Carboxamides and Hydrazide of Glycopeptide Antibiotic Eremomycin

Synthesis and Antibacterial activity

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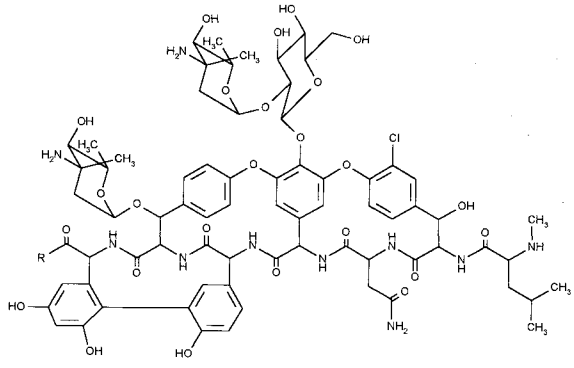
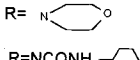
Carboxamides and hydrazide of glycopeptide antibiotic eremomycin were obtained by a direct reaction of the carboxy group of eremomycin with an appropriate amine or hydrazine using diphenyl phosphorazidate as a condencing agent. Eremomycin hydrazide was also obtained by hydrazinolysis of the eremomycin methyl ester. Use of dicyclohexylcarbodiimide or 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide for amidation led to the corresponding eremomycin ureides. The ESI-MS data indicate that eremomycin and its amides exist as dimers. The carboxamide, methylamide and benzylamide of eremomycin were as active against Gram-positive bacteria as the parent antibiotic, and the methylamide, benzylamide and hydrazide were almost an order of magnitude more active than eremomycin against *Staphylococcus epidermidis* clinical isolates *in vitro*. Amide of eremomycin as well as ureides were devoid of histamine liberating properties, which demonstrates that protection of the carboxyl group leads to a decrease in the allergenic properties.

Antibiotic eremomycin (**1**) is a potent antibacterial antibiotic of the dalbaheptide group closely related to vancomycin[1]. The molecular basis for the antibacterial activity of the group arises from the specific binding of the glycopeptide to the nascent bacterial cell wall bearing the *C*-terminal sequence D-Ala-D-Ala. Although the affinity of these antibiotics for the bacterial cell wall analogues in some cases correlates directly with the *in vitro* antibiotic activity, eremomycin , which is more active than vancomycin against a range of staphylococcal strains, has a lower affinity for the cell wall analogue di-*N*-acetyl-L-Lys-D-Ala-D-Ala. Recently it was demonstrated that eremomycin forms stable dimers (K_{dim} 3×10^6, for vancomycin K_{dim} 700) and that the dimerization plays a role in the mechanism of action of these antibiotics[2,3]. This peculiarity of eremomycin makes investigation of structure-activity relationships among eremomycin derivatives both of practical and theoretical interest.

The model of the eremomycin dimer formation and interaction with the D-Ala-D-Ala moiety suggests that the carboxyl group of the antibiotic participates neither in the dimerization nor in the interaction with the target and thus affords an opportunity for valuable chemical modifications. Eremomycin analogs modified at the carboxyl group may retain the antibacterial properties, whereas the modification may favorably influence other pharmacological properties, first of all, allergenicity. Methyl ester of eremomycin (**2**) demonstrated high



R=OH (1)
R=OCH_3 (2)
R=NH_2 (4)
R=$NHNH_2$ (5)
R=$NHCH_3$ (6)
R=$NHCH_2C_6H_5$ (7)
R=  (8)

R=  (9)
R=NCONH  (10)

R=NCONH$(CH_2)_3N(CH_3)_2$ (11)
|
C_2H_5

R=$NCONHC_2H_5$ (12)
|
$(CH_2)_3N(CH_3)_2$

antibacterial activity though lower than the parent antibiotic[4]. Methyl ester of *N,N*-dimethyleremomycin (**3**) displayed antibacterial activity *in vitro* comparable with or even superior to that of eremomycin[5].

For glycopeptide antibiotic teicoplanin and its deglycosylated derivatives, transformation into carboxamides led in some cases to compounds with antibacterial activity superior to the starting antibiotic; of special interest is some *in vitro* activity of the carboxamides against VanA enterococci highly resistant to both teicoplanin and vancomycin[6]. In the synthesis of carboxamides of teicoplanine and its deglycosylated products, diphenyl phosphorazidate (DPPA) as a condensing agent or activated cyanomethyl eaters were used. For the synthesis of amides of the glycopeptide antibiotics ardacin, parvodicin or its derivatives, the *N*-protected glycopeptides were condensed with the corresponding amines in the presence of alkylchloroformates[7]. Recently, two methods for the synthesis of vancomycin carboxamide derivatives by using DCC or HBTU (2-(1-hydroxybenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) were reported[8,9].

