Synthesis and Mode of Action of Hydrophobic Derivatives of the Glycopeptide Antibiotic Eremomycin and Des-(*N*-methyl-D-leucyl)eremomycin against Glycopeptide-Sensitive and -Resistant Bacteria

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Des-(*N*-methyl-D-leucyl)eremomycin was obtained by Edman degradation of eremomycin. Derivatives with a hydrophobic substituent at the exterior of the molecule were then synthesized, and their antibacterial activities were compared with similar derivatives of eremomycin. Comparison of derivatives of eremomycin containing the *n*-decyl or *p*-(*p*-chlorophenyl)benzyl substituent in the eremosamine moiety (*N*′) and *n*-decyl or *p*-(*p*-chlorophenyl)benzylamides with similar derivatives of eremomycin possessing the damaged peptide core (a defective binding pocket) showed that compounds of both types are almost equally active against glycopeptide-resistant strains of enterococci (GRE), whereas eremomycin derivatives are more active against staphylococci. Hydrophobic 7*d*-alkylaminomethylated derivatives of eremomycin (**9**, **10**) demonstrated similar antibacterial properties. Since the basic mode of action of glycopeptide antibiotics involves binding to cell wall intermediates terminating in -D-Ala-D-Ala and this interaction is seriously decreased in the hexapeptide derivatives (lacking the critical *N*-methyl-D-leucine), we suggest that these hydrophobic derivatives may inhibit peptidoglycan synthesis in the absence of dipeptide binding. NMR binding experiments using Ac-D-Ala-D-Ala show that binding constants of these hexapeptide derivativies are decreased in comparison with the corresponding heptapeptides with intact binding pocket. This is in agreement with the decreased biological activity of the hexapeptide derivatives against vancomycin-sensitive strains in comparison with the activity of parent compounds. Binding to the lactate cell wall analogue Ac-D-Ala-D-Lac with decylamide of eremomycin **8** was not observed, demonstrating that the interaction with this target in GRE does not occur. While hydrophobic glycopeptide derivatives retain the ability to inhibit the synthesis of peptidoglycan in manner of natural glycopeptides, biochemical investigation supports the hypothesis that they inhibit the transglycosylase stage of bacterial peptidoglycan biosynthesis even in the absence of dipeptide or depsipeptide binding.

Introduction

Synthesis and investigation of novel semisynthetic antibacterial glycopeptide antibiotics is important because emergence of vancomycin or teicoplanin resistance poses a serious threat to human health. Chemical modification of natural glycopeptide antibiotics is directed to the preparation of new compounds active against glycopeptide-resistant strains. Understanding the mechanism of action of these derivatives would provide a strong stimulation for the rational design of derivatives active against resistant microorganisms.[1,2] The search for derivatives of glycopeptide antibiotics active against glycopeptide-resistant enterococci (GRE) resulted in the discovery of the anti-GRE activity of hydrophobic derivatives of eremomycin,[3–5] vancomycin,[6] and chloreremomycin[7] among which the most active is *N*′-*p*-(*p*-chlorophenyl)benzylchloreremomycin (LY 333328).[7] The antibacterial activity of natural glycopeptide antibiotics is based on their ability to bind peptidoglycan precursors terminating in the sequence -D-Ala-D-Ala[8] (Figure 1). In the glycopeptide-resistant enterococci (VanA and VanB types), the terminal D-alanine is substituted with D-lactate, leading to a 1000-fold decrease in affinity of a glycopeptide antibiotic to its target.[9] Eremomycin (**1**) belongs to the same group of glycopeptide antibiotics as vancomycin (**2**) (Figures 2 and 3)—the most important structural feature of **1** in comparison to **2** is the presence of an additional aminosugar at the hydroxylic group in benzylic position of aminohydroxy acid no. 6 and the absence of chlorine substituent in the aromatic ring of this acid. Furthermore, the aminosugar eremosamine is an epimer of vancosamine at position 1E4.

The biological properties of eremomycin have some peculiarities compared with vancomycin: eremomycin is several times more active than vancomycin against

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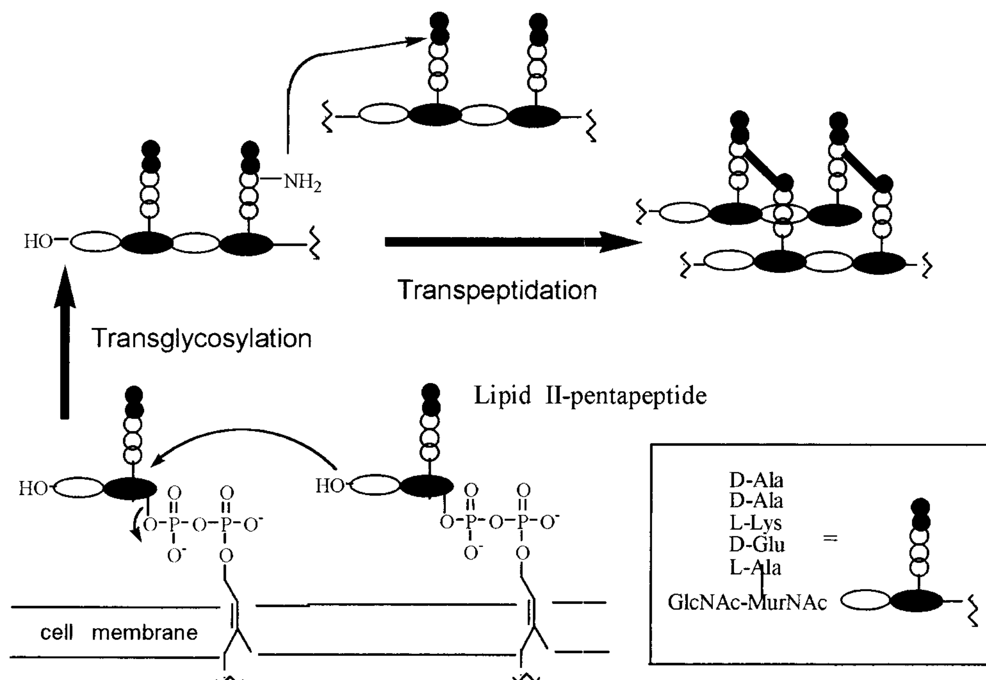
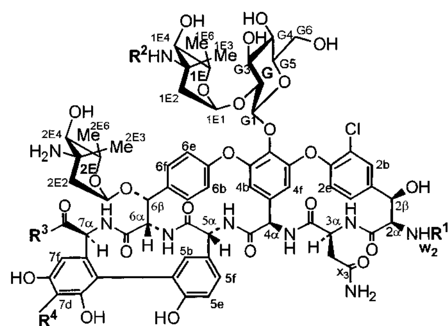


