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Synthesis of δ-(α-Aminoadipyl)cysteinylvaline and its Role in Penicillin Biosynthesis

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1. The stereoisomers of δ-(α-aminoadipyl)-L-cysteinylvaline (LLD, LLL and DLD) were synthesized from valine labelled with ^3H in its methyl groups or in the α position. L-Cysteinyl-D-[4,4′-^3H]valine was also synthesized. 2. ^3H was incorporated into a compound that behaved like penicillin N when the LLD tripeptide containing either a methyl- or an α-labelled valine residue was incubated with a cell-free system prepared by lysis of protoplasts of *Cephalosporium acremonium*. 3. Incorporation was not observed under these conditions from the labelled all-L- or DLD-tripeptide, from L-cysteinyl-D-[4,4′-^3H]valine, or from DL-[4,4′-^3H]valine. 4. δ-(α-Aminoadipyl)cysteinylvaline extracted from the mycelium of *Penicillium chrysogenum* appeared to be the LLD isomer, like that from *C. acremonium*. 5. These findings are discussed in relation to penicillin biosynthesis.

After the isolation of penicillin N, with a δ-(D-α-aminoadipyl) side chain, from culture filtrates of *Cephalosporium acremonium* (Newton & Abraham, 1954), Arnstein & Morris (1960) found that a tripeptide, δ-(α-aminoadipyl)cysteinylvaline, was present in very small amounts in the mycelium of *Penicillium chrysogenum*. Loder & Abraham (1971*a*) showed that a similar peptide in the mycelium of the *Cephalosporium* sp. was δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. A peptide that behaved like the latter on paper electrophoresis and chromatography in the sulphonic acid form was synthesized from δ-(L-α-aminoadipyl)-L-cysteine and DL-valine in the presence of an extract of the *Cephalosporium* sp. and an energy-generating system (Loder & Abraham, 1971*b*).

Circumstantial evidence has been obtained that the tripeptide is a precursor of penicillin N and cephalosporin C (Warren *et al.*, 1967; Lemke & Nash, 1972; Huang *et al.*, 1975), but a direct demonstration of its role in biosynthesis did not appear to be possible with intact mycelium of the *Cephalosporium* sp., because it was not taken up by the cells. The present paper describes the synthesis of ^3H-labelled tripeptides and their behaviour in a cell-free system obtained by lysis of protoplasts of the *Cephalosporium* sp. (Fawcett *et al.*, 1973). Some of the results have already been briefly reported (Fawcett *et al.*, 1976).

Experimental

Materials

ATP (sodium salt) was from the Sigma Chemical Co., London S.W.6, U.K. Phosphoenolpyruvate (tricyclohexylammonium salt) and pyruvate kinase were from the Boehringer Corp. (London) Ltd., London W.C.2, U.K. Hydroxybenzotriazole was from Fluka A.-G., Buchs S.G., Switzerland. L- and D-α-Aminoadipic acid were prepared as described by Usher *et al.* (1975). Other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of AnalaR grade.

General methods

Protein was determined by a micro biuret procedure (Goa, 1953), and DNA by the method of Burton (1968). Oxidation of thiol compounds to the corresponding sulphonic acids was carried out as described by Smith *et al.* (1967).

Paper electrophoresis and chromatography. Electrophoresis at pH 7.0 was carried out as described by Fawcett *et al.* (1975). At other pH values it was carried out on Whatman no. 1 paper at 70 V/cm (unless otherwise stated) in an apparatus similar to that used by Katz *et al.* (1959). The buffers used were those of Smith *et al.* (1967), at pH 1.8 and 4.5, and of Corran & Waley (1974), at pH 3.5 and 6.5.

Paper chromatograms were run on Whatman no. 1 paper (at 4°C with penicillin N) for 18–24h in butan-1-ol/acetic acid/water (4:1:5, by vol., upper phase, or 4:1:4, by vol.).

Penicillin N and amino acids and peptides were detected on paper by spraying with a ninhydrin reagent (Woiwod, 1949).

Measurements of radioactivity. Aqueous solutions of ^3H-labelled compounds were counted for radioactivity in a Nuclear–Chicago Unilux 11A liquid-scintillation system as described by Usher *et al.*

(1975). The efficiency of counting was between 23 and 29%. To determine the distribution of ^{3}H-labelled compounds after paper chromatography and electrophoresis, the paper was cut into segments (1cm× 3cm) and each immersed in 3ml of scintillation fluid (Usher *et al.*, 1975). The efficiency of counting varied from 11 to 17%, and was not detectably affected by prior spraying of the paper with ninhydrin.

Biosynthesis in a cell-free system from protoplasts

Preparation of a cell-free enzyme system. Protoplasts from *C. acremonium* C91 were prepared as described by Fawcett *et al.* (1973). The protoplast pellet (containing protoplasts that had usually been prepared in 8–10% yield, on the basis of DNA content, from 4g of damp-dry mycelium harvested after 48h) was resuspended in 0.05M-Tris/HCl buffer, pH7.0, containing 0.01M-$MgSO_4,7H_2O$ and 0.01M-KCl (0.5ml). Observation under the light microscope showed that the protoplasts had undergone osmotic lysis and the resulting preparation will hereafter be referred to as the 'protoplast lysate'. A protoplast lysate containing 316μg of DNA/ml contained 43mg of protein/ml.

