MM 47761 AND MM 49721, GLYCOPEPTIDE ANTIBIOTICS PRODUCED BY A NEW STRAIN OF *AMYCOLATOPSIS ORIENTALIS*

ISOLATION, PURIFICATION AND STRUCTURE DETERMINATION

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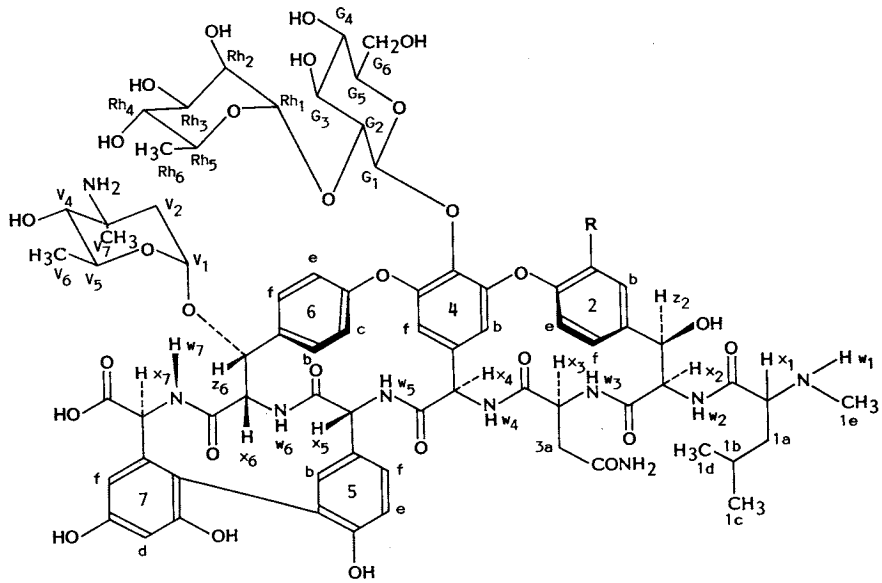
Two glycopeptide antibiotics MM 47761 and MM 49721 have been isolated from *Amycolatopsis orientalis* NCIB 12608. Fermentation conditions for their production, and methods for their isolation are described. The metabolites have been characterised by physico-chemical and biological properties and the structure determined by a combination of chemical degradation, COSY and NOE NMR studies. Both metabolites showed good antibacterial activity against Gram-positive organisms.

During a programme of screening soil microorganisms for antibiotics, the production of two glycopeptide antibiotics, designated MM 47761 and MM 49721, has been detected. The antibiotics were produced by *Amycolatopsis orientalis* NCIB 12608.

MM 47761 and MM 49721 are novel members of the vancomycin series of glycopeptide antibiotics and their structures are shown in Fig. 1. They differ only in chlorine content and contain the sugars 4-*epi*-vancosamine, glucose and rhamnose. Related antibiotics such as A82846[1] and eremomycin[2] have been reported by other groups but these differ in the constitution of the disaccharide unit attached to amino acid 4 of the heptapeptide nucleus. A42867 reported by Lepetit workers[3] appears to be similar in

Fig. 1. Structures of MM 47761 and MM 49721.



MM 47761 R=Cl
MM 49721 R=H

structure to MM 47761, but contains vancosamine rather than 4-*epi*-vancosamine.

This paper describes the isolation, physico-chemical properties and structure determination of MM 47761 and MM 49721.

Materials and Methods

Fermentation Conditions

A. orientalis NCIB 12608 was maintained on agar slopes consisting of yeast extract 0.4%, malt extract 1.0%, glucose 0.4%, agar 2.0% in deionised water pH 7.3. After inoculation, slopes were incubated at 28°C for 7 days before use.

A suspension of spores and mycelium (10 ml) in sterilised water containing 0.005% Triton X-100 (BDH Chemicals Ltd., Poole, UK) was prepared from an agar slope contained in a Universal bottle, and a portion (1 ml) used to inoculate the seed stage 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 7.0 before sterilisation in an autoclave at 121°C for 15 minutes. The inoculated flasks were incubated on a gyratory shaking table at 240 rpm for 48 hours at 28°C.

