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Modifications of the Acyl-D-alanyl-D-alanine Terminus Affecting Complex-Formation with Vancomycin

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Vancomycin forms complexes with peptides terminating in D-alanyl-D-alanine that are analogous to the biosynthetic precursors of bacterial mucopeptides. The specificity of complex-formation has been studied by means of many synthetic peptides, prepared by both solid-phase and conventional methods. The following conclusions can be drawn: (*a*) three amide linkages are required to form a stable complex; (*b*) the terminal carboxyl group must be free; (*c*) the carboxyl terminal and subterminal residues must be either glycine or of the D-configuration; (*d*) the size of the side chain in these residues greatly influences the affinity for vancomycin, a methyl group being the optimum in each case; (*e*) the nature of the side chain in the third and fourth residues has a smaller effect on complex-formation, but an L-configuration was somewhat better than a D-configuration in the third position. In addition to acyl-D-alanyl-D-alanine, other peptides that occur in bacterial cell walls will combine with vancomycin, although less strongly, e.g. acyl-D-alanyl-D-α-amino acid (where the terminal D-residue may form the cross-link in mucopeptide structure) and acyl-L-alanyl-D-glutamylglycine (a sequence found in the mucopeptide of *Micrococcus lysodeikticus* and related organisms). These results throw some light on the specificity of the uptake of vancomycin by living bacteria.

The antibiotics vancomycin and ristocetin bind strongly peptides related to precursors of bacterial cell-wall mucopeptide (Chatterjee & Perkins, 1966; Perkins, 1969; Nieto & Perkins, 1971), and the ability to form complexes with peptide has been shown to correlate with the antibiotic power under a variety of circumstances (Perkins, 1969; Nieto & Perkins, 1971). In the present work we have studied the specificity of peptide structure that will form complexes with vancomycin and have attempted to discover how this specificity might be achieved. A narrow specificity would further support the idea of involvement of complex-formation in antibiotic action. On the other hand, many of the enzymes involved in the metabolism of the cell-wall mucopeptide (and hence in growth control) have to recognize structures identical with those recognized by the vancomycin family of antibiotics. Any insight gained in the mechanism of vancomycin specificity could help the understanding of binding by these enzymes.

MATERIALS AND METHODS

Antibiotics. Vancomycin hydrochloride (Vancocin HCl) was kindly given by Eli Lilly and Co. Ltd. (Basingstoke, Hants., U.K.), and fractionated as described by Nieto & Perkins (1971). The major fraction (fraction IV of Nieto & Perkins, 1971) was used in the present work.

Paper chromatography, t.l.c. and electrophoresis. Whatman no. 3 paper was used and if required for preparative purposes it was first exhaustively washed by irrigation with M-ammonium acetate followed by water. Glass plates for t.l.c. were coated with a 250μm layer of Silicagel G (Merck, Darmstadt, Germany). The following solvents were used: solvent 1, butanol–acetic acid–water (6:2:2, by vol.); solvent 2, butanol–acetic acid–water–pyridine (15:3:12:10, by vol.); solvent 3, chloroform–methanol–acetic acid (17:2:1, by vol.); solvent 4, ethanol–water–conc. NH_3 (sp.gr. 0.88) (75:25:0.5, by vol.); solvent 5, ethanol–1M-ammonium acetate (5:2, v/v); solvent 6, isobutyric acid–aq. 0.5M-NH_3 (5:3, v/v).

Phenylthiohydantoin–amino acids were identified by t.l.c. as described by Smith & Murray (1968).

The following buffers were used for electrophoresis: buffer A, collidine–acetate, pH 7 (Newton & Abraham, 1954); buffer B, 0.25M-formic acid, pH 1.9.

Peptides were located by means of the ninhydrin reaction (0.1% in acetone) or by the method of Rydon & Smith (1952). Peptide hydroxamates were visualized by spraying with a dilute aqueous solution of ferric chloride, and hydrazides by spraying with ammoniacal 2% silver nitrate–ethanol–butanol (2:1:9, by vol.). Vancomycin or vancomycin complexes were observed by u.v. absorption or by spraying with the acid-diazosulphanilic acid reagent of Ames & Mitchell (1952).

Rotation measurements. Rotations at the sodium *D* line were measured in a Bendix NPL automatic polarimeter type 143 (New Basford, Notts., U.K.) with 1 or 2cm light-path cells. Specific rotation was calculated as

$[\alpha]_D^t = 100\alpha/cl$, where α is the rotation measured (degrees), c the concentration in g/dl and l the light-path in dm.

U.v. difference spectroscopy. The tandem-cell arrangement was used (Perkins, 1969). The cells were 2 cm light-path fused tandem cells constructed by the Thermal Syndicate Ltd. (Wallsend, Northumb., U.K.). A 5 ml sample of vancomycin solution (0.037 mg/ml in 0.02 M-sodium citrate buffer, pH 5.1) was used. At this concentration no appreciable aggregation occurs. For the peptides ending in D-leucine the same volume of a stronger solution (0.12 mg/ml) was used. When the peptide was insoluble in water, combination was carried out in 50% (v/v) ethanol (concentration of vancomycin 0.16 mg/ml). The concentration of vancomycin solutions in 0.02 M-citrate buffer, pH 5.1, was calculated from extinction measurements by using a value of $E_{1cm}^{1\%}$ 45.0 at 280 nm (Nieto & Perkins, 1971). The spectrophotometer was a Unicam SP.700. The settings were: scale, −0.3, +0.8 (extinction); damping selector, 2; resolution, 3. The difference in extinction developing upon addition of peptide (0–50 μl of 0.01–0.03 M solutions) was measured at 242–243 nm (concentration of vancomycin 0.037 mg/ml), 283–284 nm or 293 nm (concentration of vancomycin 0.12–0.16 mg/ml), the slit widths being from 0.2 mm to 0.5 mm. The difference in extinction was measured with an accuracy of ±0.002 extinction unit, the relative error being ±1.5–±2.5% near the equivalence point. The temperature in the cell compartments was 25±1°C. Additions and dilutions in the sample cell were carefully compensated for in the reference beam. Association constants were determined from these results by means of Scatchard plots (Edsall & Wyman, 1958) as described by Nieto & Perkins (1971). All concentrations of peptides were established by amino acid analysis.

Amino acid derivatives. ϵ-Benzyloxycarbonyl-L-lysine (ϵ-Z*-L-Lys) was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. All the other amino acid derivatives were synthesized as follows: ϵ-Z-D-Lys was prepared by a modification of the procedure of Neuberger & Sanger (1943) as described by Kjaer & Oleseri-Larsen (1961). *O*-Benzyl-L-tyrosine (*O*-Bzl-L-Tyr) was prepared as described by Wünsch, Fries & Zwick (1958). *tert.*-Butyloxycarbonyl derivatives of amino acids (Boc-amino acids) were prepared by acylation with *tert.*-butyl-azidoformate (Carpino, 1959) as described by Schnabel (1967). In the preparation of Boc-α-aminoisobutyric acid the reaction mixture in dioxan–water (3:2, v/v) was titrated to pH 11 for 2 days at room temperature. Under these conditions the yield was very poor but better than at lower pH values. $\alpha\delta$-di-Boc-L-ornithine, $\alpha\gamma$-di-Boc-L-2,4-diaminobutyric acid and $\alpha\epsilon$-di-Boc-D-lysine were prepared as $\alpha\epsilon$-di-Boc-L-lysine (Schnabel, 1967). All Boc derivatives were purified by silica-gel column chromatography with chloroform as eluent. Benzyl esters of L- and D-alanine were prepared as described by Erlanger & Hall (1954). D-Glutamic acid-γ-Bzl ester was prepared by the method used by Guttmann & Boissonnas (1958) for the L-isomer. Boc-D-glutamic acid α-Bzl-ester was prepared by the method of Schröder & Klieger (1964), who prepared the L-isomer. *N*-Hydroxysuccinimide esters of Boc derivatives were prepared by the general method of Anderson, Zimmerman & Callahan (1964).

Elementary analysis and other properties of new or unusual compounds are given in Table 1. All the compounds utilized were homogeneous in t.l.c. in solvents 1, 2 and 3.

Peptides. Tetraglycine was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. D-Ala-D-Ala from International Chemical and Nuclear Corp., City of Industry, Calif., U.S.A., and Gly-D-Ala, D-Ala-Gly and L-Ala-Gly-Gly from Sigma Chemical Co., St Louis, Mo., U.S.A. D-Ala-D-Ala-D-Ala was a gift from Dr I. Schechter. These peptides were acetylated as described in the Experimental section. All other peptides listed in Table 3 were synthesized in the course of the present work by solid-phase or conventional methods. D-Glu(α-Bzl)-γ-L-Lys-D-Ala-D-Ala was obtained as a by-product in the solid-phase synthesis of the tetrapeptide D-Glu-γ-L-Lys-D-Ala-D-Ala.

