Penicillin Biosynthesis: Enzymatic Synthesis of New Cephams

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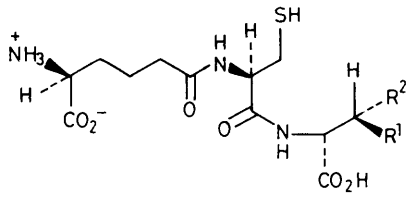
Preparations of the enzyme isopenicillin N synthetase from *Cephalosporium acremonium* CO 728 convert the modified substrates δ-(L-α-aminoadipoyl)-L-cysteinyl-D-(−)-isoleucine† and δ-(L-α-aminoadipoyl)-L-cysteinyl-D-norvaline into cepham-type products, which have been isolated and their structures established.

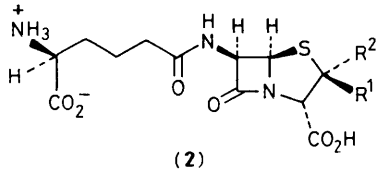
The conversion of the natural precursor δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine† (**1a**) into isopenicillin N (**2a**) by a cell-free extract of *Cephalosporium acremonium* is well established.[1] Recently[2] we have reported that the conversion of a modified substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-D-(α-aminobutyrate) (**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 δ-(L-α-aminoadipoyl)-L-cysteinyl-D-(−)-isoleucine (**1c**) occurs by both the reported[3] penam (**2c**) and novel cepham (**3b**) and (**3c**) pathways, and that the tripeptide δ-(L-α-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‡ were observed by direct ^{1}H n.m.r. spectroscopy (250 MHz)[3a] after protein precipitation to reveal three β-lactam-containing products. The sole penam product was the expected[3] 2-α-methyl penicillin (**2c**) which was purified by preparative electrophoresis (at pH 3.5) and h.p.l.c. (reverse phase ODS column), δ_H§ (D_2O, 500 MHz) 1.04 (3H, t, *J* 7.5 Hz, CH_3CH_2), 1.48 (3H, s, 2-CH_3), 1.70—1.95 (6H, m, $CH_2CH_2CH_2CO$, CH_3CH_2), 2.42 (2H, m, CH_2CO), 3.75 (1H, m, $^+NH_3CHCO_2^-$), 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^+, positive argon fast-atom bombardment (F.A.B.)]. The remaining β-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[4] (Figure 1) established the connectivities S–CH(Me)–CH(Me)–CH(CO_2H) for both (**3b**) and (**3c**) while the observation that saturation of only one of the four CH(*Me*) resonances, δ 1.49 p.p.m. gave a significant nuclear Overhauser enhancement (n.O.e.)¶ 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**) δ_H (D_2O, 500 MHz) 1.24 (3H, d, *J* 7 Hz, 3-CH_3), 1.49 (3H, d, *J* 7 Hz, 2-CH_3), 1.60—1.95 (4H, 2 × m, $CH_2CH_2CH_2CO$), 2.45 (2H, m, CH_2CO), 2.45—2.52 (1H, m, 3-H), 2.91 (1H, dq, *J* 4, 7 Hz, 2-H), 3.75 (1H, m, $^+NH_3CHCO_2^-$), 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**) δ_H (D_2O, 500 MHz), 1.09 (3H, d, *J* 7 Hz, 3-CH_3), 1.23 (3H, d, *J* 7 Hz, 2-CH_3), 1.60—1.90 (4H, 2 × m, $CH_2CH_2CH_2CO$), 2.38—2.46 (1H, m, 3-H), 2.44 (2H, m, CH_2CO), 3.35 (1H, dq, *J* 2, 7 Hz, 2-H), 3.75 (1H, m, $^+NH_3CHCO_2^-$), 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_3 (δ_H 1.49) enhanced 6-H, 2-H, and 3-H while irradiation of 3-CH_3 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_3



