MM 55266 AND MM 55268, GLYCOPEPTIDE ANTIBIOTICS PRODUCED BY A NEW STRAIN OF *Amycolatopsis*

ISOLATION, PURIFICATION AND STRUCTURE DETERMINATION

STEPHEN J. BOX, NIGEL J. COATES, CHRIS J. DAVIS, MARTIN L. GILPIN*, CATHERINE S. V. HOUGE-FRYDRYCH and PETER H. MILNER

SmithKline Beecham Pharmaceuticals, Chemotherapeutic Research Centre, Brockham Park, Betchworth, Surrey RH3 7AJ, UK

(Received for publication March 14, 1991)

Two novel glycopeptide antibiotics MM 55266 and MM 55268 containing fatty acid acyl functions, and of molecular formula $C_{86}H_{89}N_8O_{35}Cl_5$ and $C_{87}H_{91}N_8O_{35}Cl_5$, respectively, have been isolated and identified from a complex produced by *Amycolatopsis* sp. NCIB 40089. Fermentation conditions for their production, and methods for their isolation are described. Structures have been deduced by use of COSY and NOE NMR techniques and supported by chemical degradation studies. Both glycopeptides possessed good antibacterial activity against Gram-positive organisms.

We report on the production of two novel glycopeptide antibiotics, designated MM 55266 and MM 55268, as major components in a glycopeptide complex produced by *Amycolatopsis* sp. NCIB 40089. The structures are outlined in Fig. 1.

MM 55266 and MM 55268 both possess a lipophilic side-chain and in this respect resemble a number of other glycopeptides namely, the aridicins[1], the kibdelins[2], the parvodicins[3] and teicoplanin[4]. However, unlike these antibiotics, in MM 55266 and MM 55268 the acyl-bearing sugar is attached to amino acid 6 rather than to amino acid 4. The latter also appear to be more highly chlorinated than the aridicins and possess three attached sugars rather than two.

This paper describes the isolation, physico-chemical properties and structure determination of MM 55266 and MM 55268.

