Conversion of $^{17}O/^{18}O$-Labelled δ-(L-α-Aminoadipyl)–L-cysteinyl–D-valine into $^{17}O/^{18}O$-Labelled Isopenicillin N in a Cell-free Extract of *C. acremonium*

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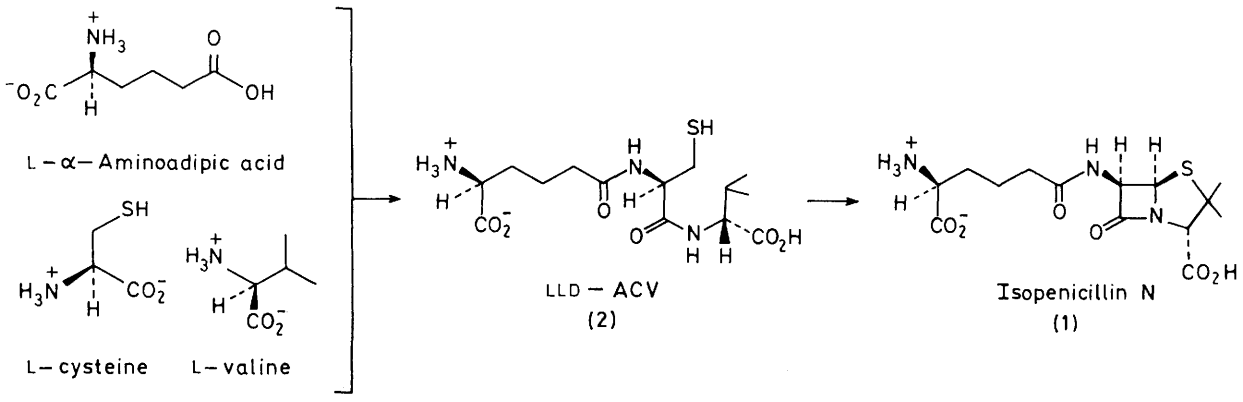
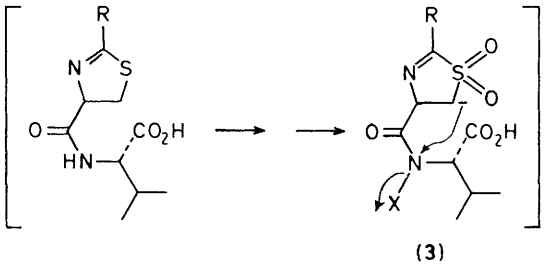
δ-(L-α-Amino[1,1,6-$^{17}O/^{18}O$]-adipyl)–L-cysteinyl–D-valine was converted into isopenicillin N in cell-free extracts of *Cephalosporium acremonium* with *no* loss of $^{17}O/^{18}O$ label as shown by ^{17}O n.m.r. spectroscopy and mass spectrometry; incubation of unlabelled tripeptide in a cell-free system containing $^{17}O/^{18}O$-enriched water produced isopenicillin N with *no* incorporation of $^{17}O/^{18}O$.

The biosynthesis of isopenicillin N (**1**) from L-valine, L-cysteine, and L-α-aminoadipic acid proceeds *via* the tripeptide, δ-(L-α-aminoadipyl)–L-cysteinyl–D-valine [LLD-ACV, (**2**)].[1] A number of proposed mechanisms for the formation of isopenicillin N involved intermediate thiazolines, orthothioamides, and related dehydration products of LLD-ACV.

