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Synthesis of Optical Isomers of Cystinylvaline

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It has been shown (Arnstein & Grant, 1954*a, b*; Arnstein & Crawhall, 1957) that L-cystine, presumably after reduction to cysteine, is incorporated intact into the β -lactam-thiazolidine nucleus of penicillin; L-valine can also be utilized without deamination or transamination in the biosynthesis of the nucleus (Stevens & De Long, 1958).

The elucidation of the mode of formation of the β -lactam-thiazolidine ring system from cystine and valine is of considerable interest and it has been suggested (Arnstein, 1957) that cystinylvaline or its disulphide form may function as a precursor. The present paper deals with the synthesis of several optical isomers of cystinylvaline, including two labelled with ^{14}C in the valine carboxyl group.

The main purpose of the synthesis with unlabelled cystine and valine was to find methods which would yield L-cystinyl-L-valine and L-cystinyl-D-valine in an optically pure state. A number of diastereoisomeric amino acids and peptides have been separated by ion-exchange chromatography on Dowex 50 (Blackburn & Lee, 1955; Blackburn & Tetley, 1956; Blackburn & Schöberl, 1956), and it was thought possible that L-cystinyl-DL-valine could be separated into its three isomers in this way. Accordingly, this peptide was synthesized by the route shown in Fig. 1; benzyloxycarbonyl-S-benzyl-L-cysteine was coupled with DL-valine ethyl ester by the mixed-anhydride method of Vaughan (1951) and, after saponification of the resulting protected dipeptide ester, both the

benzyloxycarbonyl and S-benzyl groups were removed simultaneously with sodium in liquid ammonia (pathway B, Wood & Du Vigneaud, 1939). Crystallization of the crude product, as described in the Experimental section, gave, surprisingly, optically pure L-cystinyl-D-valine. The mother liquors were evaporated to dryness and the residue was placed on a column of Zeo-Karb 225 (The Permutit Co. Ltd., London) and subjected to gradient elution with hydrochloric acid (0.5–3.0*N*); at an acid concentration of 2.6*N* a component, running as one spot in solvent A, was obtained which, after hydrolysis and separation of the cystine and valine, was shown to be L-cystinyl-DL-valine. No evidence for the separation of L-cystinyl-L-valine from the mixture by this method was found. This approach to the synthesis of the two peptides was therefore abandoned. L-Cystine was found to be racemized to a considerable extent under conditions of acid hydrolysis; the optical configurations of the cystine and valine, isolated after hydrolysis of the peptides, were determined by comparison of their specific rotations with those of cystine and valine obtained from synthetic mixtures of optically pure amino acids, which had undergone identical treatment with acid. Cystinylvaline is fairly resistant to hydrolysis, complete reaction requiring 64 hr. with 6*N*-hydrochloric acid at 110°.

Synthesis of the two required peptides was commenced with L-cystine and either L- or D-valine. The coupling method with dicyclohexylcarbodi-

imide (Sheehan & Hess, 1955) was successful; yields of 80–90% were obtained. Removal of the protecting benzyloxycarbonyl and *S*-benzyl groups, after saponification of the fully protected dipeptide esters, however, gave some difficulty. It was found that removal of the two groups simultaneously with sodium in liquid ammonia gave rise to considerable racemization of the cystine moiety, the free peptide exhibiting several spots on paper chromatography even though the valine was optically pure. This difficulty was largely overcome by removal of the benzyloxycarbonyl group with hydrogen bromide in acetic acid before reduction in liquid ammonia (pathway A). Even so, racemization of cystine did occur in the reduction of *S*-benzyl-L-cysteinyl-D-valine. It is essential that the reduction is carried out under absolutely anhydrous conditions with fresh bright sodium and liquid ammonia that has been dried by distillation from sodium in a closed system.

Synthesis of the peptides labelled with ^{14}C in the valine carboxyl group was achieved via the cyclohexylcarbodi-imide-coupling method, the protecting groups being removed by pathway A (Fig. 1). Desalting of the peptides after the reduction was facilitated to some extent by the use of lithium instead of sodium, since lithium bromide, formed on addition of ammonium bromide at the end of the reduction, is soluble in ethanol and was largely removed from the peptide-salt mixture by extraction of the latter with hot ethanol. The overall conversion of L-[carboxy- ^{14}C]valine and D-[carboxy- ^{14}C]valine into the corresponding radiochemically pure peptides was 35 and 31% respectively.

