Chemical Modification of the Eremomycin Antibiotic

Formation of a Cyclic Covalent Dimer

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Recently considerable efforts have been devoted to the chemical modification and total synthesis[1~4] of the representatives of the vancomycin group of antibiotics[5] with a glycopeptide structure[6] and possessing antibacterial activity. This is clearly because that for the treatment of the continually threatening multi and methycillin-resistant *Staphylococcus aureus* (MRSA) infections, emerged in the past few decades, the only presently known effective medicines[7,8] are the antibiotics vancomycin and/or theicoplanin. Furthermore, the appearance and increasingly spreading of the vancomycin-resistant *Enterococci* (VRE) also promoted related research.

The first reports on the structure and antibiotic activity of the glycopeptide-type antibiotic eremomycin (**1**) were published[9,10] in about ten years ago, and then later chemical modifications[11~13] at the *N*- and *C*-terminal portions of the heptapeptide aglycone of **1** were also carried out.

The present paper deals with some novel chemical modifications of the eremomycin antibiotic (**1**).

Eremomycin (**1**) readily reacted at the *N*-terminal secondary amino group with 2-chloroethylisocyanate (**2**) to furnish the *N*-(2-chloroethylureido) derivative **3**, which—under the conditions of isolation—transformed into the cyclic 2-oxazoline compound **4** in an intramolecular SN2 reaction (Scheme 1). The oxazoline structure of **4** was justified by mass spectrometric measurements (Table 1), as well as by NMR spectral data. Thus, the 500/125 MHz (ref: ext TMS) D_2O/300K 2D HETCOR spectrum unequivocally proved the presence of an extra NCH_2 (4.00/44.02 ppm) and an OCH_2 (4.97/72.88 ppm) unit as compared to the spectrum of the parent eremomycin (**1**). Also characteristic is a nearly 1 ppm shift (4→5 ppm) of ×1 proton, demonstrating that the modification has occurred at the *N*-terminus. Based on the deuteration rate of the 5-N*H* proton, the dimerization constant of **4** is higher than that of eremomycin (**1**), and perhaps this explains the biological activity of this compound.

The reaction of **1** with 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosylisocyanate (**5**) afforded *N*-(2′,3′,4′,6′-tetra-*O*-acetyl-α,β-glucopyranosyl)thioureidoeremomycin (**6**). Zemplén *O*-deacetylation[14] of **6** and subsequent column chromatography allowed to isolate *N*-(β- and α-D-glucopyranosyl)thioureidoeremomycin (**7** and **8**) in pure form.

The α-anomer quantity was insufficient for NMR. For the attached glucose unit in the β-anomer **7** the Gl anomeric signal was found at 5.89/86.3 ppm coordinates in the HETCOR spectrum. The ×1 proton is further shifted upfield, and was found at the 6.35/62.1 ppm cross-peak coordinate. This compound is a dimer, too.

A most recent field of the research for fighting against MRSA and VRE is the synthesis of the covalent dimers of the glycopeptide antibiotics. American scientists[15,16] coupled two vancomycin molecules into a covalent "head-to-head" dimer through the *C*-terminals, and English chemists[17] preferred the preparation of a "head-to-tail" dimer of the antibiotic by means of the covalent dimerization involving the *C*- and *N*-terminals. With these in mind we planned the synthesis of the covalent "tail-to-tail" dimers of the eremomycin antibiotic linked through the *N*-terminal of the molecule (Scheme 1).

Considering the number of the primary and secondary amino groups of eremomycin (**1**), the intermolecular and intramolecular coupling with the bis-isocyanate derivative **9** could lead to the formation of various dimers. The reaction of **1** with **9** at low temperature (5°C in methanol) furnished the covalent dimer **10** of eremomycin in a moderate (20%) yield. Chromatographic purification of the synthesized