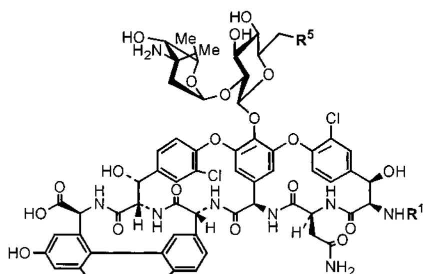
Figure 1. Peptidoglycan biosynthesis.



1 R^1=N-Me-D-Leu; R^2=H; R^3=OH; R^4=H
5 R^1=N-Me-D-Leu; R^2=p-(p-ClPh)Bn; R^3=OH; R^4=H
6 R^1=N-Me-D-Leu; R^2=$C_{10}H_{21}$; R^3=OH; R^4=H
7 R^1=N-Me-D-Leu; R^2=H; R^3=p-(p-ClPh)BnNH; R^4=H
8 R^1=N-Me-D-Leu; R^2=H; R^3=$C_{10}H_{21}$NH; R^4=H
9 R^1=N-Me-D-Leu; R^2=H; R^3=OH; R^4=CH_2-NH-[p-(p-ClPh)Bn]
10 R^1=N-Me-D-Leu; R^2=H; R^3=OH; R^4=CH_2-NH-$C_{10}H_{21}$
11 R^1=N(Me)CH_2-CH$(CH_3)_2$; R^2=H; R^3=OH; R^4=H
S=C-NH-Ph

1a R^1=H; R^2=H; R^3=OH; R^4=H
5a R^1=H; R^2=p-(p-ClPh)Bn; R^3=OH; R^4=H
6a R^1=H; R^2=$C_{10}H_{21}$; R^3=OH; R^4=H
7a R^1=H; R^2=H; R^3=p-(p-ClPh)BnNH; R^4=H
8a R^1=H; R^2=H; R^3=$C_{10}H_{21}$NH; R^4=H

Figure 2. Eremomycin derivatives.



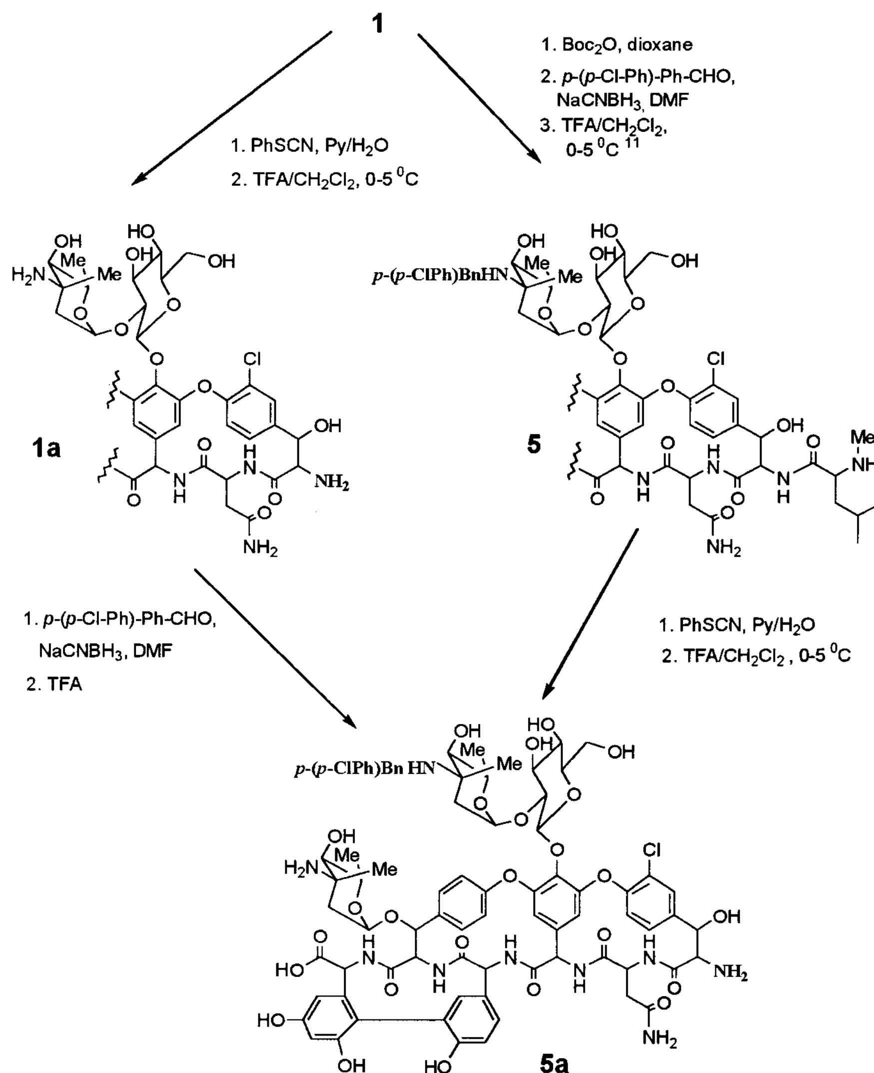
2 R^1=*N*-Me-*D*-Leu; R^2=H; R^5=OH
3 R^1=*N*-Me-*D*-Leu; R^2=*p*-(*p*-ClPh)Bn; R^5=OH **3a** R^1=H; R^2=*p*-(*p*-ClPh)Bn; R^5=OH
4 R^1=*N*-Me-*D*-Leu; R^2=H; R^5=*p*-(*p*-ClPh)BnNH **4a** R^1=H; R^2=H; R^5=*p*-(*p*-ClPh)BnNH