Incubation of labelled peptides with the enzyme system. The peptides used were: (1) δ-(L-α-aminoadipyl)-L-cysteinyl-D-[4,4′-^{3}H]valine (20mCi/mmol); (2) the same tripeptide with ^{3}H in the α position of the valine residue (10mCi/mmol); (3) δ-(L-α-aminoadipyl)-L-cysteinyl-L-[4,4′-^{3}H]valine (20mCi/mmol); (4) δ-(D-α-aminoadipyl)-L-cysteinyl-D-[4,4′-^{3}H]valine (28mCi/mmol); and (5) L-cysteinyl-D-[4,4′-^{3}H]valine (28mCi/mmol). In some experiments DL-[4,4′-^{3}H]-valine (4.3μCi, 20mCi/mmol) was used in place of a labelled peptide. The labelled compounds were added to portions (0.5ml) of the whole protoplast lysate, or to fractions (0.5ml) of the lysate obtained by centrifugation. ATP (5μmol/ml) and an energy-generating system composed of phosphoenolpyruvate (9μmol/ml) and pyruvate kinase (200μg/ml) (all final concentrations) were also added.

Incubation was carried out at 28°C in stoppered tubes for 2h. Controls containing buffer and the radioactive compound, but lacking the cell extract, were incubated under the same conditions.

Analysis of incubation mixtures. After incubation the mixtures were centrifuged at 15000*g* for 10min and the supernatants removed and freeze-dried. Amino acids, peptides and penicillin N were extracted into 70% (v/v) ethanol (2×200μl) and the extracts diluted with water (2ml) and freeze-dried.

(*a*) One-half of the extract was treated with performic acid to convert peptides containing cysteine into their sulphonic acids and to degrade penicillins to penicillaminic acid (D-$\beta\beta$-dimethylcysteic acid). Unlabelled penicillaminic acid was added as a marker and located by ninhydrin spray after a sample of the mixture had been subjected to electrophoresis on paper at pH1.8, or to electrophoresis at pH1.8 for 2.5h followed by chromatography in a second dimension in butan-1-ol/acetic acid/water (4:1:4, by vol.).

A control experiment showed that performic acid oxidation of penicillin N labelled with ^{3}H in its 2-β-methyl group (Usher *et al.*, 1975) gave ^{3}H-labelled penicillaminic acid in less-than-quantitative yield (60–70%). Nevertheless, the degradation to penicillaminic acid had the advantage that the latter was stable and well resolved, on electrophoresis at pH1.8, from compounds that did not contain sulphur and from δ-(α-aminoadipyl)cysteicylvaline.

(*b*) In some experiments the radioactivity in a position corresponding to penicillin N itself was determined after electrophoresis of a portion of the extract for 45min at pH3.5, followed by chromatography in a second dimension in butan-1-ol/acetic acid/water (4:1:5, by vol.) at 4°C. Penicillin N migrated 10.7cm towards the anode, and the tripeptide migrated 6.9cm.

(*c*) In other experiments, electrophoresis on paper was carried out at pH7.0, and measurements were made of the loss of radioactivity in the position of penicillin N and the gain in that of the penicilloate after treatment of the extracts with a solution of penicillinase (Neutrapen, 10000 units/25μl from Riker Laboratories, Loughborough, Leics., U.K.), at room temperature (about 20°C) for 30min.

Extraction of peptides from P. chrysogenum. Strain SC 6140 of *P. chrysogenum* was grown as described by Fawcett *et al.* (1975) and the washed damp-dry mycelium was resuspended in 7.5% (w/v) trichloroacetic acid (1g damp-dry weight/2ml). The mixture was blended in a Waring blender for 30s and then allowed to stand at room temperature for 30min. The mycelium was filtered off and washed on the filter with 5% (w/v) trichloroacetic acid (0.5ml/1g damp-dry weight). The combined filtrate and wash were extracted three times with an equal volume of diethyl ether. The aqueous phase was freeze-dried after removal of dissolved ether *in vacuo*. The residue was dissolved in water and the solution added to a column (8cm×1cm diam.) of Bio-Rad AG-50W X4 (H^+ form). After the column had been washed with water (15ml), the amino acids and peptides [including δ-(α-aminoadipyl)cysteinylvaline] were eluted with 1M-pyridine. The ninhydrin-positive eluate was freeze-dried and the residue treated with performic acid to convert thiol compounds into the corresponding sulphonic acids.

Synthesis of labelled compounds

D- *and* DL-[4,4′-^{3}*H*]*Valine.* Residues from the synthesis of ^{3}H-labelled isopenicillin N and 6-

aminopenicillanic acid from phenoxymethylpenicillin sulphoxide 4-methoxybenzyl ester (Usher *et al.*, 1975) were dissolved in trifluoroacetic acid and the solution was evaporated to give a dark-brown residue. The latter was mixed with water (10ml), and saturated $NaHCO_3$ (approx. 60ml) was added until there was no further effervescence. Raney nickel (three heaped spatulas) was added and the mixture heated under reflux for 30min in an oil bath at 145°C. D-[4,4′-^{3}H]-Valine was isolated from the mixture by the procedure of Abraham & Newton (1956). It was crystallized from water (2ml) and acetone (50ml) at −15°C to give 62mg of product (20mCi/mmol) which had $[\alpha]_D^{20}$ −24.5°C ($c = 3$ in 6M-HCl).

A sample (1mmol) of D-[4,4′-^{3}H]valine was converted into DL-[4,4′-^{3}H]valine hydrochloride by the racemization procedure of Greenstein & Winitz (1961*a*).

DL-[2-^3H]Valine. This was prepared from DL-valine (1mmol) by the method of Brundish *et al.* (1971), except that the *N*-acetyl-DL-valine was produced *in situ*.