Carboxamides and hydrazide of eremomycin were obtained by a direct reaction of the carboxyl group of eremomycin with the amine or hydrazine using DPPA as a condencing agent; if the NH_2-compound was used as a salt, the reaction was carried out in the presence of Et_3N. Preliminary protection of amino groups was not necessary in this case. Using this method eremomycin carboxamide (**4**), hydrazide (**5**), methylamide (**6**), benzylamide (**7**), 1-methylpyperazide (**8**), and morpholide (**9**) were synthesized. Hydrazide **5** was also obtained by the hydrazinolysis of ester **2**[4].

Use of dicyclohexylcarbodiimide for the amidation led to 1,3-dicyclohexylureide **10**. With the use of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, a mixture of isomeric ureides **11** and **12** was isolated (~1:1 as shown by HPLC). Isolation of the carboxamides, hydrazide and ureides **4**~**12** was performed by column chromatography on CM-cellulose. The modification in the eremomycin molecule was localized in the peptide moiety after acid hydrolysis (yielding unmodified eremosamines) and EDMAN's degradation.

For the analysis of eremomycin derivatives electrospray mass spectrometry (ESI-MS) was used, which is more straightforward and fast than FAB method[5]. ESI-MS is capable of providing both molecular weight and structural information of this class of antibiotics and, due to its soft nature, prevents artifacts resulting from thermal degradation and/or from the ionization process itself. In the ESI-MS spectrum of the eremomycin standard, in addition to the base peak (*m/z* 1557.6, lowest isotope), which corresponds to the monoprotonated molecule ($[M+H]^+$), other high-mass peaks are present. In particular, the peaks at *m/z* 1039.4 and *m/z* 774.4 correspond to the triply charged triprotonated dimer ($[2M+3H]^{+3}$) and to the doubly charged diprotonated monomer ($[M+2H]^{+2}$), respectively. The peaks at *m/z* 1485.5, 1413.5, 991.4, and 707.3 correspond to the loss of the eremosamine moiety (144 Da) from the doubly charged diprotonated dimer ($[2M+2H-erem]^{2+}$), from the monoprotonated monomer ($[M+H-erem]^+$), from the triply charged triprotonated dimer ($[2M+3H-erem]^{3+}$) and from the doubly charged diiprotonated monomer ($[M+2H-erem]^{2+}$), respectively. All the investigated eremomycin amides investigated exhibited the same mass spectrometric behavior. Results of the ESI-MS investigation give an independent evidence of existence of eremomycin derivatives in dimeric forms. It is noteworthy that modifications of the carboxyl group do not prevent dimerization (at least, as it follows from the ESI-MS data).

Antibacterial properties of the compounds obtained were investigated *in vitro* and *in vivo*. Derivatives **4**, **6**, and **7** were as active as the parent antibiotic against Gram-positive bacteria. It should be noted that the carboxamides **6** and **7** and hydrazide **5** are almost by an order of magnitude more active than eremomycin against the *Staphylococcus epidermidis* clinical isolates (Table 1). This type of antibacterial activity is of special interest as pathogenicity of *S. epidermidis* is associated with indwelling foreign devices, prosthetic valve endocarditis, surgical wound infections and is especially typical in immunosupressed patients. The compounds investigated did not inhibit growth of the *Enterococcus faecalis* clinical isolates resistant to vancomycin (MIC > 128 mcg/ml). An *in vitro* investigation of derivatives **10** and **11**+**12** in comparison with eremomycin and vancomycin showed that the ureides are less active than **1** and comparable with vancomycin (for *S. aureus*, MIC_{50} values for **10** and **11**+**12** are 4 and 2 μg/ml, respectively; for eremomycin and vancomycin, 0.5 and 2 μg/ml, respectively).

A study of the antibacterial activity of amides in mice with acute lethal staphylococcal infection *in vivo* demonstrated that carboxamide **4** and ester **3** when given sc are close to the parent antibiotic whereas methylamide **6**, is two-fold less active. Hydrazide **5** is four-fold less active than eremomycin and close to vancomycin (Table 2) when administered iv, amide **4** was tree-fold less active than eremomycin (ED_{50} 1.25 and 0.44 mg/kg, respec-

Table 1. Antibacterial evaluation of eremomycin amides and hydrazide in comparison with eremomycin (**1**); MIC values (μg/ml).

