Tetrahedron, Vol. 37, pp. 1589 to 1595, 1981
Printed in Great Britain.

0040-4020/81/081589-07$02.00/0

PENICILLIN BIOSYNTHESIS

RETENTION OF CONFIGURATION AT C-3 OF VALINE DURING ITS INCORPORATION INTO THE ARNSTEIN TRIPEPTIDE

JACK E. BALDWIN* and TERENCE S. WAN
Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, England

(*Received in UK 30 October* 1980)

Abstract—[3-^{3}H]-valine was efficiently synthesised from sodium α-ketoisovalerate. With a β-lactam negative mutant of *C. acremonium*, L-[1-^{14}C-3-^{3}H]-valine and DL-[1-^{14}C-3-^{3}H]-valine were independently incorporated into the Arnstein tripeptide dimer, i.e. Bis-δ-(L-α-aminodipyl)-L-cystinyl-bis-D-valine, with full retention of tricium at C-3 of the D-valine residue. This result strongly suggested retention of configuration at C-3 of valine when the tripeptide was biosynthesised, and further limited the number of possible mechanisms for the biosynthesis of penicillins.

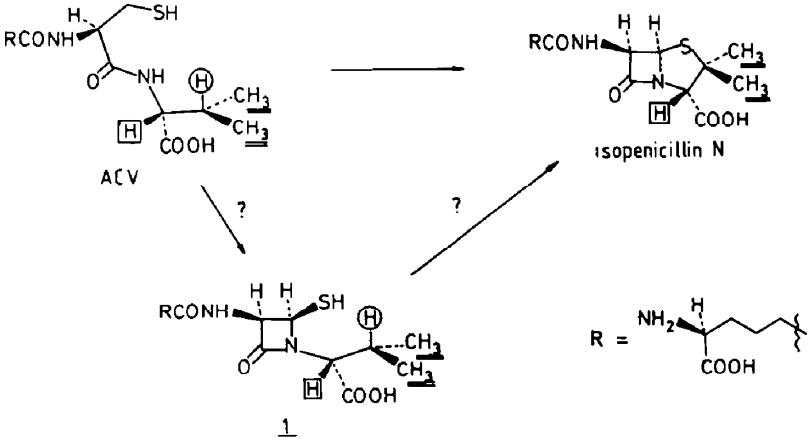
The tripeptide δ - (L - α - aminoadipyl) - L - cysteinyl - D - valine (ACV) has been incorporated into isopenicillin N.[1-4] However mechanistic details of this *in vivo* ring formation are virtually unknown.[5] Labelling studies with cell-free systems suggested that the hydrogens at C-2 and C-4 of the D-valine residue of ACV were retained in isopenicillin N by *Cephalosporium acremonium*[2,3] or *Penicillium chrysogenum*.[4] It was further suggested, in the case of *P. chrysogenum*,[4] that the valine C-3 hydrogen of ACV was lost during the formation of isopenicillin N. In addition, the isolation of a labelled intermediate was claimed,[4] whose labelling pattern as well as chromatographic behaviour were consistent with the structure of a monocyclic β-lactam 1; however proof of the suggested structure has not been given (Scheme 1).

Of great interest is the stereochemical outcome of the diastereotopic Me groups in the D-valine moiety of ACV during this transformation (Scheme 2). The incorporation of chiral valines into penicillins has been known[6-8] to proceed with an *overall* retention of configuration at C-3 of valine. With the intermediacy of ACV firmly established between valine and isopenicillin N,[9] whether those Me groups of ACV were incorporated with retention (or inversion) into penicillins could in principle be determined by examining the stereochemical outcome of the first half of the transformation (i.e. valine to ACV).

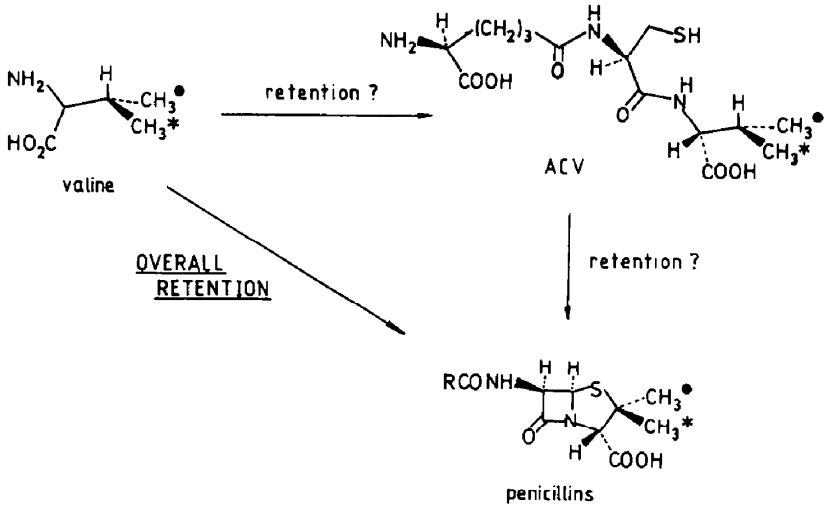
We reasoned that if the β-hydrogen of valine was fully retained during the biosynthesis of ACV, this transformation must proceed with retention at the β-carbon, which would then require another retention of configuration from ACV to penicillins as well. Previous investigations with both *C. acremonium*[10] and *P. chrysogenum*[11] seemed to provide such an observation. However both studies were conducted with [2,3,4-^{3}H]-valine, which complicated the accurate interpretation of the incorporation result. Moreover, in the study with *C. acremonium*[10] there was no ^{14}C-valine cotracer and in the *P. chrysogenum* study[11] the locations of the remaining tritium in ACV were not established. Because of these uncertainties, we have carefully re-examined the fate of the C-3 hydrogen of valine during the biosynthesis of ACV.