EXPERIMENTAL

Solid-phase peptide synthesis

Solid-phase synthesis was carried out by the method of Marshall & Merrifield (1965) with the following modifications. (*a*) The amount of the first amino acid attached was determined by the method of Esko, Karlsson & Porath (1968). Substitutions of 0.2–0.4 mmol/g were used. (*b*) Anhydrous trifluoroacetic acid (10 ml/mmol of amino acid attached to the resin, 30 min) was used to eliminate the *tert.*-butyloxycarbonyl group. (*c*) Chloroform was used as solvent in the neutralization step and in the washes before and after this step (Stewart, Young, Benjamini, Shimizu & Leung, 1966). (*d*) In the cleavage step, dry hydrogen bromide was bubbled for 15 min through the suspension of peptide–resin in anhydrous trifluoroacetic acid (5 ml/g), filtered, and this treatment repeated. The resin was further washed twice with trifluoroacetic acid (5 ml). Cleavage by ammonolysis was carried out as described by Bodansky & Sheehan (1964).

In the preparation of H-L-Lys-D-Ala-Gly-NHOH and diAc-L-Lys-D-Ala-Gly-NHOH cleavage was carried out as follows. To 1.6 g of peptide–resin (containing a total of 0.68 mmol of peptide) was added 10 mmol of hydroxylamine hydrochloride in 2 ml of water followed by 20 mmol of triethylamine in 12 ml of ethanol. The suspension was stored for 3 h at 40°C, filtered, and the resin was washed three times with 10 ml portions of 50% (v/v) ethanol.

Determination of the amount of Boc-amino acid linked to the resin. It is important to know this value precisely since it determines the amounts of all the other protected amino acids and reagents to be used through the synthesis. The method of Esko *et al.* (1968) was used because it is faster and more accurate than other conventional methods.

Coupling reaction. The coupling mediated by carbodiimide proceeded satisfactorily in all cases except in the synthesis of L-Lys-Aib-Gly. Yields were not increased by using *N*-hydroxysuccinimide esters. The overall yield of this peptide was low, lysylglycine being the main product. The introduction of a fatty acid, myristic acid, by carbodi-imide coupling in methylene chloride proved very satisfactory.

The yield of these solid-phase syntheses, as shown in

* Abbreviations: Z, benzyloxycarbonyl; Aib, α-aminoisobutyric acid; Boc, *tert.*-butyloxycarbonyl; Bzl, benzyl; Dbu, $\alpha\gamma$-diaminobutyric acid; SucNOH, *N*-hydroxysuccinimide; MurNAc, *N*-acetylmuramic acid.

Table 1. *Properties of amino acid derivatives*

Melting points were determined with a Kofler apparatus and are uncorrected. All yields are of final purified derivative: (*a*) introduction of Boc; (*b*) benzyloxycarbonylation; (*c*) esterification.

	Elementary analysis									
	Calc.			Found						
Compound	C	H	N	C	H	N	m.p. (°C)	$[\alpha]_D^t$	Yield (%)	Recrystallized from
Boc-D-Ala	50.7	7.95	7.41	50.7	7.98	7.67	83–84	+23.5 at 24.0°C *c* 2 in acetic acid	70–85 (*a*)	Ethyl acetate–*n*-hexane
Boc-D-Leu-H_2O	53.1	9.23	5.62	53.1	9.17	5.47	58–60	+16.2 at 20.0°C *c* 1 in ethanol	65–75 (*a*)	Ethanol–water
Di-Boc-L-Dbu	an oil						—	—	85–90 (*a*)	—
Boc-Aib	53.2	8.37	6.91	53.16	8.15	6.69	118–119	—	10 (*a*)	Ethyl acetate–*n*-hexane
Boc-D-Glu	48.6	6.89	5.66	48.5	7.13	5.54	111–113	+14.4 at 23.0°C *c* 1 in methanol	54–65 (*a*)	Ethyl acetate–*n*-hexane
Boc-D-Glu-α-Bzl dicyclohexylammonium salt	67.2	8.89	5.42	67.1	9.05	5.50	115–168 (decomp.)	+15.9 at 22.5°C *c* 1 in methanol	61 (*c*)	Ethanol
Boc-D-Glu-α-Bzl	60.5	6.83	4.15	60.6	6.80	4.17	97–98	+29.0 at 23.0°C *c* 1 in methanol	40 (*c*)	Ethanol–water
D-Glu-γ-Bzl	60.8	6.40	5.90	60.5	6.30	5.75	189–190	−26.9 at 20.0°C *c* 2 in M-HCl	55 (*c*)	Water–pyridine (95:1, v/v)
ε-Z-D-Lys	60.0	7.14	10.0	59.2	7.33	9.94	218–221 (decomp.)	−12.7 at 26.0°C *c* 1 in M-HCl	51 (*b*)	Water
α-Boc,ε-Z-D-Lys	an oil								80–90 (*a*)	—
Boc-D-Ala-SucNOH	50.4	6.29	9.79	50.8	6.39	9.61	147–149	+43.7 at 19.5°C *c* 1 in acetone	80 (*c*)	Propan-2-ol or ether
Di-Boc-L-Lys-SucNOH	54.3	7.44	9.49	54.4	7.44	9.19	78–81	−23.2 at 26.0°C *c* 1 in acetone	67 (*c*)	Ether
Di-Boc-L-Orn-SucNOH	53.1	7.22	9.79	53.7	7.21	9.73	130–132	−20.4 at 28.5°C *c* 1 in acetone	85 (*c*)	Ether
α-Boc,ε-Z-L-Lys-SucNOH	57.8	6.50	8.80	57.2	6.55	8.59	60–63	−21.9 at 25.0°C *c* 1 in acetone	45 (*c*)	Ether–*n*-hexane
HCl-D-Ala(Bzl)	55.8	6.52	6.52	55.6	6.69	6.46	142–144	+9.6 at 22.0°C *c* 1 in 0.1 M-HCl	50–70 (*c*)	Ethanol–*n*-hexane

Table 2. *Properties of protected peptide intermediates*

All compounds were recrystallized from ethyl acetate–*n*-hexane except Boc-Gly-D-Ala(Bzl) and di-Boc-L-Lys-Gly-D-Ala(Bzl), which were obtained as oils. All the rotations were measured as 1% (w/v) solutions in ethanol at the temperature indicated. The melting points were determined with a Kofler apparatus and are uncorrected.

	Elementary analysis								
	Calc.			Found					
Compound	C	H	N	C	H	N	m.p. (°C)	$[\alpha]_D^t$	t (°C)
Boc-D-Ala-D-Ala(Bzl)	61.7	7.44	8.00	61.9	7.65	7.95	71–72	+49.5	25.0
Boc-D-Ala-L-Ala(Bzl)	—	—	—	61.8	7.51	7.97	74–76	− 2.9	24.5
Boc-L-Ala-D-Ala(Bzl)	—	—	—	61.9	7.59	7.91	72–74	− 2.3	26.5
Boc-L-Ala-L-Ala(Bzl)	—	—	—	61.9	7.66	7.98	69–71	−48.0	24.2
Boc-D-Ala-ε-Z-D-Lys(Bzl)	64.3	7.20	7.77	64.1	6.54	7.73	112–114	+24.5	22.5
Boc-Gly-D-Ala(Bzl)			an oil					—	
Di-Boc-L-Lys-Gly-D-Ala(Bzl)			an oil					—	
Di-Boc-L-Lys-D-Ala-D-Ala(Bzl)	60.3	7.80	9.72	60.3	7.84	9.70	100–103	+32.7	28.0
Di-Boc-L-Lys-L-Ala-D-Ala(Bzl)	—	—	—	60.5	7.85	9.76	114–116	− 9.8	28.7
Di-Boc-L-Lys-D-Ala-L-Ala(Bzl)	—	—	—	60.3	7.83	9.78	109–111	− 0.7	28.5
Di-Boc-L-Lys-L-Ala-L-Ala(Bzl)	—	—	—	60.2	7.79	9.75	114–116	−44.5	28.5
α-Boc-ε-Z-L-Lys-D-Ala-D-Ala(Bzl)	62.7	7.2	9.15	62.6	7.42	9.03	126–127	+30.4	24.0
Boc-Gly-D-Ala-D-Ala(Bzl)	59.0	7.12	10.30	58.5	7.29	9.98	104–107	+52.5	28.2
Di-Boc-L-Orn-D-Ala-D-Ala(Bzl)	59.7	7.63	9.94	59.0	8.04	9.69	146–148	+35.3	23.0

Table 3, is not as high as could be expected when compared with similar peptides synthesized by conventional methods. Although the total yield of the correct peptide was usually about 80%, the removal of small amounts of peptidic contaminants that were generally present proved difficult, so the yield of pure compound was relatively small. The yield shown in Table 1 reflects the difficulty of removal of the contaminants, which were as a rule very similar to the derived product. Peptides containing leucine were exceptions. L-Lys-D-Ala-D-Leu, obtained as free peptide, showed only traces of contaminants and diAc-L-Lys-D-Leu-D-Leu and diAc-L-Lys-D-Leu-D-Ala were contaminated exclusively by the monoacetyl derivatives. This fact suggests that some of the contaminants arose during the cleavage of the peptide from the resin by hydrogen bromide–trifluoroacetic acid treatment. Other contaminants, such as those present as 5–15% of the product in the synthesis of peptides containing γ-linked glutamic acid, arose from incomplete coupling, confirming the observations of Krumdieck & Baugh (1969).