Strain	Compound						
	1	**4**	**5**	**6**	**7**	**8**	**9**
Staphylococcus aureus Tour	0.13	0.13	0.25	0.25	0.13	0.5	0.5
S. aureus Tour 30% bovine serum	0.25	0.5	0.25	0.25	0.5	1	2
S. aureus Smith	0.13	0.13	0.5	0.13	0.13	0.5	0.5
S. aureus clinical isolate	0.5	0.5	1	0.5	0.25	2	2
S. epidermidis ATCC 12228	0.25	0.25	0.13	0.25	0.25	0.25	0.25
S. epidermidis clinical isolate	1	0.25	0.13	0.13	0.13	0.25	0.25
S. haemoliticus clinical isolate	0.25	0.13	0.13	0.5	0.13	1	0.25
Streptococcus pyogenes C203	0.13	0.13	0.13	0.13	0.06	0.13	0.13
S. pneumoniae UC41	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Enterococcus faecalis ATCC 7080	0.25	0.25	0.5	0.25	0.13	0.25	0.13

Table 2. Chemotherapeutic activity of eremomycin and its derivatives in mice with acute lethal infection induced by *Staphylococcus aureus* No. 5.

Compound	ED_{50} (mg/kg, sc)
Eremomycin **1**	2.0
Vancomycin	10.0
Carboxamide **4**	3.0
Methylamide **6**	5.0
Hydrazide **5**	10
Ester **3**	2.25

Table 3. Changes of surface of stomach mucosa after administration of eremomycin or its derivatives.

Compound	Intensity of changes in balls (average of the treated rats)
Eremomycin **1**	2.5
Methyl ester **2**	0.2
Methyl ester of *N,N*-dimethyleremomycin **3**	0.7
Carboxamide **4**	0
Ureide **10**	0.1
Ureide **11**+**12**	0

tively).

Allergenic properties of the antibiotics of the vancomycin group represent the most important side-effect of these compounds. They depend on the endogenous histamine liberation which induces in rats destructive lesions of the gastrointestinal tract mucosa. This test has been previously used for determining the histamine liberation by various substances[10]. The lesions were measured after intraperitoneal administration of **1** and its derivatives **2**, **3**, **4**, **10**, **11**+**12** in doses of 150 and 300 mg/kg (0.05 and 0.1 of the LD_{50} dose). Carboxamide **4** as well as ureides **10**, **11**+**12** were devoid of the histamine liberating properties; esters **2** and **3** in this test were very weakly allergenic. It demonstrates that the protection of the carboxyl group leads to a decrease in the allergenicity (Table 3).

Experimental

Eremomycin sulfate (**1**) was produced at the pilot plant of Institute of New Antibiotics. NMR spectra were recorded on a Varian VXR-400 spectrometer in D_2O at 70°C. Paper electrophoresis was performed in 0.05 AcOH-pyridine buffer (pH 5.6) at 900 V for 3 hours on the Filtrak FN-12 paper (Germany). CM-cellulose (CM-32 Whatman) column chromatography was performed with LKB Ultragrad Gradient Mixer 11300 supplied with Uvicord 2138 and Recorder 2065. TLC was performed on the Merck Silica Gel $60F_{254}$ plates in the systems EtOAc-PrOH-25% NH_4OH 2:2:1 (A), 2:2:5 (B), and 3:3:4 (C). The samples were analyzed by loop injection electrospray mass spectrometry using the conditions listed below: HPLC: Instrument Waters 625 LC System; eluent 0.1% aqueous TFA-CH_3CN 70:30; flow rate 50 μl/minute. ESI mass spectra were obtained using a Finnigan TSQ700 instrument equipped with a Finnigan Electrospray ion source; capillary temperature 180°C; spray voltage 4.5 kV; sheath gas (N_2) pressure 70 psi, auxiliary gas (N_2) flow 20 ml/minute; scan range/rate 300~1800 m/3 sececonds. In order to obtain maximun sensitivity and accuracy, the instrument was previously tuned and calibrated using a mixture of MRFA-myoglobin. Prior to analysis, specific tune parameters were optimized using repeated injection of the eremomycin standard (*ca.* 4 mg/ml). About 0.5 mg of each sample was dissolved in 250 μl of mobile phase immediately before the analysis.