Scott and co-workers,[2] for example, have investigated thiazoline-sulphones [*e.g.* (**3**)] as potential intermediates in





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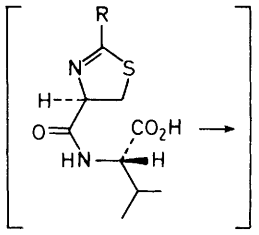

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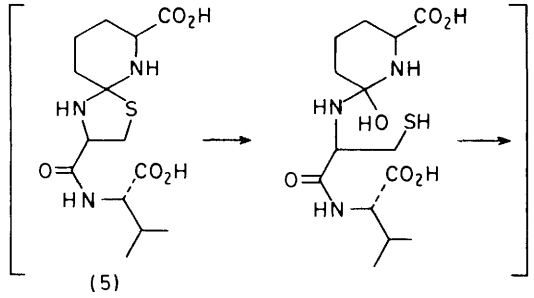


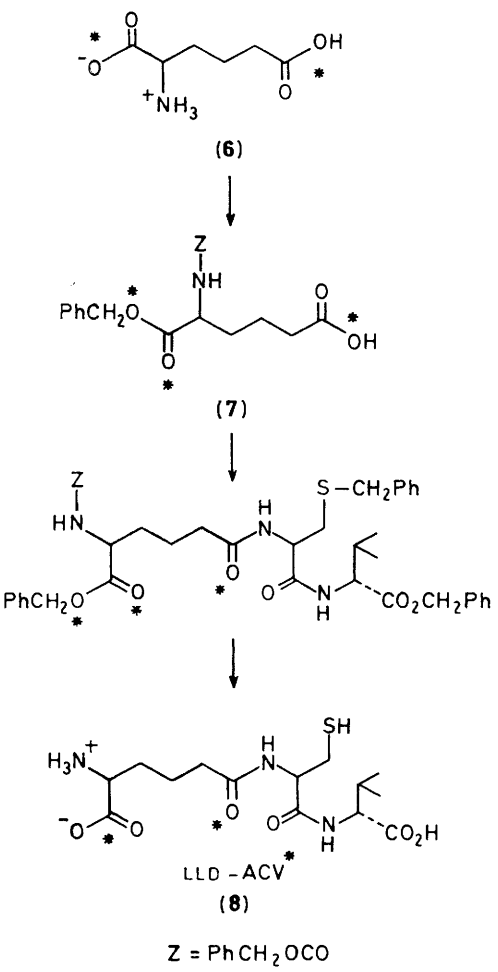
(5)

biomimetic syntheses of β-lactam antibiotics *via* peptide hydroxamate derivatives. Cooper[3] has advanced a scheme involving thiazoline intermediates [*e.g.* (**4**)] to provide structural rigidity during the cyclisation to give the azetidinone ring of the penam nucleus and the feasibility of this approach for 'biogenetic-type' syntheses of penam and cepham ring systems has been demonstrated by Kishi and co-workers.[4] Following the isolation of 6-oxo-piperidine-2-carboxylic acid from the fermentation broth of *Penicillium chrysogenum*, Morin and co-workers[5] have proposed a cyclic orthothioamide (**5**) as a biogenetic precursor of isopenicillin N.

In continuation of our studies into the mechanism of penicillin biosynthesis, we have investigated the possibility of dehydration–hydration steps in the conversion of LLD-ACV into isopenicillin N, by conducting the biosynthesis with $^{17}O/^{18}O$ isotopically labelled precursors and also by incubating unlabelled LLD-ACV in a $^{17}O/^{18}O$ isotopically enriched medium.

$^{17}O/^{18}O$-Enriched *N*-benzyloxycarbonyl-L-α-aminoadipic acid α-benzyl ester (**7**) was synthesised[6] from isotopically enriched L-α-aminoadipic acid (**6**).† The ^{17}O n.m.r. spectrum of (**7**) (Figure 1) indicated isotopic enrichment at both α- and δ-sites (*ca.* 30% ^{17}O at each site). The acid (**7**) was coupled[7] with *S*-benzyl-L-cysteinyl-D-valine benzyl ester and the tripeptide was deprotected (Na–liquid NH_3) to give LLD-ACV* (**8**) with $^{17}O/^{18}O$ labels in both the carboxy and amide groups of the L-α-aminoadipyl fragment.

