Structure Elucidation of a Glycopeptide Antibiotic, OA-7653

Siau-Gek Ang, Michael P. Williamson, and Dudley H. Williams*
University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW

The structure of a vancomycin-type antibiotic, OA-7653, isolated from *Streptomyces hygroscopicus* subsp. *hiwasaensis* subsp. nov. Nishida, has been elucidated by a combination of classical chemical methods, mass spectrometry, and nuclear magnetic resonance spectroscopy. The interaction of OA-7653 with the peptide cell wall analogues *N*-acetyl-D-alanyl-D-alanine, and di-*N*-acetyl-L-lysyl-D-alanyl-D-alanine, in solution in aqueous dimethyl sulphoxide, has been examined by n.m.r. and u.v. difference spectroscopy.

A Japanese group at the Laboratory of Fermentation Research, Otsuka Pharmaceutical Co. Ltd., reported a new antibiotic produced by *Streptomyces* sp. No. FB-5, which was isolated from soil collected near Hiwasa-cho, Tokushima Prefecture, in Japan.[1] This antibiotic, referred to as OA-7653, inhibited bacterial cell wall synthesis, and its chemical properties showed that it belongs to the glycopeptide family of antibiotics, like vancomycin.[2]

Of all the known members of this family of glycopeptides, OA-7653 appears to be most analogous to vancomycin, primarily because they both contain only glucose as the neutral carbohydrate component. OA-7653 also shows some resemblances to other glycopeptide antibiotics like ristocetin,[3] the azureomycins,[4] A35512B,[5] and teicoplanin.[6] However, while the isoelectric point of OA-7653 is acidic (P_i = 5—6),[1] it is neutral or basic for the others. This, together with t.l.c. analysis, amino acid analysis, and optical rotation results, confirmed that OA-7653 is a new antibiotic.

We began our studies on OA-7653 with the information that it contains L-glutamic acid and D-glucose.[1] In determining the structure of OA-7653, we have used a combination of chemical degradation, ^{1}H and ^{13}C n.m.r., and mass spectrometric studies.

Results and Discussion

Fast Atom Bombardment (F.a.b.) Mass Spectral Studies.—Analytical h.p.l.c. was used to assess the purity of the sample of OA-7653 obtained from the Japanese workers. In the presence of 20mM ammonium acetate, OA-7653 was successfully separated into two components, the one eluted later (the major component) being designated OA-7653 A and the other OA-7653 B.

F.a.b. mass spectral studies showed the molecular weights of OA-7653 A and B to be 1 274 and 1 275, respectively. The presence of two chlorine atoms in each component was suggested by the observed isotope pattern of the molecular ion cluster, accompanied by characteristic fragmentations in both the positive and negative mode f.a.b. mass spectra.

Amino acid analysis carried out on a sample of OA-7653 before separation confirmed the presence of a glutamine (Gln) or a glutamic acid (Glu) residue. Esterification of OA-7653 A with a solution of HCl in methanol resulted in both esterification of the carboxy terminus and methanolysis of a primary amide group (f.a.b. mass spectrometry showed ions with increases in mass of 14 and 29 u). Analytical h.p.l.c. and f.a.b. mass spectral studies also showed that OA-7653 A is converted into OA-7653 B in 0.1M HCl. This suggested that in this conversion the amide group of Gln was hydrolysed, but it was not clear if there were any other structural differences between the two components.

Mild hydrolysis of OA-7653 (following the procedure used in converting vancomycin into aglucovancomycin[7]) gave rise to incomplete removal of the sugar, even on prolonging the reaction, unlike the case of vancomycin in which removal of the disaccharide went cleanly to give aglucovancomycin. This suggests that the glucose in OA-7653 is not attached to a phenolic oxygen.

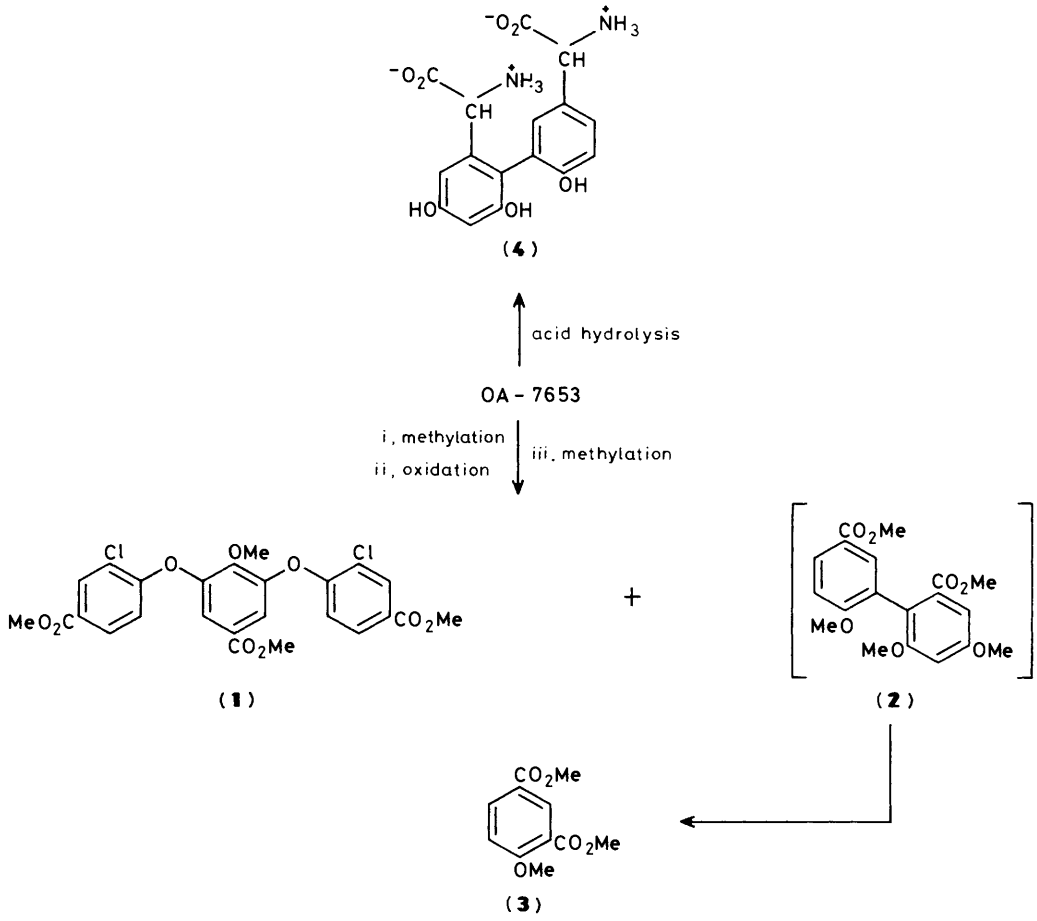
Chemical Degradation Studies.—Acid hydrolytic and oxidative degradation reactions were used to identify the aromatic moieties present in OA-7653. The fragments isolated account for the presence of five different aromatic rings (Scheme 1).