15 litres of seed stage medium, 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. 200 ml of the flask seed stage were used as inoculum and the fermentation was incubated at 26°C for 48 hours. The fermenter was stirred by an agitator, fitted with three, vaned disc impellers, at 200 rpm and supplied with sterile air at 0.5 vol/vol/minute. An overpressure of air 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. 8 litres of vegetative inoculum from the 20-litre fermenter were used as inoculum and the fermentation was incubated at 26°C until harvest at 72 hours. The fermenter was stirred by an agitator fitted with three, vaned-disc impellers at 50 rpm and supplied with sterile air at 0.5 vol/vol/minute. An overpressure of air of 0.5 bar was maintained throughout.

The harvested broth was clarified by centrifugation.

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 absorbed glycopeptides were eluted from the resin with 50% aqueous acetonitrile - 0.1 M ammonia. The eluate was evaporated *in vacuo* and the residue taken up in water prior to HPLC analysis.

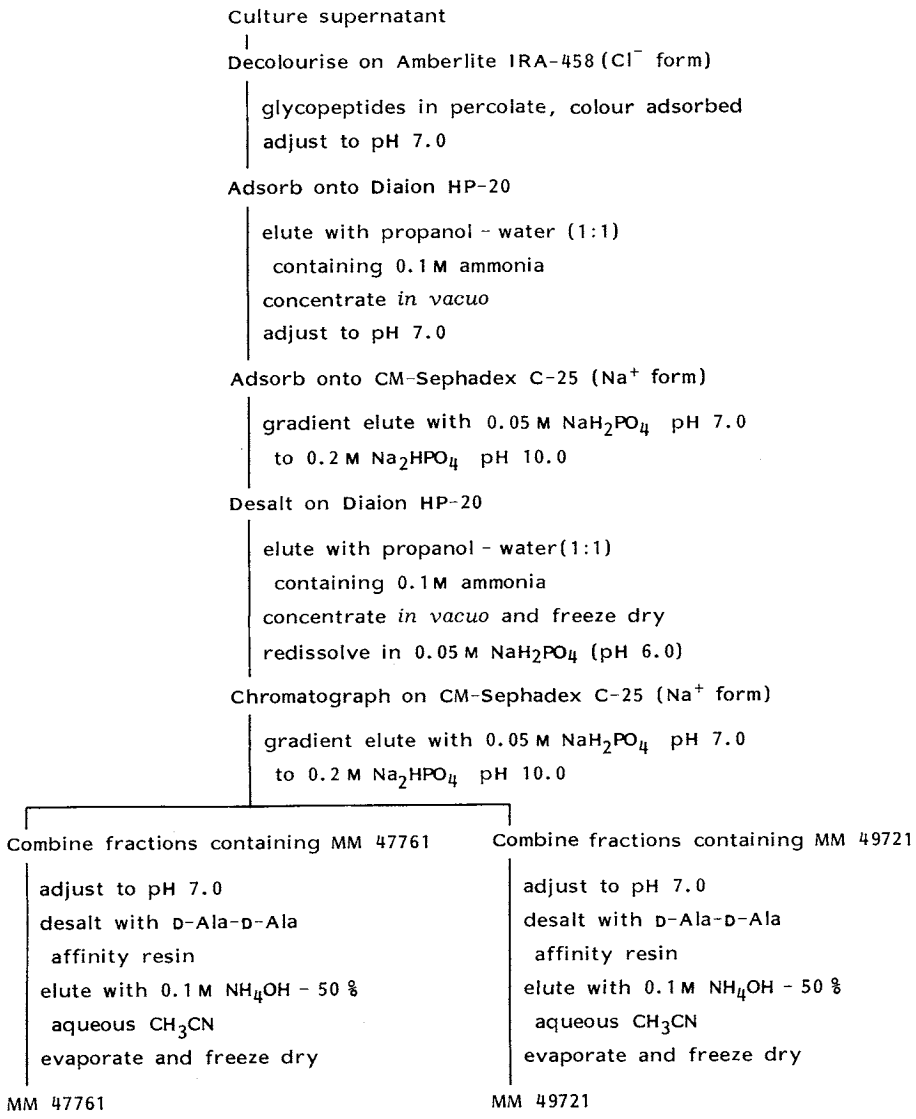
Preparations of the glycopeptides were assayed on a Waters HPLC column (3.9 × 300 mm) containing μBondapak C18 reverse phase material (Waters Associates, 34 Maple Street, Milford, Mass. U.S.A.), using a Waters Associates Model 6000A solvent delivery system. Monitoring was by a Cecil Model CE2112 UV spectrophotometer, (Cecil Instruments, Cambridge, UK), at 210 nm. The injection volume was 20 μl. The glycopeptides were eluted from the column with 0.1 M sodium phosphate buffer pH 6.0, containing 10% acetonitrile at a flow rate of 2 ml/minute. The eluate was monitored at 210 nm. Under these conditions MM 47761 had a Rt of 4.6 minutes and MM 49721 had a Rt of 5.8 minutes (*cf.* vancomycin Rt 11.4 minutes).

