

## Isolation and Structure Determination of a Novel Complex of the Teicoplanin Family

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A new teicoplanin-like antibiotic was discovered when using *Actinoplanes teichomyceticus* strain 3/W, the fermentation medium containing Alburex, and the fermentation time 275 hours. The new product was separated from teicoplanin complex by polyamide resin chromatography and purified by Amberlite XAD-7 and affinity resin chromatographies. The structure was established on the basis of the physico-chemical characteristics of the complex and of its aglycone. The new structure is that of teicoplanin with a carbonyl group substituting for the CHNH<sub>2</sub> group of amino acid 1.

We hypothesize that the novel complex is a transformation product of teicoplanin due to a simple transamination reaction, as supported by its structure and by the concomitant decrease in teicoplanin concentration during its production.

During studies aimed at improving the production of teicoplanin by modification of the fermentation medium and of the culture conditions, the presence of a novel complex of antibiotics was noticed in the broth. The formation of this complex, named teico 2, showed a retention time higher than that of teicoplanin using reverse phase gradient HPLC analysis. Its presence depended both on the inclusion of Alburex N in the medium and on the increased time of fermentation. Conditions were found in which the novel complex was the major product, thus allowing its easy isolation with minimum teicoplanin content. The crude material was purified by affinity chromatography and further chromatographic procedures yielding a white amorphous powder, which was sufficiently pure for structural and biological activity studies.

We report here the fermentation conditions, the recovery procedure, and the results of the structure determination by NMR and MS spectroscopies on the intact molecules and on the aglycone obtained by acid hydrolysis. The teico 2 complex consisted of the analogues of teicoplanin's five main components in which the terminal CH-NH<sub>2</sub> is substituted by a C=O group. The structure was confirmed by the synthesis of the teico 2 aglycone starting from teicoplanin aglycone.

### Experimental

#### Strain

*Actinoplanes teichomyceticus* strain 3/W, an improved teicoplanin producing strain, was used.

#### Culture Media

Agar medium SM: (g/liter) dextrose 10, Bacto-peptone 4, Bacto-yeast extract 4, MgSO<sub>4</sub> · 7H<sub>2</sub>O 0.5, KH<sub>2</sub>PO<sub>4</sub> 2, K<sub>2</sub>HPO<sub>4</sub> 4, Bacto-agar 20, deionized water up to.

Vegetative medium E/25: (g/liter) dextrose 25, meat extract 4, yeast autolysate 1, soybean meal 10, peptone 4, NaCl 2.5, CaCO<sub>3</sub> 5, deionized water up to.

Production medium T/2: (g/liter) dextrose 10, malt extract 20, yeast extract 4, Alburex N (Roquette Frères) 10, deionized water up to.

#### Fermentation Conditions

The strain was stored in lyophilized state. The slants were prepared from lyophilized vials on agar medium SM. The mycelium from a slant was homogenized in 10 ml of saline and inoculated (1% v/v) in 500-ml Erlenmeyer flasks each containing 100 ml of medium E/25. The cultures were grown for 60 hours on a rotative shaker at 250 rpm and at 28°C, and used as inoculum (0.14% v/v) of a second pre-culture in a fermentor containing 8 liters of medium E/25. The culture was grown for 40 hours at 28°C with an air flow of 0.7 liter/liter/minute and stirred at 600 rpm. 240 ml of this culture was used as inoculum of 8 liters of medium T/2 and the fermentation was carried on at 28°C, with an air flow of 0.7 liter/liter/minute and under a stirring of 600 rpm.

#### Analytical HPLC

Apparatus: two Waters mod 510 pumps, with the Automated Gradient Controller mod. 680; detector: Waters Lambda-Max, set at 254 nm; column: Beckman Ultrasphere ODS, 4.6 × 250 mm; mobile phase: A: 0.2% HCO<sub>2</sub>NH<sub>4</sub>-CH<sub>3</sub>CN (9:1), B: 0.2% HCO<sub>2</sub>NH<sub>4</sub>-CH<sub>3</sub>CN (3:7); gradient: linear from 8% B to 40% B in

40 minutes; flow rate: 1.5 ml/minute.

