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Synthesis of Hydrophobic N'-Mono and N',N''-Double Alkylated Eremomycins Inhibiting the Transglycosylation Stage of Bacterial Cell Wall Biosynthesis

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A series of hydrophobic N'-mono and N',N''-double alkylated derivatives of the glycopeptide antibiotic eremomycin were synthesized by reductive alkylation after preliminary protection of the N-terminal amino group of the peptide backbone. The investigation of the antibacterial activity *in vitro* showed that N'-$C_{10}H_{21}$- and N'-*p*-(*p*-chlorophenyl)benzyl derivatives of eremomycin are the most active against vancomycin-resistant enterococci among the compounds obtained though they are less effective than the corresponding lipophilic derivatives of vancomycin. The introduction of two hydrophobic substituents led to a decrease in activity against both susceptible and resistant bacteria. The biochemical evaluation of the mode of action revealed that in addition to binding to D-Ala-D-Ala these compounds also have an alternative mechanism of action that does not require substrate binding.

Vancomycin resistance of Gram-positive bacteria which is an increasingly emerging health problem worldwide stimulated search for new semisynthetic glycopeptides active against these resistant bacteria. In the last years chemical modifications of vancomycin-related glycopeptides and study of SAR for these derivatives led to glycopeptides with hydrophobic alkyl or alkylaryl substituents active against vancomycin-resistant enterococci (VRE).[1~4] For the evaluation of new semisynthetic glycopeptides active against VRE the elucidation of the role of side chain on the mechanism of action of these antibiotics is extremely important. The role of a hydrophobic substituent in a glycopeptide antibiotic was hypothesized to mediate anchoring into bacterial cell membrane.[5] Recently it was demonstrated that the hydrophobic N'-alkylated derivatives of chloroeremomycin[6] and vancomycin, active against VRE, retain the ability to bind the D-Ala-D-Ala of sensitive bacteria and act on VRE by a mechanism without binding the target D-Ala-D-Lac which substitutes for D-Ala-D-Ala in VRE.[7~9] This suggests that the derivatives of other glycopeptide antibiotics (*e.g.* eremomycin, teicoplanin aglycone) containing hydrophobic substituents in various positions of the molecule may also have a new mechanism of action on VRE.

In this paper we report a new synthetic route for preparation of N'-mono and N',N''-double substituted derivatives of eremomycin that involves the preliminary protection of the N-terminal amino group of the peptide backbone. Several of these analogs possess antibacterial activity *in vitro* against VRE, suggesting that they have a novel mode of action.

Chemistry

A series of mono-, di- and tri-alkylated (on the amino groups) derivatives of the A82846 family of glycopeptides to which eremomycin (I) belongs was previously obtained

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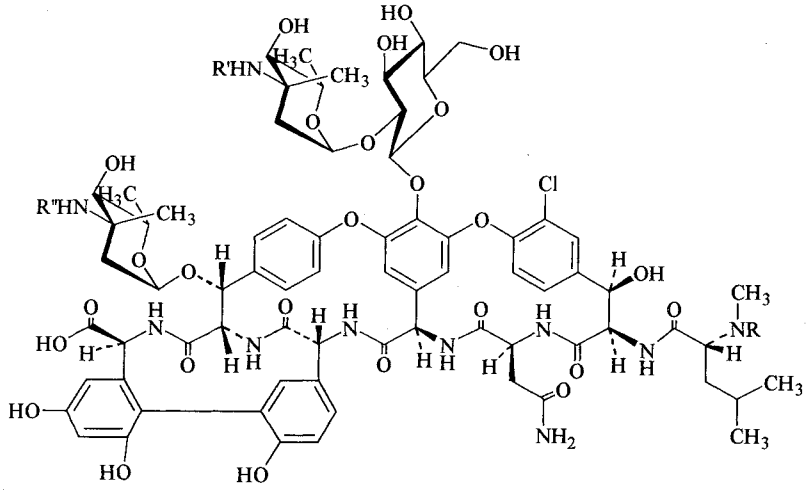
by the reductive alkylation of the parent antibiotic with an aldehyde and $NaBH_3CN$.[10] In the case of the benzyl or bulky aldehydes, the most perspective *N'*-alkylated analogs were synthesized in 20~40% yields, while for aliphatic aldehydes the yields were significantly lower. In our hands less than a 10% yield of *N'*-decyleremomycin was obtained by this method. Previously, we showed that the acylation of eremomycin occurs regioselectively under strict conditions at the *N*-terminal amino group, and some of the acylated derivatives such as *N*-Cbz- or *N*-Boc-eremomycin (II) were obtained in 50% yields (90% on reacted I).[11] This method allows preliminary protection of the *N*-terminal amino group of eremomycin for the selective modification of the amino groups of the disaccharide and monosaccharide moieties especially when aliphatic aldehydes are used. The high lability of the eremomycin molecule considerably limits the choices of a suitable *N*-protective group. We have found that the Fmoc protective group was one of the best: the Fmoc group is used widely in peptide chemistry and can be removed under mild conditions. *N*-Fmoc-eremomycin (III) was obtained by the treating eremomycin with FmocOSu at pH 7 with a yield of 75% (90% on reacted I). The higher yield observed for III compared to the corresponding Boc-derivative II can be explained by the lower solubility of III and lack of additional purification of crude III, which was pure enough for further synthesis. To obtain *N'*-monosubstituted derivatives, compounds II or III were treated with the appropriate aldehyde in the presence of $NaBH_3CN$. *N',N''*-disubstituted derivatives were obtained by $NaBH(OAc)_3$ treatment followed by $NaBH_3CN$. The

