Penicillin Biosynthesis: Active Site Mapping with Aminoadipoylcysteinylvaline† Variants

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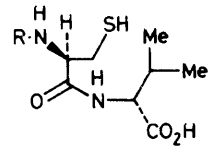
A series of structural variants on the aminoadipoyl moiety of the natural precursor of penicillins, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine,† have been synthesised and their effectiveness as substrates for the enzyme isopenicillin N synthetase has been determined.

Although the enzyme isopenicillin N synthetase has been shown to convert tripeptides[1,2] other than the natural substrate[3] δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine ACV (**1a**) into isopenicillin N (**2a**), these modifications have concentrated upon variants within the valinyl moiety. We now report‡ that tripeptides suitably modified in the δ-(L-α-aminoadipoyl)-moiety act as substrates for this enzyme to yield penicillin products.

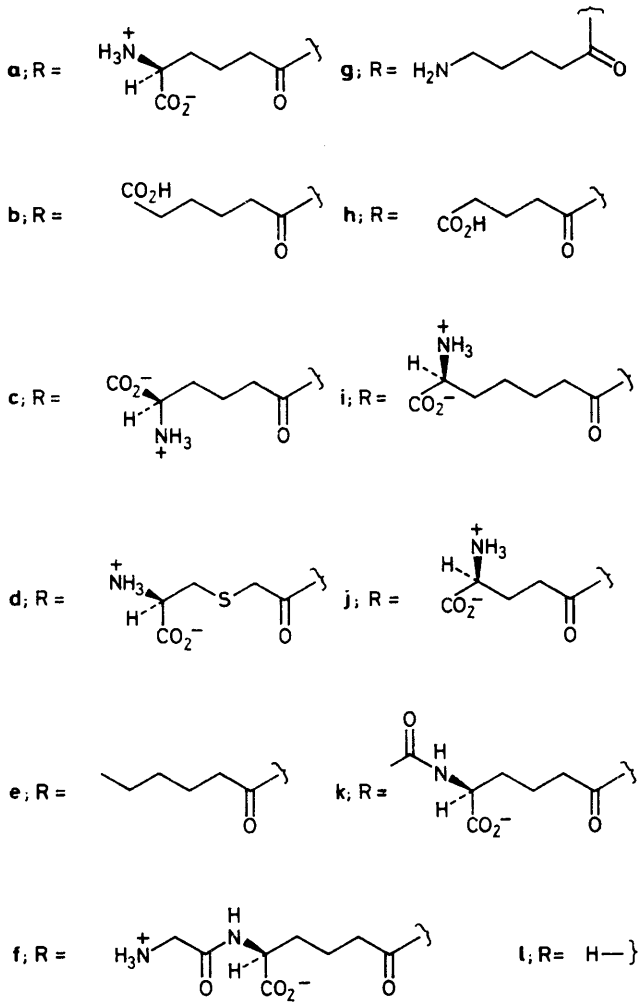
† δ-(α-aminoadipoyl) = 5-amino-5-carboxypentanoyl.

‡ The first reports of testing of the peptides (**1a—l**) as substrates for a purified sample of isopenicillin N synthetase were given by one of us (J. E. B.) at the Gordon Conference on Natural Products, New Hampton, New Hampshire, U.S.A., 20 July, 1981, and by another (E. P. A.) at a Symposium on the Biosynthesis of β-Lactam Antibiotics at the 21st Interscience Conference on Antimicrobial Agents and Chemotherapy, 4—6 November, 1981, Chicago, U.S.A.

The first of these modified tripeptides,[4] adipoyl-L-cysteinyl-D-valine (**1b**) gave, upon incubation with a highly purified sample of isopenicillin N synthetase[5] from *Cephalosporium acremonium* CO 728, a product which showed penicillinase-sensitive activity against *Staphylococcus aureus* N.C.T.C. 6571. Purification by h.p.l.c. [reverse phase ODS column, methanol: 50 mM ammonium hydrogen carbonate (9:1) as eluant] gave the penicillin[6] (**2b**) δ_H (^2H_2O, 300 MHz) 1.35—1.55 (4H, m, $[CH_2]_2CH_2CO$), 1.36 (3H, s, 2-CH_3), 1.48 (3H, s, 2-CH_3), 2.0—2.3 (4H, 2 × m, CH_2CO), 4.08 (1H, s, 3-H), 5.29 (1H, d, *J* 4 Hz, β-lactam-H), and 5.39 (1H, d, *J* 4 Hz, β-lactam-H), identical with an authentic sample. Alternatively acidification of the crude incubation mixture to pH 2, extraction into ethyl acetate and reaction with diazomethane gave the dimethyl ester (**3**) δ_H (C^2HCl_3, 300 MHz) 1.51 (3H, s, 2-CH_3), 1.60—1.85 (4H, m, $[CH_2]_2CH_2CO$), 1.68 (3H, s, 2-CH_3), 2.25—2.40 (4H, m, CH_2CO), 3.68 (3H, s,