A preliminary account of some of this work was given by Arnstein & Morris (1959).

EXPERIMENTAL

Radioactivity measurements. Compounds were mounted on disks 1 cm.² and counted at infinite thickness, a thin end-

window Geiger-Müller tube being used (Popják, 1950). The specific radioactivities were obtained by direct comparison with a poly[^{14}C]methyl methacrylate standard (1 $\mu\text{C/g.}$) obtained from The Radiochemical Centre, Amersham, Bucks. The standard error in all determinations is not greater than 5%.

Paper chromatography. All R_F values refer to descending chromatograms on Whatman no. 1 paper, unless otherwise stated. The solvent systems had the following composition (by vol.): A, butanol-acetic acid-water, 63:10:27; B, amyl alcohol-pyridine-water, 7:7:6; C, phenol-water, 5:2 (with NH_3).

Synthesis of peptides. The routes to the peptides are outlined in Fig. 1. The descriptions L-DL, etc. refer to the optical configurations of the cystine and valine portions of the dipeptide respectively. All melting points are uncorrected.

Synthesis involving separation of diastereoisomers

Benzyloxycarbonyl-*S*-benzyl-L-cysteine (II; 6.0 g.), prepared from L-cystine via *S*-benzyl-L-cysteine (Wood & Du Vigneaud, 1939), was dissolved in dry tetrahydrofuran (50 ml.) and triethylamine (1.78 g.; 1 mol.prop.) was added. After cooling to -5° , isobutylchloroformate (2.4 g.; 1 mol.prop.) was added and the mixture was shaken at -5° for 8 min.

Fischer-Speier esterification of DL-valine (11.7 g.) gave DL-valine ethyl ester hydrochloride (III) (18.1 g., 100%), m.p. 107–108° (Found: C, 46.5; H, 8.9; N, 8.0. $\text{C}_7\text{H}_{18}\text{ON}_2\text{Cl}$ requires C, 46.3; H, 8.8; N, 7.7%). This ester hydrochloride (3.15 g.) was suspended in dry tetrahydrofuran (20 ml.), triethylamine (1.78 g., 1 mol.prop.) was added and the suspension was added to the mixed anhydride solution described above. The mixture was refluxed (1 min.), cooled and poured into aq. 2% (w/v) NaHCO_3 (400 ml.). After standing for 48 hr. at room temperature the solid product was filtered off, dissolved in ethyl acetate (100 ml.) and washed successively with *N*-HCl (25 ml.), aq. saturated NaHCO_3 (2×10 ml.) and water. Benzene was added and the solvent was evaporated *in vacuo*; crystallization of the resulting gum from ethyl acetate-light petroleum (b.p. 100–120°) gave benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-DL-valine ethyl ester (IVa; $\text{R} = \text{C}_2\text{H}_5$) (5.0 g., 61%), m.p.

Fig. 1. Synthesis of dipeptides ($\text{R} = \text{C}_2\text{H}_5$ or $\text{CH}_2 \cdot \text{C}_6\text{H}_5$). The suffixes *a*, *b*, *c* and *d* in the text refer to the L-DL, LD, LL and DL-D forms of the peptide respectively.

109–113°, $[\alpha]_D^{20} - 23.7^\circ$ (c, 2.01 in dioxan) (Found: C, 63.8; H, 6.7; N, 6.2. $C_{25}H_{33}O_6N_3S$ requires C, 63.6; H, 6.8; N, 5.9%). The R_F in solvents A and B was 0.95.

A similar preparation with DL-valine benzyl ester (III, $R = CH_2 \cdot C_6H_5$) (Ben-Ishai & Berger, 1952) gave *benzyloxycarbonyl-S-benzyl-L-cysteinyl-DL-valine benzyl ester* (IVa; $R = CH_2 \cdot C_6H_5$), m.p. 98–99° (Found: C, 67.3; H, 6.3; N, 5.4. $C_{30}H_{34}O_6N_3S$ requires C, 67.4; H, 6.4; N, 5.2%). The R_F in solvent A was 0.97.