Figure 3. Vancomycin derivatives.

Gram-positive bacteria, though the binding constants (M^{-1}) of eremomycin for the peptidoglycan model Ac_2-Lys-D-Ala-D-Ala is 1 order of magnitude lower than that of vancomycin. In addition, in aqueous solution eremomycin is known to be almost completely dimerized,[10,20] whereas vancomycin dimerizes only <3% at 50 μM concentration since dimerization constants are ca. 10^5 and 700 M^{-1}, respectively.[10] Recently we synthesized a series of hydrophobic eremomycin derivatives and demonstrated that activity on GRE is based on a different mode of action, which does not necessarily involve binding to cell wall intermediates containing -D-Ala-D-Ala, and that inhibition of the transglycosylase step of peptidoglycan biosynthesis represents an additional or alternative mechanism of action of the hydrophobic eremomycin derivatives.[11] It was shown earlier[12] that considerable antibacterial activity is retained for vancomycin derivatives substituted on the vancosaminyl sugar (compound **3**) even when the peptide binding pocket is damaged (**3a**), whereas a derivative of vancomycin containing hydrophobic substituents at the glucose moiety (compound **4**) loses activity when the binding pocket is damaged (compound **4a**). It was hypothesized that the amino group in the disaccharide branch of vancomycin represented the position for obtaining hydrophobic vancomycin derivatives with novel mechanism of action whereas other hydrophobic compounds overcome vancomycin resistance due to membrane anchoring.[12] The evidence that vancomycin containing hydrophobic substituents at the aminosugar moiety can directly inhibit the transglycosylation process in bacteria was obtained when *N*-*p*-[*p*-(chlorophenyl)benzyl]vancomycin (**3**) and its derivative with the damaged heptapeptide core (**3a**) were compared.[13,14] In addition, a new gene specifically regulating susceptibility of *Escherichia coli* cells to killing by hydrophobic glycopeptide derivatives was identified, establishing a genetic basis for activity differences between these compounds and vancomycin.[15]

We have recently shown that the activity of hydrophobic derivatives of eremomycin (e.g., containing decyl and other alkyl substituents) depends strongly on the

Scheme 1. Synthesis of *N'*-[*p*-(*p*-ClPh)Bn]-derivatives of Eremomycin and Its Des-(*N*-methyl-D-leucyl)-derivatives



size of the hydrophobic substituent and is less dependent on the position of the substituent.[3–5] We evaluated the ability of various hydrophobic derivatives (including those with damaged binding pockets) to bind -D-Ala-D-Ala and potentially weakly bind D-Ala-D-lactate in order to assess the role of possible dipeptide binding in more detail. In addition, we attempted to clarify the importance of the position of a hydrophobic substituent in the molecule for the demonstration of anti-GRE properties.

The goal of our research was to synthesize eremomycin derivatives substituted at various positions of the molecule with hydrophobic substituents and similar hydrophobic derivatives of des-(*N*-methyl-D-leucyl)eremomycin. We evaluated their antibacterial activities in an attempt to determine the contribution of the interaction of the binding pocket with the target and that of the structure and the position of the hydrophobic substituents to antibacterial activity. To support these data, ligand binding constants were measured for selected hydrophobic compounds using NMR methods. This comparison could help us to understand the biochemical basis of their anti-GRE properties.

Chemistry

We compared derivatives of eremomycin (**1**) and des-(*N*-methyl-D-leucyl)eremomycin (**1a**) containing *p*-(*p*-chlorophenyl)benzyl (**5**, **5a)** or *n*-decyl substituent (**6**, **6a**) at the amino group of the disaccharide branch (*N'*), in the 7-carboxamide moiety (**7**, **7a** and **8**, **8a)** and alkylaminomethyl derivativies with the same substituents at position 7*d* of amino acid no. 7 (**9** and **10**) (Figure 2).

The syntheses of *N'*-*p*-(*p*-chlorophenyl)benzyleremomycin (**5**),[11] *N'*-*n*-decyleremomycin (**6**),[11] eremomycin decylamide (**8**),[5] and 7*d*-(*n*-decylaminomethyl)eremomycin (**10**)[3] have been previously described. The *p*-(*p*-chlorophenyl)benzylamide of eremomycin (**7**) was obtained by the standard method[5] from the antibiotic and *p*-(*p*-chlorophenyl)benzylamine in the presence of HBTU. Mannich alkylaminomethylation of eremomycin with the use *p*-(*p*-chlorophenyl)benzylamine and formaldehyde producing compound **9** was performed as previously described for the compounds of this type.[3] Unfortunately alkylaminomethylation of des-(*N*-methyl-D-leucyl)eremomycin (**1a**) led to unstable compounds. The preparation of des-(*N*-methyl-D-leucyl)-*N'*-*p*-(*p*-chlorophenyl)benzyleremomycin (**5a**) has followed two alternative schemes (Scheme 1). Edman degradation[16] of eremomycin (**1**) was performed under the conditions in which all three glycosyl bonds were untouched. In the first step, *N*-phenylaminothiocarbonyleremomycin (**11**) was obtained in 68% yield by the interaction of the antibiotic