#### Column Chromatography

**Polyamide resin:** the filtered broth was adsorbed on a polyamide resin (volume ratio 8:1), which was washed with 0.2 M sodium phosphate pH 7.7 and then with 0.2 M sodium phosphate brought to pH 4.9 with NaOH. Elution of teicoplanin was performed with a 80/20 mixture of 0.2 M sodium phosphate pH 4.9/acetone. The elution of teico 2 was obtained with a 1/1 mixture of 0.2 M sodium phosphate pH 4.9/acetone.

**Amberlite XAD-7:** the acetone in the eluate containing teico 2 was removed under vacuum and the aqueous solution was adsorbed on an Amberlite XAD-7 resin (volume ratio 15:1). The resin was washed with water and eluted with a 1/1 mixture of acetone/water. Precipitation was obtained by adjusting the pH to 7.3 and adding 10 volumes of acetone.

**Affinity resin:** the crude material was dissolved in water and passed through an affinity column of Sepharose-acyl-D-alanyl-D-alanine resin (50 ml/g)<sup>11</sup>. The column was washed with 2 volumes of a 0.2% HCO<sub>2</sub>NH<sub>4</sub> solution and eluted with an aqueous solution of NH<sub>4</sub>OH (1%, w/v). The fractions (10 ml each) were pooled according to their composition.

**Ultrafiltration:** the pool containing teico 2 was neutralized with HCO<sub>2</sub>H and concentrated in a 62 mm Amicon Stirred Cell, supporting a Diaflo ultrafiltration membrane with a nominal molecular limit of 1000 Da. The retentate was then lyophilized.

#### Fast Atom Bombardment Mass Spectrometry

FAB-MS was obtained with a Kratos MS-50 instrument in positive ion mode using  $\alpha$ -thioglycerol containing 0.6% w/v of acetic acid as matrix. For FAB Xe gas was used, the gun was operated at 6 kV potential. Calibration was obtained using a mixture of CsI and NaI<sup>2</sup>.

#### Proton NMR Spectroscopy

Proton NMR spectra were recorded in DMSO-*d*<sub>6</sub> solution at 22°C on a Bruker AM-500 or a Bruker AM-250 spectrometer, both equipped with an Aspect 3000 computer. The phase-sensitive double quantum filter <sup>1</sup>H-<sup>1</sup>H-COSY spectra were run using time-proportional-phase-increment in fl<sup>3</sup>.

#### UV Spectroscopy

The UV spectra were recorded with a Perkin-Elmer spectrophotometer mod. 320 in methanol/water (1/24 v/v) solutions at various pH values.

#### Synthesis of Teico 2 Aglycone

Teicoplanin aglycone (3 g, containing 10% H<sub>2</sub>O) was dissolved in *n*-butanol and the solvent evaporated to dryness under vacuum; this operation was repeated twice to remove the crystallization water. The aglycone was suspended under stirring in DMF (40 ml) and a

solution of mesityl-glyoxal<sup>4</sup>) (480 mg) in DMF (10 ml) was slowly added, keeping the temperature at 5°C. After 10 minutes, the reaction was allowed to reach room temperature and the aglycone went in solution<sup>5</sup>). The mixture was stirred overnight and the reddish solution was evaporated under high vacuum at 50°C. The residue was washed twice with ethyl ether and the crude product was purified by column chromatography (silanized Silicagel Merck, CH<sub>3</sub>CN/H<sub>2</sub>O, 3/7). The fractions containing the product were pooled and evaporated under vacuum. The residue was washed with ethyl ether and dried under vacuum (0.5 g).

#### MIC Determination

Minimal inhibitory concentrations (MIC) were determined by microbroth dilution methodology. Inocula were approximately 5 × 10<sup>5</sup> CFU/ml. All microorganisms were cultured at 37°C. MIC were read at 18 ~ 24 hours, except for *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Bacteroides fragilis* (48 hours). *N. gonorrhoeae* and *H. influenzae* were incubated in a 5% CO<sub>2</sub> atmosphere. *B. fragilis* was incubated in an anaerobic gas mixture. Media used were: Oxoid Iso-sensitest broth for staphylococci, *E. faecalis*, and Gram-negative bacteria; Difco Todd-Hewitt broth for streptococci; Difco GC base broth with 1% BBL Iso-Vitalex for *N. gonorrhoeae*, and Difco Brain-Hearth Infusion broth with 1% Difco supplement C for *H. influenzae*.