The protected dipeptide ester (IVa; $R = C_2H_5$) (4.65 g.) was dissolved in dioxan (10 ml.) by warming. After cooling to room temperature ethanolic 2N-KOH (10 ml.) was added and the reaction was allowed to proceed for 30 min. After this time the mixture was diluted with water (200 ml.) and extracted with ethyl acetate (3 × 40 ml. portions); the organic phase was back-extracted with aq. saturated $NaHCO_3$ (3 × 10 ml. portions) and the combined aqueous portions were acidified with 2N-HCl and extracted further with ethyl acetate (3 × 30 ml. portions). The latter extract was washed with a little water, benzene was added and the solution was evaporated to dryness *in vacuo*; the resulting gum (3.72 g., 84%) was chromatographically pure, with R_F 0.81 in solvent B. A portion of this compound (0.7 g.) was dissolved in dry ether (15 ml.) and cyclohexylamine (0.185 g., 1 mol.prop.) was added, leading to the immediate formation of *benzyloxycarbonyl-S-benzyl-L-cysteinyl-DL-valine cyclohexylamine salt* (0.68 g., 80%), m.p. 136–138°. Recrystallization from ethyl acetate-cyclohexane gave analytically pure material, m.p. 139–140° (Found: C, 63.9; H, 7.4; N, 7.5. $C_{25}H_{41}O_6N_3S$ requires C, 64.1; H, 7.5; N, 7.5%).

The protected dipeptide (Va; 3.72 g.) was dissolved in liquid ammonia (100 ml.) in a three-necked 250 ml. flask fitted with a mechanical stirrer. Sodium was added in small portions, with stirring, until a permanent blue colour was obtained; 1 g. was required. Excess of sodium was destroyed with $(NH_4)_2SO_4$ and the liquid NH_3 was evaporated as rapidly as possible, last traces being removed by evacuating the flask at 40° for 2 hr. followed by 3 hr. in a desiccator over conc. H_2SO_4 at 37°. The residue was dissolved in 0.5N- H_2SO_4 (75 ml.) and a solution of mercuric sulphate (10% in 5% H_2SO_4) was added until precipitation was complete (12 ml.). After standing overnight at 0° the precipitate was washed with water, made into a fine powder by grinding and treated in aqueous suspension with H_2S to decompose the mercury salt. The mixture was filtered from mercury sulphide and the filtrate was evaporated to dryness *in vacuo* to yield 2.2 g. of a crude product which was de-salted on a column of Zeo-Karb 215 (50 g.) (The Permutit

Co. Ltd., London) by adsorption on to the H^+ form and elution with aq. 0.05N- NH_3 soln. to give crude L-cystinyl-DL-valine (VIIIa; 1.6 g., 62%). Chromatography in solvent A revealed the presence of three components of R_F 0.19, 0.26 and 0.35 respectively, presumably representing the diastereoisomeric forms of the peptide. The peptide (1.3 g.) was suspended in water (20 ml.) and dissolved by the drop-wise addition of dilute aq. NH_3 soln.; excess of NH_3 was removed in a desiccator and on addition of ethanol and ether 320 mg. of solid was obtained which ran as one spot, R_F 0.20, in solvent A. The product was dried for 6 hr. at 100° over P_2O_5 . A sample (100 mg.) was hydrolysed in 6N-HCl for 64 hr. at 105° and evaporated to dryness *in vacuo*; the residue was dissolved in water and adjusted to pH 8 with aq. 0.15N- NH_3 soln. to precipitate the cystine liberated by hydrolysis. The latter was filtered off and the filtrate, after treatment with bromine-water to oxidize any remaining cystine to cysteic acid, was placed on a Zeo-Karb 215 (H^+ form) column and eluted with water until the eluate was neutral. Valine was displaced from the column with aq. 0.15N- NH_3 soln. and isolated. Optical rotations of both the cystine and valine were determined and compared with the rotations of cystine and valine isolated after treatment of synthetic equimolar mixtures under identical conditions of hydrolysis. The results, shown in Table 1, indicated that the peptide isolated by crystallization was L-cystinyl-D-valine dihydrate (VIIIb), m.p. > 300°, $[\alpha]_D^{25} + 21.2^\circ$ (c, 0.8 in 5N-HCl) (Found: C, 40.7; H, 7.2; N, 11.8. $C_{16}H_{30}O_6N_2S_2 \cdot 2H_2O$ requires C, 40.5; H, 7.2; N, 11.8%). The R_F in solvent A was 0.20. Attempts to separate the other optical isomers from the mother liquors by ion-exchange chromatography on Zeo-Karb 225 were unsuccessful.

