MICROBIAL DE-MANNOSYLATION AND MANNOSYLATION OF TEICOPLANIN DERIVATIVES

ANGELO BORGHI, PIETRO FERRARI, GIAN GUALBERTO GALLO, MARGHERITA ZANOL, LUIGI FRANCO ZERILLI and GIAN CARLO LANCINI

MMDRI, Centro Ricerche Lepetit,
Via R. Lepetit 34, 21040 Gerenzano (VA), Italy

(Received for publication May 2, 1991)

The single components of the teicoplanin complex, glycopeptide antibiotics active against Gram-positive bacteria, can be converted in the corresponding de-mannosyl derivatives by cultures of *Nocardia orientalis* NRRL 2450 or *Streptomyces candidus* NRRL 3218. Conversely, teicoplanin aglycone and other teicoplanin de-mannosyl derivatives can be converted in the corresponding teicoplanin mannosyl derivatives by cultures of *Actinoplanes teichomyceticus* ATCC 31121. The biological transformation yields are approximately 40% for de-mannosylation and 90% for mannosylation. The processes allow for the preparation of gram quantities of the de-mannosyl derivatives of teicoplanin and of teicoplanin mannosyl derivatives. De-mannosyl teicoplanin and teicoplanin mannosyl-pseudoaglycone were not amenable to preparation by either acidic or basic chemical hydrolysis.

Teicoplanin is a member of a complex of glycopeptide antibiotics produced by *Actinoplanes teichomyceticus* ATCC 31121 as a complex of substances[1,2]. The most interesting ones, originally named teichomycin A-2[3], are five closely related compounds designated T-A2-1, 2, 3, 4, and 5[4], whose structures were elucidated by various groups[5~7] (see Figs. 1 and 2). 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 pseudoaglycone, produced as a single substance by hydrolysis of group

Fig. 1. HPLC gradient chromatogram of teicoplanin complex.

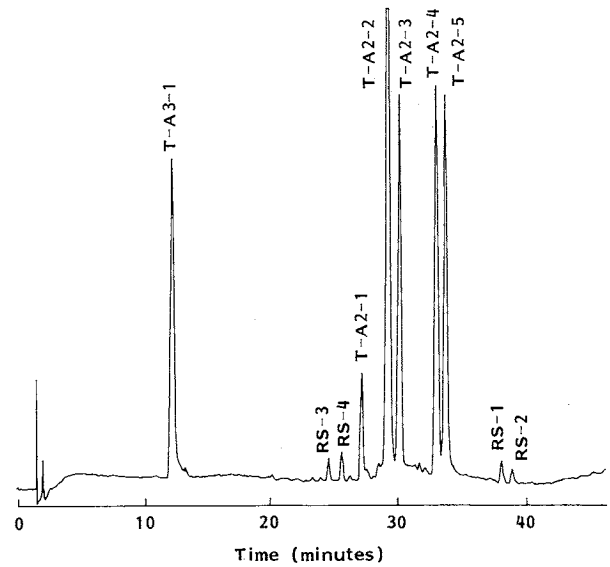
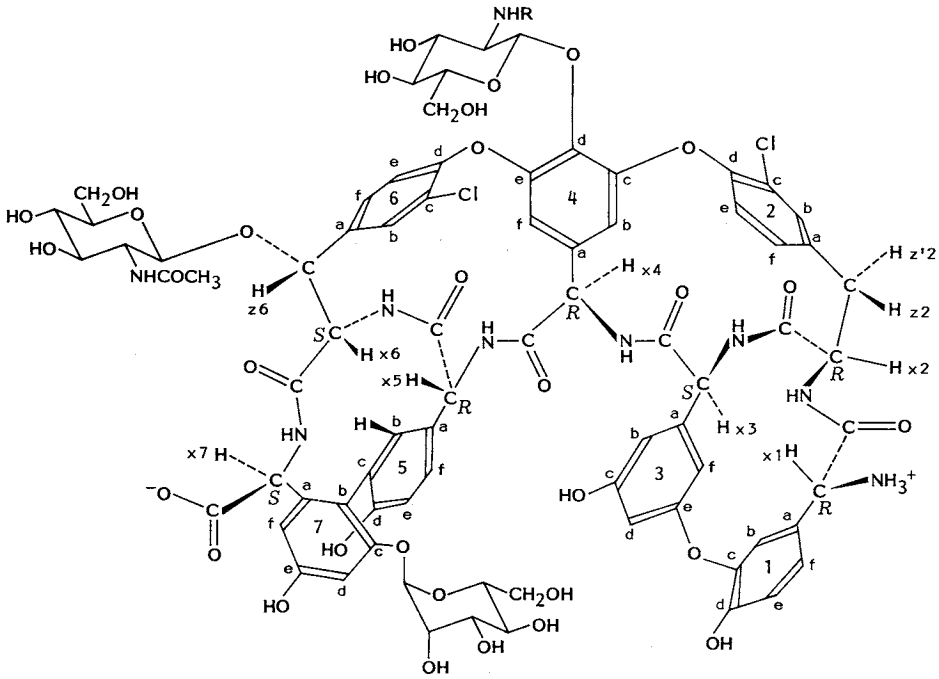
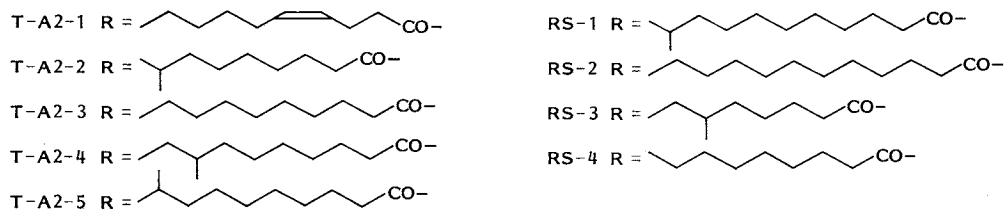


