Penicillin Biosynthesis. Dual Pathways from a Modified Substrate

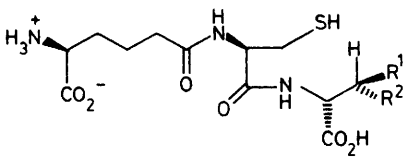
Jack E. Baldwin,*[b] Edward P. Abraham,[a] Robert M. Adlington,[b] Bulbul Chakravarti,[b] Andrew E. Derome,[b] John A. Murphy,[b] and (in part) Leslie D. Field,[b] Nicholas B. Green,[b] Hong-Hoi Ting,[b] and John J. Usher[b]

[a] *Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K.*
[b] *Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.*

Preparations of the enzyme isopenicillin N synthetase from *Cephalosporium acremonium* convert the modified substrate (L-α-amino-δ-adipyl)-L-cysteinyl-D-(α-aminobutyrate)† into both penam and cepham products, which have been isolated and their structures established.

Recently we reported the conversion of modified tripeptides, related to the natural precursor (L-α-amino-δ-adipyl)-L-cysteinyl-D-valine† (**1a**) into substituted derivatives of isopenicillin N (**2a**) with a cell-free extract of *Cephalosporium acremonium*.[1] Since we have now obtained a highly purified sample of the single enzyme, isopenicillin N synthetase,[2] MW 37 000, we have studied one of these conversions in more detail, with surprising results. Incubation of the substrate (L-α-amino-δ-adipyl)-L-cysteinyl-D-(α-aminobutyrate)†

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

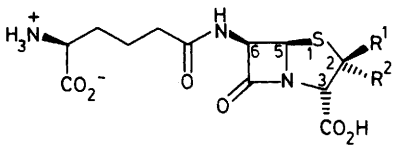


(1)

a; $R^1 = R^2 = Me$

b; $R^1 = Me, R^2 = H$

c; $R^1 = {}^2H, R^2 = Me$

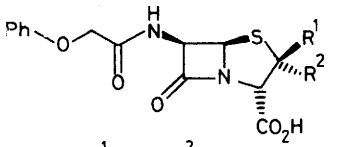


(2)

a; $R^1 = R^2 = Me$

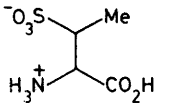
b; $R^1 = Me, R^2 = H$

c; $R^1 = H, R^2 = Me$

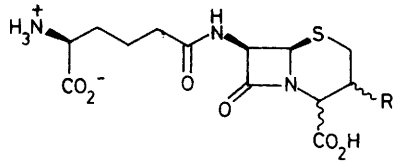


(3) $R^1 = Me, R^2 = H$

(4) $R^1 = H, R^2 = Me$



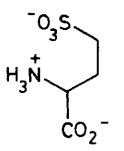
(5)



(6)

a; R = H

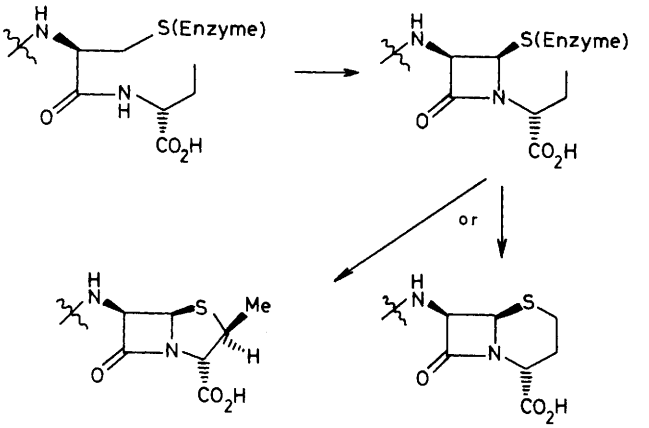
b; $R = {}^2H$



(7)

(**1b**) with this enzyme, in the presence of the cofactors $FeSO_4$ (0.1 mM), dithiothreitol (3 mM), ascorbic acid (1.5 mM), catalase, and oxygen[3] resulted in a transformation into *two* β-lactam-containing products. The first of these was the penam (**2b**) as previously reported. This substance‡ showed, in the ^1H n.m.r. spectrum, similar chemical shifts for the methyl group and both C(2)-H and C(3)-H to those of the known norpenicillins (**3**) and (**4**).[4] However the *J* value (3.2 Hz) for C(3)-H favoured the β-methyl formulation, as in (**3**). Double-irradiation experiments established the connectivity Me–CH–CH and the β-configuration of the methyl group was shown by oxidation (aqueous $NaIO_4$) to the β-sulphoxide[5] of (**2b**), which upon irradiation of the methyl group showed nuclear Overhauser enhancements (n.O.e.s) on the C(2)-H (23%) and C(3)-H (17%). There was no n.O.e. observed on either C(5)-H or C(6)-H. Virtually identical n.O.e.s were observed for the β-sulphoxide of (**3**), (19.5%) and (16%) respectively, whereas the β-sulphoxide derived from (**4**) gave only n.O.e.s on C(2)-H (18%) and C(5)-H

‡ The penam (**2b**) and cephams (**6a, b**) were purified by preparative electrophoresis at pH 3.5 and subsequently by h.p.l.c.



