Penicillin Biosynthesis: Multiple Pathways from a Modified Substrate

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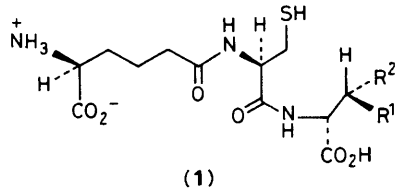
Preparations of the enzyme isopenicillin N synthetase from *Cephalosporium acremonium* CO 728 convert the modified tripeptide substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylgycine† into penam, homoceph-3-em, hydroxycepham, and hydroxyhomocepham type products; these have been isolated and their structures established.

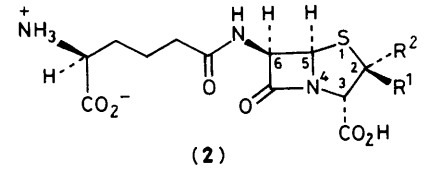
The conversion of the natural precursor δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (**1a**) into isopenicillin N (**2a**) by a cell-free extract of isopenicillin N synthetase from *Cephalosporium acremonium* is established.[1] Recently we reported[2] that the conversion of a modified substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-D-(α-aminobutyrate) (**1b**) with a highly purified sample of the enzyme isopenicillin N synthetase occurs *via* dual pathways into both penam (**2b**) and cepham (**3a**) type products. We now report that the conversion of a similarly modified tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-allylglycine (**1c**) with this purified enzyme at pH 7—8 leads

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



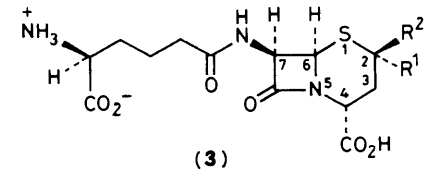
(1)

a; $R^1 = R^2 = Me$
b; $R^1 = H$, $R^2 = Me$
c; $R^1 = H$, $R^2 = CH{=}CH_2$



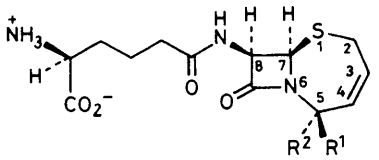
(2)

a; $R^1 = R^2 = Me$
b; $R^1 = Me$, $R^2 = H$
c; $R^1 = CH{=}CH_2$, $R^2 = H$
d; $R^1 = H$, $R^2 = CH{=}CH_2$



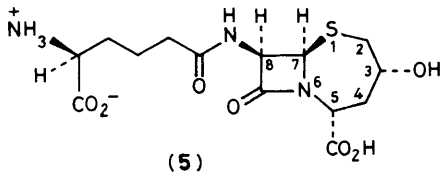
(3)

a; $R^1 = R^2 = H$
b; $R^1 = CH_2OH$, $R^2 = H$



(4)

a; $R^1 = H$, $R^2 = CO_2H$
b; $R^1 = CO_2H$, $R^2 = H$



(5)

simultaneously to six distinct β-lactam containing products (**2c**), (**2d**), (**3b**), (**4a**), (**4b**), and (**5**) (Figure 1).

The first of these products was the vinylpenicillin (**2c**) which showed penicillin type antibacterial activity‡ against Gram-positive organisms (*Staphylococcus aureus* N.C.T.C.6571, *Sarcina lutea* 400E, and *Bacillus megaterium* 1571E) but had no effect upon Gram-negative species (*Alcaligenes faecalis* DS.367, *Pseudomonas aeruginosa* N.C.T.C.10701, *Escherischia coli* 2110E, and *Salmonella typhi* DS.48) at a concentration of 50 μg ml^{-1}. The structure§ (**2c**) follows from its ^1H n.m.r. spectrum, δ_H (^2H_2O, 500 MHz)¶ 1.70—1.93 (4H, m, $CH_2CH_2CH_2CO$), 2.43—2.45 (2H, m, CH_2CO), 3.74—3.76 (1H, m, $CH[CH_2]_3$), 4.63 (1H, d, J 3.3 Hz, 3-H), 5.18 (1H, d, J 10 Hz, vinyl-H), 5.36 (1H, d, J 17 Hz, vinyl-H), 5.45, 5.47 (2H, AB q, J 3.8 Hz, 5,6-H), and 5.88—5.95 (1H, m, vinyl-H), (2-H obscured by ^2HOH). The coupling constant $J_{2,3}$ 3.3 Hz for 3-H was in agreement with a β-configuration for the vinyl group at C(2) and the penam (**2c**) also gave the expected molecular ion MH^+ *m/z* 358 upon positive argon fast-atom bombardment (F.A.B.) mass spectrometry. The vinylpenicillin (**2d**) with an α-configuration was also isolated as a minor product [ratio (**2c**):(**2d**) = 4:1], δ_H (^2H_2O, 500 MHz) 1.71—1.93 (4H, m, $CH_2CH_2CH_2CO$), 2.42—2.44 (2H, m, CH_2CO), 3.75—3.77 (1H, m, $CH[CH_2]_3$), 5.20 (1H, d, J 10.5 Hz, vinyl-H), 5.34 (1H, d, J 17.5 Hz, vinyl-H), 5.47, 5.58 (2H, AB q, J 3.8 Hz, 5,6-H), and 5.75—5.81 (1H, m, vinyl-H), (2,3-H obscured by ^2HOH). (**2d**) showed the expected penicillin type antibacterial activity against *S. aureus* N.C.T.C.6571.