The procedure of Merrifield (1963) for acetylation of amino groups on peptide–resin compounds is not very efficient on ω-amino groups, leaving 20–30% of these unacetylated and making the repetition of the treatment necessary.

Cleavage from the resin. Under our conditions the cleavage by hydroxylamine to yield the peptide hydroxamate was rather inefficient. No attempt to use other conditions was made.

Conventional peptide synthesis

The general procedure used was as follows. The benzyl ester (free base) of the *C*-terminal amino acid was coupled to the *tert.*-butyloxycarbonyl derivative of the next amino acid in the sequence by means of dicyclohexylcarbodiimide in dichloromethane at 0°C overnight. Dicyclohexylurea was removed by filtration and the protected dipeptide was purified by chromatography on a column of silica gel packed in chloroform, with the same solvent as eluent. The protected peptides were detected by the typical u.v. absorption of the benzyl group. Yields were 80–90% of the theoretical. The *tert.*-butyloxycarbonyl groups were removed by treatment with anhydrous trifluoroacetic acid at 0°C for 15 min. The trifluoroacetic acid was evaporated under vacuum and the excess was removed by repeated dissolution in 80% acetic acid and concentration *in vacuo* at 35–40°C. Finally the trifluoroacetate salt was dried *in vacuo* over KOH and P_2O_5. Coupling of the dipeptidebenzyl ester trifluoroacetate with the *tert.*-butyloxycarbonyl derivative of the *N*-hydroxysuccinimide ester of the following amino acid was carried out as described by Anderson *et al.* (1964). Yields were about 80% of the theoretical value. The protected tripeptides were purified by the same procedure as the dipeptides. Properties of protected di- and tri-peptides are given in Table 2. All the intermediates were shown to be homogeneous on t.l.c. in solvents 1 and 2. Boc-D-Ala-D-(ϵ-Z)-Lys(Bzl) represents an unsuccessful attempt to synthesize diAc-L-Lys-D-Ala-D-Lys by using the benzyl ester of D-Lys. For a reason unknown to us the yield in the carbodi-imide-mediated coupling of Boc-D-Ala with NH_2-D-(ϵ-Z)Lys(Bzl) was only 6–10%.

Removal of the *tert.*-butyloxycarbonyl group as described above followed by treatment of the peptide benzyl ester with excess of acetic anhydride in pyridine afforded the fully acetylated peptide benzyl ester. The benzyl group was removed by hydrogenation in 80% acetic acid, with 10% Pd/C as catalyst, to give the *N*-acetylated peptide. When the free peptide was required the benzyl group was removed first and then the *tert.*-butyloxycarbonyl group.

In the synthesis of L-Ala-D-Glu-Gly and diAc-L-Lys-D-Ala-D-Lys the carboxyl terminus was unprotected and only *N*-hydroxysuccinimide ester coupling in acetonitrile–water mixtures was used (Anderson *et al.* 1964). The intermediates were isolated, purified by silica gel column chromatography as above and characterized only by their solubility and u.v.-absorption properties (benzyl absorption in ϵ-benzyloxycarbonyl and γ-benzyl ester protecting groups) as compared with those of the starting materials. The final peptides were fully characterized and their properties are shown in Table 3.

Acetylation of peptides. When the complete acetyl peptide was synthesized by the solid-phase method, acetylation was carried out as described by Merrifield (1963). On the other hand, the following acetyl derivatives, diAc-L-Lys-Gly-D-Ala, α-Ac-L-Lys-Gly-D-Ala, diAc-L-Lys-D-Ala-Gly, α-Ac-L-Lys-D-Ala-Gly, *N*-Ac-L-Tyr-D-Ala-D-Ala, diAc-L-Lys-Aib-Gly, were prepared by acetylation in solution of the peptide obtained by the solid-phase method. The procedure described below was used, too, in the acetylation of some of the peptides obtained by conventional synthesis. Acetylation as described by Perkins (1969) gives with very good yield only the *N*-α-acetyl-peptide. No contamination with the diacetylated peptide was observed and less than 5% unchanged material. The following is a typical procedure for acetylation of both α- and ω-amino groups. L-Lys-Gly-D-Ala (10 μmol) was dissolved in dioxan–water (1:1, v/v) (100 μl), cooled at 0°C (ice bath), and triethylamine (50 μmol) was added followed by acetic anhydride (24 μmol). The mixture was kept in the cold for 2 h, boiled for 3 min to destroy any excess of acetic anhydride, evaporated to dryness, redissolved in water and evaporated again. Yields of diacetyl-peptide are 60–70% of the theoretical. There is always 30–40% of *N*-α-acetylated material present. This contaminant along with the triethylamine salt was eliminated by chromatography on a column (1 cm × 10 cm) of Zeo-Karb 225 or Dowex 50 (H^+ form), elution being carried out with water.

L-Tyr-D-Ala-D-Ala was acetylated by the latter procedure. No *O*-acetyl groups were produced, as shown by spectrophotometric titration of the phenolic group.

Purification of peptides. Peptides synthesized by the conventional method did not require any further purification. On the other hand, small amounts of contaminants were usually present in the peptides synthesized by the solid-phase method. Purification of basic peptides was carried out by using a continuous-flow paper electrophoresis apparatus (Beckman Instruments Inc. Spinco Division, model CP) with the following conditions: electrolyte, 0.1 M-formic acid; overflow knob, 12.0; wick knob, 4; feed rate, 0.75 ml/h of solution containing 2–3 mg/ml of peptide in the electrolyte; constant voltage, 410 V. Peptide was found in tubes 6–10.

Table 3. *Optical rotations and yields of synthetic peptides*

Yields for solid-phase syntheses refer to the amount of the first amino acid fixed to the resin; yields for conventional syntheses are products of the yields from the individual steps and include purification of the intermediates. To measure the rotation the concentration of peptide was determined either by weight (wt.) or amino acid analysis (aa).

Peptide	$[\alpha]_D^t$	Concn. determined by	Yield (%)	Method of synthesis	Purification procedure
Ac-D-Ala-D-Ala	+102.0 at 23.1°C *c* 0.8 in water, pH 2.5	aa	93	Acetylation of commercial sample	Ion-exchange (Dowex 50)
Ac-Gly-D-Ala	+42.5 at 22.5°C *c* 0.7 in water, pH 2.4	aa	89	As above	As above
Ac-D-Ala-Gly	+63.3 at 22.5°C *c* 0.8 in water, pH 2.5	aa	96	As above	As above
Ac-L-Ala-Gly-Gly	−39.3 at 23.2°C *c* 0.7 in water, pH 2.8	aa	97	As above	As above
Gly-D-Ala-D-Ala	+97.5 at 25.5°C *c* 0.2 in 2M-HCl	wt.	40	Conventional	Purification of intermediates
Ac-Gly-D-Ala-D-Ala	+87.5 at 22.7°C *c* 0.3 in water, pH 1.9	aa	90	Acetylation of free peptide	Ion-exchange (Dowex 50)
L-Lys-L-Ala-L-Ala	−45.0 at 25.5°C *c* 0.2 in 2M-HCl	wt.	51	Conventional	Purification of intermediates
αε-DiAc-L-Lys-L-Ala-L-Ala	−69.0 at 24.2°C *c* 0.5 in water, pH 5.3	aa	60	Acetylation of free peptide	Ion-exchange (Dowex 50)
L-Lys-L-Ala-D-Ala	+23.0 at 25.5°C *c* 0.2 in 2M-HCl	wt.	44	Conventional	Purification of intermediates
αε-DiAc-L-Lys-L-Ala-D-Ala	−37.1 at 23.2°C *c* 0.4 in water, pH 2.0	aa	53	Acetylation of free peptide	Ion-exchange (Dowex 50)
L-Lys-D-Ala-L-Ala	+49.2 at 25.5°C *c* 0.2 in 2M-HCl	wt.	46	Conventional	Purification of intermediates
αε-DiAc-L-Lys-D-Ala-L-Ala	−2.0 at 24.2°C *c* 0.5 in water, pH 1.7	aa	66	Acetylation of free peptide	Ion-exchange (Dowex 50)
L-Lys-D-Ala-D-Ala	+116.5 at 25.8°C *c* 0.4 in water, pH 3.0	aa	49	Conventional	Purification of intermediates
L-Lys-D-Ala-D-Ala	+121.0 at 22.0°C *c* 0.4 in water, pH 2.5	aa	70	Solid-phase	Continuous flow electrophoresis
DiAc-L-Lys-D-Ala-D-Ala	+37.7 at 26.0°C *c* 0.9 in water, pH 2.6	aa	60	Solid-phase	Ion-exchange (Dowex 50) followed by recrystallization from ethanol–*n*-hexane
DiAc-L-Lys-D-Ala-D-Ala	+37.3 at 26.0°C *c* 0.9 in water, pH 2.9	aa	30–60	Conventional; acetylation of free peptide or of the benzyl ester followed by hydrogenation	Ion-exchange (Dowex 50 followed by Dowex 1)
α-Ac-L-Lys-D-Ala-D-Ala	+31.2 at 20.8°C *c* 0.4 in 2M-HCl	wt.	73	Solid-phase	Continuous flow electrophoresis followed by recrystallization from ethanol–water
α-Ac-L-Lys-D-Ala-D-Ala	+38.0 at 21.2°C *c* 0.5 in 2M-HCl	wt.	48	Conventional	Ion-exchange (Dowex 1) followed by recrystallization as above
L-Orn-D-Ala-D-Ala	+107.9 at 24.5°C *c* 0.2 in 2M-HCl	wt.	48	Conventional	Purification of intermediates
αδ-DiAc-L-Orn-D-Ala-D-Ala	+39.8 at 22.5°C *c* 0.5 in water, pH 1.8	aa	72	Acetylation of free peptide	Ion-exchange (Dowex 50)
L-Lys-D-Ala-D-Leu-2HBr	+61.9 at 22.5°C *c* 0.5 in 2M-HCl	wt.	80	Solid-phase	Homogeneous as synthesized
αε-DiAc-L-Lys-D-Ala-D-Leu	+40.4 at 25.5°C *c* 0.8 in water, pH 1.9	aa	72	Acetylation of free peptide	Ion-exchange (Dowex 50 followed by Dowex 1)