The distribution of the protonated molecular ions (MH^+) in the ammonia chemical ionisation (C.I.) mass spectrum of



(6)

(7)

(8)

Z = $PhCH_2OCO$

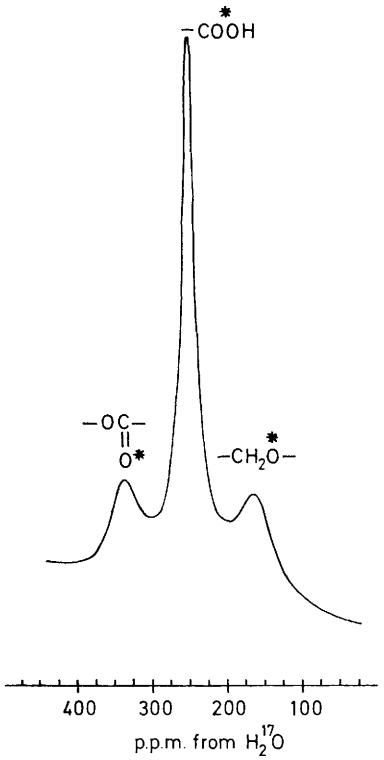
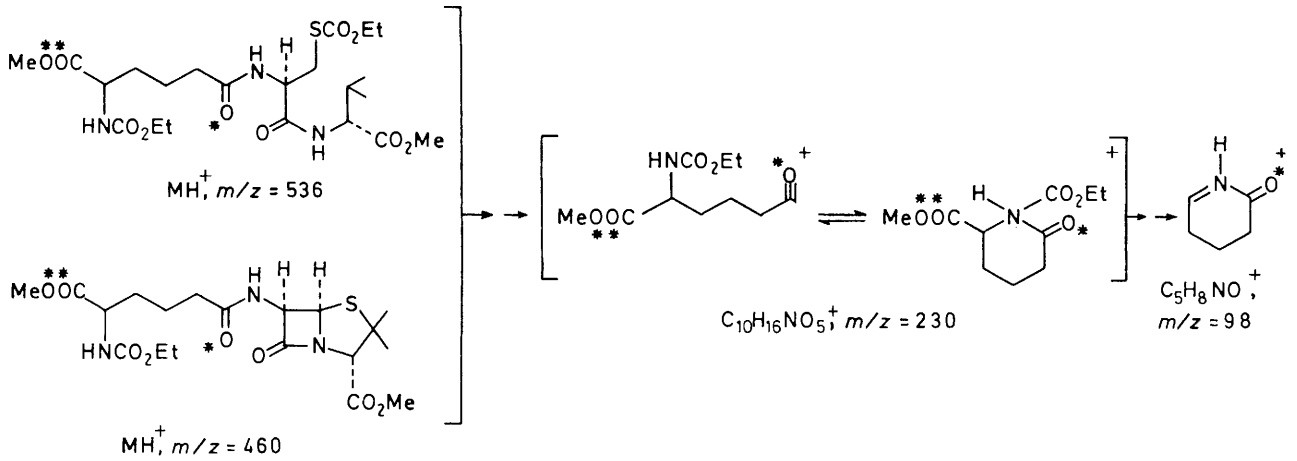


Figure 1. ^{17}O N.m.r. spectrum of (**7**) (40.7 MHz, $CDCl_3$ solvent 20 °C) referenced to internal MeOH = δ −38 p.p.m.

† L-α-Aminoadipic acid was dissolved in hydrochloric acid (3 M in $H_2{}^{16}O:H_2{}^{17}O:H_2{}^{18}O$ *ca.* 10:50:40) and allowed to stand at 20 °C for 48 h. The aqueous solution was neutralised (Na_2CO_3) and used directly without further purification.



