VOL. 52, NO. 3, MAR. 1999 THE JOURNAL OF ANTIBIOTICS pp. 319 — 324

Chemical Modification of Antibiotic Eremomycin at the Asparagine Side Chain

EUGENIA N. OLSUFYEVA, TATIANA F. BERDNIKOVA, OLGA V. MIROSHNIKOVA, MARINA I. REZNIKOVA and MARIA N. PREOBRAZHENSKAYA

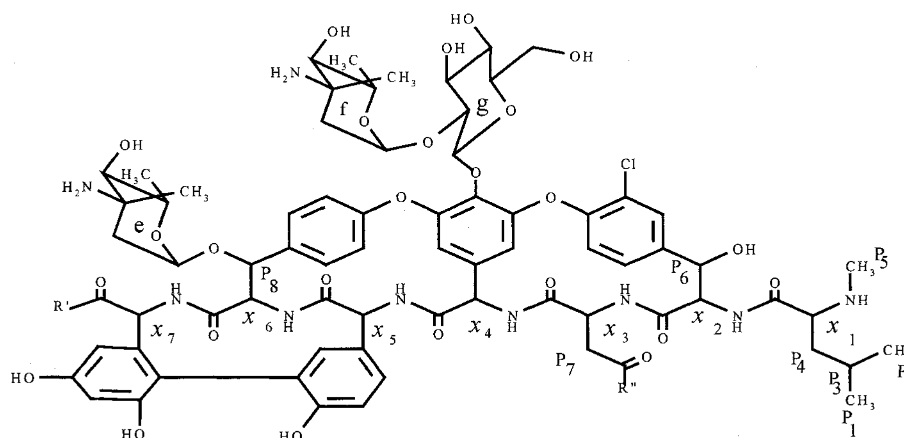
Institute of New Antibiotics, Russian Academy of Medical Sciences, 119867, Moscow, B. Pirogovskaya, Russia

(Received for publication October 21, 1998)

AA3-Carboxyeremomycin **2**, obtained by selective hydrolysis of antibiotic eremomycin was used as a starting compound for the eremomycin chemical modifications at the asparagine side chain to be transformed into eremomycin AA3, AA7 bis-amides (**3a**~**c**). Bis-benzylamide **3b** displayed an activity (8 μg/ml) against an *E. faecium* VanA strain.

Glycopeptide antibiotics display a high activity against Gram-positive bacteria including pathogens resistant to β-lactams, tetracyclines, and fluoroquinolones. They are the drugs of last choice in treatment of severe bacterial infections. Recently, the widespread use of glycopeptide antibiotics such as vancomycin and teicoplanin in clinical practice has given rise to the bacterial strains resistant to these antibiotics. This requires new semisynthetic glycopeptides active against highly resistant clinical strains, especially against vancomycin-resistant enterococci[1~3].

The chemical modifications of the side chain of asparagine, the third amino acid residue in the eremomycin backbone, is a promising approach to overcoming the bacterial resistance to vancomycin. The importance of the asparagine residue was recently demonstrated by the X-ray study of the vancomycin acetate dimer[4]. It was shown that one of the binding pockets of this dimer is occupied by an acetate ion, which mimics the *C*-terminus of the nascent cell wall peptide; at the same time another pocket is closed by the asparagine side chain, which in the absence of ligand occupies its place. It was sup-



Eremomycin (**1**) and its derivatives

1	R′=OH; R″=NH_2
2	R′=R″=OH
3a	R′=R″=NHMe
3b	R′=R″=$NHCH_2C_6H_5$
3c	R′=R″=$NH(CH_2)_3NMe_2$
4a	R′=NHMe; R″=NH_2
4b	R′=$NHCH_2C_6H_5$; R″=NH_2
4c	R′=$NH(CH_2)_3NMe_2$; R″=NH_2

Table 1. Properties and yields of eremomycin derivatives (**2**, **3a**∼**c**, **4c**).

