ISOLATION AND STRUCTURE DETERMINATION OF TWO NEW ANALOGS OF TEICOPLANIN, A GLYCOPEPTIDE ANTIBIOTIC

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Teicoplanin is an antibiotic produced by fermentation of *Actinoplanes teichomyceticus* as a complex formed by five closely related glycopeptides characterized by different fatty acid chains of ten and eleven carbon atoms. In addition, minor quantities of related substances are present. Two of them, named RS-1 and RS-2, were shown to be teicoplanins having as fatty acid chains 10-methylundecanoic acid and *n*-dodecanoic acid, respectively. Other two related substances, named RS-3 and RS-4, have now been isolated and purified starting from fermentation broths of a mutant of the same microorganism producing them in substantial amounts. This was achieved by semipreparative reversed-phase liquid chromatography carried out on high-pressure scale.

The structures were assigned on the basis of ^{1}H NMR spectra and homonuclear COSY 2D experiments and fast atom bombardment MS spectrometry, in comparison with the large mass of data till now accumulated on teicoplanin. RS-3 and RS-4 are teicoplanins having as fatty acid chains 6-methyloctanoic acid and *n*-nonanoic acid, respectively.

Teicoplanin[1,2] is a glycopeptide antibiotic, produced by *Actinoplanes teichomyceticus* ATCC 31121, active against aerobic and anaerobic Gram-positive bacteria[3]. It has been found to be very effective and well tolerated when administered iv or im in extended clinical trials[4].

Teicoplanin is produced by the Actinoplanes as a complex of substances, as demonstrated by the HPLC obtained under reversed-phase gradient conditions. The most interesting ones, originally named teichomycin A-2[5], are five closely related compounds designated T-A2-1, 2, 3, 4, and 5[6] (see Fig. 1). A sixth active component, named T-A3-1, not found in fermentation broths, is always present in small amounts in crude or purified extracts. This compound was shown to be a degradation (hydrolysis) product of group T-A2[7].

The structural work carried out by various groups[7~10] has elucidated the structures of the

Fig. 1. HPLC gradient chromatogram of a sample of teicoplanin complex.

