

## Penicillin Biosynthesis: Enzymatic Synthesis of New Cephams

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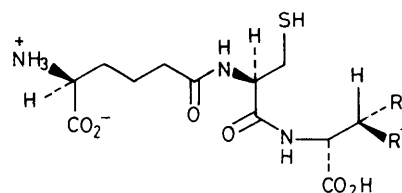
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Preparations of the enzyme isopenicillin N synthetase from *Cephalosporium acremonium* CO 728 convert the modified substrates  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-(-)-isoleucine<sup>†</sup> and  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-norvaline into cepham-type products, which have been isolated and their structures established.

The conversion of the natural precursor  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-valine<sup>†</sup> (**1a**) into isopenicillin N (**2a**) by a cell-free extract of *Cephalosporium acremonium* is well established.<sup>1</sup> Recently<sup>2</sup> we have reported that the conversion of a modified substrate  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-( $\alpha$ -aminobutyrate) (**1b**) with a highly purified sample of the enzyme isopenicillin N synthetase occurs via dual pathways into both penam (**2b**) and cepham (**3a**) products. We now report that the conversion of the similarly modified tripeptide  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-(-)-isoleucine (**1c**) occurs by both the reported<sup>3</sup> penam (**2c**) and novel cepham (**3b**) and (**3c**) pathways, and that the tripeptide  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-norvaline (**1d**) is converted into the novel cephams (**3d**) and (**3e**).

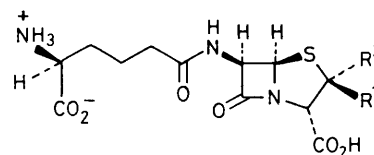
The first of these modified tripeptides (**1c**) was incubated with a cell-free extract of isopenicillin N synthetase from *C. acremonium* CO 728 and the crude products<sup>‡</sup> were observed by direct <sup>1</sup>H n.m.r. spectroscopy (250 MHz)<sup>3a</sup> after protein precipitation to reveal three  $\beta$ -lactam-containing products. The sole penam product was the expected<sup>3</sup> 2- $\alpha$ -methyl penicillin (**2c**) which was purified by preparative electrophoresis (at pH 3.5) and h.p.l.c. (reverse phase ODS column),  $\delta_{\text{H}}^{\S}$  (D<sub>2</sub>O, 500 MHz) 1.04 (3H, t, *J* 7.5 Hz, CH<sub>3</sub>CH<sub>2</sub>), 1.48 (3H, s, 2-CH<sub>3</sub>), 1.70—1.95 (6H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CO, CH<sub>3</sub>CH<sub>2</sub>), 2.42 (2H, m, CH<sub>2</sub>CO), 3.75 (1H, m, +NH<sub>3</sub>CHCO<sub>2</sub><sup>-</sup>), 4.30 (1H, s, 3-H), 5.42 (1H, d, *J* 4 Hz, 6-H), and 5.55 (1H, d, *J* 4 Hz, 5-H), *m/z* 374 [MH<sup>+</sup>, positive argon fast-atom bombardment (F.A.B.)]. The remaining  $\beta$ -lactam-containing products were purified by preparative electrophoresis (at pH 3.5) and by h.p.l.c. (reverse phase ODS column) to give the cephams (**3b**) and (**3c**) as a mixture in ca. 1:1 ratio. A Jeener n.m.r. spectrum<sup>4</sup> (Figure 1) established the connectivities S-CH(Me)-CH(Me)-CH(CO<sub>2</sub>H) for both (**3b**) and (**3c**) while the observation that saturation of only one of the four CH(*Me*) resonances,  $\delta$  1.49 p.p.m. gave a significant nuclear Overhauser enhancement (n.o.e.)<sup>¶</sup> to 6-H implied that the two compounds were epimeric at C-2. The mixture was then separated by repeated h.p.l.c. into the more mobile cepham (**3b**) and less mobile cepham (**3c**); for (**3b**)  $\delta_{\text{H}}$  (D<sub>2</sub>O, 500 MHz) 1.24 (3H, d, *J* 7 Hz, 3-CH<sub>3</sub>), 1.49 (3H, d, *J* 7 Hz, 2-CH<sub>3</sub>), 1.60—1.95 (4H, 2  $\times$  m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CO), 2.45 (2H, m, CH<sub>2</sub>CO), 2.45—2.52 (1H, m, 3-H), 2.91 (1H, dq, *J* 4, 7 Hz, 2-H), 3.75 (1H, m, +NH<sub>3</sub>CHCO<sub>2</sub><sup>-</sup>), 4.03 (1H, d, *J* 2.5 Hz, 4-H), 5.26 (1H, d, *J* 4 Hz, 6-H), and 5.41 (1H, d, *J* 4 Hz, 7-H); for (**3c**)  $\delta_{\text{H}}$  (D<sub>2</sub>O, 500 MHz), 1.09 (3H, d, *J* 7 Hz,