Table 3. *Cont.*

Peptide	$[\alpha]_D^t$	Concn. determined by	Yield (%)	Method of synthesis	Purification procedure
αε-DiAc-L-Lys-D-Leu-D-Ala	+39.9 at 22.0°C *c* 0.6 in water, pH 3.1	aa	63	Solid-phase	Paper electrophoresis in buffer A
αε-DiAc-L-Lys-D-Leu-D-Leu	+34.3 at 24.0°C *c* 0.5 in 2M-HCl	wt.	51	Solid-phase	Continuous flow electrophoresis
L-Lys-D-Ala-Gly	+60.2 at 22.5°C *c* 0.5 in 2M-HCl	wt.	70	Solid-phase	Continuous flow electrophoresis
L-Lys-Gly-D-Ala	+46.5 at 23.0°C *c* 0.5 in 2M-HCl	wt.	71	Solid-phase	Continuous flow electrophoresis
αε-DiAc-L-Lys-Gly-D-Ala	+14.0 at 22.8°C *c* 0.9 in water, pH 1.2	aa	25	Conventional; acetylation of benzyl ester	Ion-exchange (Dowex 50 followed by Dowex 1)
L-Dbu-D-Ala-D-Ala	+133.6 at 23.8°C *c* 0.3 in water, pH 5.4	aa	48	Solid-phase	Continuous flow electrophoresis
αγ-DiAc-L-Dbu-D-Ala-D-Ala	+38.6 at 20.4°C *c* 0.7 in water, pH 2.8	aa	58	Solid-phase	Ion-exchange (Dowex 50)
L-Lys-Aib-Gly	+48.6 at 22.4°C *c* 0.3 in water, pH 2.6	aa	15	Solid-phase	Continuous flow electrophoresis
L-Lys-D-Ala-D-Lys	+59.8 at 21.8°C *c* 0.4 in water, pH 5.4	aa	20	Solid-phase	Paper electrophoresis in buffer B
αε-DiAc-L-Lys-D-Ala-D-Lys	+15.4 at 20.8°C *c* 1.3 in water, pH 4.8	aa	10	Conventional	Purification of intermediates and paper electrophoresis in buffer A
L-Lys-D-Ala-Gly-NHOH	+79.8 at 24.0°C *c* 1.6 in water, pH 6.0	aa	21	Solid-phase	Ion-exchange (Dowex 1) followed by paper electrophoresis in buffer A
α-Ac-L-Lys-D-Ala-D-Ala-NH_2	+25.7 at 24.7°C *c* 1 in water, pH 6.1	aa	60	Solid-phase	Paper electrophoresis in buffer B
L-Ala-D-Glu-Gly	+53.4 at 23.0°C *c* 0.6 in water, pH 3.8	aa	30	Conventional	Purification of intermediates
Ac-L-Ala-D-Glu-Gly	−6.24 at 23.0°C *c* 1 in water, pH 2.18	aa	96	Acetylation of free peptide	Ion-exchange (Dowex 50)
L-Tyr-D-Ala-D-Ala	+142.5 at 21.7°C *c* 0.5 in water, pH 3.7	aa	21	Solid-phase	Continuous flow electrophoresis
N-Ac-L-Tyr-D-Ala-D-Ala	+76.5 at 22.8°C *c* 0.7 in water, pH 2.3	aa	70	Acetylation of free peptide	Ion-exchange (Dowex 50)
D-Glu-γ-L-Lys-D-Ala-D-Ala	+16.2 at 25.0°C *c* 0.6 in water, pH 3.8	aa	65	Solid-phase	Paper electrophoresis (buffer B) followed by paper chromatography (solvent 1)
D-Glu-γ-L-Lys-D-Ala-D-Ala	+14.3 at 26.0°C *c* 1 in water, pH 5.2	aa	10	Conventional	Purification of intermediates
L-Ala-D-Glu-γ-L-Lys-D-Ala-D-Ala	+6.83 at 22.7°C *c* 0.6 in water, pH 5.2	aa	47	Solid-phase	Continuous flow electrophoresis followed by paper chromatography in solvent 1
L-Tyr-D-Glu-γ-L-Lys-D-Ala-D-Ala	+55.6 at 25.0°C *c* 0.6 in water, pH 5.7	aa	45	Solid-phase	As preceding peptide

For neutral peptides the overflow knob was set at 10.5 and a constant voltage of 500 V was used. The peptide was found in tubes 12–15. With acidic peptides the voltage was changed to 600 V, the peptide then appearing in tubes 23–27. Neutral peptides still showed neutral contaminants after this purification procedure. Separation was effected by preparative paper chromatography in solvent 1. This procedure was also used in the separation of ^{125}I-labelled Tyr-D-Glu-γ-L-Lys-D-Ala-D-Ala from the non-iodinated peptide.

Small amounts of peptide (up to 10 mg) were purified by paper electrophoresis in buffer A or B, on washed Whatman

no. 3 paper, voltages in the range 560–800 V and times of 2 or 3 h being used.

Myristoyl-D-Ala-D-Ala was purified by silica gel column chromatography with chloroform as eluent.

All peptides described here were considered pure when they gave a single spot on t.l.c. in solvents 1 and 2, in paper chromatography in solvents 5 and 6 and paper electrophoresis in buffers A and B.

Identity of peptides. Peptides were hydrolysed in 6M-HCl for 20 h at 103°C in a sealed tube and the amino acid composition was measured in an automatic analyser (Beckman, Spinco model 120c) or on paper electrophoretograms as described by Chatterjee & Perkins (1966).

Sequence of tripeptides was established by dinitrophenylation (Bailey, 1967), hydrolysis and comparison of the DNP-amino acid obtained with standards on t.l.c. in solvent 3. Monoacetyl-lysine derivatives were shown to be acetylated on the α-amino group by dinitrophenylation, hydrolysis of the DNP-peptide and comparison of the mono-DNP-lysine obtained with standards by paper chromatography in the system of Levy (1954). The *C*-terminal amino acid of each peptide was identified by hydrazinolysis (Ito & Strominger, 1964).

Ondetti, Deer, Sheehan, Pluščec & Kocy (1968) reported the formation of succinimide derivatives during the attempt to synthesize peptides containing the sequence Asp-α-Gly or Asp-β-Gly by the solid-phase method. To ensure that no glutarimido derivatives or α-linked glutamic acid was present in the peptides synthesized in solid phase in our work, all of them were subjected to hydrazinolysis (Ito & Strominger, 1964) and Edman degradation (Holmgren, 1968). The former afforded only glutamyl-γ-hydrazine; the Edman degradation failed in the tetrapeptide Glu-γ-L-Lys-D-Ala-D-Ala and only the first cycle succeeded in cleaving the phenylthiohydantoins of tyrosine and alanine from L-Tyr-D-Glu-γ-L-Lys-D-Ala-D-Ala and L-Ala-D-Glu-γ-L-Lys-D-Ala-D-Ala respectively. The aqueous residue after failure of the second cycle was hydrolysed and amino acid analysis showed it to contain glutamic acid, alanine and lysine in the proportions 1:2:1. A further check for the tetrapeptide was dinitrophenylation and photolysis of the DNP-peptide (Perkins, 1967). No DNP-glutamic acid could be found in a hydrolysate of the photolysed DNP-peptide. Last of all, the same tetrapeptide was synthesized by conventional methods and shown to be identical with the peptide synthesized by the solid-phase method (Table 3). The presence of any cyclic glutarimide derivative was further discounted by the electrophoretic mobility of the peptides. The peptides containing γ-linked glutamic acid were also heated at 50°C for 16 h at pH 8.0. Under these conditions any cyclic imide present should open to give a mixture of α- and γ-linked glutamyl-peptides. Only the starting material was found by electrophoresis at pH 4.0 (Ito & Strominger, 1964).

