Synthesis and incorporation of [3-^{3}H, U-^{14}C]-L-valine into penicillin V using *Penicillium chrysogenum*

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The title compound was synthesized by exchange of dimethylpyruvic acid with NaOH–HTO, followed by reductive amination using cyanoborohydride in methanol solvent, resolution of the *N*-chloroacetyl derivative, and admixture with [U-^{14}C]-L-valine. Degradation of the compound revealed that 94.5% of the tritium was attached to C3, and 5.5% was located on the methyl groups. Incorporation of this doubly labelled valine into penicillin V, purified as the dibenzylamine salt, proceeded with 100.7% loss of the tritium originally present at C3. Commercial [2,3-^{3}H, U-^{14}C]-L-valine, which was found to have 12% of the tritium on the methyl groups, was also incorporated into penicillin V, purified as its methyl ester. Degradation to remove the hydrogen attached to C3 of this ester revealed that no tritium was present at this position. Therefore, in agreement with an earlier result and other related studies, the β-hydrogen of valine is not transferred to C3 of penicillin during the biosynthesis of the latter compound.

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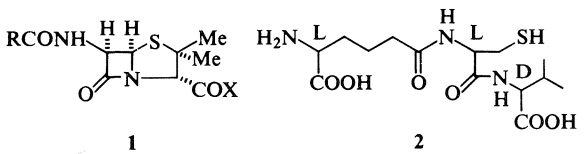
On a synthétisé le composé mentionné dans le titre grâce à un échange de l'acide diméthylpyruvique avec une solution de NaOH–HTO, suivi d'une amination réductrice utilisant le cyanoborohydrure dans le méthanol, d'une résolution du dérivé *N*-chloroacétyle et finalement d'un mélange avec la [U-^{14}C]-L-valine. La dégradation du composé a révélé que 94,5% du tritium est attaché au carbone en position 3 et 5,5% se retrouve au niveau des groupes méthyles. L'incorporation de cette valine doublement marquée dans de la pénicilline V, purifié sous forme de sel de dibenzylamine, se fait avec une perte de 100,7% du tritium originalement présent au niveau du carbone en position 3. On a également incorporé la [^{3}H-2,3, U-^{14}C] L-valine commerciale qui contient 12% de tritium dans les groupes méthyles à la péniciline V purifiée sous forme d'ester méthylique. La dégradation en vue d'enlever le tritium du carbone en position 3 a révélé qu'il n'y en avait pas dans cette position. Par conséquent, en accord avec un résultat antérieur et d'autres études apparentées, l'hydrogène en position β de la valine n'est pas transférré sur le carbone en position 3 de la pénicilline pendant la biosynthèse de ce dernier composé.

[Traduit par le journal]

Both eukaryotic (1) and prokaryotic (2) organisms synthesize the penicillin nucleus (**1**, X = OH) from the tripeptide precursor **2**, δ-L-(α-aminoadipyl)-L-cysteinyl-D-valine (ACV). The overall process **2** → **1** includes a two-equivalent oxidation in the cysteinyl moiety, and a two-equivalent oxidation in the valinyl moiety of ACV. Although the stereochemical consequences of these processes at the β carbon atoms of cysteine and valine are known (3), their relative timing and detailed chemical mechanisms are not, despite much effort and ingenious speculation.

It has been known for some time that L-valine is a more efficient precursor of the D-valinyl moiety of penicillin than is D-valine (4), and that both enantiomers are incorporated with loss of their α-hydrogen atoms. More recently, it has been found that this loss occurs during the attachment of valine to an aminoadipylcysteine dipeptide, since some (5) or all (6) of the α-valinyl hydrogen of ACV is retained during the oxidative stages of the biosynthesis. This fact suggests (7) that the oft-considered dehydrovalinyl-containing peptide **3** (8) can no longer be regarded as a viable post-ACV intermediate in the overall process.



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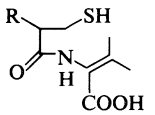

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The present work originated from some mechanistic considerations, based on literature precedents, which suggested that retention of the α-valinyl hydrogen of ACV need not be incompatible with the postulate of **3** as a post-ACV intermediate.