3-CH<sub>3</sub>), 1.23 (3H, d, *J* 7 Hz, 2-CH<sub>3</sub>), 1.60—1.90 (4H, 2  $\times$  m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CO), 2.38—2.46 (1H, m, 3-H), 2.44 (2H, m, CH<sub>2</sub>CO), 3.35 (1H, dq, *J* 2, 7 Hz, 2-H), 3.75 (1H, m, +NH<sub>3</sub>CHCO<sub>2</sub><sup>-</sup>), 4.25 (1H, d, *J* 1.5 Hz, 4-H), 5.26 (1H, d, *J* 4 Hz, 6-H), and 5.37 (1H, d, *J* 4 Hz, 7-H). The stereochemistry of (**3b**) follows from further n.o.e. experiments. Thus irradiation of 2-CH<sub>3</sub> ( $\delta_{\text{H}}$  1.49) enhanced 6-H, 2-H, and 3-H while irradiation of 3-CH<sub>3</sub> enhanced 2-H, 3-H, and 4-H. Assuming that no n.o.e. would be observed between *trans*-diaxial substituents, these observations are consistent with a chair-like conformation of stereochemistry (**3b**). In the case of (**3c**), which must be epimeric at C-2, irradiating the 3-CH<sub>3</sub>



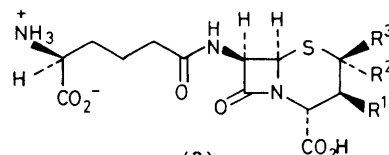
(1)

- a; R<sup>1</sup> = R<sup>2</sup> = Me
- b; R<sup>1</sup> = H, R<sup>2</sup> = Me
- c; R<sup>1</sup> = Et, R<sup>2</sup> = Me
- d; R<sup>1</sup> = H, R<sup>2</sup> = Et



(2)

- a; R<sup>1</sup> = R<sup>2</sup> = Me
- b; R<sup>1</sup> = Me, R<sup>2</sup> = H
- c; R<sup>1</sup> = Et, R<sup>2</sup> = Me



(3)

- a; R<sup>1</sup> = R<sup>2</sup> = R<sup>3</sup> = H
- b; R<sup>1</sup> = R<sup>2</sup> = Me, R<sup>3</sup> = H
- c; R<sup>1</sup> = R<sup>3</sup> = Me, R<sup>2</sup> = H
- d; R<sup>1</sup> = R<sup>3</sup> = H, R<sup>2</sup> = Me
- e; R<sup>1</sup> = R<sup>2</sup> = H, R<sup>3</sup> = Me

<sup>†</sup>  $\delta$ -( $\alpha$ -aminoadipoyl) = 5-amino-5-carboxypentanoyl.

<sup>‡</sup> The ratio of penam (**2c**) to combined cephams (**3b**), (**3c**) in the crude product prior to work up was greater than 10:1.

<sup>§</sup> Chemical shifts are referenced to internal sodium 3-trimethylsilylpropionate. [2,2,3,3-<sup>2</sup>H<sub>4</sub>]TSP = 0.00 p.p.m.

<sup>¶</sup> N.O.e.s observed in this and subsequent experiments were in the range 5—15%.