(1)



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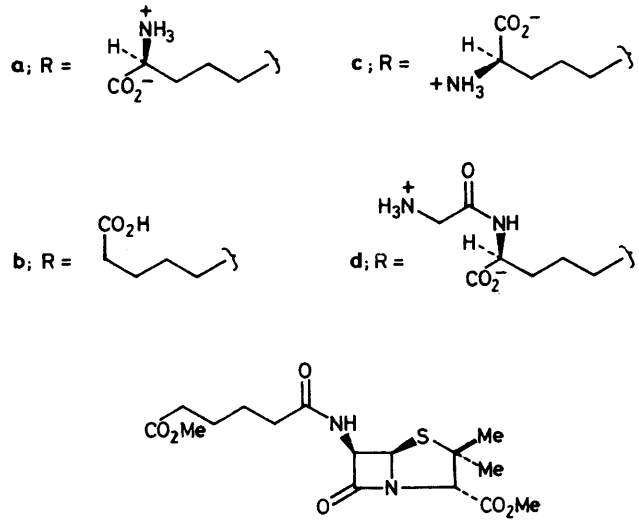

(3)

CO_2CH_3), 3.79 (3H, s, CO_2CH_3), 4.44 (1H, s, 3-H), 5.55 (1H, d, *J* 4 Hz, 5-H), 5.73 (1H, dd, *J* 4, 9 Hz, 6-H), and 6.14 (1H, d, *J* 9 Hz, NH), *m/z* (ammonia desorption chemical ionisation) 373 (MH^+, 40%) and 174 (100%), identical (^{1}H n.m.r., *m/z*, t.l.c.) with a sample prepared from authentic (**2b**).

The second of these tripeptides, δ-(D-α-aminoadipoyl)-L-cysteinyl-D-valine (**1c**) gave, upon incubation with isopenicillin N synthetase, a penicillinase-sensitive product whose spectrum of antibacterial activity against *Salmonella typhi*,[7] *S. aureus* N.C.T.C. 6571,[7] and *Escherichia coli* ESS[8] was in agreement with the product as penicillin N (**2c**). Purification of the crude product by h.p.l.c. (reverse phase ODS column, pH 7.5 potassium dihydrogen-, dipotassium hydrogen-phosphate, potassium chloride buffer as eluant) gave penicillin N (**2c**) identical with an authentic sample (^{1}H n.m.r., h.p.l.c.) which upon incubation with a partially purified sample of ring expansion enzyme[9] gave a mixture of deacetoxy- and deacetylcephalosporin C (antibacterial activity against *E. coli* ESS in the presence or absence of β-lactamase I from *Bacillus cereus*).

A control in which L,L,D-ACV (**1a**) was incubated with the isopenicillin N synthetase gave no penicillin N (**2c**) proving that the isopenicillin N synthetase was free of any epimerase activity.[10] D,L,D-ACV (**1c**) is a poorer substrate for the synthetase than L,L,D-ACV (**1a**) and for this reason its conversion into penicillin N (**2c**) was not detected by Fawcett *et al.*[11] in early experiments with a crude cell-free extract obtained by lysis of protoplasts of *C. acremonium*. However, our present results are in contrast to a more recent report by Wolfe[2] that D-α-aminoadipoyl-containing peptides did not cyclise when incubated with purified preparations of the synthetase.

Other peptides (**1d**—**l**)§ were tested as substrates for isopenicillin N synthetase, but antibacterial activity against *S. aureus* N.C.T.C. 6571 was only detected in the product[12] from (**1d**); similar negative results for (**1j**) have been found elsewhere.[1f,2,13] The lack of conversion of the glycinyl tetrapeptide (**1f**) into glycinylisopenicillin N (**2d**) was confirmed by the n.m.r. method[1d] (no β-lactam protons observable in the incubation mixture) and by independent chemical synthesis of (**2d**) which was shown to give antibacterial activity against *S. aureus* N.C.T.C. 6571; this result should be compared with the report[13] that incubation of (**1f**) with a purified sample of isopenicillin N synthetase from *C. acremonium* CW-19 (containing a suspected exoaminopeptidase activity as a contaminant) gave isopenicillin N (**2a**).

In summary these results indicate that the minimal structural requirement for *N*-acyl-L-cysteinyl-D-valine peptides to be converted into penicillin products by the enzyme isopenicillin N synthetase is that the *N*-acyl group has a six carbon, or equivalent chain, terminating in a carboxy group. The implication of these results is that a binding site is separated from the catalytically active site (which may itself bind to the cysteinyl sulphur atom of the substrate) by a distance

§ For (**1l**) the crude product was acylated ($PhCH_2COCl$–pyridine) prior to bioassay.

equivalent to one of the conformations of the adipoyl side chain. This binding has previously been shown not to involve exchange of the carboxy oxygen atoms during the conversion of the natural substrate.[14]

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