TEICOPLANIN, ANTIBIOTICS FROM *ACTINOPLANES TEICHOMYCETICUS* NOV. SP.

VIII. OPENING OF THE POLYPEPTIDE CHAIN OF TEICOPLANIN AGLYCONE UNDER HYDROLYTIC CONDITIONS†

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Hydrolysis of teicoplanin (a complex of five closely related factors plus one, more polar component) under selected conditions (acids in a biphasic hydroalcoholic medium) gives the single aglycone with good yields. When the reaction is carried out in homogeneous hydroalcoholic phase the removal of the sugars yields two new compounds.

On the basis of fast atom bombardment mass spectra (FAB-MS), acid-base titration, IR, UV and ^{1}H NMR analyses it has been demonstrated that these compounds are two diastereoisomers; they differ from the teicoplanin aglycone in having additional carboxyl and amino groups derived from the hydrolysis of an amide bond.

Although the molecular shape of the new aglycones is greatly modified, they still maintain some antibacterial activity which might be correlated with residual binding ability towards the terminal D-alanyl-D-alanine residue of the cell-wall mucopeptides.

Teicoplanin (I), a new glycopeptide antibiotic produced by *Actinoplanes teichomyceticus* ATCC 31121[1], is formed by a complex of five closely related factors T-A2-1 ~ 5 (formerly called teichomycin A_2) plus one, more polar component T-A3-1[2]. The aglycone moiety carries one D-mannose, one *N*-acetyl-D-glucosamine and one *N*-acyl-D-glucosamine unit whose *N*-acyl chains differentiate the components of the complex[3,4] (Fig. 1). Selected hydrolysis[5] gives the pseudo-aglycones T-A3-1 by removing the *N*-acyl-glucosamines and T-A3-2 by removing D-mannose. By complete hydrolysis the true aglycone (II) is obtained[6] (Scheme 1). All these compounds maintain the antibacterial activity[5]. In teicoplanin chemistry, as for many other glycopeptides, the aglycone is of great importance both for structure and activity studies and as an intermediate for chemical modifications.

The different hydrolytic approaches to the aglycone were investigated previously[6]. In this paper, an unusual opening of the polypeptide chain not yet described for the glycopeptides of the vancomycin-ristocetin family is discussed.

Chemistry

The hydrolytic removal of the sugar moieties from glycopeptides of the vancomycin-ristocetin family has been the subject of many studies (see the papers cited in ref 6). No general procedures can be established since the strength of the semi-acetalic bonds depends upon the type of the hydroxyls on

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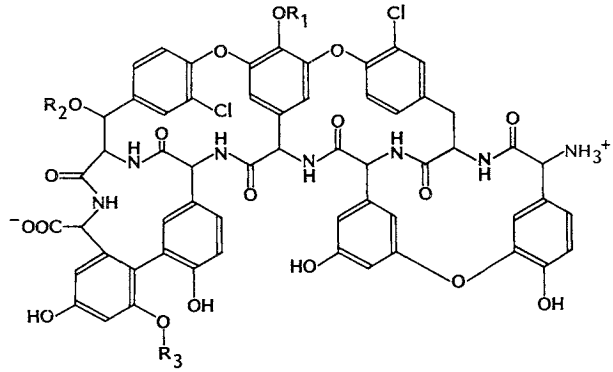
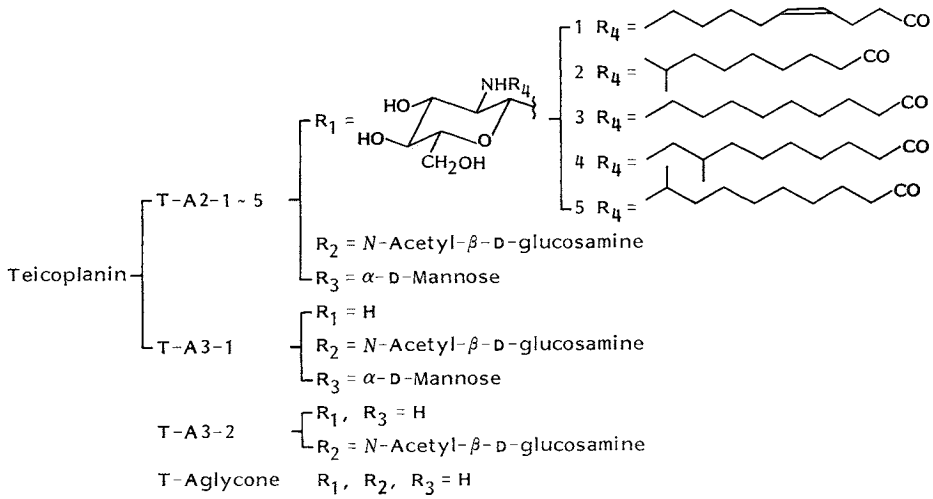
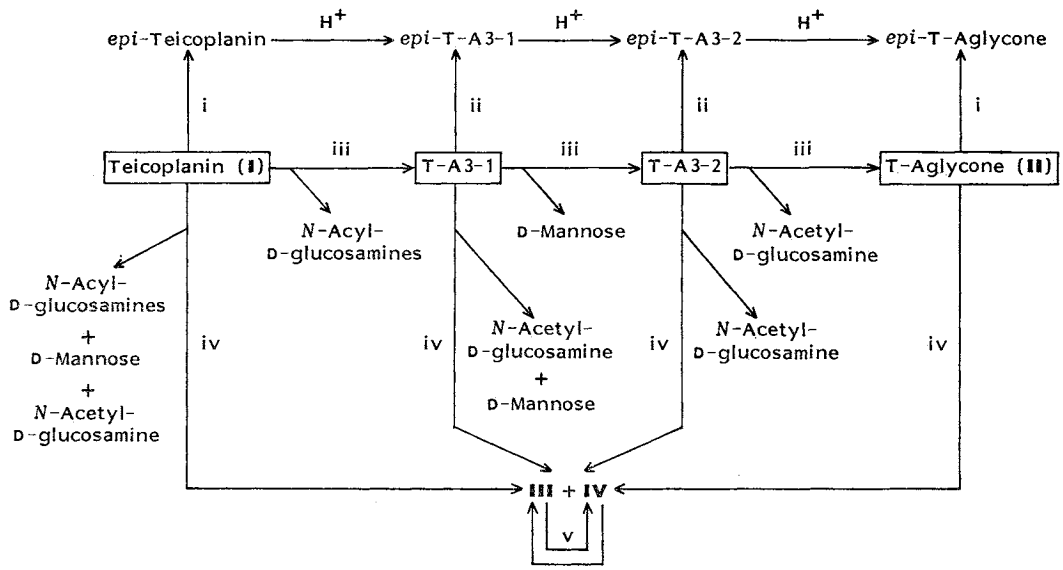
Fig. 1. Structures of the components of teicoplanin, the pseudo-aglycones and the aglycone.