Fig. 2. Structures of the teicoplanin components.



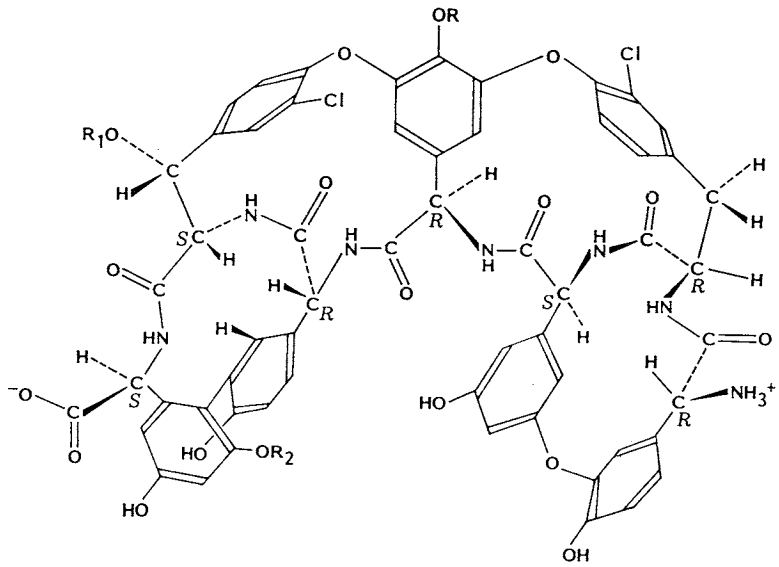


Nomenclature according to WILLIAMS[6]: Residues are numbered starting at the *N*-terminus; α carbons/protons are designated x_n; β carbons/protons z_n; lettering of aromatic carbons/protons is such that substituted carbons are reached first in the lettering system and in the case of symmetry the lettering is counterclockwise.

T-A2[8]. In addition, other related substances, named RS-1, RS-2, RS-3, and RS-4, are usually present in minute amounts[9,10] (see Figs. 1 and 2). Successively, during the extensive chemical work following the development of this antibiotic two other acidic hydrolysis products were obtained, *i.e.*, the pseudoaglycone containing acetyl-β-D-glucosamine (named T-A3-2) and the true aglycone (T-Ag)[8,11] (structures shown in Fig. 3).

The pseudoaglycone containing the two glucosamines and lacking mannose could not be obtained by chemical methods. In fact, while acid hydrolysis yielded the products mentioned above, basic hydrolysis led to epimerization at the chiral center of the third amino acid (starting from the *N*-terminus) resulting in a marked decrease in the activity[12]. Similarly, for the same reasons the pseudoaglycone lacking the two glucosamines and containing mannose, and the two pseudoaglycones containing mannose and only one glucosamine could not be obtained by chemical methods (structures shown in Fig. 3).