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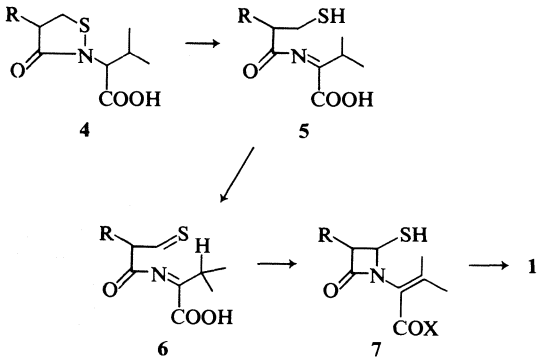
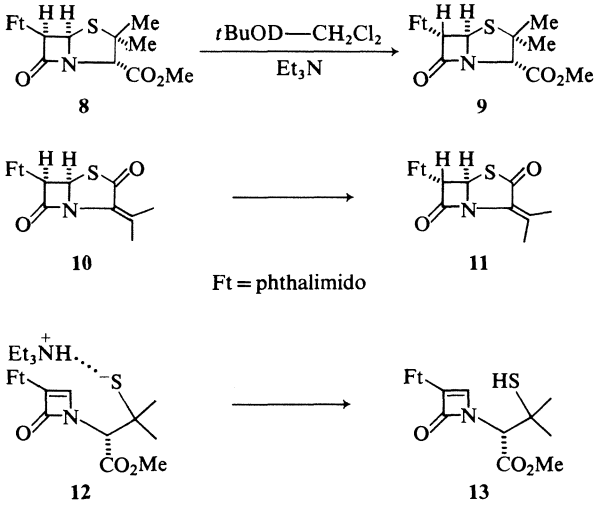
In one version of the Morin–Baldwin hypotheses (9–11), oxidation of ACV at sulfur, followed by intramolecular dehydration, leads to the isothiazolidinone **4**. A β-elimination to the acylimine **5** (12), followed by a second oxidation at sulfur, then affords **6** which, by an ene reaction, is transformed to the mercaptoazetidinone **7**, which cyclizes to penicillin. Although the reported examples of the latter reaction (13, 14) have not been confirmed (15), such cyclizations are known in model systems (16), and the reverse reaction, i.e., **1** (X = Cl, OCH_3, OCOR) → **7** (X = Cl, OCH_3, OCOR) also exists (17, 18).

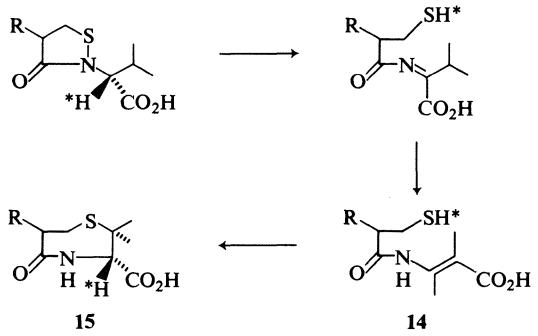


During an investigation of the C6 epimerization of the penicillin derivative **8** (19), it was observed that exposure of this compound to triethylamine in a mixture of *tert*-butanol-*d* and methylene chloride led to **9** *without incorporation of deuterium from the solvent*. Under the same conditions, the anhydropenicillin **10** was epimerized to **11**, again without incorporation of deuterium. To account for these observations, it was proposed that triethylamine promotes a β-elimination to form an ion-pair (e.g., **12**), which is transformed to the mercaptan **13**. This corresponds to an intramolecular 1 → 3 shift of hydrogen from C6 to sulfur. Ring closure by a concerted *cis*-addition of S—H to the β-face of the C5—C6 double bond would then explain the labelling results. The role of ion-pairs in base-catalyzed intramolecular 1,3-proton transfer reactions has since been examined in some detail (20). In addition, there is now good evidence for intramolecular 1,3-hydrogen shifts under enzymatic control, e.g., the C4 to C6 hydrogen transfer during the conversion of cholesterol into cholestenone (21), and the intramolecular hydrogen transfer observed during the enzymatic conversion of 3-carboxymuconolactone into 3-oxoadipic acid (22).

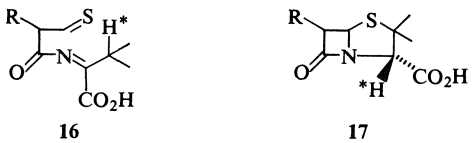
On the basis of these precedents, it was considered that the postulated conversion of **4** to **5** might proceed with transfer of hydrogen from the α-valinyl carbon to sulfur. Prototropic rearrangement



to **14**, followed by *cis*-addition to the carbon–carbon double bond would then lead to the thiazepine **15** (23) with retention of some or all of the α-valinyl hydrogen. This mechanism is consistent with the labelling results, predicts that the β-hydrogen of the valinyl moiety is lost, and provides a new route to the putative (23) intermediate **15**.