A sample of OA-7653 was methylated, oxidised, then esterified according to the procedure reported by Roberts *et al.*[8] The mixture of products was then separated by preparative t.l.c. and analysed by electron impact mass spectrometry. Two major products were isolated and were found to have molecular weights of 534 and 224. In the light of this information, subsequent ^{1}H n.m.r. studies on the intact antibiotic, and the fact that the same products have been isolated and identified among the oxidation products of vancomycin, the two products are identified as (**1**) and (**3**), respectively (Scheme 1). The isolation of dimethyl 4-methoxyisophthalate (**3**) provides evidence for the presence of the biphenyl moiety (**2**) in OA-7653. This was further substantiated by the isolation of the mono- and di-methyl esters of the di-*N*-acetyl derivative of (**4**), the bis(amino acid) (**4**) being obtained on acid hydrolysis of OA-7653 (Scheme 1).

The isolation of the triester (**1**) confirms the presence of two organic chlorine atoms. It also indicates that the glucose moiety of OA-7653 is not attached to the phenolic oxygen of the triphenyl diether portion of the antibiotic. To confirm this, the entire oxidative degradation sequence was repeated, this time with CD_3I in the first step to give amongst other products, a compound with molecular weight 537 as detected by e.i. mass spectrometry. The incorporation of a CD_3 group confirmed the presence of a free phenolic group on the triphenyl diether portion of the antibiotic.

Linkage of the Glu/Gln *Residue.*—The nature of the Gln residue was checked by subjecting OA-7653 A to a Hofmann rearrangement reaction with (diacetoxyiodo)benzene after protection of the phenolic groups with diazomethane. Gas chromatography was then carried out on *N*-trifluoroacetyl *O*-isopropyl derivatives of the total acid hydrolysate, comparing retention times with suitable standards. The appearance of an amino acid with the same retention time as 2,4-diaminobutyric acid indicated that the residue is Gln and not iso-Gln. G.l.c.–mass spectrometry performed on the ester derivatives confirmed the presence of 2,4-diaminobutyric acid in the hydrolysate.

The linkage of the Glu residue (α or γ) in OA-7653 B was investigated using a published method[9] (treatment with acetic anhydride in aqueous pyridine) with the modification that D_2O was used instead of T_2O. The reaction selectively deuteriates an



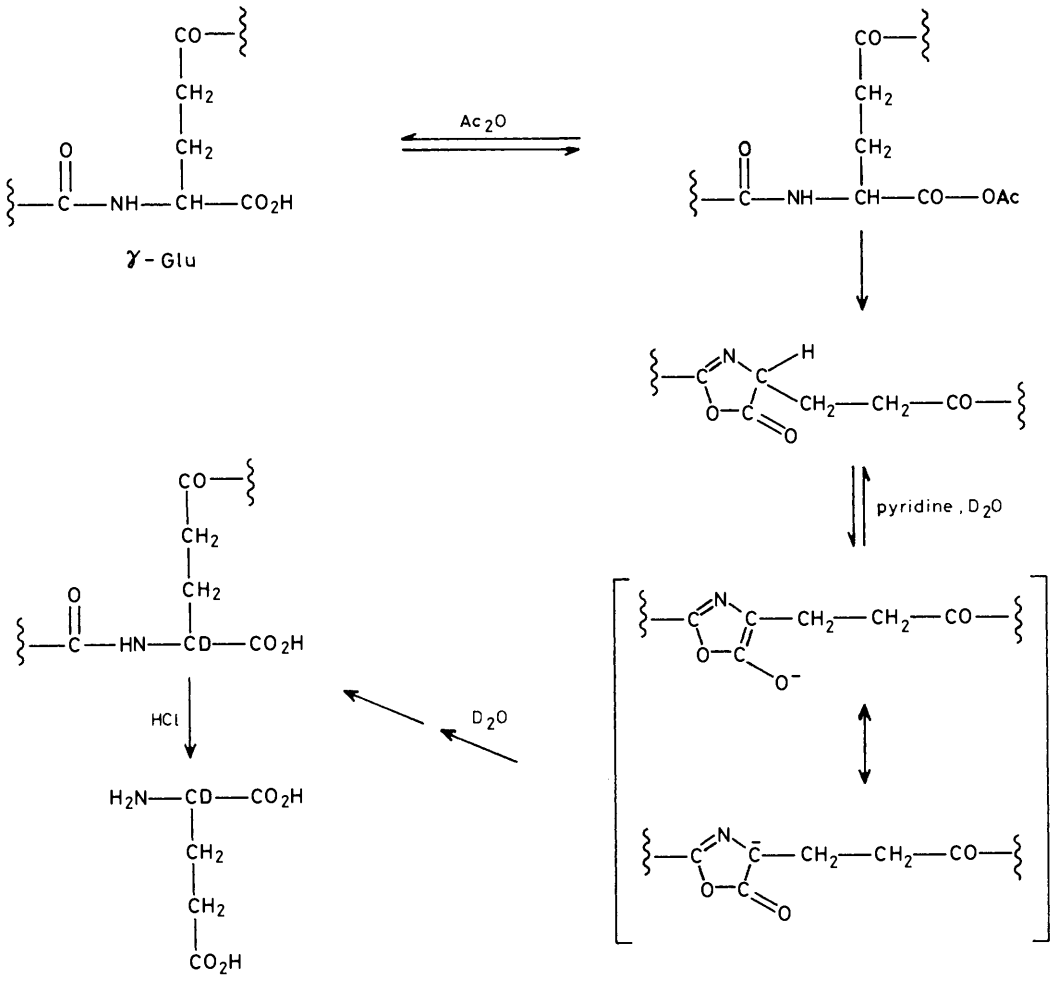
Scheme 1. Chemical degradation products of OA-7653

amino acid residue containing a free α-carboxy group (Scheme 2). The Glu residue in OA-7653 was not deuteriated, whereas a commercial sample of L-glutamic acid was successfully deuteriated. This suggests that the Glu residue is linked to the rest of the peptide backbone through the α-carboxy group. Confirmation of the α-Glu linkage was also obtained from ^{1}H n.m.r. studies in which the secondary amide proton in Glu was shown to be close to the amide protons of its two neighbouring amino acid residues.