Fig. 3. Structures of the aglycone and pseudoaglycones of teicoplanin obtained by * chemical transformation and ** microbial transformation.



T-A3-1 * and ** R=H R_1=*N*-acetyl-glucosamine R_2=mannose
T-A3-2 * R=H R_1=*N*-acetyl-glucosamine R_2=H
T-Ag * R=H R_1=H R_2=H
DM-T-A2-2 ** R=*N*-8-methyl-nonanoyl-glucosamine R_1=*N*-acetyl-glucosamine R_2=H
M-T-Ag ** R=H R_1=H R_2=mannose

The subject of the present paper is the description of the microbiological transformation of teicoplanin to obtain compounds that could not be prepared by chemical hydrolysis.

Materials and Methods

Strain and Culture Media

Nocardia orientalis NRRL 2450, *Streptomyces candidus* NRRL 3218 and *Actinoplanes teichomyceticus* ATCC 31121. Medium S/bis: Yeast extract 4 g, peptone 4 g, glucose 10 g, $MgSO_4$ 0.5 g, KH_2PO_4 2 g, K_2HPO_4 4 g and distilled water to 1,000 ml, pH after sterilization 7. Medium C: Glucose (a) 20 g/liter, yeast extract 5 g/liter, asparagine 1.5 g/liter, $MgSO_4$ 0.5 g/liter, $CaCO_3$ 5 g/liter, $CaCl_2 \cdot 2H_2O$ 2 g/liter, NaCl 2 g/liter and mineral supplement (b) 1 ml/liter, pH after sterilization 6.9. (a) Glucose was sterilized separately. (b) 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 molibdate 0.20.

Materials

T-A2-2, 3, 4, and 5 were prepared as described in ref 4.

Fermentation Conditions

For all the strains, lyophilized cultures were aseptically transferred onto a slant of oatmeal agar and incubated at 28°C for 7 days. The culture was suspended in distilled water and inoculated into an Erlenmeyer flask containing 100 ml of medium S/bis. This was incubated for 48 hours at 28°C on a rotary shaker at 200 rpm, frozen and stored for further use. 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 S/bis. The culture was incubated at 28°C for 48 hours on a shaker at 200 rpm and in the 5-cm throw.

With *N. orientalis* or *S. candidus*, 5 ml of the culture was used to inoculate 100 ml of productive

medium C in a 500-ml flask and it was incubated under the same conditions. Substances to be transformed were added after 48 hours and the fermentation was continued under the conditions described above. In some experiments the substances were added to 48 hour-old mycelium, which had been washed and resuspended in sterile saline. With *A. teichomyceticus*, fermentation was carried out as described above in medium C. At 48 hours the mycelium of each flask was collected by centrifugation, washed with sterile saline and resuspended in 100 ml of sterile saline to which the substances to be transformed were added and incubated for 24 hours.

Recovery and Purification

The culture broth from the combined flasks was brought to pH 10.5 with 1 N NaOH, filtered in the presence of a filter aid, and the pH was adjusted to 7.5 with HCl. A suitable amount of Sepharose-acyl-D-alanyl-D-alanine affinity resin[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 five resin volumes of Tris-HCl buffer (0.05 M, pH 7.5) and then with the same volume of Tris base solution. Teicoplanin derivatives were eluted with an aqueous solution of NH_4OH (1%, w/v). The fractions pooled according to their content were neutralized with formic acid.

Ultrafiltration

The aqueous solutions of teicoplanin derivatives were concentrated in a 90 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 × 4.6 mm (Carlo Erba). Mobile phases: 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), 48 (10), 50 stop. Flow rate: 1.5 ml/minute. Injection volume: 20 μl loop. External standard: teicoplanin ARS, from Lepetit Research Center, was dissolved in water at the concentration of about 1 mg/ml.