We wish to report here a novel efficient synthesis of [3-^{3}H]-valine and the intact incorporation of L- and DL - [1-^{14}C-3-^{3}H] - valine into Bis - δ - (L - α - aminoadipyl) - L - cystinyl - bis - D - valine (ACV dimer) with full retention of tritium at C-3 of the D-valine residue, by a β-lactam negative mutant of *C. acremonium*.

Synthesis of [3-^{3}H]-*valine.* The direct synthesis of [3-^{3}H]-valine has never been reported. An indirect synthesis of DL-[3-^{3}H]-valine by the removal of tritium from C-2 of



Scheme 1.



Scheme 2.

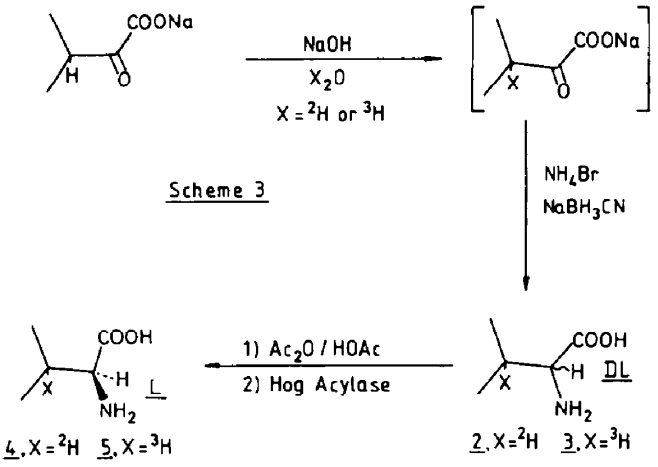
DL-[2, 3-^{3}H]-valine has been claimed.[12] However the DL-[2, 3-^{3}H]-valine used was derived (in 8.8% radiochemical yield) from the catalytic reduction of 2-phenyl-4-isopropylidine-oxazol-5-one with tritium gas followed by hydrolysis. This catalytic reduction gave rise to different distributions of tritium between successive preparations; the majority of tritium in each case was located at C-2 and there was a minor amount at C-4. Hence the DL-[3-^{3}H]-valine reported is actually DL-[3, 4-^{3}H]-valine. Clearly, this is a poor route to valine specifically labelled at C-3 with tritium.†

Sodium α-ketoisovalerate was rapidly deuterated at C-3 with deuterium oxide in the presence of sodium hydroxide. After neutralisation and evaporation of solvent, the crude sodium [3-^{2}H] - 2 - oxoisovalerate was reductively aminated[14] to DL-[3-^{2}H]-valine (2) in 33% overall yield (Scheme 3). NMR (^{1}H, ^{2}H and ^{13}C) study of 2 revealed about 95% of deuterium at C-3 and no deuterium at C-2 or C-4. Using tritiated water, DL-[3-^{3}H]-valine (3) was similarly obtained (6.30 mCi/mmole). Enzymatic resolution (with hog acylase) of 2 *via* its N-acetyl derivative[15] gave L-[3-^{2}H]-valine (4), $[\alpha]_D^{20} = +5.5°$ ($c = 1$ in H_2O). Similarly, L-[3-^{3}H]-valine (5), 6.32 mCi/mmole, $[\alpha]_D^{20} = +5.6°$ ($c = 0.5$ in H_2O), was obtained from 3.

β-Tritiated amino acids have been recognised[12] as more desirable for certain biological studies than the more readily available α-tritiated amino acids,[16] since the α-tritium atom is more labile in enzymatic processes. In view of the availability of inexpensive, easily-handled tritiated water of very high specific activity, the above method represents a convenient one-step procedure for the regiospecific (see below) synthesis of highly-active [3-^{3}H]-valine. Furthermore since reductive amination of other keto acids has been reported,[14] it is expected that our method can be extended to the efficient syntheses of a variety of other β-tritiated amino acids with high specific activities.

Incorporation of valine into ACV dimer. The β-lactam negative mutant, N-2, of *C. acremonium*, which accumulates the disulfide of ACV (ACV dimer), was reported recently.[17] We chose to do our feeding experiment with this mutant because of the ease of isolation

†The same approach to DL-[3-^{3}H]-valine from commercial L-[2, 3-^{3}H]-valine was reported[13] but again it contained 0.46% of tritium at C-4.



Scheme 3

Scheme 3.

and identification of ACV dimer. Thus, after preliminary purification the culture broth of the above mutant which had been fed with L-[1-^{14}C-3-^{3}H]-valine (^{3}H/^{14}C = 4.052) was subjected to hplc isolation.[18] The hplc profiles of synthetic ACV dimer[19] and the pre-purified reaction mixture were shown in Fig. 1 and Fig. 2 respectively. The fact that primary dialkyl disulphides have an extinction coefficient of *ca* 480 at around 250 nm[20] resulted in easy detection of the ACV dimer by absorbance at 254 nm. By calibrating the area of the peak with known amounts of synthetic material, the culture broth was estimated to have accumulated *ca* 1.1 mg/mL of ACV dimer. The tripeptide was isolated in 0.45% radiochemical yield based on ^{14}C-valine. The ^{3}H/^{14}C ratio of the isolated and purified ACV dimer was also 4.052, suggested that L-[1-^{14}C-3-^{3}H]-valine was incorporated intact with 100% retention of tritium. Similarly DL-[1-^{14}C-3-^{3}H]-valine (^{3}H/^{14}C=4.035) was incorporated intact into ACV dimer (^{3}H/^{14}C=4.024) with 99.7% retention of tritium (0.83% radiochemical yield based on ^{14}C-valine). The result of 2-dimensional cellulose tlc of the starting doubly-labelled valine and the isolated ACV dimer in each case was shown in Figs. 3 or 4. All four compounds are reasonably chemically pure as judged by ninhydrin reaction and essentially radiochemically pure as judged by fluorography.