Scheme 1.



i; 20% Methanolic 1-$BuNH_2$, 80°C, ii; 3% aq $NaHCO_3$, 80°C, iii; $C_5H_{11}OH$ - 37% HCl (8: 2), 65°C, iv; 2-PrOH - 37% HCl (8: 2), 80°C, v; saturated aq $NaHCO_3$, room temp.

Fig. 2. HPLC profile of the reaction products.

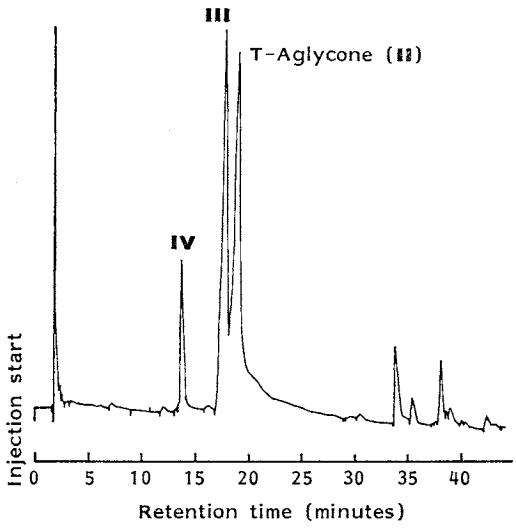


Table 1. Analytical data.

		III·2HCl	IV·2HCl
HPLC retention time[a] (minutes)		18.03	13.92
Weight loss (%)[b]		9.9	10.6
Analysis for $C_{58}H_{47}Cl_2N_7O_{19}\cdot 2HCl$, MW=1,289.90			
Found[c]	C	53.25	53.87
	H	4.64	4.31
	N	7.03	7.43
	Cl	10.03	9.42
	Cl^-	4.10	4.24
Calcd	C	54.00	
	H	3.83	
	N	7.60	
	Cl	10.99	
	Cl^-	5.44	
FAB-MS (MH^+)		1,216	1,216

[a] See the Experimental section. T-aglycone (II) shows retention time 19.35 minutes.
[b] Determined by TGA.
[c] On a sample dried at 140°C in inert atmosphere.

the core-peptide, the type of sugars, the steric hindrance, *etc.*

Teicoplanin (I) or its pseudo-aglycones T-A3-1 and T-A3-2 can be transformed into the aglycone (II) by acid treatment under different conditions, whereas basic treatment gives rise to the corresponding epimers[7] (Scheme 1). In particular, fairly good yields were obtained by using trifluoroacetic acid or other acids in a biphasic hydroalcoholic medium or in anhydrous alcoholic medium (some water is originally present as residual solvent in teicoplanin)[6].