Optically specific syntheses

Benzyloxycarbonyl-S-benzyl-L-cysteine (II; 6 g.) and L-valine ethyl ester hydrochloride (Vaughan & Eichler, 1953) (3.15 g., 1 mol.prop.) were shaken together in dry ethylene dichloride (50 ml.) for 40 min. to give a clear solution. Triethylamine (1.76 g., 1 mol.prop.) and dicyclohexylcarbodi-imide (3.86 g., 1 mol.prop.) (Sheehan & Hess, 1955) were added, with cooling; the mixture was shaken mechanically for 2½ hr. and allowed to stand at room temperature overnight. The insoluble dicyclohexylurea formed during the reaction was filtered off, the solvent was evaporated *in vacuo* and the residue was dissolved in ethyl acetate (100 ml.). The latter solution was extracted successively with water (2 × 30 ml.), N-HCl (2 × 30 ml.), water (2 × 30 ml.), aq. 2% $NaHCO_3$ (2 × 30 ml.) and water (2 × 30 ml.); the organic phase was evaporated to dryness *in vacuo* in the presence of benzene to yield an oil which on subsequent evaporation with ethanol yielded a crystalline solid. After standing overnight under dry light petroleum (b.p. 100–120°) the solid was filtered off and dried *in vacuo* to give *benzyloxycarbonyl-S-benzyl-L-cysteinyl-L-valine ethyl ester* (IVc; $R = C_2H_5$) (7.52 g., 92%), m.p. 78°, $[\alpha]_D^{24} - 38.0^\circ$ (c, 1.0 in dioxan) (Found: C, 63.4; H, 6.8; N, 6.1%).

A similar preparation with L-valine benzyl ester hydrobromide (prepared by the method of Ben-Ishai & Berger, 1952), m.p. 118° (Found: C, 49.9; H, 6.6; N, 4.7. $C_{15}H_{18}O_2NBr$ requires C, 50.0; H, 6.3; N, 4.8%), gave *benzyloxycarbonyl-S-benzyl-L-cysteinyl-L-valine benzyl ester* (IVc; $R = CH_2 \cdot C_6H_5$), m.p. 62–64°, $[\alpha]_D^{24} - 24.4^\circ$ (c, 1.56 in benzene) (Found: C, 67.7; H, 6.3; N, 5.3%).

Table 1. Effect of acid hydrolysis on the optical rotation of cystine and valine

Cystine and valine were isolated from the peptide after hydrolysis with 6N-HCl at 105° for 64 hr. and from synthetic mixtures which had been submitted to the same hydrolytic procedure. The optical rotations refer to 1% solutions in 5N-HCl.

	$[\alpha]_D^{22}$	
	Cystine	Valine
Synthetic peptide	-74°	-27.4°
L-Cystine + DL-valine	-57	0
L-Cystine + L-valine	-60	+24.0
L-Cystine + D-valine	-83	-25.6

The ethyl ester (IVc; R = C₂H₅; 7.2 g.) was dissolved in acetone (105 ml.) and N-NaOH (75 ml., 5 mol.prop.) was added. The mixture was shaken mechanically at room temperature for 30 min., filtered and the filtrate was poured into water (850 ml.); the aqueous solution was extracted with ethyl acetate (3 × 200 ml.), the organic phase then being back-extracted with aq. 2% NaHCO₃ (3 × 100 ml.). The combined aqueous portions were acidified to Congo red with 2N-HCl in the usual way and the acidified solution was extracted thoroughly with ethyl acetate (3 × 200 ml.). After drying overnight (MgSO₄) the solvent was removed *in vacuo* to yield a gum (6.74 g.) which gave one spot, *R_F* 0.85 in solvent B. For analysis a small quantity of material was converted into the cyclohexylamine salt in the manner described above. Several recrystallizations from ethyl acetate-cyclohexane (containing 1 drop of cyclohexylamine) gave *benzyloxycarbonyl-S-benzyl-L-cysteinyl-L-valine cyclohexylamine salt*, m.p. 145–146° (Found: N, 7.53. C₂₆H₄₁O₆N₂S requires N, 7.7%).