N.m.r. Studies.—Most n.m.r. work on the structure of OA-7653 has been carried out on OA-7653 A, shown to be the major component by h.p.l.c. The ^{1}H n.m.r. spectrum of OA-7653 A is reproduced in Figure 1. The approach adopted in the assignment of the spectrum was based on that used previously for vancomycin,[2] ristocetin,[3] and teicoplanin,[10] using decoupling and nuclear Overhauser effect (n.O.e.) experiments. In addition, COSY and NOESY were also used to confirm many of the ^{1}H assignments. The derived structure is shown in Figure 2 and the proton assignments are in Table 1, together with the chemical shifts and coupling constants of analogous protons in vancomycin. We have observed that the patterns of n.O.e.s for the nest of protons on the left of the molecule (Figure 2; protons z_6, 6b, x_6, x_5, 5b, and w_7) are very similar to those for vancomycin[11] and ristocetin.[3] In fact, the chemical shifts of x_5, x_6, x_7, 5b, 5e, 5f, 6b, 6e, 6f, 7d, and 7f agree to within 0.2 p.p.m. with those of vancomycin. It appears therefore that the stereochemistries of residues 5, 6, and 7 are the same as those of analogous asymmetric centres in vancomycin.

In the course of establishing the nature of the aromatic moieties present in OA-7653 by oxidative degradation reactions, it was observed that the glucose residue is not attached to the phenolic oxygen of ring 4, where the disaccharide in vancomycin is located.[12] An n.O.e. between z_6 and the glucose anomeric proton G_1 clearly indicated the attachment of the sugar through the benzylic oxygen atom on the left-hand side of the molecule (Figure 2). The anomeric proton G_1 also showed an n.O.e. to G_2 indicating that the glucose is attached as the α-anomer. This is further supported by the magnitude of the coupling constant between G_1 and G_2 [$J(G_1G_2)$ 2.9 Hz].

In the course of assigning the sugar protons, coupling was observed between protons resonating at δ 2.79 and 3.36; both protons were coupled to x_2, which thus gives rise to an octet. The chemical shifts of this pair of protons are comparable with those of a similar pair in teicoplanin ψ-aglycone (δ 2.84 and 3.31) which shows a similar coupling pattern.[10] The DEPT ^{13}C n.m.r. spectrum of OA-7653 showed a methylene group at δ 36, similar to the value (δ 37) for a methylene group in teicoplanin. The resonances at δ 2.79 and 3.36 are thus assigned to a pair of methylene protons z_2 and z_2'. Earlier work[11] had shown that under the conditions used in the present studies, two protons would be about 2.0—2.7 Å apart if the half-lives for the exponential build-up of their mutual n.O.e.s lie in the range 0.2—1 s. Without actually calculating interproton distances, we were able to compare proton proximities by the observation of such kinetic n.O.e.s. The assignments of z_2 and z_2' could thus be confirmed through the use of time-dependent n.O.e.s.