Racemization. Small differences in rotation of peptides synthesized by both solid-phase and conventional methods (Table 3) reflect rather the inaccuracy of the estimation of the concentration than more or less racemization in either technique. Most of the rotations were measured in solutions whose concentration was determined by amino acid analysis. Some of the concentrations were determined by weight and since many of these compounds are very hygroscopic these rotations could be low by 5–10%. Racemization during these syntheses must be very low since many of the peptides were completely digested by a bacterial carboxypeptidase specific for D–D linkages (Leyh-Bouille *et al.* 1970*a*).

RESULTS

Combination of peptides with vancomycin

To try to establish the specificity for complex-formation with vancomycin, a large number of peptides were prepared. The structure in which variations were introduced was that of the peptidic portion of the mucopeptide precursor of *Staphylococcus aureus*:

H-L-Ala-D-Glu-γ-L-Lys-D-Ala-D-Ala-OH
5 4 3 2 1

The different residues have been numbered to facilitate the description and discussion of the results. The word 'substrate' is used to describe any peptide showing specific change in the u.v. absorption, the optical rotatory dispersion or the chromatographic or electrophoretic mobility of vancomycin. Combination was studied quantitatively by u.v. difference spectroscopy and the results confirmed qualitatively by independent methods, i.e. t.l.c. in solvent 4, electrophoresis in buffer A and paper chromatography in solvents 5 and 6.

Requirement for a free terminal carboxyl group. Perkins (1969) showed that esterification of the carboxyl group of a good substrate prevented combination. This could be due either to a strong steric restriction at this point of the vancomycin molecule or to a requirement for a strongly polar carboxyl group or both. To clarify this point we synthesized a peptide amide (α-Ac-L-Lys-D-Ala-D-Ala-NH_2) and a peptide hydroxamate ($\alpha\epsilon$-diAc-L-Lys-D-Ala-Gly-NHOH). Neither of them combined with vancomycin, indicating that steric restriction is not the main reason, since the -CO-NH_2 group is smaller than the -CO_2H group. The -CO-NHOH group on the other hand is both bulkier and more polar than a methyl ester. Its polarity, however, is not strong enough or has not the appropriate geometry to be useful for complex-formation. The stability of the complex decreased sharply with decreasing pH below 3 (Nieto & Perkins, 1971), which, together with the present results, suggests that ionized carboxyl groups in the peptide and possibly also in vancomycin are required for complex-formation to take place.

Variations in residue 1. Table 4 shows the variation of the affinity of peptides for vancomycin on changing the configuration, size and/or polarity of residue 1. As already shown (Perkins, 1969), altering the configuration of residue 1 completely

Table 4. *Association constants and free enthalpy changes for the combination of vancomycin with peptides*

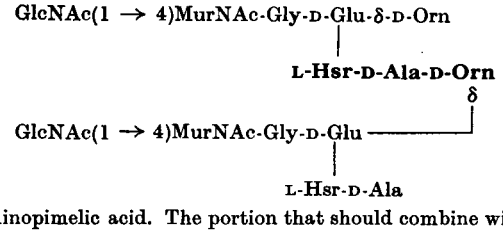
The experiments were carried out as indicated in the Materials and Methods section. Temperature was 25±1°C.

Peptide	K_A (l·mol^{-1})	ΔG (cal/mol)
Changes in residue 1		
(1) αε-DiAc-L-Lys-D-Ala-D-Ala	1.5×10^6	−8400
(2) αε-DiAc-L-Lys-D-Ala-Gly	1.3×10^5	−6950
(3) αε-DiAc-L-Lys-D-Ala-D-Leu	9.20×10^3	−5390
(4) αε-DiAc-L-Lys-D-Ala-D-Lys	1.37×10^4	−5620
(5) αε-DiAc-L-Lys-D-Ala-L-Ala	No combination	
(6) Ac-D-Ala-D-Ala	1.99×10^4	−5840
(7) Ac-D-Ala-Gly	5.40×10^3	−5070
Changes in residue 2		
(8) αε-DiAc-L-Lys-D-Leu-D-Ala	2.9×10^5	−7420
(9) αε-DiAc-L-Lys-Gly-D-Ala	9.4×10^4	−6760
(10) αε-DiAc-L-Lys-L-Ala-D-Ala	No combination	
(11) αε-DiAc-L-Lys-Aib-Gly	No combination	
(12) Ac-Gly-D-Ala	1.12×10^4	−5500
Changes in residue 3		
(13) Ac-Gly-D-Ala-D-Ala	9.40×10^4	−6760
(14) Ac-L-Ala-D-Ala-D-Ala	3.06×10^5	−7450
(15) αγ-DiAc-L-Dbu-D-Ala-D-Ala	7.7×10^5	−8000
(16) αδ-DiAc-L-Orn-D-Ala-D-Ala	1.3×10^6	−8320
(17) *N*-Ac-L-Tyr-D-Ala-D-Ala	1.9×10^5	−7180
(18) Myristoyl-D-Ala-D-Ala*	5.9×10^4	−6500
(19) αε-DiAc-L-Lys-D-Ala-D-Ala*	1.1×10^6	−8220
(20) Ac-D-Ala-D-Ala-D-Ala	4.95×10^4	−6380
Influence of free amino groups		
(21) L-Lys-D-Ala-D-Ala	1.16×10^4	−5510
(22) α-Ac-L-Lys-D-Ala-D-Ala	4.7×10^5	−7700
Influence of peptide chain length		
(23) D-Glu-γ-L-Lys-D-Ala-D-Ala	7.62×10^5	−8000
(24) D-Glu(α-Bzl)-γ-L-Lys-D-Ala-D-Ala	3.20×10^5	−7670
(25) L-Ala-D-Glu-γ-L-Lys-D-Ala-D-Ala	6.25×10^5	−7870
(26) L-Tyr-D-Glu-γ-L-Lys-D-Ala-D-Ala	8.25×10^5	−8050
(27) UDP-MurNAc-L-Ala-D-Glu-γ-L-Dap-D-Ala-D-Ala | D-CO_2H	7.2×10^5	−7960
Simultaneous changes in residues 1, 2 and 3		
(28) Ac-L-Ala-Gly-Gly	4.9×10^3	−5200
(29) Ac-L-Ala-Gly-Gly†	7.2×10^3	−5750
(30) Ac-Gly-Gly-Gly-Gly	1.50×10^3	−4300
(31) Ac-L-Ala-D-Glu-Gly	4.75×10^5	−7720
(32) αε-DiAc-L-Lys-D-Leu-D-Leu	5.00×10^3	−5000
(33) αε-DiAc-L-Lys-L-Ala-L-Ala	No combination	
(34) *C. poinsettiae* dimer‡	9.4×10^4	−6760

* Experiment carried out in 50% ethanol.

† Experiment carried out with a concentration of vancomycin of 0.8 mg/ml.

‡ The dimer from *C. poinsettiae* has the following structure (Leyh-Bouille *et al.* 1970*a*; Perkins, 1967):



Hsr, homoserine; Dap, αα′diaminopimelic acid. The portion that should combine with vancomycin is in **bold** type.

prevents the peptide from being a substrate. On the other hand, when this residue is glycine the peptide is still a good substrate, showing that there is a strong steric restriction for side chains (even a methyl group) in the L-configuration. For side chains in the D-configuration it is clear from Table

4 that a methyl group is the optimum size. When the size of the side chain is increased the affinity decreases, being slightly higher for an unbranched side chain (D-lysine) than for a branched one (D-leucine). The presence of a positively charged group in the side chain of residue 1 (D-lysine) has no effect on combination, at least when it is separated by four methylene groups from the peptide backbone.

