A Modification of the *N*-Terminal Amino Acid in the Eremomycin Aglycone

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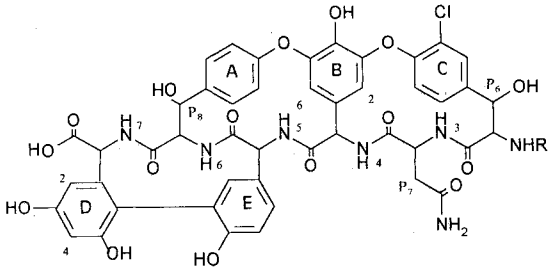
An Edman degradation of the antibiotic eremomycin aglycone produced the corresponding hexapeptide, which was aminoacylated with D-lysine, D-histidine or D-tryptophan derivatives to give new heptapeptide analogs of the eremomycin aglycone. The aminoacylation of the eremomycin aglycone produced an octapeptide analog. The substitution of D-lysine for the *N*-terminal *N*-methyl-D-leucine does not seriously affect the *in vitro* antibacterial properties of the eremomycin aglycone whereas the heptapeptides with the *N*-terminal D-tryptophan or D-histidine moieties and the octapeptide with the *N*-terminal D-lysine are practically devoid of the antibacterial properties.

Antibacterial glycopeptide antibiotics (dalbaheptides) inhibit biosynthesis of bacterial cell walls by forming a complex with the terminal D-alanyl-D-alanine of the peptidoglycan precursors. The cases of bacterial resistance to clinically used vancomycin and teicoplanin that became more and more frequent nowadays, make the search for glycopeptides active against dalbaheptides resistant microorganisms a problem of great importance[1]. For the directed design of semisynthetic glycopeptides capable to overcome resistance to vancomycin or teicoplanin it is important to elucidate the structural factors that are responsible for the binding to bacterial cell wall, which results in the antibacterial activity of antibiotics.

The structure of the natural glycopeptides backbone is rather conserved: in the antibiotics of the teicoplanin family the *N*-terminal amino acid is represented by a substituted phenylglycine connected through an oxygen bridge with the phenyl nucleus of the third amino acid; in the vancomycin family of antibiotics the *N*-terminal amino acid is most often represented by *N*-methyl-D-leucine (vancomycin, eremomycin). *N*-Terminal residues are 4-hydroxyphenyl-D-sarcosine in *β*-avoparcin[1] and 3-chloro-4-hydroxyphenyl-D-glycine or 4-hydroxyphenyl-D-glycine in actinoidins[2].

N-Terminal methylleucine residue of vancomycin was removed by an Edman degradation[3,4] and the exposed amino group of the second amino acid was acylated with *N*-methylglycine, or *N*-methyl-D-alanine



I. R = –CO–CH(α1)–NHCH3 ; CH2 ; CH ; H3C, CH3

II. R = –H

III. R = –CO–CH(α1)–NH2 ; CH2–CH2–CH2–CH2–NH2 (P4 P3 P2 P1)

IV. R = –CO–CH(α1)–NH2 ; P4 CH2 ; HN, N

V. R = –CO–CH(α1)–NH2 ; CH2 P4 ; N H

VI. R = –CO–CH(α1)–NH(CH3)–CO–CH(α0)–NH2 ; P4 CH2 ; CH ; H3C P3 CH3 ; P2 P2 ; CH2–CH2–CH2–CH2–NH2 (P'4 P'3 P'2 P'1)

to yield new dalbaheptides[5]. A strengthening of the $-CO_2-...NH_2Me^+$ interaction in the presence of the $-CHCH_2(CH_3)_2$ side chain moiety responsible for the highly efficient sequestering of the D-Ala-D-Ala *C*-terminal carboxylate from the aqueous solution was postulated.

To obtain more information about the role of the *N*-terminal amino acid in the interaction with D-Ala-D-Ala and hence in the antibacterial activity of antibiotics of the vancomycin family, an investigation of analogs of various types containing an additional basic group (Lys, His) at the *N*-terminus, was needed. Introduction of D-tryptophan to the *N*-terminus could influence the antibacterial properties, since tryptophan can be considered as a polar yet hydrophobic residue[6].