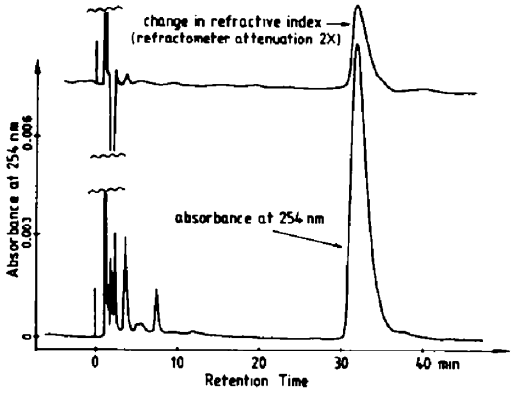


Fig. 1. Hplc profile of 160 μg of synthetic ACV dimer.

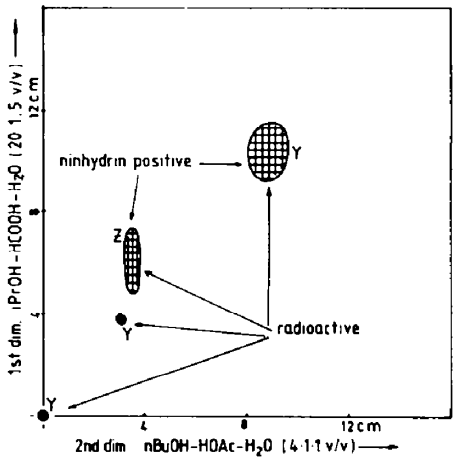


Fig. 3. Two-dimensional thin layer chromatography on cellulose. Y: L-[1-^{14}C-3-^{3}H]-valine, ~4 μg, (~220 nCi ^{3}H, ~55 nCi ^{14}C); Z: ACV dimer isolated after feeding Y, ~8 μg, (~0.56 nCi ^{3}H, ~0.14 nCi ^{14}C).

Location of tritium. As shown above the DL-[3-^{2}H]-valine (2) contains no deuterium at C-2 and C-4. Hence the DL-[3-^{3}H]-valine (3) obtained similarly should also contain no tritium at C-2 and C-4. The absence of tritium at C-2 of 3 was confirmed by the identical specific activity of the L-[3-^{3}H]-valine (5) obtained from 3. Any tritium at C-2 of 3 would have been washed out during the acetic anhydride-acetic acid treatment[21] and a lower specific activity of 5 would have been observed. The absence of tritium at C-4 of 3 was confirmed by the following experiment: DL-[1-^{14}C-3-^{3}H]-valine (532 nCi ^{3}H and 130 nCi ^{14}C) was treated with D-amino acid oxidase. The mixture was then passed through a column of Dowex-50 cation exchange resin (H^+ form). The water eluate, which contained α-ketoisovalerate has respectively 94% and 90% of the total ^{3}H and ^{14}C activity from the D-antipode. Further, elution of the resin with ammonium hydroxide recovered respectively 87% and 90% of the total ^{3}H and ^{14}C activity from the remaining unoxidized L-[1-^{14}C-3-^{3}H]-valine. Treatment of the α-ketoisovalerate fraction with aqueous sodium hydroxide followed by neutralisation and freeze-drying resulted in

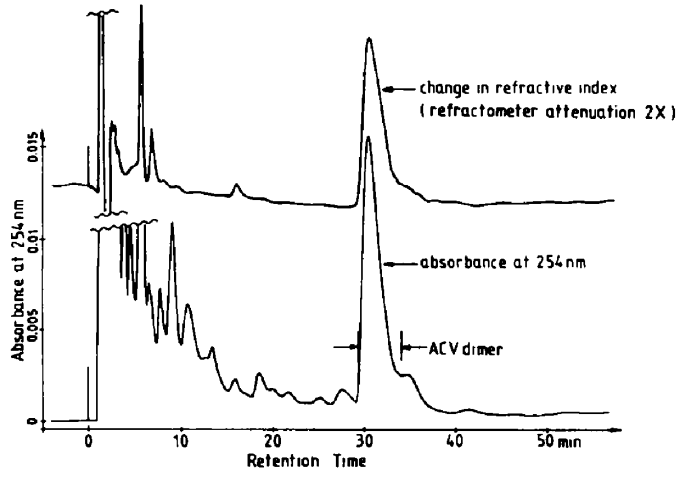


Fig. 2. Hplc profile of 100 μL of Fraction B (Experimental), obtained from 400 μL of culture broth fed with L-[1-^{14}C-3-^{3}H]-valine.

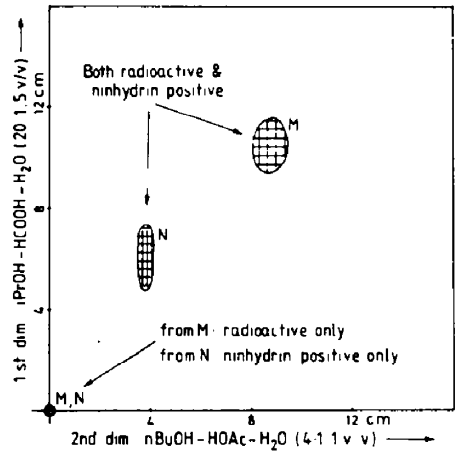


Fig. 4. Two-dimensional thin layer chromatography on cellulose. M: DL-[1-^{14}C-3-^{3}H]-valine, ~2 μg, (~112 nCi ^{3}H, ~28 nCi ^{14}C); N: ACV dimer isolated after feeding M, ~20 μg, (~1.9 nCi ^{3}H, ~0.48 nCi ^{14}C).

the loss of *all* ^{3}H activity, while retaining 87% of the total ^{14}C activity from the D-antipode. Hence the DL-[1-^{14}C-3-^{3}H]-valine should contain no tritium at C-4. From these results we concluded that our DL-[3-^{3}H]-valine (3) as well as the L-[3-^{3}H]-valine (5) derived from it have no detectable amount of tritium at C-2 and C-4.

