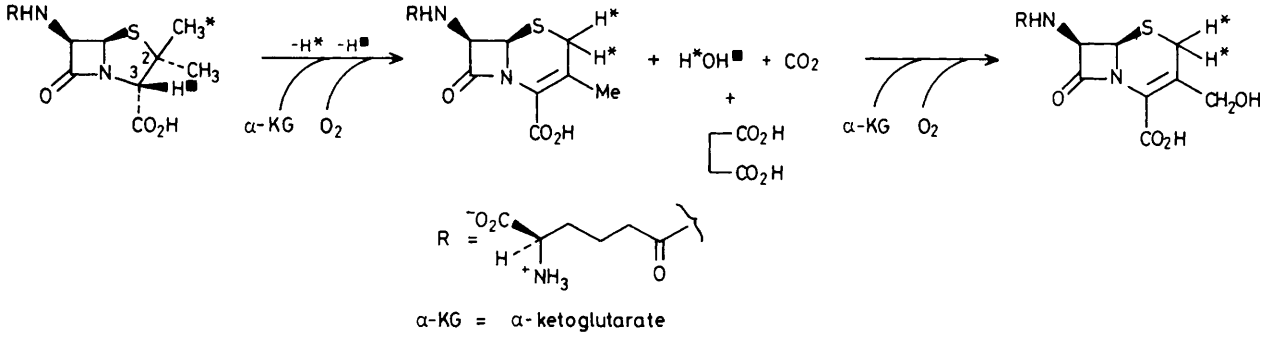
Stepwise Hydrogen Removal in the Enzymic Ring Expansion of Penicillin N to Deacetoxycephalosporin C

Jack E. Baldwin,* Robert M. Adlington, Robin T. Aplin, Nicholas P. Crouch, Christopher J. Schofield, and Hong-Hoi Ting

The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.

Reaction of a 1 : 1 mixture of penicillin N and 2,2-bis(trideuteriomethyl)penicillin N with partially purified deacetoxycephalosporin C/deacetylcephalosporin C synthetase from *Cephalosporium acremonium* CO 728 resulted in preferential conversion of the protiated substrate, whereas in similar experiments with a 1 : 1 mixture of penicillin N and 3-deuteriopenicillin N both substrates were converted at the same rate; these results accord with a stepwise process for hydrogen removal during the ring expansion in which the methyl hydrogen is first removed.

Enzymic ring expansion of penicillin N (**1a**) to deacetoxycephalosporin C (DAOC) (**2a**) occurs with loss of two hydrogen atoms, from the 2β-methyl group[1] (H*) and the C-3 position (H■) (Scheme 1). At present it is unknown whether these events occur in a stepwise or a concerted manner. Experiments, *in intact cell* systems, with L-valine bearing a chiral methyl group at the 3-pro-*R* site, as precursor gave, *via* the corresponding penicillin N, cephalosporin C (**2b**) with equal



Scheme 1

Table 1. Enzymic conversion of (**1a**) + (**1b**).

Expt.	% Conversion of penicillin Ns	Relative intensities of molecular ions (MH^+)[a] for (**3a**)/(**3b**) *m/z* 459	460	461	462	463	464	465	466	467	468	469	Ratio (**3a**) : (**3b**)
1	0	1	94	23	9	2	0	2	100	25	10	2	0.94 : 1
	30	1	50	12	5	2	0	2	100	25	10	2	0.50 : 1
2	0	2	100	26	10	6	2	3	100	26	11	3	1.00 : 1
	40	0	51	14	7	4	3	2	100	28	10	1	0.51 : 1
	60	8	41	16	8	7	7	8	100	30	20	10	0.41 : 1[b]

[a] Analysis by ammonia desorption chemical ionisation (error ± 2%). [b] This value represents a *maximum* ratio which is artificially increased by a high noise level resulting from low penicillin recovery.

Table 2. Enzymic conversion of (**1a**) + (**1c**).

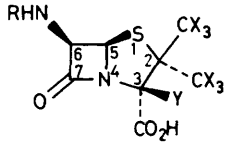
Expt.	% Conversion of penicillin Ns	Relative intensities of molecular ions (MH^+)[a] for (**3a**)/(**3c**) *m/z* 459	460	461	462	463	464	465	Ratio (**3a**) : (**3c**)
1	0	1	78	100	27	9	2	0	0.95 : 1
	30	3	78	100	27	10	2	0	0.95 : 1
	65	3	76	100	27	10	2	0	0.93 : 1
2	0	1	96	100	28	10	2	0	1.24 : 1
	30		94	100	28	9	0		1.22 : 1
	80	6	93	100	29	11	2		1.20 : 1

[a,b] See Table 1.

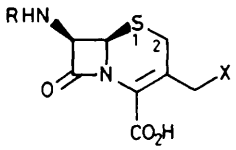
tritium content at both C-2α and C-2β sites.[2,3] Utilising a partially purified sample of deacetoxycephalosporin C/deacetylcephalosporin C (DAOC/DAC) synthetase activities† from *Cephalosporium acremonium* CO 728, which requires Fe^{2+}, ascorbate, and α-ketoglutarate for activity, we have examined the deuterium isotope effect on the conversions in Scheme 1 with the following results.