The second class of β-lactam products isolated was the homoceph-3-ems (**4a**,**b**) [ratio (**4a**): (**4b**) = 10:1], whose structures were established by a combination of n.m.r. and mass spectrometry. For (**4a**), δ_H (^2H_2O, 500 MHz) 1.71—1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.43—2.47 (2H, m, CH_2CO), 3.06 (1H, dd, J 8, 16 Hz, 2-H), 3.53 (1H, dd, J 6, 16 Hz, 2-H), 3.73—3.76 (1H, m, $CH[CH_2]_3$), 5.20 (1H, m, 5-H), 5.30, 5.48 (2H, AB q, J 4 Hz, 7,8-H), 5.78 (1H, dd, J 5, 11.5 Hz, 4-H), and 6.04—6.10 (1H, m, 3-H); *m/z* (positive argon F.A.B.) MH^+ 358; for (**4b**) as (**4a**) except δ_H 5.24, 5.31 (2H, AB q, J 4 Hz, 7,8-H), and 5.95—6.03 (2H, m, 3,4-H); a Jeener n.m.r. spectrum** established the connectivity S–CH_2–CH=CH–$CH(CO_2H)$– for (**4a**) and (**4a**) showed no antibacterial activity against *S. aureus* N.C.T.C.6571 at a concentration of 400 μg ml^{-1}. A similar lack of antibacterial activity has been reported for a homoceph-4-em.[3] When a homogeneous sample of (**4a**) was resuspended in buffer (pH 7—8) some equilibration to (**4b**) occurred (by ^1H n.m.r. and h.p.l.c.); likewise, equilibration of (**4b**) to (**4a**) was also observed. A similar experiment in deuteriated media at pH 8—9 gave the 5-[^2H]-labelled homoceph-3-em (**4a**), δ_H 5.36, 5.56 (2H, AB q, J 4 Hz, 7,8-H), 5.85 (1H, d, J 11 Hz, 4-H), and 6.12—6.16 (1H, m, 3-H), (5-H absent), and 5-[^2H]-labelled homoceph-3-em (**4b**), δ_H 5.30 (1H, d, J 4 Hz, β-lactam-H) and 6.03 (1H, d, J 11 Hz, 4-H), (other resonances obscured), ratio (**4a**):(**4b**) *ca.* 9:1. A preference for an α-carboxylate over a β-carboxylate under equilibrating conditions has precedent.[4]

The structure of (**3b**), representing the third class of β-lactams was established as a hydroxymethylcepham from its n.m.r. and mass spectra, δ_H (^2H_2O, 500 MHz), 1.71—1.95 (4H, m, $CH_2CH_2CH_2CO$), 2.14—2.22 and 2.35—2.40 (2H, m, 3-H), 2.43—2.46 (2H, m, CH_2CO), 3.21—3.26 (1H, m, 2-H), 3.73—3.76 (3H, m, $CH[CH_2]_3$ and CH_2OH), 4.36—4.38 (1H, m, 4-H), and 5.24—5.34 (2H, AB q, J 4 Hz, 6,7-H); *m/z* (positive argon F.A.B.) MH^+ 376. A Jeener n.m.r. spectrum established the connectivity S–$CH(CH_2OH)$–CH_2–$CH(CO_2H)$; irradiation of the C(2)–CH_2OH group, δ_H 3.74 generated a nuclear Overhauser enhancement (n.O.e.) of 6-H consistent with an α-configuration at the methylenehydroxy

‡ The specific activity of (**2c**) against *Staphylococcus aureus* N.C.T.C.6571 was comparable to that of isopenicillin N, and was destroyed by β-lactamase I from *Bacillus cereus*.

§ For (**2c**), (**2d**), (**3b**), and (**4a**), an α-configuration for the carboxyl group is assumed, not proven.