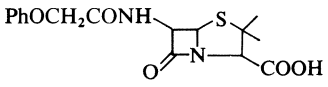


Alternatively, an intramolecular hydrogen transfer during the step **6** → **7**, followed by *cis*-addition of S—H* to the carbon–carbon double bond (*cf.* **16** → **17**) would lead to penicillin, with loss of the α-valinyl hydrogen of ACV, and a transfer of the β-valinyl hydrogen of ACV to the α-valinyl carbon atom of penicillin.



Although this latter pathway is inconsistent with the observations of refs. 5–7, it seemed desirable to reexamine the fate of the β hydrogen atom of valine during the incorporation of this amino acid into penicillin by the eukaryotic *P. chrysogenum*. An experiment of this type has been reported previous-

ly by Adriaens *et al.* (24). In the latter work, DL-[3-^{3}H]valine was prepared by racemization of L-[2,3-^{3}H]valine, and was found to contain 0.46% tritium on the methyl groups; after admixture with DL-[4,4′-^{14}C]valine to a ^{3}H/^{14}C ratio of 3.0103, the compound was incorporated into penicillin V (**18**), using *P. chrysogenum*. The latter compound exhibited a ^{3}H/^{14}C ratio of 0.05184 (average of four runs), somewhat higher than the value (0.01385) expected for complete loss of the 3-^{3}H and retention of only the 0.46% tritium on the methyl groups (25). The specific objectives of the present work were, therefore, to develop an alternative synthesis of DL-[3-^{3}H]valine, and a degradation of the labelled penicillin to locate the position(s) of any residual tritium.



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Experimental

General experimental procedures have been summarized in ref. 23. Enzymes used in the present work were purchased from Sigma Chemical Company. [2,3-^{3}H]-L-valine was supplied by Schwarzmann, Inc., Division of Mediscience, Spring Valley, NY 10977, U.S.A.

[3-^{3}H]-Dimethylpyruvic acid

A solution of dimethylpyruvic acid (1.06 g, 9.1 mmol), sodium hydroxide (600 mg, 15 mmol), and HTO (5.0 mL, specific activity 4 mCi/mmol) was stirred at room temperature for 17 h. The solution was then acidified by addition of concentrated hydrochloric acid (1.6 mL) and was continuously extracted with methylene chloride for 3 h. The organic extract was evaporated and the residual oil dissolved in methanol (40 mL) to exchange the carboxylic acid proton. This solution was evaporated and the residue was again dissolved in methanol (40 mL). After evaporation of the solvent a colourless oil (994 mg) was obtained, with specific activity 25.5 μCi/mmol.

Reductive amination of dimethylpyruvic acid (26)

A solution of dimethylpyruvic acid (106 mg, 0.9 mmol) and ammonium acetate (385 mg, 50 mmol) in methanol (20 mL) was treated with sodium cyanoborohydride (126 mg, 2.0 mmol) and stirred at 20°C for 46 h. The reaction was quenched by addition of concentrated hydrochloric acid (5.0 mL), and the solvent was removed under reduced pressure. The white, solid residue was dissolved in water (3.0 mL) and added to an ion exchange column, which had been prepared as follows: Rexyn RG50 (H+) (24 g, 100 mequiv.) was stirred with water (50 mL) and decanted. This process was repeated several times to remove small particles of resin. The slurry was then poured into a glass column and washed with 2 *N* hydrochloric acid (150 mL), followed by water until the eluate was neutral.

The solution of the product (3 mL) was added to the column. The acidic impurities were removed by a distilled water wash (500 mL), after which the product was eluted with 1 *N* ammonium hydroxide (300 mL). Evaporation of the eluate gave a light brown solid (60 mg, 50%), which gave a purple colour with ninhydrin. Analysis by paper chromatography (Whatman 3M; *n*-butanol – acetic acid – water; 27:10:33) showed a single spot, R_f 0.73, identical to that of an authentic sample of valine; ir (Nujol): 3360–1830 (s, br, NH_3^+), 2140 (s, NH_3^+), 1620 (s, br, CO_2^-) cm^{-1}; ^{3}Hmr (D_2O/D_2SO_4) δ: 0.97 (3H, d, J = 7 Hz, CH_3), 1.03 (3H, d, J = 7 Hz, CH_3), 1.80–2.52 (1H, m, C3 methine), 3.57 (1H, d, J = 5 Hz, C2 methine).