The saponified material (Vc; 5.39 g.) was suspended in dry acetic acid (108 ml.) containing HBr (17.5 g.) and allowed to stand at room temperature for 45 min.; the resulting clear solution was freeze-dried to yield 5.1 g. of crude product (VIc), *R_F* 0.77 in solvent A, which was dissolved in water (50 ml.) and extracted with ether to remove benzyl bromide. The aqueous solution was then applied to a Zeo-Karb 215 (30 g.) column in the H⁺ form; the column was eluted with water until the eluate was neutral and the product was finally displaced from the column with aq. 0.15N-NH₃ soln.; evaporation of the solution to dryness, followed by crystallization of the residue from ethanol-ether, gave 2.9 g. (77%) of *S-benzyl-L-cysteinyl-L-valine* (VIc), m.p. 198–200°, [α]_D²⁵ + 16.0° (c, 1.0 in ethanol) (Found: C, 58.2; H, 7.39; N, 9.05. C₁₅H₂₃O₃N₂S requires C, 58.0; H, 7.1; N, 9.05%). The *R_F* values were 0.85 in solvent A and 0.53 in solvent B.

Liquid NH₃ was treated with sodium until a permanent blue colour was obtained and was then distilled in a closed system into a flask cooled in liquid air. When 50 ml. had been collected, the *S*-benzyl peptide (620 mg.) was added and sodium was introduced into the flask in small quantities, with mechanical stirring, until a permanent (7 min.) blue colour was obtained; a total of 154 mg. was required. Ammonium sulphate (450 mg.) was added and the liquid NH₃ was evaporated as quickly as possible, last traces being removed in a desiccator over conc. H₂SO₄. The residue was suspended in water, the pH adjusted to pH 8 with N-HCl, a trace of dil. aq. FeCl₃ was added and air was bubbled through the solution until the blue colour was discharged (15 min.). The peptide was absorbed on a Zeo-Karb 215 column (30 g., 2 mol.prop.), which was eluted with water to neutrality in the usual way and the peptide was then displaced from the column with aq. 0.15N-NH₃ soln. Evaporation of the eluate *in vacuo* gave 0.35 g. (79.5%) of crude product. Purification was effected by dissolving the product in the minimum of water containing a trace of dil. NH₃ soln. and then adding ethanol and ether; the product was dried at 100° for 6 hr. over P₂O₅ to give pure *L-cystinyl-L-valine monohydrate* (VIIIc), m.p. > 300°, [α]_D²⁷ - 21.8° (c, 0.78 in 5N-HCl) (Found: C, 42.1; H, 6.91; N, 12.7. C₁₆H₂₀O₆N₂S₂H₂O requires C, 42.1; H, 7.04; N, 12.3%). The peptide gave one spot, *R_F* 0.30 in solvent A. The optical purity of this material was confirmed by hydrolysis, the isolated cystine having [α]_D²⁴ - 77.0° (c, 1.0 in 5N-HCl) and the valine [α]_D²⁵ + 24.0° (c, 1.0 in 5N-HCl).

