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Peptide Inhibitors of *Streptomyces* DD-Carboxypeptidases

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1. Peptides that inhibit the DD-carboxypeptidases from *Streptomyces* strains *albus* G and R61 were synthesized. They are close analogues of the substrates of these enzymes. The enzymes from *albus* G and R61 strains are in general inhibited by the same peptides, but the enzyme from strain R39 differs considerably. 2. The two *C*-terminal residues of the peptide substrates and inhibitors appear to be mainly responsible for the initial binding of the substrate to the enzymes from *albus* G and R61 strains. The side chain in the third residue from the *C*-terminus seems critical in inducing catalytic activity. 3. Experimental evidence is presented suggesting that the amide bond linking the two *C*-terminal residues has a *cis* configuration when bound to the enzymes from strains *albus* G and R61. 4. The peptide inhibitors are not antibiotics against the same micro-organisms.

Bacterial DD-carboxypeptidases catalyse the cleavage of the *C*-terminal D-Ala-D-Ala dipeptide in the un-cross-linked peptide chains of the cell-wall mucopeptide (peptidoglycan). In most bacteria these enzymes appear to be bound to the cell membrane, but many *Streptomyces* strains excrete them into the culture medium in a soluble form. Advantage of this fact has been taken to purify and study the substrate specificity of the DD-carboxypeptidases from *Streptomyces* strains *albus* G (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970), R61 (Leyh-Bouille *et al.*, 1971) R39 and K11 (Leyh-Bouille *et al.*, 1972). Although in general, all these enzymes show similar specificity profiles, their kinetic parameters (K_m and $V_{max.}$) differ considerably. They also differ in their sensitivity to the penicillin and cephalosporin group of antibiotics; whereas the enzyme from strain *albus* G is not inhibited by the antibiotics, the DD-carboxypeptidases from strains R61, K11 and R39 are very sensitive although their kinetics of inhibition differ. We now report the synthesis of peptides that are analogues of the substrates of the carboxypeptidases and that inhibit both penicillin-resistant and penicillin-sensitive types of enzyme. These substrate analogues are shown to be better inhibitors of the penicillin-resistant enzyme.

Materials and Methods

Enzymes

The enzyme preparations and units of enzymic activity used in this work have been previously described (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1971, 1972). D-Alanine, liberated during enzymic hydrolysis of the standard substrate $\alpha\epsilon$-Ac_2-L-Lys-D-Ala-D-Ala, was converted into the Dnp derivative and measured as previously described. (Ghuysen *et al.*, 1970) or by using $\alpha\epsilon$-Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala. In the latter case after incubation with the enzyme, the D-[^{14}C]alanine cleaved was separated by paper electrophoresis (0.25M-formic acid, pH1.9, 10V/cm for 2–3h), eluted with water (0.3–0.4ml) and radioactivity was measured in a Packard Tri-Carb liquid-scintillation counter, with 10ml of a solution in dioxan (final volume 1 litre) of 180g of naphthalene, 4.0g of 2,5-diphenyloxazole and 1.0g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene as scintillant. Efficiency of counting was 70–73%.

Amino acids

These were puriss quality, from Koch–Light Laboratories Ltd. (Colnbrook, Bucks., U.K.). Dicyclohexylcarbodi-imide was purchased from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and *N*-hydroxysuccinimide, benzyloxycarbonyl chloride and D-cycloserine (D-4-amino-3-isoxazolidone) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. D-[^{14}C]Alanine (30mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals used in this work were of the best quality commercially available.

Amino acid derivatives

The synthesis of the t-butoxycarbonyl derivatives of D-alanine and L-lysine and their *N*-hydroxysuccinimide esters, as well as that of the γ-benzyl ester

of D-glutamic acid have been described (Nieto & Perkins, 1971). Dibenzyloxycarbonyl-L-lysine was prepared as described by Schröder *et al.* (1961). Benzyloxycarbonyl-D-alanine was prepared as described by Greenstein & Winitz (1961, p. 895) for L-threonine. It recrystallizes easily from ethyl acetate–hexane, yield 70% of theoretical, m.p. 72–75°C, $[\alpha]_D^{25}$ +16.8 (*c* 2 in acetic acid) (Found: C, 59.2; H, 5.8; N, 6.3; $C_{11}H_{13}NO_4$ requires C, 58.9; H, 5.9; N, 6.9%). The *N*-hydroxysuccinimide ester of dibenzyloxycarbonyl-L-lysine and benzyloxycarbonyl-D-alanine were prepared by the general method of Anderson *et al.* (1964) in a 86–90% yield and recrystallized from propan-2-ol. Dibenzyloxycarbonyl-L-lysine *N*-hydroxysuccinimide ester had m.p. 111–113°C and $[\alpha]_D^{22.5}$ −15.4 (*c* 1 in acetone) (Found: C, 60.9; H, 5.7; N, 8.1; $C_{26}H_{29}N_3O_8$ requires C, 61.1; H, 5.7; N, 8.2%). The *N*-hydroxysuccinimide ester of benzyloxycarbonyl-D-alanine had m.p. 114–117°C, $[\alpha]_D^{25}$ +32.7 (*c* 1 in acetone) (Found: C, 56.1; H, 5.3; N, 8.5. Calc. for $C_{15}H_{16}N_2O_6$: C, 56.2; H, 5.0; N, 8.7%).