Table 1. The structure and overall yields of the derivatives of eremomycin II~X.



Eremomycin: R=R'=R''=H

Compound	R	R'	R''	Yields, % (method)	Molecular formula	ESI MS, M.W. Calculated	Found
II	Boc	H	H	50	$C_{78}H_{98}ClN_{10}O_{28}$	1656.60	1656.6
III	Fmoc	H	H	75	$C_{88}H_{99}ClN_{10}O_{28}$	1778.63	1778.6
IV	H	p-ClBn	H	55 (A), 30*	$C_{80}H_{94}Cl_2N_{10}O_{26}$	1680.57	1680.6
V	H	p-ClBn	p-ClBn	40 (B)	$C_{87}H_{99}Cl_3N_{10}O_{26}$	1804.58	1804.6
VI	H	$C_{10}H_{21}$	H	45 (A), 20*	$C_{83}H_{109}ClN_{10}O_{26}$	1696.62	1696.6
VII	H	$C_{10}H_{21}$	$C_{10}H_{21}$	20 (A), 50 (B)	$C_{93}H_{129}ClN_{10}O_{26}$	1836.88	1836.9
VIII	H	p-PhBn	H	50 (A), 30*	$C_{86}H_{99}Cl_2N_{10}O_{26}$	1722.64	1722.7
IX	H	p-PhBn	p-PhBn	30 (A), 40 (B)	$C_{99}H_{109}ClN_{10}O_{26}$	1888.72	1888.7
X	H	p-(p-ClPh)Bn	H	40 (A), 25*	$C_{86}H_{98}Cl_2N_{10}O_{26}$	1871.38	1871.4

* from compound II

deprotection of the *N*-Fmoc-eremomycin derivatives was performed by the treating with the 10% diethylamine in DMF at room temperature for 30 minutes. The structures and overall yields (starting from I) of the final compounds IV~X are presented in Table 1. We have also elaborated the conditions for mild removal of the *N*-Boc-protective group in a solution of 30% TFA in CH_2CI_2 at 0°C for 1 hour. The use of II as an intermediate instead of III, however, gave a 2 fold lower overall yields of the desired *N'* and *N'*,*N''* derivatives (Table 1). The final compounds were purified by reverse phase column chromatography on silanized silica gel, and the homogeneity was demonstrated by TLC and HPLC analysis. For compounds III~X, peaks corresponding to their molecular ions were observed by ESI mass-spectra. In addition, the positions of the introduced substituents for compounds III~X were validated by the acidic hydrolysis and Edman degradation as previously described.[11]