The same method was conveniently extended to the location of tritium in the ACV dimer isolated. In each case the doubly-labelled ACV dimer isolated from feeding L- or DL-[1-^{14}C-3-^{3}H]-valine was hydrolysed† with 6 M HCl. After removal of acid, the hydrolysate was directly treated with D-amino acid oxidase and analysed as above. The results, summarised in Tables 1 and 2, clearly indicated that the location of tritium in the ACV dimer isolated from either incorporation experiment was entirely at C-3 of the D-valine residue.

Discussions on penicillin biosynthesis

With a mutant of *C. acremonium*, doubly-labelled L-valine was incorporated intact into ACV dimer with full retention of tritium at C-3. The use of DL-[1-^{14}C-3-^{3}H]-valine gave essentially identical result, indicating that *either* the D-antipode of the doubly-labelled valine was never utilised *or*, more likely (since ACV dimer was obtained in a higher radiochemical yield), the incorporation of D-valine into ACV dimer, presumably via L-valine,[5] also proceeded without any loss of the C-3 hydrogen. This complete retention of tritium strongly suggested that ACV was biosynthesised with a retention of configuration at C-3 of valine (Scheme 4). Hence, together with previous results, we concluded that the *in vivo* formation of ACV from intact valine only results in the loss of its α-hydrogen;‡ and also that the biosynthesis of isopenicillin N (and subsequently other penicillins) from ACV must proceed with retention of configuration at C-3 of the D-valine residue. The latter conclusion puts further restrictions on the possible mechanisms for the formation of the bicyclic system from ACV.

Tetrapeptides structurally related to ACV but containing β-hydroxyvaline in place of valine have been isolated[13,24] in β-lactam producing organisms. This led to the suggestion that ACV might be hydroxylated to 6 or 7 and a SN_2 displacement (with inversion) of the OH group by sulphur would establish the S–C [S(1)-C(2)] bond in penicillins. However this hypothetical transformation would most likely result in an overall *inversion* of the C-3 configuration of the D-valine residue, since microbial hydroxylation of unactivated carbons generally proceeds with retention of configuration.[25] The present finding therefore strongly disfavours this sort of mechanism.

It is possible that hydroxylated peptides, such as those isolated,[23,24] were only side products indirectly related to the biosynthesis of penicillins from ACV. On the other hand, our earlier hypothesis[26] of a radical cyclisation of the disulphide of 1[4] is still consistent with all known facts.

EXPERIMENTAL

Optical rotations were obtained on a Perkin–Elmer 241 automatic polarimeter in a 10 cm cell. F.T.NMR spectra were obtained on a Bruker WH-300 multinuclear magnetic resonance spectrometer; chemical shifts were reported with respect to TMS. The mass spectrum was obtained from a VG Micromass ZAB-16F spectrometer.

Chromatography. Ascending thin layer chromatography was carried out at r.t. on cellulose sheets (Eastman 13254, 20 cm × 20 cm × 160 μm). Detection of amino acids and peptides was

†No correction was made for any racemisation of D-valine to L-valine during the hydrolysis.[22]

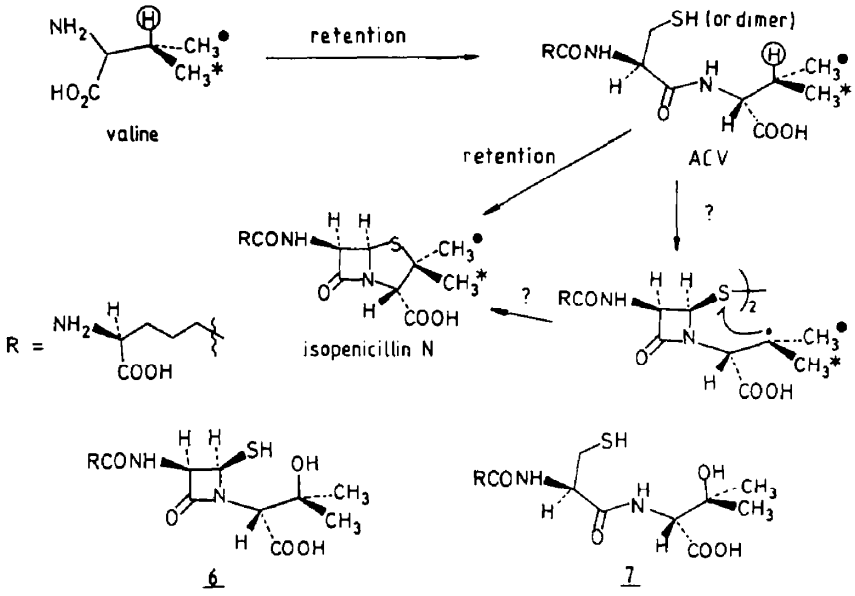
‡Whether the nitrogen of D-valine was retained or not still has not been established.

Table 1. Percentages of radioactivity recovered from the degradation of ACV dimer (9.633 nCi ^{3}H, 2.344 nCi ^{14}C) incorporated from L-[1-^{14}C-3-^{3}H]-valine

Water Eluate	Water Eluate after Base Treatment	NH_4OH Eluate
82% ^{3}H	3% ^{3}H	2% ^{3}H
70% ^{14}C	74% ^{14}C	3% ^{14}C

Table 2. Percentages of radioactivity recovered from the degradation of ACV dimer (14.25 nCi ^{3}H, 3.490 nCi ^{14}C) incorporated from DL-[1-^{14}C-3-^{3}H]-valine

Water Eluate	Water Eluate after Base Treatment	NH_4OH Eluate
90% ^{3}H	0% ^{3}H	2% ^{3}H
81% ^{14}C	89% ^{14}C	2% ^{14}C



Scheme 4.

carried out by dipping the chromatograms in 0.2% ninhydrin/ethyl acetate (w/v) followed by heating.