Semi-preparative HPLC

Each crude product was purified and separated by this chromatographic procedure under the following conditions:

Apparatus; Waters liquid chromatograph model 6000A, equipped with two pumps, an absorbance UV detector model 440 set at 254 nm, and a solvent programmer model 660. Column: HIBAR Lichrosorb RP-18, 7 μm, 250 × 10 mm (Merck). Mobile phases: A: 0.02 M $HCOONH_4$-CH_3CN (9 : 1), B: 0.02 M $HCOONH_4$-CH_3CN (3 : 7). Gradient: linear from 5% of B to 45% of B in 45 minutes. Flow rate: 6 ml/minute. Injection volume: 0.2 ml. Sample preparation: 100 mg of product dissolved in 1 ml mobile phase A.

^{1}H NMR Spectrometry

The instrument was a Bruker model AM-250, operating at 250 MHz, with the console equipped with an array processor and an Aspect 3000 computer. The spectra were obtained in DMSO-d_6 solutions at 22°C with tetramethylsilane as reference. ^{1}H-^{1}H COSY spectra were run by phase-sensitive double quantum filter (PHDQ) mode using time-proportional-phase-incrementation in f1.

FAB-MS

The instrument was a VG 70/250, using as a matrix thioglycerol for M-T-Ag and the mixture thioglycerol-glycerol (2 : 1) for DM-T-A2-2. 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 microbiological de-mannosylation of teicoplanin was carried out by the selective hydrolysis

Fig. 4. Transformation of T-A2-2 in DM-T-A2-2 by *Nocardia orientalis*. HPLC gradient chromatogram of the fermentation broth.

Time after the addition of 200 mg/liter: (A) 0 hour, (B) 48 hours, (C) 120 hours.

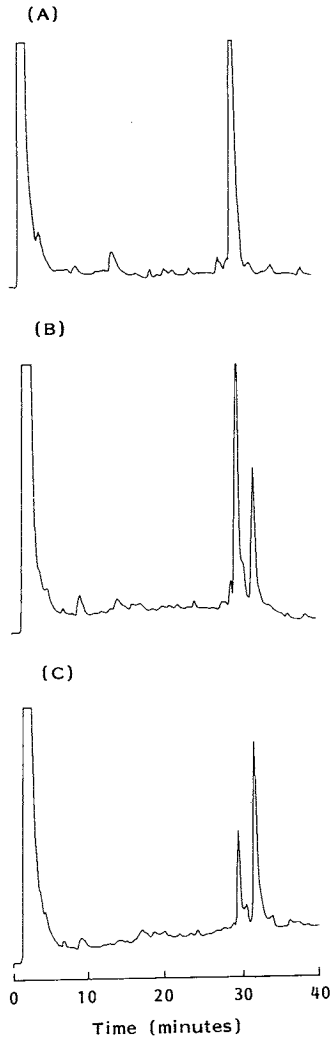
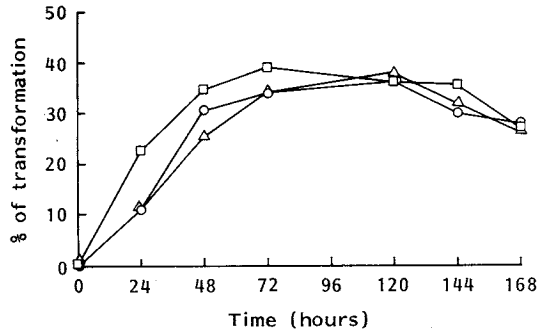


Fig. 5. Time course of the formation of DM-T-A2-2 by washed mycelium of *Nocardia orientalis* upon addition of different concentrations of T-A2-2.

□ 0.25 g/liter, ○ 0.5 g/liter, △ 1.0 g/liter.



The concentration of DM-T-A2-2 was determined by HPLC as described in the Materials and Methods.

of the mannose moiety by cultures of *N. orientalis* or *S. candidus*. Teicoplanin component T-A2-2 (100 mg) was added to five flasks of the *N. orientalis* culture in medium C at a concentration of about 0.2 g/liter and fermented under the conditions described in the Materials and Methods. A time dependent decrease of T-A2-2 (Rt 29.2 minutes) and the appearance of a new product at a longer Rt (30.9 minutes) was observed by HPLC analysis (Fig. 4). The percentage of transformation was about 40%. For the identification of the transformation product the filtered broth from the five flasks was worked up as described in the Materials and Methods and, after semi-preparative chromatography, 20 mg of product was obtained and submitted to structure determination by spectroscopic techniques. As described below, the transformation product was identified as de-mannosyl teicoplanin component A2-2 (DM-T-A2-2).

