Direct N.m.r. Observation of Cell-free Conversion of (L-α-Amino-δ-adipyl)-L-cysteinyl-D-valine† into Isopenicillin N

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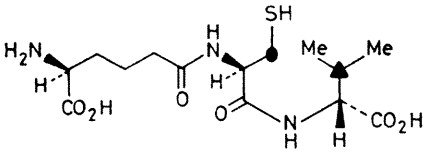
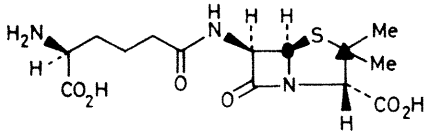
Summary Carbon-13 n.m.r. spectroscopy has been used to observe the efficient conversion of (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine into isopenicillin N in cell-free extracts of *Cephalosporium acremonium*.

† α-Amino-δ-adipyl = 5-amino-5-carboxypentanoyl.

RECENT investigations with *Cephalosporium acremonium* by O'Sullivan *et al* [1] and Konomi *et al* [2] into the cell-free conversion of (L-α amino-δ-adipyl)-L-cysteinyl-D-valine (LLD-ACV) (**1a**) into the corresponding penicillins have shown that the first-formed penicillin is isopenicillin N (**2a**) Furthermore, the results of O'Sullivan *et al* [1] were consistent with the hypothesis that the carbon skeleton of the LLD-ACV tripeptide was incorporated intact into the isopenicillin N molecule We now report the direct observation of this conversion by ^{13}C n m r spectroscopy, which entirely confirms the previous work



(1)



(2)

a ● = ^{12}C, ▲ = ^{12}C
b ● = ^{13}C, ▲ = ^{12}C
c ● = ^{13}C ▲ = ^{13}C

When labelled tripeptide, (L-α-amino-δ-adipyl)-L-[3-^{13}C]-cysteinyl-D-valine[3] (**1b**) was incubated with cell-free extracts[4] of *C acremonium* at 5 °C in the probe of an n m r spectrometer (Bruker WH 300, 25 mm n m r tube), the intensity of the ^{13}C n m r signal‡ at 26 7 p p m due to the labelled atom of the tripeptide decreased with time, whilst a new signal appeared at 67 7 p p m and showed a corresponding increase in intensity with time The latter signal appeared as a doublet in the off-resonance proton-decoupled spectrum and was ascribed to C-5 of the isopenicillin N (**2b**) A plot of the change in peak height of this signal *versus* time at 5 °C, and subsequently 9 °C, is shown in Figure 1B Bioassay[4] of samples of the incubation mixture showed a similar increase in β-lactam antibiotic activity with time (Figure 1A)

The yield of the conversion was high after completion of the incubation the height of the ^{13}C n m r signal due to labelled substrate (**1b**) was less than 20% of that due to the product (**2b**) It was not possible under these conditions to calculate molar proportions from the signal intensities but bioassay of the final mixture indicated that the conversion was 80 ± 8%

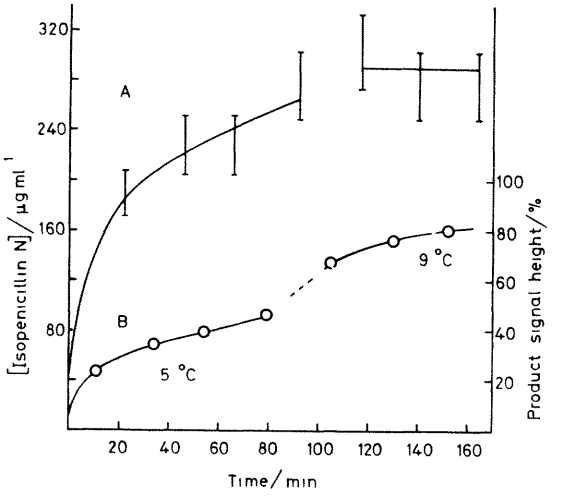


FIGURE 1 Incubation of a mixture of the LLD-ACV isotopomer (**1b**) with cell free extract of *C acremonium* at a probe temperature of 5 °C and subsequently 9 °C Curve A Level of antibacterial activity of samples removed from the incubation mixture as determined by a hole plate assay[4] against *Staphylococcus aureus* Error bars represent a reading of the assay plate to ± 0 5 mm Curve B Increase in the height of the ^{13}C n m r signal at 67 7 p p m due to C 5 of isopenicillin N (**2b**) The signal height is expressed as a percentage of the combined signal heights of reactant and product The change in bioactivity with time (Curve A) shows that the change in n m r signal height is proportional to concentration change of isopenicillin N

Entirely consistent results were obtained in an analogous experiment using the doubly labelled tripeptide, (L-α-amino-δ-adipyl)-L-[3-^{13}C]cysteinyl-D-[3-^{13}C]valine[3] (**1c**) During incubation the ^{13}C n m r signals at 26 7 and 31 8 p p m of the labelled atoms of the substrate (**1c**) showed a uniform decrease in intensity whilst two new signals which appeared at 67 7 and 65 6 p p m showed a corresponding increase in intensity with time (see, for example, Figure 2) The signals at 67 7 and 65 6 p p m were assigned§ to C-5 and C-2, respectively, of the isopenicillin *N* isotopomer (**2c**) Off-resonance proton-decoupled ^{13}C n m r spectra of the final incubation mixture, after removal of the protein,¶ confirmed these assignments the signal at 67·7 p p m occurred as a doublet, whilst that at 65 6 p p m was a singlet Geminal ^{13}C–^{13}C coupling between C-2 and C-5 of (**2c**) was not observed under conditions where the width at half-height of the signals was 2·5 Hz