When the reaction is carried out in water or in hydroalcoholic medium in homogeneous phase (or in the presence of co-solvents) with mineral acids at a temperature between 80 and 90°C the formation of a new compound (III) having shorter HPLC retention than II was observed (Fig. 2), together with minor amounts of an additional compound IV. The reaction may be carried out also starting from the pseudo-aglycones T-A3-1 and T-A3-2 with shorter times of heating. Compounds III and IV are formed also when II is treated under the same reaction conditions and the relative amount of IV is clearly dependent on the reaction time. Compound IV is formed also during the purification of crude III by reverse-phase chromatography on silanized silica gel. Compounds III and IV were isolated by chromatography. It was verified that III is relatively unstable in aqueous solution and transforms into IV. Also by heating III in inert atmosphere at 140°C until the residual water is removed (checked by thermal gravimetric analysis, TGA) a partial transformation into IV (up to 16%) occurs. An equilibrium is reached when either III or IV is treated with a $NaHCO_3$ aqueous solution for 5 minutes at room temperature.

By examining the elemental analysis data (Table 1) it results that both compounds III and IV (containing approximately 10% of residual water) are dihydrochloride salts, although the salification does not appear complete. Ionization data (Table 2) show the presence of two carboxyls and two amino groups. The empirical formulas obtained from the combustion data are consistent with the MW's obtained from high resolution fast atom bombardment mass spectra (FAB-MS). In fact, both compounds III and IV show the same MH^+ ion at *m/z* 1,216, that corresponds to a MW of 1,215, whereas aglycone II shows MH^+ *m/z* 1,198 corresponding to a MW of 1,197 ($C_{58}H_{45}Cl_2N_7O_{18}$).

Table 2. Ionization data[a].

pK_{MCS}		Attribution
T-aglycone·HCl	III·2HCl	
—	2.40	COOH of an α-amino acid ($^-$OOC-C(7)H-NH_3^+)
4.65	3.85	COOH ending group of a peptidic chain ($^-$OOC-C(7)H-NH) and ($^-$OOC-C(6)H-NH)
6.90	6.90	NH_2 ending group of a peptidic chain (C(1)H-NH_3^+)
—	~10[b]	NH_2 of an α-amino acid ($^-$OOC-C(7)H-NH_3^+)

[a] See Introduction to Experimental.
[b] The pK_{MCS} value can not be clearly detected because of the interference of the phenolic hydroxyls.

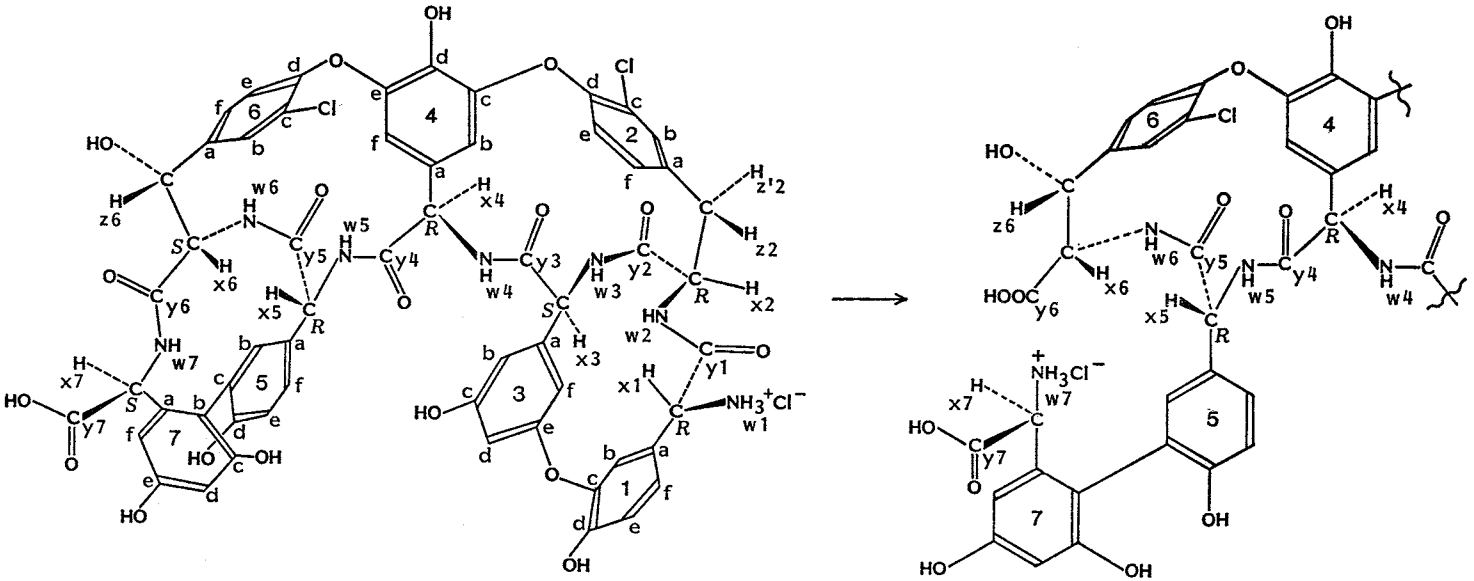
The clusters of the FAB-MS spectra of **III** and **IV** (which are identical) by comparison with those calculated from isotope distribution confirm the presence of two chlorine atoms and are in agreement with the molecular formula $C_{58}H_{47}Cl_2N_7O_{19}$. The UV spectrum (identical for **III** and **IV**) does not differ from that of **II**[5], showing an absorption maximum at 280 nm (ε 11,000) in neutral and acidic media with a shift to 300 nm (ε 20,000) in basic media. These data lead to the hypothesis that compounds **III** and **IV** are isomers.