Scheme 2. Labelling of C-terminal amino acids or γ-Glu in peptides

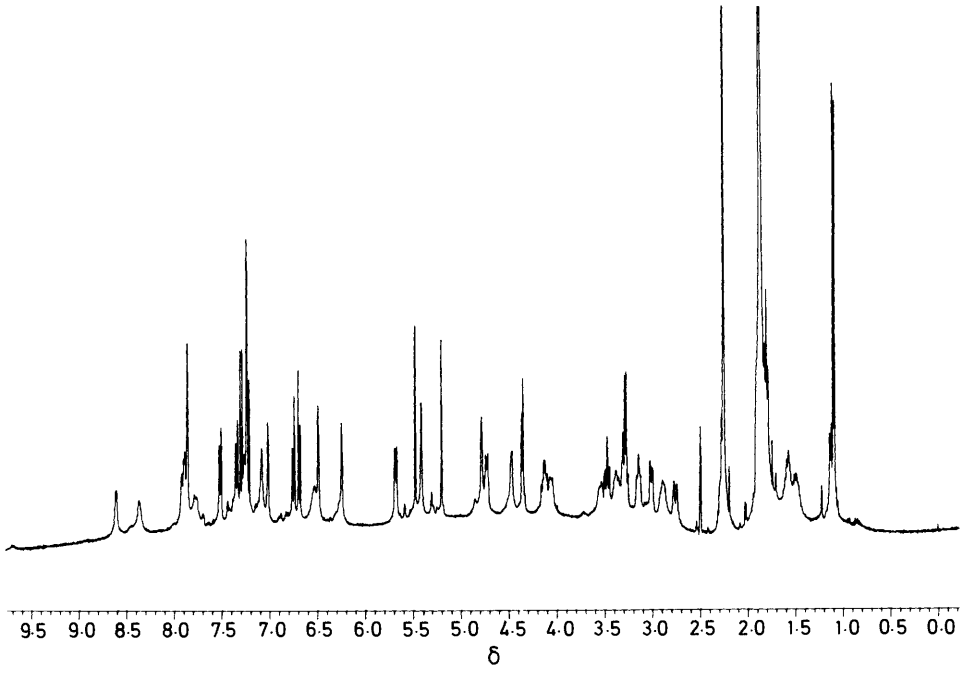


Figure 1. 400 MHz ^{1}H n.m.r. spectrum of OA-7653 A in $(CD_3)_2SO$ at 20 °C

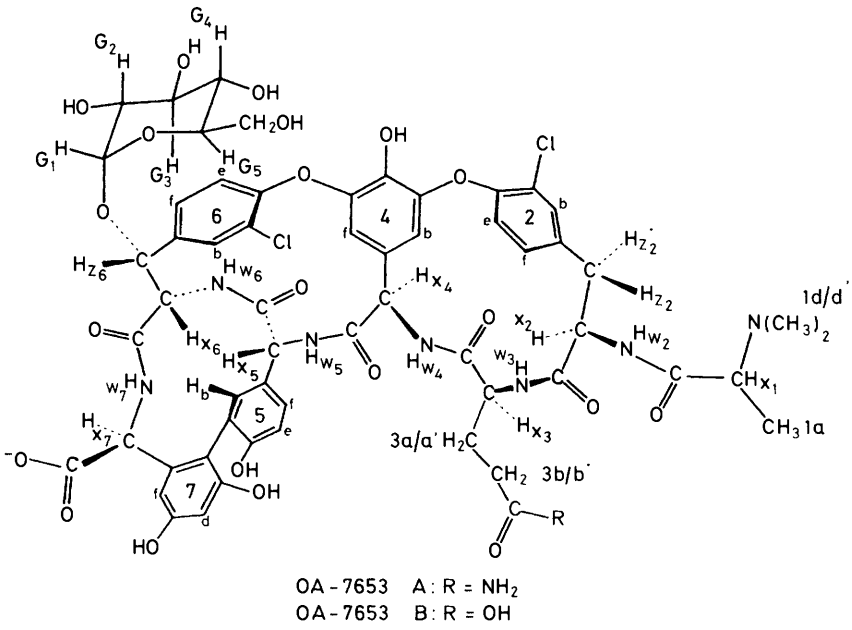


Figure 2. Structure of OA-7653, showing the proton designations

The identification of the methylene group on residue 2 of the antibiotic facilitated the determination of the N-terminus of the peptide chain in OA-7653. In the ^{1}H n.m.r. spectrum of both OA-7653 A and B, an intense peak was observed at δ 2.30. The spectrum also showed a methyl doublet at δ 1.14 coupled to a proton resonating at δ 3.29, indicating that the N-terminus contains a CH_3CH group attached to some electronegative group. The singlet at δ 2.30 was twice as intense as the methyl doublet at δ 1.14. The chemical shift of the singlet suggested the presence of methyl groups attached to nitrogen. The DEPT ^{13}C n.m.r. spectrum of OA-7653 also showed the presence of possibly two methyl groups (δ 41). All this evidence combined to indicate that the N-terminus contains dimethylated nitrogen. The intense singlet was resolved into a pair of doublets at δ 2.81 and 2.85 on acidification of the solution of OA-7653 in $(CD_3)_2SO$. Decoupling experiments showed coupling of both doublets to an NH proton signal at δ 10.60. These results suggested an *N,N*-dimethylalanine residue at the N-terminus, which would account for the remaining 100 u of the molecular weight.

The N-Terminus of OA-7653.—To confirm that the N-terminus in OA-7653 is an *N,N*-dimethylalanyl residue, an authentic sample of *N,N*-dimethylalanine was prepared, by a modified version of the procedure reported by Borch *et al.*[13] (see Experimental section), which has been successfully used for the reductive alkylation of amino acids and proteins.[14] G.l.c. analysis of the isopropyl esters of the total acid hydrolysate of OA-7653 and authentic *N,N*-dimethylalanine confirmed the identity of the N-terminus.

Attempts were made to determine the absolute configuration of the N-terminus. It was found that isopropyl esters of *N,N*-dimethyl-DL-alanine could not be resolved on a Chirasil-Val column, commonly used for determining absolute configuration of amino acids by chiral g.l.c. Similarly, diastereoisomers formed by esterification of *N,N*-dimethyl-DL-alanine with (*S*)-(+)-butan-2-ol and (*R*)-(−)-octan-2-ol were not resolved by normal or chiral g.l.c. Two methods involving n.m.r. have been used successfully to resolve derivatives of *N,N*-dimethyl-DL-alanine. The first involves coupling the amino acid with 3,5-dinitroaniline and subsequent n.m.r. analysis with a chiral solvating agent.[15] The agent used in the present work is (*R*)-(−)-2,2,2-trifluoro-1-(9-anthryl)ethanol [(*R*)-(−)(TFAE]. A seven-fold excess of (*R*)-(−)-TFAE added to a solution of *N*-(*N,N*-dimethyl-DL-alanyl)-3,5-dinitroaniline in $CDCl_3$ produces two sets of resonances for the α-, *C*-methyl, and *N*-methyl protons of the *N,N*-dimethylalanyl portion. In the second method, diastereoisomeric esters of *N,N*-dimethyl-DL-alanine with (*S*)-(+)-methyl mandelate were prepared, and these gave rise to non-equivalent n.m.r. signals. The obstacle to applying these methods to OA-7653 lies in the acid hydrolysis of the glycopeptide antibiotic. As a result of degradation of the amino acids during acid hydrolysis, insufficient amounts of free amino acids are produced to enable the necessary derivatisation and subsequent isolation of the relevant products to be carried out. Although the question of the absolute configuration of the N-terminus is not resolved, it is likely that it is a D-amino acid, as has been found for all other members of the vancomycin family so far investigated.

Binding of OA-7653 *to Bacterial Cell-wall Analogues.*—The binding constants of OA-7653 A with Ac-D-Ala-D-Ala and Ac_2-L-Lys-D-Ala-D-Ala are given in Table 2 alongside values previously reported[16] for vancomycin and ristocetin A. In all three cases, results show that the tripeptide binds more strongly to the antibiotics than the dipeptide. It has been observed from n.m.r. studies[17] of the binding of Ac_2-L-Lys-D-Ala-D-Ala to vancomycin that the lysyl side-chain lies over ring 7 of vancomycin, and that the lysyl α-acetyl group and side-chain contribute towards completing the hydrophobic wall round the binding cleft. This presumably accounts in part for the increase in binding constant as the peptide was changed from Ac-D-Ala-D-Ala to Ac_2-L-Lys-D-Ala-D-Ala. It is noteworthy that this increase is less for ristocetin A than for vancomycin (Table 2; see also ref. 18). We conclude that this is because the mannose residue of risocetin A interferes with the most favoured intermolecular interactions between the lysine side-chain and ring 7.