Distribution of ^2H in valine from phenoxymethylpenicillin sulphoxide ester ^2H-labelled in its 2-β-methyl group

Phenoxymethylpenicillin sulphoxide 4-methoxybenzyl ester (1.458g, 3mmol), ^2H_2O (0.6ml, 30mmol) and dioxan (15ml) were heated at 100°C under reflux for 48h. The residues from the isolation of the ^{2}H-labelled product (1.5mmol) were degraded to D-[^{2}H]valine (0.057g, 32% yield) by a procedure similar to that used for preparation of D-[^{3}H]valine.

The p.m.r. (proton-magnetic resonance) spectrum of non-^{2}H-labelled *N*-benzoyl-D-valine (recorded in [^{2}H]chloroform at 100MHz) showed integrated areas beneath the N*H* (3.25τ, doublet, $J = 9$Hz), NH·C*H* (5.17τ, multiplet, $J = 5$Hz, 9Hz), C*H*$(CH_3)_2$ (7.63τ, crude septet) and gem-dimethyl groups (8.96, 8.98τ, overlapping pair of doublets each with $J =$ 7Hz) of 1:1:1:7 respectively. The spectrum of the *N*-benzoyl derivative of the [^{2}H]valine showed corresponding integrated areas of 1:1:1:1:5.2. The ratio of the areas corresponding to the gem-dimethyl groups of the two compounds was thus approx. 6:4.5. The spectra also showed that the pair of doublets arising from the gem-dimethyl groups were symmetrical in the non-^{2}H-labelled *N*-benzoylvaline and essentially symmetrical in the *N*-benzoyl[^{2}H]-valine. The fine structure of the C*H*$(CH_3)_2$ proton was lost in the ^{2}H-labelled compound, owing to the coupling of this proton with both the ^{1}H and ^{2}H nuclei of the methyl groups.

N - Benzyloxycarbonyl - S - acetamidomethyl - L - cysteine. *S*-Acetamidomethylcysteine hydrochloride, m.p. 160°–163°C, was prepared by the method of Veber *et al.* (1972). It was converted into its *N*-benzyloxycarbonyl derivative by treatment with benzyloxycarbonyl chloride in 50% (v/v) acetone in the presence of $NaHCO_3$ (Greenstein & Winitz, 1961*b*). The product was obtained as an oil which crystallized as needles (62% yield) when stored under ether at room temperature. It had m.p. 100°–101°C (Found: C, 51.5; H, 5.5; N, 8.4; $C_{14}H_{18}N_2O_5S$ requires: C, 51.5; H, 5.5; N, 8.6%).

N - Benzyloxycarbonyl - S - acetamidomethyl - L - cysteinyl[4,4′-^3H]valine ethyl ester. *N*-Benzyloxycarbonyl-*S*-acetamidomethyl-L-cysteine (1mmol), hydroxybenzotriazole (1mmol) and dicyclohexylcarbodi-imide (1.1mmol) were mixed at 0°C in chloroform (5ml). The mixture was stirred at 0°C for 60min and then at 20°C for 60min. DL-[4,4′-^{3}H]Valine ethyl ester (1mmol, 20mCi/mmol) in chloroform (5ml) was added and the mixture kept at 20°C for 10min. The resulting solution was added to a column of silica gel G, type 60 (from E. Merck, Darmstadt, Germany) and elution carried out with ethyl acetate. Fractions (4.5ml) were collected, and samples (0.01ml) of alternate fractions were mixed with 3ml of water-miscible scintillation fluid and counted for radioactivity (see above). Fractions 56–68 (containing LL-dipeptide), and 85–105 (containing the LD dipeptide), were pooled and evaporated to dryness to give white solids that were purified separately by chromatography as described above. The LL dipeptide was obtained as a white crystalline solid (0.104g; 20.2mCi/mmol) which gave a single spot on t.l.c. on silica gel in ethyl acetate. The LD dipeptide was obtained as a similar white crystalline solid.

The labelled LD-dipeptide showed the same R_F value (0.29) on t.l.c. in ethyl acetate as an unlabelled sample synthesized from D-valine ethyl ester and had m.p. 132°–133°C after crystallization from propan-2-ol at −15°C. (Found: C, 55.8; H, 6.8; N, 9.2; S, 7.2; $C_{21}H_{31}N_3O_6S$ requires C, 55.6; H, 6.8; N, 9.3; S, 7.1%). The LL dipeptide showed R_F 0.34.

S - Acetamidomethyl - L - cysteinyl - [4,4′ - ^3H]valine ethyl ester. The fully protected dipeptide (45mg) was treated with 45% (w/v) HBr in acetic acid (1ml) for 30min. The product was precipitated as a white hydrobromide by the addition of dry diethyl ether. It was washed with ether, taken up in chloroform containing an excess of triethylamine, and the solution evaporated to dryness. The white solid was dissolved in chloroform and the solution used immediately for synthesis of tripeptide. On t.l.c. (silica gel G) in butan-1-ol/acetic acid/water (4:1:4, by vol.) it showed a single ninhydrin-positive spot with R_F 0.31.

