Stepwise Ring Closure in Penicillin Biosynthesis. Initial β-Lactam Formation

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Incubation of a 1 : 1 mixture of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine† (L,L,D-ACV) and L,L,D-A[3,3-$^2H_2$]CV with a cell-free extract of isopenicillin N synthetase from *Cephalosporium acremonium* results in preferential conversion of the fully protiated substrate into isopenicillin N; alternatively a similar experiment with a 1 : 1 mixture of L,L,D,-ACV and L,L,D-AC[3-^{2}H]V gave isopenicillin N without isotopic discrimination between the two substrates.

Although the conversion of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine† L,L,D-ACV (**1a**) into isopenicillin N (**2**) by cell-free extracts of isopenicillin N synthetase from *Cephalosporium acremonium* has been known for several years,[1] no reliable evidence has been forwarded regarding the order of ring formation and indeed no intermediates have been isolated.[2] Recently we reported[3] that the conversion of a modified substrate, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-aminobutyrate (**3**) occurred *via* dual pathways into both penam (**4**) and cepham (**5**) products, presumably by a common monocyclic β-lactam intermediate. Further evidence[4] from a modified substrate follows from studies upon the tripeptide δ-(L-α-aminoadipoyl)-L-[3-^{3}H]cysteinylglycine (**6**) in which loss of tritium upon incubation with isopenicillin N synthetase suggested the formation of a β-lactam type intermediate. We now report direct evidence for a similar monocyclic β-lactam intermediate during the enzymatic conversion of the natural substrate, L,L,D-ACV (**1**) into isopenicillin N (**2**).

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

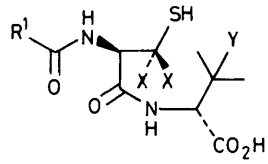
The conversion of a 1 : 1 mixture of L,L,D-ACV (**1a**) and L,L,D-A[3,3-$^2H_2$]CV (**1b**)‡ with a cell-free extract of isopenicillin N synthetase in the presence of β-lactamase I (from *Bacillus cereus*) was observed§ and samples removed and

‡ *S*-Benzyl-(2*S*)-[3,3-$^2H_2$]cysteine was prepared from dideuterioparaformaldehyde (MSD isotopes, distributed by Merck and Co. Inc., Rahway, New Jersey, U.S.A.) by the methods of Murray and Wood[5] and the deuterium content was shown to be >98% at the cysteinyl-3-position (^{1}H 300 MHz n.m.r. spectroscopy). (2*R*)-[3-^{2}H]Valine was prepared by the method of Baldwin *et al.*[6] and the deuterium content shown to be >98% at the valinyl-3-position (^{1}H 300 MHz n.m.r. spectroscopy). The amino acids were converted into the tripeptides (**1b**) and (**1c**) by the standard method.[7]

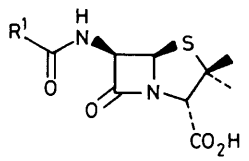
§ In separate experiments we have shown that the enzymatic hydrolysis of the penicillin to penicilloate is not rate limiting under our conditions. We have found this coupled enzyme system to be a convenient method for following the rate of penicillin synthesis by titrating the so formed penicilloate using a pH stat (further details of these experiments will appear elsewhere).

Table 1

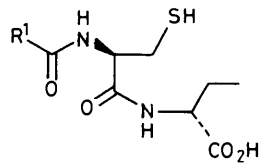
Experiment	% Conversion of ACV	Molecular ion (MH^+) intensity (**7a,b**)					Molecular ion (MH^+) intensity (**8a,b**)				Ratio (**7a**) : (**7b**)	Ratio (**8a**) : (**8b**)
		536	537	538	539	540	564	565	566	567		
1	0	98	30	100	27	9	—	—	—	—	1.08 : 1	—
	60	69	19	100	26	9	100	92	30	11	0.73 : 1	1.54 : 1
	90	55	17	100	26	9	90	100	32	12	0.57 : 1	1.20 : 1
2	0	100	29	97	25	9	—	—	—	—	1.14 : 1	—
	60	71	23	100	27	9	100	90	29	9	0.76 : 1	1.60 : 1