Results and Discussion

The *in vitro* antibacterial activity of compounds IV~X, in comparison to eremomycin (I), vancomycin (XI) and *N'*-*p*-(*p*-chlorophenyl)benzylvancomycin (XII), are shown in Table 2. Compounds VI, X and XII are the most active against vancomycin resistant enterococci (VRE) though VI and X are less active than eremomycin against staphylococci. It is interesting to note that whereas eremomycin is several times more active than vancomycin, the introduction of *N'*-*p*-(*p*-chlorophenyl)benzyl substituent into vancomycin molecule leads to the enhancement of antibacterial activities both against sensitive and resistant strains, and the introduction of the same substituent into eremomycin molecule leads to decrease of activity against sensitive bacteria, and though activity against VanA enterococci is rather high it is lower than for the vancomycin derivative XII. In chloreremomycin series the introduction of *N'*-*p*-(*p*-chlorophenyl)benzyl substituent (LY 333328) leads to high antibacterial activity against VanA, but LY 333328 is less active than the parent antibiotic against sensitive staphylococci.[3] Eremomycins with two hydrophobic substituents (compounds V, VII and IX) have low antibacterial activity against both sensitive and resistant bacteria in contrast to deacylated teicoplanin with two lipophilic substituents which are highly active both against sensitive and resistant bacteria.[12] It seems that differences in SAR for relative glycopeptide antibiotics are connected with a complicated type of interaction of these antibiotics with cell targets in sensitive and resistant bacteria, and also with differences in total lipophilicity and bioavailability of these derivatives. Distinctions in SAR for various glycopeptide antibiotics are connected with peculiarities of their mechanisms of action and

Table 2. Antibacterial activity *in vitro* (MIC, μg/ml) of the derivatives of eremomycin IV~X in comparison to eremomycin (I), vancomycin (XI) and *N'*-*p*-(*p*-chlorophenyl)benzylvancomycin (XII).

Microorganism	I	IV	V	VI	VII	VIII	IX	X	XI	XII
Staphylococcus aureus ATCC 29213 (MSSA)	0.78	1.56	1.56	3.12	25	1.56	12.5	3.12	1.56	0.06
S. aureus ATCC 33591 (MRSA)	1.56	1.56	1.56	1.56	6.25	0.78	6.25	3.12	1.56	0.06
Enterococcus faecalis ATCC 29212 (VanS)	0.78	1.56	1.56	1.56	25	0.78	25	1.56	3.12	0.06
E. faecalis CL 4877 (VanA)	>25	>25	>25	3.12	25	25	12.5	6.12	>25	3.12
E. faecium ATCC 49624 (VanS)	0.25	0.25	0.25	0.78	6.25	0.25	3.12	0.78	0.39	0.01
E. faecium CL 4931 (VanB)	>25	>25	>25	12.5	6.25	25	25	12.5	>25	6.25

understanding of the reasons of this distinctions may help to decode these mechanisms.

It was demonstrated recently that hydrophobic vancomycin derivatives inhibit transglycosylation step of peptidoglycan synthesis differently from vancomycin which activity is based on the interaction with D-Ala-D-Ala of sensitive cells.[8,9] Eremomycin itself did not inhibit peptidoglycan synthesis when UDP-MurNAc-tetrapeptide (a substrate of transglycosylation, which lacks D-Ala terminal residue) was used as substrate (IC_{50} >640 μM), but did inhibit (IC_{50} 0.27 μM) when UDP-MurNAc-pentapeptide was used, and the ratio IC_{50} (μM) for peptidoglycan synthesis inhibition when tetrapeptide or pentapeptide were used as precursors was >2300. In contrast, lipophilic derivatives did inhibit when either the penta- or tetrapeptide substrates were used. The *p*-(*p*-chlorophenyl)benzyl derivative X was the most potent compound for inhibition of both UDP-MurNAc-pentapeptide (0.12 μM) and UDP-MurNAc-tetrapeptide (2.67 μM) incorporation into peptidoglycan, compound VI inhibited UDP-MurNAc-pentapeptide and UDP-MurNAc-tetrapeptide incorporation at 1.83 μM and 13.32 μM respectively. For compounds IV and VIII the ratios of IC_{50} (tetrapeptide/pentapeptide) were 9.5 and 15 respectively. These data show that the inhibition of transglycosylation step of peptidoglycan biosynthesis represents an additional or alternative mechanism of action of the hydrophobic eremomycin derivatives, as it was shown for the hydrophobic derivative of vancomycin.[8,9]

Material and Methods/Experimental

Eremomycin sulfate was produced at the 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 synthesized at Advance Medicine East, Inc. *N*-Boc-eremomycin (II)[11] and *N'*-*p*-(*p*-chlorophenyl)benzyl-vancomycin[8] were prepared as described earlier. The progress of the reactions, column eluates and all final samples were analyzed by TLC using Merck Silica Gel $60F_{254}$ plates in systems containing EtOAc - PrOH - 25% - NH_4OH, 3 : 2 : 2 , or 1 : 1 : 1. In addition, purity of the final compounds IV~X was demonstrated by HPLC using the conditions described earlier.[4] Reaction products were purified by reversed-phase column chromatography on Merck Silanized Silica Gel (0.063~0.2 mm). Mass spectra were determined by Electrospray Ionization (ESI) on a Finnigan SSQ7000 single quadrupole mass spectrometer.