The time course of the transformation by the washed mycelium of *N. orientalis*, at different concentrations of T-A2-2, is shown in Fig. 5.

Separate additions of the teicoplanin single components T-A2-3 (Rt 30.1 minutes), T-A2-4 (Rt 33.1 minutes), and T-A2-5 (Rt 33.8 minutes) resulted in the formation of the corresponding de-mannosyl derivatives DM-T-A2-3 (Rt 31.8 minutes), DM-T-A2-4 (Rt 34.7 minutes), and DM-T-A2-5 (Rt 35.4 minutes).

Similar results were obtained by using *S. candidus* cultures (data not shown).

The microbiological mannosylation of teicoplanin aglycone (T-Ag) (200 mg) was carried out by

adding it to the mycelium of ten flasks of an *A. teichomyceticus* culture resuspended in sterile saline, at a concentration of about 0.2 g/liter under the conditions described in the Materials and Methods. After 24 hours incubation the HPLC analysis revealed a 90% transformation of the starting material (Rt 16.5 minutes) to a new product with a lower Rt (14.6 minutes). The filtered broth from the ten flasks was worked up as described in the Materials and Methods and, after affinity resin chromatography, 114 mg of product was obtained, which was identified as teicoplanin mannosyl pseudoaglycone (M-T-Ag).

Addition of the pseudoaglycone bearing acetyl-glucosamine (T-A3-2, Rt 13.8 minutes) or DM-T-A2-2 (Rt 30.9 minutes) resulted in an almost quantitative formation of the known compounds T-A3-1 (Rt 11.8 minutes) and T-A2-2, respectively.

A general comment on the transformation is that these biological methods give good yields and that they appear suitable for scaling up studies.

The structures were determined by ^{1}H NMR and FAB-MS spectroscopies. The ^{1}H NMR spectra of DM-T-A2-2 and of M-T-Ag are shown in Figs. 6 and 7, respectively. By reference to the present knowledge on the correlations established between NMR signals and chemical structures in the field of teicoplanins[6,7,11,14~16], it can be clearly deduced that DM-T-A2-2 is the de-mannosyl derivative of component A2-2 of teicoplanin, and that M-T-Ag is the mannosyl-pseudoaglycone of teicoplanin. By

Fig. 6. ^{1}H NMR spectrum of DM-T-A2-2 in DMSO-d_6.

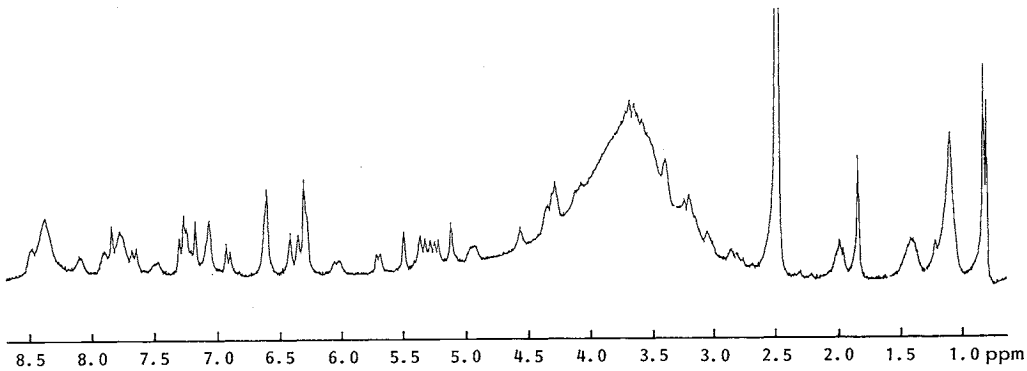


Fig. 7. ^{1}H NMR spectrum of M-T-Ag in DMSO-d_6, added with CF_3CO_2D.