with PhNCS, and **11** gave des-(*N*-methyl-D-leucyl)-eremomycin (**1a**) under the action of CH_2Cl_2/TFA mixture at 0−5 °C in 91% yield. Edman degradation of **5** similarly produced des-(*N*-methyl-D-leucyl)eremomycin derivative (**5a**) in 54% yield via the intermediate *N*-phenylaminothiocarbonyl-*N*′-[*p*-(*p*-chlorophenyl)benzyl]-eremomycin. Following the second scheme, reductive alkylation of **1a** gave intermediate borate complexes which produced a derivative identical with **5a** after the hydrolysis with TFA (30% yield).

The compounds obtained were characterized by HPLC in three systems. The structures of **1a** and **8a** were confirmed by NMR study (Table 1). Assignment of eremomycin derivatives was based on earlier NMR assignments for eremomycin.[17] The structures were also confirmed by mass spectral (ESI MS) analysis and by chemical degradation methods[18] (Table 2, Figure 4). Mild hydrolysis of eremomycin in 1 N HCl at 100 °C for 10 min leads to the splitting off of eremosamine (1E) from the disaccharide branch. The presence of the unsubstituted eremosamine and des-(eremosaminyl)-eremomycin can be easily demonstrated with paper chromatography by comparison with authentic compounds.[18] Under such conditions, hydrolysis of compounds **5**, **6**, **5a**, and **6a** do not produce the unsubstituted eremosamine and do produce des-(eremosaminyl)eremomycin, whereas hydrolysis of all other derivatives with the unsubstituted eremosamine moiety (**1a**, **7**, **7a**, **8**, **8a**, **9**, **10**) revealed the presence of the unsubstituted eremosamine and did not reveal the presence of des-(eremosaminyl)eremomycin. After drastic hydrolysis (concentrated HCl, room temperature, 4 h) when all sugars of a glycopeptide antibiotic are split off, the formation of the unmodified eremomycin aglycone was determined for the derivatives **5** and **6**, as well as the presence of the unmodified eremosamine (2E) formed after the splitting of the monosaccharide branch. Hydrolysis of compounds **1a**, **5a**, and **6a** under identical conditions also showed the presence of the unsubstituted eremosamine (1E plus 2E for **1a**, and 2E for **5a** and **6a**), but instead of eremomycin aglycone,[18] the des-(*N*-methyl-D-leucyl) eremomycin aglycone was present.[19] Compounds **7a**, **8a**, **9**, and **10**, after hydrolysis with concentrated HCl, also gave the unsubstituted eremosamine (1E plus 2E), whereas the unmodified eremomycin aglycon was absent.

NMR Study of the Interaction with Ligand

HSQC spectra reveal that compound **1a** is dimeric, since α_4, G1, 2e, and some more signals are doubled at slow exchange measurement conditions[20] (278 K), while compounds **8** and **8a** did not exhibit signal doubling. However, a characteristic pattern for the *head-to-tail* dimers (unusual proton chemical shifts of the aromatic 6e signal at ∼ 5 ppm because of the orthogonal $\sigma-\pi$ interaction) is observed for both **8** and **8a**, most probably due to dimer formation. Measured binding constants for Ac-D-Ala-D-Ala are 6230 M^{-1} (**8**) and 120 M^{-1} (**8a**). While the published binding constant[21] for eremomycin (**1**) is 2800 M^{-1}, hexapeptide (**1a**) showed 1120 M^{-1} affinity to Ac-D-Ala-D-Ala. For the vancomycin series, removal of *N*-methyl-D-leucine is known to dramatically reduce -D-Ala-D-Ala binding.[22] Attempts to observe any useful chemical shifts for (**8**) when titrating with Ac-D-

Table 1. ^1H and ^{13}C NMR Parameters of HSQC Spectra of Compound **1a** (50 mM phosphate buffer, pH 4.5, H_2O/D_2O, 9:1) and Compound **8a** (278 K, 50 mM phosphate buffer, pH 4.5, H_2O/D_2O, 9:1, + 23 v/v % of DMSO-d_6) (HSQC spectra)