Scheme 1. Possible enzyme-bound species involved in the enzymatic conversion of peptide (**1b**).

(16.5%), as expected. Final proof for these assignments was obtained by oxidation (HCO_3H–HCO_2H) of (**2b**), (**3**), and (**4**) to the corresponding norpenicillaminic acids (**5**)[6] which, on paper electrophoresis (5 kV, 1 h, pH 1.8), showed that the acid from (**2b**) comigrated with that from (**3**) only. The penam (**2b**) was also converted[7] into the *N*-ethoxycarbonyl dimethyl ester derivative which on mass spectral analysis [NH_3, desorption chemical ionisation (D.C.I.)] gave ions at *m/z* 446 (MH^+, 80%), 287 (16%), and 160 (100%) (electron impact, M^+, Found, *m/z* 445.1520. Calc. for $C_{18}H_{27}N_3SO_8$ 445.1519). These data, taken together, rigorously establish the complete structure and stereochemistry of (**2b**).

The structure of the second product as (**6a**)‡ was established by a combination of n.m.r. data and degradation. This n.m.r. spectrum was simplified by generating the monodeuteriocepham (**6b**) from the corresponding peptide (**1c**).[8] Double-irradiation experiments§ on (**6b**) proved the connectivity –S–CH_2–CHD–CH– and hydrolysis–oxidation (6 M HCl, 5 h, 90 °C followed by HCO_3H–HCO_2H) gave an aminosulphonic acid which comigrated on paper electrophoresis (1.5 kV, 1 h, pH 1.8) with (**7**), derived by oxidation of homocysteine. The cephams (**6a, b**) were converted as before into the *N*-ethoxycarbonyl dimethyl ester derivatives for mass spectral analysis (NH_3, D.C.I.) which gave *m/z* 446 (MH^+, 40%), 287 (15%), and 160 (100%) for (**6a**); and 447 (MH^+, 60%), 287 (25%), and 161 (100%) for (**6b**).

Thus, both these products result simultaneously from the reaction of the substrate (**1b**) with the single enzyme, isopenicillin synthetase, isolated from *C. acremonium* CW19. The ratio of penam to cepham is *ca.* 3:1.¶ There was no ring expansion activity since this was separated during the purification procedure.[9] Furthermore, the enzyme converted the natural substrate (**1a**) only into isopenicillin N (**2a**), and the modified substrate (**1b**) gave no isolable amounts of the α-methyl isomer (**2c**).** We conclude that the enzyme iso-

§ Irradiation of a signal at δ 4.3 [d, originally a dd in (**6a**)] simplified a complex multiplet around δ 1.7 (partly obscured by signals due to the α-amino-δ-adipyl methylene protons). Irradiation at δ 1.7 caused the collapse of the signal at δ 4.3 to a singlet and the simplification of an eight-line pattern at δ 2.6 (AB part of an ABMX system) to an AB quartet. Full details of these experiments will be published elsewhere.

¶ Ratio estimated by direct ^1H n.m.r. (300 MHz) of the crude product after 5 h incubation at 20 °C.

** Although our original report, ref. 1, indicated that a minor isomer (**2c**) was present *via* electrophoresis of the norpenicillaminic acids we have been unable to isolate and purify this substance since the β-isomer (**2b**) predominates in a ratio of at least 10:1.

penicillin N synthetase is able to effect both 5 and 6 ring oxidative cyclisations with the modified substrate. A possible stepwise pathway is illustrated in Scheme 1 in which formation of the β-lactam precedes the 5 or 6 ring closure. The balance between the last two pathways might well be dependent on the conformations available to the n-butyric acid moiety in the enzyme-bound intermediate.

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8 Synthesised from (*R*)-[1-^{2}H]ethanol, J. E. Baldwin, R. M. Adlington, and J. J. Usher, unpublished results.
9 J. E. Baldwin, P. D. Singh, M. Yoshida, Y. Sawada, and A. L. Demain, *Biochem. J.*, 1980, **186**, 889. Note: This ring expansion occurs at a higher oxidation state than the conversion of (**2b**) into (**6a**).