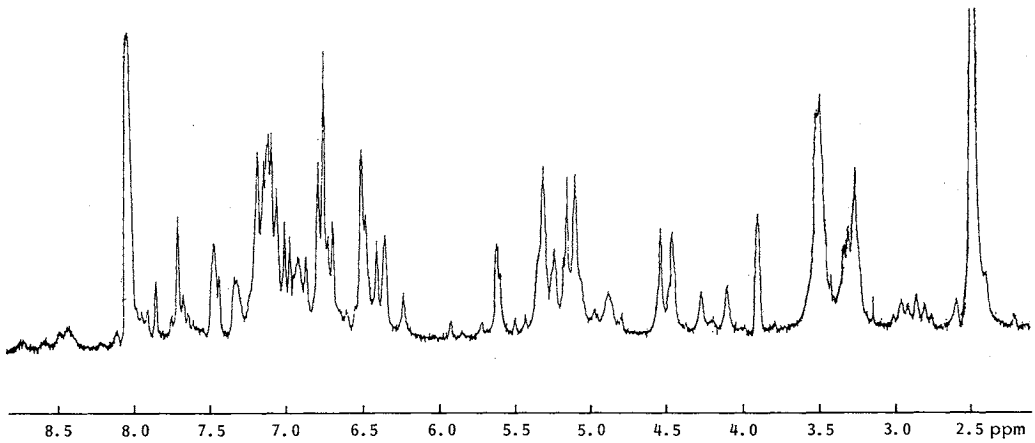


Table 1. Assignments of the main signals of the ^1H NMR spectra of T-A2-2, DM-T-A2-2, and M-T-Ag, δ (ppm) in DMSO-d_6.

Proton	m[a]	T-A2-2	DM-T-A2-2	M-T-Ag[b]
Isopropyl CH_3 groups	d	0.84	0.82	Absent
Various CH_2 groups of the chain	m	1.13~1.24	1.10	Absent
CH_2 α to C=O and isopropyl CH	m	1.47	1.42	Absent
Acetyl group of glucosamine	s	1.90	1.84	Absent
CH_2 groups β to C=O	m	2.10	1.99	Absent
CH_2 of mannose	m	3.50	Absent	3.50
C_2-H of acyl-glucosamines	m	3.71	3.68	Absent
x6	dd	4.11	4.10	4.10
x5, x7, and anomeric H of acetyl-glucosamine	d	4.30~4.45	4.30~4.40	4.15, 4.54, and absent
x2	m	4.80	4.93	4.88
4f	s	5.11	5.11	5.10
x1	s	4.50	4.57	5.31
Anomeric H of mannose	s	5.23	Absent	5.16
z6	s	5.29	5.22	5.10
x3	d	5.34	5.34	5.32
Anomeric H of acyl-glucosamines	d	5.40	5.26	Absent
4b	s	5.56	5.50	5.62
x4	d	5.62	5.69	5.61
7f	s	6.44	6.28	6.50
7d	s	6.75	6.36	6.78

[a] m: Multiplicity.
[b] Added with CF_3CO_2D.

inspection of the NMR table where the main NMR signals of T-A2-2, DM-T-A2-2 and M-T-Ag are reported, the following considerations can be drawn.

The presence in M-T-Ag and the absence in DM-T-A2-2 of D-mannose is revealed by the presence or the absence, respectively, of the characteristic mannose signals at 3.50 ppm (CH_2) and ~5.20 ppm (anomeric proton). The attachment of D-mannose to the oxygen atom in position c of benzene ring 7 is consistent with the expected downfield shift of ~0.4 ppm for the 7d proton and of ~0.2 ppm for the 7f proton[7], passing from DM-T-A2-2 to T-A2-2, and from T-Ag to M-T-Ag. D-Mannose must be linked as the α anomer (the configuration produced by the microorganism) because this configuration brings about a small vicinal coupling constant involving the anomeric proton, whose signal appears as a singlet. A doublet with a large coupling constant displayed by the anomeric proton of acetyl- and acyl-glucosamines in the teicoplanin derivatives in question is consistent with a *trans* diaxial configuration between the anomeric proton and the vicinal proton and indicates a β-configuration.

FAB-MS confirmed for DM-T-A2-2 the MW (lightest mass mono-isotopic composition) of 1,715, which corresponds to 1,877 (T-A2-2) a difference of 162 (mannose = 180 minus water = 18), and for M-T-Ag a value of 1,359.