Synthesis of non-radioactive peptides

The synthesis of L-Lys-D-Ala-D-Ala, Gly-D-Ala-D-Ala and L-Ala-D-Ala-D-Ala has been described previously (Nieto & Perkins, 1971). The remaining peptides were synthesized by the method of Anderson *et al.* (1964) without protecting the carboxyl group of the amino-donor amino acid. A mixture of acetonitrile–water (1.3:1, v/v) was used as solvent. The intermediates were isolated and purified as described by Nieto & Perkins (1971) but in general they were not characterized. Removal of protecting groups and acetylation or succinylation (3-carboxypropionylation) were carried out as previously described (Nieto & Perkins, 1971). The procedure for glutarylation (4-carboxy-*n*-butyrylation) was similar to that for succinylation. Whatman no. 3 paper required for preparative purposes was first exhaustively washed by irrigation with 1M-ammonium acetate followed by water. Additional information is as follows.

(*a*) In the synthesis of L-Lys-D-Glu-D-Ala, dibenzyloxycarbonyl-L-Lys-D-Glu γ-benzyl ester was synthesized as described above and then coupled to D-alanine benzyl ester by means of dicyclohexylcarbodi-imide as described by Nieto & Perkins (1971). Carbodi-imide coupling was also used in the synthesis of myristoyl-D-Ala-D-Asp from myristic acid, t-butoxycarbonyl-D-alanine and D-aspartic acid dibenzyl ester.

(*b*) The protecting group in t-butoxycarbonyl-D-Ala-D-cycloserine was removed with 10% (v/v) trifluoroacetic acid in dichloromethane at 0°C for 15min, followed by 10min at room temperature. Even under these conditions about 15% of the D-cycloserine in the peptide was degraded. Free D-cycloserine, however, seemed stable. Also, after succinylation of D-Ala-D-cycloserine in the presence of triethylamine, paper electrophoresis at pH7 and 10V/cm, showed two close anionic bands. Their relative mobilities with respect to glutamic acid were 0.3 and 0.7 respectively. Under identical conditions the relative mobility with respect to glutamic acid of Ac-D-Ala-D-Glu was 1.4 and that of Ac-D-Ala-D-Ala, 0.78. The succinylated product was obtained as a mixture of the two bands. After acid hydrolysis of the mixture and correction for destruction of serine an alanine/serine ratio of 1:1 was obtained. No further attempt was made to identify the chemical nature of the compound.

A similar treatment as for the D-cycloserine peptide was used to remove protecting groups of di-t-butoxycarbonyl-L-Lys-racemic-cyclodiaminoadipic acid. No ring opening was observed. The rotations of most of the peptides synthesized are given in Table 1. The methods used for identification of peptides were as described by Nieto & Perkins (1971).

Table 1. *Optical rotations of synthetic peptides*

The concentration of peptide was estimated by automatic amino acid analysis in all cases. Abbreviations: Ac, acetyl; Z, benzyloxycarbonyl.

Peptide	$[\alpha]_D$
D-Ala-D-Glu	−12.68 at 24°C (*c* 0.5 in water, pH3.9)
Ac-D-Ala-D-Glu	+77.8 at 26.5°C (*c* 1 in water, pH1.6)
Z-D-Ala-D-Glu	+10.7 at 23.5°C (*c* 0.5 in acetic acid)
D-Ala-D-Asp	−11.9 at 22°C (*c* 1.6 in water, pH2.75)
Ac-D-Ala-D-Asp	+63.5 at 23.0°C (*c* 0.8 in water, pH1.8)
D-Ala-D-cycloserine	−5.5 at 22.6°C (*c* 0.2 in water, pH5.3)
L-Lys-D-Ala-D-Glu	+92.4 at 24.1°C (*c* 0.5 in water, pH4.35)
L-Lys-D-Glu-D-Ala	+103.2 at 23.0°C (*c* 0.5 in water, pH4.35)
α-Ac-L-Lys-D-Glu-D-Ala	+8.25 at 24°C (*c* 0.8 in water, pH4.2)
Ac_2-L-Lys-D-Glu-D-Ala	+17.9 at 25°C (*c* 1 in water, pH2.45)

All peptides gave correct amino acid analyses, but some were obtained in too small an amount for measurement of rotation. Acetyl- and succinyl-peptides were separated from the parent peptides by paper electrophoresis (Nieto & Perkins, 1971) and identified by their mobilities, and by their correct amino acid analysis.