Extraction and Isolation

The following column media were used in the isolation procedure outlined in Fig. 2.

Amberlite IRA-458: Acrylic based strongly basic anion exchange resin (supplied by ROHM and HAAS, Croydon, UK). Diaion HP-20: Styrene di-vinyl benzene cross-linked polymeric adsorbent (supplied by

Fig. 2. Isolation procedure for MM 47761 and MM 49721.



Mitsubishi Chemical Industries Limited, Tokyo, Japan). CM-Sephadex C-25: Weakly acidic cation exchange dextran gel (supplied by Pharmacia Fine Chemicals, Uppsala, Sweden).

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, UK) with D-alanyl-D-alanine (1.5 g) according to general coupling procedures described by the manufacturer.

The glycopeptides were bound onto the affinity resin by stirring solutions containing MM 47761 and MM 49721 with a slurry of the resin for 30 minutes, filtering off the resin and washing the resin with water. The glycopeptides were then eluted from the affinity resin by stirring the resin 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 were run at 100°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 1 U of 4-*epi*-vancosamine[4] in MM 47761 was confirmed by perbenzoylation, followed by acidic methanolysis and isolation of the sugar derivatives by silica gel chromatography in the manner of MALABARBA *et al.*[5]. Glucose and rhamnose were not detected by this means but an alternative procedure involving prior acidic hydrolysis of the glycopeptide followed by the perbenzoylation method afforded benzoylated glucose and rhamnose.

Results and Discussion

Isolation

The titres of MM 47761 and MM 49721 in tank fermentations were generally in the ratio of 95 : 5, respectively.

The glycopeptides bound strongly to the Diaion HP-20 resin and an increase in pH as well as a decrease in polarity was necessary to elute the products in high yield.

The dual ionic strength/pH gradient used to elute the CM-Sephadex cation exchange columns, gave a linear rise in pH as the column developed. The gradient gave good chromatographic separation of the two glycopeptides both from each other and from impurities.

Properties of MM 47761 and MM 49721

The physico-chemical properties of MM 47761 and MM 49721 are shown in Table 1, and the ^1H NMR data 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 less active than vancomycin limiting the chemotherapeutic utility of these compounds. They were not significantly active against a range of Gram-negative organisms.

Structure Determination

Preliminary data obtained on MM 47761 and MM 49721 such as UV absorption and binding to D-alanyl-D-alanine affinity resin, suggested that they were glycopeptide antibiotics. More specifically, the presence of aspartic acid and *N*-methylleucine in the acid hydrolysates indicated that they were related to vancomycin.

Proton assignments in MM 47761 were made by a combination of COSY and NOE NMR methods (Table 2) and indicated, in addition to 4-*epi*-vancosamine[4] and rhamnose, the presence of a third sugar which appeared to be fully oxygenated. It was not possible to determine the stereochemistry of this residue by NMR alone due to overlapping signals in the spectrum. However, recourse to acid hydrolysis of MM 47761 and suitable derivatisation of the carbohydrate residues confirmed that glucose, 4-*epi*-vancosamine and rhamnose were all constituents of MM 47761. The points of attachment of these to the heptapeptide nucleus were determined

Table 1. Physico-chemical properties of MM 47761 and MM 49721.