Results and Discussion

Previously, the vancomycin hexapeptide was obtained and the terminal amino group of the latter was selectively aminoacylated with the corresponding Boc-amino acids in the presence of DCC followed by deblocking with ammonia and then TFA[5]. As a result of the low stability of eremomycin in acid and alkaline media[7], the attempts to aminoacylate eremomycin with the Boc-amino acids hydroxysuccinimide esters led to deglycosylated eremomycin derivatives. For this reason, we used eremomycin aglycone in studies of the role of the *N*-terminal amino acid of dalbaheptides.

The Edman degradation of eremomycin aglycone **1** selectively removed the *N*-terminal *N*-methyl-D-leucine to provide the eremomycin aglycone hexapeptide **II** in 68% yield. The *N*-terminal amino group of the hexapeptide **II** was aminoacylated with N^{α},N^{ε}-di-Boc-D-Lys-OSu, N^{α},N^{im}-di-Boc-D-His-OSu or N^{α}-Boc-D-tryptophan pentafluorophenyl ester to afford the eremomycin aglycone analogs **III**~**V**.

As among the newly obtained semisynthetic heptapeptides only the compound with an *N*-terminal D-lysine **III** has demonstrated antibacterial properties, an octapeptide **VI** with an *N*-terminal D-lysine was synthesized starting from eremomycin aglycone and N^{α},N^{ε}-di-Boc-D-Lys-OSu. It was isolated after ion exchange column chromatography in 5% yield. The low yield of **VI** may be caused by a lower reactivity of the secondary terminal amino group of the aglycone compared with the primary amino group of hexapeptide **II** in the aminoacylation.

The compounds were isolated by column chromatographies and characterized by ion-spray mass spectrometry and NMR. The properties of the compounds obtained are presented in Table 1.

An expanded part of the positive ion-spray spectrum of a heptapeptide **III** is shown as an example in Fig. 1. As typical for such a soft ionization technique, only ions corresponding to the non-fragmented molecules can be usually obtained (Table 1). In particular, the base peak

Fig. 1. Ion-spray mass spectrum of a heptapeptide **III**.

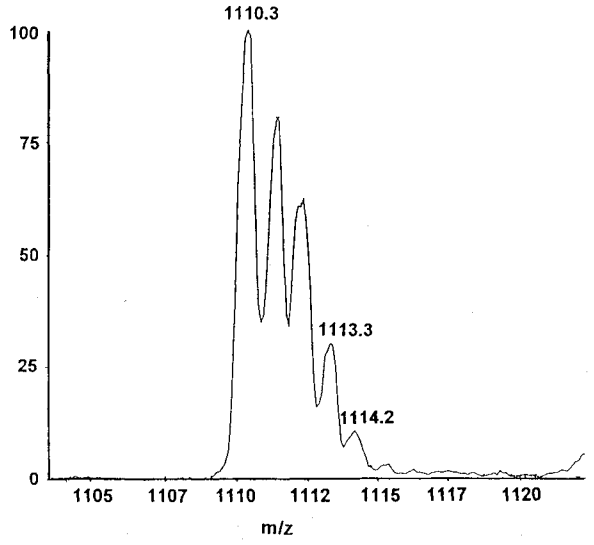


Table 1. Properties and yields of the compounds **II**~**VI** and eremomycin aglycone **1**.

Compound	Yield (%)	TLC Rf values System A	TLC Rf values System B	Electrophoretic mobility R_m	HPLC Rt minutes (System)	Molecular formula	Ion-spray MS MW Calcd	Ion-spray MS MW Found
1		0.40	0.57	1	3.12 (C)	$C_{53}H_{53}N_8O_{17}Cl$	1108.5	
II	83	0.17	0.41	1	4.97 (D)	$C_{46}H_{40}N_7O_{16}Cl$	981.2	981.3
III	40	0.12	0.28	2	5.85 (D)	$C_{52}H_{52}N_9O_{17}Cl$	1109.3	1109.3
IV	15	0.23	0.55	2	8.88 (D)	$C_{52}H_{47}N_{10}O_{17}Cl$	1118.3	1118.3
V	32	0.42	0.63	1	10.00 (D)	$C_{57}H_{50}N_9O_{17}Cl$	1167.3	1167.3
VI	5	0.21	0.37	2	7.34 (C)	$C_{59}H_{65}N_{10}O_{18}Cl$	1236.4	1236.4

corresponds to the ion cluster of the mono protonated molecule ($[M+H]^+$: m/z 1110.3).

The chemical shifts assignment in ^1H NMR spectra of the compounds **II**~**VI** has been done on the base of data obtained for eremomycin aglycone[8] using DQ COSY experiments (Table 2). Atoms numbering is given in accordance with the paper[8]

Table 2. Parameters of ^1H NMR spectra of compounds **II**~**VI**.