A similar experiment employing ammonium chloride (268 mg, 5.0 mmol) in place of ammonium acetate as the nitrogen source afforded only a 10% yield of valine.

A further experiment with methanol-*d* (99.5 at.%) in place of methanol gave a 50% yield of valine. Analysis of this product by ^{1}Hmr indicated that no deuterium had been incorporated into the product. This material was then converted to *N*-acetyl-D,L-valine, mp 149.5–151°C, with *p*-nitrophenyl acetate, and esterified with diazomethane to form *N*-acetyl-D,L-valine methyl ester as a colourless syrup which, on standing, crystallized slowly as large needles, mp 59–60°C; ir (film): 3400 (br, sh, NH), 3290 (s, br, NH), 1735 (s, ester C=O), 1650 (s, amide C=O), 1535 (s, amide II) cm^{-1}; ^{1}Hmr ($CDCl_3$) δ: 0.93 (3H, d, J = 6.5 Hz, CH_3), 0.95 (3H, d, J = 6.5 Hz, CH_3), 2.05 (3H, s, $COCH_3$), superimposed on 1.80–2.47 (1H, m, C3 methine), 3.74 (3H, s, CO_2CH_3), 4.55 (1H, q, J = 5, 8 Hz, C2 methine), 6.14 (1H, br d, J = 8 Hz, N*H*); ms (70 eV) *m/e* (relative intensity) taking *m/e* 114 ($M^+ - CO_2CH_3$) as the base peak: 116 (3.0), 115 (4.7) 114 (100). A sample prepared from D,L-valine (Aldrich) had: 116 (2.9), 115 (8.8), 114 (100).

[3-^{3}H]-D,L-Valine

In the same manner, the reductive amination of [3-^{3}H]-dimethylpyruvic acid (0.99 g, 8.6 mmol) (specific activity 25.5 μCi/mmol) gave [3-^{3}H]-D,L-valine (499 mg, 50%). The product was converted immediately to *N*-chloroacetyl-[3-^{3}H]-D,L-valine (27), mp 126–130°C, specific activity 27.9 μCi/mmol.

[3-^{3}H, U-^{14}C]-L-Valine

The *N*-chloroacetyl-[3-^{3}H]-D,L-valine (197 mg, 1.013 mmol), specific activity 28 μCi/mmol, was resolved, using hog kidney acylase (28) to give [3-^{3}H]-L-valine (12.19 μCi, 85.5%). A solution of [3-^{3}H, U-^{14}C]-L-valine was prepared by mixing a solution of [3-^{3}H]-L-valine (41.72 mg, 9.986 μCi) in water (9.5 mL) with a solution of [U-^{14}C]-L-valine (2.28 mg, 3.359 μCi, New England Nuclear) in water (3.0 mL). The solution showed ^{3}H/^{14}C = 2.91. Aliquots (3.0 mL) of this solution were used for the incorporation experiments.

Distribution of tritium in [2,3-^{3}H, U-^{14}C] or [3-^{3}H, U-^{14}C]-L-valine

Eighty μmoles of doubly labelled L-valine containing approximately 1 μCi ^{3}H and 0.3 μCi ^{14}C were absorbed onto Dowex 50 (H^+) at neutral pH, eluted with 2 *M* ammonium hydroxide solution, and the eluate was lyphilized. The eluate was reconstituted at pH 8.0 in 6 mL of a solution containing 500 μmol Tris-HCl and 500 μmol KCl, and shaken with L-amino acid oxidase (10 mg) and catalase (1 mg) at 37°C for 18 h. A further 80 μmol aliquot of unlabelled L-valine was added to the reaction mixture and the incubation was continued for 6 h. The pH of the final solution was adjusted to 5.0 with dilute acetic acid. The solution was then passed through Dowex 50 (H^+), and the resin was washed with four volumes of water. The combined eluate and wash were dried by lyophilization, and reconstituted with water. Lyophilization was repeated to ensure the complete removal of tritiated water. The resultant dimethylpyruvic acid was dissolved in water (5 mL) and the pH adjusted to 13 by the addition of 1 *N* ammonium hydroxide (1.5 mL), and the solution was maintained overnight in a sealed tube at 37°C. The alkaline solution was neutralized with dilute hydrochloric acid and dried by lyophilization. The exchange procedure was repeated to ensure the complete removal of tritium from the 3-position.