(*c*) Phenylacetylation was performed as follows. A sample of racemic-diaminoadipic acid lactam (9 μmol in 1 ml water) was adjusted to pH 9.5 by addition of triethylamine and placed in an ice bath. Then phenylacetyl chloride (5 μl) was added and the mixture was shaken, before being evaporated to dryness *in vacuo*. After acidification with HCl, excess of phenylacetic acid was removed by ether extraction. The aqueous layer was then applied to paper for electrophoresis in acetic acid–collidine–water (2.65:9.1:1000, by vol.), pH 7, at 10 V/cm for 2 h. Apart from a little unchanged lactam, the main product was an anionic band that reacted strongly with the chlorine/starch–iodide procedure of Rydon & Smith (1952). The peptide was eluted from the paper. On acid hydrolysis (4 M-HCl, 105°C, 1 h) this material yielded diaminoadipic acid and was therefore taken to be the *N*-phenylacetyl-cyclodiaminoadipic acid (racemic mixture of DD- and LL-isomers).

Synthesis of peptides of D-[^{14}C]*alanine*

D-*Ala*-D-[*U*-^{14}C]*Ala*. To 50 μCi of D-[U-^{14}C]alanine in water (100 μl) were added $NaHCO_3$ (3.2 mg) and t-butoxycarbonyl-D-Ala *N*-hydroxysuccinimide ester (3.2 mg) in acetonitrile (120 μl), and the mixture was agitated for 2 h at room temperature. The clear solution was concentrated to dryness and treated with 50% (v/v) trifluoroacetic acid in dichloromethane (300 μl) for 5 min at 0°C followed by 10 min at room temperature. The solution was concentrated to dryness, redissolved in 80% (v/v) acetic acid (300 μl) and evaporation was repeated. Finally the reaction mixture was dissolved in 0.25 M-formic acid and applied to washed Whatman no. 3 paper for purification by paper electrophoresis in 0.25 M-formic acid (10 V/cm, 3 h). Under these conditions the ratio of mobilities (D-Ala-D-Ala)/(D-Ala) is 1.22. Better separation was obtained in acetic acid–pyridine–water (10:1:1000, by vol.), pH 3.5 (53 V/cm, 1 h), the ratio of mobilities being 3.0 in this case. The position of the peptide was determined by radioautography. After elution of the peptide with water a conversion of radioactivity from D-[^{14}C]Ala into D-Ala-D-[^{14}C]-Ala of 93% was obtained.

Similarly, by reaction between di-t-butoxycarbonyl-L-Lys *N*-hydroxysuccinimide ester and D-Ala-D-[U-^{14}C]Ala the peptide L-Lys-D-Ala-D-[U-^{14}C]Ala was obtained and purified. Acetylation was carried out as described by Nieto & Perkins (1971). The overall recovery of radioactivity in acetylated tripeptide was 78% and the specific radioactivity of the peptide should be identical with that of the starting labelled amino acid.

2,5-*Diaminoadipic acid lactam*. The parent acid was synthesized via dimethyl $\alpha\alpha$-diphthalimidoadipate as described by Greenstein & Winitz (1961, p. 2510). The *meso* and racemic forms of the intermediate were separated by fractional crystallization, the final m.p. of recrystallized samples being 210°C (Kofler) for the *meso*-isomer (lit. 211°C) and 178.5°C for the racemic mixture of DD- and LL-isomers. The value of 165°C given by Greenstein & Winitz (1961, p. 2510) for the latter compound is evidently too low. The free diaminoadipic acids obtained by hydrazinolysis and subsequent acid hydrolysis of the above intermediates were examined by paper chromatography in solvent A [methanol–water–pyridine–conc. HCl (32:7:4:1, by vol.) (Rhuland *et al.*, 1955)] or solvent B [methanol–water–pyridine–98% (w/v) formic acid (80:19:10:1, by vol.) (Perkins, 1965)]. The *meso*-isomer was rather insoluble but gave a single ninhydrin-positive spot in each solvent with $R_{meso\text{-diaminopimelic acid}}$ 0.79 (solvent A) or 0.91 (solvent B), whereas the racemic mixture of DD- and LL-isomers gave two spots with $R_{meso\text{-diaminopimelic acid}}$ 0.79 and 0.97 (solvent A) or 0.91 and 1.11 (solvent B). Thus optical isomers of diaminoadipic acid separate in these solvents, as first described for diaminopimelic acid (Rhuland *et al.*, 1955). On chromatograms developed in solvent A and then treated with ninhydrin and heated at 105°C, diaminoadipic acids gave cherry-red spots like that given by ornithine (Perkins & Cummins, 1964). The identity of the various compounds was confirmed by elution of samples chromatographed in solvent B followed by conversion into the di-Dnp derivatives. The derivatives from both slow- and fast-moving spots found in the racemic mixture ran in one position (R_F 0.39) on a chromatogram (Whatman no. 1 paper) in solvent C [butan-1-ol–water–aq. NH_3 (sp.gr. 0.880) (20:19:1, by vol.)] whereas the di-Dnp derivative of the *meso*-isomer ran with R_F 0.50 (cf. the di-Dnp derivatives of diaminopimelic acid in the same solvent *meso*-isomer, R_F 0.13; LL- or DD-isomer, R_F 0.19).