Proton	Compound[a]				
	II	**III**[b]	**IV**[c]	**V**[d]	**VI**
B-2	5.45	5.48	5.62	5.65	5.60
B-6	5.34	5.41	5.35	5.30	5.31
D-2	6.27	6.37	6.39	6.40	6.26
D-4	6.37	6.34	6.26	6.26	6.40
P-6	5.08	5.20	5.12	5.12	5.10
P-8	5.08	5.16	5.15	5.15	5.16
α-6	4.18	4.17	4.18	4.20	4.20
α-1	—	3.20	4.28	4.20	5.16
α-2	3.61	4.67	4.71	4.78	4.68
α-7	4.42	4.40	4.44	4.44	4.44
α-5	4.45	4.47	4.47	4.53	4.55
α-4	5.61	5.68	5.70	5.74	5.70
α-3	4.66	4.40	4.28	4.20	4.22
P-7	2.30; 2.15	2.50; 2.15	2.54; 2.20	2.64; 2.13	2.50; 2.10
H-3	7.78	*	*	*	*
H-4	8.09	7.90	*	8.10	8.14
H-5	8.56	8.71	8.73	8.77	8.74
H-6	6.56	6.67	6.59	6.58	6.60
H-7	8.44	8.54	8.59	8.64	8.62
P-8-OH	5.73	*	5.76	5.77	5.76
P-6-OH	5.73	*	5.92	5.93	5.80
P-1	—	2.75	—	—	—
P-2	—	1.35	—	—	0.93; 0.86
P-3	—	1.46	—	—	1.30
P-4	—	1.71; 1.55	3.10; 3.02	3.33; 3.09	1.80; 1.70

[a] Aromatic protons of A, C and E rings are in the area of 6.60~7.74 ppm. [b] Signals of the *N*-terminal D-lysine: P-1′ 2.74; N-Me 3.02; P-2′, and P-3′, and P-4′ 1.60. [c] Signals of imidazole ring: 7.25 and 7.15. [d] Signal of indole NH 10.99; indole C-H protons 7.74~6.70 ppm. * Signal was not observed.

Antibacterial activities *in vitro* of new heptapeptides **III**~**V** and octapeptide **VI** in comparison with hexapeptide **II** and the eremomycin aglycone **1** are presented in Table 3. It is well known that the deglycosylation of eremomycin leads to a strong decrease in the antibacterial activity[7] whereas deglycosylation of teicoplanin does not seriously affect the antibacterial properties *in vitro*[9]. Transition from the eremomycin aglycone **1** to hexapeptide **II** leads to a further decrease in the antibacterial activity. Aminoacylation of hexapeptide with D-tryptophan or D-histidine (compounds **IV** and **V**) does not improve the antibacterial properties of **II** whereas introduction of D-lysine into the *N*-terminal position (compound **III**) gives a heptapeptide with antibacterial properties comparable to those of the natural aglycone. The antibacterial activity of the octapeptide is lower than that of the aglycone. It is noteworthy that the influence of D-lysine introduced to the *N*-terminus of the eremomycin aglycone is different than in the case of the teicoplanin octapeptides containing D- or L-lysine in *N*-terminal position, which are more active than the starting teicoplanin aglycone[9]. None of the compounds obtained was active against VanA enterococci or *E. coli*.

Experimental

Eremomycin was produced at the pilot plant of the Institute of New Antibiotics. The eremomycin aglycone was obtained by the method[7]. All reagents and solvents used were commercial products. Activated esters of *N*-Boc-amino acids were synthesized with the use of DCC. TLC was performed on the precoated Merck silica gel $60F_{254}$ plates in an *n*-propanol - ethyl acetate - 25% aq. NH_3 10:10:13 mixture (system A) or in an *n*-butanol - acetic acid - water 20:7:13 mixture (system). Paper electrophoresis was performed in a 0.05 M acetic acid-pyridine buffer (pH 5.6) at 900 V for 3 hours using Filtrak FN-12 paper (Germany). The CM-cellulose CM-32 (Whatman) column chromatography was performed using LKB Ultragrad Mixer 11300 supplied with

Table 3. Antibacterial activities of compounds **II**~**VI** *in vitro* in comparison with eremomycin aglycone **1**.