		compound **1a**		compound **8a**	
amino acids	atom no.	^1H	^{13}C	^1H	^{13}C
no. 2	2b	7.082	130.15	6.984	129.06
		7.033	130.15		
	2e	7.257	125.23	7.170	124.68
		7.082	125.23		
	2f	7.365	126.87	7.423	127.96
	2α	4.260	59.88	4.065	62.89
	2β	5.246	69.85	ND[a]	ND
no. 3	3α	4.856	52.70	ND	ND
	3β	2.337	36.64	ND	ND
no. 4	4b	5.168	108.00	5.031	104.45
		5.002	105.27		
	4f	5.246	104.17	5.070	104.72
		5.149	103.63		
	4α	6.203	54.68	6.164	54.69
		6.115	54.68		
no. 5	5b	6.799	135.89	6.906	135.90
		6.760	135.89		
	5e	6.672	118.12	6.730	117.85
	5f	6.574	127.96	6.984	129.06
	5α	4.231	54.68	4.231	54.41
		4.163	54.96		
no. 6	6b	7.296	129.06	7.296	129.06
		7.160	127.96		
	6c	6.838	123.04	ND	ND
		6.711	123.31		
	6e	5.27	122	5.32	122.4
		5.03	121.4		
	6f	6.574	127.96	ND	ND
	6α	3.958	62.89	3.752	60.42
	6β	5.168	74.96	ND	ND
no. 7	7d	6.242	103.08	6.242	103.08
	7f	6.144	107.73	6.105	107.18
	7α	4.397	58.24	4.368	57.96
		compound **1a**		compound **8a**	
sugars	atom no.	^1H	^{13}C	^1H	^{13}C
glucose	G1	5.432	100.07	5.227	103.90
		4.963	102.81		
	G2	3.918	79.84	3.557	73.82
		3.421	77.92		
	G3	4.055	75.46	ND	ND
		3.850	75.46		
	G4	3.352	67.81	3.264	68.08
		3.098	68.08		
	G5	3.352	76.56	3.411	76.01
	G6	3.596	60.97	3.294	61.52
		3.255	60.97		
		2.766	60.15		
eremosamine 1E	1E1	5.012	97.34	5.28	97.9
		4.973	97.61		
	1E2	2.102	38.28	2.278	38.28
	1E3	0.999	17.50	1.029	17.77
	1E4	3.157	74.37	3.167	74.64
	1E5	4.387	66.44	ND	ND
		4.133	66.44		
	1E6	0.833	17.77	0.970	17.50
eremosamine 2E	2E1	5.04/ 4.98	92.8	4.97	93.0
	2E2	2.044	38.55	2.182	38.82
		1.780	39.10		
	2E3	1.302	18.04	1.351	18.04
	2E4	3.079	74.92	3.108	74.64
	2E5	3.479	66.17	3.391	66.71
	2E6	1.058	17.22	1.097	17.50
decyl amide	N-CH_2			3.118	39.92
				2.854	
	9-CH_2			0.970	22.69
	10-CH_3			0.570	13.94
	$(CH_2)_7$			0.990	28.98
				1.263	28.43
				1.204	33.36

[a] ND: not determined because of H_2O overlap or could not be assigned by comparison.

Table 2. Products of Partial Degradation of Eremomycin Derivativies

hydrolysis products	compounds investigated				
	N-R²-eremomycin (**5** or **6**)	*N*-R²-de(methyl-D-leucyl)-eremomycin (**5a** or **6a**)	carboxyamides (**7** or **8**)	carboxyamides (**7a** or **8a**)	7*d*-R⁴-eremomycin (**9** or **10**)
Mild Hydrolysis (1 N HCl, 100 °C, 10 min)					
eremosamine	−	−	+	+	+
de-(eremosaminyl)-eremomycin	+	−	−	−	−
Drastic Hydrolysis (35% HCl, 22 °C, 4 h)					
eremosamine	+	+	+	+	+
des-(*N*-methyl-D-leucyl)-eremomycin aglycon	−	+	−	−	−
eremomycin aglycon	+	−	−	−	−

Figure 4. Hydrolysis of eremomycin derivatives.

Ala-D-Lactate were unsuccessful. Consequently, the lactate affinity to the compound (**8**) is unmeasurably small. It was shown earlier that *N*-substituted hydrophobic derivatives of chloroeremomycin have weak binding with Ac_2-D-Ala-D-Lac so the substantial lactate binding was not expected based on literature data.[23] This demonstrates that anti-GRE activity of these compounds is not based on their interaction with the modified target D-Ala-D-lactate.

Biological Evaluation

In vitro antibacterial activities of compounds **5**−**10** and **5a**−**8a** in comparison with eremomycin (**1**) and des-(*N*-methyl-D-leucyl)eremomycin (**1a**) are presented in Table 3. All eremomomycin derivatives with hydrophobic substituents (**5**−**10**, **5a**−**8a**) demonstrated rather good activity against enterococci, both sensitive (GSE) and resistant (GRE) strains. Minimum inhibitory concentration (MIC) values for hexapeptide derivatives (**5a**−**8a**) are very close to MIC values of the corresponding heptapeptides (**5**−**8**), the pair **6**−**6a** being an exception. Compounds **9** and **10** also demonstrated definite activity against GSE and GRE strains. In general, MIC values for staphylococci are very close to MIC values for GRE strains, in which the -D-Ala-D-Ala moiety in nascent peptidoglycan is substituted by -D-Ala-D-lactate, whereas *n*-decyl-containing derivatives **6**, **8**, **8a**, **9**, and **10**, which are several times more active against staphylococci than against GRE, represent an exception. It is important to focus on the activity of these derivatives against strains of *Staphylococcus aureus* with intermediate susceptibility to glycopeptides (GISA strains). These strains have a mechanism of resistance to glycopeptide antibiotics that does not involve substitution of -D-Ala-D-Ala by -D-Ala-D-lactate.[24] Hydrophobic derivatives of eremomycin are slightly more active than the parent antibiotic against GISA, whereas des-(*N*-methyl-D-leucyl)eremomycin (**1a**) has a rather low activity against GISA strains. However, hydrophobic derivatives (**5a**−**8a**) are several times more active.