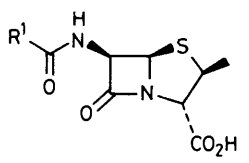
(1) a; X = Y = ^{1}H
b; X = ^{2}H, Y = ^{1}H
c; X = ^{1}H, Y = ^{2}H



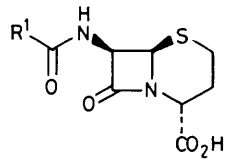
(2)



(3)



(4)



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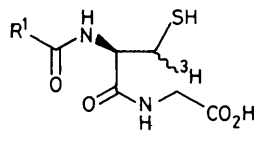
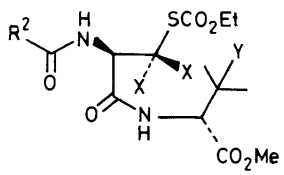
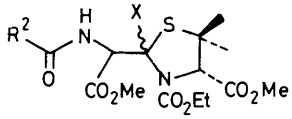


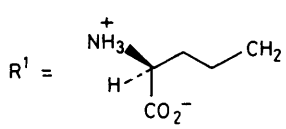
(6)

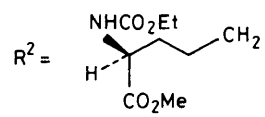


(7) a; X = Y = ^{1}H
b; X = ^{2}H, Y = ^{1}H
c; X = ^{1}H, Y = ^{2}H



(8) a; X = ^{1}H
b; X = ^{2}H





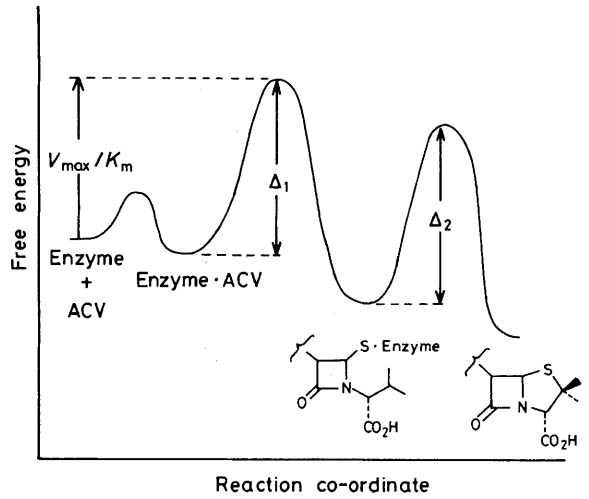


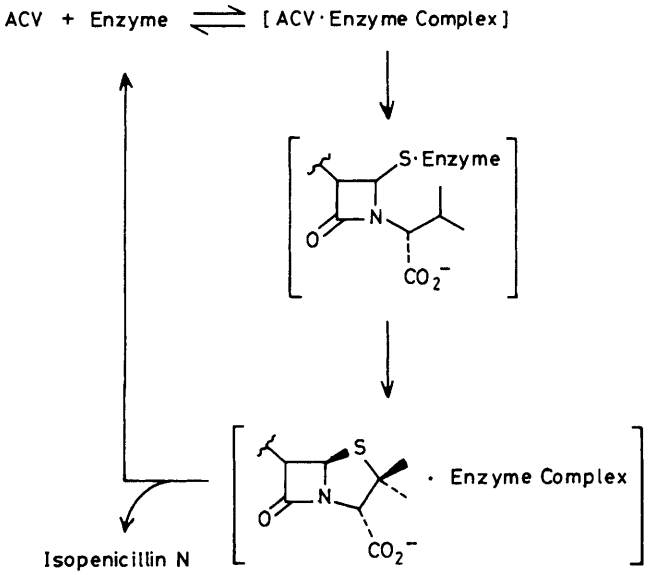
Figure 1. Suggested energy level diagram for the sequence of events occurring on the enzyme in the biosynthesis of isopenicillin N. Only cysteinyl C–H(D) bond cleavage exhibits a V_{max}/K_m isotope effect, so the transition state for that step must be predominant. This requirement limits the maximum free energy of the transition states for substrate binding and valinyl C–H(D) cleavage. The activation energies Δ_1 and Δ_2 must be comparable to rationalise the observation of V_{max} isotope effects at the cysteinyl- and valinyl-3-positions.