Strain No.	MIC (μg/ml) values of the compounds					
	1	**II**	**III**	**IV**	**V**	**VI**
Staphylococcus aureus Smith L 819	8	32	8	64	64	16
Staphylococcus aureus L 561	16	>128	32	>128	128	128
Staphylococcus epidermidis L 533	16	>128	64	>128	>128	64
Staphylococcus haemolyticus L 602	32	128	32	>128	>128	64
Enterococcus faecalis L 659	16	>128	32	128	128	64
Enterococcus faecium L 658	32	>128	32	128	>128	128
Streptococcus pyogenes L 49	8	8	8	64	>128	32

Uvicord 2138 and Recorder 2065. Ion-exchange resin SDW-3, an analog of Dowex 50 × 2, was obtained from BioLar (Olaine, Latvia).

Analytical reversed phase HPLC was performed on a Shimadzu HPLC instrument of LC 10 series on a Zorbax C-8 DuPont column (4.6 × 250 mm i.d.; particle size 5 μm; gradient elution; injection volume 5 μl). Concentrations of the sample solutions were about 0.05~0.2 mg/ml, flow rate 1.5 ml/minute. A variable wavelength UV detector set at 280 nm was used. The mobile phase for the eremomycin aglycone **1** and its derivative **VI** was 0.2% ammonium formate solution adjusted to pH 3.8 with formic acid. Percentage of acetonitrile changed from 20 to 50% for 20 minutes (system C). For hexapeptide **2** and heptapeptides **III**~**V**, the mobile phase was acetonitrile-0.2% ammonium formate, pH 6.4. Percentage of acetonitrile changed from 15 to 36 for 20 minutes (system D).

An API **III**+ triple quadrupole mass spectrometer (PE-Sciex, Thornhill, ON, Canada) equipped with an articulated ion spray interface was employed. Tuning and calibration were performed on both the first (Q1) and the third quadrupole using a solution of polypropylene glycols (PPG's) in 3 mM ammonium acetate. Samples were infused *via* a 75 μm i.d. fused silica capillary tubing to the ion spray tip which was held at a potential of +5.5 kV. A syringe pump (Model 22, Harvard Apparatus, MA, U.S.A.) controlled the delivery of the sample at the rate of 5.0 μl/minute. Zero grade compressed air was used as nebulizer gas (pressure set at 55 psi). A curtain gas (99.999% UHP nitrogen) flow of 0.8 liter/minute was employed. The interface heater was set at 60°C. Mass spectra were obtained at a dwell time of 1.00 msec (Q1 scan range 300~1900 u, 10 scans averaged) and a step size of 0.10 u. The orifice voltage was maintained at 65 V. All the samples (*c.a.* 100 μg/ml) were dissolved, immediately prior to analysis, in a 1 : 1 mixture of water, added with a 0.1% of trifluoroacetic acid, and methanol.

^{1}H NMR spectra were recorded on AM-500 Brucker spectrometer operating at 500 MHz, using signals of solvent (d_6-DMSO, 2.50 ppm) as internal standard.

De-(*N*-methyl-D-leucine)eremomycin Aglycone (**II**)

To a stirred solution of 600 mg (0.6 mmol) of eremomycin aglycone (**1**) in 20 ml of pyridine-H_2O 1 : 1 mixture was added 0.26 ml (2 mmol) of phenylisothiocyanate and the stirring continued for 3 hours. Afterwards, the reaction mixture was evaporated under reduced pressure, 25 ml of water was added and the solution was adjusted to pH 2 with 1 N HCl. The precipitate was collected, rinsed with water and dried in vacuum to yield 608 mg (91%) of *N*-phenylthiocarbamoylheptapeptide, which was incubated in 6 ml of TFA at 55°C for 1 hour. Afterwards TFA was evaporated in vacuum and the solid product was precipitated with 50 ml of ether, then dissolved in 80 ml of water (adjusted to pH 4 with 1 N HCl) and the by-products were extracted with *n*-butanol. The water solution was evaporated to dryness under vacuum, the residue was dissolved in methanol and the product was precipitated with ether and dried in vacuum at room temperature to yield 365 mg (68%) of **II**.