The incorporation of labeled peptidoglycan precursors into peptidoglycan was studied using either UDP-Mur*N*Ac-pentapeptide (containing -D-Ala-D-Ala moiety) or UDP-Mur*N*Ac-tetrapeptide (which lacks the *C*-terminal -D-Ala, so the dipeptide binding with an antibiotic is impossible) by the method described[14] (Table 4) in order to demonstrate that hydrophobic *N*-*p*-[*p*-(chlorophenyl)benzyl] derivatives **5** and **5a**, *N*-decyl eremomycin (**6**), and eremomycin decylamide (**8**) inhibit peptidoglycan synthesis in the absence of dipeptide binding. Eremomycin failed to inhibit peptidoglycan synthesis when UDP-Mur*N*Ac-tetrapeptide was used as substrate (IC_{50} >640 μM); however, it did inhibit (IC_{50} 0.27 μM) when UDP-Mur*N*Ac-pentapeptide was used.[11] More interestingly, all hydrophobic eremomycin derivatives studied did inhibit peptidoglycan synthesis regardless of whether pentapeptide or tetrapeptide substrates were used. Although eremomycin derivative **5** inhibited UDP-Mur*N*Ac-pentapeptide incorporation at lower concentrations than the corresponding hexapeptide derivative (**5a**), IC_{50} values for both compounds for UDP-Mur*N*Ac-tetrapeptide incorporation are of the same order. This demonstrates that hydrophobic derivatives of eremomycin directly inhibit polymerization of lipid-II-tetrapeptide into peptidoglycan, while eremomycin derivatives with the damaged binding pocket do the same despite dramatically decreased -D-Ala-D-Ala binding.

If the hexapeptide analogue **5a** is inhibiting the transglycosylation reaction directly in the absence of dipeptide binding (as indicated by the above data), it should directly inhibit the isolated transglycosylation reaction. An in situ reaction system was developed[25] to follow the conversion of Glc*N*Ac-β-1,4-Mur*N*Ac-tetrapeptide-pyrophosphoryl-undecaprenyl [lipid II-tetrapeptide, which differs from usual lipid II-pentapeptide (Figure

Table 3. Antibacterial Activity of Glycopeptide Analogues

strain	533 *S. epidermidis*	602 *S. haemolyticus*	3797 *S. aureus* (GISA)	3798 *S. aureus* (GISA)	568 *E. faecium* (GSE)	559 *E. faecalis* (GSE)	569 *E. faecium* (GRE)	560 *E. faecalis* (GRE)
1	0.25	0.25	8	8	0.25	0.25	>128	>128
1a	16	16	64	64	16	16	>128	>128
2	1	1	16	8	1	1	>128	>128
			N-Alkylated Derivatives					
5	2	4	4	4	2	4	4	8
5a	4	4	8	8	4	4	8	8
6	0.5	0.5	4	4	0.5	0.5	4	8
6a	4	8	8	16	8	8	16	16
			Amides					
7	2	1	4	4	1	2	4	4
7a	4	4	8	8	4	4	2	4
8	0.13	0.13	4	4	0.5	1	2	4
8a	0.5	1	4	8	2	2	2	4
			7*d*-Aminomethyl Derivativies					
9	0.5	0.25	2	2	1	1	8	16
10	0.25	0.25	2	2	0.5	0.5	8	8

Table 4. Inhibition of Cell Wall Synthesis by Glycopeptide Analogues

compd	IC_{50} for inhibition (μM) using[a] pentapeptide	tetrapeptide	ratio tetrapeptide/ pentapeptide
1	0.3 (0.19−0.40)	>640	>2300
2	0.9 (0.3)	123.7 (45.5)	141
5	0.12 (0.095−0.14)	2.7 (2.17−3.23)	23
5a	7.7 (5.55−10.5)	9.2 (7.67−12.2)	1.2
6	1.8 (0.42−7.7)	13.3 (5.42−32.5)	7.3
8	1.3 (0.56−3.13)	6.4 (2.2−18.6)	5

[a] Concentration response curves were analyzed by nonlinear regression using a four-parameter logistic model fitted and plotted with GraphPad Prism (v. 2.0, GraphPad Software, Inc., San Diego, CA). IC_{50} values were determined using at least six concentrations of drug in duplicate. Single values in parentheses are standard deviations; value ranges represent the 95% confidence levels for data sets.

1) in the absence of *C*-terminal -D-Ala, so dipeptide binding is impossible] directly into peptidoglycan. The assay involves the preferential synthesis and accumulation of lipid II in a reaction mixture containing the cell wall membrane material isolated from *Escherichia coli*, exogenously supplied UDP-Mur*N*Ac-pentapeptide, and radiolabeled UDP-Glc*N*Ac. In the presence of Triton X-100, the radiolabeled product formed is almost exclusively lipid II, while the subsequent formation of peptidoglycan is inhibited. Removal of the detergent results in the synthesis of peptidoglycan (25% incorporation of radiolabeled material) from the accumulated lipid II. This reaction was inhibited by moenomycin, a known transglycosylase inhibitor. Hexapeptide **5a** inhibited incorporation of Glc*N*Ac-β-1,4-Mur*N*Ac-tetrapeptide-pyrophosphoryl-undecaprenyl (lipid II-tetrapeptide) into peptidoglycan with an IC_{50} value of 43 μM, which is within 5-fold of its potency of inhibition of peptidoglycan synthesis (9.19 μM) when UDP-Mur*N*Ac-tetrapeptide was used as substrate and the reaction started with UDP-*N*-acetyl-[^{14}C]-D-glucosamine.

Summary and Conclusions

A series of hydrophobic derivatives of eremomycin and des-(*N*-methyl-D-leucyl)eremomycin were synthesized and evaluated for antibacterial activity against vancomycin- or eremomycin-sensitive and -resistant Gram-positive bacteria. All hydrophobic derivatives exhibited rather good activity against vancomycin-resistant strains of enterococci while retaining activity against sensitive strains. Loss of residue no. 1 in the glycopeptide core of the compounds with hydrophobic substituents does not diminish antibacterial activities. The position of a hydrophobic substituent on the periphery of the glycopeptide does not seriously influence its antibacterial properties. Earlier[12] it was shown that the hydrophobic derivative of vancomycin substituted on the vancosaminyl moiety (**3**) is active even when the binding pocket is damaged (**3a**), and it was suggested that **3** and **3a** have an additional biological activity that cannot be simply due to membrane localization. The activity of the vancomycin derivative substituted on the glucose moiety (**4**) was explained by the membrane localization and increased binding to lipid II since the activity was lost when binding pocket was damaged (compound **4a**).[12] The absence of activity in this case may be connected with the position of the substituent. Generally, ring 4 oligosaccharides may have a capping effect at the binding site.[26] Substitution at the glucose moiety may disturb this capping and may render the hydrophobic side chain unavailable for membrane contact.