Production of penicillin V by Penicillium chryosogenum *ATC-C26818*

Surface spores were developed on tube slants on an agar

medium containing 0.75% glycerol, 0.75% molasses, and 0.5% Bactopeptone. Spores from a 10 day culture were suspended in distilled water and inoculated at a density of 10^4/mL into a seed medium of composition 3% corn steep liquor, 1% distillers' solubles, 2% sucrose, and 0.5% calcium carbonate adjusted to pH 6.5 before sterilization. The seed was cultured in 100 mL volume in a 500 mL Erlenmeyer flask by shaking at 250 rpm at 24°C for 72 h. Production medium consisted of 6% corn steep liquor, 6% lactose, 1.0% $CaCO_3$, 0.3% $MgSO_4 \cdot 7H_2O$, 0.5% KH_2PO_4, 0.5% soybean oil, and 0.2% phenoxyacetic acid, adjusted to pH 6.5 before sterilization. Production fermentations were carried out in 100 mL of medium in 500 mL Erlenmeyer flasks shaken at 250 rpm at 24°C for 72 h. A 10% cross volume from the third day of seed was used to inoculate the production medium. Bioassay results using *Bacillus subtilis* indicated that penicillin V was produced at a titre of 1.1 mg/mL filtrate at 72 h.

Incorporation of doubly labelled L-*valine into penicillin V*

Doubly labelled L-valine (10 mg) was dissolved in distilled water (15 mL) and sterilized by membrane filtration. Equal aliquots were added to *P. chrysogenum* growing in three flasks of production medium at 52 h and the fermentation was continued for a further 20 h, after which time the contents of the flasks were pooled, unlabelled penicillin V (1.5 g) was added, and the whole broth filtered. The pH of the filtrate was adjusted to 2.5 with dilute sulfuric acid and the mixture was then extracted with an equal volume of ethyl acetate. The organic phase was separated, washed with water, and dried over sodium sulfate. Crude penicillin V was precipitated from the ethyl acetate as the potassium salt by titration to pH 7.5 with 2 *M* potassium 2-ethylhexanoate in *n*-butanol, collected by filtration, and finally washed with ethyl acetate.

Separation of penicillin V from phenoxyacetic acid via the dibenzylamine salt

A solution of potassium penicillin V (646 mg, 1.67 mmol) and potassium phenoxyacetate (253 mg, 1.33 mmol), in water (10 mL), was stirred vigorously at room temperature, and a warm solution of dibenzylamine hydrochloride (767 mg, 3.3 mmol), in water (23 mL), was added to give a thick white paste. The mixture was cooled overnight at 5°C and then filtered, washed with ice-cold water (6 mL), and air-dried. Drying under high vacuum gave 894 mg of material. This was suspended in acetonitrile (5.0 mL) and water (8.0 mL), and it dissolved readily upon heating. The hot solution was filtered through a short stemmed, preheated filter funnel, containing a small plug of tissue paper, into a flask containing hot water (8.0 mL). The product crystallized smoothly as the solution cooled. After cooling of the mixture overnight, the product was collected to give a white microcrystalline solid (692 mg) mp 98–100°C; ir (Nujol): 3442, 3355, 3218 (s, NH), 2615, 2445 (s, NH_2^+), 1770 (s, β-lactam C=O), 1675 (s, amide C=O), 1628 (s, CO_2^-), 1576 (s, amide II and NH_2^+) cm^{-1}; ^{1}Hmr ($CDCl_3$) δ: 1.56 (3H, s CH_3), 1.46 (3H, s, CH_3), 3.83 (4H, s, $PhCH_2$), 4.49 (2H, s, $PhOCH_2$), 4.23 (1H, s, C3 methine), 5.36 (1H, d, J = 4 Hz, C5 methine), 5.56 (1H, q, J = 4, 8 Hz, collapses to a doublet, J = 4 Hz on exchange of NH with D_2O, C6 methine), 5.99–6.39 (3H, exch D_2O, N*H* and NH_2^+), 7.28 (10H, s, Ph) overlapping with 6.76–7.49 (5H, m, PhO). *Anal.* calcd. for $C_{30}H_{33}N_3O_5S$: C 65.79, H 6.07, N 7.67, S 5.85; found: C 65.78, H 6.04, N 7.51, S 5.70.