	MM 47761	MM 49721
Molecular formula	$C_{72}H_{86}N_9O_{28}Cl$	$C_{72}H_{87}N_9O_{28}$
FAB-MS (MH^+)	1,560	1,526
UV (H_2O) nm	280 (ε 6,403)	280 (ε 5,844)
IR (KBr) cm^{-1}	1653, 1587, 1501	1653, 1587, 1500
$[\alpha]_D^{20}$	−79° (*c* 1% in H_2O)	
Acid hydrolysis	Aspartic acid, *N*-methylleucine	Aspartic acid, *N*-methylleucine

Table 2. ^{1}H NMR data on MM 47761 at 400 MHz and 100°C in DMSO-d_6.

NH	w_7	8.36	d, J=6.1 Hz		1a	1.44	m
		7.98	d, J=6.0 Hz			1.55	m
α-CH	x_1	3.03	dd, J=7.0 and 6.5 Hz		1b	1.80	m
	x_2	4.73	overlapped		1c	0.94	d, J=6.6 Hz
	x_3	4.37	br		1d	0.92	d, J=6.6 Hz
	x_4	5.70	br		z_2	5.16	d, J=3.2 Hz
	x_5	4.54	overlapped		3a	2.61	dd, J=15.7 and 3.6 Hz
	x_6	4.24	br			2.21	dd, J=15.7 and 7.1 Hz
	x_7	4.54	overlapped		z_6	5.26	s
ArH	2b	7.41	br s	Sugar H	G_1	5.54	
	2e	7.26	d, J=8.4 Hz		$G_2 \sim G_6$	3.3~3.8	overlapping m
	2f	7.55	dd, J=8.4 and 1.0 Hz		Rh_1	5.18	s
	4b	5.69	s		Rh_2	3.78	m
	4f	5.43	s		Rh_3	*ca.* 3.5	overlapping
	5b	7.15	br s		Rh_4	3.25	dd, J=9.3 and 9.3 Hz
	5e	6.73	d, J=8.4 Hz		Rh_5	4.11	m
	5f	6.82	dd, J=8.4 and 2.1 Hz		Rh_6	1.10	d, J=6.1 Hz
	6b	7.66	dd, J=7.7 and 1.0 Hz		V_1	4.73	overlapping
	6c	7.06	dd, J=8.4 and 1.0 Hz		V_2	1.98	br m
	6e		v br			1.72	br dd
	6f	7.33	br d		V_4	2.96	d, J=9.4 Hz
	7d	6.40	s		V_5	3.68	dq, J=9.2 and 6.0 Hz
	7f	6.40	s		V_6	1.21	d, J=6.0 Hz
	1e	2.34	s		V_7	1.21	s

Assignments made by COSY and NOE experiments.

Table 3. Antibacterial activity of MM 47761 and MM 49721.

Organsim	MIC (μg/ml)		
	MM 47761	MM 49721	Vancomycin
Bacillus subtilis ATCC 6633	1	2	1
Corynebacterium xerosis NCTC 9755	1	2	2
Micrococcus luteus NCTC 8340	2	4	1
Staphylococcus aureus Oxford	1	1	1
S. aureus Russell	8	8	4
S. aureus V573 MR[a]	8	8	4
S. saprophyticus FL1	4	8	
S. epidermidis 60137	2	4	2
S. epidermidis 54815	4	8	4
Streptococcus pyogenes CN10	0.25	1	1
S. agalactiae Hester	0.50	2	1
S. sanguis ATCC 10556	4	8	2
S. faecalis I	0.50	1	1

[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.