In conclusion, we have shown that specific hydrophobic derivatives of eremomycin demonstrate antibacterial properties despite decreased -D-Ala-D-Ala binding and in the absence of -D-Ala-D-lactate binding. We conclude that these compounds do inhibit bacterial cell wall synthesis and show that they do inhibit the transgycosylation step of peptidoglycan biosynthesis. Recent data, using mutants selected for resistance to lipophilic glycopeptide derivatives and monenomycin (a known transglycosylase inhibitor), have revealed that resistance is due to the alteration of a novel gene (*yfgL* in *E. coli*) that is involved in the regulation of peptidoglycan synthesis and the cell death response that is triggered upon exposure to inhibitors of peptidoglycan synthesis, specifically those that inhibit transglycosylation.[15]

Experimental Section

Eremomycin sulfate was produced at a pilot plant of the Gause Institute of New Antibiotics, Moscow. All reagents and solvents were purchased from Aldrich, Fluka, and Merck. *p*-(*p*-Chlorophenyl)benzaldehyde was kindly provided by Advanced Medicine East, Inc. (NJ). *p*-(*p*-Chlorophenyl)benzylamine was obtained by the reduction of *p*-(*p*-chlorophenyl)benzaldehyde

oxime with the use of $LiAlH_4$. The progresses of the reactions, column eluates, and all final samples were analyzed by TLC using Merck silica gel $60F_{254}$ plates in EtOAc/*n*-PrOH/25% NH_4OH (1.75:1:1) with UV detection. Reaction products were purified by reverse-phase chromatography on Merck silanized silica gel (0.063 ~ 0.2 mm).

NMR Study. A Bruker DRX-500 NMR spectrometer was used at 500.13 and 125.79 MHz frequency for ^1H and ^{13}C, respectively. An $^1H/^{13}C/^{15}N$ triple resonance probehead equipped with triple (*x*, *y*, *z*) gradient facility was applied. NMR titration was followed by ^1H NMR using the watergate technique for water suppression. ^{13}C NMR spectra were acquired using 2D-HSQC (heteronuclear single-quantum correlation) method. Ninety degree pulses for $^1H/^{13}C$ were 10.5/16 μs, respectively.

Sample Preparation and Measuring Conditions. A total of 600 μL of 8−10 mM solutions of the antibiotics **1a**, **8**, and **8a** were prepared in 9:1 H_2O/D_2O mixture and 50 mM phosphate buffer (pH 4.5). Poor solubility of compounds **8** and **8a** required the addition of 23% v/v of DMSO-d_6. The antibiotic solution (100 μL) was taken out, and sufficient amount of ligand was added to have a ca. 100 mM solution of ligand concentration. Titration was carried out with constant antibiotic concentration, and the final amount of added ligand generally exceeded 2 equiv. Measuring temperature was always 5 ± 1 °C in order to slow exchange processes. Titration was monitored using the w_2 chemical shift changes upon adding ligands (Ac-D-Ala-D-Ala or Ac_2-Lys-D-Ala-D-Ala). A + L ↔ AL equilibrium is supposed to be a fast ligand exchange and was evaluated with computer fitting.

Chemistry. Edman Degradation of Eremomycin. 1. *N*-Phenylaminothiocarbonyleremomycin (11). Duolite basic anion-exchanger A 30 (OH^- form) was added to a solution of eremomycin sulfate (500 mg, ~0.3 mmol) in water (100 mL), and the mixture was left at room temperature for 3 h. Eremomycin base solution was filtered off and concentrated in vacuo with the addition of *n*-BuOH, and then acetone (50 mL) was added to give a precipitate. The latter was filtered off, washed with acetone, and dried in vacuo to give eremomycin base (410 mg, ~0.26 mmol). It was dissolved in pyridine/water 1:1 mixture (5 mL), and PhNCS (0.02 mL, 0.2 mmol) was added under argon. The reaction mixture was stirred at room temperature for 16 h, concentrated in vacuo with the addition of *n*-BuOH, and applied to a column with the silanized silica gel (2 × 100 mL), previously equilibrated with 0.001 M acetic acid. Acetic acid (0.001 M) was used for elution to give fractions containing nonreacted eremomycin. A mixture of MeOH/0.001 M CH_3COOH (2:8) with the rate 30 mL/h was used to give fractions containing compound **11**. The fractions were pooled and concentrated with the addition of *n*-BuOH in vacuo, and acetone (50 mL) was added to yield the precipitate, which was filtered off, washed with acetone, and dried to yield 303 mg (68%) of **11**.

2. Des-(*N*-methyl-D-leucyl)eremomycin (1a). A solution of *N*-phenylaminothiocarbonyl-eremomycin (**11**) (260 mg, 0.15 mmol) in 3 mL of CH_2Cl_2/TFA (1:1) was stirred at 0−5 °C for 1 h, and then water (3 mL) was added. The mixture was neutralized by 25% NH_4OH and then washed with EtOAc (5 mL × 3), and the aqueous fraction was concentrated in vacuo. The precipitate obtained after the addition of acetone (50 mL) was filtered off, washed with acetone, and dried in vacuo to give 200 mg (91%) of compound **1a**.