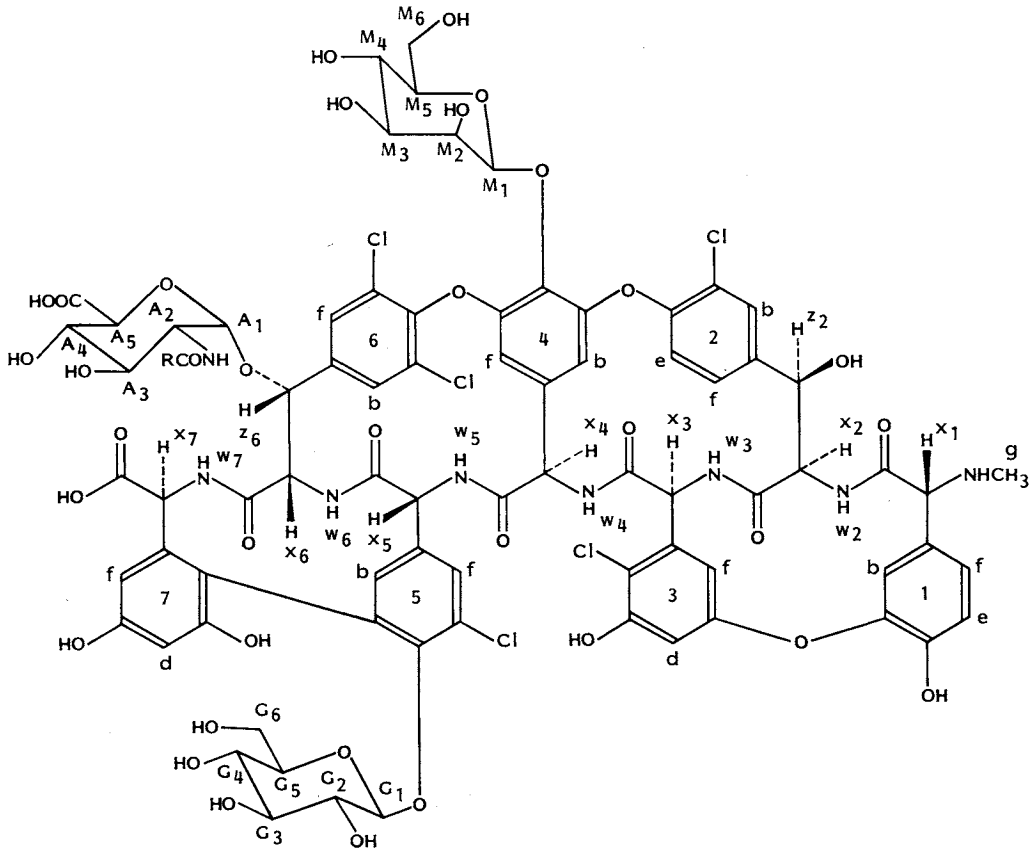
Materials and Methods

Fermentation Conditions

Amycolatopsis sp. NCIB 40089 was maintained as a vegetative cell suspension stored in glycerol 20% and lactose under liquid nitrogen 10%. Vegetative cell suspension (1 ml) was used to inoculate seed medium (100 ml) contained in a 500-ml Erlenmeyer flask. The seed stage medium consisted of soya bean flour 1.0%, glycerol 2.0%, maltose 0.2%, $CoCl_2 \cdot 6H_2O$ 0.0005%, and stock trace elements 1.0% in deionised water. The stock trace element solution contained, $CaCl_2 \cdot 2H_2O$ 1.0%, $MgCl_2 \cdot 6H_2O$ 1.0%, NaCl 1.0%, $FeCl_3$ 0.3%, $ZnCl_2$ 0.05%, $CuCl_2 \cdot 2H_2O$ 0.05%, $MnSO_4 \cdot 4H_2O$ 0.05%. The medium was adjusted to pH 7.3 before sterilisation in an autoclave at 121°C for 15 minutes. After inoculation the flask was then incubated on a gyratory shaking table at 240 rpm at 28°C. After 72 hours aliquots (4 ml) were transferred to 500-ml Erlenmeyer flasks containing seed medium (100 ml) with the same composition as above. These flasks were then incubated at 28 °C and 240 rpm for a further 48 hours.

Seed stage medium (15 litres) with the same composition as above but with the addition of 0.1% antifoaming agent (polypropylene glycol P2000) was sterilised in a 20-litre, fully baffled fermenter for 1 hour at 121°C. The second flask seed stage (400 ml) was used as inoculum and the fermentation was incubated at 28°C for 45 hours. The fermenter was stirred by an agitator, fitted with three vaned disc

Fig. 1. Structures of MM 55266 and MM 55268.



MM 55266 $R=(CH_2)_5CH(CH_3)_2$
MM 55268 $R=C_9H_{19}$

impellers, at 200 rpm and supplied with sterile air at 0.23 v/v/m and an overpressure of 0.5 bar was maintained throughout.

For the final fermentation 300 litres of medium with the same composition as the 20-litre seed stage were sterilised in a 450-litre fully baffled fermenter for 1 hour at 121 °C. Vegetative inoculum (12 litres) from the 20-litre fermenter was used as inoculum and the fermentation was incubated at 28°C until harvest at 94 hours. The fermenter was stirred by an agitator, as described above, at 100 rpm and supplied with sterile air at 0.5 v/v/m. An overpressure of air of 0.5 bar was maintained throughout.

The fermenter was harvested in 50-litre portions which were adjusted to pH 10.9 by addition of 5 M NaOH solution prior to centrifugation. The resulting supernatant was adjusted to pH 6~8 by addition of 5 M HCl.