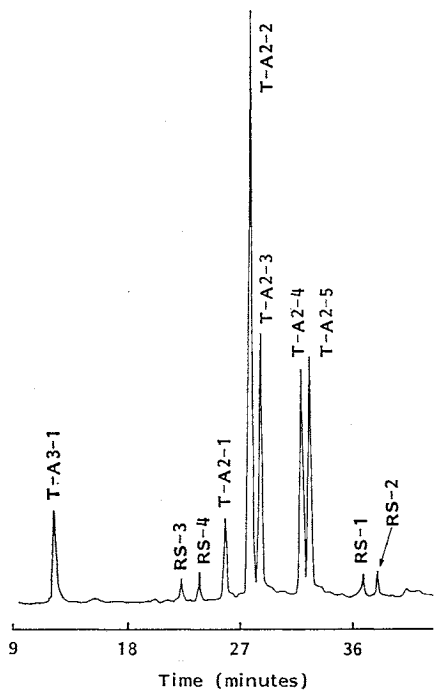
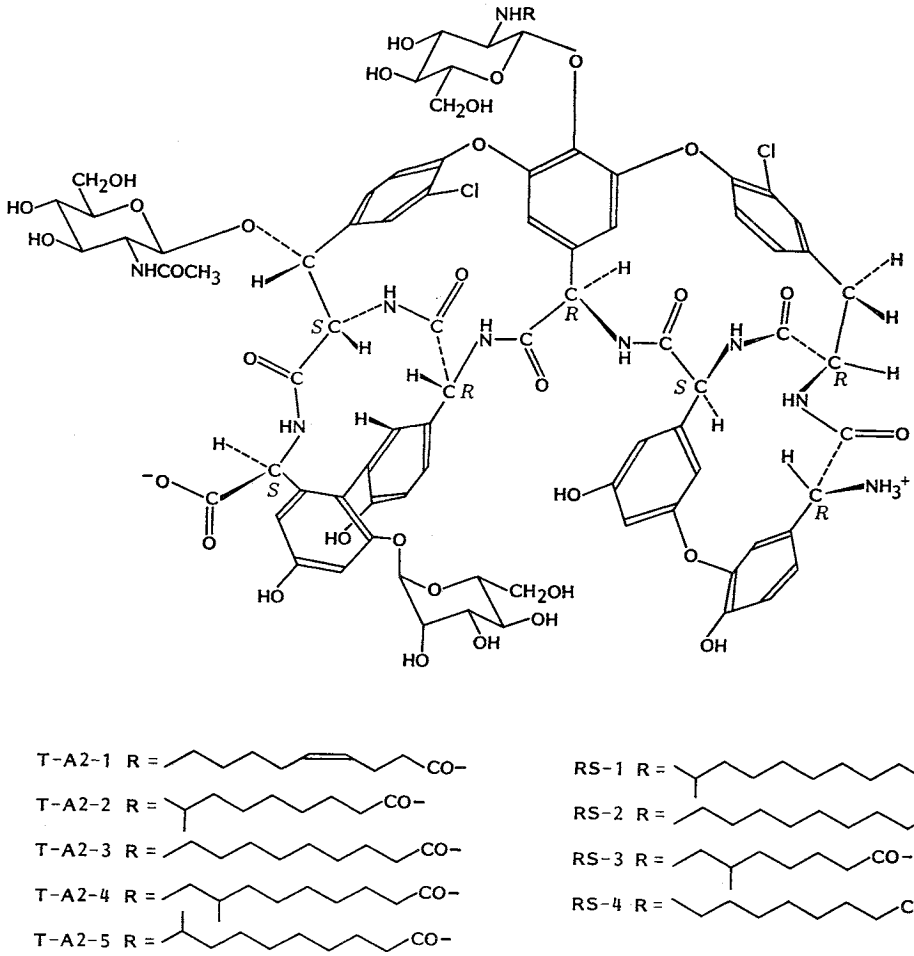


Fig. 2. Structures of the teicoplanin components.



six main components, *i.e.*, A3-1 and A2-1 to 5, shown in Fig. 2. However, an accurate analysis of the batches deriving from large scale fermentations revealed the presence of additional components, two of which, more lipophilic and designated related substances RS-1 and RS-2 (see Fig. 1) were shown to be teicoplanins having as fatty acid chains 10-methylundecanoic acid and *n*-dodecanoic acid, respectively[11].

Two less lipophilic related substances are also present in teicoplanin complex and they were named RS-3 and RS-4. During a mutagenesis program for selection of higher producing strains of *A. teichomyceticus* carried out in the Lepetit Development Department (L. PALUMBO, *et al.*; internal communication), a strain was found which showed higher levels of production of these two components. This gave us the opportunity to obtain, with a minor effort, enough material for their structure determination, which is the subject of the present paper. The structures of RS-3 and RS-4 for convenience are already shown in Fig. 2.

Experimental

Strain and Culture Media

Strain A-184, a mutant of *A. teichomyceticus*, was obtained from Dr. L. PALUMBO (Lepetit - Brindisi)

(internal communication).

Vegetative Medium (g/liter): Glucose 10, Bacto Peptone (Difco) 4, Bacto yeast extract (Difco) 4, $MgSO_4$ 0.5, KH_2PO_4 2, K_2HPO_4 4, pH 7 after sterilization.

Medium C (g/liter): Glucose (glucose was sterilized separately) 20, yeast extract 5, asparagine 1.5, $MgSO_4$ 0.5, $CaCO_3$ 5, NaCl 0.1, $CaCl_2 \cdot 2H_2O$ 0.1, mineral supplement 1 ml/liter, pH 6.9 after sterilization. Mineral supplement composition (g/liter); boric acid 0.50, $CuSO_4 \cdot 5H_2O$ 0.04, KI 0.10, $FeCl_3 \cdot 6H_2O$ 0.20, $MnSO_4 \cdot H_2O$ 0.40, $FeSO_4 \cdot 7H_2O$ 0.40, ammonium molybdate 0.20.