δ-(N-4-Methoxybenzyloxycarbonyl-α-aminoadipyl-α-4-methoxybenzyl ester)-S-acetamidomethyl-L-cysteinyl-[4,4′-^3H]valine ethyl esters. 4-Methoxybenzyloxycarbonyl-D-α-aminoadipic acid α-4-methoxybenzyl ester [0.545mmol, prepared as described by Usher *et al.* (1975) for the L isomer] was mixed with

dicyclohexylcarbodi-imide (0.6mmol) and hydroxybenzotriazole (0.545mmol) in chloroform (5ml). The solution was stirred at 0°C for 1h and then at 20°C for 1h. It was then added to a solution of *S*-acetamidomethyl-L-cysteinyl-D-[4,4′-^{3}H]valine ethyl ester (from 0.39mmol of hydrobromide) in chloroform (5ml). After 3h the solution was evaporated to dryness. The residue was dissolved in a mixture of chloroform (1ml) and ethyl acetate (1ml) and purified by chromatography on a column (45cm×1cm diam.) of silica gel G in ethyl acetate. The product was crystallized from ethyl acetate (yield 55%, m.p. 149–151°C; 28mCi/mmol). It showed a single radioactive spot (R_F 0.94) after paper chromatography in butan-1-ol/acetic acid/water (4:1:4, by vol.) and detection by spraying with aqueous trifluoroacetic acid (70% v/v), to remove the *N*-protective group, and then with ninhydrin. An unlabelled sample of this fully protected tripeptide was prepared similarly and had m.p. 155°–156°C (Found: C, 58.2; H, 6.7; N, 7.6; $C_{36}H_{50}N_4O_{11}S$ requires: C, 58.0; H, 6.7; N, 7.5%).

The corresponding labelled L-α-aminoadipyl compounds containing a D-[4,4′-^{3}H]valine residue and a D-[2-^{3}H]valine residue respectively were synthesized in a similar way.

δ - (α - Aminoadipyl) - S - acetamidomethyl - L - cysteinyl-[4,4′-^{3}H]valine. A solution of the fully protected, labelled DLD tripeptide (62μmol) in pyridine (1ml) and 0.2M-NaOH (1ml) was stirred for 1h at room temperature and then diluted with water (20ml). The pH was adjusted to 2.0 with H_2SO_4 and the product extracted into ethyl acetate (20ml). The extract was dried over $MgSO_4$ and evaporated to yield a colourless gum (42.6μmol) which gave a single radioactive peak of material, 13cm from the origin towards the anode, on paper electrophoresis (70V/cm, 1h) at pH3.5.

The *N*- and *S*-protected tripeptide (37μmol) was dissolved in a mixture of trifluoroacetic acid (1.8ml) and anisole (0.2ml). The solution was evaporated *in vacuo* and the residue partitioned between ethyl acetate (5ml) and water (5ml). The aqueous layer was added to a column of Dowex-1 X8 (acetate form; 38cm×1cm diam.). The column was washed with water and the product eluted with 0.5M-acetic acid, 9ml fractions being collected. Fractions 10 and 11 were evaporated to dryness, and the residue was dissolved in water (5ml) and freeze-dried to yield a white solid (yield 22%) which gave a major radioactive peak of material (R_F0.5) on paper chromatography in butan-1-ol/acetic acid/water (4:1:4, by vol.), and on paper electrophoresis at pH1.8 (70V/cm for 60min) migrated 15.5cm towards the cathode.

An unlabelled sample of this *S*-protected tripeptide, prepared similarly, was crystallized twice from aqueous acetone. It had m.p. 135°–136°C (Found: C, 45.1; H, 6.9; N, 11.9; $C_{17}H_{30}N_4O_7S,H_2O$ requires: C, 45.1; H, 7.1; N, 12.4).

The corresponding LLD and all-L *S*-protected tripeptides, each containing a residue of [4,4′-^{3}H]-valine, were synthesized in a similar manner.

δ-(α-Aminoadipyl)-L-cysteinyl-[4,4′-^{3}H]valine. Mercuric acetate (20μmol) was added to the *S*-protected labelled DLD tripeptide (7.9μmol) in water (500μl), and the solution stirred for 2h at room temperature. The white precipitate was removed by centrifugation, washed with water (2×5ml), resuspended in water (2ml) and decomposed with H_2S. Most of the HgS was removed by centrifugation and the supernatant passed through a 3μm Millipore filter. After removal of excess of H_2S *in vacuo*, the solution was freeze-dried and the residue dissolved in water. The radioactivity of the solution, in conjunction with the specific radioactivity of the [4,4′-^{3}H]-valine used, indicated that 4.5μmol of unprotected tripeptide had been obtained (yield 58%).

After electrophoresis on paper at pH1.8, the labelled product showed two spots with relative mobilities of 1:1.16 towards the cathode and relative radioactivities of about 4:1. A sample of a preparation of the corresponding unlabelled tripeptide (Found: C, 43.0; H, 6.9; N, 10.4; $C_{14}H_{25}N_3O_6S,1.5H_2O$ requires C, 43.0; H, 7.2; N, 10.8), behaved similarly. However, after a stream of air had been passed through a solution of this product in 0.2M-Tris/HCl buffer, pH7.8, for 1h, only the component with the higher mobility towards the cathode was observed on electrophoresis at pH1.8. After oxidation of the product with performic acid, it behaved as a single compound on electrophoresis at pH1.8, and migrated towards the anode in a position corresponding to that of δ-(α-amino-adipyl)cysteicylvaline (Loder & Abraham 1971*b*). The minor, faster moving, component observed on paper chromatography and electrophoresis of the preparation of δ-(D-α-amino-adipyl)-L-cysteinyl-D-[4,4′-^{3}H]valine was thus the corresponding disulphide.

