plates (Merck) in systems containing EtOAc-PrOH-25%-NH₄OH, 30:30:22 or 1:1:1. In addition, purity of the final compounds was demonstrated by HPLC (column Kromasil 100-5C8, 4.6 × 250 mm, size 5 μm; Ekzo Nobel, Sweden) with loop 20 μL using 2 systems: A – 0.2% HCOONH₄ (pH 4.2) and MeCN 5→60%, 30 min; B – 0.6% HCOONH₄, pH 7.8 and MeCN 8→70%, 40 min. Reaction products were purified by reversed-phase column chromatography on Merck Silanized Silica Gel (0.063–0.2 mm). Melting points were determined using a Buchi SMP-20 apparatus and are uncorrected. Optical rotation was measured by AA-55 series polarimeter (Optical Activity Ltd., Huntingdon, UK). UV-spectra were determined by UNICO 2804 single-beam infrared scanning spectrophotometer (Unico, Inc., Franksville, WI, USA). The IR spectra were obtained on a Nicolet-iS10 Fourier transform IR spectrometer (DTGS detector, splitter – KBr) (Thermo Fisher Scientific, Waltham, MA, USA) with a Smart Performer module equipped with a ZnSe-crystal. The spectra were run on the range of 3,000–650 cm⁻¹ with a resolution of 4 cm⁻¹. The spectra were preceded using the OMNIC-7.0 program package. High resolution mass spectra were recorded with electron spray ionization (ESI) on a Bruker Daltonics microOTOF-QII instrument (Bruker Daltonics GmbH Life Sciences, Bremen, Germany). NMR spectra were recorded on a Varian VXR-400 instrument (Varian Inc., Palo Alto, CA, USA) operated at 400 and 100 MHz for ¹H and ¹³C, respectively. Chemical shifts were measured in D₂O using

3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS) as an internal standard or MeOH as an internal standard.

Several properties of the compounds 3–6

Vancomycin pyrrolidide (**3**). Yield 48%. White powder. Melting temperature 215°C–220°C (decomposition). TLC on Merck Silica Gel 60F₂₅₄ plates in system EtOAc-PrOH-25%-NH₄OH, 30:30:22, R_f = 0.51. HPLC: Rt = 19.07 min (A system). [α]_D²² = –16°, c = 0.25, H₂O. UV-spectra, H₂O: λ_{max} = 279 nm, E_{1 cm}^{1%} = 25.0. IR-spectra, strong bands: 3,323, 1,644, 1,504, 1,229, 1,045 cm⁻¹. ESI MS: found, 1,501.49, calculated for C₇₀H₈₂Cl₂N₁₀O₂₃, 1,501.50.

Vancomycin piperidide (**4**). Yield 65%. White powder. Melting temperature >220°C (decomposition); TLC on Merck Silica Gel 60F₂₅₄ plates in system EtOAc-PrOH-25%-NH₄OH, 30:30:22, R_f = 0.58. HPLC: Rt = 20.73 min (A system). [α]_D²² = –24°, c = 0.25, H₂O. UV-spectra, H₂O: λ_{max} = 279 nm, E_{1 cm}^{1%} = 23.0. IR-spectra, strong bands: 3,289, 1,645, 1,504, 1,228, 1,045 cm⁻¹. ESI MS: found 1,515.46, calculated for C₇₁H₈₄Cl₂N₁₀O₂₃, 1,515.52.

Eremomycin pyrrolidide (**5**). Yield 66%. White powder. Melting temperature >220°C (decomposition). TLC on Merck Silica Gel 60F₂₅₄ plates in system EtOAc-PrOH-25%-NH₄OH, 30:30:22. R_f = 0.36. HPLC: Rt = 27.31 min (B system). [α]_D²² = –64°, c = 0.25, H₂O. UV-spectra, H₂O: λ_{max} = 282.5 nm, E_{1 cm}^{1%} = 29.4. IR-spectra, strong bands: 3,286, 1,644, 1,504, 1,212, 1,063 cm⁻¹. ESI MS: found 1,610.62, calculated for C₇₇H₉₆ClN₁₁O₂₅, 1,610.63.