To determine which of the racemic pair of isomers of diaminoadipic acid ran faster in solvents A and B, a sample of the di-Dnp derivative of the faster spot was dissolved in 0.125 M-$NaHCO_3$ and its optical rotatory dispersion was compared with that of the di-Dnp derivative of LL-diaminopimelic acid. The shape of the curve was identical; hence the faster spot was the LL-isomer of diaminoadipic acid. The molar rotations of the di-Dnp derivatives, $[M]^{25}_{350}$, were −35000° for LL-diaminopimelic acid and −79700° for LL-diaminoadipic acid.

An attempt was made to cyclize racemic-diaminoadipic acid by using a soluble carbodi-imide. Racemic

acid (23mg) in water (5ml) was treated with triethylamine (36μl) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide methotoluene-*p*-sulphonate (33mg), and left in the dark at room temperature. After 3 days a sample chromatographed in solvent A and treated with ninhydrin showed the cherry-red spots of the unchanged DD- and LL-isomers, but also a faster-running purple spot ($R_{DD\text{-isomer}}$ 2.25) and one attributable to the reagents ($R_{DD\text{-isomer}}$ 2.7). The corresponding spot ($R_{DD\text{-isomer}}$ 2.25) from a chromatogram run in solvent B was eluted. On t.l.c. on silica gel G in solvent D [propan-1-ol–water–aq. NH_3 (sp.gr. 0.880) (14:3:3, by vol.)] it gave a spot at R_F 0.43, yellow on heating with ninhydrin, contrasting with the purple spot of diaminoadipic acid, R_F 0.12. That this product was the lactam sought was shown by the following procedures. (*a*) On hydrolysis in acid it yielded the parent diaminoadipic acid (later the optimum conditions were shown to be 4M-HCl at 105°C for 0.5–1h); (*b*) on treatment with fluorodinitrobenzene in the presence of triethylamine it gave a Dnp derivative extractable into diethyl ether from acid solution. After t.l.c. on silica gel G in chloroform–methanol–acetic acid (95:5:1, by vol.) it gave a spot at R_F 0.08. This was eluted and hydrolysed in 4M-HCl for 3h. During ether extraction the yellow colour now remained in the aqueous layer. On t.l.c. as before, it now had R_F 0.41, the same value as authentic mono-Dnp-diaminoadipic acid, and on spraying with ninhydrin the spot turned brown, indicating a free amino group.

It was observed that old marker solutions of diaminoadipic acid in propan-2-ol–water (1:4, v/v) contained a small amount of the cyclic compound identified by the above criteria. Further investigation showed that heating an aqueous solution (or suspension) of racemic-diaminoadipic acid (2mg/ml) at 90°C in a sealed tube for 22h led to considerable conversion into the lactam. Heating the dry compound or refluxing under dry benzene was not effective. To check that heating at 90°C had not resulted in a change of configuration at the asymmetric carbon atoms, a sample of the lactam was hydrolysed to diaminoadipic acid and converted into its di-Dnp derivative. Chromatography in solvent C showed that no more than a trace of *meso*-compound had been formed. Longer periods of heating caused increasing racemization, no doubt via the lactam itself.

Racemic-diaminoadipic acid (200μmol) was heated in water as described above, and the resulting lactam was separated from unchanged diaminoadipic acid by chromatography on a cation-exchange resin. A column (21cm×1.5cm) of Zeo-Karb 225 (H^+ form) was equilibrated at 2°C with 0.2M-pyridine–acetate buffer, pH3 (16.1ml of pyridine added to 500ml of water, adjusted to pH3 with acetic acid and diluted to 1 litre), and the sample containing diaminoadipic acid lactam was applied. The column was eluted with the same buffer and 3.5ml fractions were collected. Fractions 36–41 contained lactam and no diaminoadipic acid, as shown by t.l.c. in solvent D. The buffer solution was then changed to 0.2M-pyridine–acetate buffer, pH5, which soon eluted the diaminoadipic acid. The combined fractions containing lactam were concentrated to dryness by rotary evaporation and dried *in vacuo* over conc. H_2SO_4 and NaOH. The sample was dissolved in water and shown by t.l.c. in solvent D to contain no diaminoadipic acid. The concentration was determined by hydrolysis to diaminoadipic acid as described above, followed by measurement of E_{510} in the acid-ninhydrin procedure of Work (1957), a reaction not given by the lactam. The final yield of purified lactam was 90μmol (45%).

Crystalline lactam was also prepared as follows. Racemic-diaminoadipic acid (100mg) was dissolved in water (35ml) and heated in a sealed tube at 90°C for 22h. After cooling, the solution was evaporated successively to 20ml, 5ml and 1.5ml and after each evaporation it was kept at 2°C for 2h, during which time unchanged diaminoadipic acid was precipitated. It was removed by centrifuging. The final supernatant solution contained only lactam (t.l.c. in solvent D). It was evaporated to dryness, redissolved in water (5ml) with warming, and a small residue was removed by centrifuging. The solution was again concentrated to 1.5ml, and crystallization of the lactam was initiated by dropwise addition of 80% (v/v) ethanol and completed at 2°C. The crystals were washed with ethanol and ether and finally dried *in vacuo* over P_2O_5. On t.l.c. in solvent D a sample showed no diaminoadipic acid. The lactam had m.p. 171°C (Found: C, 40.3; H, 6.7; N, 16.1%. This is the analysis required by diaminoadipic acid, or the lactam monohydrate).