quenched throughout the reaction. The samples were then converted into their *N,S*-ethoxycarbonyl methyl ester derivatives[8] which were then directly analysed by desorption chemical ionisation mass spectrometry. The intensity of the protonated molecular ions (MH^+) of the derivatised unchanged starting material (**7a,b**) and derivatised penicilloate product (**8a,b**) clearly show a preferential conversion of the fully protiated substrate (**1a**) when it is in direct competition with the 3,3-dideuteriated cysteinyl substrate (**1b**) (Table 1, experiments 1,2). A control experiment using boiled enzyme with the same 1 : 1 mixture showed *no* isotopic discrimination of the two substrates over the normal incubation period, and an individual conversion of the 3,3-dideuteriated cysteinyl substrate (**1b**) with active enzyme in H_2O gave a fully monodeuteriated penicilloate derivative (**8b**) [MH^+ *m/z* (intensity) = 565 (100), 566 (28), 567 (12), 568 (3); calculated for $C_{23}H_{37}{}^2HN_3O_{11}S$, 565 (100), 566 (28), 567 (10), 568 (2); for (**8a**) found 564 (100), 565 (27), 566 (11), 567 (1)].

By similar means, the conversion of a 1 : 1 mixture of L,L,D-ACV (**1a**) and L,L,D-AC[3-^{2}H]V (**1c**) was studied. However in this experiment the derivatised unchanged starting materials (**7a,c**) showed *no* isotopic discrimination between protiated (**1a**) and deuteriated substrates (**1c**) (Table 2, experiments 3,4).

Table 2

Experiment	% Conversion of ACV	Molecular ion (MH^+) intensity (7a,c)				Ratio (7a) : (7c)
		536	537	538	539	
3	0	81	100	29	9	1.04 : 1
	60	81	100	29	9	1.04 : 1
4	0	94	100	29	9	1.27 : 1
	25	95	100	30	9	1.28 : 1
	50	93	100	30	10	1.25 : 1



Scheme 1

Isotopic discrimination in competitive mixed-label experiments is a V_{max}/K_m effect[9] (V_{max} = maximum velocity, K_m = Michaelis constant) and reflects events only up to the first irreversible step. The deuteriated cysteinyl and valinyl substrates each show significant V_{max} deuterium isotope effects as measured in separate, non-competitive experiments.¶ That only one of the bond cleavages shows a V_{max}/K_m isotope effect implies that the step involved [cysteinyl C(3)–H bond cleavage] occurs as the first chemical step in the reaction sequence. If valinyl C(3)–H bond cleavage preceded that occurring at the cysteinyl site, then both steps would show V_{max}/K_m effects, or there would only be one such effect and that at the valine 3-position, or neither substrate would show an effect. Assuming a single isotopically-sensitive step for each C–H cleavage, we suggest an energy level diagram such as Figure 1, for the sequence of events occurring on the enzyme in the biosynthesis of isopenicillin N. This form of free energy profile agrees with our unpublished observations on both V_{max}/K_m and V_{max} isotope effects in the two ring formations.

In summary, we have used competitive experiments to show that there is isotopic discrimination at the cysteinyl-3-position and not the valinyl-3-position in the biosynthesis of isopenicillin N from its precursor ACV. These experiments support a mechanism in which initial β-lactam formation precedes thiazolidine formation during the biosynthesis of isopenicillin N (Scheme 1).

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¶ Further details of these experiments will appear elsewhere.

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