Eremomycin piperidide (**6**). Yield 52%. White powder. Melting temperature >220°C (decomposition). TLC on Merck Silica Gel 60F₂₅₄ plates in system EtOAc-PrOH-25%-NH₄OH, 30:30:22, R_f = 0.46. HPLC: Rt = 28.04 min (B system). [α]_D²² = –64°, c = 0.25, H₂O; UV-spectra, H₂O: λ_{max} = 282 nm, E_{1 cm}^{1%} = 31.2. IR-spectra, strong bands: 2,949, 1,683, 1,652, 1,515, 1,102, 1,028 cm⁻¹. ESI MS: found 1,624.65, calculated for C₇₈H₉₈ClN₁₁O₂₅, 1,624.65.

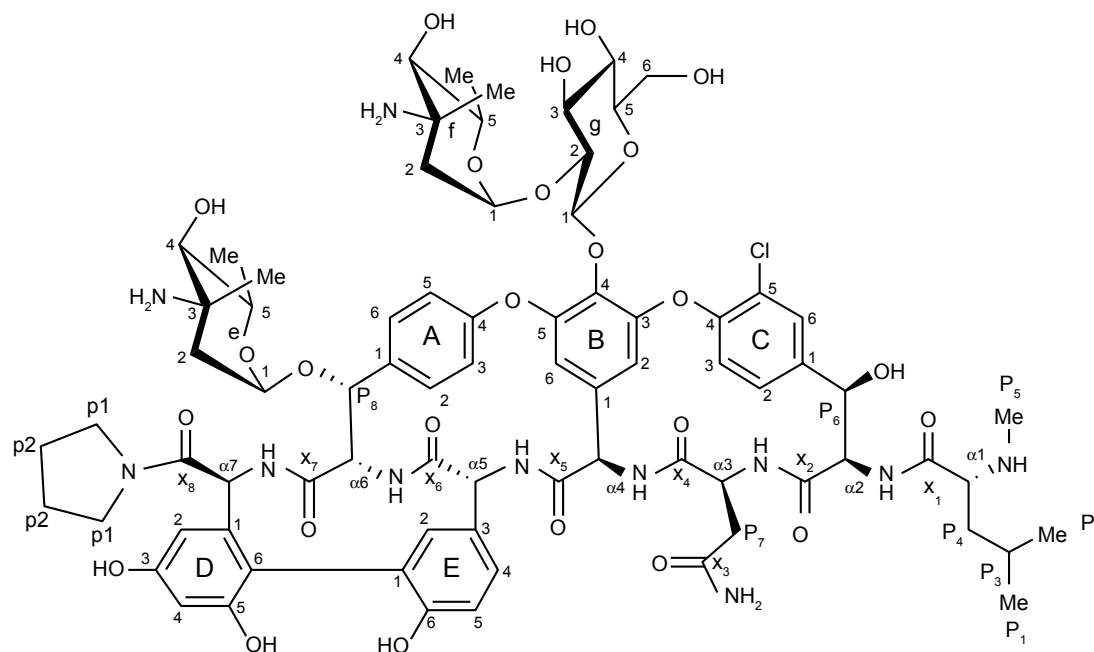


Figure S1 Atom's numeration for ^{13}C nuclear magnetic resonance spectra of eremomycin pyrrolidide (**5**).