Variation in residue 2. The general specificity characteristics for residue 2 are seen to be the same as for residue 1 (Table 4). Present results suggest that a methyl group represents the optimum size for chains in the D-configuration at position 2. Increasing the side chain of residue 2 as in $\alpha\epsilon$-diAc-L-Lys-D-Leu-D-Ala produces a smaller decrease in the association constant than a similar change at residue 1. Correspondingly, there is a higher favourable free energy for the interaction of the methyl group in residue 2 of $\alpha\epsilon$-diAc-L-Lys-D-Ala-D-Ala (when compared with $\alpha\epsilon$-diAc-L-Lys-Gly-D-Ala) than for the same group in residue 1. This implies that the combining site is 'wider' around residue 2 than about residue 1. The combining site, though, is equally strict in both positions 1 and 2, for side chains in the L-configuration. Site 2 does not accept L-side chains even when accompanied simultaneously by a D-side chain as in the peptide containing α-aminoisobutyric acid (peptide 11). Apparently the favourable interaction with the D-methyl group in peptide 11 is not enough to counterbalance the strong steric repulsion of the methyl group in the L-position, which must be higher than 6950 cal/mol (value of ΔG for $\alpha\epsilon$-diAc-L-Lys-D-Ala-Gly, Table 4).

Variations in residue 3. The effect of the size, shape and configuration of the side chain in residue 3 on the affinity of peptide for vancomycin is also shown in Table 4. A side chain in the D-configuration in this residue is seen to be better than none (compare with Ac-D-Ala-D-Ala), but presumably it also introduces a weak unfavourable interaction because the affinity of vancomycin for Ac-Gly-D-Ala-D-Ala is higher than the affinity for Ac-D-Ala-D-Ala-D-Ala. When the side chain is of the L-configuration the affinity is increased, this increase being for aliphatic side chains roughly proportional to their length. An optimum is reached for L-ornithine or L-lysine, the difference in the association constants for these peptides being within experimental error. An aromatic side chain, on the other hand, introduces an unfavourable interaction (compare K_A for Ac-L-Tyr-D-Ala-D-Ala and Ac-L-Ala-D-Ala-D-Ala), possibly attributable to steric hindrance. This is, however, much weaker than the apparent steric repulsion for a chain in the D-configuration. A long aliphatic acyl chain, as in the myristoyl peptide, also improves combination to some extent, as compared with Ac-D-Ala-D-Ala. For reasons of solubility the combination experiment for myristoyl-D-Ala-D-Ala was carried out in 50% ethanol and as a control the affinity of $\alpha\epsilon$-diAc-L-Lys-D-Ala-D-Ala for vancomycin was measured in the same solvent (Table 4, peptides 18 and 19).

Throughout this work we have assumed a molecular weight for vancomycin of 1800 (see Nieto & Perkins, 1971) and the Scatchard plot gives one combining site per mol of vancomycin for this value. However, there remains some uncertainty in the precise value of the molecular weight, but if the association constants and free enthalpies of combination of the peptides were calculated by assuming mol.wt. of 1700 only small differences resulted.

Influence of free amino groups in residues 2 *and* 3. Perkins (1969) showed that D-Ala-D-Ala did not combine with vancomycin at all. However, when the amino group was blocked by acetylation Ac-D-Ala-D-Ala was a fairly good substrate (Table 4). The two following alternatives were possible: (*a*) combination was prevented because of electrostatic repulsion due to the free amino group, (*b*) the interaction with the carbonyl group of the acetyl radical was essential for combination. DNP-D-Ala-D-Ala was synthesized as a peptide devoid of both carbonyl group and basicity at the amino group and shown not to combine by u.v. difference spectroscopy and t.l.c. in solvent 4. However, 2,4-dinitrobenzoyl-D-Ala-D-Ala did not combine either, hence steric hindrance was the dominant factor and this experiment did allow a decision between the two alternatives.

The effect of α- and ω-amino groups at residue 3 is shown in Table 4. A free α-amino group decreases considerably the affinity of peptide for vancomycin. This was already found for L-Ala-D-Ala-D-Ala (Perkins, 1969), and is also true for all the tripeptides. The same alternative explanations as for residue 2 were possible and again $\alpha\epsilon$-di-DNP-D-Lys-D-Ala-D-Ala was found to combine (t.l.c.) much more weakly than the diacetyl derivative, but as for residue 2, the result could be misleading because of the large size of the dinitrophenyl radical. The influence of the ω-amino group when the amino acid in residue 3 is dibasic was more clear-cut. Such groups decreased the stability of the complex and the nearer they were to the backbone the poorer was the combination. In fact the combination of L-Dbu-D-Ala-D-Ala as seen by u.v. difference spectroscopy was so weak and the spectral change so small that it was not possible to measure it with any accuracy. All these facts seem to point to electrostatic repulsion as the source of the effect of the free amino groups. Comparison of the ΔG for L-Lys-D-Ala-D-Ala (peptide 21) and Ac-D-Ala-D-Ala

(peptide 6), bearing in mind that a side chain in the L-configuration improves interaction (Table 4), suggests that free amino groups introduce an unfavourable interaction rather than merely lacking a favourable one.

Effect of residues beyond residue 3. Residues beyond residue 3 hardly interact with vancomycin (Table 4). The improvement that D-Glu-γ-L-Lys-D-Ala-D-Ala represents over α-Ac-L-Lys-D-Ala-D-Ala is probably due to the presence of a free carboxyl group on the D-glutamic acid residue, because when this carboxyl group is esterified in the α-Bzl ester the affinity is again decreased. This, again, points to electrostatic forces playing a main role in the decrease in stability caused by free amino groups.

Simultaneous variations in residues 1, 2 *and* 3. When more than one residue is changed with respect to the standard peptide the results are consistent with the results already described (Table 4). It is noteworthy that Ac-L-Ala-D-Glu-Gly (peptide 31), with a relatively large, unbranched, negatively charged side chain in residue 2, is a good substrate, better than αε-diAc-L-Lys-D-Ala-Gly (peptide 2), indicating that a negative charge in residue 2 favours combination. This is still a further indication that a positively charged group in vancomycin is located in the vicinity of this group. The affinity of vancomycin for the dimer from *Corynebacterium poinsettiae* (peptide 34) is comparable with that for αε-diAc-L-Lys-D-Ala-D-Lys (peptide 4), emphasizing that there is little steric restriction at residue 1 beyond the first three or four methylene groups in the side chain.

Source of the u.v. difference spectrum. Figs. 1–4 show the u.v. difference spectra induced by combination between vancomycin and peptides having side chains of different sizes in residues 1, 2 and 3. A peptide devoid of any side chains, Ac-Gly-Gly-Gly-Gly, produced the negative difference with extremes around 274nm and 283nm but not the positive difference at about 294nm (Figs. 1 and 3). The main source of negative difference may thus be attributed to interaction of the peptide backbone with the vancomycin chromophores. Residue 3, however, contributes to the minimum at 274nm when the side chain is in the L-configuration (Figs. 3 and 4). This contribution, however, is only appreciable when the side chain is the aromatic one of tyrosine and is very small for aliphatic side chains. In fact, the difference spectra in dilute solution (0.03–0.1 mg/ml) for peptides terminating in D-Ala-D-Ala and having an aliphatic side chain of L-configuration in residue 3 are identical, within experimental error. The side chains that modify the shape of the difference spectrum more are those of residues 1 and 2. As can be seen in Figs. 1 and 2, they are responsible for the positive difference at about 294nm. They do not contribute equally, however,

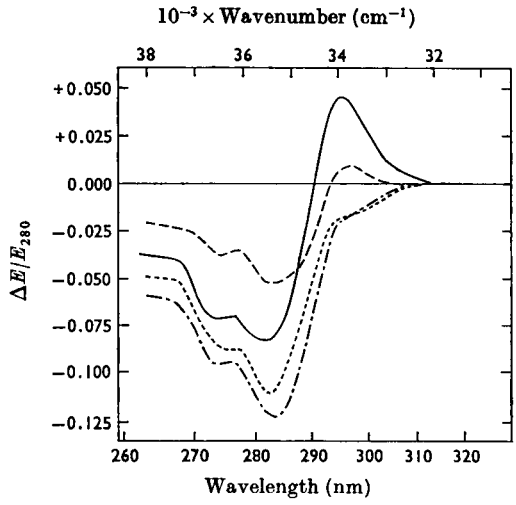


Fig. 1. U.v. difference spectra induced on vancomycin by complex-formation with Ac-D-Ala-D-Ala (——), Ac-D-Ala-Gly (– – –), Ac-Gly-Gly-Gly-Gly (····), Ac-Gly-D-Ala (– · –). The concentration of vancomycin was 0.10–0.16 mg/ml in 0.02M-sodium citrate buffer, pH5.1, and a large excess of peptide was added to ensure that all the vancomycin was in complex form. The temperature was 25°C and the spectrophotometer settings were: damping, 2; resolution, 2; slit adjustment, automatic.