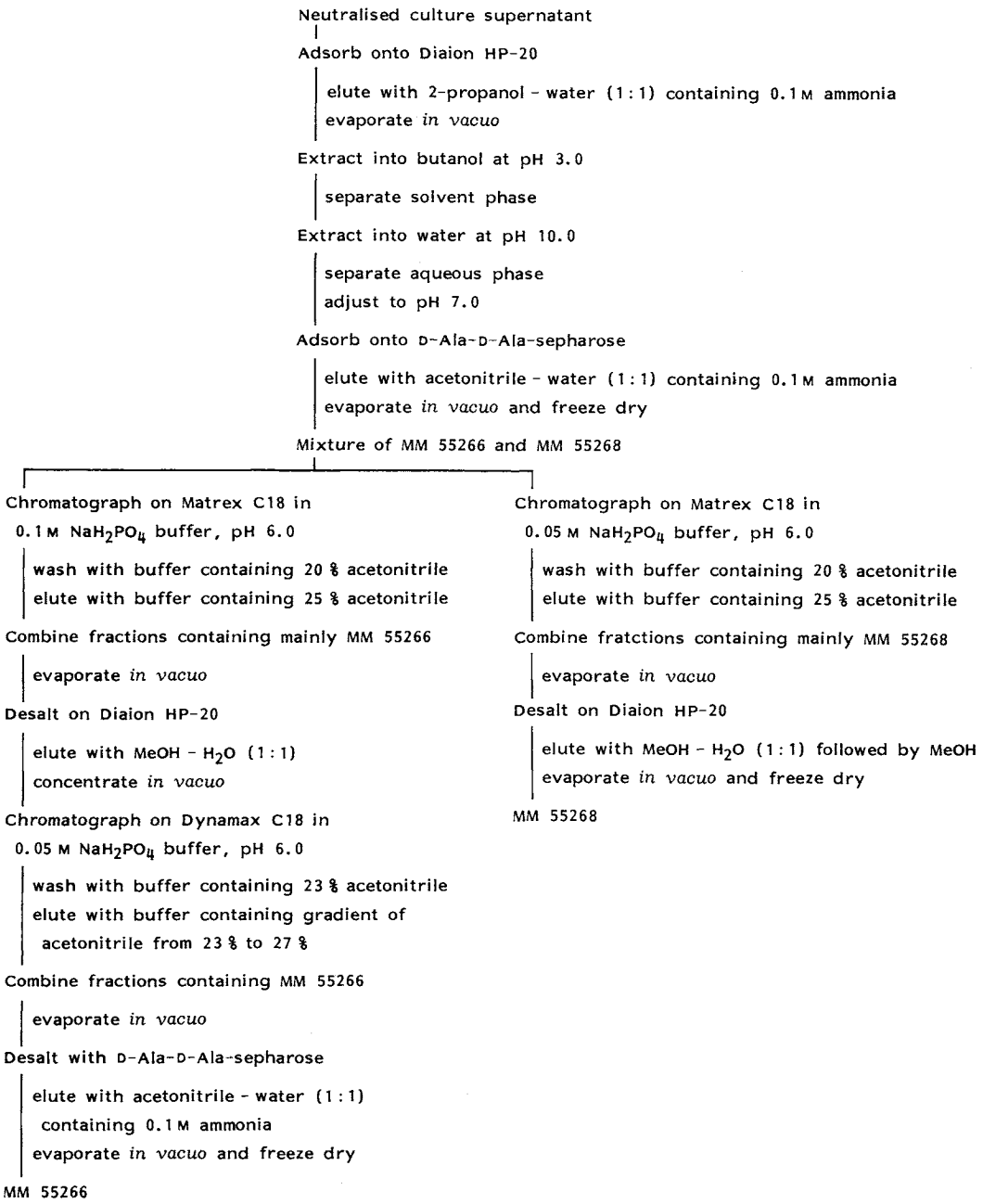
Detection Methods

Fermentation samples were monitored for antibiotic activity by the agar diffusion method using *Staphylococcus aureus* V573.

The relative titres of the glycopeptides could be monitored in the culture broth by stirring clarified broth samples with D-alanyl-D-alanine affinity resin. The adsorbed glycopeptides were eluted from the resin with 50% acetonitrile - 0.1 M ammonia. The eluate was evaporated *in vacuo* and the residue taken up in water prior to HPLC analysis.

Preparations of the glycopeptides were assayed on a Waters HPLC column (3.9 × 150 mm) containing Novapak C18 reverse phase material (Waters Associates, Milford, MA, U.S.A.) using a Waters

Fig. 2. Isolation procedure for MM 55266 and MM 55268.



600 multisolvent delivery system. Monitoring was by a Waters Lambda Max Model 481 LC spectrophotometer at 220 nm. The injection volume was 25 μl. The glycopeptides were eluted from the column with 0.1 M sodium phosphate buffer at pH 6.0 containing 25% acetonitrile at a flow rate of 2 ml/minute. Under these conditions MM 55266 had a Rt of 4.4 minutes and MM 55268 had a Rt of 9.2 minutes.

Both MM 55266 and MM 55268 have characteristic UV maxima at 280 nm. Purified samples can be assayed using direct measurement of the absorbance.