It was also observed that neither the di- nor the tri-peptide binds as strongly to OA-7653 A as to vancomycin, although the

Table 1. Comparison of ^{1}H n.m.r. data of vancomycin and OA-7653 A[a]

		Vancomycin[2]		OA-7653 A	
		δ	*J*/Hz	δ	*J*/Hz
NH	w_2	8.00		7.90	8.8
	w_3	6.59	7	7.25	7.8
	w_4	8.14	8	7.87	7.8
	w_5	8.43	6	8.60	5.9
	w_6	6.50	12	7.76	9.8
	w_7	8.39	7	8.36	7.8
α-CH	x_1			3.28	5.9
	x_2	4.86	4	4.73	8.8, 3.9, 3.9
	x_3	4.38	7, 7, 7	4.13	7.8, 7.8, 7.8
	x_4	5.71	8	5.67	7.8
	x_5	4.50	6	4.47	5.9
	x_6	4.22	12	4.06	9.8
	x_7	4.50	7	4.36	7.8
ArH	2b	7.42		7.23	
	2e	7.57	8	7.21	7.8
	2f	7.20	8	7.34	7.8
	4b	5.64		5.47	
	4f	5.21		5.20	
	5b	7.19		7.08	1.0
	5e	6.73	8	6.68	8.8
	5f	6.78	8, 1	6.75	8.8, 1.0
	6b	7.87		7.85	1.0
	6e	7.28	8	7.29	8.8
	6f	7.48	8	7.51	8.8, 1.0
	7d	6.44	2	6.49	1.0
	7f	6.30	2	6.24	1.0
	1a	N.c.[b]		1.10	6.8
	z_2	N.c.		2.76	11.7
	z_2'	N.c.		3.30	11.7
	3a	N.c.		1.59	7.8
	3a′	N.c.		1.50	7.8
	z_6	5.13		5.41	
	1d	2.41			
	1d′			2.26	
	3b				
	3b′			1.81	7.8
Sugar H	G_1	5.35	8	4.78	2.9
	G_2	3.62		3.01	8.8, 2.9
	G_3			3.47	8.8, 8.8
	G_4			2.90	8.8, 7.8
	G_5			3.15	7.8, 7.8
	G_6			3.55	11.7, 7.8
	G_6'			3.38	11.7, 7.8

[a] See Figure 2 for proton designations. [b] Not comparable.

structures of these two antibiotics are similar. One factor that could contribute to this difference is the lack of sugar residues on ring 4 in OA-7653 A. It has been proposed[19] that the vancosaminyl residue on ring 4 of vancomycin provides a portion of the hydrophobic walls round the carboxylate binding pocket, its methyl group having a hydrophobic interaction with the methyl of the C-terminal alanyl residue of the peptide. The absence of this hydrophobic interaction and of the resulting hydrophobic wall would be expected to contribute to the reduced stability of the OA-7653 complex. An additional factor is that residues 1 and 3 of OA-7653 A are together apparently less suited for strong peptide binding than the corresponding residues of vancomycin.

Preliminary n.m.r. binding studies conducted on solutions of OA-7653 A and Ac-D-Ala-D-Ala in $(CD_3)_2SO$ showed that OA-7653 is either not binding or binding extremely weakly with

Table 2. Association constants (*K*) for antibiotic–peptide complexes measured by u.v. difference spectroscopy[a]

	10^{-4} *K*/l mol^{-1}		
Peptide	OA-7653 A	Vancomycin	Ristocetin A
Ac-D-Ala-D-Ala	0.4	2.0	7.2
Ac_2-L-Lys-D-Ala-D-Ala	2.5	150	59

[a] Measurements were carried out in 0.02M sodium citrate solution at pH 5.0 and ambient temperature.

Table 3. Intermolecular n.O.e.s observed in the 1:1 complex of OA-7653 A with Ac_2-L-Lys-D-Ala-D-Ala in 1:2 $(CD_3)_2SO$–D_2O (v/v)

Signal of peptide	Signal of OA-7653 A	Size of n.O.e.[a]
Lys γ-CH_2	7f	w
Lys δ-CH_2	7f	w
Lys α-CH_3CO	z6	w
Ala^1 CH_3	2e	m
Ala^1 α-CH	1d/d′	m

[a] m: n.O.e. 30—70% of that between two aromatic *ortho*-protons which do not show strong n.O.e.s to any other protons; w: less than 30%.

the dipeptide in this solvent. That the two do form a complex in water is obvious, on the basis of results of measurement of binding constants by u.v. difference spectroscopy. One possible explanation is that hydrophobic interactions in the complex are relatively strong in water. However, because of solubility problems, ^{1}H n.m.r. binding studies could not be carried out in D_2O alone, and therefore were carried out in mixed solvents [5:8 $(CD_3)_2SO$—D_2O for Ac-D-Ala-D-Ala binding, and 1:2 $(CD_3)_2SO$—D_2O for Ac_2-L-Lys-D-Ala-D-Ala binding]. COSY and NOESY techniques were used to assign partially the ^{1}H n.m.r. spectra of the complexes with the dipeptide and the tripeptide.

In both cases, the data show distinctly the upfield shifts characteristic of the two alanyl methyl groups of the bound peptide. For example, in the case of binding to the tripeptide, the C-terminal alanyl methyl signal moves from δ 1.4 to 0.55, and the non-C-terminal alanyl methyl group from δ 1.3 to 0.9 (data for a 1:1 mixture of the two components, each 10mM, at 20 °C). Another interesting point is that the $N(CH_3)_2$ peak at δ 2.75 is shifted to δ 2.88 after addition of the dipeptide, indicative of a protonated N-terminal amino group in both cases. The N-terminal amino group has previously been observed to be unprotonated in Me_2SO before and after addition of the peptide. The antibiotic thus exists as a zwitterion in aqueous Me_2SO, but not in Me_2SO. The fact that the N-terminus of OA-7653 is not protonated even after addition of Ac-D-Ala-D-Ala in Me_2SO is probably the major reason that no significant complex formation occurs in this solvent. This situation is in contrast to that for vancomycin in Me_2SO, where addition of 1 equiv. of the acid Ac-D-Ala-D-Ala leads to formation of the bound anion of the cell wall analogue and protonation of the N-terminus of bound vancomycin. We emphasise that although vancomycin hydrochloride exists as a zwitterion in D_2O solution (with the N- and C-terminal amino acids both charged), both the terminal functionalities are neutral in Me_2SO solution (see the chemical shifts reported in ref. 2). Our observations are in accord with a recently reported study of the pK_a values of glycine and its *N*-methylated derivatives.[20] When the extent of methylation of the basic nitrogen atom is increased from one (*cf.* vancomycin) to two (*cf.* OA-7653), the pK_a of the amino group falls by *ca.* 1.5 units.