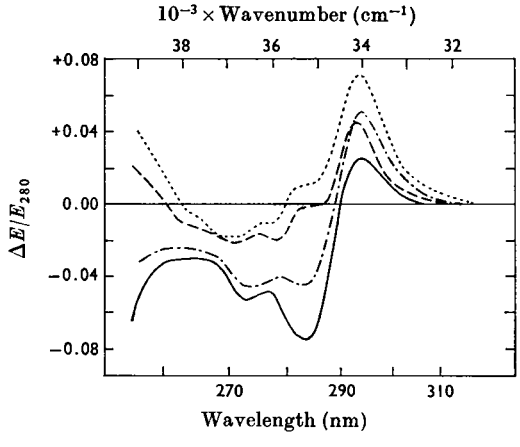


Fig. 2. U.v. difference spectra induced on vancomycin by complex-formation with αε-diAc-L-Lys-D-Ala-D-Ala (concn. of vancomycin 0.05mg/ml) (——), αε-diAc-L-Lys-D-Leu-D-Leu (····), αε-diAc-L-Lys-D-Ala-D-Leu (– – –), αε-diAc-L-Lys-D-Leu-D-Ala (– · –). The concentration of vancomycin for the three last-named was 0.120mg/ml. All spectra were recorded in 0.02M-sodium citrate buffer, pH5.1, at 25°C. A large excess of peptide was added to ensure that all the vancomycin was in the form of complex. Spectrophotometer settings were as given for Fig. 1.

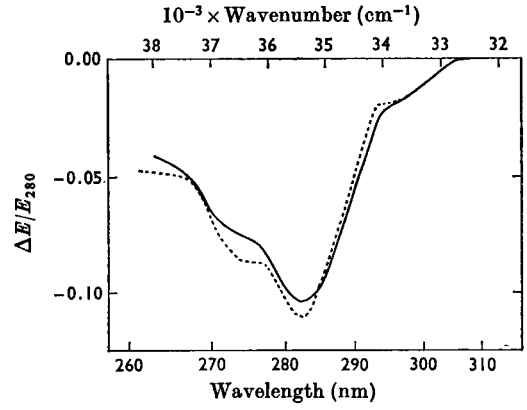


Fig. 3. U.v. difference spectra induced on vancomycin by complex-formation with Ac-L-Ala-Gly-Gly (——), Ac-Gly-Gly-Gly (----). Conditions of the experiment were as given in Fig. 1.

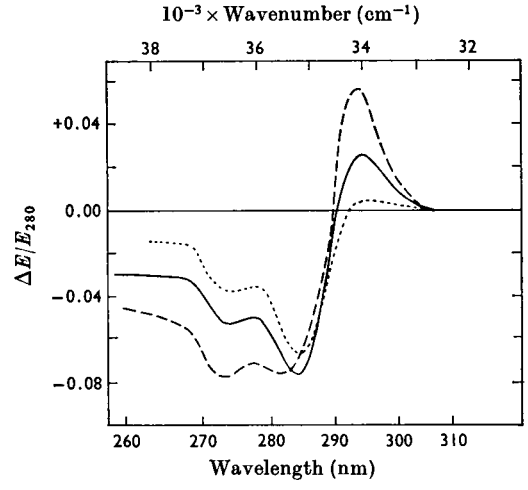


Fig. 4. U.v. difference spectra induced on vancomycin by complex-formation with *N*-Ac-L-Tyr-D-Ala-D-Ala (–––), Ac-D-Ala-D-Ala-D-Ala (----), αε-diAc-L-Lys-D-Ala-D-Ala (——). The vancomycin concentration was 0.05 mg/ml for the last-named and 0.157 mg/ml for the other two peptides. Other conditions were as given in Fig. 1.

when the side chain is a methyl group (Fig. 1). When the size is increased both of them are seen to produce a positive difference of similar size (Fig. 2). It is noteworthy, however, that although qualitatively we can ascribe individual parts of the difference spectrum to interaction with particular regions of the peptide, quantitatively the difference spectra are not additive. Thus Ac-D-Ala-D-Ala is seen (Fig. 1) to induce a positive difference at 294 nm that is bigger than the sum of Ac-Gly-Gly-Gly-Gly, Ac-Gly-D-Ala and Ac-D-Ala-Gly. It seems then

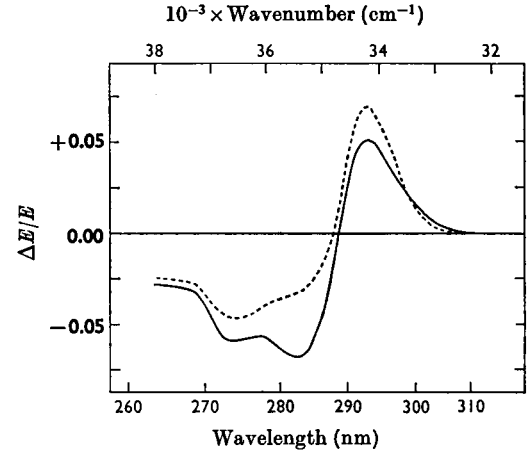


Fig. 5. U.v. difference spectra induced on vancomycin by complex-formation with αε-diAc-L-Lys-D-Ala-D-Ala (——), L-Lys-D-Ala-D-Ala (----). The concentration of vancomycin in each was 0.230 mg/ml. Other conditions were as given in Fig. 1.

that a methyl side chain in residue 1 contributes to this positive difference only when there is another methyl side chain in residue 2 (Fig. 1). Also an aromatic side chain in residue 3 (Ac-L-Tyr-D-Ala-D-Ala, Fig. 4) contributes to the positive difference whereas an aliphatic one does not.

Chromatographic mobility of vancomycin–peptide complexes and free amino groups in the peptide

Chatterjee & Perkins (1966) first discovered UDP-*N*-acetylmuramyl-pentapeptide–vancomycin complexes when attempting to purify the nucleotide precursors by paper chromatography in solvents 5 and 6. The complex gave a spot moving more slowly than both vancomycin and free nucleotide in both solvents in paper chromatography and the same is true of t.l.c. in solvent 4. We have now found that the chromatographic separation depends upon the presence of free amino groups in the peptide. None of the fully acetylated peptides gave a slow spot and all the peptides having either an α- or ω-amino group, or both amino groups, free, gave a slow spot independently of the length of the peptide and the presence or absence of the UDP-*N*-acetylmuramyl moiety. On the other hand when the difference spectra induced by identical peptides differing only in the presence or absence of free amino groups are compared (Fig. 5) the most prominent difference is the increased positive difference at 294 nm for the peptides having free amino groups. For a given peptide sequence, the value of this difference has been shown by Nieto & Perkins (1971) to correlate with the amount of vancomycin–peptide

complex present in aggregated form and the present data indicate that free amino groups favour aggregation of the complex. The difference spectrum induced by combination with Ac-L-Ala-D-Glu-Gly (not shown), the only negatively charged peptide synthesized in the present work, shows no maximum at 294, suggesting a lack of aggregation and supporting the view that positive charges in the complex induce aggregation. The implication of these results is that the slow spot is aggregated vancomycin–peptide complex.

DISCUSSION

Interactions contributing to the binding. The change in free enthalpy (Gibbs free energy) for the combination of peptides with vancomycin has been calculated from the association constants and presented in Table 4. Since the contributions of residues beyond residue 3 are very small or none we can consider that an acetylated tripeptide is fully responsible for the interactions leading to binding. If ΔG for combination of Ac-Gly-Gly-Gly-Gly is accepted as the contribution of the backbone (4300cal/mol), it represents a substantial proportion of the total free enthalpy of combination of other peptides. The rest must be contributed by the interaction of the side chains. When we try to estimate the contribution of side chains in residue 1 and 2 we find, however, that this contribution depends upon the length of the peptide. Thus comparison of the ΔG values for diAc-L-Lys-D-Ala-D-Ala and diAc-L-Lys-D-Ala-Gly gives a value of −1450cal/mol for the interaction with vancomycin of the methyl group in residue 1. If we compare Ac-D-Ala-D-Ala with Ac-D-Ala-Gly we obtain only −770cal/mol. The same situation occurs when residue 2 is considered. Since the differences are far beyond experimental error the consequence is that the interaction of peptide with vancomycin is a property of the whole sequence or, in other words, the presence of residue 3 (in the L-configuration) and its associated favourable interaction makes the interactions at residues 1 and 2 stronger. A similar situation was observed in comparing the u.v. difference spectra for different peptides. Although qualitatively we can ascribe individual contributions to the backbone and the side chains a fair amount of the free enthalpy of binding depends upon the presence of the peptide as a whole.

Since the length of the peptide is important for a strong binding we can conclude that the backbone is in contact with vancomycin. The same could be deduced by comparing ΔG for the combination of Ac-D-Ala-D-Ala and Ac-Gly-D-Ala-D-Ala (Table 4, peptides 6 and 13). On the other hand, since peptides with the terminal carboxyl group blocked do not combine at all and this is not dependent upon steric hindrance we can conclude that a large amount of the energy of binding comes from a polar interaction (ionic or hydrogen bond) of this group with some group in vancomycin. The poor additivity of the free enthalpies could be explained if the presence of side chains or substituents on the terminal dipeptide had the effect of isolating the polar bond from the aqueous environment. This would result in a large increase in the strength of this polar bond depending upon the hydrophobic interactions of the side chains with the antibiotic.