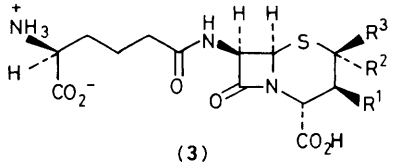
(**1**)

a; $R^1 = R^2 = Me$
b; $R^1 = H$, $R^2 = Me$
c; $R^1 = Et$, $R^2 = Me$
d; $R^1 = H$, $R^2 = Et$



(**2**)

a; $R^1 = R^2 = Me$
b; $R^1 = Me$, $R^2 = H$
c; $R^1 = Et$, $R^2 = Me$



(**3**)

a; $R^1 = R^2 = R^3 = H$
b; $R^1 = R^2 = Me$, $R^3 = H$
c; $R^1 = R^3 = Me$, $R^2 = H$
d; $R^1 = R^3 = H$, $R^2 = Me$
e; $R^1 = R^2 = H$, $R^3 = Me$

† δ-(α-aminoadipoyl) = 5-amino-5-carboxypentanoyl.

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

§ Chemical shifts are referenced to internal sodium 3-trimethylsilylpropionate, [2,2,3,3-$^2H_4$]TSP = 0.00 p.p.m.

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

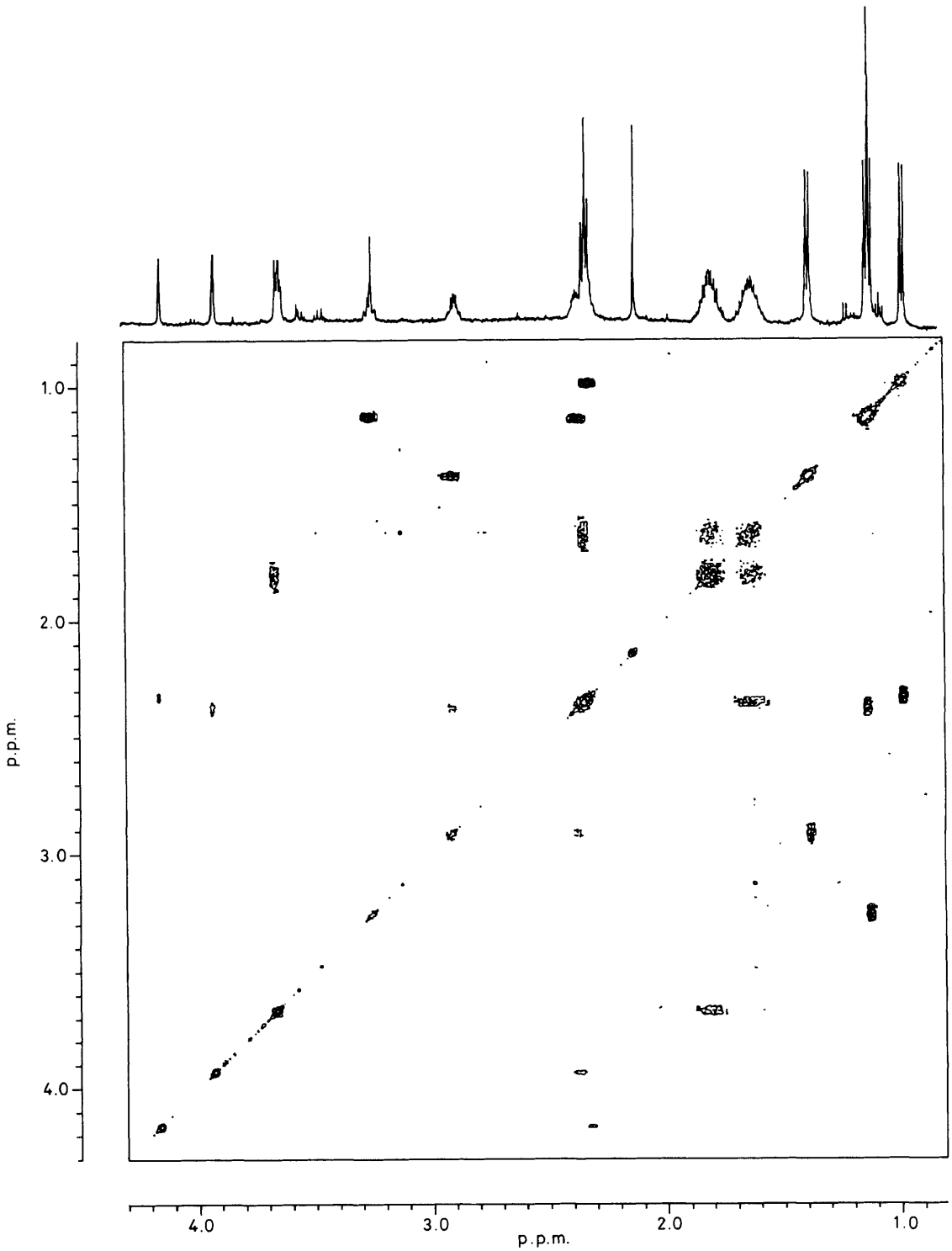