Extraction and Isolation

The following column media were used in the isolation procedure outlined in Fig. 2. Diaion HP-20: Styrene divinyl benzene cross-linked polymeric adsorbent (supplied by Mitsubishi Chemical Industries Limited, Tokyo, Japan). Matrex C18: Reverse phase silica (30 μm particles, 60Å pore diameter) (supplied by Amicon, Upper Mill, Stonehouse, Gloucestershire, UK). Dynamax 150-A preparative HPLC column, 10 × 300 mm, containing 12 μm particles of C18 reverse phase silica (supplied by Rainin Instrument Co., Woburn, MA, U.S.A).

Sepharose-D-alanyl-D-alanine was prepared by reacting the *N*-hydroxysuccinimide ester of 6-aminohexanoic acid Sepharose 4B (60 g) (supplied by Sigma, Poole, Dorset, UK) with D-alanyl-D-alanine according to general coupling procedures described by the manufacturer.

The glycopeptides were bound onto the affinity resin by stirring solutions containing MM 55266 and/or MM 55268 with a slurry of the resin for 1 hour, filtering off the resin, and washing it with water. The glycopeptides were then eluted from the affinity resin by stirring it for 5 minutes with 0.1 M ammonia in 50% aqueous acetonitrile and filtering off the eluate, which was evaporated *in vacuo* and freeze dried.

Spectroscopic Methods

NMR spectra on MM 55266 were run at 80°C on a Bruker AM 400 operating at 400 MHz in DMSO-d_6 with TMS as an internal standard. FAB-MS were obtained on a VG ZAB 1F instrument in a mixture of glycerol, thioglycerol and TFA.

Sugar Analysis

The presence of glucose and mannose in MM 55266 was confirmed by acidic hydrolysis followed by perbenzoylation and methanolysis of the sugar units in the manner of MALABARBA *et al.*[5]. The derivatised sugars were separated by silica gel chromatogaphy and analysed by ^{1}H NMR.

Results and Discussion

Isolation

Culture broth (270 litres) from *Amycolatopsis* sp. NCIB 40089, when treated as outlined in Fig. 2, afforded approximately 1 g of MM 55266 and 3.7 g of MM 55268. The adoption of a lengthy purification procedure to obtain these two antibiotics was largely aimed at removing small quantities of related antibiotics that are not reported here. We hope to publish details of minor components in due course.

Properties of MM 55266 and MM 55268

The physico-chemical properties of MM 55266 and MM 55268 are shown in Table 1 and the ^{1}H NMR data obtained on MM 55266 in Table 2. Both compounds have a UV absorption maximum of 280 nm, typical of glycopeptides.

Both glycopeptides show a Gram-positive spectrum of antibacterial activity and typical MICs are shown in Table 3. Both were marginally less active than vancomycin.

Structure Determination

The binding of MM 55266 and MM 55268 to D-alanyl-D-alanine affinity resin gave a strong indication as to their glycopeptide nature. Furthermore, hydrolysis studies on these glycopeptides followed by TLC and HPLC examination, indicated the absence of actinoidinic acid but the presence of a component co-eluting with chloroactinoidinic acid obtained from glycopeptide A41030[6]. In addition,

Table 1. Physico-chemical properties of MM 55266 and MM 55268.

	MM 55266	MM 55268
Molecular formula	$C_{86}H_{89}N_8O_{35}Cl_5$	$C_{87}H_{91}N_8O_{35}Cl_5$
FAB-MS $(M+H)^+$	1,969	1,983
UV $\lambda_{max}^{H_2O}$ nm	280 (ε 8,680)	280 (ε 8,430)
IR (KBr) cm^{-1}	1662, 1600, 1506	1661, 1595, 1505
$[\alpha]_D^{20}$	−68°	−72° (*c* 0.1, H_2O)

Table 2. ^{1}H NMR data on MM 55266 at 400 MHz and 80°C in DMSO-d_6.