Bis-benzyloxycarbonyl-L-cystine (5.1 g.) (Bergmann & Zervas, 1932) and D-valine ethyl ester hydrochloride (3.63 g.) were suspended in dry ethylene dichloride (40 ml.) and a clear solution was obtained after addition of dry ethyl acetate (20 ml.). Triethylamine (2.02 g.) and dicyclohexylcarbodi-imide (4.53 g., 1.1 mol.prop.) were added and the reaction was allowed to proceed in the usual way. The precipitated product was separated from dicyclohexylurea by dilution of the reaction mixture with warm ethyl acetate (100 ml.), and after removal of the urea by filtration the solution was treated as described in previous experiments to give 6.98 g. (90%) of crude peptide. Recrystallization from ethyl acetate-ethanol-light petroleum (b.p. 80–100°) gave *bis-benzyloxycarbonyl-L-cystinyl-D-valine ethyl ester* (5.5 g.; 78%), m.p. 179–180°, [α]_D²⁵ + 6.0° (c, 0.5 in dioxan) (Found: C, 56.9; H, 6.6; N, 7.3. C₃₆H₅₀O₁₀N₄S₂ requires C, 56.7; H, 6.6; N, 7.3%). This material could not be saponified and therefore this route to the peptides via cystinyl intermediates was abandoned.

Benzyloxycarbonyl-S-benzyl-L-cysteine (11; 6 g.) and D-valine ethyl ester hydrochloride were coupled under conditions identical with those described for the fully protected L-peptide (IVc), to give *benzyloxycarbonyl-S-benzyl-L-cysteinyl-D-valine ethyl ester* (IVb; R = C₂H₅), 7.63 g., (93%), m.p. 120°, [α]_D²⁵ + 19.0° (c, 1.0 in dioxan) (Found: C, 63.6; H, 6.5; N, 5.9%). The *R_F* in solvent B was 0.96.

The ester (IVb; 6.15 g.) was saponified for 40 min. in acetone (160 ml.) and N-NaOH (65 ml., 5 mol.prop.) and treated in the usual way to give *benzyloxycarbonyl-S-benzyl-L-cysteinyl-D-valine* (Vb) (4.6 g., 80%), m.p. 112–114°, [α]_D²⁴ - 31° (c, 1.0 in ethanol) (Found: C, 62.2; H, 6.55; N, 6.5%. The *R_F* in solvent B was 0.85. C₂₃H₂₈O₆N₂S requires C, 62.2; H, 6.3; N, 6.3%).

The above product (Vb; 4.48 g.) was treated with a 2M solution of HBr in acetic acid at room temperature for 45 min.; the reaction mixture was treated as described for the L-isomer (VIc) to give fine needles of *S-benzyl-L-cysteinyl-D-valine* (VIb) (2.0 g., 64%), m.p. 207–209°, [α]_D²³ + 17.6° (c, 1.6 in 2.5N-HCl) (Found: C, 57.7; H, 7.2; N, 8.8. Calc. for C₁₅H₂₂O₃N₂S: C, 58.04; H, 7.1; N, 9.0%). Holley, Peel, Luz & Folkers (1952) found m.p. 204–209°, [α]_D²³ + 23.5° (c, 1.6 in 2.5N-HCl).

The *S*-benzyl peptide (VIb; 2.03 g.) was reduced with sodium in liquid NH₃ (720 mg. in 75 ml.) in the usual way and the product was obtained as described above for the L-isomer (VIIIc); recrystallization gave *cystinyl-D-valine dihydrate* (800 mg., 56%), m.p. > 300°, [α]_D²⁵ + 29.6° (c, 1.01 in 5N-HCl) (Found: C, 40.0; H, 7.2; N, 11.6%). The product ran as one spot, *R_F* 0.19, on Whatman no. 4 paper in solvent A. Attempts to remove completely the water of crystallization by rigorous drying *in vacuo* at 100° were unsuccessful; a loss in weight of 7.5% was obtained (theory 9%). The optical configuration of the peptide was obtained by hydrolysis and subsequent isolation of the cystine and valine. The cystine gave [α]_D²⁵ 0° (c, 1.0 in 5N-HCl), and the valine gave [α]_D²⁵ - 28.0° (c, 1.0 in 5N-HCl); the peptide was therefore *DL-cystinyl-D-valine* (VIIId).