Compound	Yield	HPLC Rt (min)	Electrophoretic mobility relative to Ere[a]	Molecular formula	Ion-spray MS[b] MW $[M+H]^+$	
					Calcd	Found
1		9.0	1.00			
2	33%	8.4	0.60	$C_{73}H_{88}ClN_9O_{27}$	1558.54	1558.5
3a	75%	9.8	1.15	$C_{75}H_{94}ClN_{11}O_{25}$	1584.61	1584.6
3b	73%	21.5	1.15	$C_{87}H_{102}ClN_{11}O_{25}$	1736.67	1736.7
3c	63%	3.4	1.35	$C_{83}H_{112}ClN_{13}O_{25}$	1726.76	1726.7
4c	22%	8.1	1.25	$C_{78}H_{101}ClN_{12}O_{25}$	1641.67	1641.7

[a] In 2 N CH_3COOH. Ere: eremomycin.
[b] Mass numbers refer to the lowest mass isotope of the cluster.

posed that the asparagine side chain keeps the binding pocket in its conformation suitable for peptide docking and swings out of the way when the peptide enters the pocket[4].

Structurally eremomycin (**1**) is closely related to vancomycin. The binding pockets of both antibiotics are similar; however, eremomycin forms more stable dimers[5]. The first and the third amino acids directly participate in the formation of the antibiotic-target complex. The replacement of the *N*-terminal D-MeLeu in vancomycin[6] or in eremomycin aglycon[7] with various amino acids was not effective, whereas the new teicoplanin aglycon derivatives obtained by removing the native AA1 and AA3 constituents and incorporating new ones, exhibited a pronounced activity against VanA enterococci[8]. Recently, a free energy perturbation study was performed on vancomycin derivatives by means of molecular dynamics simulation. Its results suggested that the modification of the asparagine side chain of the antibiotic, which enhances its hydrophobicity, would increase the affinity of glycopeptide antibiotics for depsipeptide ligands and act synergetically with other modifications that enhance the efficacy of these agents against VanA-positive bacteria[9].

In this communication we report a method of direct chemical modification of the asparagine side chain in eremomycin (**1**). The antibiotic was transformed into carboxyeremomycin (**2**) by selective hydrolysis with saturated $Ba(OH)_2$ solution at 37°C for 4 hours. An attempt to hydrolyze, under the same conditions, vancomycin to carboxyvancomycin, which was earlier described as a minor component of the vancomycin complex[10], failed, probably, due to the transformation of vancomycin into an inactive isoasparagine analogue of vancomycin CDP-1[11]. This indicates that the additional sugar moiety at amino acid 6 in eremomycin, which is absent in vancomycin, stabilizes the core of eremomycin. The acid hydrolysis of the asparagine amide group in eremomycin is accompanied with hydrolysis of the glycoside bonds[12].

Carboxyeremomycin (**2**) reacted with methylamine, benzylamine, or 3-(*N*,*N*-dimethyl-amino)propylamine in the presence of benzotriazolyl-tris-pyrrolidinophosphonium-hexafluorophosphate (PyBOP) giving the corresponding bis-amides at amino acids 3 and 7 (**3a**, **3b**, **3c**) in 63∼75% yields. The eremomycin monoamide (**4c**) was obtained by the method described for the eremomycin methylamide (**4a**) or benzylamide (**4b**)[13].

New derivatives **2**, **3a**∼**c**, and **4c** were structurally elucidated with the use of ^1H NMR spectroscopy, electrospray mass spectrometry (ES MS), and paper electrophoresis (Table 1). The ^1H NMR assignments (Table 2) were based on the DQCOSY proton-proton correlations and on a comparison with the data reported for eremomycin (**1**)[12,14]. ^1H NMR spectra of **3a**∼**c** and **4c** demonstrate the presence of all signals of the eremomycin (**1**) backbone protons as well as of the groups introduced. The ^1H NMR spectrum of carboxyeremomycin (**2**) is very close to the spectrum of **1**. The ES MS of **2**, **3a**∼**c**, and **4c** demonstrate the presence of the corresponding mono- or double-charged protonated molecular ions. In the ES MS of **2**, **3b**, and **3c**, triple-charged molecular ions of dimers are also present as in the case of eremomycin and its amides[13].

Table 2. ^{1}H NMR spectra of eremomycin derivatives (**2**, **3a**$\sim$**c**, **4c**) in comparison with **1**.