Des-(*N*-methyl-D-leucyl)-*N*'-[*p*-(*p*-chlorophenyl)benzyl]-eremomycin (5a). A. *N*'-*p*-(*p*-Chlorophenyl)benzyl]eremomycin **5**[11] (20 mg, 0.011 mmol) was dissolved in MeOH (5 mL), Duolite basic anion-exchanger A 30 (OH^- form) was added, and the mixture was incubated at room temperature for 1 h. After filtration, the solution of compound **7** (in base form) was evaporated in a vacuum with the addition of *n*-BuOH, and the residue (15 mg, 0.009 mmol) was dissolved in a mixture of pyridine/water (1:1) and phenylisothiocyanate (1.1 μL, 0.009 mmol) under argon. After 16 h of being stirred at room temperature, the mixture was concentrated in vacuo and the product precipitated with ether. The intermediate *N*-phenylaminothiocarbonyl-*N*'-[*p*-(*p*-chlorophenyl)benzyl]-eremomycin was dissolved in CH_2Cl_2/TFA (1:1) (1 mL) and stirred at 0−5 °C for 1 h, then 2 mL of water was added, and the mixture was neutralized with 25% NH_4OH. The solution was extracted with EtOAc (1 mL × 2) and concentrated in vacuo. Ether addition gave a precipitate, which was washed with ether and filtered. After drying in vacuo, **5a** was obtained (8 mg, 54%) as a white solid.

B. To a solution of **1a** (200 mg, 0.14 mmol) in dry DMF (4 mL) heated to 70 °C, *p*-(*p*-chlorophenyl)benzaldehyde (91 mg, 0.42 mmol) was added in portions while stirring over a course of 4 h. $NaCNBH_3$ (27 mg, 0.42 mmol) was added and the reaction mixture stirred for 2 h at room temperature. Acetone (70 mL) addition gave a precipitate, which was filtered off and dried. It was then dissolved in MeOH with the addition of TFA to pH 3. Silanized silica gel was added, the mixture was evaporated in vacuo and applied to a column with the silanized silica gel (2 × 100 cm) preliminary equilibrated with 0.001 M acetic acid. Elution was first performed with the use of 0.001 M CH_3COOH to give the starting **1a**, then changed to a 30−70% of MeOH gradient in 0.001M CH_3COOH, and finally to a mixture MeOH/1 M CH_3COOH (6:4). The fractions containing the target compound **5a** were pooled, concentrated in vacuo with the addition of *n*-BuOH, and precipitated with ether (50 mL) to give a white solid, which was washed with ether and dried in vacuo to give **5a** (44 mg, 19%), identical by HPLC, TLC, and ESI-MS to the compound obtained by method A.

Des-(*N*-methyl-D-leucyl)-*N*'-*n*-decyleremomycin (6a) was obtained by Edman degradation of compound **6** according to the procedure for **5a** (method A) in 40% yield.

***p*-(*p*-Chlorophenyl)benzylamides of Eremomycin (7) and of Des-(*N*-methyl-D-leucyl)eremomycin (7a), *n*-Decylamide of Des-(*N*-methyl-D-leucyl)eremomycin (8a). General Procedure.**[5] To a solution of eremomycin or des-(*N*-methyl-D-leucyl)eremomycin (0.03 mmol) in DMSO (2 mL) were added hydrochlorides of *p*-(*p*-chlorophenyl)benzylamine or *n*-decylamine (0.3 mmol), Et_3N (0.3 mmol), and HBTU [*O*-benzotriazol-1-yl-*N,N,N',N'*-bis(tetramethylene)uronium hexafluorophosphate] (0.06 mmol) at room temperature in three portions with stirring over 1 h. After 4 h, acetone (100 mL) was added to give a colorless solid, which was washed with acetone and dried in vacuo to give the corresponding amide in ca. 90% yield.

Determination of Antibacterial Activity. Minimum inhibitory concentrations (MICs) were determined by broth microdilution method using Mueller Hinton broth as recommended by NCCLS procedure. Results were usually identical and always within 2-fold. The strains tested were kindly provided by Dr. R. Ciabatti and G. Romano from Biosearch Italia SpA (Gerenzano, Italy). Resistant strains with the confirmed genotype for vancomycin-resistant enterococci are the same as used in the previously published paper.[27] 533 *Staphylococcus epidermidis* and 602 *Staphylococcus haemoliticus* are clinical isolates. Glycopeptide intermediate strains are 3797 *Staphylococcus aureus* (GISA HIP-5836 New Jersey) and 3798 *Staphylococcus aureus* (GISA HIP-5827 Michigan).

Peptidoglycan Polymerization Assay. Peptidoglycan synthesis was conducted in 96-well GFC filter plates (Millipore Corp. #MAFCNOB) using membranes from *E. coli* OV58 (pTA9), UDP-MurNAc-pentapeptide, UDP-MurNAc-tetrapeptide, and UDP-*N*-acetyl-[^{14}C]-D-glucosamine as substrates, as previously described.[14] Direct inhibiton of the transglycosylation reaction was evaluated using *N*-acetylglucosamine-β-1,4-MurNAc-tetrapeptide-pyrophosphoryl-undecaprenyl (lipid II-tetrapeptide) as substrate.[25] The in situ transglycosylase system allows synthesis and accumulation of radiolabeled lipid II in membranes in the presence of Triton X-100.[25] When the Triton is removed with detergent binding resin, the accumulated lipid II is polymerized into peptidoglycan. Addition of moenomycin, a known transglycosylase inhibitor, likewise blocks conversion of lipid II into peptidoglycan, as did des(*N*-methyl-D-leucyl)-*p*-(*p*-chlorophenyl-benzyl)-vancomycin.[15]

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Supporting Information Available: TLC, HPLC, molecular formula, and mass spectra data (ESI MS) for compounds **1a**, **5a**−**8a**, and **5**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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