## Results

### Fermentation

Fermentation of strain 3/W was performed in a fermentor containing 8 liters of medium T/2 as described in Experimental. When the time course of antibiotic was prolonged to 275 hours, the HPLC chromatogram showed, besides teicoplanin complex, the presence of a new complex at higher retention time (Fig. 1). The time course of antibiotic production (Fig. 2) indicates that the formation of teicoplanin reaches a maximum at 150 hours and then decreases, and the formation of the second complex steadily increases up to 275 hours.

### Recovery and Purification

The fermentation broth was brought to pH 11 with concentrated NaOH, filtered on Clarcel and the filtrate brought to pH 7.8 with 10% HCl, and adsorbed on the polyamide resin. Fractions containing the new complex, obtained by the elution described in Experimental, were fractionated by the Amberlite XAD-7 column chromatography, yielding 1.3 g of crude material. A portion of this material (500 mg) was submitted to the affinity chromatography. The pooled fractions containing the new complex yielded, after ultrafiltration and lyophilization, 85 mg of pure product, named teico 2.

Fig. 1. HPLC of the fermentation broth after 275 hours.

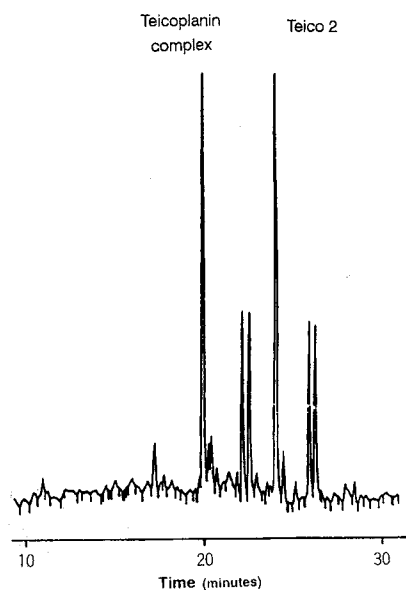
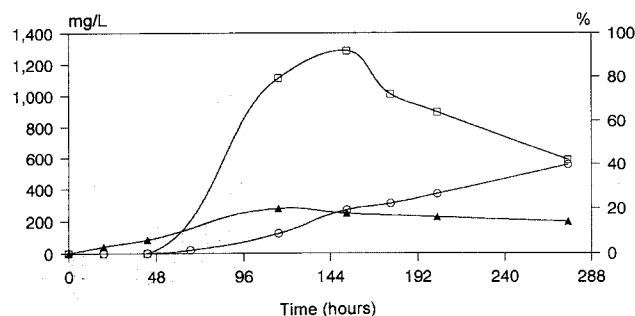


Fig. 2. Time course of the antibiotic production.

▲ pmv, □ teicoplanin complex, ○ teico 2.



### Structure Determination

#### Spectroscopic Properties of Teico 2 Complex

The HPLC chromatogram of teico 2 (Fig. 3) strongly suggests that it is a complex formed by components similar to those of teicoplanin and differing somewhere in the molecule. The structure was then determined on the basis of the acquired knowledge on the relationships between structure and physicochemical characteristics in the field of glycopeptides<sup>5~9)</sup>.

The FAB-MS spectrum of teico 2 (Fig. 4) shows several peaks corresponding to a mixture of compounds, each having a molecular weight one unit lower than the corresponding component of the teicoplanin complex<sup>6)</sup>. These findings strongly suggest that the number of nitrogen atoms in teico 2 must be different from that of teicoplanin, whereas the fatty acid chains appear unchanged. The whole picture of all the relevant ions

Fig. 3. HPLC of teico 2 complex.