In a previous paper[6], the complete assignment of ^{1}H NMR spectrum of T-aglycone (**II**) hydrochloride, whose structure is depicted in Fig. 3 (the numbering system is that proposed by D. H. WILLIAMS group[4]), was reported. The structure of compound **III** (Fig. 3) was assigned on the basis of ^{1}H NMR studies on its dihydrochloride, mainly homonuclear correlation spectroscopy (^{1}H,^{1}H-COSY), using the same approach and in comparison with T-aglycone·HCl, taking into account that molecular weight and ionization data suggest that **III** is derived from **II** through a hydrolytic cleavage of one peptidic bond. All the ^{1}H NMR assignments are reported in Table 3.

In practice, the resonances were sorted into sub-structure groupings by ^{1}H,^{1}H-COSY experiments. Only five out of the six amidic NH's signals present in **II** are found in **III** through their coupling with the adjacent CH's. One of these (δ 8.73 ppm) is coupled to a CH (δ 4.83 ppm) which is also coupled to a CH_2 (δ 2.96 and 3.35 ppm). These data account for the fragment NHCH(CH_2)CO of the phenylalanine derived moiety 2, present also in compound **II**, in an environment which appears well comparable. Another fragment NHCH(CHOH)CO is relevant to an NH (δ 7.35 ppm) which is coupled to a CH (δ 4.66 ppm) which in turn is coupled to another CH group (δ 5.37 ppm). This fragment corresponds to the phenylalanine derived moiety 6, and shows consistent differences in chemical shifts in respect to compound **II**. In fact, in **II** this grouping has the amide group in the unusual *cis* conformation, as deduced from the high field value of the NH chemical shift (δ 6.62 ppm), while the chemical shift (δ 7.35 ppm) of this NH in compound **III** suggests the normal *trans* conformation of the 5–6 amide group.

One broad singlet is present in the CH's peptidic region at δ 5.48 ppm and is easily attributed to x1 because it has the same chemical shift and coupling to NH_3^+ as the corresponding proton in compound **II**. Of the remaining amidic groups, that 7.79 ppm is attributed to amino acid 4 on the basis of its coupling with x4 and of the long range coupling of this latter to 4b and 4f. It is worthwhile to recall that these resonances are unambiguously attributed due to their chemical shift at a particularly high field for an aromatic proton, caused by the shielding effect of rings 2 and 6, respectively. Differently from **II**, there is another singlet at 4.46 ppm, which is attributed to x7 and is of particular im-

Fig. 3. Structure of teicoplanin aglycone (II) hydrochloride and of compound III; configuration (*R* or *S*) of the asymmetric centers and nomenclature[4].



T-Aglycone (II)·HCl

III · 2HCl

Table 3. Assignments of the ^1H NMR signals of compound **III** dihydrochloride in comparison with those of T-aglycone hydrochloride.