Proton	**1**[a] D_2O, 70°C	**2**[b] D_2O, 80°C	**3a** DMSO-d_6, 80°C	**3b** DMSO-d_6, 35°C	**3c** DMSO-d_6, 80°C	**4c** D_2O, 35°C
Amide substituents						
CONMe			2.52 (6H, s)			
$CONCH_2Ph$				3.25 (4H, m), 7.30 (10H, m)		
α-NCH_2[c]					3.25 (4H, m)	3.25 (2H, m)
β-CH_2					1.89 (2H, m), 1.95 (2H, m)	2.25 (2H, m)
γ-NCH_2					2.85 (2H, m), 3.05 (2H, m)	3.25 (2H, m)
NMe_2					2.80 (12H, s)	2.97 (6H, s)
Hexapeptide core protons[d]						
P-1, P-2	0.96 (3H, d), 0.98 (3H, d)	0.96 (3H, d), 0.98 (3H, d)	0.90 (3H, d), 0.92 (3H, d)	0.90 (3H, d), 0.92 (3H, d)	0.89 (3H, d), 0.91 (3H, d)	0.89 (3H, d), 0.90 (3H, d)
P-4, P-3	1.75 (2H, m), 1.78 (1H, m)	1.80 (2H, m), 1.82 (1H, m)	1.62 (2H, m), 1.70 (1H, m)	1.88 (2H, m), 1.90 (1H, m)	1.58 (2H, m), 1.70 (1H, m)	1.70 (2H, m), 1.92 (1H, m)
P-5	2.68 (3H, s)	2.78 (3H, s)	2.38 (3H, s)	2.55 (3H, s)	2.80 (3H, s)	2.95 (3H, s)
P-6	5.53 (1H, m)	5.55 (1H, m)	5.45 (1H, m)	5.40 (1H, m)	5.35 (1H, m)	5.59 (1H, m)
P-7	2.65 (2H, m)	2.73 (2H, m)	2.36 (2H, m)	2.60 (2H, m)	2.60 (2H, m)	2.73 (2H, m)
P-8	5.48 (1H, m)	5.40 (1H, m)	5.36 (1H, m)	5.32 (1H, m)	5.35 (1H, m)	5.17 (1H, m)
x-1[e]	3.88 (1H, m)	4.03 (1H, m)	4.01 (1H, m)	4.02 (1H, m)	4.05 (1H, m)	4.45 (1H, m)
Carbohydrate protons[f]						
e-1	5.09 (1H, m)	5.15 (1H, m)	5.10 (1H, m)	5.15 (1H, m)	5.12 (1H, m)	5.41 (1H, m)
e-2_{ax}	2.38 (1H, m)	2.35 (1H, m)	2.25 (1H, m)	2.35 (1H, m)	2.45 (1H, m)	2.45 (1H, m)
e-2_{eq}	2.56 (1H, m)	2.55 (1H, m)	2.40 (1H, m)	2.50 (1H, m)	2.75 (1H, m)	2.45 (1H, m)
e-4	3.50 (1H, d)	3.50 (1H, d)	3.42 (1H, m)	3.38 (1H, m)	3.25 (1H, m)	3.57 (1H, d)
e-5	3.77 (1H, m)	3.90 (1H, m)	3.60 (1H, m)	3.65 (1H, m)	3.50 (1H, m)	3.91 (1H, m)
Me-e_3	1.70 (3H, s)	1.70 (3H, s)	1.50 (3H, s)	1.72 (3H, s)	1.39 (3H, s)	1.65 (3H, s)
Me-e_5	1.45 (3H, d)	1.45 (3H, d)	1.25 (3H, d)	1.54 (3H, d)	1.39 (3H, d)	1.43 (3H, d)
f-1	5.35 (1H, m)	5.30 (1H, m)	$\sim$5.0 (1H, m)	$\sim$5.0 (1H, m)	4.25 (1H, m)	5.43 (1H, m)
f-2_{ax}	2.13 (1H, m)	2.15 (1H, m)	2.12 (1H, m)	2.12 (1H, m)	2.35 (1H, m)	2.25 (1H, m)
f-2_{eq}	2.35 (1H, m)	2.35 (1H, m)	2.22 (1H, m)	2.35 (1H, m)	2.70 (1H, m)	2.45 (1H, m)
f-4	3.46 (1H, d)	3.50 (1H, d)	3.30 (1H, m)	3.32 (1H, m)	3.25 (1H, m)	4.47 (1H, d)
f-5	4.58 (1H, m)	4.40 (1H, m)	4.50 (1H, m)	4.42 (1H, m)	4.25 (1H, m)	4.57 (1H, m)
Me-f_3	1.40 (3H, s)	1.30 (3H, s)	1.25 (3H, s)	1.40 (3H, s)	1.39 (3H, s)	1.45 (3H, s)
Me-f_5	1.28 (3H, d)	1.28 (3H, d)	1.15 (3H, d)	1.25 (3H, d)	1.13 (3H, d)	1.25 (3H, d)
g-1	$\sim$5.4 (1H, d)	$\sim$5.5 (1H, d)	5.41 (1H, d)	5.47 (1H, d)	5.32 (1H, d)	5.25 (1H, d)