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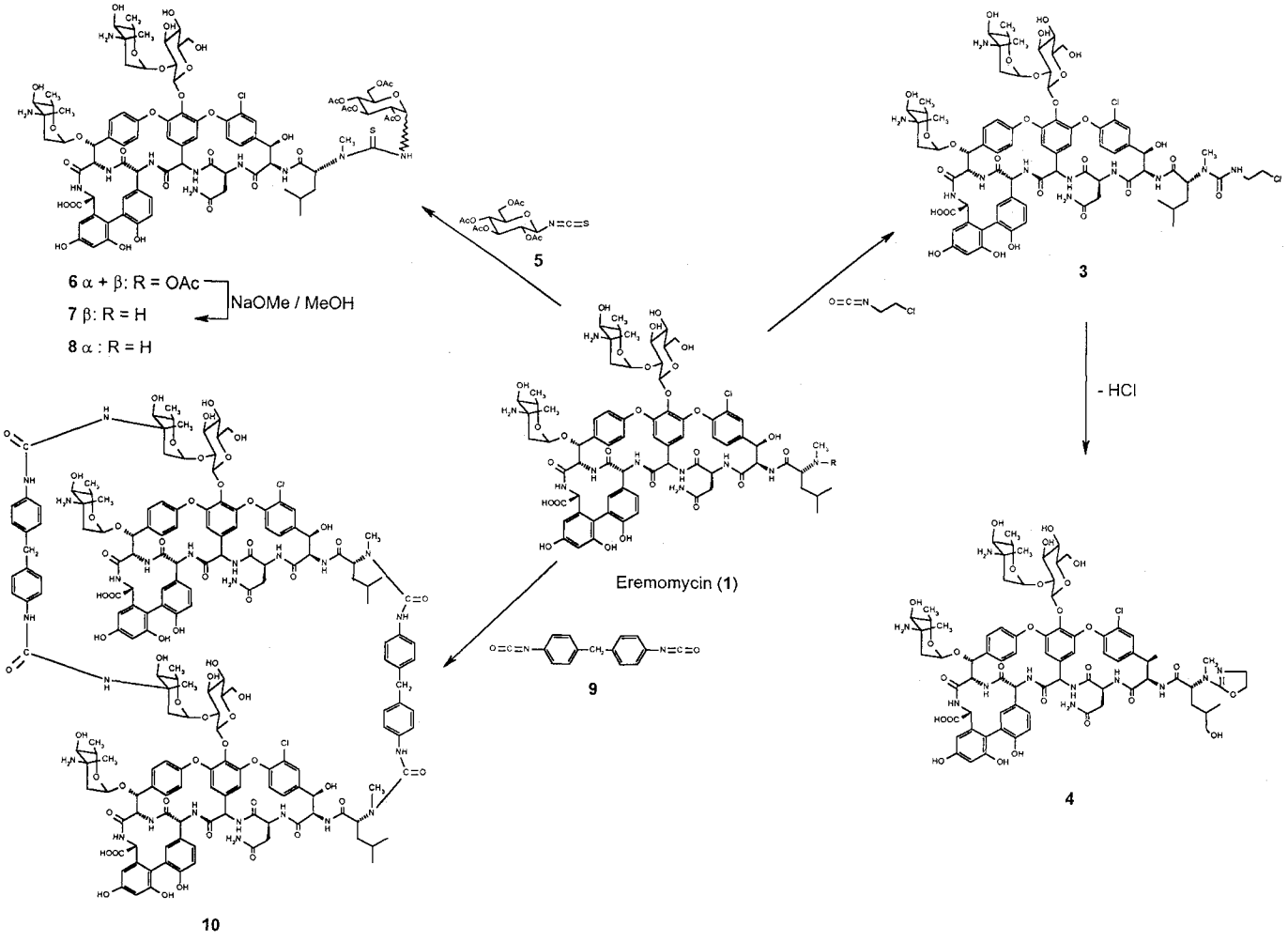
Scheme 1. Chemical modification of the eremomycin antibiotic.



eremomycin derivatives **4**~**10** was performed on silanized silica gel columns with two-step gradient elution using 0.01 M aqueous acetic acid and 50% methanol. In the case of **10** this purification protocol was extended with subsequent elutions employing 40% methanol containing 0.04 M acetic acid and 50% methanol containing 0.05 M acetic acid. The homogenity of the compound was represented by HPLC (Table 1).

The position of the introduced ureido and thioureido groups was determined after Edman degradation and acid hydrolysis. Edman degradation showed that in all derivatives the *N*-methylamino group is substituted: TLC demonstrated the absence of 5-isopropyl-1-methyl-3-phenyl-2-thiohydantoin by comparison with an authentic sample of this latter, which was obtained in a parallel experiment by the Edman degradation of eremomycin.[18]

We have shown[19] previosly that hydrolysis of eremomycin in 0.2 N HCl at 100°C for 10 minutes leads to the splitting of the glycosidic bond between two sugars in the disacharide moiety, and gives rise to eremosamine. TLC comparison of the hydrolysis products of **4**, **6**~**8** with an authentic sample of eremosamine revealed that eremosamine is not substituted in these compounds. The hydrolysis products of compound **10** contained aminosugars different from eremosamine. TLC and HPLC comparison of the hydrolysis products of **10**, formed under more drastic conditions (37% HCl, 3 hours., *i.e.* when the aglycone of eremomycin was obtained in a parallel hydrolysis of eremomycin), whith an authentic sample of the aglycone of eremomycin demonsrated the absence of this latter.