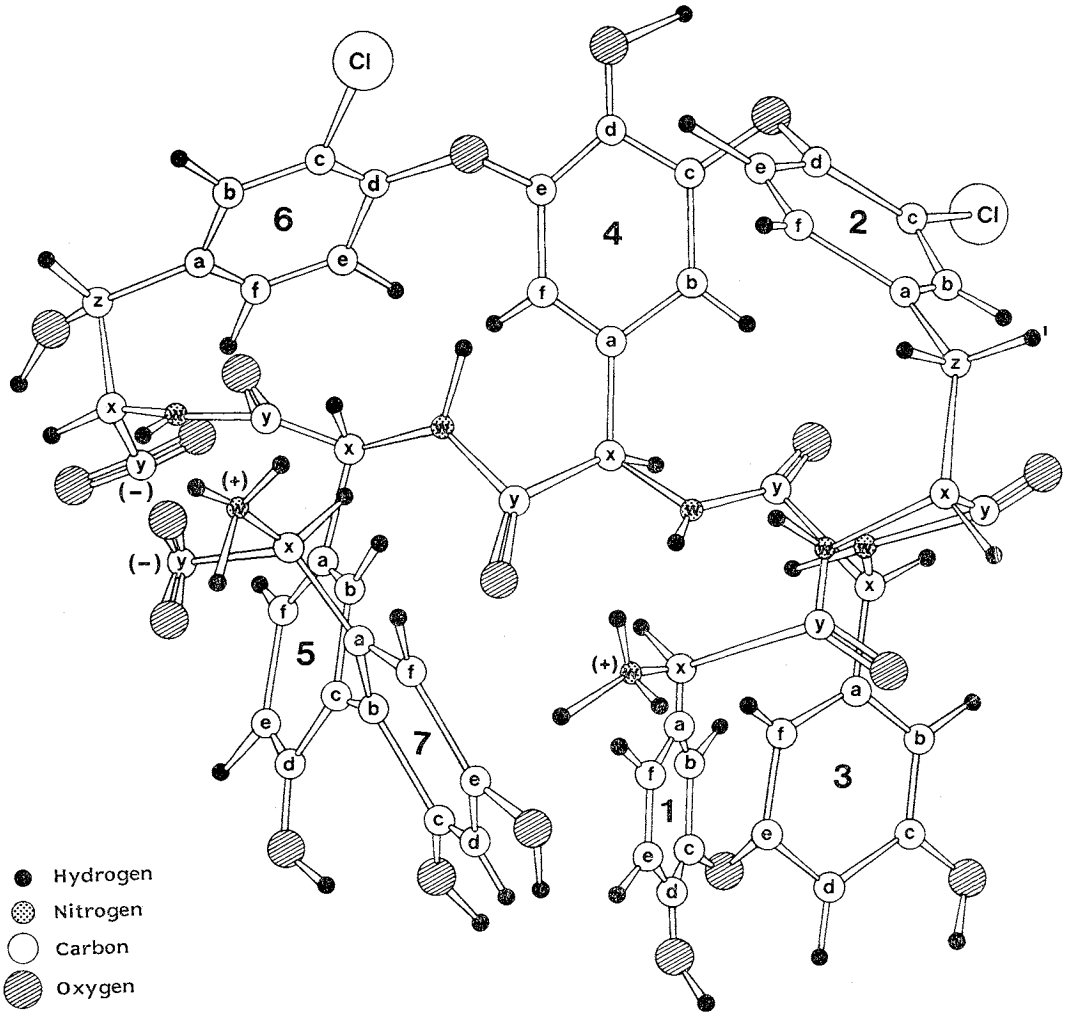
Proton	T-aglycone·HCl		III·2HCl		
	δ (ppm)	^3J (Hz)	δ (ppm)	^3J (Hz)	$\Delta\delta$
x1	5.47	nd	5.48	nd	+0.01
x2	4.92	nd	4.83	7.5 and 3.5	−0.09
x3	5.35	10	5.39	10	+0.04
x4	5.60	8	5.26	8.5	−0.34
x5	4.33	5	5.27	9.5	+0.94
x6	4.10	12 and 2	4.66	8 and 3.5	+0.56
x7	4.42	6	4.46	—	+0.04
z2	2.87	14 and 3	2.96	10 and 2	+0.09
z'2	3.35	14 and 5	3.35	nd	0
z6	5.10	nd	5.37	nd	+0.27
w1	8.53	nd	8.64	nd	+0.11
w2	8.10	8	8.73	7.5	+0.63
w3	7.66	10	8.08	10	+0.42
w4	7.53	8	7.79	8.5	+0.26
w5	8.38	5	8.82	9.5	+0.44
w6	6.62	12	7.35	nd	+0.73
w7	8.40	6	8.64	nd	+0.24
1b	6.77	2	7.08	2	+0.31
1e	7.02	8	7.04	8.5	+0.02
1f	7.20	8 and 2	7.24	8.5 and 2	+0.04
2b	7.20	2	7.20	2	0
2e	7.16	8	7.29	8.5	+0.13
2f	7.86	8 and 2	7.92	8.5 and 2	+0.06
3b	6.32	2	6.38	2	+0.06
3d	6.34	2	6.48	2	+0.14
3f	6.39	2	6.65	nd	+0.26
4b	5.50	2	5.47	2	−0.03
4f	5.08	2	5.75	2	+0.67
5b	7.07	2	7.08	2	+0.01
5e	6.65	8	6.78	8.5	+0.13
5f	6.68	8 and 2	6.74	8.5 and 2	+0.06
6b	7.77	2	7.53	2	−0.24
6e	7.19	8	7.31	8.5	+0.12
6f	7.43	8 and 2	7.58	8.5 and 2	+0.15
7d	6.39	2	6.39	2	0
7f	6.24	2	6.44	2	+0.20

nd: Not determined.