Figure 1. Combined one-dimensional and Jeener n.m.r. spectra (500 MHz) of (**3b**) and (**3c**).

enhanced 4-H but not 2-H.** The low value of J(2-H,3-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 molecular ion MH^+ at m/z 374 upon positive argon F.A.B. mass spectrometry, and both showed no antibacterial activity against *Escherischia coli* ESS or *Staphylococcus aureus* N.C.T.C. 6571 at a concentration of 100 μg 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

** In view of the small quantities of material available this may not be safely taken to imply that the 3-CH_3 and 2-H substituents are *trans*.

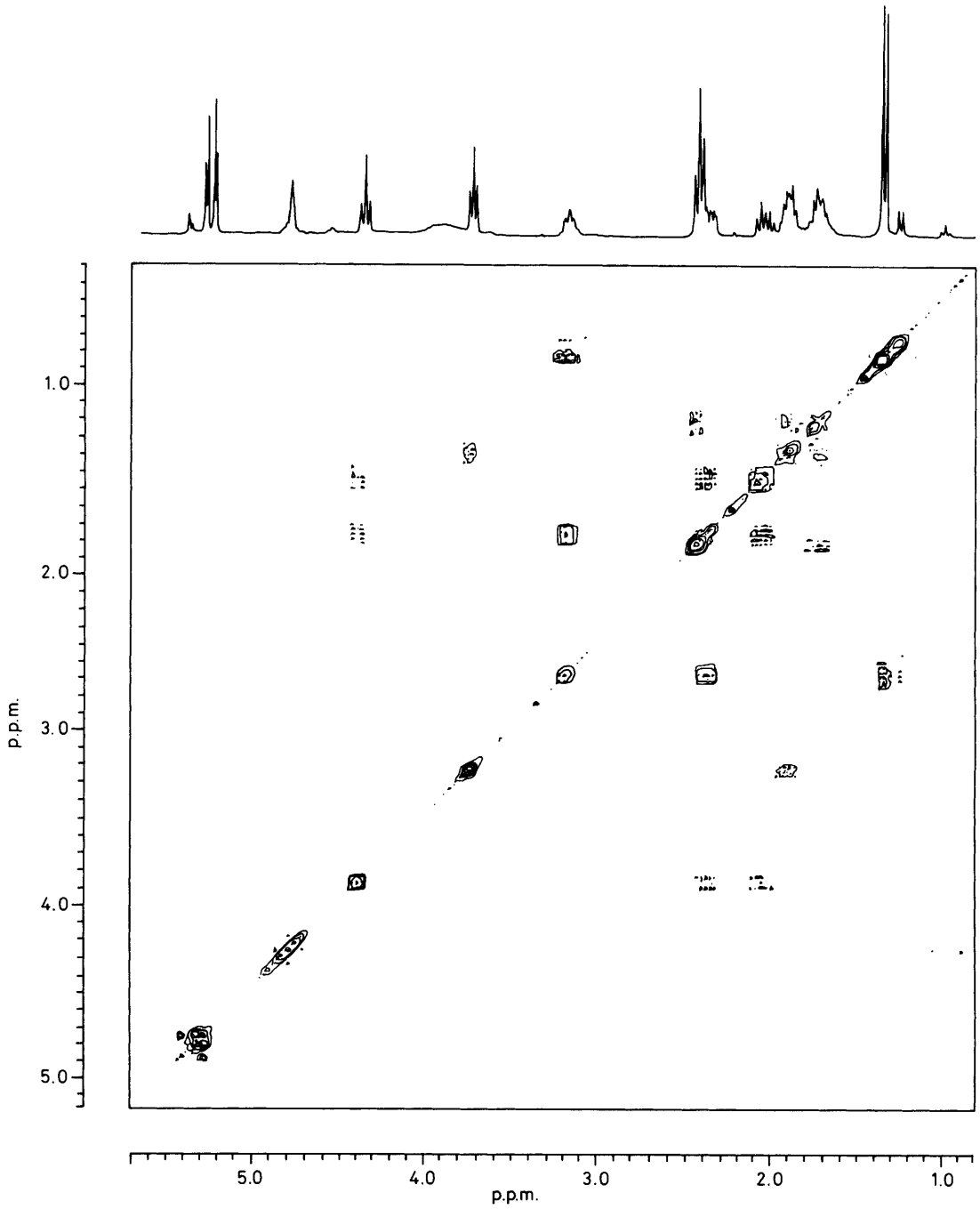
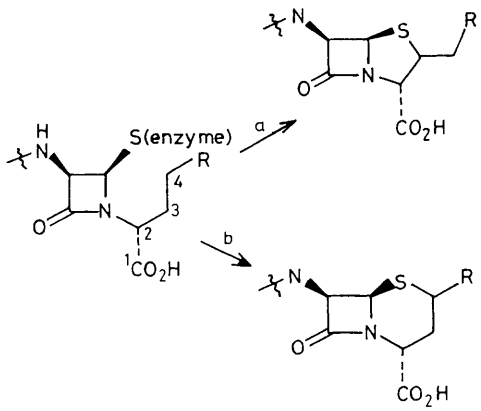