¶ Chemical shifts are referenced to internal sodium (2,2,3,3-$^2H_4$)-3-trimethylsilylpropionate (TSP) = 0.00 p.p.m.

** Further details of these experiments will be reported elsewhere.

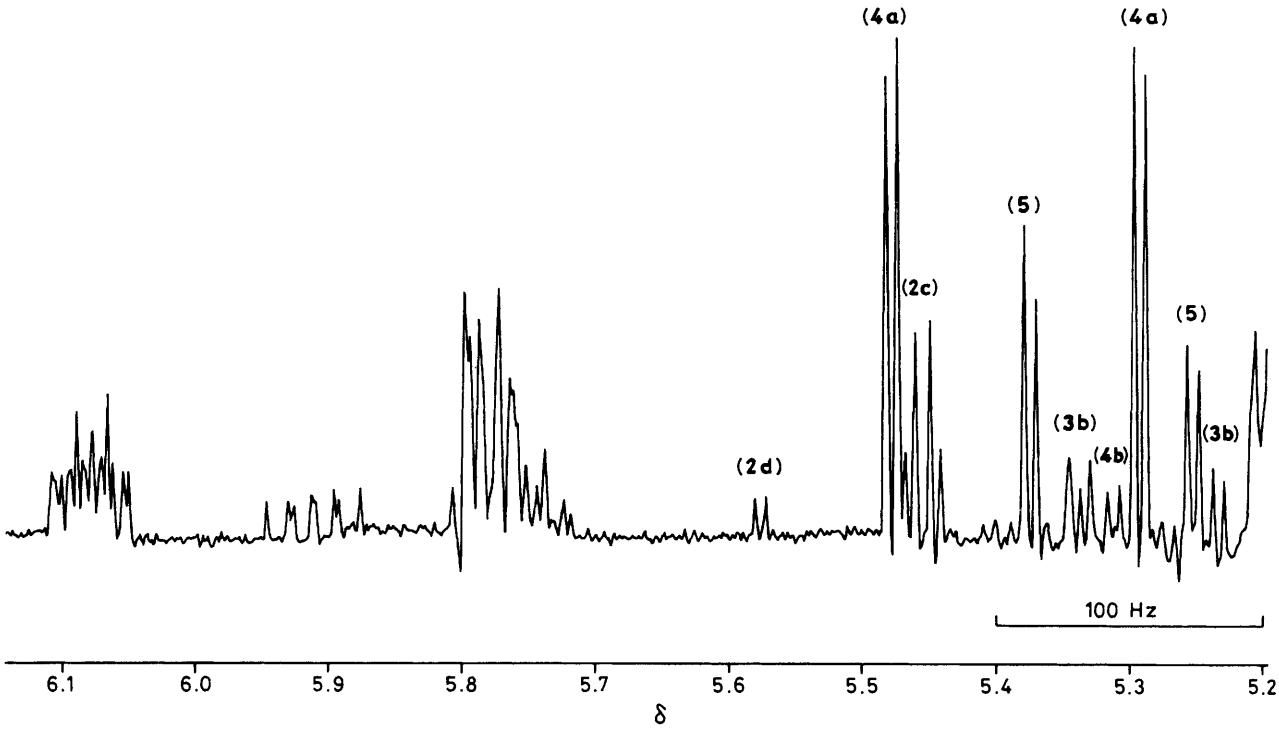
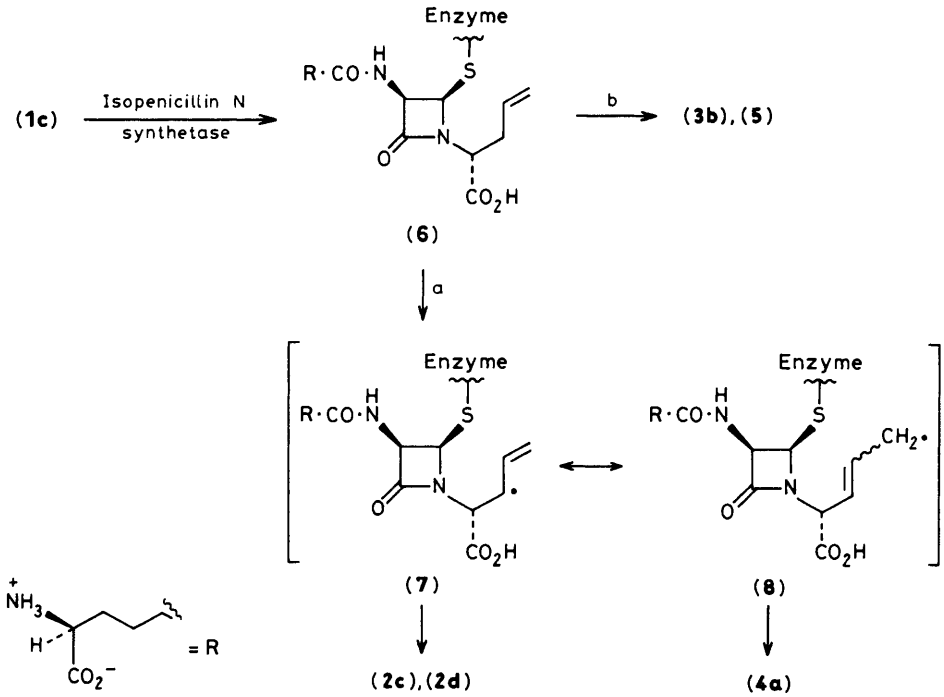