portance because it localizes the hydrolytic cleavage in the peptidic chain between amino acids 6 and 7. The attribution lies on the fact that this signal shows no coupling with an amidic NH and that the ionization pattern indicates the presence of an amino acidic moiety. The last two CH's in the peptidic region give rise to two signals at δ 5.27 and 5.39 ppm and are attributed by exclusion to x5 and x3, respectively, in agreement with the remarkable shift for x5 passing from **II** to **III**, as x5 belongs to the left part of the molecule which is strongly modified. Consequently, the signals at δ 8.82 and 8.08 ppm are assigned to w5 and w3, respectively.

Analogously, the signals of all the aromatic protons were assigned and they are reported in Table 3. It may be concluded that compound **III** differs from T-aglycone for the presence of an additional carboxyl on C-6 and an additional amino group on C-7, originated by the opening of the amidic bond

Fig. 4. Stereo-structure of compound III obtained from a Dreiding model.
The interactions through space found by the NOE experiment (Table 4) can be seen.



between these two carbons. The structure assigned to compound **III** is reported in Fig. 3. It is important to point out that the configurations of the asymmetric centers involved in the hydrolytic cleavage (*i.e.*, amino acids 6 and 7) have been assumed the same as those of the original aglycone **II**, but no direct confirmation has been tried.

Information on the dipolar interactions through space between relevant protons of **III** was obtained by 2D nuclear Overhauser effect spectroscopy phase sensitive (NOESYPH) experiments[8]. The results are reported in Table 4.

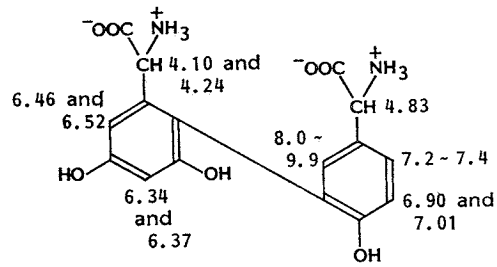
This information confirms the structure attributed and gives a picture of the shape of **III**, which appears substantially modified in the left part. Fig. 4 shows a stereochemical Dreiding model, where the conformation of the molecule can be visualized. Furthermore, the interactions through space reported in Table 4 can be seen.

It is noteworthy to comment the chemical shift variations of **III** in respect to **II**. Even if the right part of the molecule is the same for the two compounds for **III** a change in conformation has to be

Table 4. Selected qualitative difference NOE observations for III·2HCl protons (labeling system Fig. 3).

Proton irradiated	Resonance involved
x2	z2, z'2
x3	3b
x5	5b, w5
x6	z6, 6b
x7	5b, x5
w2	x1
w3	3f
w4	4b
1b	3f
1e	1f
2b	z'2
2e	2f
5e	5f
6b	z6
6e	6f
7d	1e, 1f

Fig. 5. Compound V and ^{1}H NMR assignments.



V

deduced because this is reflected by the variation for 1b and 3f and by the strong NOE observed for them. Furthermore, w2, w3 and w4 show decreasing variations of δ, which can be interpreted as a modified availability of these groups to the solvent as a consequence of the changed conformation. The left part of the molecule has a different structure for **II** and **III**, and the differences in the NMR signals are in complete agreement. Finally, the NOE observed between 7d and 1e, 1f indicates that rings 1 and 7 approach one over the other.

The ^{1}H NMR experiments performed on compound **IV** led to the conclusion that this latter is a diastereoisomer of **III** because of the presence of a different enantiomeric form at x7. In fact, the NMR spectrum is very similar for **III** and **IV** ($\Delta\delta$ 0.1 ppm) and the only difference is the δ value of x7, which is 4.46 ppm in **III** and 4.29 ppm in **IV**. A diastereoisomeric relationship of this type was

Table 5. *In vitro* antibacterial activity[a].

Organism	MIC (μg/ml)			
	Teicoplanin (I)	II	III	IV
Staphylococcus aureus ATCC 6538	0.125	0.063	0.5	4.0
S. aureus TOUR	0.125	0.063	0.25	2.0
S. aureus TOUR[b]	0.5	0.125	1.0	8.0
S. aureus TOUR[c]	0.5	0.25	4.0	16.0
S. epidermidis ATCC 12228	0.25	0.016	0.12	0.25
Streptococcus pyogenes C203	0.063	0.125	2.0	8.0
S. pneumoniae UC41	0.063	0.125	1.0	8.0
S. faecalis ATCC 7080	0.125	0.125	1.0	16.0
S. mitis L 796	0.125	0.125	1.0	2.0
Escherichia coli SKF 12140	>128	64	>128	>128
Proteus vulgaris X 19 H ATCC 881	>128	128	>128	>128
Pseudomonas aeruginosa ATCC 10145	>128	>128	>128	>128

[a] Minimum inhibitory concentration (MIC) was determined using the 2-fold dilution method in microtiter system. The media used were: Iso-Sensitest broth (Oxoid) for Staphylococci, *S. faecalis* and Gram-negative bacteria; Tood-Hewitt broth (Difco) for Streptococci. The final inoculum was about 10^4 cfu/ml. MIC was read as the lowest concentration which showed no visible growth after 18~24 hours incubation at 37°C.