L-Cysteinyl-D-[4,4′-^{3}H]valine. Removal of the protective groups from the fully protected L-cysteinyl-D-[4,4′-^{3}H]valine (approx. 10mg, 28mCi/mmol) was carried out by the methods used in the synthesis of the tripeptides. When subjected to electrophoresis on paper at pH6.5 (35V/cm, 2h), *N*-benzyloxycarbonyl-*S*-acetamidomethyl-L-cysteinyl-D-[4,4′-^{3}H]valine behaved as a single radioactive compound, migrating 14.5cm from the origin towards the anode with a mobility of 0.6 relative to that of D-α-aminoadipic acid. The preparation of *S*-acetamidomethyl-L-cysteinyl-D-[4,4′-^{3}H]valine (yield 33%) was found by electrophoresis on paper at pH1.8 (70V/cm for 1h) to consist of a major component (73% of the total radioactivity), which migrated 20cm towards the cathode (1.09 times as far as α-aminoadipic acid), and two minor compounds, with 24 and 3% of the total radioactivity, which migrated 28 and 3cm respectively towards the cathode.

The impure material was used for removal of the *S*-acetamidomethyl group and the dipeptide then isolated as described by Loder & Abraham (1971*b*). Its radioactivity (52.9 μCi) corresponded to 0.41 mg (yield 31%).

When subjected to electrophoresis on paper at pH1.8 (70V/cm, 1h) the preparation of the LD dipeptide gave a major component, corresponding to 75% of the total radioactivity, and a minor one, corresponding to 16%, which migrated 26 and 22cm respectively towards the cathode. The major component behaved on paper electrophoresis and chromatography as the L-cysteinyl-D-valine synthesized by Loder & Abraham (1971*b*).

Electrophoresis at pH1.8 of the product obtained by treatment of a sample of the crude preparation with a stream of air in Tris/HCl buffer, pH7.8 (0.2M), for 1.5h revealed that the major component had disappeared and that 87% of the radioactivity was in a position corresponding to the former minor component. Electrophoresis at pH1.8 of the product obtained after oxidation with performic acid revealed that it contained a major component (86% of the radioactivity) with the mobility of L-cysteicyl-D-valine (Loder & Abraham, 1971*b*). It thus appeared that the original product contained about 75% of L-cysteinyl-D-[4,4′-^{3}H]valine and 16% of L-cystinyl-bis-D-[4,4′-^{3}H]valine.

An unlabelled sample of L-cysteicyl-D-valine, which was synthesized in a similar way, but on a 1mM scale, showed the same mobility at pH1.8 as the corresponding labelled product. (Found: C, 36.1; H, 6.0; N, 10.1; $C_8H_{16}N_2O_6S$ requires: C, 35.8; H, 6.0; N, 10.4.)

^{3}H-Labelled 6-aminopenicillanic acid. This compound, labelled in its 2-β-methyl group (371mCi/mmol), was synthesized as described by Usher *et al.* (1975).

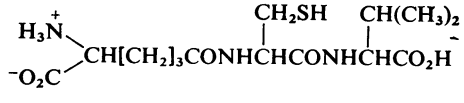
Results

Synthesis of ^{3}H-labelled isomers of δ-(α-aminoadipyl)-L-cysteinylvaline and of L-cysteinyl-D-[^{3}H]valine

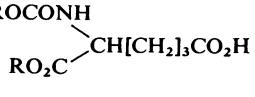
Methyl-labelled valine used in these syntheses was prepared from the residues remaining from the synthesis of penicillins labelled with ^{3}H in the 2-β-methyl group (Usher *et al.*, 1975). In some cases the D-valine obtained was racemized to the DL-isomer.

The finding that about one-sixth of the radioactivity was lost as water during hydrogenolysis with Raney nickel suggested that desulphurization of the penicillamine fragment of the penicillin occurred by a process of β-elimination, as had been found in the conversion of benzyl- and phenoxymethyl-penicillin into the corresponding desthiopenicillins (Wolfe & Hasan, 1970). To obtain further information on this question, the residues from an experiment in which ^{2}H was incorporated into the 2-β-methyl group of phenoxymethylpenicillin sulphoxide ester (Cooper, 1970; Usher *et al.*, 1975) were degraded to D-[^{2}H]valine. In the p.m.r. spectrum of the *N*-benzoyl derivative of this valine, the pair of doublets formed by coupling of both methyl groups with the adjacent proton was essentially symmetrical, as it was with the *N*-benzoyl derivative of non-^{2}H-labelled valine. Hence, it appeared that the ^{2}H was distributed equally between the two methyl groups.

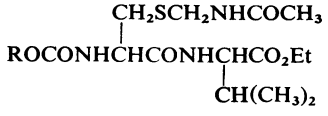
Essentially the same procedures were used for the synthesis of ^{3}H-labelled LLD, all-L and DLD diastereoisomers of δ-(α-aminoadipyl)-L-cysteinylvaline (I). The dicyclohexylcarbodi-imide/hydroxybenzotriazole procedure (König & Geiger, 1970) was used for coupling. Aminoadipic acid was protected by conversion into 4-methoxybenzyloxycarbonyl-α-aminoadipic acid α-4-methoxybenzyl ester (II, R = 4-methoxybenzyl). The thiol and amino groups



(I) δ-(α-Aminoadipyl)cysteinylvaline



(II) 4-Methoxybenzyloxycarbonyl-α-aminoadipic acid α-4-methoxybenzyl ester (R = 4-methoxylbenzyl)