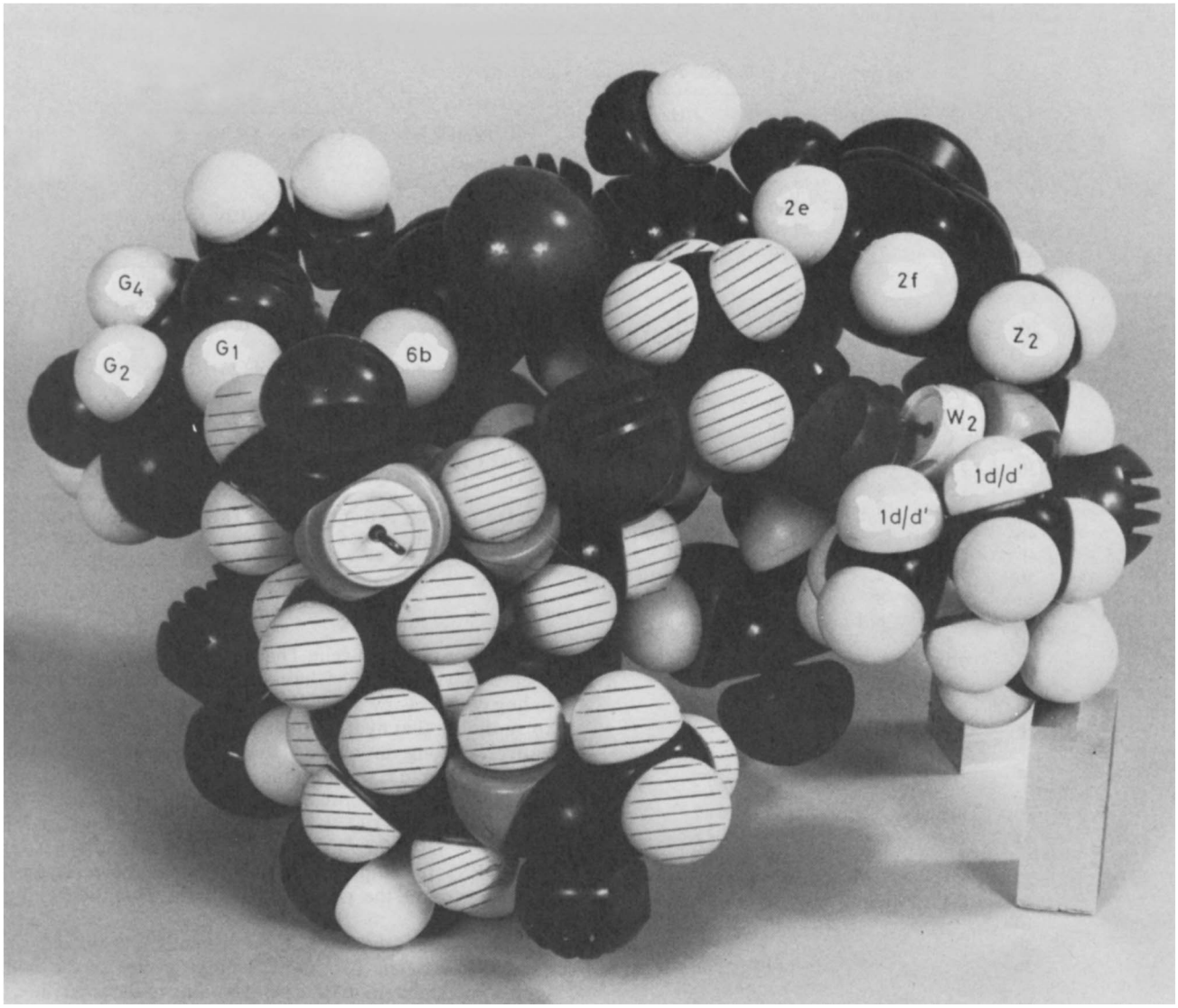


Figure 3. CPK model of the proposed complex of OA-7653 A and Ac_2-L-Lys-D-Ala-D-Ala in aqueous Me_2SO (protons of the latter component indicated by hatching)

In the NOESY spectrum of the antibiotic–dipeptide complex, only one intermolecular n.O.e. was observed, that between 2e and the C-terminal Ala-CH_{3B} [refer to Figure 2 for the proton nomenclature of the antibiotic (the peptide residues are numbered from the C-terminus); the subscript B indicates a bound proton(s)]. This could be because many of the signals were inherently broad in aqueous solution. All the intramolecular n.O.e.s observed in the bound complex are consistent with the conformation the antibiotic adopts in Me_2SO solution. The C-terminal Ala-CH_{3B} resonance of the antibiotic–tripeptide complex is at higher field (δ 0.55) than in the antibiotic–dipeptide complex (δ 0.88), supporting the formation of a stronger complex with the tripeptide. Table 3 shows the intermolecular n.O.e.s observed in the NOESY spectrum of the antibiotic–tripeptide complex. Just as in the vancomycin–tripeptide complex in aqueous solution, the lysyl sidechain lies over ring 7. The n.O.e. observed between the C-terminal Ala-α-CH and the *N*-methyl protons suggests that the role of the methyl groups on the N-terminal amino group is to contribute towards providing a hydrophobic environment round the carboxylate binding pocket. A similar role has been suggested for the N–CH_3 group of vancomycin.[19] Figure 3 shows a CPK model of the proposed complex of OA-7653 A with Ac_2-L-Lys-D-Ala-D-Ala.

Conclusion.—The structure of OA-7653 is thus determined as shown in Figure 2. The presence of a di-*N*-methylated amino acid in this group of antibiotics is, so far as we are aware, without precedent. These two methyl groups appear to contribute to the binding properties of the antibiotic to cell wall analogues terminating in D-Ala-D-Ala by providing a hydrophobic environment round the carboxylate binding pocket.

Experimental

All f.a.b. mass spectra were recorded with a Kratos MS50TC spectrometer fitted with a standard Kratos f.a.b. source, an Ion Tech neutral atom gun, and a magnet of mass range 10 000 u at an accelerating voltage of 8 kV. The matrices used were 1:1 (v/v) thioglycerol–glycerol and thioglycerol–diglycerol with 1M HCl added. E.i. mass spectra were recorded with an A.E.I. MS902 or MS30 (with data system DS30) spectrometer. G.l.c.–

mass spectrometry was performed with a Finnigan 4000 instrument, with 6110 data system in the negative ion (ammonia) chemical ionisation mode, using a SE54 fused silica column.