A similar procedure was used for preparing the lactam of *meso*-diaminoadipic acid except that, since the parent compound was so much less soluble than the racemic-isomer mixture, the suspension after heating at 105°C was evaporated under vacuum to a small volume and adjusted to pH3 with acetic acid, when virtually all the lactam present was in solution. The final separation was on cation-exchange resin as described above.

Measurement of inhibition constants

K_i values were calculated from plots of $1/v$ versus inhibitor concentration in the presence of different concentrations of standard substrate $\alpha\epsilon$-Ac_2-L-Lys-D-Ala-D-Ala (range 0.4–1.7mM). With enzyme from strain *albus* G, the concentration of the inhibitor Ac-D-Ala-D-Glu ranged from 0.3 to 0.9mM and the experiments were carried out in 0.02M-Tris–HCl buffer (pH7.5)–2mM-$MgCl_2$. With enzyme from

strain R61, concentration of the inhibitor Ac-D-Ala-D-Asp ranged from 2.8 to 6.3 mM and the experiments were carried out in 0.01 M-Tris–HCl buffer, pH 7.5. Incubations were performed at 37°C.

Results

To facilitate the description of the results, the amino acid residues in the peptides are numbered as H-3-2-1-OH.

Inhibition of DD-*carboxypeptidase from S. albus G*

G DD-carboxypeptidase from *S. albus* G, an enzyme that is not inhibited by penicillins and cephalosporins (Ghuysen *et al.*, 1970), can be inhibited by peptide analogues of the standard substrate αε-Ac_2-L-Lys-D-Ala-D-Ala. Table 2 summarizes the results obtained. The inhibition by Ac-D-Ala-D-Glu seems kinetically competitive, with K_i 2.1×10^{-4} M. When one compares the behaviour of Ac-D-Ala-D-Glu or Ac-Gly-D-Ala-D-Glu with that of αε-Ac_2-L-Lys-D-Ala-D-Glu it is striking that the acetylated side chain of L-lysine can change a non-substrate, inhibitory peptide into a very good substrate. The same is observed for Ac-D-Ala-D-Ala, compared with the standard substrate αε-Ac_2-L-Lys-D-Ala-D-Ala. Also, succinylation instead of acetylation of the above lysine tripeptides or lack of substitution of the ε-amino group decreased or suppressed their substrate activity and made the peptides inhibitors. The most straightforward conclusion one can draw from these results is that the *C*-terminal dipeptide is the main portion of the molecule concerned with the initial binding to the enzyme surface, whereas a side

Table 2. *Activity of the* DD-*carboxypeptidase from Streptomyces albus G in the presence of peptide inhibitors*

Ac_2-L-Lys-D-Ala-D-Ala (15–20 nmol) was incubated at 37°C for 30 min in the presence of inhibitor and enough enzyme to liberate 75% of its terminal D-alanine in the absence of inhibitor. The final volume was 30 μl and the buffer was 0.02 M-Tris–HCl–2 mM-Mg^{2+}, pH 7.5. % Inhibition is $100(A_0 - A/A_0)$, A being the alanine liberated in the presence of the peptide inhibitor and A_0 that in its absence. Activities as substrate were measured by incubating the inhibitors (150–200 nmol) under the same conditions as described above and comparing the amount of terminal amino acid liberated with that cleaved under identical conditions from the standard substrate (Ac_2-L-Lys-D-Ala-D-Ala). With standard substrate this represented a saturating concentration. Abbreviation: Suc, succinyl (3-carboxypropionyl).

Peptide	Activity as substrate (%)	% Inhibition	Molar ratio inhibitor/substrate
Ac_2-L-Lys-D-Ala-D-Ala	100	0	
Ac-D-Ala-D-Ala	0	10–20	10
αε-Suc_2-L-Lys-D-Ala-D-Ala	2–8	50	10
Ac-D-Ala-D-Glu	0	88	10
Suc-D-Ala-D-Glu	0	52	11.5
α-Ac-L-Lys-D-Ala-D-Glu	0	45	10
Ac_2-L-Lys-D-Ala-D-Glu	100	—	
αε-Suc_2-L-Lys-D-Ala-D-Glu	0	88–96	10
Ac-Gly-D-Ala-D-Glu	0	84	12
Ac-D-Ala-D-Asp	0	59	11
Myristoyl-D-Ala-D-Asp	0	70	11
Ac-racemic-cyclodiaminoadipic acid	0	52	17
Ac-*meso*-cyclodiaminoadipic acid	0	0	8.6
Glutaryl-racemic-cyclodiaminoadipic acid	0	17	11
Ac_2-L-Lys-racemic-cyclodiaminoadipic acid	0	43	11
D-Cycloserine	0	0	10
Suc-D-cycloserine	0	0	10
Suc-D-Ala-D-cycloserine*	0	88–100	10
Ac-D-Ala-D-cycloserine	0	8	6
Ac_2-L-Lys-D-Ala-D-cycloserine	3.5	59	5
α-Ac-L-Lys-D-Glu-D-Ala	0	67	11.6
Ac_2-L-Lys-D-Glu-D-Ala	0	87	14

* For discussion of the purity of this peptide see the text.