(III) *N*-Benzyloxycarbonyl-*S*-acetamidomethylcysteinylvaline ethyl ester (R = benzyl)

of L-cysteine were protected by the acetamidomethyl group (Veber *et al.*, 1972) and the benzyloxycarbonyl group respectively. Coupling of the protected cysteine with labelled valine ethyl ester gave *N*-benzyloxycarbonyl-*S*-acetamidomethyl-L-cysteinylvaline ethyl ester (III, R = benzyl). When DL-valine ethyl ester was used (either methyl- or α-labelled), the diastereoisomers of the protected dipeptide (LL and LD) were separated by chromatography in ethyl acetate on a column of silica gel. The benzyloxycarbonyl group was removed from the appropriate isomer of compound III by treatment with HBr in acetic acid, and the product coupled with compound (II). Cleavage of the two ester groups from the resulting fully protected tripeptide by alkaline hydrolysis and of the 4-methoxybenzyloxycarbonyl group by treatment of the product with trifluoroacetic acid containing 10% anisole yielded δ-(α-aminoadipyl)-*S*-acetamidomethyl-L-cysteinylvaline. Removal of the acetamidomethyl group by treatment of the *S*-protected tripeptide with mercuric acetate in aqueous solution then led to δ-(α-aminoadipyl)-L-cysteinylvaline (I) in about 8.5% yield, based on the fully protected tripeptide.

The labelled LLD, all-L and DLD-tripeptides behaved on paper electrophoresis like the synthetic unlabelled peptides, all appearing to be contaminated with a small amount of the corresponding disulphide, which was presumably formed by atmospheric oxidation. After oxidation with performic acid the synthetic tripeptides behaved like δ-(α-aminoadipyl)cysteicylvaline when analysed by two-dimensional paper electrophoresis and chromatography (Loder & Abraham, 1971*a*). A preparation of labelled L-cysteinyl-D-valine, obtained by removal of the protective groups from compound III, appeared to be identical with the unlabelled dipeptide synthesized by Loder & Abraham (1971*b*).

Identification of stereoisomers of δ-(α-aminoadipyl)-L-cysteinylvaline

δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine could be distinguished from the all-L isomer by oxidation of the peptides to the corresponding sulphonic acids and electrophoresis of the latter on paper at pH1.8 (70V/cm) for 8h. Under these conditions the LLD-cysteicyl peptide migrated 31.3cm towards the anode,

Table 1. *Incorporation of ^3H from labelled peptides into penicillin N*

Each peptide was added to 0.5ml of the crude enzyme system, obtained by lysis of protoplasts and the addition of an energy-generating system, and also to 0.5ml of a control solution containing no enzyme. For the determination of radioactivity in compounds corresponding to penicillaminic acid and penicillin N, see the Experimental section.

Expt.	DNA in lysed protoplasts (μg)	^3H-labelled peptide (Nature*)	(μCi/μmol)	(μCi added)	Penicillaminic acid (nCi)	(nmol†)
1	370	LLD (γ)	20	3.5	27	1.3
	Control	LLD (γ)	20	3.5	0.6	—
	370	LD (γ)	28	2.5	0.3	—
	Control	LD (γ)	28	2.5	0.9	—
2	238	LLD (γ)	20	4.8	8	0.4
	Control	LLD (γ)	20	4.8	1	—
	238	LLD (α)	10	4.8	8	0.7
	Control	LLD (α)	10	4.8	1	—
	238	LLL (γ)	20	4.8	0.6	—
	Control	LLL (γ)	20	4.8	1	—
3	305	LLD (γ)	20	4.8	6	0.3
	Control	LLD (γ)	20	4.8	0.2	—
	305	DLD (γ)	28	6.5	0.9	0.03
	Control	DLD (γ)	28	6.5	0.1	—
					Penicillin N	
					(nCi)	(nmol)
4	67	LLD (γ)	20	4.8	30	1.3
	67	LLL (γ)	20	4.8	6	—
	Control	LLD (γ)	20	4.8	5	—

* LLD signifies δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and LLL and DLD the corresponding diastereoisomers. LD signifies L-cysteinyl-D-valine. ^3H in the methyl groups of the valine residue is denoted by 'γ' and in the α position by 'α'.

† The amount of endogenous unlabelled peptide in the crude enzyme system (<6nmol/0.5ml) was virtually negligible in comparison with the amount of labelled peptide added (175–240nmol).

the all-L peptide 30.4cm, and the DLD peptide 30.6cm. When the LLD and all-L peptides were applied to the paper together (in 5μl of solution) the two spots were clearly resolved. This resolution enabled it to be shown that δ-(α-aminoadipyl)cysteinylvaline from the mycelium of *Penicillium chrysogenum* strain SC 6140 (see the Methods section) was not the all-L isomer and behaved like the LLD isomer from *C. acremonium* (Loder & Abraham, 1971*a*).

Incorporation of ^3H from labelled peptides into penicillin

When δ-(L-α-aminoadipyl)-L-cysteinyl-D-[4,4'-^{3}H]-valine (4.8μCi, 240nmol) was added to a suspension (0.5ml) of intact protoplasts of *C. acremonium* in 0.8M-NaCl in 10ml shake flasks (Fawcett *et al.*, 1973), there was no detectable loss of radioactivity from the extracellular fluid after 2h and no incorporation of ^3H into the penicillin N produced.

Typical results of a number of experiments in which ^3H-labelled peptides were incubated with a lysed protoplast system from *C. acremonium* are given in Table 1, different experiments being carried out with different preparations of crude enzyme. On the assumption that the labelled penicillaminic acid was derived from penicillin N, the Table shows that ^3H was incorporated into this antibiotic from δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine labelled in either the methyl groups or the α-position of its valine residue (Expts. 1, 2 and 3), but not to a significant extent from the methyl-labelled all-L or DLD-tripeptides. No incorporation from the methyl-labelled LLD tripeptide was detected when the crude enzyme system was heated for 20min at 90°C before use. The incorporation was decreased by more than 50% when the energy-generating system was omitted from the system.