Eremomycin Carboxamide (**4**)

DPPA (0.043 ml, 0.2 mmol) was added to a stirred solution of 165 mg (0.1 mmol) of eremomycin sulfate (**1**), 22 mg (0.4 mmol) of NH_4Cl and 0.07 ml (0.5 mmol) of

Et_3N in 8 ml dimethylsulfoxide. The reaction mixture was stirred for about 8 hours at 20°C, and then another portion of DPPA (0.043 ml) were added. The stirring was continued for 14 hours at 20°C, then 150 ml of acetone was added, and the precipitate was collected, dissolved in 5 ml of 0.2 M CH_3COONH_4 (pH 6.7) and applied to a chromatographic column with CM-cellulose (1 × 45 cm) preequilibrated with 0.2 M CH_3COONH_4 (pH 6.7). The column chromatograpy was carried out with 0.2 M CH_3COONH_4 with a linear pH gradient (pH 6.7→9.5). The fractions containing **4** were acidified with 6 N H_2SO_4 to pH 6 and desalted by stirring with XAD-resin (50 ml) for 24 hours followed by elution with 150 ml of H_2O-CH_3OH (1 : 1) mixture. The eluate was concentrated to a small volume (about 2 ml) *in vacuo* at 50°C and 100 ml of acetone was added. The precipitate formed was collected, washed with acetone and dried *in vacuo* at 20°C overnight to give 39 mg (23%) of **4**. Rf 0.14 (A); 0.62 (B), 0.42 (C). $E^{1\%}_{1cm}$ 40 (280 nm). $[\alpha]^{20}_D$ −100° (*c* 1, water). ESI-MS: 1555 $[M]^+$, $C_{73}H_{90}N_{11}O_{25}Cl$, Calc. 1555.

Eremomycin Hydrazide (**5**)

A. **5** was obtained from **1** and $NH_2NH_2 \cdot HCl$ similarly to **4** in 20% yield.

B. Hydrazine hydrate (0.04 ml, 0.6 mmol) was added to a stirred solution of 83 mg (0.05 mmol) of **2** in 5 ml of methanol. After **5** hours at 37°C, the formed precipitate was filtered off, washed with methanol and dried *in vacuo*. The purification was performed as described for **4** to give 29 mg (17%) of **5**. Rf 0.64 (B); 0.43 (C). $E^{1\%}_{1cm}$ 40 (280 nm). $[\alpha]^{20}_D$ −98° (*c* 1, water). ESI-MS: 1570 $[M]^+$, $C_{73}H_{91}N_{12}O_{25}Cl$, Calc. 1570.

Eremomycin Methylamide (**6**)

DPPA (0.043 ml, 0.2 mmol) was added to a stirred solution of 165 mg (0.1 mmol) of **1** in 8 ml of DMSO and 0.05 ml of 30% aqueous CH_3NH_2. The reaction mixture was stirred for 8 hours at 20°C, and another portion (0.043 ml) of DPPA and 0.1 ml of 30% aqueous CH_3NH_2 were added. After 14 hours of stirring, additional 0.086 ml of DPPA was added, and in 20 hours the product was precipitated with 150 ml of acetone. Purification was performed as described for **4** to yield 33 mg (20%) of **6**. Comparison of the ^1H NMR spectra of **6** and eremomycin demonstrates the presence of a six-proton singlet at 2.86 ppm, which corresponds to two methyl groups (*N*-terminal and methylamide ones). Rf 0.18 (A); 0.68 (B); 0.48 (C). $E^{1\%}_{1cm}$ 41 (280 nm). $[\alpha]^{20}_D$ −98° (*c* 1, water). ESI-MS: 1569 $[M]^+$, $C_{74}H_{92}N_{11}O_{25}Cl$, Calc. 1569.

Eremomycin Benzylamide (**7**)

DPPA (0.043 ml, 0.2 mmol) was added to a stirred solution of 165 mg (0.1 mmol) of **1** and 0.043 ml (0.4 mmol) of benzyl amine in 8 ml of DMSO. The reaction mixture was stirred for 8 hours at 20°C, and two portions (0.086 ml each) of DPPA were added with a 14 hours interwal. The stirring was continued for 14 hours, acetone (150 ml) was added and the precipitate was purified as described for amide **4** to give 35 mg of **7** (20%). In the ^1H NMR spectrum, five-proton multiplet in low field at 7.6 ~ 7.3 ppm, corresponding to phenyl protons, is present. Rf 0.47 (A); 0.66 (C); $E^{1\%}_{1cm}$ 38 (280 nm). $[\alpha]^{20}_D$ −96° (*c* 1, water). ESI-MS: 1645 $[M]^+$, $C_{80}H_{96}N_{11}O_{25}Cl$, Calc. 1645.