Fermentation Conditions

A frozen stock culture of the strain (2.5 ml) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of vegetative medium. The culture was incubated at 28°C for 48 hours on a shaker at 200 rpm and 5 cm throw.

This culture (400 ml) was used to inoculate a fermentor containing 4 liters of production medium (Medium C). The jar was aerated with sterile air at a flow rate of 2 liters/minute and stirred at 900 rpm, while maintaining the temperature at 28°C.

Recovery and Purification

The culture broth, harvested 4 days after inoculation, was filtered at pH 11 and then the pH of the filtered broth was adjusted to 7.5. A suitable amount of Sepharose-acyl-D-alanyl-D-alanine affinity resin[12,13] was added and stirred overnight at 4°C. The resin was then separated from the exhausted broth and poured into a chromatographic column. The column was washed with 5 resin-volume of Tris-HCl buffer (0.05 M, pH 7.5) and then with the same volume of Tris base solution (0.05 M). Teicoplanin complex was eluted with an aqueous solution of NH_4OH (1%, w/v). The fractions, collected according to their composition, were neutralized with formic acid.

Ultrafiltration

Aqueous solutions of teicoplanin were concentrated in a 142-mm Hi-Flux U-F Cell Millipore apparatus supporting a PCAC Pellicon ultrafiltration membrane with a nominal molecular weight limit (NMWL) of 1,000 dalton.

Analytical HPLC

Apparatus: Hewlett-Packard liquid chromatograph, model 1084 B; the UV detector was set at 254 nm. Column: Erbasil C18 5 μm, 150×4 mm (Carlo Erba). Mobile phase: A; 0.02 M NaH_2PO_4 - CH_3CN (95:5), B; 0.02 M NaH_2PO_4 - CH_3CN (25:75). Gradient: minutes (%B), 0 (10), 40 (40), 45 (55), 49 (10), 50 stop. Flow rate: 1.5 ml/minute. Column pressure: 200 atm. Injection volume: 20 μl. Attenuation: 8. Chart speed: 0.5 cm/minute. Standard: teicoplanin, ARS batch B/7/84 from Lepetit Research Center, was dissolved in water to give a solution at the concentration of 1,156.5 μg/ml.

Semipreparative HPLC

Apparatus: Hewlett-Packard liquid chromatograph, model 1084 B; the UV detector was set at 254 nm. Column: LiChrosorb RP-18 7 μm, 250×10 mm (Merck). Mobile phase: A; 0.02 M $HCOONH_4$ - CH_3CN (95:5), B; 0.02 M $HCOONH_4$ - CH_3CN (25:75). Gradient: minutes (%B), 0 (25), 18 (25), 22 (65), 29 (65), 30 (25), 31 stop. Flow rate: 4 ml/minute. Column pressure: 130 atm. Injection volume: 200 μl. Attenuation: 1,024. Chart speed: 0.5 cm/minute. Sample preparation: Aliquots of 300 mg of lyophilized material were dissolved in 1 ml of DMF and added with 1 ml of a mixture of H_2O - CH_3CN (1:1).

NMR Spectrometry

The instrument was a Bruker model AM-250 with an array processor, a magnet at 250 MHz, and a computerized console Aspect 3000. The spectra were obtained in DMSO-d_6 solutions at 25°C with TMS as a reference.

Fast Atom Bombardment Mass Spectrometry (FAB-MS)

The instrument was a VG 70/250, using the mixture thioglycerol - glycerol (2:1) as a matrix.

Bombardment gas: Xe; kinetic energy 6~8 keV; accelerating voltage 6 kV. Positive ion spectra were collected from m/z 600 to 2,000.