[b] Inoculum 10^6 cfu/ml.

[c] Determined in Iso-Sensitest broth supplemented with 30% bovine serum.

not unexpected[9,10]. In fact, treatment of teicoplanin (I) with 6 N HCl at reflux for 8 hours gave compound **V**, which originated from the diphenyl moiety, as a mixture of two diastereoisomers, as revealed by the doubling in the ^{1}H NMR spectrum (in DMSO-d_6) of one of the two methine groups and of all the aromatic signals[11] (see Fig. 5).

Antibacterial Activity

In Table 5 the MICs of compounds **III** and **IV** are compared with those of teicoplanin (**I**) and the T-aglycone (**II**). Compound **III** still retains activity against the Gram-positive bacteria tested, although to a lesser extent in respect to **II** and to **I**. A greater decrease is shown by compound **IV**.

Discussion

The common feature of the glycopeptides of the vancomycin-ristocetin group is the presence of a linear heptapeptide chain made by aromatic amino acids whose phenyl rings are linked together by ether or carbon-carbon bonds forming three or four condensed dipeptide rings which can be visualized as part of a larger cycle. Although many glycopeptides have been submitted to controlled hydrolysis for the selective removal of sugars, the cleavage of the heptapeptide skeleton with the formation of an additional terminal amino acid is new. Teicoplanin, as the other glycopeptide antibiotics, inhibits the biosynthesis of the bacterial cell-wall by interfering with the peptides terminating in the sequence D-alanyl-D-alanine at the free carboxyl end[12,13]. Studies with the model peptide *N*-Ac-D-Ala-D-Ala showed that hydrogen bonds between the NH's groups and one carbonyl of the glycopeptide and the carboxyl group, one carbonyl, and one NH group of the *N*-Ac-D-Ala-D-Ala together with hydrophobic interactions are responsible for the binding and that the initial interaction between the peptide model carboxylate and the terminal protonated amine in the antibiotic is electrostatic[14]. Recently, it was shown that the complete removal of sugars from teicoplanin causes a variation of the free energies of binding with the model *N*-Ac-D-Ala-D-Ala peptide (ΔG) from 35.0 to 32.3 kJ/mol (measured by UV difference spectroscopy)[15].

A different way to compare the affinity binding ability of glycopeptides to a D-Ala-D-Ala terminal is the solid-phase enzyme-receptor assay (SPERA)[16], which is based on the competition of the antibiotics and horseradish peroxidase-labeled teicoplanin for a synthetic analog of the biological receptor, *e.g.*, albumine-ε-aminocaproyl-D-alanyl-D-alanine. In this test T-aglycone (**II**) shows 50% of the affinity binding ability of teicoplanin, and compounds **III** and **IV**, 2.1% and 3.4%, respectively. These data show that the structural modifications in the left side of the molecule, *i.e.* the opening of the ring with the introduction of an amino acid center and the reversal of the amide bond, strongly affect the binding ability. The residual binding activity of compounds **III** and **IV** may be attributed to the unmodified right part of the peptide chain that in all the members of the class forms a "binding pocket" for the D-Ala-D-Ala terminal carboxylate ion. Taking into account that also the epimerization at C-3 of T-aglycone gives a dramatic decrease of the activity[7], we can confirm that the geometry of the glycopeptides, together with the protonation state[14,15], is determinant for the binding activity, whereas fatty acid residues and sugars have a minor influence.

The correlation between the binding capabilities observed with models and the activity in the bacterial cells is only qualitative and this may be attributed to transport and/or cell surface recognition[15] as is often the case with antibiotics rather than to an other mode of action as suggested for vancomycin[17].

Experimental

Evaporation was carried out, after addition of BuOH to prevent foaming, with a rotary evaporator at room temp under vacuum.

HPLC were run with a chromatograph Hewlett-Packard 1084A equipped with a UV detector at 254 nm and a column Brownlee RP-18 (250 mm, 5 μm). Injection volume; 30 μl. Flow rate; 1.5

ml/minute. Mobile phases: (A) CH_3CN - 0.02 M aq NaH_2PO_4 (5 : 95); (B) CH_3CN - 0.02 M aq NaH_2PO_4 (75: 25); linear step-gradient as follows:

Minutes:	0	10	20	30	35	40
% B:	8	15	25	40	40	40

The *pK* values were determined potentiometrically in Methyl Cellosolve (MCS) - water (4 : 1) solution by titration with 0.1 N KOH.