Eremomycin 4-Methylpiperazide (**8**)

It was obtained as described above in 18% yield. In ^1H NMR spectrum of **8** three-proton singlet of N-Me group and signals of four CH_2 groups at 4.10 ~ 3.37 and 3.15 ~ 2.90 ppm are present. Rf 0.23 (A); 0.57 (C). $E^{1\%}_{1cm}$ 40 (280 nm). $[\alpha]^{20}_D$ −101° (*c* 1, water). ESI-MS: 1638 $[M]^+$, $C_{78}H_{99}N_{12}O_{25}Cl$, Calc. 1638.

Eremomycin Morpholide (**9**)

It was obtained as described above in 17% yield. Rf 0.25 (A); 0.59 (C). $E^{1\%}_{1cm}$ 39 (280 nm). $[\alpha]^{20}_D$ −98° (*c* 1, water). In the ^1H NMR spectrum of **9**, signals of four CH_2 groups at 3.68 ~ 3.21 ppm are present. ESI-MS: 1625 $[M]^+$, $C_{77}H_{96}N_{11}O_{26}Cl$, Calc. 1625.

Eremomycin 1,3-Dicyclohexyl Ureide (**10**)

DCC (21 mg, 0.1 mmol) was added to a solution of 165 mg (0.1 mmol) of **1** in 8 ml of DMSO at 20°C, after 8 hours addition of 21 mg of DCC was repeated. Dicyclohexylurea was filtered off after 14 hours, and the reaction product was precipitated with ethyl acetate and purified as described above to give 54 mg (29%) of **10**. Rf 0.50 (A), 0.31 (B). $E^{1\%}_{1cm}$ 38 (280 nm). $[\alpha]^{20}_D$ −93° (*c* 1, methanol). In the ^1H NMR spectrum of **10** signals of the dicyclohexylureido group are present. ESI-MS: 1762 $[M]^+$, $C_{86}H_{111}N_{12}O_{26}Cl$, Calc. 1762.

A Mixture of Eremomycin 1-Ethyl-3-(3-dimethylaminopropyl)ureide (**11**) and Eremomycin 3-Ethyl-1-(3-dimethylaminopropyl)ureide (**12**)

It was obtained from 165 mg (0.1 mmol) of **1** and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (76 mg) as described above in a yield of 112 mg (60%). Rf 0.41 (A); 0.20 (B). $E^{1\%}_{1cm}$ 40 (280 nm). $[\alpha]^{20}_D$ −95° (*c* 1, water). In the ^1H NMR spectrum of **11**+**12**, signals of the *N*-ethyl-*N'*-(3-dimethylaminopropyl)ureido group are present. ESI-MS: 1711 $[M]^+$, $C_{81}H_{106}N_{13}O_{26}Cl$, Calc. 1711.

Study of Biological Properties

Experiments *In Vitro*

Microtiter method; overnight incubation 37°C; inocula were at a final dilution of 1/500 of the overnight broth culture. Todd-Hewitt broth was used for all enterococci and strepococci, nutrient broth was used for all other cultures.

Experiments *In Vivo*

Therapeutical effects of the amides in comparison with eremomycin and its *N*,*N*-dimethyl methyl ester (**3**) were

determined against an acute lethal staphylococcal infection in albino mice (BALB/c, 18+2 g) challenged by intraperitoneal injection with bacterial cells suspended in gastric mucin sufficient to kill nontreated control within 24~48 hours. The test compounds were administrated sc 30 minutes post infection and 24 hours later. In each test five mice were treated per one dose level. On the 7th day ED_{50} were calculated on the basis of percentage of surviving mice of each dose.

Histamine Liberating Properties

Singular intraperitoneal injection of the compounds in the dose of 150 and 300 mg/kg (0.05 and 0.1 of LD_{50} for eremomycin) were given to rats. Each group consisted of 6~10 white rats weighting 110~130 g each. The stomach was opened 2~3 hours later after administration of the drugs. Intensity of changing of stomach mucosa was macroscopically marked in four ball system and was also investigated hystologically. Hemorrhages and ulcers were marked with four balls if they covered 50~85% of stomach mucosa. Three balls correspond to changes of 30~50% of surface and two balls to 10~30%, smaller changes were marked with one ball. (Table 3).

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