Fluorography. Dried cellulose chromatograms (20 cm × 20 cm) were dipped in toluene (BDH scintillation grade) containing 2,5-diphenyloxazole (20% w/v) and air dried. Kodak X-Omat-H film was pre-exposed with a Metz Mecablitz III flash unit at a distance of 90 cm as described.[27] The surface of the film which had been nearest the light source was placed in contact with the chromatogram and stored at −70° in the dark. After 16 days, spots were visible on the developed film (Figs. 3 and 4).

Radioactivity measurement. Radioactivity was measured by a LKB Rackbeta-1215 liquid scintillation counter. About 10 mL of a universal colloidal scintillation liquid (Lumagel or, for high salt content, Lumagel SB: all from Lumac Systems) was mixed with 0.3–2.0 ml of the sample in HClaq or water. The homogeneous samples were counted after overnight equilibration inside the counter, together with suitable blanks and standards. Standardisation was carried out with LKB internal standard capsules for water samples containing [U-^{14}C]-sucrose or [6,6'-^{3}H(n)]-sucrose. The reported values were usually the mean of two or more determinations. Comparisons of radioactivities or ^{3}H/^{14}C ratios were made only when samples were counted under identical conditions.

DL-[3-^{2}H]-*Valine* (2) Sodium α-ketoisovalerate (Sigma, 618 mg, 4.475 mmole) and NaOH (79 mg, 2 mmole) were dissolved in 1.44 ml of 99.8% D_2O (Flurochem. Ltd). Under similar condition, deuteration of the β-hydrogen of sodium α-ketoisovalerate was judged complete in less than 10 min by ^{1}H NMR spectroscopy. After 2 hr at r.t., the soln was neutralised with dil HBr and freeze-dried. The crude sodium α-keto-β-deuteroisovalerate so obtained was dissolved in 45 ml anhyd MeOH; to which were added 0.9 g (9 mmole) NH_4Br and 280 mg (4.5 mmole, Aldrich) sodium cyanoborohydride. After stirring at r.t. under N_2 for 4 days, the cloudy mixture was carefully treated with 12 ml conc HCl, stirred for a while, concentrated *in vacuo* and freeze-dried. The dried powder was then taken up in 25 ml EtOH, filtered and the residue was washed with 5 ml EtOH. Aniline (3 mL) was added to the combined filtrate and wash. After 3 days, the crystals were collected, washed with EtOH (3 times) and dried *in vacuo*, giving 173 mg (33%, yield not optimised) of 2. tlc (nBuOH/HOAc/H_2O (3:1:1, v/v), silical gel): R_f 0.2, ninhydrin positive. ^{1}H NMR (300 MHz, D_2O): δ(H_2O internal ref) 0.81 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 2.1 (m, 0.05 H, residual β-H), 3.43 (s, 1H, α-H). ^{2}H NMR (46 MHz, fully decoupled, H_2O): δ(D_2O internal reference) 2.1 (s, β-^{2}H). ^{13}C NMR (75 MHz, broad-band ^{1}H-decoupled and off-resonance continuous wave ^{1}H decoupled, 15% D_2O); δ(p-dioxane internal ref) 16.5 (q, CH_3), 17.8 (q, CH_3), 28.6 (t, $^{1}J_{CD}$ = 19.8 Hz, β-C), 29.0 (d, residual $(CH_3)_2$CH-), 60.4 (d, α-C), 174.1 (s, C=O). MS (70 eV) *m/e* 119 (M+H), 73 (M–COOH).

DL-[3-^{3}H]-*Valine* (3). The procedure was similar to that for the preparation of 2. Thus a soln of sodium α-ketoisovalerate (620 mg, 4.49 mmole) and NaOH (90 mg, 2.2 mmole) in 1.44 ml of 1.2 Ci of tritiated water (Radiochemical Centre–Amersham, 5 Ci/ml, diluted before use) was kept at r.t. for 4 days. After neutralisation and freeze-drying, the residue was treated again with water followed by freeze-drying to remove the last trace of T_2O. Reductive amination with 285 mg (4.5 mmole) sodium cyanoborohydride was done as above. Conc HCl (15 ml) was then added cautiously and the cloudy mixture was stirred for 0.5 hr before removal of solvent. The dried residue was taken up in 10 ml abs EtOH, heated briefly to boil. On cooling, the mixture was filtered and washed with 4 + 1 ml abs EtOH. The filtrate and washes were combined and treated with 4 ml aniline. After 3 days, the crystals were collected and washed with 2 × 1 ml absEtOH to give 109 mg (21%, yield not optimised) of 3 after drying *in vacuo* (6.31 mCi/mmole). ^{1}H NMR (300 MHz, D_2O): L δ(H_2O internal ref) 0.82 (d, J = 7.0 Hz, 3H, CH_3), 0.87 (d, J = 7.2 Hz, 3H, CH_3), 2.11 (m, 1H, $(CH_3)_2$CHN), 3.44 (d, J = 4.4 Hz, 1H, –NHCHCO–). Part of it was recrystallised in aqueous EtOH: 6.30 mCi/mmole (specific activity expected if complete exchange had occurred: 7.2 mCi/mmole). Two-dimensional cellulose tlc (see Fig. 4).