Figure 2. Combined one-dimensional and Jeener n.m.r. spectra (250 MHz) of (**3d**) and (**3e**).

major†† new β-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 cephams (**3d**), δ_H (D_2O, 300 MHz) 1.36 (3H, d, *J* 7 Hz, 2-CH_3), 1.69—1.97 (4H, m, $CH_2CH_2CH_2CO$), 2.01—2.11 and 2.34—2.39 (2H, 2 × m, 3-H), 2.41—2.46 (2H, m, CH_2CO), 3.14—3.25 (1H, m, 2-H), 3.74—3.78 (1H, m, $^+NH_3CHCO_2^-$), 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**) δ_H (D_2O, 300 MHz) as for (**3d**) except 1.26 (3H, d, *J* 7 Hz, 2-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), $\nu_{max.}$ (CaF_2 cells, D_2O), 1745 cm^{-1}, ratio (**3d**) : (**3e**) = 7 : 1, as an inseparable mixture. The connectivity S–CH(Me)–CH_2–CH(CO_2H) 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 δ 3.19 (2-H) collapsed the doublet due to the major isomer at δ 1.36

†† 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 μg of isopenicillin N (**2a**). The ratio of cephams (**3d**), (**3e**) to 2-ethyl-penicillin(s) was greater than 10 : 1 (500 MHz n.m.r.).



Scheme 1

(2-CH_3) to a singlet. Similar irradiation of the multiplet at δ 3.09 (2-H) reduced the minor doublet at δ 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-α-methyl epimer was established by an n.O.e. experiment. Thus irradiation of the major 2-methyl doublet, δ 1.36, produced an enhancement of 6-H (δ 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^{-1} 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.[2] 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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