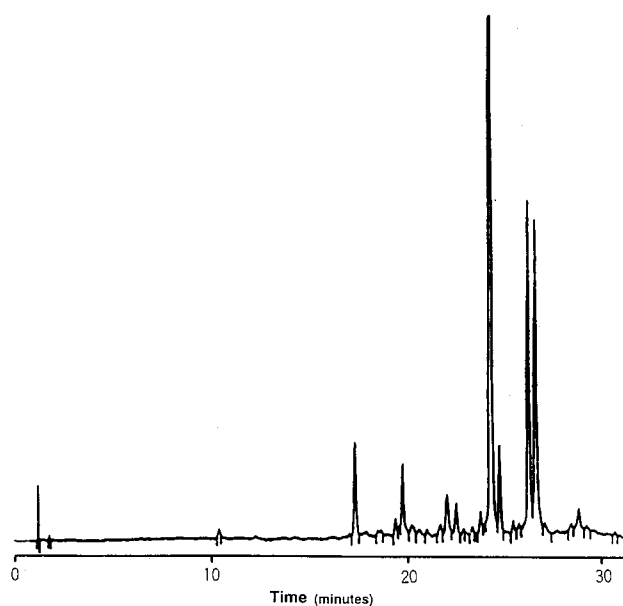
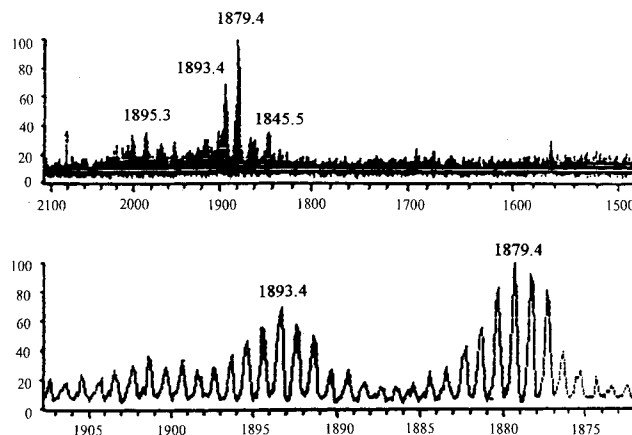


Fig. 4. Positive ion FAB-MS spectrum (top) and expanded quasi-molecular ions region (bottom) of teico 2 complex.



observable is reported in Table 1 together with the assignments.

The general appearance of the proton NMR spectrum of teico 2 corresponds to that of teicoplanin<sup>5~8)</sup> and the minor but significant differences emerged after the complete attribution are shown in Table 2. The variation of the structure of teico 2 resides in the right-hand side of the molecule, namely in the amino acid 1, as indicated by the absence of the signal of x1 and by the differences in the signals of x2, 1b, 1e, and 1f. Thus, the combined results of MS and NMR analyses suggest that the difference in structure between teico 2 and teicoplanin is due to the presence of a keto group instead of the CH-NH<sub>2</sub> group of amino acid 1. The structure of teico

Table 1. Ions observed from teico 2 in positive ion Fast Atom Bombardment Mass Spectra.

$m/z$	Rel. int.	Inference
1999	0.1	(M+H+TG) <sup>+</sup> for components 4 and 5
1985	0.2	(M+H+TG) <sup>+</sup> for components 2 and 3
1891	0.6	(M+H) <sup>+</sup> for components 4 and 5
1877	1.0	(M+H) <sup>+</sup> for components 2 and 3
1875	0.1	(M+H) <sup>+</sup> for components 2 and 3
1857	0.1	(M+H) <sup>+</sup> for component 1 and dehydrogenation products from 2 and 3
1857	0.1	(M+2H-Cl) <sup>+</sup> for components 4 and 5
1843	0.2	(M+2H-Cl) <sup>+</sup> for components 2 and 3

$m/z$  refers to the lowest isotope peak of the cluster.

TG =  $\alpha$ -thioglycerol.

Table 2. Relevant proton NMR data for teico 2 and teicoplanin.

Proton	Teico	Teico 2	Diff.
x1	4.7	—	—
x2	4.8	5.0	+0.2
x3	5.3	5.3	0
x4	5.7	5.8	+0.1
x5	4.4	4.3	-0.1
x6	4.1	4.1	0
x7	4.4	4.5	+0.1
1b	6.6	7.0	+0.4
1e	6.9	6.5	-0.4
1f	7.1	7.5	+0.4
4b	5.6	5.5	-0.1
4f	5.1	5.2	+0.1
z6	5.3	5.3	0
6b	7.8	7.8	0
5f	6.7	6.7	0
5e	6.7	6.7	0