[a] HCl salt, [b] H_2SO_4 salt, [c] the protons in $N(CH_2)_3NMe_2$ groups of the compounds **3c** and **4c** were marked as α, β, and γ. [d] aromatic protons signals (14H) are at 5.5$\sim$8.0 ppm, [e] the signals of six x-CH-groups (x-2$\sim$x-7) of the peptide core are at $\sim$4.0$\sim$6.5 ppm, [f] signals g-2$\sim$g-6 of the glucose residue (6H) are at $\sim$ 3.6$\sim$4.1 ppm.

The antibacterial activity of these compounds against clinical isolates of Gram-positive bacteria was compared with that of **1** and the corresponding eremomycin monoamides **4a**$\sim$**4c** (Table 3). Hydrolysis of the asparagine amide group of eremomycin led to a compound with a reduced antibacterial activity against all the strains tested, whereas amidation of both carboxyl groups of amino acids 3 and 7 in **2** to give bisamides **3a**$\sim$**3c** led to an increase in activity. However, the antibacterial activity of **3a**$\sim$**3c** was 2$\sim$8 times lower than that of the parent antibiotic **1** or the corresponding monoamides **4a**$\sim$**4c**. The most interesting is the activity

Table 3. Antibacterial activity (MIC μg/ml) of new eremomycin derivatives (**2**, **3a**~**c**, **4a**~**c**) in comparison with **1**.

Microorganism	Compounds							
	1	**2**	**3a**	**3b**	**3c**	**4a**	**4b**	**4c**
L 819 *S. aureus* Smith	0.13	4	0.5	0.5	0.5	0.13	0.13	0.13
L 561 *S. aureus* clinical isolate	0.5	16	2	4	2	0.5	0.25	1
L 533 *S. epidermidis* clinical isolate	0.13	2	0.25	0.5	1	0.13	0.13	0.13
L 602 *S. haemoliticus* clinical isolate	0.13	4	0.25	0.13	0.25	0.5	0.13	0.13
L 560 *E. faecalis* Van A	>128	>128	>128	64	>128	>128	>128	>128
L 559 *E. faecalis* (isogenic L 560)	0.5	4	1	0.25	0.5	0.25[a]	0.13[a]	0.25
L 569 *E. faecium* VanA	>128	>128	>128	8	32	ND	ND	>128
L 568 *E. faecium* (isogenic L 569)	0.13	ND	0.5	0.25	0.13	ND	ND	0.13
L 49 *S. pyogenes* C 203	0.13	1	0.13	0.13	0.13	0.13	0.06	0.13

[a] Determined for *E. faecium* ATCC 7080.

of bisamide **3b** against VanA strain of *E. faecium* L 569 (VanA) (MIC 8 μg/ml). Recently it was demonstrated that the derivatives of eremomycin at amino acid 7 that contain hydrophobic substituents (*e.g.*, benzyl or decyl groups) overcome the resistance to vancomycin[15]. Our data show that the search for compounds active against VanA enterococci among bisamides of type **3** containing hydrophobic substituents is promising. It is remarkable that although strategies involving deamidation and functionalization of the asparagine side chain seemed to be conceivable only after suitable protection of other reactive moieties or with suitable direction of the derivatizing reagents[9], we succeeded in the selective deamidation of the asparagine moiety in hydrolytic conditions.

Experimental

General

Eremomycin was produced at the pilot plant of the Institute of New Antibiotics. Paper electrophoresis was performed in a 2 N acetic acid at 700 V for 3 hours using Filtrak FN-12 paper (Germany). The CM-cellulose CM-32 (Whatman) column chromatography was performed using LKB Uvicord 2138 supplied with Recorder 6520. Ion-exchange resin SDW-3, an analog of Dowex 50×2, was obtained from Biolar (Olaine, Latvia). HPLC analyses were performed on a Shimadzu HPLC LC 10 instrument equipped with a Diasorb C-16 column (4.0×250 mm, 7 μk, BioChem Mack, Russia) and variable wavelength UV detector set at 280 nm with an injection volume 10 μl. The mobile phases were: (A) 0.1 M $NH_4H_2PO_4$, pH 3.75; and (B) acetonitrile; the eluent was a linear gradient of phase A in phase B (5 to 45%, 25 minutes) at a flowrate of 1.0 ml/minute.