The ^1H NMR spectra and the various experiments were obtained with Bruker instruments equipped with Aspect 3000 console at 500 and at 250 MHz. The spectra were recorded at 40°C in DMSO-d_6 solution (internal standard TMS, δ 0.00 ppm). The samples were previously lyophilized twice from DMSO-d_6 solutions.

Preparation of Compounds III and IV

Two mmol of teicoplanin (I)*, containing 15% by weight of crystallization water, was added to 150 ml of a mixture of 2-propanol and 37% HCl, 8: 2 preheated at 80°C. The powder dissolved. Heating was continued for 75 minutes, then the reaction mixture was evaporated to dryness under vacuum.

The residue was dissolved with 30 ml of H_2O and the solution was applied to a column (280 × 3.6 mm) prepared with LiChroprep RP-8 40~63 μm (Merck) (Column A) slurried in H_2O. The column was eluted with H_2O until the eluate had pH 7, then with a mixture of 0.02 M aq NaH_2PO_4 - CH_3CN (87: 13) under a slight pressure. Fractions of 20 ml were collected at a rate of 16 ml/minute. Fractions 100~180 contained **IV**.

Fractions 181~250 containing essentially **III** were combined, the pH was brought to 2 with 1 N HCl and the solution was evaporated to dryness.

The residue was dissolved with 50 ml of H_2O and the solution was re-chromatographed (Column A) eluting with a mixture of 0.02 M NaH_2PO_4 - CH_3CN (85: 15). Fractions of 25 ml were collected. Fractions 30~75 contained a mixture of **III** and **IV**. Fractions 76~120, containing **III**, were combined, acidified to pH 1 with 1 N HCl and evaporated to dryness. The residue was dissolved in H_2O and the solution was passed through a column containing 80 g of LiChroprep RP-8, followed by H_2O until the eluate was free of Cl^-. The product was then eluted with a mixture of CH_3CN - H_2O (3: 7) collecting 200 ml of a solution which was acidified to pH 2 (1 N HCl). BuOH was added and the solution was concentrated under vacuum to give a butanolic solution (about 20 ml). By adding 200 ml of EtO_2 a precipitate formed which was collected, washed with EtO_2 and dried at 40°C under vacuum (yield 0.8 g of pure compound **III**).

Fractions 100~180 (from 1st column) and 30~75 (from 2nd column) were combined, adjusted to pH 2 (1 N HCl) and evaporated to dryness. The residue was dissolved with 50 ml of H_2O and the solution was applied to column A. Water was passed through until a neutral eluate was collected, then the column was developed with a mixture of 0.02 M aq NaH_2PO_4 - CH_3CN (85 : 15), collecting 25 ml fractions at a rate of 16 ml/minute. Fractions 35~60 contained pure **IV** and were combined and worked up as described above obtaining 0.2 g of the product.

Isomerization of III and IV

To a solution of 1 mg of **III** in a mixture of 0.5 ml of CH_3CN and 0.5 ml of 0.02 M aq NaH_2PO_4 0.2 ml of a saturated aq solution of $NaHCO_3$ was added. The reaction mixture was stirred for 5 minutes at room temp.

By HPLC the presence of 54% of **IV** was detected. When a solution of pure compound **IV** was treated as described above, 42% and 44% of **III** was revealed by HPLC after 5 and 15 minutes, respectively.

Evaluation of the Affinity Binding Ability by the SPERA Method

The preparation of the reagents and the assay procedures have been previously described[16]. A solution of teicoplanin aglycone (**II**, 6.2 mg) in 2.5 ml of DMF was diluted to a concentration of 4

* The reaction may be done also starting from T-A3-1, T-A3-2 or T-aglycone (**II**). In these cases heating was continued for 70, 60 and 40 minutes, respectively.

μg/ml with the analysis buffer; compounds **III** (3.5 mg) and **IV** (2.8 mg) were separately dissolved each in 0.25 ml of DMF and diluted to 40 μg/ml as above. These solutions were tested according to the SPERA method by using teicoplanin (**I**) as reference. The concentrations of the antibiotics obtained, expressed as teicoplanin equivalents, were 2.02, 0.85 and 1.36 for T-aglycone, compound **III** and compound **IV**, respectively. The ratios between the amount of the antibiotic "measured" and the amount "added" (%), taken as a measure of the relative affinity of each compound for the immobilized D-Ala-D-Ala, resulted 50 for T-aglycone, 2.1 for compound **III** and 3.4 for compound **IV**.

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