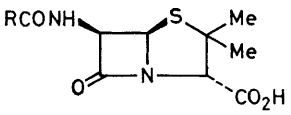
Partial Elimination of Valine-oxygen During Penicillin Biosynthesis

By JAMES S. DELDERFIELD, ELI MTETWA, ROBERT THOMAS,* and THEMBA E. TYOBEKA
(*Chemistry Department, University of Surrey, Guildford, Surrey* GU2 5SX)

Summary G.c.–m.s. analysis of the methyl ester of [^{18}O]-penicillin V obtained on incubating *Penicillium chrysogenum* mycelium with L-[$^{18}O_2$]valine demonstrated the elimination of one of the two carboxy-oxygen atoms.

PENICILLIN biosynthesis has been shown to involve the utilisation of L-valine *via* a pathway requiring retention of the intact C_5-skeleton, together with the amino-nitrogen and all six methyl hydrogen atoms.[1] Of the eight carbon–hydrogens, only those present at the α- and β-carbons are eliminated, the former[2] during incorporation of the L-amino-acid into the intermediate tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) and the latter as a pre-requisite of the subsequent oxidative cyclisation step leading to formation of the penam S–C(2) bond.

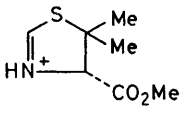
The fate of the two valine oxygen atoms remains to be determined and is of interest in view of possible alternative mechanisms for the incorporation of this carboxy-terminal amino-acid constituent of the tripeptide. Feeding studies with L-[$^{18}O_2$]valine in conjunction with gas chromatography and mass spectrometric analysis (g.c.–m.s.) of methyl esters of the acidic metabolites have now shown that the carboxy-group of valine is incorporated with the elimination of one of its oxygen substituents.



(1)

a; R = PhO·CH$_2$—

b; R = H$_2$NCH[CH$_2$]$_3$—
CO$_2$H



m/z 174

(2)

By means of a simple biosynthetic system using re-suspended mycelium as described in a previous study,[3] [1-^{14}C]valine was readily incorporated into penicillin V (**1a**) on incubating washed mycelium from a 72 h culture of *Penicillum chrysogenum* at pH 6·5 in 0·05 M phosphate buffer (2 g/6 ml) containing 0·1% phenoxyacetic acid for 20 h at 24 °C. Extraction with ethyl acetate at pH 2 and methylation with CH_2N_2 yielded a mixture of ^{14}C-labelled esters which was examined autoradiographically following silica t.l.c. (ethyl acetate: light petroleum = 2:3). The predominant radioactive component (R_f 0·5) corresponded to the methyl ester of penicillin V, the presence of which was confirmed by g.c.–m.s. analysis and assayed relative to a penicillin G methyl ester standard, using a 2·6 m 1·0% Dexsil 300 column heated from 80 °C to 240 °C at 5 °C/min (retention times of methyl esters of penicillins G and V were 42·0 and 42·6 min, respectively). On repeating the above incubation in the presence of predominantly doubly labelled L-[$^{18}O_2$]valine (Prochem Ltd., *ca.* 80·5 atom %) fed at 17·0 mg/6 ml, the resulting penicillin V samples both showed retention of only one ^{18}O-atom.

Direct m.s. examination of the [^{18}O]valine precursor yielded three isotopically-labelled fragment ions [NH_2:$CH.CO_2H$]$^+$ at *m/z* 74, 76, and 78 of relative intensities 2:30:100, consistent with a mixture of *ca.* 3 molecules of doubly ^{18}O-labelled valine to each singly labelled molecule. These ratios were unchanged in valine recovered from a control incubation (24 h) of the labelled precursor in pH 6·5 buffer; furthermore, only minor changes in the relative ^{18}O-abundancies were observed on conversion of this recovered valine into its *N*-benzyloxycarbonyl methyl ester in which esterification was effected with CH_2N_2 (M^+ 265:267:269 = 5:37:100).

The loss of a single carboxy-oxygen atom in the course of the incorporation of valine into penicillin V was evident on examination of the ^{18}O-content of the methoxycarbonyl group of the resulting labelled ester. Thus, relative intensities of the $^{16}O_2$-, $^{16}O^{18}O$-, and $^{18}O_2$-containing ions at M^+ 364, 366, and 368, respectively, (100:31:3) showed considerable enhancement compared with the ratios of the corresponding ions observed in the mass spectrum of unlabelled penicillin V methyl ester (100:7:0). The apparent absence of doubly ^{18}O-labelled ions relative to the singly labelled species contrasts with the corresponding 3:1 ratio present in the parent valine. Specific labelling of the methoxycarbonyl substituent was evident from the similar ratios of the fragment ions (**2**) at *m/z* 174, 176, and 178 (100:30:2) relative to the predominant methoxycarbonyl-deficient ions [(**2**) — CO_2Me]$^+$ seen at *m/z* 114, 116, and 118 (100:11:2), the marginal enrichment possibly representing a contribution from the minor isobaric ^{18}O-containing fragment (Me_2C:$CHCO_2Me$)$^+$.

While this finding does not exclude the possible indirect partial elimination of valine-carboxy-oxygen through covalently bound intermediates formed by enzymes not directly involved in penicillin biosynthesis, the most likely interpretation would appear to be that the mechanism of incorporation of valine into the penicillanic acid-carboxy-group requires the elimination of one of its oxygen substituents. At present the only established intermediate between the constituent amino-acid precursors and the fully formed penam, isopenicillin N (**1b**),[4] is the LLD-ACV tripeptide and the elimination of a valine-oxygen atom could take place either in the course of biosynthesis of this tripeptide or its subsequent oxidative cyclisation to the penam nucleus.

The ACV-tripeptide is structurally analogous to glutathione (γ-L-glutamyl-L-cysteinylglycine) the formation of which, *via* the glutathione cycle,[5] does not appear to require activation of the glycine-carboxy-group *via* a covalently linked acyl derivative. A glutathione-type biosynthetic pathway for the incorporation of valine into LLD-ACV would therefore be expected to lead to the retention of both carboxy-oxygen atoms. This is in contrast with the known mechanism of amino-acid incorporation into polypeptides involving either aminoacyl transfer RNA or alternatively the more recently reported non-ribosomal pathway leading to peptide antibiotics such as gramicidin S *via* a thioester multi-enzyme complex analogous to the normal fatty acid synthetase system.[6] The non-ribosomal thioester mechanism which is known to effect the conversion of L-phenylalanine into the corresponding D-amino-acid residue in gramicidin S could similarly account for the observed formation of the D-valine moiety of the LLD-ACV tripeptide from L-valine, which apparently takes place without intermediate formation of the LLL-epimer.[4] Possible different mechanisms of formation of glutathione and the ACV tripeptide are indicated by studies with fractionated enzymes of *Cephalosporium spp.*, in which the former tripeptide was formed from its constituent amino-acids by supernatant enzymes, whereas ACV was formed by enzymes of the particulate fraction for which the AC dipeptide was apparently an obligatory precursor.[7]

If, however, both valine-carboxy-oxygens are retained in the biosynthesis of the tripeptide, the loss of one of its carboxy-oxygens could occur during the subsequent cyclisation to isopenicillin N or any earlier penam intermediate.

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