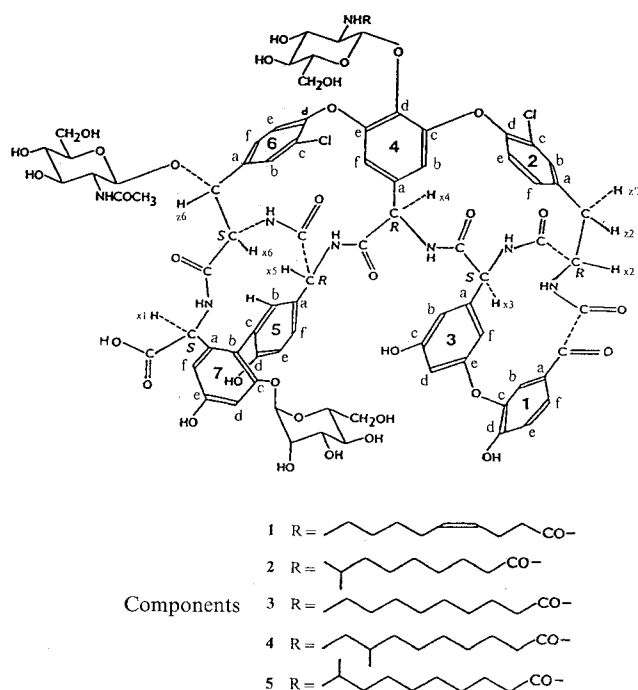
$\delta$  chemical shift, ppm ( $\delta$  difference) (see Fig. 5 for structure and nomenclature).

2 complex is shown in Figure 5.

### Preparation of Teico 2 Aglycone

Confirmation of the structure was obtained by acid hydrolysis of teico 2 to the single teico 2 aglycone and its spectrometric analysis. Teico 2 (50 mg) was suspended in 1.5 ml of CF<sub>3</sub>CH<sub>2</sub>OH saturated with HCl and kept in a sealed tube at 80°C overnight. The reaction mixture was evaporated to dryness under vacuum, dissolved in 6 ml of a mixture of H<sub>2</sub>O-CH<sub>3</sub>CN, 3:1 and submitted to affinity chromatography<sup>1)</sup>. The fractions containing the aglycone and the pseudoaglycone (HPLC analysis) were pooled and acidified with HCO<sub>2</sub>H. The precipitate collected by centrifugation was dried under vacuum (25 mg). Successive separation and purification were obtained by preparative TLC on silanized Silicagel plates Merck RP-8 and 5% Na<sub>2</sub>SO<sub>4</sub>-CH<sub>3</sub>CN, 7:3 as mobile phase. The relevant bands were scraped and extracted with CH<sub>3</sub>CN-H<sub>2</sub>O, 1:1. After evaporation under vacuum 14 mg of aglycone and 6 mg of pseudo-

Fig. 5. Structure of teico 2 complex.



Configuration (*R* or *S*) of the asymmetric centers and nomenclature proposed by D. H. WILLIAMS for teicoplanin<sup>6)</sup>.

aglycone were obtained.

### Spectroscopic Properties of Teico 2 Aglycone

The various peaks present in the FAB spectrum of teico 2 aglycone (Fig. 6) correspond to the protonated molecule ( $m/z$  1197) and to the adduct ions with sodium ( $m/z$  1219), potassium ( $m/z$  1235), and cesium ( $m/z$  1329) of the aglycone of teico 2.

The proton NMR spectrum of teico 2 aglycone (Fig. 7) allowed the complete attribution of all the signals (displayed in Table 3 in comparison with the signals of teicoplanin aglycone) and confirmed the structure.

The UV spectra of teico 2 aglycone at various pH values confirm the structure attribution (Table 4). In fact, in addition to the absorption typical of teicoplanin

Fig. 6. Positive ion FAB-MS spectrum (top) and expanded quasi-molecular ions region (bottom) of teico 2 aglycone.

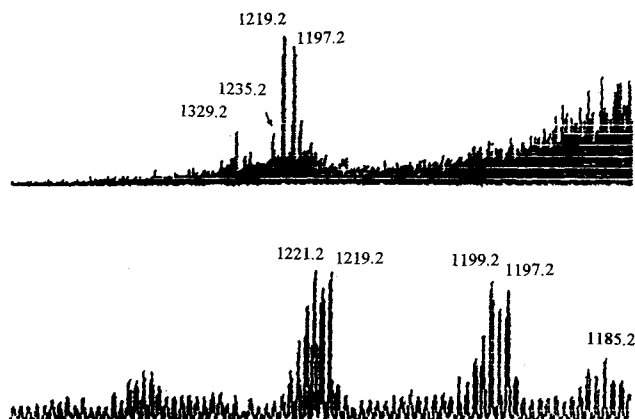


Fig. 7. Proton NMR spectrum of teico 2 aglycone at 250 MHz.