References

1) CORONELLI, C.; G. G. GALLO & B. CAVALLERI: Teicoplanin: chemical, physicochemical and biological aspects. Il Farmaco, Ed. Sci. 42: 767~787, 1987 and references therein
2) CAVALLERI, B. & G. C. LANCINI: Glycopeptide antibiotics of the vancomycin group. *In* Biochemistry of Peptide Antibiotics. *Eds.*, H. KLEINKAUF & H. VAN DÖREN, pp. 178~199 and references therein, de Gruyter, Berlin, 1990
3) BARDONE, M. R.; M. PATERNOSTER & C. CORONELLI: Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* nov. sp. II. Extraction and chemical characterization. J. Antibiotics 31: 170~177, 1978

4) BORGHI, A.; C. CORONELLI, L. FANIUOLO, G. ALLIEVI, R. PALLANZA & G. G. GALLO: Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* nov. sp. IV. Separation and characterization of the components of teichomycin (teicoplanin). J. Antibiotics 37: 615~620, 1984
5) CORONELLI, C.; M. R. BARDONE, A. DEPAOLI, P. FERRARI, G. TUAN & G. G. GALLO: Teicoplanin, antibiotics from *Actinoplanes teichomyceticus* nov. sp. V. Aromatic constituents. J. Antibiotics 37: 621~626, 1984
6) BARNA, J. C. J.; D. H. WILLIAMS, D. J. M. STONE, T.-W. C. LEUNG & D. M. DODDRELL: Structure elucidation of the teicoplanin antibiotics. J. Am. Chem. Soc. 106: 4895~4902, 1984
7) HUNT, A. H.; R. M. MOLLOY, J. L. OCCOLOWITZ, G. G. MARCONI & M. DEBONO: Structure of the major glycopeptide of the teicoplanin complex. J. Am. Chem. Soc. 106: 4891~4895, 1984
8) MALABARBA, A.; P. STRAZZOLINI, A. DEPAOLI, M. LANDI, M. BERTI & B. CAVALLERI: Teicoplanin, antibiotics from *Actinoplanes teichomyceticus* nov. sp. VI. Chemical degradation: Physico-chemical and biological properties of acid hydrolysis products. J. Antibiotics 37: 988~999, 1984
9) COMETTI, A.; G. G. GALLO, J. KETTENRING, G. B. PANZONE, G. TUAN & L. F. ZERILLI: Isolation and structure determination of the main related substances of teicoplanin, a glycopeptide antibiotic. Il Farmaco, Ed. Sci. 43: 1005~1018, 1988
10) BORGHI, A.; P. ANTONINI, M. ZANOL, P. FERRARI, L. F. ZERILLI & G. C. LANCINI: Isolation and structure determination of two new analogs of teicoplanin, a glycopeptide antibiotic. J. Antibiotics 42: 361~366, 1989
11) MALABARBA, A.; P. FERRARI, G. G. GALLO, J. KETTENRING & B. CAVALLERI: Teicoplanin, antibiotics from *Actinoplanes teichomyceticus* nov. sp. VII. Preparation and NMR characteristics of the aglycone of teicoplanin. J. Antibiotics 39: 1430~1442, 1986
12) BARNA, J. C. J.; D. H. WILLIAMS, P. STRAZZOLINI, A. MALABARBA & T.-W. C. LEUNG: Structure and conformation of epimers derived from the antibiotic teicoplanin. J. Antibiotics 37: 1204~1208, 1984
13) CORTI, A. & G. CASSANI: Synthesis and characterization of D-alanyl-D-alanine-agarose: a new bioselective adsorbent for affinity chromatography of glycopeptide antibiotics. Appl. Biochem. Biotechnol. 11: 101~109, 1985
14) TRANI, A.; P. FERRARI, R. PALLANZA & R. CIABATTI: Thioureas and isothiouronium salts of the aglycone of teicoplanin. I. Synthesis and biological activity. J. Antibiotics 42: 1268~1275, 1989
15) HEALD, S. L.; L. MUELLER & P. W. JEFFS: Structural analysis of teicoplanin A2 by 2D NMR. J. Magn. Res. 72: 120~138, 1987
16) WALTHO, J. P.; D. H. WILLIAMS, E. SELVA & P. FERRARI: Structure elucidation of the glycopeptide antibiotic complex A40926. J. Chem. Soc. Perkin Trans. I 1987: 2103~2107, 1987