by NOE experiments. Thus, the anomeric proton of glucose, assigned to a signal at δ 5.54 in the spectrum of MM 47761 showed a large negative NOE effect to the 2e and 6c resonances, clearly positioning the glucose residue on ring 4. A similar effect was noted between the anomeric proton of rhamnose and G_2 demonstrating the nature of the disaccharide linkage between rhamnose and glucose. The anomeric proton of 4-*epi*-vancosamine was observed at unusually high field (δ 4.73) for an α-glycosidic linkage and suggested impingement on the shielding zone of an aromatic nucleus. A similar effect has been noted[6] in

other cases where the sugar in question was situated at the benzylic position of amino acid 6. That this was the case in MM 47761 also was readily shown by irradiation of z_6 whereupon negative NOE was observed not only in the protons involved in the 'NOE nest'[7] (*i.e.* 6b, x_5, 5b, x_6 and w_7) but also to the anomeric proton of 4-*epi*-vancosamine. The aromatic proton, 6b, appears as a double doublet ($J=7.7$ and 1.0 Hz) in the NMR spectrum and therefore cannot be adjacent to chlorine. The positioning of the chlorine atom in ring 2 was deduced from irradiation of the hydroxytyrosine β-proton, z_2. An NOE effect to two other protons was noted, an aromatic singlet, 2b, clearly *ortho* to chlorine, and the adjacent amino acid α-proton, x_2.

A recent report by the WILLIAMS' group[8] refers to a number of unusually broad signals namely; 6e, 6f, x_4 and x_3, in the NMR spectrum of ristocetin in DMSO-d_6. A theory has been proposed to explain this phenomenon in terms of a back-to-back dimerisation of the ristocetin molecule which leaves the binding faces of the glycopeptide free to interact will cell-wall precursors in the usual way. It would appear that a similar situation is occuring in MM 47761 where broad resonances are observed for 6f, x_4 and x_3 and again the position of the 6e resonance cannot be ascertained with certainty presumably because of excessive line-broadening. In addition, the V_2 protons in MM 47761 are noticeably broadened in contrast to the sharpness of all other carbohydrate signals, suggesting that they experience different environments in the monomer and dimer forms. Fast exchange between these states would be expected at 100°C, the temperature of the experiment, and this could explain the broadness of the lines. Evidence that the V_2 protons are involved in dimer formation can be drawn from the ristocetin case[8] where the corresponding protons in ristosamine show an intermolecular NOE to 2c on dimer formation.

MM 49721 gave a molecular ion 34 mass units down from that of MM 47761 indicating that it was likely to be a dechloro derivative. Further evidence was obtained from ^{1}H NMR, in particular the greater complexity of the aromatic region and the absence of the singlet at δ 7.41, corresponding to proton 2b in the spectrum of MM 47761. In other respects the two spectra were similar.

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References

1) NAGARAJAN, R.; D. M. BERRY, A. H. HUNT, J. L. OCCOLOWITZ & A. A. SCHABEL: Conversion of antibiotic A82846B to orienticin A and structural relationships of related antibiotics. J. Org. Chem. 54: 983~986, 1989
2) GAUSE, G. F.; M. G. BRAZHNIKOVA, N. N. LOMAKINA, T. F. BERDNIKOVA, G. B. FEDOROVA, N. L. TOKAREVA, V. N. BORISOVA & G. Y. BATTA; Eremomycin—new glycopeptide antibiotic: Chemical properties and structure. J. Antibiotics 42: 1790~1799, 1989
3) RIVA, E.; L. GASTALDO, M. G. BERETTA, P. FERRARI, L. F. ZERILLI, G. CASSANI, E. SELVA, B. P. GOLDSTEIN, M. BERTI, F. PARENTI & M. DENARO: A42867, A novel glycopeptide antibiotic. J. Antibiotics 42: 497~505, 1989
4) HUNT, A. H.; R. M. MOLLOY, M. DEBONO & J. L. OCCOLOWITZ: Isolation and characterization of 4-*epi*-vancosamine. Tetrahedron Lett. 29: 1223~1226, 1988
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) ANG, S.-G.; M. P. WILLIAMSON & D. H. WILLIAMS: Structure elucidation of a glycopeptide antibiotic, OA-7653.

J. Chem. Soc. Perkin Trans. I. 1988: 1949~1956, 1988

7) 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

8) 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