Experimental Synthetic Procedures

N-Fmoc-eremomycin (III)

To a stirring solution of eremomycin sulfate (330 mg, 0.2 mmol) in 15 ml of H_2O - THF, 1 : 1 (v/v) adjusted to pH 7 with 0.1 N NaOH, the solution of FmocOSu (750 mg, 0.3 mmol) in 5 ml of THF was added dropwise over 2 hours, while pH 7 was maintained by adding 0.1 N NaOH. After stirring for 6 hours, 50 ml of H_2O was added to the milky reaction mixture. The precipitated solid was filtrated and washed with cool H_2O. The filtrate was evaporated *in vacuo* with *n*-BuOH to dryness and treated with FmocOSu (660 mg, 0.2 mmol) and then H_2O as described above. The two portions of III were combined, dissolved in 10 ml DMSO, followed by addition of 100 ml acetone. The precipitated solid was collected, washed with acetone and dried *in vacuo* to yield 280 mg (75% yield) of III. After purification of water filtrates on a Dowex 50×16 column (H^+-form), 50 mg (15%) of unreacted I was obtained.

Reductive Alkylation

Method A. A solution of II or III (0.2 mmol) and an appropriate aldehyde (0.4 mmol) in 15 ml of DMSO - DMF - MeOH, 1 : 1 : 1 (v/v/v) was stirred for 2 hours at room temperature. To the resulting solution $NaBH_3CN$ (0.6 mmol) was added in aliquots over 4 hours. The reaction mixture was stirred for 2 hours and then 100 ml of acetone was added. The precipitated solid was filtered, washed with acetone and dried *in vacuo*. When II was used as a starting compound the derivatives were treated with a solution of 30% TFA in CH_2CI_2 at 0°C for 1 hour. The organic layer was separated following addition of 100 ml of cool H_2O. The water solution was washed with EtOAc, adjusted to pH 4 with 1 N NaOH, evaporated to minimal volume and precipitated with 100 ml of acetone. In the case of compound III, its derivatives were treated with 10 ml of 10% $(CH_3)_2NH$ in DMF for 30 minutes at room temperature. After acidification to pH 4 with glacial AcOH, 100 ml of acetone was added. The precipitated solid was filtered, dissolved in minimal amount of H_2O (~4 ml) and applied to a silanized silica gel column (100×3 cm), pre-equilibrated with 0.1 M AcOH. Column chromatography was performed using 0.1 M AcOH. The fractions with pure final compound were collected, evaporated to the minimal volume and 100 ml of acetone added. The precipitate was filtered, washed by acetone and dried *in vacuo* to give the desired compound as an acetate salt.

Method B. The solution of III (0.2 mmol) and an

aldehyde (1 mmol) in 15 ml of DMSO - DMF - MeOH, 2 : 1 : 1 (v/v/v) was stirred for 2 hours at room temperature. To the resulting solution $NaBH(OAc)_3$ (1 mmol) was added in aliquots over 2 hours. The reaction mixture was stirred for 1 hour and then $NaBH_3CN$ (0.4 mmol) was added in aliquots over 2 hours. After stirring an additional 1 hour, 100 ml of Et_2O - acetone, 1 : 1 (v/v) was added. Following steps were carried out as described above in Method A.

Determination of Antibacterial Activity

Minimum inhibitory concentrations (MICs) were determined by broth microdilution method in Brain Heart Infusion media (BBL, Cockeysville, MD) supplemented with 0.1% casamino acids (BHI/CAA) at 37°C. Results were usually identical, and always within two fold. The strains tested were either clinical isolates from the Advanced Medicine East, Inc. culture collection with the confirmed genotypes for vancomycin-resistant enterococci, or were reference strains obtained from the American Type Culture Collection (Manassas, VA).

Peptidoglycan Polymerization Assay

Peptidoglycan synthesis was conducted in 96-well GFC filter plates (Millipore Corp. #MAFCNOB) using membranes from *E. coli* OV58 (pTA9) and UDP-MurNAc-pentapeptide (UDP-MurNAc-pp) and UDP-*N*-acetyl-[^{14}C]-*D*-glucosamine (UDP-GlcNAc) as substrates, as previously described.[8)]

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