A 1 : 1 mixture of non-deuteriated (**1a**) and hexadeuteriated (**1b**)‡ penicillin N was enzymically transformed§ into a mixture of DAOC (**2a**) and DAC (**2c**) along with unchanged penicillin Ns (**1a** and **b**). After purification (h.p.l.c.; reverse phase octadecylsilane, 25mM-ammonium hydrogen carbonate buffer) these penicillins were derivatised [*N*-ethoxycarbonyl dimethyl esters (**3**)][5] and their deuterium content was determined by mass spectrometry (Table 1). As can be seen in these two experiments, faster transformation of the unlabelled penicillin N (**1a**) was obtained, leading to an enrichment of the deuteriated species (**1b**) in the penicillin N pool, as a result of an isotope effect.¶ In the presence of boiled enzyme no change in the ratio (**1a**) : (**1b**) was observed over the same time period. In contrast to this result, when a 1 : 1 mixture of non-deuteriated penicillin N (**1a**) and [3-^{2}H]penicillin N (**1c**)‡ was similarly transformed then *no* discrimination was observed (Table 2), both being consumed at the same rate.

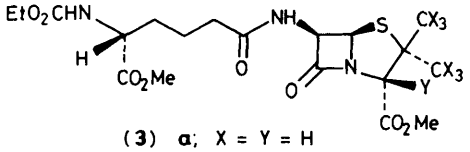
The expression of an isotope effect, as a discrimination, in competitive mixed-label experiments is a V_{max}/K_m effect[6] ($V_{max.}$ = max. velocity, K_m = Michaelis constant) and reflects events up to and including the first irreversible step. As no such isotope effect was observed when the deuterium was at C-3 [*i.e.* (**1c**)], and since the presence of this deuterium causes a marked changed in the product composition, with the formation of (**4**) as a major enzymic product,[7] then together these results are most easily explained if the enzymic reaction proceeds in *two steps* and the loss of the 2-methyl hydrogen



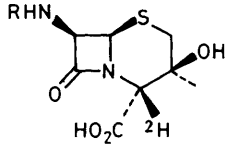
(**1**) **a**; X = Y = H
b; X = ^{2}H, Y = H
c; X = H, Y = ^{2}H



(**2**) **a**; X = H
b; X = OAc
c; X = OH



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



(**4**)

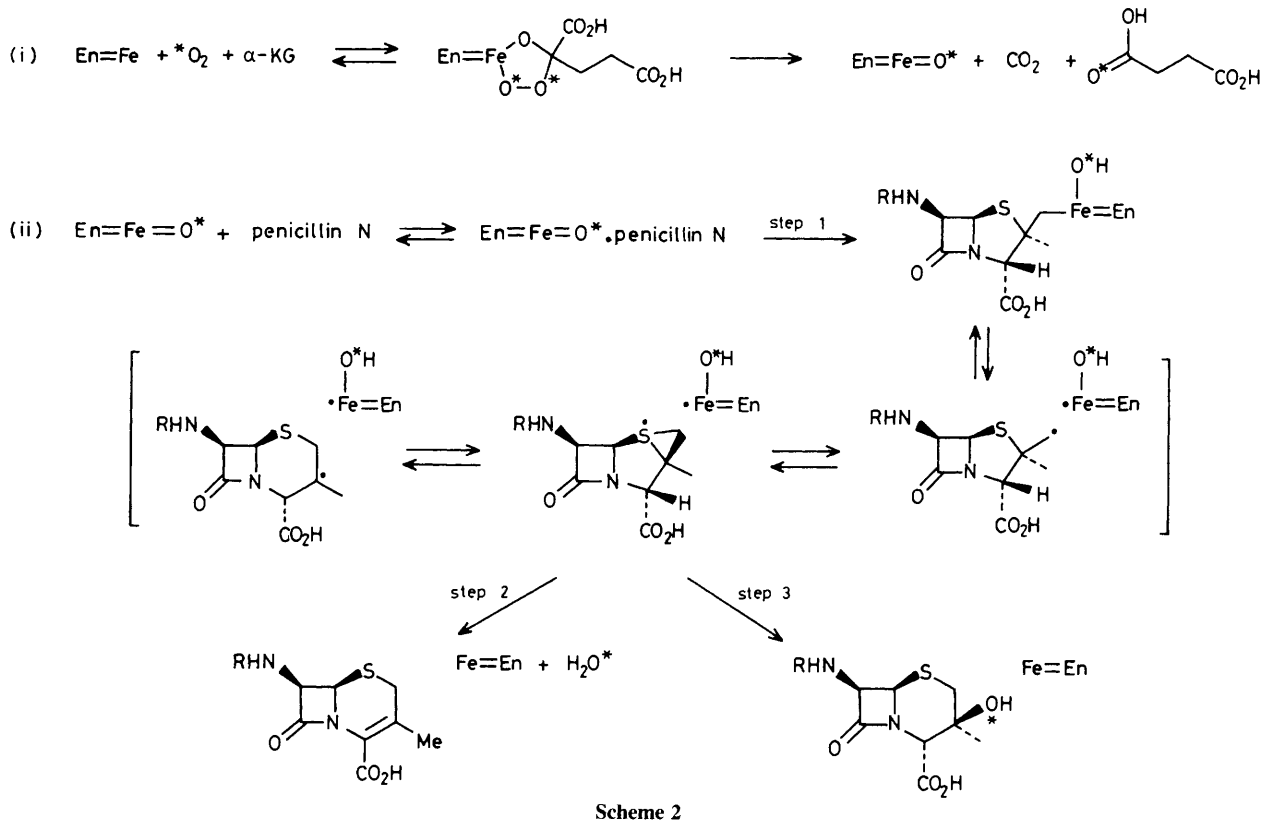
precedes the loss of the C-3 hydrogen. Presumably the entity responsible for hydrogen removal results from the interaction of Fe^{2+}, dioxygen, and α-ketoglutarate as has been suggested for proline hydroxylase.[8] A formal representation of these events is shown in Scheme 2, where the intermediate lying between the two C–H bond-breaking steps, as an equilibrating set of radicals, is derived by initial oxidative addition to an enzyme-iron oxo species.[9] The chemical ring expansion of such a radical has recently been confirmed in a penicillin system.[10] The intermediacy of such a radical formed at the 2β-methyl group of penicillin N provides an explanation of the loss of chiral methyl stereochemistry observed in this transformation.[2,3] Furthermore, as the isotopically sensitive branching pathways (steps 2 and 3, Scheme 2), emanate from this intermediate radical, then no $V_{max.}/K_m$ isotope effect at the C-3 hydrogen in (**1c**) would be expected, as these steps follow step 1, in which the $V_{max.}/K_m$ isotope effect was expressed. Instead the isotope effect of the deuterium at C-3 in (**1c**) is expressed as a change in the relative flux through the