L-[3-^{2}H]-*valine* (4). The compound 2 (118 mg, 1 mmole) was refluxed with 0.2 mL (excess) Ac_2O in 1 ml glacial AcOH under N_2 for 40 min. On cooling, the mixture was concentrated *in vacuo* to a syrup at 35°. The syrup was then treated twice with water followed each time by evaporation *in vacuo*. Crude solid N-acetyl-DL-[3-^{2}H]-valine was thus obtained in 91% yield (146 mg). It was dissolved in 4 ml water and adjusted to pH 7.0 with dil LiOH aq. Hog kidney acylase I(Koch–Light, 10 mg, 900 units/mg) was added and the mixture was incubated at 37° for 21 hr, then heated briefly to boil in the presence of a pinch of activated charcoal. On cooling, it was filtered and washed with water. The filtrate and wash were combined and freeze-dried. The dry solid was re-dissolved in 0.4 ml water, adjusted to pH 6 with a drop of dil HCl, filtered and washed with 0.2 ml water. The combined filtrate and wash was treated with 2.4 ml EtOH and placed at 0° for 2.5 days. The crystals were collected, washed with EtOH and diethyl ether, and dried *in vacuo* to give 17 mg (32%, yield not optimised) of 4, $[\alpha]_D^{20} = +5.5°$ (*c* = 1 in H_2O), ^{1}H NMR (300 MHz, D_2O) identical to that of 2.

L-[3-^{3}H]-*Valine* (5). This preparation was similar to that of 4. Thus 3 (83.6 mg, 0.71 mmole) was refluxed with 0.5 ml Ac_2O in

1.5 ml glacial AcOH for 1 hr under N_2. After concentration to dryness as above, 106 mg (93% yield) crude DL-[3-^{3}H]-N-acetylvaline was obtained (~6.4 mCi/mmole). This compound (105 mg, 0.66 mmole), which gave a negative ninhydrin test, was dissolved in 5 ml water, similarly adjusted to pH 7.5, and treated with 10 mg of the acylase. After incubation with shaking at 37° for 2 days, another 5 mg of the acylase was added and the reaction was continued for 1 more day. The mixture was heated briefly to boil. On cooling a pinch of activated charcoal was added and the mixture was filtered through Celite 503 (Koch–Light). The white solid after freeze-drying was re-dissolved in 0.8 ml water, filtered and the filtrate was treated with 3.2 ml abs EtOH. After standing at 0° for 1.5 day, 21.5 mg (56%, yield not optimised) crystalline **5** (~6.0 mCi/mmole) was isolated as above. Recrystallisation from aqueous EtOH gave 17.3 mg of **5**, 6.32 mCi/mmole, $[\alpha]_D^{20} = +5.6°$ ($c = 0.5$ in H_2O), ^{1}H NMR (300 MHz, D_2O) identical to that of **3**, 2-dimensional cellulose tlc shown in Fig. 3.

Organism. The organism used in this study was a β-lactam negative mutant, N-2, derived from *C. acremonium* ATCC 14553 as described.[17] This mutant was available to us as a generous gift from the Takeda Chemical Industries, Japan.

Culture media and conditions. The growth condition was derived from a procedure kindly suggested by Dr. Kanzeki[28] of the Takeda Chemical Industries, Japan. A slant culture was prepared by propagating the mutant at 28° for 10-14 days on the following medium: yeast extract 0.25%, casamino acid 0.1%, malt extract 0.5%, sucrose 2.0%, glycerin 1.0%, agar 1.5%, pH (before autoclaving) 6.8. A seed culture was obtained by cultivating the slant culture at 25° for 5 days (30 ml medium in 250 ml conical flask, 250 rpm rotary shaker with 2 in. eccentric throw) using the following conidiation medium: sucrose 3.0%, meat extract 1.5%, corn steep liquor 0.5%, calcium carbonate 0.15%, pH (before autoclaving) 6.8. About 3 ml of the seed culture was transferred to the following fermentation medium: sucrose 3.0%, soy bean flour 3.2%, DL-methionine 0.5%, $CaCO_3$ 0.15%, pH (before autoclaving) 6.8. Fermentation was carried out at 26° (30 ml medium in 250 ml flask, 250 rpm rotary shaker with 1 in. eccentric throw).

Incubation of doubly-labelled valines. After 3 days of fermentation, 3.0 ml of the culture broth was transferred to each of the two 10 ml conical flasks. To one was added L-[1-^{14}C-3-^{3}H]-valine (90 μl, 6.8 μg/ml, final concentration 200 μg/ml; 31.47 μCi ^{3}H, 7.767 μCi ^{14}C, ^{3}H/^{14}C = 4.052; prepared by mixing the appropriate amounts of aqueous solns of **5** and L-[1-^{14}C]-valine). To the other was added 85 μl of DL-[1-^{14}C-3-^{3}H]-valine (7.0 μg/ml, final concentration 190 μg/ml; 28.92 μCi ^{3}H, 7.167 μCi ^{14}C, ^{3}H/^{14}C = 4.035; prepared similarly from **3** and DL-[1-^{14}C]-valine as above). The L- and DL-[1-^{14}C]-valine were obtained from Radiochemical Centre, Amersham (2% ethanol solns, freeze-dried before use). Fermentation was allowed to continue for 3 more days at 26°, 250 rpm on a rotary shaker with 1 in. eccentric throw.

Treatment of incubation mixtures. The following procedure was applicable to both samples. The incubation mixture was treated with 3 ml acetone, and the precipitated protein was removed by centrifugation at 7000 g for 10 min. The supernatent fluid was freeze-dried and treated with 1.5 ml deionised water. One third of it (Fraction A, 0.5 ml) was set aside for direct hplc analysis. The remaining 1.0 ml sample was poured onto a column (0.4 cm dia) containing a prewashed (H_2O) mixture of 200 mg Celite 503 (Koch–Light) and 100 mg activated decolourising charcoal powder (BDH, acid-washed). Elution was achieved by applying 5 psi N_2 to the column.

After washing the column with deionised water (3 × 1 ml), it was eluted with 4 ml 50% acetone. The acetone eluate was diluted with deionised water and freeze-dried. The dried material was then taken up in 500 μl deionised water (Fraction B) and analysed by hplc.