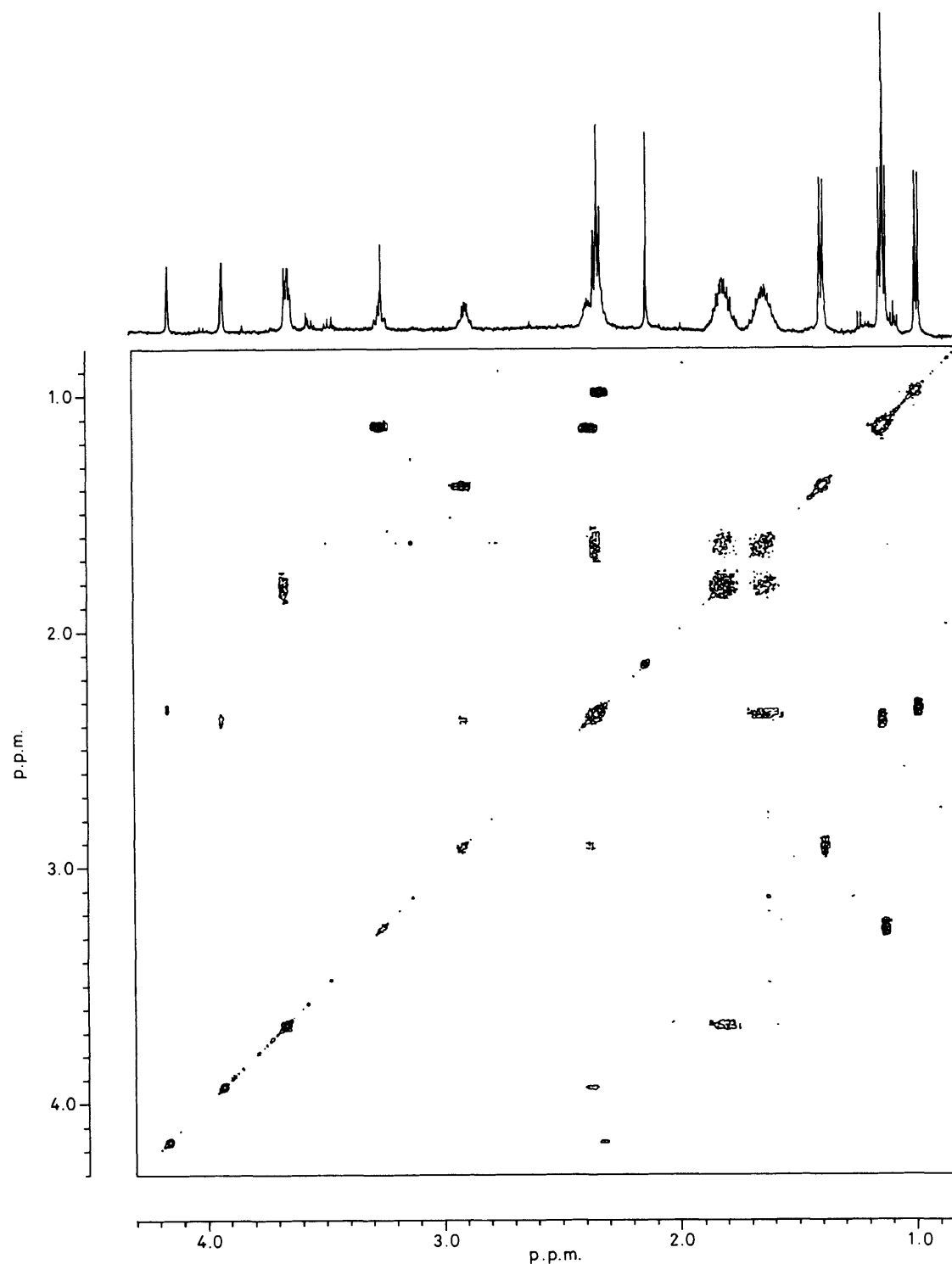


Figure 1. Combined one-dimensional and Jeener n.m.r. spectra (500 MHz) of (3b) and (3c).

enhanced 4-H but not 2-H.\*\* The low value of  $J(2\text{-H},3\text{-H})$  (2 Hz) excludes a diaxial relationship between these protons, so for a chair-like conformation, (3c) is the only possible stereochemistry. The cephams (3b) and (3c) gave the expected

\*\* In view of the small quantities of material available this may not be safely taken to imply that the 3-CH<sub>3</sub> and 2-H substituents are *trans*.

molecular ion  $M\text{H}^+$  at  $m/z$  374 upon positive argon F.A.B. mass spectrometry, and both showed no antibacterial activity against *Escherichia coli* ESS or *Staphylococcus aureus* N.C.T.C. 6571 at a concentration of 100  $\mu\text{g ml}^{-1}$  in individual experiments.