Results and Discussion

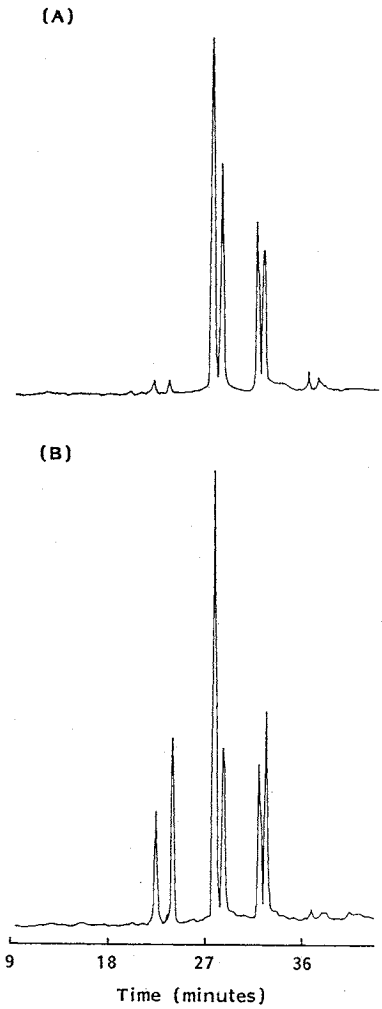
The *A. teichomyceticus* strain A-184 (ATCC 53649) was obtained after mutagenic treatment with nitrosoguanidine of the parental *A. teichomyceticus* ATCC 31121. Mutant A-184 shows substantially the same morphological and physiological characteristics as the parent strain but an HPLC analysis of the fermentation broth revealed the presence of substantial amounts of two teicoplanin-like substances (Fig. 3), named related substances 3 and 4 (RS-3 and RS-4), which were present only in trace amounts in the normal teicoplanin batches.

To isolate the new compounds, fermentations were carried out, as described under Experimental, in four 4-liter fermentors for 96 hours. Fourteen liters of the filtered broth were treated with 200 ml of D-alanyl-D-alanine affinity resin which was eluted with ammonia (for details see Experimental). Fractions of 200 ml were collected and analyzed. Fractions 6 to 10, containing the bulk of RS-3 and RS-4, were pooled, neutralized and concentrated by ultrafiltration to 70 ml. A crude of 2.53 g was obtained by lyophilization.

Chromatography of this material on the preparative column by repetitive runs gave several fractions of which fraction 1 (12.9 mg) and fraction 3 (15.1 mg) on HPLC analysis revealed to contain essentially RS-3 and RS-4, respectively. They were considered sufficiently pure for being submitted to structure investigation.

The ^{1}H NMR spectra of RS-3 and RS-4 are reported in Figs. 4 and 5, respectively. The attribution of the most significant peaks was given by comparison with the teicoplanin spectrum[14] and on the basis of two dimensional spectroscopy, namely ^{1}H homonuclear correlation spectroscopy. The spectra show that RS-3 and RS-4 differ from that of teicoplanin components only in the aliphatic chain region. In fact, in RS-3 two methyl groups are found as shown by a triplet due to the (CH_2)-CH_3 moiety and a doublet due to the (CH)-CH_3 moiety nearly coincident at δ 0.8 (J=6.4 Hz). In addition, there

Fig. 3. HPLC profile of teicoplanin from fermentation broth of: (A) Wild *Actinoplanes teichomyceticus* ATCC 31121, (B) mutant *A. teichomyceticus* ATCC 53649.



The fermentations were carried out with medium C (see Experimental). Under these conditions T-A2-1 is not produced.

Fig. 4. ^{1}H NMR spectrum of RS-3.