Chromatograms. These were obtained by using Waters M-6000A solvent delivery system and U6K septumless injector. Detection was carried out with Waters 440 absorbance detector at 254 nm and R-401 differential refractometer connected in series. The stationary phase was bonded-phase propylamine on 10 μm silica (Waters μBondapak-NH_2, 4 mm internal diam × 30 cm). The guard column contained Waters Bondapak AX/Corasil. The solvent system was a degassed mixture of glacial AcOH/MeOH/acetonitrile/deionised water (2:4:7.5:86.5, v/v). The MeOH and acetonitrile were of hplc grade (Rathburn Chemical). All samples were filtered through millipore before injections. Separations were done at *ca* 1700 psi with a flow rate of 2 ml/min and a chart speed of 0.5 cm/min.

Analysis of reaction mixtures by hplc. The hplc profile of synthetic ACV dimer[19] was shown in Fig. 1 (The retention time of ACV dimer ranged from 70 min to 30 min, depending on the life of the column). Part of Fraction B (200 μL) from feeding L-[1-^{14}C-3-^{3}H]-valine was purified by hplc in 2 injections; a typical hplc profile was shown in Fig. 2. The fraction corresponding to ACV dimer was collected in each run, combined and freeze-dried: 37.59 nCi ^{3}H, 9.276 nCi ^{14}C, ^{3}H/^{14}C = 4.052, projected radiochemical yield based on ^{14}C-valine = 0.45%, 2-dimensional cellulose tlc shown in Fig. 3. Part of fraction B (300 μl) from feeding DL-[1-^{14}C-3-^{3}H]-valine was similarly purified in 3 injections. The isolated ACV dimer gave: 95.746 nCi ^{3}H, 23.794 nCi ^{14}C, ^{3}H/^{14}C = 4.024, projected radiochemical yield based on ^{14}C-valine = 0.83%, 2-dimensional cellulose tlc shown in Fig. 4.

Part of Fraction A in each case (42 μL from L-[1-^{14}C-3-^{3}H]-valine experiment and 30 μl from DL-[1-^{14}C-3-^{3}H]-valine experiment) was analysed by hplc. Here ACV dimer was again well-resolved in each case. The amount of ACV dimer produced in the culture broth in each case was estimated to be *ca* 1.1 mg/ml by calibrating its peak area (half-width X peak height) of absorbance at 254 nm with that of synthetic ACV dimer under the same hplc conditions. Similar peak area comparison between Fraction A and Fraction B in each case also revealed that 31–33% of ACV dimer was lost during carbon chromatography (see above). Hence theoretically the isolated radio chemical yields (based on Fraction A) could be 0.67% from L-[1-^{14}C-3-^{3}H]-valine and 1.2% from DL-[1-^{14}C-3-^{3}H]-valine; however, hplc isolation of any appreciable amount of ACV dimer directly from Fraction A (i.e: without preliminary clean up by carbon chromatography) will have to require an impracticable number of injections with our analytical column.

Location of tritium. A soln containing 9.91 μmole D-valine (Calbiochem) and 0.09 μmole of DL-[1-^{14}C-3-^{3}H]-valine (531.8 nCi ^{3}H, 129.5 nCi ^{14}C, ^{3}H/^{14}C = 4.11†), 1 ml of 0.1 M sodium pyrophosphate buffer at pH 8.3, 2 μl of bovine liver catalase (2300 units; Sigma aqueous crystalline suspension with 0.1% thymol, 50 mg protein in 1.3 ml, 30,000 units/mg protein) and 20 μl of hog kidney D-amino acid oxidase (1.9 units; Sigma crystalline suspension in 3.2 M $(NH_4)_2SO_4$, pH 6.5, 19 units/mg protein, 5 mg protein/ml) in water (final volume 2.7 ml) was shaken at 250 rpm in air at 37° for 4 hr. Glacial AcOH (0.1 ml) was added to stop the reaction. The soln was then applied to a pre-washed (1 M LiOH, 2M HCl and then water till neutral) column (2.5 × 0.5 cm) of Dowex 50 (Sigma 50 × 8-400, H^+ form) cation exchange resin. The column was washed with 2 + 3 ml of water. The combined water eluate was neutralised with dil NaOH (to prevent decomposition of the unstable α-ketoisovaleric acid) and then freeze-dried. The column, which contained unoxidised L-[1-^{14}C-3-^{3}H]-valine, was eluted with 4 ml of 1M NH_3. The ammonia eluate was freeze-dried and counted (232.6 nCi ^{3}H, 58.34 nCi ^{14}C, ^{3}H/^{14}C = 3.99). The freeze-dried water eluate was dissolved in 2.0 ml water. One half of it (1.0 ml), which contained [1-^{14}C-3-^{3}H]-2-oxoisovalerate, was counted (125.5 nCi ^{3}H, 29.15 nCi ^{14}C, ^{3}H/^{14}C = 4.30). The other half (1.0 ml) was treated with 1 ml of 2M NaOH overnight and neutralised with dil HCl. This fraction, which contained [1-^{14}C]-2-oxoisovalerate, was freeze-dried and counted (−0.01 nCi ^{3}H, 28.30 nCi ^{14}C).

Doubly-labelled ACV dimer (9.633 nCi ^{3}H, 2.344 nCi ^{14}C, ^{3}H/^{14}C = 4.11†) isolated from feeding L-[1-^{14}C-3-^{3}H]-valine was

†The measured ^{3}H/^{14}C ratios here were slightly different from that obtained previously since the liquid scintillant used here was Lumagel SB (for high salt content) rather than Lumagel, which was used for the feeding experiment.

mixed with 900 μg synthetic ACV dimer and hydrolysed in 900 μl 6M HCl in a sealed tube at 105° for 23 hr. The freeze-dried hydrolysate together with 800 μg D-valine was treated with D-amino acid oxidase for 4 hr as above. A control experiment using the hydrolysate of 1.2 mg of synthetic ACV dimer mixed with 780 μg D-valine was oxidatively deaminated (final pH = 8.0) in less than 1 hr, as judged by the disappearance of valine on cellulose tlc (nBuOH–HOAc–H_2O 4:1:1 v/v). The mixture was analysed as above. The freeze-dried ammonia eluate, which contained unoxidised L-amino acids, had 0.173 nCi ^3H and 0.0746 nCi ^{14}C. Half of the freeze-dried water eluate had 3.948 nCi^3H, 0.817 nCi^{14}C, $^3H/^{14}C$ = 4.83. The other half, which had been treated with base to remove the β-tritium, gave 0.149 nCi ^3H and 0.863 nCi ^{14}C (Table 1).