The second of these modified tripeptides (1d), derived from norvaline, gave upon similar incubation with a cell-free extract of isopenicillin N synthetase from *C. acremonium* CO 728, two

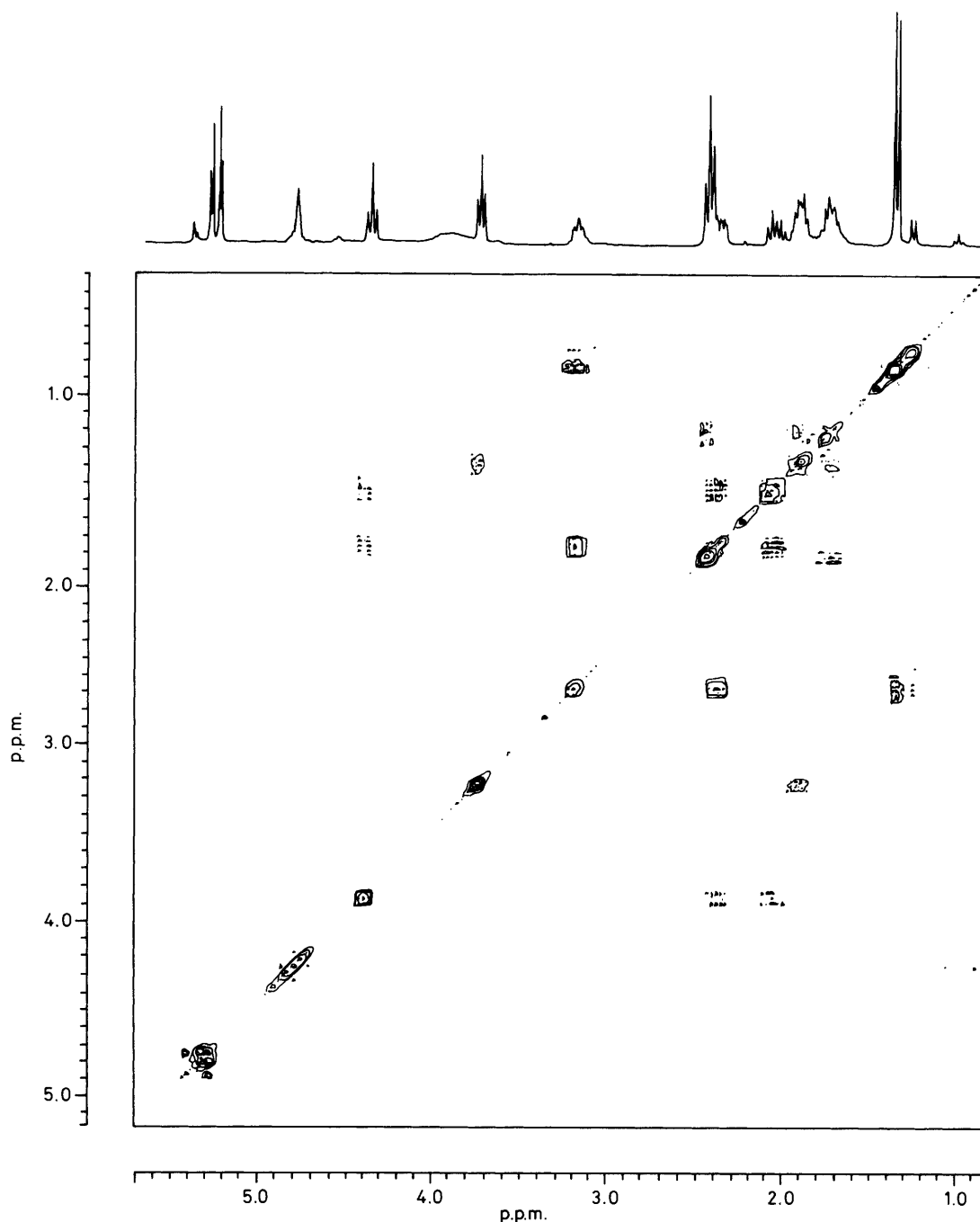
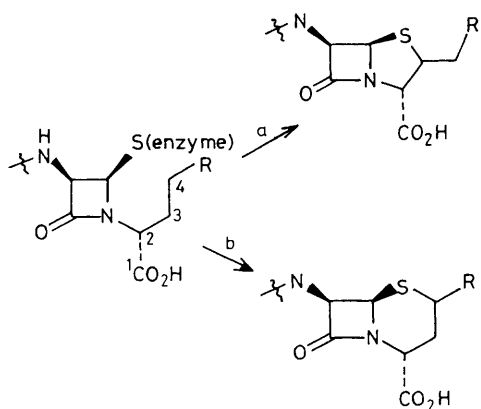