*Isolation of [^{3}H, ^{14}C]-penicillin V dibenzylamine salt from the crude penicillin isolate obtained after incubation of [3-^{3}H, U-^{14}C]-*L-*valine*

In a similar manner, the crude penicillin V isolate (824 mg) from the incubation of [3-^{3}H, U-^{14}C]-L-valine (^{3}H/^{14}C 2.91) with *Penicillium chrysogenum* was dissolved in water (9.0 mL) and treated with a solution of dibenzylamine hydrochloride (700 mg) in hot water (24.0 mL). The precipitated [^{3}H, ^{14}C]-penicillin V dibenzylamine salt was collected and dried under high vacuum, mp 91–95°C; specific activity: ^{3}H, 0.0043; ^{14}C, 0.031 μCi/mmol. Recrystallization from aqueous acetonitrile gave a fluffy white microcrystalline solid, mp 97–100°C; specific activity: ^{3}H, 0.0052; ^{14}C, 0.0355 μCi/mmol (^{3}H/^{14}C 0.146, 5.03% retention of tritium).

A second penicillin V isolate (751 mg) was also treated with dibenzylamine hydrochloride (640 mg) in water. The precipitated salt was recrystallized twice from aqueous acetonitrile to give a fluffy white microcrystalline solid, mp 98.5–100.5°C (mixture mp with an authentic sample, 98–100°C). Specific activity: ^{3}H, 0.0045; ^{14}C, 0.033 μCi/mmol (^{3}H/^{14}C 0.136, 4.7% retention of tritium).

*Isolation of [^{3}H, ^{14}C]-penicillin V methyl ester from the incubation of [2,3-^{3}H, U-^{14}C]-*L-*valine with* Penicillium chrysogenum

The crude penicillin V isolate (401.5 mg) was dissolved in water (4.0 mL), and the solution was cooled to 0°C. The pH was adjusted to 2.0 with 6 *N* hydrochloric acid and the product was extracted into ethyl acetate (4 × 10 mL). The combined extracts were dried and evaporated to leave a pale yellow gum (340 mg). The product was dissolved in absolute ether (20 mL and esterified at 0°C with alcohol-free diazomethane. The excess diazomethane was destroyed by the dropwise addition of acetic acid and the solvent was evaporated. The residue was dissolved in methylene chloride (20 mL) and washed with 7% sodium bicarbonate (10 mL). The organic layer was dried and evaporated to leave a golden yellow syrup (363 mg). Chromatography on silica gel (7.0 g) (methylene chloride – ethyl acetate, 19:1) separated methyl phenoxyacetate from the penicillin V methyl ester, which was isolated as two samples; the first, 195.5 mg, was homogenous on tlc, R_f 0.58 (methylene chloride – ethyl acetate, 19:1) and identical by ^{1}Hmr with an authentic sample. Specific activity: ^{3}H, 0.011; ^{14}C, 0.023 μCi/mmol; ^{3}H/^{14}C 0.46.

Rearrangement of methyl 6-phenoxyacetamido-[^{3}H, ^{14}C]-penicillanate-1β-oxide (29)

Methyl 6-phenoxyacetamidopenicillanate-1β-oxide (118 mg, 0.311 mmol, specific activity: ^{3}H, 0.011 μCi/mmol; ^{3}H/^{14}C 0.46) was reacted with trimethyl phosphite (40 mg, 0.32 mmol) in benzene (10 mL). The crude product was purified by column chromatography on 5 g silica gel (methylene chloride – ethyl acetate, 1:1) to give 56 mg of material that was homogenous on tlc (R_f 0.55), and identical by ^{1}Hmr with an authentic sample (29). Specific activity: ^{3}H, 0.008; ^{14}C, 0.027 μCi/mmol; ^{3}H/^{14}C 0.3.

Isomerization of the β,γ-isomer (29)

A solution of the β,γ-isomer (173 mg, 0.5 mmol) in methanol (2.5 mL) was stirred with triethylamine (0.07 mL, 51 mg, 1 equiv.) at room temperature for 20 min. The solvent was then evaporated and the residue dissolved in methylene chloride (15 mL) and reevaporated. Repetition of this process with methylene chloride (15 mL) gave a syrup which was dried under high vacuum (174 mg); ir ($CDCl_3$): 1769 (s, β-lactam C=O), 1720 (s, ester C=O) cm^{-1}; ^{1}Hmr ($CDCl_3$) δ: 1.77 (3H, s, CH_3), 2.23 (3H, s, CH_3), 3.74 (3H, s, CO_2CH_3), 4.95 (2H, s, $PhOCH_2$), 5.83 (1H, d, J = 4 Hz, β-lactam *H*), 6.03 (1H, d, J = 4 Hz, β-lactam *H*), 6.77–7.43 (5H, m, Ar*H*).