^{1}H NMR spectra were recorded on Varian VXR-400 spectrometer, with signals of the solvent used as an internal standard (DMSO-d_6, 2.5 ppm). Chemical shifts were assigned using the DQCOSY experiments (proton chemical shift correlation with double quantum filtration) and on the basis of the data obtained for eremomycin[12,14].

ES mass spectra were obtained on a Finnigan TSQ 700 instrument equipped with a Finnigan Electrospray ion source and an API III+ triple quadrupole mass

spectrometer (PE-Sciex, Thornhill, ON, Canada) equipped with an articulated ion spray interface using the conditions described earlier[7,13].

Antibacterial activity was performed in Lepetit Research Center, Gerenzano (Varese), Italy. Methods of determination of antibacterial activity *in vitro* and the bacterial strains used were described in a previous paper[16].

Carboxyeremomycin (2)

A solution of 3,000 mg (1.8 mmol) of **1** was incubated in 60 ml of a saturated aqueous $Ba(OH)_2$ solution at 37°C for 4 hours. The reaction mixture was then adjusted to pH 4 with 6 N H_2SO_4 and kept at 0°C for 12 hours. The resulting suspension was centrifuged; the solution was concentrated *in vacuo* to final volume of ~20 ml and loaded on a CM-32-cellulose column (3.5×35 cm) preequilibrated with 0.05 M $AcONH_4$ - EtOH 9 : 1 (pH 7), which was developed with a linear gradient of $AcONH_4$ (0.02 to 0.2 M, pH 7, 10 ml fractions). The fractions containing **2** were pooled, acidified with 6 N H_2SO_4 to pH 2, and passed through a column (2×10 cm) of CDW-3 (H^+) resin with 0.25 N NH_4OH as eluent. The eluates were concentrated under reduced pressure to a final volume of 10 ml to give, after storing at 0°C for 12 hours, a precipitate, which was collected, rinsed with water, and EtOH and dried *in vacuo*. Yield of **2** was 720 mg, 33%. Additionally, 900 mg (33%) of starting **1** was regenerated.

Carboxyeremomycin bis-Methylamide (3a)

32 mg (0.06 mmol) of PyBOP was added portion-wise during 1 hour to a stirred solution of 31 mg (0.02 mmol) of **2**, 0.1 ml *N*-methylmorpholine and 47 mg $CH_3NH_2 \cdot HCl$ (1.00 mmol) in 1 ml DMSO. The reaction mixture was stirred at room temperature for 3 hours. The addition of ether (~15 ml) gave an oily residue, which was washed with ether (15 ml×2) and acetone (~15 ml) and acidified to pH 5 with 0.05 HCl. After addition of 15 ml of acetone a precipitate of **3a** (25 mg, 75%) was obtained.

Carboxyeremomycin bis-Benzylamide (3b)

Carboxyeremomycin bis-benzylamide (**3b**) was obtained as a precipitate (23 mg, 63%) from 31 mg (0.02 mmol) of **2**, and 0.02 ml (0.2 mmol) of benzylamine in 1 ml DMSO and 32 mg (0.06 mmol) of PyBOP.

Carboxyeremomycin bis-3-(*N,N*-Dimethylamino)propylamide (3c)

Carboxyeremomycin bis-3-(*N,N*-dimethylamino)propylamide (**3c**) was obtained as a precipitate (23 mg, 65%) from 31 mg (0.02 mmol) of **2**, 0.05 ml *N*-methylmorpholine and 86 mg (0.5 mmol) 3-(*N,N*-dimethylamino)propylamine dihydrochloride, and 32 mg (0.06 mmol) of PyBOP.

Eremomycin 3-(*N,N*-Dimethylamino)propylamide (4c)

Eremomycin 3-(*N,N*-dimethylamino)propylamide (**4c**) was obtained by the method described for eremomycin monoamides[13] in the 22% yield.

Acknowledgments

The authors are grateful to Dr. R. Ciabatti and Dr. A. Malabarba for fruitful discussions, to Dr. L. Colombo and Dr. C. Sottani (all from Lepetit Research Center, Gerenzano, Varese, Italy) for studying the ES MS, and to Dr. E. I. Lazhko (Institute of New Antibiotics, Russia) for studying the ^1H NMR spectra.