Table 3. *Kinetics of hydrolysis of peptides with different side chains at residue 3 by* DD-*carboxypeptidase from S. albus G*

$V_{max.}$ is expressed in units of μmol of D-Ala-D-Ala linkage cleaved/h per mg of enzyme. Experimental conditions are as described in Leyh-Bouille *et al.* (1970) and in the text. ΔG (Gibbs free energy) $= -RT\ln(1/K_m)$. Abbreviations: A_2bu, 2,4-diaminobutyric acid; R, UDP-MurNAc-Gly-D-isoGlu.

Peptide	K_m (mM)	$V_{max.}$	$-\Delta G$ (J/mol)
Ac-Gly-D-Ala-D-Ala	1.1	1.9	17500
Ac-L-Ala-D-Ala-D-Ala	3.3	0.7	14700
R-L-Hse-D-Ala-D-Ala*	1.0	16	17800
Ac_2-L-A_2bu-D-Ala-D-Ala	0.6	42	19000
Ac_2-L-Lys-D-Ala-D-Ala	0.33	132	20600
α-Ac-L-Lys-D-Ala-D-Ala*	6.0	20	13100

* Taken from Leyh-Bouille *et al.* (1970). The value of $V_{max.}$ reported there for Ac_2-L-Lys-D-Ala-D-Ala was slightly smaller (100).

chain of very definite molecular characteristics is required in residue 3 to induce enzymic action of the molecules bound to the enzyme. To test these views the K_m and $V_{max.}$ values for a series of tripeptides differing in the side chain at residue 3 were determined (Table 3). If as a first approximation we accept the Michaelis constant K_m as the dissociation constant of the enzyme–peptide complex, it is clear that although residue 3 makes some contribution to the binding, most of the free enthalpy (Gibbs free energy) of binding is supplied by the interaction of residues 1 and 2. On the other hand, an uncharged aliphatic side chain in residue 3 longer than one carbon atom induces striking increases in the $V_{max.}$ value. Positive charges in this position decrease both $-\Delta G$ and $V_{max.}$ (α-Ac-L-Lys-D-Ala-D-Ala). Negative charges in the same position do not prevent peptides from binding but greatly decrease enzymic activity and hence presumably the value of $V_{max.}$. Thus $\alpha\epsilon$-Suc_2-L-Lys-D-Ala-D-Ala is a poorer substrate than the diacetyl peptide, and whereas $\alpha\epsilon$-Ac_2-L-Lys-D-Ala-D-Glu is a good substrate the corresponding disuccinyl-peptide is no longer a substrate but an effective inhibitor. It is, of course, difficult to decide whether the effect of succinyl groups is due to their negative charge or to the increase in the length of the side chain at residue 3. The values for K_m and $V_{max.}$ for Ac-L-Ala-D-Ala-D-Ala are slightly anomalous, without any obvious explanation.

The lactam of diaminoadipic acid (cyclodiaminoadipic acid in Table 2) was synthesized as an analogue of the Ala-Ala peptide in which the two methyl side chains have been linked covalently. Racemic-cyclodiaminoadipic acid would correspond to a mixture of L-Ala-L-Ala and D-Ala-D-Ala and *meso*-cyclodiaminoadipic acid to a mixture of D-Ala-L-Ala and L-Ala-D-Ala. Peptides with the two latter *C*-terminal sequences are neither substrates nor inhibitors for the streptomyces enzymes (Leyh-Bouille *et al.*, 1970, 1971). Ring formation makes it obligatory for the peptide bond to be *cis* in all cases.

As could be predicted the acetyl-*meso*-compound does not bind to the enzyme, whereas the racemic-compound does, although presumably only the DD-isomer (Table 2). The mixture designated as Suc-D-Ala-D-cycloserine is the most inhibitory of the peptides listed in Table 2, and that is the reason why it is reported although the inhibitory compound(s) was not characterized.