‡ Chemical shifts are given in p p m downfield from Me_4Si and were measured relative to the high field signal of the buffer, sodium 3-(*N*-morpholino)propanesulphonate, which had been calibrated relative to dioxan (δ 67 4 p p m)

§ The chemical shifts of C-2 and C-5 of penicillin N are given as δ 65 1 and 67 4 p p m , respectively, in ref 5

¶ An aqueous extract of the separated protein did not show bioactivity and gave negligible signal intensity in the ^{13}C n m.r spectrum recorded under conditions similar to those of all other spectra

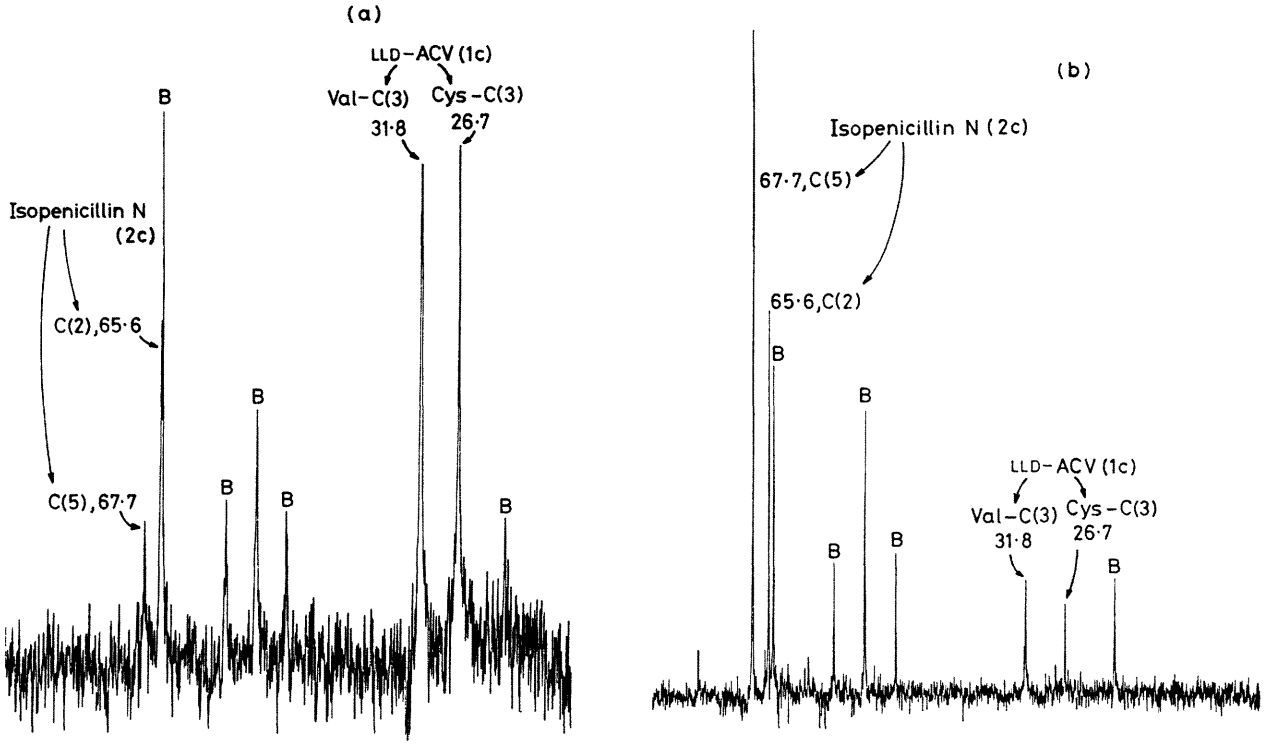


FIGURE 2. 75·47 MHz Broad-band proton-decoupled ^{13}C n.m.r. spectra (25 mm tube) of a mixture of cell-free extract and (L-α-amino-δ-adipyl)-L-[3-^{13}C]cysteinyl-D-[3-^{13}C]valine (**1c**) (initial concentration 1mM): (a) after incubation at 5 °C for 25 min; (b) after incubation for a further 65 min, precipitation of the protein with acetone, lyophilisation of the supernatant, and resuspension in D_2O. The incubation mixture also contained dithiothreitol (2mM), L-ascorbic acid (1mM), and $FeSO_4$ (0·05mM) in sodium 3-(*N*-morpholino)-propanesulphonate buffer at pH 7·2 (10mM) (B). Transients (1000) were accumulated in a data length of 16,384 with a sweep-width of 18·5 kHz.

It is significant that no other ^{13}C n.m.r. signals were observed in any of these experiments. If enzyme-free intermediates are involved in the cell-free conversion of LLD-ACV tripeptide to isopenicillin N, their concentrations do not appear to reach levels in the present system sufficient to permit detection by this method.

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