The results of this paper and the preceding one (Nieto & Perkins, 1971) do much to define the specificity of the *C*-terminal peptide sequences that will combine with vancomycin and the conditions under which such combination can occur. In particular, it is clear that the optical configuration of the three terminal amino acid residues controls the specificity to a large extent. Discrimination between carbon optical isomers requires interaction with three or the four substituents of the asymmetric carbon atom. Vancomycin recognizes in an all or none manner two centres of asymmetry (residues 1 and 2) and strongly discriminates at a third (residue 3). This necessitates interaction at two points on each of the three amino acid residues. Our results allow these interactions to be ascribed as follows: for residue 1 one of the interactions is a strong steric repulsion for side chains in the L-configuration; a hydrogen or an ionic bond between the ionized carboxyl group and some group in vancomycin accounts for the other. There is certainly also, however, an interaction with the side chain of D-amino acids, which is favourable when this group is a methyl group and unfavourable when it is bigger. In residue 2 one of the interactions is again a strong steric discrimination against L-side chains and another favourable interaction occurs with a D-side chain. The combining site must be 'wider' around this residue than around residue 1 since the repulsive free enthalpy for a D-leucine in residue 2 is +980cal/mol (comparison of ΔG for diAc-L-Lys-D-Ala-D-Ala and diAc-L-Lys-D-Leu-D-Ala, peptides 1 and 8) compared with +3010cal/mol in residue 1. In residue 3 the interactions are a weak repulsion for side chains of the D-configuration and a fairly strong favourable interaction with L-side chains. The peptide backbone also clearly contributes to the binding in this residue.

Van der Waals' repulsive forces and hydrophobic bonds seem thus to contribute largely to the specificity shown by vancomycin. The peptide backbone could on the other hand form up to four hydrogen bonds, which would account for a fair amount of the free enthalpy of interaction for Ac-Gly-Gly-Gly-Gly. All these interactions would co-operate to give the overall ΔG.

Since the vancomycin molecule apparently has

very limited conformational flexibility (Nieto & Perkins, 1971) the binding can be considered in terms of a lock and key model. The peptide may be assumed to be lying in a cleft in the vancomycin molecule, this cleft being of the appropriate geometry so as to provide for the necessary steric restrictions. Phenolic groups may be supposed to line this cleft and they may be involved in hydrogen bonding (Nieto & Perkins, 1971). When the peptide combines with vancomycin it will 'fill' the cleft, blocking the access of the solvent to at least one face of the phenolic rings.

Shape of the difference spectrum. The most prominent feature of the difference spectra of complexes between vancomycin and peptide with side chains bigger than a methyl group in residue 1 is the positive difference having a maximum at about 294nm. This is reminiscent of the changes which appear in the difference spectrum of diAc-L-Lys-D-Ala-D-Ala–vancomycin complex when aggregation takes place (Nieto & Perkins, 1971). The peptides eliciting this maximum as the main feature in the difference spectrum show an affinity for vancomycin much lower than the standard peptide ending in D-Ala-D-Ala, and it is reasonable to conclude that the perturbation giving rise to it is an interaction of the side chains with the phenolic rings in vancomycin. This interaction would be a repulsion for side chains bigger than a methyl group. The fact that this steric repulsion appears for the standard peptide ($\alpha\epsilon$-diAc-L-Lys-D-Ala-D-Ala) only on aggregation (Nieto & Perkins, 1971) could be explained if aggregation had as a consequence a generalized or local narrowing of the cleft holding the peptide. If this were true, aggregated molecules of vancomycin should show a higher affinity for a peptide like Ac-L-Ala-Gly-Gly, as was in fact observed (Table 4, peptides 28 and 29). A local narrowing of the cleft of about 1 Å would be enough and it is unnecessary to invoke any extensive conformational changes for which there is little evidence (Nieto & Perkins, 1971).

Interaction of vancomycin with bacterial cell walls. Perkins & Nieto (1970) found that living cells and isolated cell walls of *M. lysodeikticus* took up much more radioactive iodovancomycin than bacilli, and indeed more than their possible content of uncross-linked peptide chains ending in D-Ala-D-Ala. The [^{125}I]iodovancomycin thus fixed to the walls was slowly extracted by diAc-L-Lys-D-Ala-D-Ala and also exchanged slowly with non-radioactive iodovancomycin. Salts did not remove any vancomycin from these walls. A possible explanation was that vancomycin was able to combine with the peptide chains having a glycine residue amidating the α-carboxyl group of the glutamic acid, and in fact Ac-L-Ala-D-Glu-Gly had a high affinity for vancomycin (Table 4, peptide 31). Possibly the limit to the amount of vancomycin taken up by *M. lysodeikticus* and other bacteria of the chemotype III of Ghuysen (1968) is set by the restrictions imposed by the wall structure (such as steric and ionic repulsions) as well as by vancomycin molecules previously attached to it, rather than by the availability of specific sites with which to form stable complexes.

The fact that the dimer from *C. poinsettiae* combined with vancomycin is also relevant to the interaction of vancomycin with cell walls *in vitro* or *in vivo*. It means that vancomycin will bind to the peptide cross-bridges of the bacterial mucopeptides provided that a sequence R-CO-D-Ala-D(R_2)-CO_2H is present. The binding will be two to three orders of magnitude weaker than for peptides ending in D-Ala-D-Ala but the number of sites available will be very high. This will be true for mucopeptides of the chemotypes I and IV of Ghuysen (1968). Binding will only be prevented if the terminal carboxyl group is amidated as for *B. licheniformis* (Hughes, 1970), where the heptapeptide diamide was found not to combine with vancomycin at all. In chemotype II, on the other hand, it has been reported that peptide chains which are not involved in cross-linking terminate in D-Ala-D-Ala (Tipper & Berman, 1969). Since the cross-linking is about 75% in *S. aureus* Copenhagen and lower in other bacteria of the same chemotype, the uncross-linked chains supply a large number of potential binding sites for vancomycin, just like the cell-wall mucopeptides of other Gram-positive bacteria.

Inhibition in vivo *and in cell-free systems.* The present work clearly shows that variations in the sequence of the peptide commonly found in natural mucopeptides (variations in the nature of residue 3 conserving its L-configuration) do not decrease its affinity for vancomycin. This adds strength to the hypothesis that complexes are involved in the mechanism of inhibition of mucopeptide synthesis by these antibiotics (Perkins, 1969; Perkins & Nieto, 1970). Inhibition of synthesis *in vivo* must, however, be clearly distinguished from inhibition of synthesis in the test tube by particulate membrane preparations. Perkins & Nieto (1970) showed *in vivo* that [^{125}I]iodovancomycin was bound primarily to the wall and then transferred to the membrane, and very little, if any, of the radioactivity was found in the cytoplasm. This seems to rule out any involvement of UDP-MurNAc-pentapeptide–vancomycin complex in inhibition of biosynthesis *in vivo*. On the other hand, binding of vancomycin to accessible chains ending in D-Ala-D-Ala in the cell wall is not only possible but inevitable given the high affinity of the vancomycin for peptides with this sequence. The growth points or zones in the mucopeptide are generally believed to contain

uncross-linked peptide chains ending in D-Ala-D-Ala. If vancomycin can reach these points (and since it is able to reach the membrane it is very likely that it can), it will combine with the peptide chains and presumably prevent cross-linking. Studies on the inhibition by vancomycin of a soluble D-Ala-D-Ala carboxypeptidase from *Streptomyces albus* G (Leyh-Bouille *et al.* 1970*b*) showed that vancomycin was able to block the access of this type of enzyme to the D-Ala-D-Ala terminus.

In the systems *in vitro*, on the other hand, permeability barriers have been diminished or totally removed and vancomycin or ristocetin will be able to combine with almost any appropriate peptide chain present in the system. In such experiments, the number of available specific peptide chains and their relative affinities for vancomycin will influence the conclusions about inhibition by any particular concentration of antibiotic.

Complex-formation as a general mechanism for antibiotic action. There is a whole family of antibiotics including the ristocetins, ristomycins and actinoidins similar to vancomycin and although their specificities differ (M. Nieto & H. R. Perkins, unpublished work) all that have been examined seem to show the same optimum affinity for the R_4-CO-L-(R_3)D-Ala-D-Ala sequence. Another completely different antibiotic, bacitracin, also seems to act through specific complex-formation, this time with the polyisoprenoid alcohol pyrophosphate that acts as carrier of disaccharide pentapeptide building blocks across the membrane (J. L. Strominger, personal communication). Complex-formation with intermediates in metabolic pathways could be one type of general mechanism of antibiotic action. Such antibiotics would compete with one or more particular enzymes depriving them of substrate. One condition for this type of inhibition would be an association constant of the antibiotic–intermediate higher than the association constant for the enzyme–intermediate complex. Since the enzyme also has a catalytic action and the reaction product would no longer combine with the antibiotic, this type of inhibition would be time-dependent.

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