Inhibition of the DD-*carboxypeptidase from strain R*61

The carboxypeptidase from strain R61 is inhibited by penicillins or cephalosporins, and this inhibition is kinetically competitive (Leyh-Bouille *et al.*, 1971). As shown in Table 4 it is also inhibited by peptide analogues of its standard substrate Ac_2-L-Lys-D-Ala-D-Ala. The kinetics of the inhibition of the enzyme by Ac-D-Ala-D-Asp have been studied. The inhibition is competitive, with a K_i value of 3.2mM. In general the behaviour of all the peptide inhibitors on the activity of carboxypeptidases from *S.* strains *albus* G and R61 is similar, although inhibition of the latter is usually smaller. This can be correlated with the high K_m values reported by Leyh-Bouille *et al.* (1971). As with the enzyme from strain *albus* G, residues 1 and 2 appear to be the ones mainly concerned with the binding to the enzyme, whereas size, shape and charge on residue 3 appear to be decisive for enzymic activity. Also, as with the enzyme from strain *albus* G, Ac-racemic-cyclodiaminoadipic acid is an inhibitor of carboxypeptidase from strain R61.

Table 4. *Activity of the* DD-*carboxypeptidase from Streptomyces strain R61 in the presence of peptide inhibitors*

Conditions were as described in Table 2 except that the buffer was 0.01 M-tris–HCl, pH 7.5. The following compounds were neither substrates nor inhibitors at a concentration of greater than 10 times that of the standard substrate: Suc-D-Ala-D-Glu; α-Ac-L-Lys-D-Ala-D-Glu; Ac-*meso*-cyclodiaminoadipic acid; Ac_2-L-Lys-racemic-cyclodiaminoadipic acid; D-cycloserine; Suc-D-cycloserine; Ac-D-Ala-D-cycloserine; Suc-D-Ala-D-cycloserine. When D-glutamic acid was liberated experiments were repeated in sodium phosphate buffer (0.01 M, pH 8) to avoid confusion between Dnp-glutamic acid and Dnp-Tris.

Peptide	Activity as substrate (%)	% Inhibition	Molar ratio inhibitor/substrate
Ac_2-L-Lys-D-Ala-D-Ala	100	—	
Ac-D-Ala-D-Ala	1	9	10
Suc_2-L-Lys-D-Ala-D-Ala*	16	43	12
Ac-D-Ala-D-Glu	0	25	10
Ac_2-L-Lys-D-Ala-D-Glu	10	—	
Suc_2-L-Lys-D-Ala-D-Glu	0	32–46	10
Ac-Gly-D-Ala-D-Glu	0	25	10
Ac-D-Ala-D-Asp	0	38	10
Ac-racemic-cyclodiaminoadipic acid	0	26	17
Ac_2-L-Lys-D-Ala-D-cycloserine	16	43	5
α-Ac-L-Lys-D-Glu-D-Ala	0	33–35	11.6
Ac_2-L-Lys-D-Glu-D-Ala	0	34–36	14

* In this experiment the substrate was Ac_2-L-Lys-D-Ala-D-[^{14}C]Ala and hence alanine liberated from it was measured as radioactivity and could be distinguished from alanine liberated from the inhibitor.

Table 5. *Activity of the* DD-*carboxypeptidase from Streptomyces strain R39 on several peptides*

Peptide (200 nmol) was incubated with the enzyme (150 units; Leyh-Bouille *et al.*, 1972) in Tris–HCl buffer (0.03 M) containing 3 mM-$MgCl_2$ at pH 7.5; final volume 30 μl. Activity is expressed as a percentage of that on the standard substrate, Ac_2-L-Lys-D-Ala-D-Ala. The following compounds were neither substrates nor inhibitors for the DD-carboxypeptidase from strain R39; Ac-D-Ala-D-Asp; Suc-D-Ala-D-Glu; phenylacetyl-D-Ala-D-Glu; L-Lys-D-Glu-D-Ala; α-Ac-L-Lys-D-Glu-D-Ala; Ac_2-L-Lys-D-Glu-D-Ala; D-Ala-D-cycloserine; D-cycloserine; Ac-D-Ala-D-cycloserine; Suc-D-Ala-D-cycloserine; racemic-cyclodiaminoadipic acid; Ac_2-L-Lys-racemic-cyclodiaminoadipic acid.

Peptide	Activity (%)
Ac-D-Ala-D-Ala	5
Suc_2-L-Lys-D-Ala-D-Ala	36
Ac-D-Ala-D-Glu	4
α-Ac-L-Lys-D-Ala-D-Glu	110
Ac_2-L-Lys-D-Ala-D-Glu	67
Suc_2-L-Lys-D-Ala-D-Glu	5

Assuming that only the DD-isomer is inhibitory, the cyclic peptide would be about three times as good an inhibitor as Ac-D-Ala-D-Ala.

DD-*Carboxypeptidase from strain R39.* The DD-carboxypeptidase from strain R39 is inhibited by penicillin in a kinetically non-competitive manner. None of the peptide inhibitors of the enzymes from strains R61 and *albus* G had any effect on the DD-carboxypeptidase from strain R39. In fact some of them were good substrates for this enzyme (Table 5).