References

1) Cooper, R. D. G. & R. C. Thompson: Semisynthetic glycopeptide antibiotics. Annual Reports in Medicinal Chemistry 31: 131~140, 1996
2) Malabarba, A.; T. I. Nicas & R. C. Thompson: Structural modification of glycopeptide antibiotics. Medicinal Research Reviews 17: 69~137, 1997
3) Trenin, A. S. & E. N. Olsufyeva: Mechanisms of bacterial resistance to glycopeptide antibiotics as a base for development of derivatives overcoming resistance. Russian Journal of Bioorg. Chem. 23: 757~772, 1997
4) Schaefer, M.; T. R. Schneider & G. M. Shedrick: Crystal structure of vancomycin. Structure 4: 1509~1515, 1996
5) Mackey, J. P.; U. Gerhard, D. A. Beaurgard, M. S. Wastwell, M. S. Searle & D. H. Williams: Glycopeptide antibiotic activity and the possible role of dimerization: a model for biology signaling. J. Am. Chem. Soc. 116: 45~73, 1994
6) Cristofaro, N. F.; D. A. Beuregard, H. Yan, N. J. Osborn & D. H. Williams: Cooperativity between non-polar and ionic forces in the binding of bacterial cell wall analogues by vancomycin in aqueous solutions. J. Antibiotics 48: 805~810, 1995
7) Miroshnikova, O. V.; T. F. Berdnikova, E. N. Olsufyeva, A. Y. Pavlov, M. I. Reznikova, M. N. Preobrazhenskaya, R. Ciabatti, A. Malabarba & L. Colombo: A modification of the *N*-terminal amino acid in the eremomycin aglycon. J. Antibiotics 49: 1157~1161, 1996
8) Malabarba, A.; R. Ciabatti, E. Gerli, F. Ripamonti, P. Ferrari, L. Colombo, E. N. Olsufyeva, A. Y. Pavlov, M. I. Reznikova, E. I. Lazhko & M. N. Preobrazhenskaya: Substitution of amino acids 1 and 3 in teicoplanin aglycon: synthesis and antibacterial activity of three first non-natural dalbaheptides. J. Antibiotics 50: 70~81, 1997

9) AXELSEN, P. H. & D. LI: A rational strategy for enhancing the affinity of vancomycin forwards depsipeptide ligands. Bioorganic & Medicinal Chemistry 6: 877~881, 1998

10) NAGARAJAN, R.: Structure-activity relationships of vancomycin antibiotics. *In* Glycopeptide Antibiotics. *Ed.*, R. NAGARAJAN, pp. 195~218. Marcel Dekker. Inc., New York, 1994

11) HARRIS, C. M.; H. KOPECKA & T. M. HARRIS: Vancomycin: Structure and transformation to CDP-1. J. Am. Chem. Soc. 105: 6915~6922, 1989

12) BERDNIKOVA, T. F.; N. N. LOMAKINA, E. N. OLSUFYEVA, L. N. ALEXANDROVA, N. P. POTAPOVA, I. V. MALKOVA & G. I. ORLOVA: Structure and antibacterial activity of eremomycin: partial degradation products. Antibiotiki i khimioterapia (Russ.) 36 (6): 28~31, 1991

13) PAVLOV, A. Y.; T. F. BERDNIKOVA, E. N. OLSUFYEVA, O. V. MIROSHNIKOVA, S. T. FILIPPOSYANZ, M. N. PREOBRAZHENSKAYA, C. SOTTANI, L. COLOMBO & B. P. GOLDSTEIN: Carboxamides and hydrazide of glycopeptide antibiotic eremomycin: Synthesis and antibacterial activity. J. Antibiotics 49: 194~198, 1996

14) BATTA, G.; F. SZTARICSKAI, K. E. KOVER, C. RUDEL & T. F. BERDNIKOVA: An NMR study of eremomycin and it's derivatives. Full ^{1}H and ^{13}C assignment, motional behaviour, dimerization and complexation with Ac-D-Ala-D-Ala. J. Antibiotics 44: 1208~1221, 1991

15) PAVLOV, A. Y.; E. I. LAZHKO & M. N. PREOBRAZHENSKAYA: A new type of chemical modification of glycopeptides antibiotics: aminomethylated derivatives of eremomycin and their antibacterial activity. J. Antibiotics 50: 509~513, 1996

16) PAVLOV, A. Y.; M. N. PREOBRAZHENSKAYA, A. MALABARBA, R. CIABATTI & L. COLOMBO: Mono and double modified teicoplanin aglycon derivatives on the amino acid No. 7; Structure-activity relationship. J. Antibiotics 51: 73~78, 1998