The structure of the covalent eremomycin dimer **10** was

Table 1. Physico-chemical properties and mass spectral data of the new eremomycin derivatives.

Cmpd.	Yield (%)	TLC R_f Solvent system A_1 A_2 B	HPLC Rt; min	Molecular formula	Mass spectrum (m/z)	
					Calcd. MW	Found MW
4	62	0.35 0.1 -	29.65	$C_{76}H_{92}N_{11}O_{27}Cl$	1625.5	1626.9[a]
6	86	0.55 0.42 -	31.00	$C_{88}H_{108}N_{11}O_{35}ClS$	1945	1947[b]
7	45	- - 0.40	11.18	$C_{80}H_{100}N_{11}O_{31}ClS$	1777.5	1778.8[b]
8	9	- - 0.50	8.29	$C_{80}H_{100}N_{11}O_{31}ClS$	1777.5	1778.8[b]
10	20	0.52 0.19	34.05	$C_{176}H_{198}N_{24}O_{56}Cl_2$	3616	3681[c]

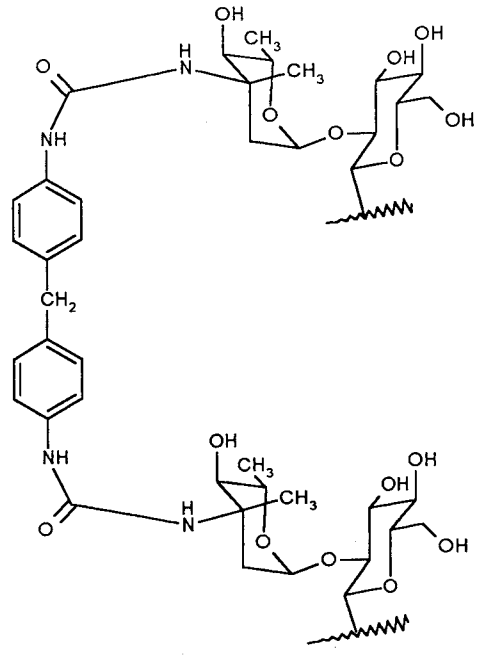
Mass spectral measurements: [a]$(M+H)^+$, [b](M+2H), [c](M+2Na+H_2O+H)
HPLC instrument: Shimadzu LC 10 with UV detector (254 nm)
Column: Diasorb C-16 (4.0 x 250 mm, 7μk, Biochem Mack)
Solvent system: linear gradient 8% → 70% MeCN in 0.2 M $HCOONH_4$ (pH: 7.6; flow rate: 1.0 ml/min)

proved by means of mass spectrometric investigations. By applying either the electrospray (ES-MS) or the MALDI-TOF technique, the molecular mass of **10** was found to be *m/z*=3681 (Table 1). The *m/z*=860 fragment ion in the MALDI-TOF spectrum corresponds to the structural unit **11** split off from **10**. A further proof for this structure is that the split disaccharide portion must be appeared at *m/z*=306. The observed molecular mass *m/z*=3681 corresponds to a dimeric structure (**10**) in which two eremomycin molecules are coupled through the *N*-terminus of the aglycone and the amino group of the disaccharide moiety. In addition, the two *C*-terminal carboxylic functions link two sodium ions, and the whole molecule is associated with one molecule of water. Consequently, protonation of this molecular complex under the electrospray or MALDI-TOF conditions leads to the formation of the peak *m/z*=3681.

The covalent dimeric structure of **10** was also proved by NMR diffusion experiments.[20] Comparison of the spectrum of **10** with that of eremomycin (**1**) (DMSO, 330K) showed a difference of 0.11 on a $\log_{10}$ diffusion rate scale. This immediately translates to a mass ratio slightly bigger than two. In the 2D HETCOR spectrum 8 aromatic C*H* protons are detected at 7.11/129.6 ppm, and 4-4 C*H* groups at 7.54/121.23 and 7.36/119.58 ppm. In addition, $2\times CH_2$ is observed at 3.84/40.88 ppm. These observations are in a good correspondence with the proposed structure **10** for the dimer.