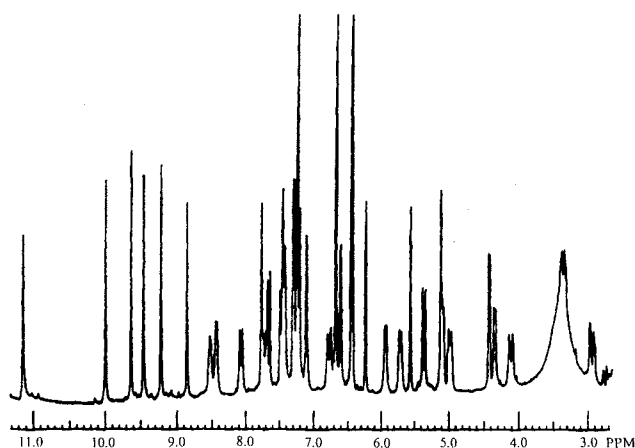


Table 3. Relevant proton NMR data for teico 2 aglycone in comparison with teicoplanin aglycone<sup>7)</sup>.

Proton	Teico aglyc	Teico 2 aglyc
x1	5.47	—
x2	4.92	5.00
x3	5.35	5.37
x4	5.60	5.66
x5	4.33	4.35
x6	4.10	4.11
x7	4.42	4.45
w1	8.53	—
w2	8.10	8.06
w3	7.66	7.70
w4	7.53	7.40
w5	8.38	8.51
w6	6.62	6.76
w7	8.40	8.42
OH-z6	5.85	5.86
OH-1	9.88	9.87
OH-3	9.63	11.00
OH-4	9.45	9.47
OH-5	9.08	9.06
OH-7c	8.78	8.71
OH-7e	9.34	9.34
z2	2.8	2.93
z'2	3.35	3.37
z6	5.10	5.10
1b	6.77	7.20
1e	7.02	7.20
1f	7.20	7.20
2b	7.20	7.20
2e	7.16	7.26
2f	7.86	7.64
4b	5.50	5.59
4f	5.08	5.14
5b	7.07	7.11
5e	6.65	6.68
5f	6.68	6.68
6b	7.77	7.77
6e	7.19	7.20
6f	7.43	7.45
7d	6.39	6.44
7f	6.24	6.22

$\delta$  chemical shift, ppm (see Fig. 5 for structure and nomenclature).

Table 4. UV data for teico 2 aglycone in aqueous solutions at various pH values.

pH of the solution	$\lambda_{\max}$ , nm	$\epsilon_{\text{mol}}$	$\lambda_{\text{sh}}$ , nm	$\epsilon_{\text{mol}}$
0.1 N HCl and 4.7	285	15,360	320 sh	5,940
6.1	280	14,700	365	6,860
7.4	276	15,960	368	16,660
8.5	276	17,914	368	17,300
0.1 N KOH	298	21,700	356	16,240

sh = shoulder

(maximum at about 280 nm in acidic solution, shifting to about 300 nm in basic solution) they show in acidic solution a low intensity shoulder at 320 nm and in basic solution a high intensity maximum at 368 nm. The shoulder is attributed to the  $n \rightarrow \pi^*$  transition of the additional C=O group, while from the latter maximum the presence of an ionizable group related to the chromophore with a pKa value of 6.3 can be deduced. This behaviour is interpreted as due to the  $\alpha$ -keto-amide group which is in conjugation with the  $\pi$  system of the aromatic ring 1 and which produces a vinylogous acid with the para hydroxyl group<sup>10)</sup>. This new maximum at 368 nm in basic solution reflects a new chromophore connected with an acidic ionizable function, which, for the sake of speculation, can be depicted as follows:

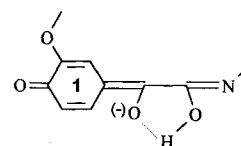


Table 5. *In vitro* activity (MIC,  $\mu\text{g}/\text{ml}$ ) of teico 2 complex in comparison with teicoplanin.