derivatised‡§ LLD-ACV* (**8**) was consistent with $^{17}O/^{18}O$ enrichment at three sites in the tripeptide (MH^+, m/z = 536; the ratio 536 : 537 : 538 : 539 : 540 : 541 : 542 = 27 : 65 : 100 : 96 : 68 : 35 : 15). The distribution of oxygen isotopes in the fragment ions ($C_{10}H_{16}NO_5^+$, $C_5H_8NO^+$) verified the location of $^{17}O/^{18}O$ enrichment *only* in the adipyl amide bond (*ca.* 51% ^{16}O, 28% ^{17}O, 21% ^{18}O) and the two carboxy-oxygen sites in the adipyl carboxy-group (*ca.* 42% ^{16}O, 33% ^{17}O, 25% ^{18}O). Incubation¶ of LLD-ACV* (**8**) with a cell-free extract of *C. acremonium*[8] produced isopenicillin N quantitatively (by ^1H n.m.r. spectroscopy[9] and bioassay[8]) with *no* loss of label from carbonyl or carboxy-sites (by ^{17}O n.m.r. spectroscopy). The isopenicillin N produced was derivatised‡ and isolated from the incubation mixture and the protonated molecular ion in the C.I. mass spectrum of the derivative showed an identical isotope distribution (MH^+, m/z = 460; the ratio 460 : 461 : 462 : 463 : 464 : 465 : 466 = 28 : 67 : 100 : 99 : 71 : 34 : 13) to that of the labelled precursor. The $^{17}O/^{18}O$ isotope distribution in the fragment ions ($C_{10}H_{16}NO_5^+$, $C_5H_8NO^+$) confirmed that the isotopic labels were in the L-α-aminoadipyl fragment in the same location and concentration as in the labelled precursor.

In a complementary experiment, unlabelled LLD-ACV was incubated with a cell-free extract of *C. acremonium* in $^{17}O/^{18}O$-enriched water ($H_2{}^{16}O : H_2{}^{17}O : H_2{}^{18}O$ *ca.* 10 : 50 : 40). The conversion into isopenicillin N was quantitative and the product showed *no* detectable label incorporation (by ^{17}O n.m.r. spectroscopy). The C.I. mass spectrum of the derivatised‡ product was identical to that of authentic unlabelled material (MH^+, m/z = 460; the ratio 460 : 461 : 462 = 100 : 26 : 12).

Thomas and co-workers[10] have reported the loss of one oxygen atom from a labelled L-valine precursor in the biosynthesis of penicillin V by intact mycelia of *Penicillium chrysogenum*. To be consistent with our findings, this oxygen atom must be lost during the biosynthesis of LLD-ACV from its constituent amino-acids or during the side chain transacylation step.

During the biosynthesis of isopenicillin N from LLD-ACV the fact that no water is incorporated from an isotopically enriched medium and no oxygen atoms are lost from a $^{17}O/^{18}O$-labelled precursor precludes any mechanism involving a dehydration–hydration step. The formation of intramolecular thiazolines, orthothioamides, *etc.*, as intermediates is clearly inconsistent with our experimental results; *i.e.* none of the proposed mechanisms cited above[2–5] is viable. Similarly, the possible formation of any intermolecular, covalently linked intermediates (*e.g.* enzyme-bound thioesters, esters, amidines) involving any of the oxygen sites of LLD-ACV is also excluded.**

Penicillin biosynthesis can involve the oxygen sites of LLD-ACV *only* in non-covalent interactions (*e.g.* hydrogen bonding). The possibility remains that the SH or NH groups of LLD-ACV may play an important role in binding the substrate to the active site of the enzyme during the cyclisation to isopenicillin N.

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‡ Peptides and penicillins were routinely derivatised for mass spectroscopy as their -*N*,*S*-ethoxycarbonyl,-carboxymethyl derivatives; see P. B. Loder and E. P. Abraham, *Biochem. J.*, 1971, **123**, 471.

§ Full details of mass spectra will be published elsewhere.

¶ Incubation of 0.03 mmol of substrate in a total volume of 5 ml of cell-free extract shaken at 27 °C for 60 min.

** Covalent linkages between the enzyme and ACV peptide which could be formed and broken without oxygen exchange are still possible, *e.g.* mixed anhydrides.