Reversed-phase h.p.l.c. was carried out with a Varian MicroPak MCH-5 analytical column, a Spherisorb 10 ODS semi-preparative column or a Whatman Partisil ODS2 preparative column on a Varian 5000 instrument. OA-7653 was resolved into two components by using solvents (A) 20mM NH_4OAc in H_2O, pH 8.0 and (B) 20mM NH_4OAc in 90% MeCN and 10% H_2O, with a gradient of 0—60% (B) in 40 min (detector wavelength 254 nm).

Analytical and preparative t.l.c. were carried out on Merck plates coated with silica GF254. Spots were located with a u.v. lamp or a ninhydrin spray. Gas chromatography was performed with a Carlo Erba 4130 instrument fitted with a split/splitless injector (splitting ratio typically 20:1) and a flame ionisation detector. The capillary column used was a SE54 fused silica column (Carlo Erba Strumentazione, 15 m × 0.32 mm i.d.). The injector temperature was 240 °C and the detector temperature 300 °C.

High-field ^1H and ^{13}C n.m.r. spectra were recorded with a Bruker WH400, AM400, or AM500 spectrometer operating in the Fourier transform mode. Solutions were approximately 10mM in $(CD_3)_2SO$ or D_2O, prepared from samples previously dried *in vacuo* over P_2O_5. A variable-temperature unit allows the aquisition of spectra from 0 to 70 °C. Typical data aquisitions were made using a spectral width of 4 000—6 000 Hz in 8 or 16 K data points, with quadrature detection and phase alternation. Appropriate Gaussian multiplication of the free induction decay (f.i.d.) was used to assist in the measurement of chemical shifts and coupling constants.

Standard microprograms were used for routine decoupling and n.O.e. difference experiments, generally with an irradiation time of 0.2 s for the latter. In studies of the rates of build-up of n.O.e.s, the irradiation time was set at 0.1, 0.2, 0.3, 0.4, 0.8, 1.6, 3.2, or 6.4 s with a 5 s delay between the end of one aquisition and the start of the next pulse for complete relaxation. Sizes of n.O.e.s (in % growth) were estimated by measuring the height of peaks in the difference n.O.e. spectra and by comparison with heights in the normal spectrum. For direct n.O.e.s, the growth of the n.O.e. with increase in irradiation time is exponential. Plots of size of n.O.e. against irradiation time for non-direct n.O.e.s give rise to sigmoid curves. COSY and NOESY (phase sensitive) spectra were performed at 400 or 500 MHz with sweep widths of 3 000—6 000 Hz in ω_2 and 1 500—3 000 Hz in ω_1. Typically 512 f.i.d.s were acquired, each with 2 K data points. *F*1 data were zero-filled once to 1 K before Fourier transformation. Sine multiplication was used in *F*1 and Gaussian multiplication in *F*2.

Solutions of diazomethane in ether were prepared by treating Diazald with KOH solution and distilling the ether–diazomethane mixture.

Esterification of OA-7653.—OA-7653 A (1 mg) was dried *in vacuo* over P_2O_5 overnight before the addition of HCl–MeOH (1 ml; 1M). The mixture was set aside at room temperature. After 4 h, a sample (500 µl) was lyophilized before analysis by f.a.b. mass spectrometry. The remaining 500 µl was left overnight before f.a.b. mass spectral analysis. After 4 h the product showed $[M + H]^+$ at *m/z* 1 289 and 1 275; after 24 h $[M + H]^+$ at *m/z* 1 304 and 1 289.

Mild Acid Hydrolysis of OA-7653.—OA-7653 (25 mg) was dissolved in boiling water (187.5 µl) and HCl (32.5 µl, 4M) was added. Boiling was continued for 2 min before a sample was removed and cooled to room temperature. Both portions were then treated in the same manner. The cooled solutions were centrifuged and the solid residues were then washed with HCl (50 µl; 0.6M). Finally the solid products were dried *in vacuo* over P_2O_5 before f.a.b. analysis. The 2 min product showed $[M + H]^+$ at *m/z* 1 275, 1 241, 1 113, and 1 079; 5 min product showed $[M + H]^+$ at *m/z* 1 276, 1 242, 1 114, and 1 080.

Strong Acid Hydrolysis of OA-7653.—OA-7653 (0.1 mg) was placed in a chromic acid-cleaned Pyrex tube. HCl (1 ml; 6M) was added and the tube cooled, evacuated, and then sealed. The mixture was heated in an oven at 110 °C for 30 h. The hydrolysate was lyophilized before analysis with a Beckman 119 C amino acid analyser, which showed the presence of Glu.

Oxidative Degradation of OA-7653.—(a) *Methylation.* OA-7653 (50 mg) was heated under reflux with a mixture of MeOH (4 ml), MeI (1 ml), and K_2CO_3 (0.05 g) for 6 h. The mixture was evaporated and the residue was then triturated with water (2 × 0.5 ml).

(b) *Oxidation.* The methylated compound was suspended in water (2 ml) and a solution of $KMnO_4$ (265 mg) in water (6 ml) and ammonia solution (1 ml; 2M) was added. The mixture was stirred at 70—75 °C for 5 h, then filtered, acidified with HCl, and extracted with EtOAc (3 × 5 ml). The organic extract was evaporated and dried *in vacuo*.

(c) *Esterification.* The product from step (b) dissolved in MeOH (625 ml) was cooled in ice and treated with an excess of ethereal diazomethane. After 10 min, the solution was evaporated and the products were separated by preparative t.l.c. [10% MeOH in $CHCl_3$ (v/v)]. The separated products were then analysed by e.i. mass spectrometry. Molecular ions corresponding to the degradation products (**1**) and (**3**) (Scheme 1) were observed at *m/z* 534 and 224.

Hydrolytic Degradation of OA-7653.—(a) *Methylation.* OA-7653 (25 mg) was dissolved in MeCN (15 ml), MeOH (15 ml), and H_2O (15 ml). The mixture was treated with ethereal diazomethane for 24 h. The solvent was then removed under reduced pressure at room temperature.

(b) *Hydrolysis.* The foregoing product was hydrolysed for 12 h in HCl (30 ml; 6M) under N_2 at 105 °C. The mixture was then freeze-dried.