De-(*N*-methyl-D-leucine)-D-lysyl Eremomycin Aglycone (**III**)

A solution of 145 mg (0.145 mmol) of hexapeptide **II**, 200 mg (0.45 mmol) of N^{α},N^{ε}-di-Boc-D-Lys-OSu and 0.08 ml (0.8 mmol) of *N*-methylmorpholine in 3 ml of a DMSO-DMF 1 : 1 mixture was stirred at room temperature for 20 hours. Afterwards the reaction mixture was diluted with 20 ml of water, adjusted to pH 3 with 1 N HCl and extracted with *n*-butanol. The organic extracts were washed with water and evaporated under reduced pressure. To the solid residue 3 ml of TFA was added and after 40 minutes the reaction product was precipitated with 20 ml of ether. The precipitate was rinsed with ether and dried in vacuum to yield 160 mg of crude heptapeptide **III**. The latter was loaded on a CM-cellulose column (2 × 20 cm) and the elution was performed with 700 ml of a mixture containing 0.1 M ammonium acetate buffer, DMSO and ethanol (75 : 10 : 15 v/v) followed by 600 ml of mixture containing 0.2 M ammonium acetate buffer, DMSO and ethanol (75 : 10 : 15 v/v) at pH 7.1. The fractions containing the pure title compound were pooled, the resulting solution was desalted on a column with ion-exchange resin SDW-3, and eluted with 0.25 N NH_4OH. The solution was evaporated to dryness under vacuum, dissolved in *n*-butanol and ether was added. The solid precipitate was collected, rinsed with ether and dried in vacuum at room temperature to yield 65 mg (40%) of heptapeptide **III**.

De-(*N*-methyl-D-leucine)-D-histidyl Eremomycin Aglycone (**IV**)

A solution of 87 mg (0.09 mmol) of hexapeptide **II**, 125 mg (0.28 mmol) of N^{α},N^{im}-di-Boc-D-His-OSu and 0.05 ml (0.5 mmol) of *N*-methylmorpholine was stirred at room temperature for 20 hours. The isolation of heptapeptide **IV** was performed as described for **3** to yield 15 mg (15%) of heptapeptide **IV**.

De-(*N*-methyl-D-leucine)-D-tryptophanyl Eremomycin Aglycone (**V**)

A solution of 98 mg (0.01 mmol) of hexapeptide **II**, 70 mg (0.15 mmol) of N^{α}-Boc-tryptophan pentafluorophenyl ester and 0.05 ml (0.5 mmol) of *N*-methylmorpholine in 1.5 ml of a DMSO-DMF 1 : 2 mixture was stirred at room temperature for 20 hours. Afterwards the solution was diluted with 10 ml of water adjusted to pH 3 with acetic acid and extracted with 20 ml of an ethyl acetate-*n*-butanol 1 : 1 mixture. The extract was washed with water three times and evaporated to a 2 ml volume. Afterwards 20 ml of ether was added, the precipitate was collected, rinsed with ether and dried in vacuum. TFA (2 ml) and mercaptoethanol (0.002 ml) were added,

followed, in 50 minutes, by 20 ml of ether, the precipitate was collected, rinsed with ether and dried in vacuum to yield 103 mg of crude heptapeptide **V**, which was purified by chromatography on a Sephadex LH-20 column (40 × 2 cm; elution with methanol - water 80 : 20) to yield 40 mg (31%) of pure **V**.

N-D-Lysyl Eremomycin Aglycone (Octapeptide) (**VI**)

To a solution of 120 mg (0.1 mmol) of the eremomycin aglycone **1** in 2.5 ml of a DMSO - DMF, 1 : 1 mixture was added 88.8 mg (0.2 mmol) of $N^{\alpha}N^{\varepsilon}$-di-Boc-D-Lys-OSu, pH of the mixture being adjust at 8 ~ 8.5 by addition of 43.5 mg (0.4 mmol) of DMAP for 20 hours. After 45 hours of stirring at room temperature 15 ml of water acidified to pH 3 with 1 N HCl was added and the product was extracted with 30 ml of an ethyl acetate - butanol 1 : 1 mixture. The extract was washed with water and evaporated to dryness under vacuum, and 1.5 ml of TFA was added to the residue. After 40 minutes incubation the product was precipitated with 20 ml of ether and the precipitate was purified by column chromatography with CM-cellulose as described for **III** to yield 6 mg (5%) of **VI**.

Experiments *In Vitro*

Microtitre method; incubation at 37°C overnight; inocula were at the final dilution of 1/500 with the overnight broth culture. Todd-Hewitt broth was used for all enterococci and strepococci, except *Streptococcus faecalis* I, for which nutrient broth (Oxford No. 2) was used. Nutrient broth was used for all other cultures.

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