NH	w_2	7.09	d, J=9.9 Hz		6b	7.80	d, J=2 Hz
	w_3	7.45	d, J=10.6 Hz		6f	7.38	d, J=2 Hz
	w_4	7.35	d, J=7.5 Hz		7d	6.41	d, J=2.0 Hz
	w_5	8.60	d, J=6 Hz		7f	6.37	d, J=2.0 Hz
	w_6	6.10	br d		1g	2.38	s
	w_7	8.12	d, J=6.4 Hz		z_2	5.15	d, J=5.1 Hz
α-CH	x_1	4.33	s		z_6	5.33	s
	x_2	5.08	m	Sugar H	G_1	5.39	d, J=7.5 Hz
	x_3	6.07	d, J=10.6 Hz		G_2	3.43	Overlapped
	x_4	5.69	d, J=7.3 Hz		G_3	3.33	Overlapped
	x_5	4.41	Overlapped		$G_4 \sim G_6$	3.7~3.0	Overlapped
	x_6	4.12	d, J=11.1 Hz		M_1	5.26	d, J=1.4 Hz
	x_7	4.53	d, J=6.2 Hz		M_2	3.92	m
ArH	1f	7.72	dd, J=9.4, 1 Hz		$M_3 \sim M_6$	3.7~3.0	Overlapped
	1e	7.37	d, J=9.4 Hz		A_1	4.41	Overlapped, J small
	1b	6.78	d, J=1 Hz		A_2	3.4	Overlapped
	2b	7.25	d, J=2 Hz		A_3	3.25	Overlapped
	2e	7.28	d, J=8.5 Hz		A_4	3.15	Overlapped
	2f	7.20	dd, J=8.5, 2 Hz		A_5	3.5	Overlapped
	3f	6.59	Overlapped		A-NH	7.50	br d
	3d	6.59	Overlapped	Side-chain		2.17	2H, m
	4b	5.81	s			1.50	2H, m
	4f	5.11	s			1.25	7H, CH_2 envelope
	5b	7.13	s			0.85	6H, d, J=6.6 Hz
	5f	6.82	d, J=2.1 Hz				

Assignments made by COSY and NOE experiments.

Table 3. Antibacterial activity of MM 55266, MM 55268 and vancomycin.

	MIC (μg/ml)		
	MM 55266	MM 55268	Vancomycin
Bacillus subtilis ATCC 6633	4	4	0.25
Corynebacterium xerosis NCTC 9755	8	4	2
Micrococcus luteus NCTC 8340	4	2	2
Staphylococcus aureus Oxford	4	4	2
S. aureus Russell	8	4	2
S. aureus V573 MR[a]	2	1	2
S. saprophyticus FL1	16	8	2
S. epidermidis 60137	8	4	2
Streptococcus pyogenes CN10	2	0.25	1
S. agalactiae Hester	4	2	1
S. sanguis ATCC 10556	4	2	2
S. faecalis I	8	4	2

[a] Multi-resistant (methicillin, tetracycline, erythromycin and gentamicin-resistant).

Tests were carried out by serial dilution in nutrient broth by microtitre. Inoculum was prepared by dilution of an overnight broth culture to give the equivalent of approx 10^6 cells/ml.

the biphenyl ether fragment obtained by hydrolysis, appeared to have similar elution properties to that isolated from aridicin A[1]. These studies gave preliminary indications as to the nature of rings 1, 3, 5 and 7 in MM 55266 and MM 55268.

Proton assignments in MM 55266 were made by a combination of COSY and NOE NMR methods (Table 2) and indicated the presence of three sugar units. It was not possible to determine the stereochemistry

of these residues entirely by NMR due to overlapping signals in the spectrum but recourse to acid hydrolysis of MM 55266 and suitable derivatisation of the carbohydrate residues confirmed that glucose and mannose were constituents of MM 55266 and unambiguous assignment of sets of signals in the 2D NMR spectrum was a facile exercise. The third sugar proved to be 2-amino-2-deoxyglucuronic acid by consideration of the connectivities and coupling constants of the related protons. The A_5 proton appeared to be coupled only to A_4 suggesting a carboxylic acid function at the C-6 position. Further evidence for this was found from a consideration of ^{13}C DEPT NMR data, only two oxygenated CH_2 groups (at δ 61.5 and 61.2) corresponding to the C-6 of glucose and mannose units being evident in the spectrum. Furthermore, isoelectric focusing experiments established a pI of approximately 4.9 for MM 55266 and MM 55268, closely matching that of aridicin[1] which also features the same acidic sugar.