The antibacterial activity of the synthesized ureido-eremomycin derivatives was tested on six *Staphylococcus aureus* strains, using oxacillin, methycillin, vancomycin and eremomycin as the reference materials (Table 2). Of the *Staphylococcus aureus* strains shown in Table 2 those marked with the numbers 14287/1 and 1665 are

Fig. 1. Structure of the mass fragment **11** of the covalent dimer of eremomycin (**10**).



11 (m/z 860)

Table 2. Comparison of the *in vitro* antibacterial activity of the ureido derivatives of eremomycin.

Strain	MIC* of the compounds (μg/ml)								
	Oxacillin	Methycillin	Vancomycin	Eremomycin	**4**	**6**	**7**	**8**	**10**
S. aureus 14287/1	0.78	3.12	≥0.39	≥0.39	3.12	50	25	25	75
S. aureus 1665	50	12.5	0.78	≥0.39	6.25	100	25	50	75
S. aureus 13851/2 (MRSA)	25	25	≥0.39	≥0.39	6.25	100	25	50	75
S. aureus 14136 (MRSA)	25	25	≥0.39	≥0.39	6.25	100	25	50	75
S. aureus 2339 (MRSA)	>100	>100	≥0.39	≥0.39	6.25	100	25	50	75
S. aureus 1931/2 (MRSA)	>100	>100	≥0.39	≥0.39	6.25	100	25	50	75

MRSA: methycillin-resistant
*Determined by the agar dilution method after an overnight incubation at 37°C
Liquid medium: Nutrient Broth (10^4 CFU/ml)

methycillin-sensitive and moderately methycillin-resistant, respectively, while all of the other strains are methycillin-resistant (MRSA). The antibacterial activity of the samples was investigated at the concentrations 100 and 0.39 μg/ml.

The biological tests revealed that the only compound which possessed higher activity than oxacillin and methycillin against the MRSA strains was the modified antibiotic **4**. At the same time, even this compound was still less effective than vancomycin and eremomycin. It is interesting to note that the β-D-glucopyranosyl-thioureido-eremomycin derivative (**7**) was two times more efficient than the corresponding α-glucoside **8**. The covalent eremomycin dimer **10**, coupled from both sides, possesses a rather rigid structure, which unfavourably influences binding of this modified antibiotic to the *N*-acyl-D-Ala-D-Ala unit of the peptidoglycan moiety of the bacterial cell-wall.

Eremomycin (**1**) and its semisynthetic derivatives (**4**, **6**, **7**, **8** and **10**) did not show platelet agglutination in the thrombocyte-enriched plasm.[21] In addition, neither of the new antibiotic derivatives inhibited platelet agglutination[22] induced by ristocetin (ristomycin) A (AGGRISTIN®), ADP, adrenalin or collagene.

Experimental

The ^{1}H (500 MHz) and ^{13}C-NMR (125 MHz) spectra were obtained with a Bruker DRX 500 spectrometer. Mass spectra were recorded with a Finnigan TSQ ion source. Electrospray (cone voltage 100 V, eluent: 1 : 1 (v/v) methanol-water). MALDI-TOF (Brucker Reflex, matrix 1.5 DMB). Thin layer chromatography was carried out on Kieselgel 60 F_{254} (Merck) precoated plates, and column chromatography was performed on a silanized Kieselgel 60 (Merck 0.063~0.2) with the following eluent systems: (A_1) 14 : 13 : 15 EtOAc - *n*PrOH - 25% NH_4OH; (A_2) 15 : 10 : 10 EtOAc - *n*PrOH - 25% NH_4OH; (B) 5 : 2 : 2 *n*PrOH - 25% NH_4OH - H_2O. Evaporations were carried out under diminished pressure (bath temperature below 45°C).

N-Oxazoline of Eremomycin (**4**)

To a solution of 165 mg (0.1 mmol) of eremomycin sulfate (**1**) in DMSO (3 ml) was added 0.0085 ml (0.1 mmol) of 2-chloroethylisocyanate and the reaction mixture was stirred for 2 hours at room temperature. Then 50 ml of acetone was added, the formed precipitate was filtered off, washed with acetone and dried in vacuum to afford 166 mg of the crude product **4**. Purification was carried out by

means of column chromatography employing a 2×30 cm column, and 400 ml of 0.01 M acetic acid (pH 4) at a rate of 0.3 ml/minute. The fractions containing the pure title compound were pooled, and this solution was concentrated and frezee-dried to obtain 102 mg (62%) of **4** as a white powder. Rf=0.35 (A_1); Rf=0.09 (A_2).