Figure 2. Combined one-dimensional and Jeener n.m.r. spectra (250 MHz) of (3d) and (3e).

major<sup>††</sup> new  $\beta$ -lactam products. Purification of the crude product by preparative electrophoresis (at pH 3.5) and by h.p.l.c. (reverse phase, ODS column) gave the cepham (3d),  $\delta_{\text{H}}$  ( $\text{D}_2\text{O}$ , 300 MHz) 1.36 (3H, d,  $J$  7 Hz, 2- $\text{CH}_3$ ), 1.69–1.97

<sup>††</sup> The entire product from incubation of the tripeptide (1d) (0.50 mg) with an active extract of isopenicillin N synthetase gave a weak antibacterial activity against *S. aureus* (–) N. C. T. C. 6571 by the 'holed plate' method. This activity, presumably, due to a 2-ethyl penicillin, was destroyed by penicillinase and was equivalent to less than 10  $\mu\text{g}$  of isopenicillin N (2a). The ratio of cepham (3d), (3e) to 2-ethyl-penicillin(s) was greater than 10:1 (500 MHz n.m.r.).

2  $\times$  m, 3-H), 2.41–2.46 (2H, m,  $\text{CH}_2\text{CO}$ ), 3.14–3.25 (1H, m, 2-H), 3.74–3.78 (1H, m,  $+\text{NH}_3\text{CHCO}_2^-$ ), 4.37–4.42 (1H, m, 4-H), 5.26 (1H, d,  $J$  4 Hz, 7-H), and 5.32 (1H, d,  $J$  4 Hz, 6-H), and (3e)  $\delta_{\text{H}}$  ( $\text{D}_2\text{O}$ , 300 MHz) as for (3d) except 1.26 (3H, d,  $J$  7 Hz, 2- $\text{CH}_3$ ), 3.04–3.13 (1H, m, 2-H), 5.27 (1H, d,  $J$  4 Hz, 7-H), and 5.40 (1H, d,  $J$  4 Hz, 6-H),  $\nu_{\text{max}}$ . ( $\text{CaF}_2$  cells,  $\text{D}_2\text{O}$ ), 1745  $\text{cm}^{-1}$ , ratio (3d):(3e) = 7:1, as an inseparable mixture. The connectivity S-CH(Me)-CH<sub>2</sub>-CH(CO<sub>2</sub>H) for the major isomer (3d) was again established by a Jeener n.m.r. spectrum (Figure 2), whilst this connectivity was also established for the minor isomer (3e), in individual decoupling n.m.r. experiments. Thus irradiation of the multiplet at  $\delta$  3.19 (2-H) collapsed the doublet due to the major isomer at  $\delta$  1.36



Scheme 1

(2-CH<sub>3</sub>) to a singlet. Similar irradiation of the multiplet at  $\delta$  3.09 (2-H) reduced the minor doublet at  $\delta$  1.26 to a singlet. Furthermore irradiation of the major and minor 2-methyl doublets in turn collapsed each respective 2-H resonance to the same multiplet (approximate double doublet). That the major isomer (**3d**) was the 2- $\alpha$ -methyl epimer was established by an n.O.e. experiment. Thus irradiation of the major 2-methyl doublet,  $\delta$  1.36, produced an enhancement of 6-H ( $\delta$  5.26) in the difference spectrum. The mixture (**3d**) and (**3e**) gave the expected molecular ion  $MH^+$  at  $m/z$  360 upon positive argon F.A.B. mass spectrometry and the mixture showed no antibacterial activity against *S. aureus* N.C.T.C. 6571 at a concentration of 1 mg ml<sup>-1</sup> by the 'holed plate' assay method.

In conclusion, we note that this work provides more examples of the operation of a dual pathway, *i.e.* penam *vs.* cepham synthesis by this enzyme.<sup>2</sup> It appears from these results that the C-H bond dissociation energy is an important determinant in the balance of the two paths, *i.e.* penam formation *via* a tertiary radical at C-3 of the terminal amino acid (route a) usually predominates (Scheme 1). However cepham formation (route b) competes if a secondary radical centre can be generated at C-4, *e.g.* (**3b-e**). When two secondary radicals may be generated at C-3 and C-4 of the terminal amino acid, *e.g.* the norvaline case (**1d**), then closure occurs predominantly to the six-membered cepham ring system. Thus the balance of the two pathways is the result of a subtle interplay between steric and radical stability effects.

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