(c) *Acetylation and esterification.* The hydrolysate was dissolved in water (15 ml), acetic anhydride (15 ml) was added, and the mixture was stirred overnight at room temperature. After removal of the solvent, methanolic HCl (30 ml; 0.1M) was added; this mixture was then left overnight. The solution was evaporated and preparative t.l.c. (10% MeOH in $CDCl_3$) was used to separate the products which were analysed by e.i. mass spectrometry. Ions were observed at *m/z* 502 and 488, corresponding to the di- and mono-methyl esters of the di-*N*-acetyl derivative of (**4**) (Scheme 1).

Determination of Gln *Linkage in* OA-7653 A *by Hofmann Degradation.*—OA-7653 A (10 mg) dissolved in MeOH–H_2O (1:1 v/v; 2 ml) was treated with an excess of ethereal diazomethane. The product was lyophilized. Methylated OA-7653 A (3 mg) was dissolved in MeCN–H_2O (1:1 v/v; 2 ml) and (diacetoxyiodo)benzene (2.5 mg) was added. The mixture was stirred in the dark at room temperature for 24 h and then washed with EtOAc (3 × 2 ml). The aqueous layer was lyophilized, then hydrolysed with HCl (1 ml; 6M) at 105 °C for 24 h.

The hydrolysis product was dried *in vacuo* and then heated with propan-2-ol (2 ml) containing HCl (3M) at 110 °C for 30 min in a screw-top vial. Most of the propan-2-ol was then blown off with oxygen-free nitrogen while the solution was still warm. The last traces of propan-2-ol were removed by vacuum pump.

The residue was dissolved in CH_2Cl_2 (2 ml) and trifluoroacetic anhydride (200 ml) was added; the mixture was then heated at 110 °C for 15 min. On cooling, the solvent and excess of reagent were removed with a gentle stream of oxygen-free nitrogen at 0 °C (ice-bath). The residue was dissolved in CH_2Cl_2 (200 ml) for g.l.c. analysis. Co-chromatography was carried out with authentic 2,4-diaminobutyric acid, derivatised similarly, using three different temperature programmes. G.l.c.—mass spectrometric analysis was also performed.

Determination of Glu *Linkage in* OA-7653 B.—OA-7653 B (0.1 mg) was dissolved in D_2O (5 ml) and pyridine (10 ml) to give a clear solution. After the addition of acetic anhydride (10 ml) at 0 °C, the mixture was kept in a parafilm-sealed tube at 0 °C for 5 min, and then at 20 °C for another 15 min. Pyridine (20 ml) and acetic anhydride (20 ml) were then added at 0 °C; the mixture was maintained at 0 °C for 5 min, and then at 20 °C for 1 h. D_2O (5 ml) was added and the solution kept at 20 °C for 1 h to decompose the excess of acetic anhydride. The resulting solution was then evaporated to dryness; exchangeable deuterium was removed by addition of acetic acid (100 ml; 10%), followed by evaporation; this process was repeated (× 5). The product thus obtained was directly hydrolysed with HCl (1 ml; 6M) in a sealed tube at 105 °C for 24 h. The *N*-trifluoroacetyl *O*-isopropyl derivatives of the vacuum-dried amino acids were prepared by using the derivatisation conditions already described previously. G.l.c.—mass spectrometric analysis showed no deuteriation in the Glu residue. A sample of commercial glutamic acid was successfully deuteriated when subjected to the same treatment.

Preparation of N,N-*Dimethylalanine*.—To a stirred solution of L-alanine (1.780 g) and aqueous HCHO (16 ml, 40%) in MeCN (120 ml) and H_2O (100 ml) was added $Na(CN)BH_3$ (4 g). A vigorous exothermic reaction ensued. The mixture was stirred for 15 min, and then glacial acetic acid was added dropwise until the solution was neutral. Stirring was continued for another 45 min, glacial acetic acid being added occasionally to maintain the pH near neutrality. The MeCN was removed under reduced pressure and then conc. HCl was added until the solution was acidic. Rapid effervescence was seen during the addition. The acidic solution was then washed with ether 2 × 200 ml) and the aqueous layer freeze-dried.

The product was taken up in water (25 ml) and applied to a Dowex 50 (H^+ form) column. The column was washed with water (250 ml) and then eluted with NH_3 (250 ml; 1M). Evaporation left essentially pure product (as established by e.i. mass spectrometry and ^1H n.m.r. analysis).

Preparation of N-(N,N-*Dimethylalanyl*)-3,5-*dinitroaniline*.—N,N-Dimethyl-DL-alanine (11.7 mg) was stirred for 3 h at room temperature with oxalyl chloride (2 ml). After rotary evaporation, the acid chloride was stirred overnight at room temperature with 3,5-dinitroaniline (18.3 mg), triethylamine (12 mg), and dichloromethane (3 ml). The product was purified by column chromatography (silica; 5% MeOH in $CHCl_3$) and characterised by ^1H n.m.r.

The product and TFAE were dissolved in a 1:7 molar ratio in deuteriochloroform for n.m.r. analysis.

Esterification of N,N-*dimethyl*-DL-*alanine with* (S)-(+)-*Methyl* 2-*Hydroxy*-2-*phenylethanoate*.—To a solution of *N,N*-dimethyl-DL-alanine (58.5 mg) and 4-dimethylaminopyridine (1.25 mg) in dichloromethane (3 ml) at −10 °C were added (*S*)-(+)-methyl 2-hydroxy-2-phenylethanoate (83 mg) and dicyclohexylcarbodi-imide (103 mg), and the mixture was stirred overnight. The precipitated urea was filtered off, solvent was removed under reduced pressure, the residue was taken up in dichloromethane (2 ml), and the solution was filtered again. This was repeated twice more before the final product was purified by preparative t.l.c. [ethyl acetate–hexane (1:2)] and then analysed by ^1H n.m.r.

U.v. Binding Studies.—D-Ala-D-Ala was purchased from Sigma Chemical Co. Ltd. and acetylated without further purification. Ac_2-L-Lys-D-Ala-D-Ala was prepared as detailed in the literature.[21] Experiments were carried out as previously described[19] with a Pye Unicam UV/VIS spectrophotometer.

Acknowledgements

We thank Dr. T. Kawaguchi for samples of OA-7653, and Dr. E. Houghton and Mr. P. Teale for g.l.c.—mass spectrometry carried out on the equipment at the Horseracing Forensic Labs., Newmarket. We acknowledge support from the S.E.R.C., (to D. H. W.) and the National University of Singapore (to S.-G. A.) for the research carried out in Cambridge.

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Received 3rd *November* 1987; *Paper* 7/1943