N-(2′,3′,4′,6′-Tetra-*O*-acetyl-D-glucopyranosyl)thioureide of Eremomycin (**6**)

A stirred solution of 50 mg (0.03 mmol) of eremomycin sulfate (**1**) and 35 mg (0.09 mmol) of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranosylisothiocyanate in 1 ml of DMSO was heated at 40~50°C for 2 hours. After cooling to room temperature the reaction product was precipitated by the addition of acetone (30 ml). The precipitate was filtered off, washed with acetone, and dried in vacuum at room temperature to yield 58 mg of the crude **6**. It was dissolved in 1 ml of water, loaded to a 1×30 cm column, and column chromatography was performed by step gradient elution from 0.01 M acetic acid in water to 50% methanol in 0.01 M acetic acid. The appropriate fractions (50% MeOH) were combined, concentrated to a small volume (~0.5 ml) and 30 ml of acetone was added. The precipitate produced was filtered off, washed with acetone, and dried in vacuum at room temperature to isolate 50 mg (86%) of pure **6**, Rf=0.55 (A_1); Rf=0.43 (A_2).

N-(β-D-Glucopyranosyl)thioureide of Eremomycin (**7**) and *N*-(α-D-Glucopyranosyl)thioureide of Eremomycin (**8**)

A stirred solution of 50 mg (0.26 mmol) of compound **6** in a 1 : 1 mixture of dry MeOH - DMF (2 ml) was treated with 0.1 M sodium methoxide in MeOH (pH=8) and stirring was continued for 3 days at room temperature. The reaction mixture was then diluted with acetone (30 ml), the precipitate formed was filtered off, washed with acetone, and dried in vacuum at room temperature to obtain 30 mg of a mixture of two products (ratio is~3 : 1) as shown by TLC and ^{1}H-NMR analyses. This mixture was submitted to column chromatography as described above for compound **4**, to furnish 20 mg (45%) of the pure β-anomer **7**, Rf=0.02 (A_1), Rf=0.4 (B).

The α-anomer **8** was isolated from the mixture by repeated column chromatogrpahy to obtain 4 mg (8%) of the product of 80~90% purity (TLC), Rf=0.02 (A_1), Rf=0.5 (B).

Preparation of the Cyclic Covalent Dimer **10** of Eremomycin

A suspension of 165 mg (0.1 mmol) of eremomycin sulfate (**1**) in 10 ml of dry MeOH was cooled to 5°C, and then 37 mg (0.15 mmol) of bis-(biphenyl)methyl-N^1,N^2-isocyanate was added. The mixture was stirred at 5°C for 20 minutes, when TLC (A_1) showed the presence of about 50% of the starting **1**, ~35% of a product with an Rf higher than that of the parent **1**, and also ~15% of a slower-moving product. The precipitated mixture was filtered off, washed with cold, dry MeOH, and then dissolved in 1.5 ml of 0.01 M acetic acid. Purification was carried out on a 2×40 cm column by means of step gradient elution from 0.01 M acetic acid (pH 4) to 50% MeOH in 0.05 M acetic acid (pH 3) to obtain three fractions.

The first fraction (from 0.01 M acetic acid) gave 80 mg of the starting eremomycin (**1**).

The second fraction (from 20% MeOH to 30% MeOH in 0.03 M acetic acid) afforded a ~2 : 1 mixture (TLC) of the faster- and slower-moving products.

The third fraction (from 40% MeOH in 0.04 M acetic acid to 50% MeOH in 0.05 M acetic acid) gave the desired faster-moving title dimeric compound **10** (6 mg).

The methanol filtrate containing some of the product **10** was evaporated to dryness, and the residue was washed with acetone (2×10 ml). It was then dissolved in 0.5 ml of water, and the product was precipitated with 20 ml of acetone. The precipitate was filtered off, washed with acetone, and dried in vacuum at room temperature to give 10 mg of the pure dimer **10**. Overall isolated yield of **10**: 16 mg (20%), Rf=0.52 (A_1), Rf=0.19 (A_2).

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