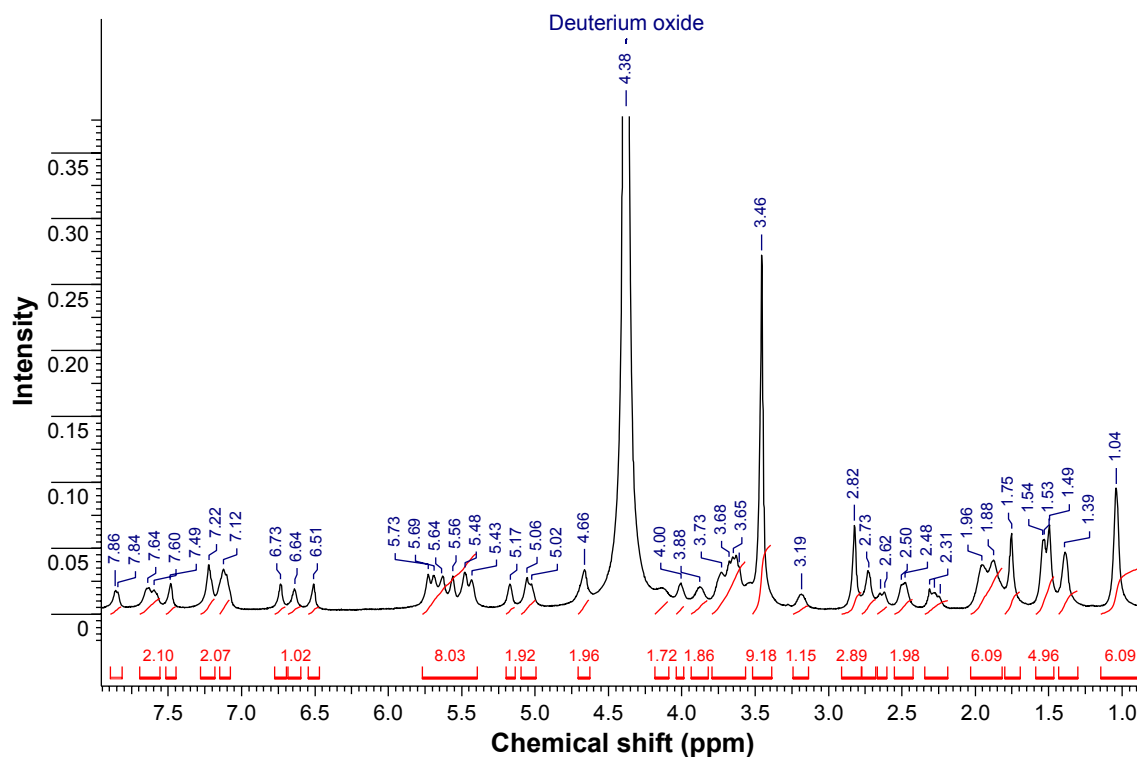


Figure S2 $^1\text{H-NMR}$ -spectra of eremomycin pyrrolidide (**5**) (D_2O , 85°C). Chemical shifts were measured in D_2O using 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS) as an internal standard.

Notes: $^1\text{H-NMR}$ (400 MHz, D_2O , 85°C) δ : 7.85 (d, 1H); 7.62 (m, 2H); 7.49 (s, 1H); 7.22 (s, 2H); 7.12 (m, 2H); 6.73 (s, 1H); 6.64 (s, 1H); 6.51 (s, 1H); 5.75–5.40 (m, 8H); 5.17 (s, 1H); 5.04 (m, 2H); 4.66 (br. s, 2H); 4.13 (br. s, 2H); 4.00 (br. s, 1H); 3.88 (br. s, 2H); 3.77–3.57 (m, 7H); 3.46 (s, 9H); 3.19 (br. s, 1H); 2.82 (c, 3H); 2.73 (br. s, 2H); 2.63 (d, 1H); 2.49 (m, 2H); 2.34–2.20 (m, 2H); 2.02–1.83 (m, 6H); 1.75 (c, 3H); 1.57–1.47 (m, 5H); 1.39 (s, 3H); 1.04 (s, 6H).

Abbreviation: NMR, nuclear magnetic resonance.