The incorporation of ^3H from the LLD isomer into a compound yielding penicillaminic acid on oxidation is also illustrated by the radioactivity profiles shown in Figs. 1 and 2. Little, if any, incorporation was observed from the all-L tripeptide, and the addition of an unlabelled sample of this isomer in 20 molar excess decreased the incorporation from the LLD-tripeptide.

The results of experiments in which radioactivity was measured in the position occupied by penicillin N

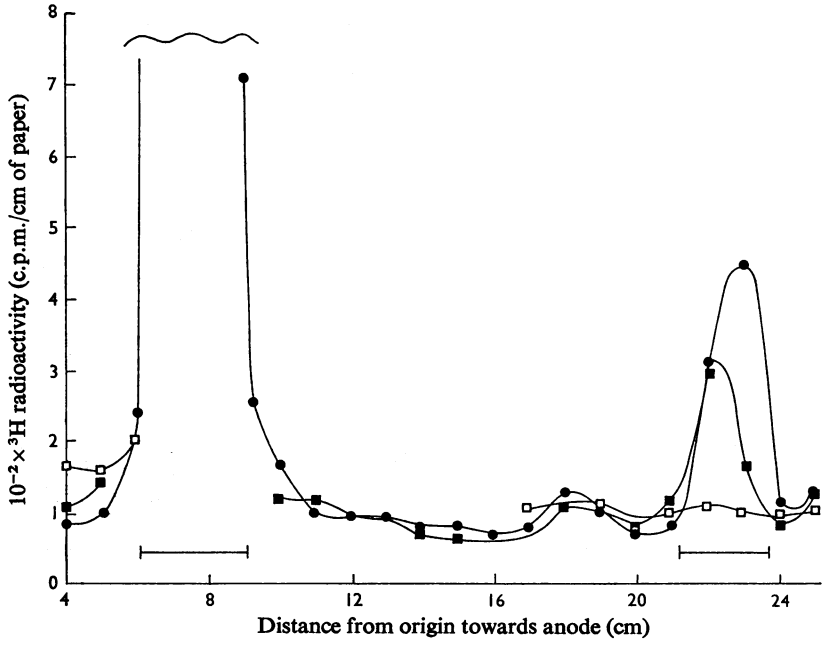


Fig. 1. *Incorporation of ^3H from δ-(L-α-aminoadipyl)-L-cysteinyl-D-[4,4'-^3H]valine into material yielding penicillaminic acid on oxidation*

Electrophoresis on paper was at pH1.8 for 2.5h with 16.6% of the total sample. The values for radioactivity (c.p.m.) were from segments of the paper (each 1cm×3cm). The enzyme system was from lysed protoplasts of *C. acremonium*. For details, see the Experimental section. The bars show the position of added markers of penicillaminic acid (right) and the tripeptide sulphonic acid (left) as revealed by ninhydrin spray. ●, Labelled LLD tripeptide; ■, labelled LLD tripeptide plus unlabelled LLL tripeptide (20:1 mol/mol); □, control.

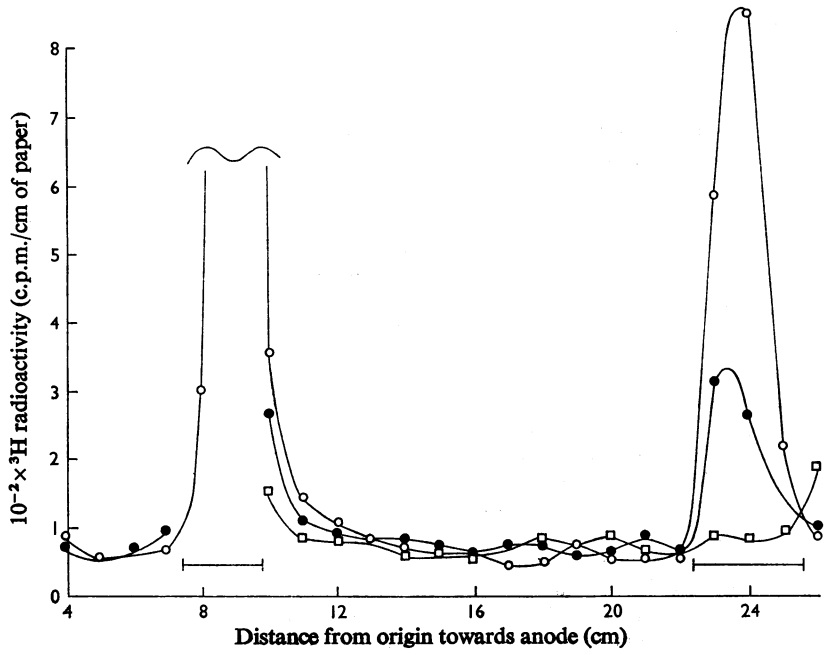


Fig. 2. *Incorporation of ^3H from δ-(L-α-aminoadipyl)-L-cysteinyl-D-[4,4′-^3H]valine into material yielding penicillaminic acid on oxidation*

Electrophoresis was carried out at pH 1.8 for 2.5 h, with 12.5% of the total sample (for details, see the Experimental section). The bars show the positions of added markers of penicillaminic acid (right) and the tripeptide sulphonic acid (left) as revealed by ninhydrin spray. ○, Whole system from lysed protoplasts of *C. acremonium* lysate; ●, 50000*g* supernatant after centrifugation for 15 min at 4°C; □, 50000*g* pellet.

itself, after two-dimensional paper electrophoresis and chromatography, again indicated that ^3H was incorporated into the antibiotic from the LLD tripeptide, but not from the all-L tripeptide (Table 1, Expt. 4). Further support for this conclusion was obtained from the behaviour to penicillinase of the product obtained in an experiment with the methyl-labelled LLD tripeptide (30 μCi; 1.5 μmol). When electrophoresis on paper at pH 7.0 was preceded by treatment with the enzyme there was a gain in radioactivity (20 nCi) in the location of penicillin N penicilloate.