*Isomerization in methanol-*d

A similar experiment using methanol-*d* (99.5 at.%) in place of methanol gave the α,β-isomer containing one atom of deuterium by nmr integration; ^{1}Hmr ($CDCl_3$) δ: 1.77 (2.8H, s, vinyl CH_3), 2.23 (2.2H, s, vinyl CH_3).

Isomerization of the β,γ-isomer at high dilution

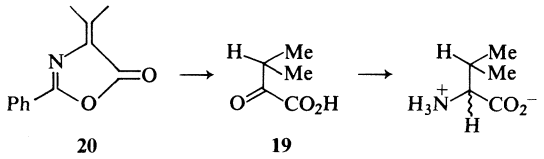
Triethylamine (0.70 mL) was dissolved in redistilled methanol and the solution was diluted to 50.0 mL in a volumetric flask. An aliquot of this solution (5.0 mL, 0.5 mmol of triethylamine) was added to a solution of the β,γ-isomer (17.3 mg, 0.05 mmol) in redistilled methanol (345 mL). After 5 h the solvent was evaporated and the residue dissolved in methylene chloride (15 mL), filtered, and evaporated to leave a pale yellow oil (17 mg) which was identical (^{1}Hmr) to material prepared previously.

Isomerization of the [^{3}H, ^{14}C]-β,γ-isomer

In the same way, the [^{3}H,^{14}C]-β,γ-isomer (27.0 mg, 0.078 mmol), was isomerized using triethylamine (10 equiv.) in redistilled methanol (500 mL). The product (27.0 mg) was purified on silica gel (1.0 g), (methylene chloride – ethyl acetate, 19:1) to give 19.0 mg of the α,β-isomer which was identical (^{1}Hmr, tlc) to material prepared previously; specific activity: ^{3}H, 0.007; ^{14}C, 0.026 μCi/mmol; (^{3}H/^{14}C 0.27).

Results and discussion

Dimethylpyruvic acid **19** was prepared by hydrolysis (30) of 2-phenyl-4-isopropylidene-5-oxazolone **20** (31), and was converted to valine in 50% yield using ammonium acetate as the nitrogen source in the reductive amination. Interestingly, the yield of valine decreased to only 10% when ammonium chloride was employed in place of ammonium acetate.



20 **19**

Exchange of **19** with 1 *M* NaOD–D_2O was complete within 5 min at room temperature. Because of the facility of this exchange, it was necessary to check for loss of isotope to the solvent during the reductive amination step. Therefore, **19** was converted to D,L-valine with cyanoborohydride and ammonium acetate in methanol-*d*. The resulting product showed no incorporation of deuterium by ^{1}Hmr and mass spectral examination of the derived *N*-acetyl methyl ester (see Experimental).

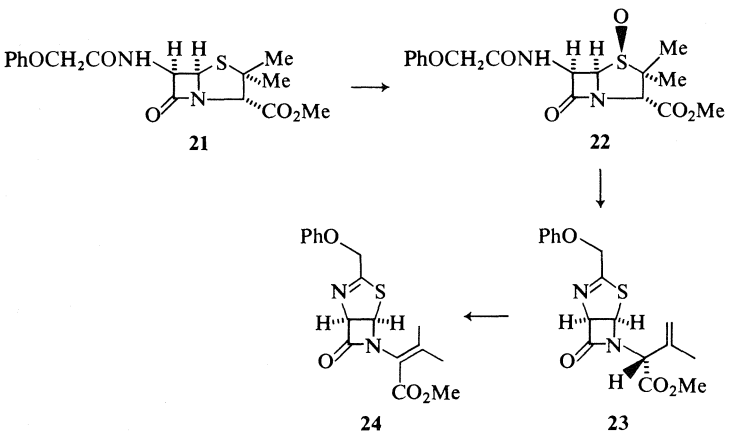
Tritiation of **19** was performed in 1 *M* NaOH–HTO and the resulting **19**-*t* was converted to [3-^{3}H]-D,L-valine in 49% yield. The *N*-chloroacetyl derivative of this compound, specific activity 28.0 μCi/mmol, was resolved, using hog kidney acylase, and the product was mixed with [U-^{14}C]-L-valine, ^{3}H/^{14}C = 2.91. The distribution of tritium in this compound was then determined. Incubation with L-amino acid oxidase afforded [3-^{3}H,U-^{14}C]-dimethylpyruvic acid, which was exposed to 1 *M* ammonium hydroxide. Reacidification, followed by repetition of the exchange procedure, afforded doubly labelled **19**, ^{3}H/^{14}C = 0.161. These results indicated that, in the doubly labelled L-valine, 94.5% of the tritium was attached to C3, and 5.5% was located in the methyl groups. The presence of tritium in the methyl groups was unexpected, and is indicative of homoenolization (32) during the exchange of **19** with NaOH in a tritiated medium.