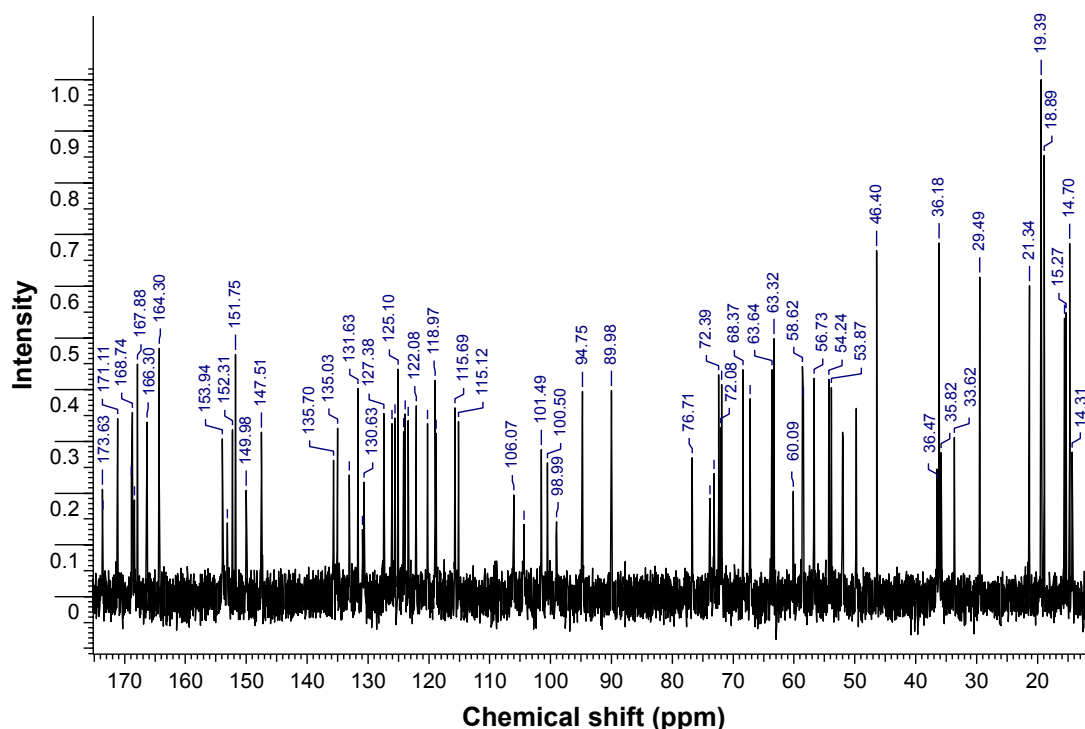


Figure S3 ¹³C-NMR-spectra of eremomycin pyrrolidide (**5**) (D₂O, 85°C). The sample concentration was ~20 mg/mL in D₂O with MeOH as an internal standard.

Notes: ¹³C-NMR (101 MHz, D₂O, 85°C, inner standard – CH₃OH) δ: 173.63 (X-8); 173.63 (X-3); 171.33 (X-5); 171.02 (X-6); 170.50 (X-4); 169.20 (X-1); 168.92 (X-2); 167.64 (X-7); 156.93 (D-3); 156.50 (B-3); 155.69 (A-4); 155.37 (D-5); 154.59 (E-4); 152.58 (B-5); 150.09 (C-4); 137.74 (C-1); 135.50 (D-1); 135.67 (E-2); 134.25 (B-1); 133.53 (B-4c); 133.11 (A-1); 129.95 (C-6); 128.61 (A-2); 128.22 (A-6); 127.75 (C-5); 127.05 (E-6); 126.41 (C-2); 125.99 (E-1); 124.68 (C-3); 122.79 (A-3); 121.09 (E-3); 121.47 (A-5); 118.14 (E-5); 117.97 (D-6); 107.46 (D-2); 107.06 (B-2); 104.09 (B-6); 103.79 (D-4); 101.64 (g-1); 97.37 (f-1); 92.68 (e-1); 79.27 (g-2); 76.44 (g-3); 75.75 (g-5); 74.68 (e-4); 74.62 (P-8); 74.53 (f-4); 70.89 (P-6); 69.82 (g-4); 66.41 (e-5); 65.95 (f-5); 62.57 (α-6); 61.51 (α-1); 61.26 (g-6); 59.30 (α-2); 56.61 (e-3); 56.24 (α-7); 56.40 (f-3); 54.66 (α-5); 54.52 (α-4); 52.39 (α-3); 46.50 (p-1); 46.28 (p-1); 39.49 (P-4); 39.14 (f-2); 38.46 (e-2); 36.37 (P-7); 32.53 (P-5); 25.18 (p-2); 24.09 (P-3); 23.51 (p-2); 22.05 (P-1); 21.56 (P-2); 18.10 (Me-e3); 17.90 (Me-f3); 17.23 (Me-e5); 16.94 (Me-f5).

Abbreviation: NMR, nuclear magnetic resonance.

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