For some experiments the crude protoplast lysate was separated into supernatant and sediment fractions, which were each tested for their ability to incorporate ^3H from the methyl-labelled LLD tripeptide into penicillin N in the presence of an energy-generating system. Fig. 2 indicates that the incorporation catalysed by a supernatant obtained after centrifugation at 50000*g* was about 50% of that obtained with the complete lysate, and that no significant incorporation was observed with the corresponding pellet. The incorporation observed with a supernatant obtained by centrifugation at 20000*g* was virtually identical with that observed with the corresponding complete lysate.

In one experiment, 6-aminopenicillanic acid labelled with ^3H in its 2-β-methyl group (5.2 μCi, 371 mCi/mmol) was incubated with the crude system from lysed protoplasts under the conditions used with the labelled tripeptides. After electrophoresis on paper at pH 4.5, no significant increase in radioactivity above background was found in the position corresponding to penicillin N. However, since the background radioactivity was high, a very low amount of incorporation of ^3H into penicillin N might not have been detected.

There was no detectable incorporation of radioactivity from L-cysteinyl-D-[4,4′-^3H]valine into a compound yielding penicillaminic acid on oxidation (Table 1, Expt. 1) and no incorporation was observed in a parallel experiment in which DL-[4,4′-^3H]valine was used.

Discussion

The results described here indicate that δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine is a precursor of a

substance which behaves like penicillin N in a cell-free system from protoplasts of *C. acremonium*, whereas the corresponding all-L and DLD tripeptides are not precursors, or are at least very much poorer ones. Isopenicillin N would not have been distinguished from penicillin N under the conditions used, and the optical configuration of the δ-(α-aminoadipyl) side chain of the labelled product remains to be determined. But penicillin N, which has a D-α-aminoadipyl side chain, is the only penicillin yet known to be produced by intact cells of the *Cephalosporium* sp.

Although further experiments with a doubly labelled tripeptide are desirable, it seems clear that the incorporation of ^3H from the valine residue of the peptide into the penicillin was not preceded by peptide hydrolysis. No significant formation of labelled valine was observed in the cell-free system, and no incorporation was detected when DL-[4,4′-^3H]-valine was added to the system, in place of the tripeptide, under the conditions used. The failure to observe incorporation of ^3H from L-cysteinyl-D-[4,4′-^3H]valine and 6-amino-[^3H]penicillanic acid into the penicillin are also consistent with the view that δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine functions intact as a precursor, and with the earlier finding that the tripeptide was not synthesized from L-α-aminoadipic acid and L-cysteinyl-D-valine in an extract of *C. acremonium* (Loder & Abraham, 1971*b*).

The incorporation of ^3H into the penicillin from the LLD tripeptide labelled in the α position of its valine residue indicates that there is no free intermediate containing an αβ-dehydrovaline residue between the tripeptide and the antibiotic. Since this tripeptide is accompanied in the mycelium of *C. acremonium* by a related peptide containing a residue of β-hydroxyvaline (Loder & Abraham, 1971*a*), the question arises of whether oxidation at the β-carbon atom of a valine residue precedes closure of the thiazolidine ring.

Adriaens *et al.* (1975*a*) and Bycroft *et al.* (1975) have shown by experiments with DL-[2-^3H]valine, DL-[3-^3H,4,4′-^{14}C]valine and D-[2-^3H]valine respectively that D-valine is not incorporated directly into penicillin produced by *P. chrysogenum*, but only after epimerization to L-valine, and that no ^3H migration from C-3 to C-2 of the valine occurs during this process. These results are consistent with the synthesis of the LLD tripeptide from L-valine, but not from the D isomer, in *C. acremonium* (Abraham, 1974). The finding of Huang *et al.* (1975) that the tripeptide synthesized in the presence of L-[2,3-^3H]-valine contained a D-valine residue with a ^3H atom at C-3 again showed that the epimerization did not involve a free αβ-dehydrovalinyl intermediate or a hydride shift from C-3 to C-2. The failure to demonstrate the incorporation of ^3H into penicillin from the all-L tripeptide indicated that the latter was not a free intermediate in the formation of the LLD isomer.

The conclusion that the δ-(α-aminoadipyl)cysteinylvaline in *P. chrysogenum* is the LLD isomer agrees with the findings of Adriaens *et al.* (1975*b*) and Chan *et al.* (1976). The present results thus suggest that the D centre at C-3 of the penicillin-ring system synthesized by both *C. acremonium* and *P. chrysogenum* arises during the formation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, although the mechanism by which it occurs remains to be established. This tripeptide could be converted into isopenicillin N in *P. chrysogenum* without further change in its asymmetric centres, but its conversion into penicillin N in *C. acremonium* would involve epimerization of the L-α-aminoadipic acid residue. Since no evidence for the conversion of the DLD tripeptide into penicillin was obtained from the experiments described here, the epimerization may occur at a later stage. Further experiments with labelled peptides may throw light on this aspect of penicillin biosynthesis.

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