Figure 1. ^{1}H N.m.r. spectrum (500 MHz, δ_H 5.1—6.1) of a crude incubation mixture [(**1c**) with isopenicillin N synthetase] after protein precipitation. The characteristic β-lactam resonances are labelled. The ratio (**2c**):(**2d**):(**3b**):(**4a**):(**4b**):(**5**) was approximately 4:1:2:10:1:5. These products were subsequently purified by preparative electrophoresis (at pH 3.5) and by h.p.l.c. (reverse phase O.D.S.column, pH 8 buffer).



Scheme 1

group. Conversely, irradiation of the C(2)-H group, δ_H 3.23 gave no n.O.e. to 6-H. The hydroxymethylcepham (**3b**) showed no antibacterial activity against *S. aureus* N.C.T.C.-6571 at a concentration of 100 μg ml^{-1}.

The fourth class of product was established as the hydroxyhomocepham (**5**) from its n.m.r. and mass spectra, δ_H (^2H_2O, 500 MHz) 1.70—1.97 (4H, m, $CH_2CH_2CH_2CO$), 2.09—2.17 and 2.30—2.34 (2H, m, 4-H), 2.42—2.45 (2H, m, CH_2CO),

2.76—2.80 and 3.03—3.06 (2H, m, 2-H), 3.73—3.76 (1H, m, C*H*[CH_2]$_3$), 4.09—4.14 (1H, m, 3-H), 4.18 (1H, dd, *J* 2.5, 12.5 Hz, 5-H), and 5.26, 5.38 (2H, AB q, *J* 4 Hz, 7,8-H); *m/z* (positive argon F.A.B.) MH^+ 376. A Jeener n.m.r. spectrum established the connectivity S–CH_2–CH(OH)–CH_2–CH(CO_2H)– and the product (**5**) showed no antibacterial activity against *S. aureus* N.C.T.C.6571 at a concentration of 300 μg ml^{-1}. The stereochemistry of the hydroxy group as 3-α-OH was established by n.O.e. experiments. Thus irradiation of the higher field C(4)-H (δ_H 2.78) gave a n.O.e. (8%) to 7-H but not to 3-H, whereas irradiation of the lower field C(4)-H (δ_H 3.05) gave no enhancement of 7-H but instead n.O.e.'s to 3-H (13%) and 5-H (14%). A similar experiment was performed on the C(2)-H's. Thus irradiation of the high field C(2)-H (δ_H 2.13) gave n.O.e.'s to 7-H (5%) and 3-H (3%) whereas irradiation of the lower field C(2)-H (δ_H 2.32) gave n.O.e.'s to 5-H (3%) and 3-H (8%) (in all these experiments large geminal n.O.e.'s were observed: 20—25%). These results are consistent with formulation of the structure as (**5**).

In summary we suggest that the diverse structural range of products formed from the allylglycine tripeptide (**1c**) with isopenicillin N synthetase may be explained by two discrete pathways (routes a, b) from a common monocyclic β-lactam intermediate (**6**)[5] (Scheme 1). From route a, a radical (or carbon–metal) type intermediate (**7**) would lead, on ring closure, to the penicillin products (**2c**) and (**2d**) whilst the rearranged radical (**8**) would give the homoceph-3-em (**4a**). The preference of (**2c**) over (**2d**) has precedent[2] in a related system, whilst the predominance of (**4a**) over (**2c**) and (**2d**) probably reflects a lower energy barrier for the second ring closure. The product (**4b**) probably arises by epimerisation of the biosynthetic (**4a**). The novel hydroxylated products (**3b**) and (**5**) are thought to arise by a different mechanism, route b. Whether the source of the oxygen atom [*e.g.* at C(3) of (**5**)] is the co-substrate dioxygen[6] or, alternatively, water is currently under investigation.

We wish to thank B.T.G. for support, and the S.E.R.C. for a studentship (N. J. T.).

Received, 7th June 1984; Com. 791

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