	Teico 2	Teicoplanin
<i>Staphylococcus aureus</i> Tour L165	4	0.5
<i>S. aureus</i> Tour 30% bovine serum	4	0.5
<i>S. aureus</i> clinical isolate L561	> 128	8
<i>S. epidermidis</i> ATCC12228 L147	128	0.5
<i>S. epidermidis</i> clinical isolate L533	> 128	8
<i>S. haemolyticus</i> clinical isolate L602	> 128	16
<i>Streptococcus pyogenes</i> C203 L49	0.125	0.063
<i>S. pneumoniae</i> UC41 L44	0.125	0.063
<i>Enterococcus faecalis</i> ATCC7080 L149	2	0.125
<i>E. faecalis</i> clinical isolate L562	> 128	64
<i>Escherichia coli</i> SKF12140 L47	> 128	> 128
<i>Pseudomonas aeruginosa</i> ATCC10145 L4	> 128	> 128
<i>Proteus vulgaris</i> X19H ATCC881 L79	> 128	> 128

Table 6. *In vitro* activity (MIC,  $\mu\text{g}/\text{ml}$ ) of teico 2 aglycone in comparison with teicoplanin aglycone and teicoplanin.

	Teico 2 aglycone	Teicoplanin aglycone	Teicoplanin
<i>Staphylococcus aureus</i> Tour L165	0.13	0.063	0.25
<i>S. aureus</i> Tour 30% bovine serum	16	0.25	1
<i>S. aureus</i> clinical isolate L561	0.5	—	16
<i>S. epidermidis</i> ATCC12228 L147	0.13	0.016	1
<i>S. epidermidis</i> clinical isolate L533	0.13	—	32
<i>S. haemolyticus</i> clinical isolate L602	4	0.25	16
<i>Streptococcus pyogenes</i> C203 L49	1	0.125	0.06
<i>S. pneumoniae</i> UC41 L44	0.5	0.125	0.13
<i>Enterococcus faecalis</i> ATCC7080 L149	2	0.125	0.13
<i>E. faecalis</i> clinical isolate L562	> 128	—	> 128
<i>Bacteroides fragilis</i> ATCC23745 L1010	> 128	—	128
<i>Neisseria gonorrhoeae</i> ISM68/126 L997	64	8	32
<i>Haemophilus influenzae</i> type b ATCC19418 L970	32	16	> 128
<i>Escherichia coli</i> SKF12140 L47	> 128	64	> 128
<i>Pseudomonas aeruginosa</i> ATCC10145 L4	> 128	> 128	> 128
<i>Proteus vulgaris</i> X19H ATCC881 L79	> 128	128	> 128

### Synthesis of Teico 2 Aglycone

As a final confirmation of the structure, the synthesis of teico 2 aglycone was carried on starting from teicoplanin aglycone. The method described for ristocetin was essentially applied<sup>11)</sup> (see Experimental). From 3 g of teicoplanin aglycone 0.5 g of teico 2 aglycone was obtained, whose analytical characteristics resulted identical to those of the product obtained by fermentation.

### Microbiological Activity

Teico 2 complex (Table 5) and teico 2 aglycone (Table 6) show a microbiological activity similar but somewhat lower than that of teicoplanin complex and teicoplanin aglycone.

### Discussion

The physico-chemical data on teico 2 aglycone and the unequivocal synthesis starting from teicoplanin aglycone clearly demonstrate that the structure corresponds to the substitution of a carbonyl group for the CH-NH<sub>2</sub> group of amino acid 1 (Fig. 5). These data are in accordance with those reported in literature for similar glycopeptide derivatives, produced either by microorganisms<sup>10,12)</sup> or by chemical reaction<sup>11)</sup>.

The MS-FAB of teico 2 complex, showing that the single components differ from the corresponding members of teicoplanin by one mass unit less, indicates that no other alterations are present. This is agreement with the findings of NMR spectroscopy, showing that the sugars are the same and the fatty acid moieties present a similar pattern.

From the structure and the time course of fermentation, in which the production of teico 2 is accompanied

by a decrease in teicoplanin concentration, it can be assumed that the former is a transformation product of the latter, due to a simple transamination reaction.

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