Incorporation of the doubly labelled L-valine into penicillin V was achieved using *P. chrysogenum* in a medium supplemented with phenoxyacetic acid. The crude penicillin was isolated as the potassium salt by filtration of the broth, acidification to pH 2.5, extraction with ethyl acetate, and treatment with potassium 2-ethylhexanoate. Three experiments were performed, with an average incorporation of L-valine of 5.5%, based on recovered ^{14}C. These crude potassium penicillin V isolates exhibited an average ^{3}H/^{14}C ratio of 0.407, and suggested retention of 14% of the tritium originally present in the valine, corresponding to ca. 8.5% of the tritium originally located at C3 of the precursor.

Since ^{1}Hmr examination of unlabelled crude potassium penicillin V isolates indicated some contamination with potassium phenoxyacetate, separation and purification of the penicillin was necessary. This was accomplished by treatment of the mixture, in water, with a hot aqueous solution of dibenzylamine hydrochloride. The dibenzylamine salt of penicillin V precipitated virtually quantitatively, and could be recrystallized to analytical purity from aqueous acetonitrile. Similar treatment of the labelled material yielded pure dibenzylamine salt of penicillin V having an average ^{3}H/^{14}C ratio of 0.136, corresponding to 100.7% loss of the tritium originally present at C3 of the valine.

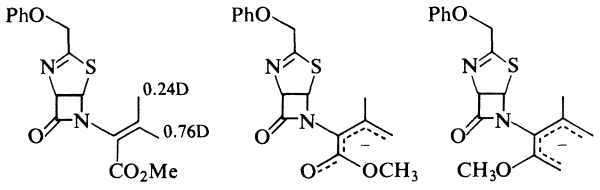
A further set of experiments was performed using commercially available [2,3-^{3}H]-L-valine. Degradation of this material, using L-amino acid oxidase as described above, revealed 12% tritium in the methyl groups. The compound was mixed with [U-^{14}C]-L-valine (^{3}H/^{14}C = 5.1) and incorporated into Penicillin V as before. Three experiments were performed. In this case, the crude potassium salts were acidified to pH 2, extracted into ethyl acetate, and treated with alcohol-free diazomethane. The pure methyl ester of penicillin V (^{3}H/^{14}C = 0.46) was isolated by column chromatography. Although this radioactivity corresponds to ca. 103% loss of tritium from the C3 position of the valine precursor, the methyl ester (**21**) was degraded to the thiazoline **24** via the known sequence **21** → **22** → **23** → **24** (29), to allow the removal of the hydrogen attached to C3.

To validate this degradative sequence, it was necessary to demonstrate that the rearrangement **23** → **24** proceeds in an intermolecular manner, with incorporation of hydrogen from the solvent.



Therefore, **23** was converted to **24**-*d* with triethylamine in methanol-*d*. The product was found, by ^{1}Hmr, to contain one deuterium atom, distributed unequally between the two methyl groups, as indicated in **25**. The assignments of the chemical shifts of these methyl groups are based on literature precedents (33). These observations indicate that the conversion of **23** to **24** in methanol solvent is intermolecular, and proceeds via a carbanionic intermediate which protonates most readily in the sickle (**26**) or U (**27**) conformations (34).

In the radioactive series, the conversion of **23** to **24** was performed with triethylamine in a large excess of methanol. The ^{3}H/^{14}C ratios were found to be: **21**, 0.46; **23**, 0.31; **24**, 0.27. This corresponds to loss of 23% tritium in the sequence **21** → **22** → **23**, slightly higher than the expected 17% and, within the experimental error, no further loss of tritium in the formation of **24**.



The two sets of experiments confirm the conclusions of Adriaens *et al.* (24), and provide no support for our hypothesis that the β-hydrogen of valine is transferred to C3 of penicillin during the biosynthesis of the latter compound. As has been reported elsewhere (35), the thiazepine **15** (R = δ-L-α-aminoadipyl) (23) is not converted to penicillin by a cell-free preparation of *C. acremonium*.

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