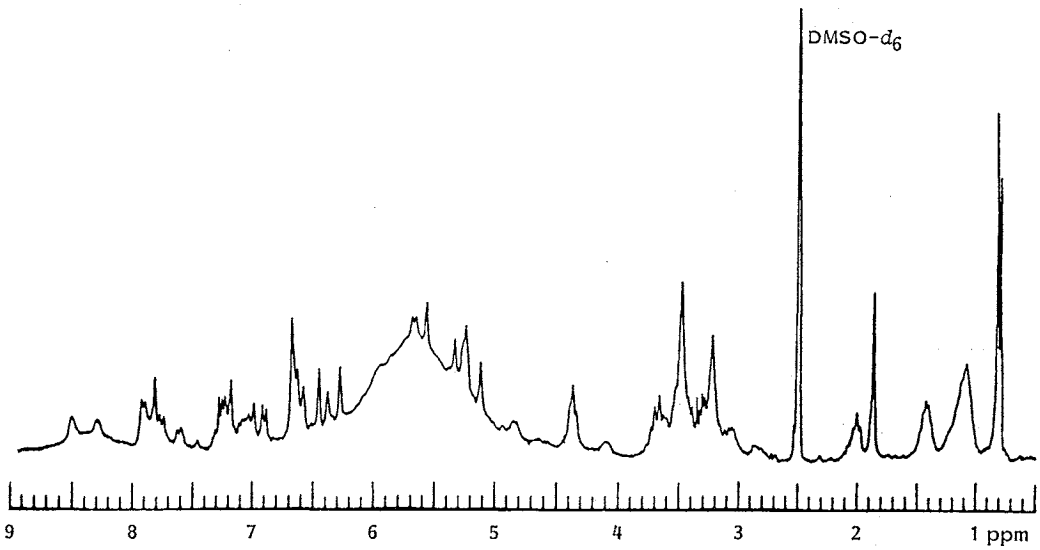
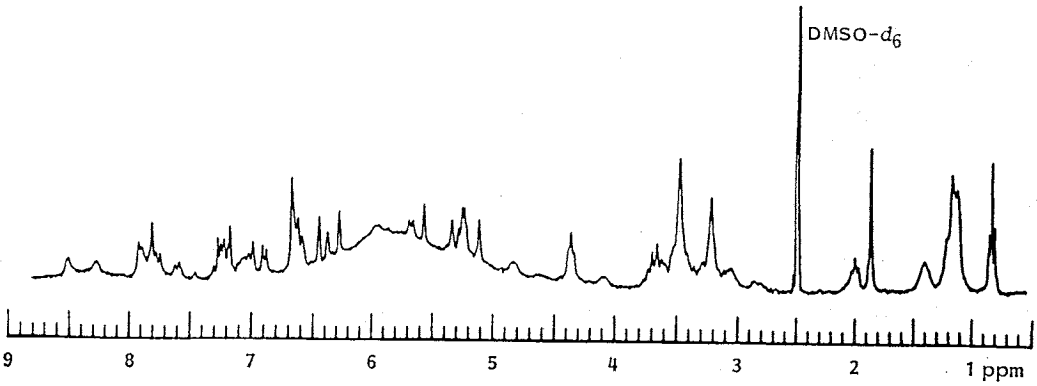


Fig. 5. ^{1}H NMR spectrum of RS-4.



are four CH_2 groups in the chain, as deduced from the spectrum integral.

In RS-4 the terminal methyl group of the chain is shown by a triplet due to the (CH_2)-CH_3 moiety at δ 0.83 (J=6.5 Hz). Seven CH_2 groups are present in the chain, as shown by the spectrum integral.

The cationized molecular ions (MH^+ or MNa^+) and the adducts with the matrix determined by FAB-MS indicated a molecular weight of 1,849 (lowest isotope composition) for both the compounds, in agreement with the NMR data.

The NMR and FAB spectra clearly demonstrate that the structure of RS-3 is that of a teicoplanin having, as the side chain, a 6-methyloctanoyl moiety and that the structure of RS-4 is that of a teicoplanin with a *n*-nonanoyl side chain.

The presence in the fermentation broth of a teicoplanin with a 6-methyloctanoyl side chain (corresponding to the *anteiso*-nonanoic acid) is not surprising since this acid can easily be formed biogenetically by β-oxidation and loss of an acetate unit from the *anteiso*-undecanoic acid, which is the moiety of component T-A2-4[1].

The presence, as a side chain, of a linear odd carbon acid (*n*-nonanoic) is rather surprising, since it was biosynthetically unrelated to the other components of the teicoplanin complex. Studies on the relationship between antibiotic fatty acid moiety and whole cell fatty acid composition are in progress and will be published elsewhere.

Acknowledgments

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