Similarly, doubly-labelled ACV dimer (14.25 nCi ^3H, 3.490 nCi ^{14}C, $^3H/^{14}C$ = 4.08†) isolated from feeding DL-[1-^{14}C-3-^{3}H]-valine was mixed with synthetic ACV dimer, hydrolysed, oxidatively deaminated and analysed in the same manner. The freeze-dried ammonia eluate had 0.244 nCi^3H and 0.0815 nCi^{14}C. Half of the water eluate had 6.443 nCi^3H and 1.415 nCi^{14}C($^3H/^{14}C$ = 4.55). The other half which had been treated with base had 0.03 nCi^3H and 1.566 nCi^{14}C (Table 2).

Acknowledgement—We wish to thank the Takeda Chemical Industries, Japan for providing us with the N-2 mutant, Dr. T. Kanzaki for providing us with its growth conditions and Mr. J. Keeping for growing the mutant. We would like to acknowledge Mr. M. Jung for a sample of L-[3-^{3}H]-valine (20 μCi/mmole synthesised by another route, and Prof. Sir E. Abraham F. R. S. for the use of his dark-room facilities.

REFERENCES

[1]T. Konomi, S. Herchen, J. E. Baldwin, M. Yoshida, N. A. Hunt and A. L. Demain, *Biochem. J.* **184**, 427 (1980).
[2]J. O'Sullivan, R. C. Bleaney, J. A. Huddleston and E. P. Abraham, *Ibid.* **184**, 421 (1980).
[3]P. A. Fawcett, J. J. Usher, J. A. Huddleston, R. C. Bleaney, J. J. Nisbet and E. P. Abraham, *Ibid.* **157**, 651 (1976).
[4]B. Meesschaert, P. Adriaens and H. Eyssen, *J. Antibiotics* **33**, 722 (1980).
[5]For an excellent review see D. J. Aberhart, *Tetrahedron* **33**, 1545 (1977).
[6]N. Neuss, C. H. Nash, J. E. Baldwin, P. A. Lemke and J. B. Grutzner, *J. Am. Chem. Soc.* **95**, 3797, 6511 (1973).
[7]H. Kluender, C. H. Bradley, C. J. Sih, P. Fawcett and E. P. Abraham, *Ibid* **95**, 6149 (1973).
[8]D. J. Aberhart and L. J. Lin, *J. Chem. Soc.* Perkin I, 2320 (1974).
[9]For incorporation of labelled valine into ACV see: P. B. Loder and E. P. Abraham, *Biochem. J.* **123**, 477 (1971); P. Adriaens, B. Meesschaert, W. Wuyts, H. Vanderhaeghe and H. Eyssen, *Antimicrob. Agents Chemother.* **8**, 638 (1975); J. A. Chan, F. C. Huang and C. J. Sih, *Biochemistry* **15**, 177 (1976).
[10]F. C. Huang, J. A. Chan, C. J. Sih, P. Fawcett and E. P. Abraham, *J. Am. Chem. Soc.* **97**, 3858 (1975).
[11]P. Adriaens, B. Meesschaert, H. Vanderhaeghe and H. Eyssen, *Arch. Int. Physiol. Biochim.* **84**, 767 (1976).
[12]J. C. Crawhall and D. G. Smyth, *Biochem. J.* **69** 280 (1958).
[13]P. Adriaens, H. Vanderhaeghe, B. Meesschaert and H. Eyssen, *Antimicrob. Agents Chemother.* **8**, 15 (1975).
[14]R. F. Borch, M. D. Bernstein, and H. D. Durst, *J. Am. Chem. Soc.* **93**, 2897 (1971).
[15]J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 3, p. 2375. Wiley, London (1961).
[16]D. E. Brundish, D. F. Elliott and R. Wade, *J. Labelled Compounds* **7**, 473 (1971).
[17]H. Shirafuji, Y. Fujisawa, M. Kida, T. Kanzaki and M. Yoneda, *Agric. Biol. Chem.* **43**, 155 (1979).
[18]R. D. Miller and Norbert Neuss, *J. Antibiotics* **29**, 902 (1976).
[19]J. E. Baldwin, S. R. Herchen, B. L. Johnson, M. Jung, J. J. Usher, T. S. Wan, submitted for publication to *J. Chem. Soc.* Perkin I.
[20]L. Field, *Organic Chemistry of Sulfur*, (Edited by S. Oae), p. 337. Plenum Press, New York (1977).
[21]P. Adriaens, B. Meesschaert and H. Vanderhaeghe, *Anal. Biochem.* **69**, 297 (1975).
[22]S. Shankman and Y. Schvo, *J. Am. Chem. Soc.* **80**, 1164 (1958).
[23]L. A. Eriquez and M. A. Pisano, *Antimicrob. Agents Chemother.* **16**, 392 (1979).
[24]P. B. Loder and E. P. Abraham, *Biochem. J.* **123**, 471 (1971).
[25]R. Bentley, *Molecular Asymmetry in Biology*. Academic Press, New York (1970); W. L. Alworth, *Stereochemistry and Its Application in Biochemistry*. Wiley-Interscience, New York (1972).
[26]J. E. Baldwin and T. S. Wan, *J. Chem. Soc.* Chem. Comm. 249 (1979).
[27]R. A. Laskey and A. D. Mills, *Eur. J. Biochem.* **56**, 335 (1975).
[28]T. Kanzaki, personal communication (1979).