DD-*Carboxypeptidase inhibitors as antibiotics.* The peptide inhibitors listed in Table 2 as well as benzyloxycarbonyl-D-Ala-D-Glu, benzyloxycarbonyl-D-Ala-D-Asp, phenylacetyl-D-Ala-D-Glu, and benzyloxycarbonyl-D-Ala-D-cycloserine were tested as antibiotics against *S.* strain *albus* G by the hole-in-the-plate method (Perkins, 1969), each being present as 50 μl of approx. 13 mM solution. In addition, Ac-D-Ala-D-Asp was tested against *S.* strain R61. None of them was active.

Discussion

Mechanism of the action of DD-*carboxypeptidases from strains S. albus G and R61*

The binding of Ac-DD-cyclodiaminoadipic acid to the DD-carboxypeptidases from strains *albus* G and R61 is stronger than that of Ac-D-Ala-D-Ala, as judged by the inhibition caused by both of them. It seems reasonable to hypothesize from this fact that these carboxypeptidases combine only with peptides having a *cis* configuration in the *C*-terminal amide

bond. Although the free peptides in solution have most likely a predominantly *trans* configuration in all their amide linkages, the combination with the enzyme can provide enough free enthalpy to alter the normal distribution of the isomers. The peptide $\alpha\epsilon$-Ac_2-L-Lys-D-Ala-D-Ala is a very good substrate for enzymes from strains R61 and *albus* G, but $\alpha\epsilon$-Ac_2-L-Lys-racemic-cyclodiaminoadipic acid is not a substrate at all, i.e. the amide linkage in the ring is not cleaved by the enzymes. This can be accounted for if we assume that after the initial binding of the peptide to the enzyme occurs, the amide bond to be broken is forced by the enzyme to adopt a configuration intermediate between *cis* and *trans*. For a normal peptide this involves overcoming an energy barrier of about 30–40kJ (Schellman & Schellman, 1964), but for the cyclic peptide the energy involved would be so much higher, owing to the restrictions imposed by the ring, that hydrolysis would not take place.

A simplified sequence of events leading to hydrolysis of peptide substrates by the DD-carboxypeptidases from strains *albus* G and R61 can be imagined as follows.

(*a*) The enzymes bind the molecules of peptide that have a *cis* configuration in the *C*-terminal amide linkage. The interaction with the enzyme of the residues 1 and 2 would supply most of the free enthalpy of binding.

(*b*) If the size, shape and charge of the side chain at residue 3 were appropriate, it would induce a conformational change in the enzyme, which, in turn, would result in the previously *cis*-amide linkage adopting a configuration intermediate between *cis* and *trans*, thus losing all double-bond character. The conformational change in the enzyme does not need to be a spectacular one, but together with the interactions between enzyme groups and peptide substrate it has to supply an energy of some 30–40kJ.

(*c*) The active intermediate of the previous step would break down into an acyl-peptide and the terminal amino acid. This final reaction is probably made up of more than one step, as suggested by the fact that transpeptidation can occur with enzyme from strain R61 if supplied with the appropriate carboxyl acceptor (Pollock *et al.*, 1972). The proposed mechanism does not necessarily apply to all DD-carboxypeptidases, since the enzyme from strain R39 was not inhibited by any of the analogues examined.

Lee (1971), in attempting to add support to the 'structure analogue' hypothesis for the mode of action of penicillin (Tipper & Strominger, 1965), has proposed that transpeptidases might bind maximally to acyl-D-Ala-D-Ala peptides when the latter have their *C*-terminal amide bond suitably distorted so as to lose most of its double-bond character. In contrast, we propose that the enzyme would simply select the small population of *cis* isomer and thus displace the configuration equilibrium. Distortion of the amide bond would then only occur once the substrate was bound to the enzyme. The corresponding change in the enzyme would only occur in the presence of bound substrate. Thus, according to our proposal, the free enzyme need not necessarily have any affinity for the transition state of the substrate.

Role of DD-*carboxypeptidases as transpeptidases*

The work of Pollock *et al.* (1972) has shown that the DD-carboxypeptidases of *Streptomyces* strains R61 and R39 can function as transpeptidases *in vitro*, and further, this transpeptidase reaction was as sensitive to penicillin as the carboxypeptidase reaction. It is evident that, if such a transpeptidase is indeed the enzyme involved in the cross-linking of nascent mucopeptide, then an inhibitor of the enzyme should also be an antibiotic. Some of the peptides described in the present paper were good inhibitors of the DD-carboxypeptidase activity of the enzyme from *S.* strain *albus* G and poorer inhibitors of that from strain R61, but the same peptides did not inhibit growth of the parent organisms on agar plates. So far as strain *albus* G is concerned, it has not been possible to demonstrate transpeptidation with the isolated enzyme and any acceptor (Pollock *et al.*, 1972; H. R. Perkins, M. Nieto, J. M. Frère, M. Leyh-Bouille & J. M. Ghuysen, unpublished work), so that in this case the transpeptidase functioning *in vivo* may well have a different inhibitor spectrum. The DD-carboxypeptidase from strain R61 was inhibited less than 50% by a high concentration of Ac-D-Ala-D-Asp, and so perhaps it is not surprising that growth of cells showed no inhibition by the same substance, even if it were able to reach the appropriate region of the cell.

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