The points of attachment of the three sugar units to the heptapeptide nucleus and also the positioning of the acyl side-chain on the amino sugar were determined by a consideration of NOE effects. Thus, the anomeric proton of the amino sugar, assigned to signal at δ4.41 in the NMR spectrum of MM 55266 showed not only the expected shielding from aromatic ring 6[7], but also large negative NOE effects to the z_6 and 6f resonances as well as to the 'NOE nest'[8] associated with amino acids 5, 6 and 7. Furthermore, the CH_2 adjacent to the acyl carbonyl in the side chain showed NOE effects to the amide NH of the amino sugar and also to other resonances such as z_6 and 6f, clearly positioning the acylated amino sugar at the benzylic position of amino acid 6. It was similarly shown by NOE experiments that the anomeric proton of mannose was in close proximity to aromatic proton 2e, placing mannose on ring 4. Unexpectedly, no NOE effect was seen between this anomeric proton and position 6c indicating that this position may be chlorinated, a fact which emerged later on detailed analysis of the 2D spectrum. The point of attachment of the glucose moiety to the glycopeptide nucleus is not so clear. Irradiation of the anomeric proton, G_1, failed to indicate any NOE elsewhere in the molecule suggesting attachment of the sugar unit to a phenolic hydroxyl with substituted *ortho* positions. The only phenolic group to fulfil this criterion is positioned at 5d.

The C-1~C-2 proton coupling constants (Table 2) for the three sugars was indicative of the stereochemistry of the linkage between the sugars and the heptapeptide nucleus and a consideration of these led to the conclusion that glucose and mannose were present as β-anomers but that the acylated amino sugar was α-linked since the coupling constant was small.

The NMR spectrum of ristocetin in DMSO is reported to show NOE effects between x_3 and both 3b and 3f due to oscillatory motion[9]. In the spectrum of MM 55266 only one such enhancement is observed providing further evidence for chlorination at position 3b.

The structure of the acyl side-chain in MM 55266 was readily deduced by a consideration of the ^1H NMR spectrum (Table 2) which clearly indicated terminal branching.

MM 55268 exhibited an almost identical ^1H NMR spectrum to MM 55266. The only differences observed related to the branching in the acyl group. Specifically, two methyl signals were observed at δ_H 0.75 (t), δ_C 10.9 and δ_H 0.75 (d), δ_C 19.0 indicating branching at a non-terminal position. The side-chain present in MM 55268 was clearly longer by one C atom than that present in MM 55266, as judged by FAB-MS.

Acknowledgements

The authors wish to thank M. GWYNN and M. LEYTON for antibacterial data, C. BURBIDGE for large scale fermentation, J. TYLER and J. HART for NMR studies and G. RISBRIDGER and D. TOLSON for mass spectral data.

We also express our thanks to D. H. WILLIAMS of the University of Cambridge for helpful discussions.

References

1) CHUNG, S. K.; P. TAYLOR, Y. K. OH, C. DEBROSSE & P. W. JEFFS: Biosynthetic studies of aridicin antibiotics. I. Labeling patterns and overall pathways. J. Antibiotics 39: 642~651, 1986
2) FOLENA-WASSERMAN, G.; B. L. POEHLAND, E. W-K. YEUNG, D. STAIGER, L. B. KILLMER, K. SNADER, J. J. DINGERDISSEN & P. W. JEFFS: Kibdelins (AAD-609), novel glycopeptide antibiotics. II. Isolation, purification and structure. J. Antibiotics 39: 1395~1406, 1986
3) CHRISTENSEN, S. B.; H. S. ALLAUDEEN, M. R. BURKE, S. A. CARR, S. K. CHUNG, P. DEPHILLIPS, J. J. DINGERDISSEN, M. DIPAOLO, A. J. GIOVENELLA, S. L. HEALD, L. B. KILLMER, B. A. MICO, L. MUELLER, C. H. PAN, B. L. POEHLAND, J. B. RAKE, G. D. ROBERTS, M. C. SHEARER, R. D. SITRIN, L. J. NISBET & P. W. JEFFS: Parvodicin, a novel glycopeptide from a new species, *Actinomadura parvosata*: Discovery, taxonomy, activity and structure elucidation. J. Antibiotics 40: 970~990, 1987
4) 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
5) 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
6) EGGERT, J. H. & K. H. MICHEL: Isolation and characterization of A41030, a complex of novel glycopeptide antibiotics. Application of the Michel-Miller high performance low pressure liquid chromatography system. J. Antibiotics 39: 792~799, 1986
7) ANG, S.; M. P. WILLIAMSON & D. H. WILLIAMS: Structure elucidation of a glycopeptide antibiotic, OA-7653. J. Chem. Soc. Perkin Trans. I 1988: 1949~1956, 1988
8) 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
9) WALTHO, J. P. & D. H. WILLIAMS: Aspects of molecular recognition: Solvent exclusion and dimerization of the antibiotic ristocetin when bound to a model bacterial cell-wall precursor. J. Am. Chem. Soc. 111: 2475~2480, 1989