Synthesis of ¹⁴C-labelled peptides

Resolution of DL-[carboxy-¹⁴C]valine. DL-[carboxy-¹⁴C]-Valine (117.1 mg., 1 mc) was converted into the chloroacetyl derivative (Birnbbaum, Levintow, Kingsley & Greenstein, 1952), diluted with unlabelled chloroacetyl-DL-valine (6.15 g.) and resolved with alyase I by the method of

Table 2. *Summary of synthesis of labelled peptides and intermediates*

The radiochemical purity has been calculated from the specific radioactivities of the original valine and products as follows:

$$\frac{\text{specific radioactivity of product} \times \text{mol.wt. of product}}{\text{specific radioactivity of valine} \times \text{mol.wt. of valine}} \times 100.$$

Optical rotations were determined under the same conditions as for the unlabelled materials (see text); —, signifies value not determined.

Compound	Yield (%)	M.p.	Specific activity ($\mu\text{C/g.}$)	Radiochemical purity (%)	$[\alpha]_D$
IVb	85	119–120°	47.4	93	–18.1°
IVc	84	75–77	48.6	94	–39.0
Vb	84	111–113	55.1	103	–29.8
Vc	90	110–111	57.6	105	—
VIb	74	206–209	70.0	92	—
VIc	61	199–201	74.0	97	—
VIIIb	59.5	> 300	104.8	96	+23.0
VIIIc	75.5	> 300	104.8	96	–21.5

Greenstein (1954). After the incubation was complete the solution was deproteinized by boiling, then filtered and concentrated *in vacuo*. The concentrated solution was loaded on to a Zeo-Karb 225 column (The Permutit Co. Ltd., London) (200 g., 60 cm. $\times$ 2 cm. diam.) and eluted with water until neutral; the acidic fractions were bulked, evaporated to dryness *in vacuo* and hydrolysed with 2.5N-HCl (100 ml.) at 100° for 4 hr. After two evaporations to dryness *in vacuo* to remove excess of HCl, the residue was freeze-dried from water (20 ml.); the product was heated at 60° for 3 hr. in a vacuum-sublimation apparatus to remove chloroacetic acid. The yield of D-[carboxy- ^{14}C]valine hydrochloride was 2.54 g. (100%), $[\alpha]_D^{25}$ –26.0° (c, 1.0 in 5N-HCl), specific activity 155.2 $\mu\text{C/g.}$ L-[carboxy- ^{14}C]valine was displaced from the Zeo-Karb 225 column with 2.5N-HCl and the eluate was fed into a fraction collector. Fractions containing valine (ascertained by paper chromatography in solvent A) were bulked and freeze-dried to give L-[carboxy- ^{14}C]valine hydrochloride (2.45 g., 84%), $[\alpha]_D^{25}$ +25.0° (c, 1.02 in 5N-HCl), specific activity 159.2 $\mu\text{C/g.}$

Summary of synthesis of ^{14}C -labelled peptides. The route followed in the synthesis of L-cystinyl-L-[carboxyl- ^{14}C]valine and L-cystinyl-D-[carboxy- ^{14}C]valine was identical with that described in the section on optically specific syntheses as far as the stage of removal of the benzyloxycarbonyl group. The liquid- NH_3 reduction stage was carried out with metallic lithium instead of sodium in an attempt to simplify the removal of salt from the product; the reduction went smoothly but desalting was only partially successful in that after a first crop of salt-free peptide had been obtained by crystallization it was necessary to use a Zeo-Karb 215 column to complete the process. The details of yields, specific activity etc. are shown in Table 2. The L-cystinyl-L-[carboxy- ^{14}C]valine was subsequently found to contain a small quantity of more acidic material which was removed as follows. The peptide (200 mg., 104.8 $\mu\text{C/g.}$) was dissolved in 0.5M-acetic acid (5 ml.) and applied to a column of De-Acidite FF (The Permutit Co. Ltd., London) in the acetate form (25 g.). The column was eluted with 0.5M-acetic acid at a rate of 5 ml./hr.; 5 ml. fractions were collected with a fraction collector. The yield of pure disulphide peptide was 180 mg. and was isolated as the diacetate, as indicated by the drop in specific activity to 89.2 $\mu\text{C/g.}$ (diacetate requires 90 $\mu\text{C/g.}$). Another sample of

peptide (104.8 $\mu\text{C/g.}$), which had not been subjected to treatment with De-Acidite FF, was dissolved in 0.5M-acetic acid, freeze-dried and then dried to constant weight at 100° *in vacuo* over P_2O_5 ; the product had a specific activity of 86.7 $\mu\text{C/g.}$, again in fair agreement with the required specific activity of the diacetate salt. Attempts to remove all the acetic acid by prolonged drying were unsuccessful; products with specific activities intermediate between those of the anhydrous and salt forms of the peptide were obtained.

SUMMARY

1. The synthesis of L-cystinyl-L-valine, L-cystinyl-D-valine and DL-cystinyl-D-valine by routes using the mixed-anhydride and dicyclohexylcarbodi-imide-coupling methods is described.
2. The problems of racemization during the removal of the protecting groups and removal of salt from the free peptides are discussed.
3. The synthesis of L-cystinyl-L-[carboxy- ^{14}C]valine and L-cystinyl-D-[carboxy- ^{14}C]valine is reported.

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The Utilization of L-Cystinyl-L-Valine for Penicillin Biosynthesis

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There is now good evidence that the thiazolidine- β -lactam ring structure of penicillin (I) is derived from L-cystine and L-valine (for recent reviews see Demain, 1959; Arnstein, 1957) and oxidative ring-closure of the dipeptide cysteinylvaline (II) or of a closely related derivative has been suggested as a possible mechanism for the biosynthesis (Arnstein & Crawhall, 1957; Birch & Smith, 1958). In order to investigate the metabolism of this dipeptide in relation to penicillin biosynthesis, the disulphide form of (II) was synthesized from ^{14}C -labelled valine (Arnstein & Morris, 1960), both L-cystinyl-L-[carboxy- ^{14}C]valine and L-cystinyl-D-[carboxy- ^{14}C]valine being prepared.

$\text{R}=\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot$ in benzylpenicillin. The broken lines indicate the metabolic origin of the thiazolidine- β -lactam ring structure from cysteine and valine.

In this paper it will be shown that *Penicillium chrysogenum* utilizes L-cystinyl-L-valine for penicillin formation without obligatory cleavage of the peptide bond. In contrast, the peptide containing D-valine was not taken up by the mycelial cells and appeared to be metabolically inert. Most of this

work has been described by Arnstein & Morris (1959).

EXPERIMENTAL

Organism and maintenance of cultures. The strain of *Penicillium chrysogenum* used was WIS51-20 F3, obtained from Dr W. J. Halliday, University of Queensland. Subcultures and spore inocula were prepared as described by Arnstein & Grant (1954).

Preparation of washed mycelium. Fermentations were carried out in the manner described by Halliday & Arnstein (1956) with the exception that the sterile potassium phenylacetate (0.1%/24 hr.) was added in two discrete portions after 24 and 40 hr. fermentations respectively. After 64–70 hr. the mycelium was harvested, washed three times by resuspension in 0.01M-potassium phosphate buffer, pH 7, once by resuspension in 0.1M-potassium phosphate buffer, pH 7, and filtered to give a mycelium of 13–15% dry wt.; the mycelium was then either used at once or stored at +1° under O_2 overnight for use on the following day. The potassium phosphate buffers referred to in this paper were prepared by diluting a M solution containing 136 g. of KH_2PO_4 and 40 g. of KOH/l .

Incubation of washed mycelium with precursors. Portions of mycelium (5 g. wet wt.) were added to duplicate 500 ml. conical flasks containing 50 ml. of ice-cold 0.01M-potassium phosphate buffer, pH 7, 0.005% of potassium phenylacetate and 1% (w/v) of glucose (hereafter referred to as the medium) in the presence of a known quantity of ^{14}C -labelled precursor. After mixing at 0°, 1 ml. was removed from each flask, filtered and the filtrate was frozen and kept for uptake estimations (see below). The flasks were warmed rapidly to 25° and incubated on a rotary shaker (approx. 170 rev./min.) at this temperature for the required period. The flasks were then removed, chilled in ice and the medium was filtered by suction; 1 ml. of filtrate was removed from each flask for uptake estimation and frozen as before. The mycelial pads were immediately stored in solid CO_2 and the filtrate from each incubation was made up to 100 ml. in calibrated flasks with 0.1M-potassium phosphate buffer, pH 7. After thorough mixing, portions (1 ml.) were