† This enzymic activity, associated with a protein mol. wt. of 41 000, contains both DAOC and DAC synthetase activities (Scheme 1). We have not been able to separate these two activities: J. E. Baldwin, R. M. Adlington, J. B. Coates, M. J. C. Crabbe, N. P. Crouch, J. W. Keeping, G. C. Knight, C. J. Schofield, H-H. Ting, C. A. Vallejo, M. Thorniley, and E. P. Abraham, *Biochem. J.*, 1987, **245**, 831; see also A. Scheidegger, M. T. Küenzi, and J. Nüesch, *J. Antibiot.*, 1984, **37**, 522.

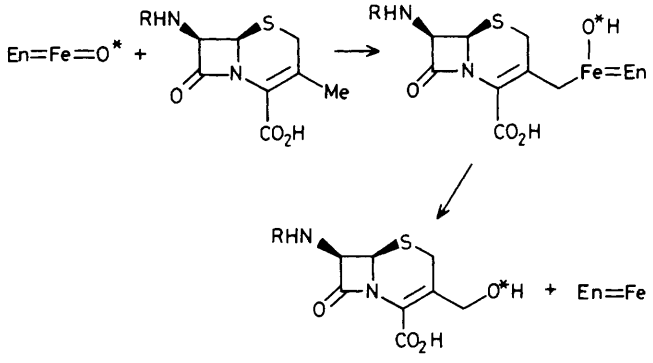
‡ The deuteriated penicillins (**1b** and **c**) were prepared from [(*R*)-5-amino-5-carboxypentanoyl]-L-cysteinyl-D-[4,4′-$^2H_6$]valine and -D-[2-$^2H_1$]valine, respectively by enzymic synthesis utilising isopenicillin N synthetase; see ref. 4.

§ For a representative procedure, see preceding paper.

¶ This same discrimination was also found in the observed cephalosporin products, *i.e.* a relative depletion of deuterium in (**2a**) and (**2c**).



Scheme 2



Scheme 3

steps 2 *versus* 3. Finally the observed incorporation into (**4**) of the label from dioxygen[7] follows naturally from Scheme 2. It also follows from the observation of the $V_{max.}/K_m$ isotope effect observed from (**1b**) that the presumably irreversibly formed active oxidising species (as En=Fe=O) is long-lived with respect to the first penicillin *N*-enzyme complex. If the second oxidation, *i.e.* the hydroxylation to (**2c**), occurs at the same active site then this step could be accommodated in the same mechanistic picture (Scheme 3). Here, the Fe-carbon species cannot obtain radical stabilisation from the ring sulphur, as in the ring-expansion step, and rapidly collapses with retention of stereochemistry to the hydroxylated product.[11]

We thank Eli Lilly, Indianapolis, U.S.A. and the S.E.R.C. (to N.P.C.) for support.

Received, 1st June 1987; Com. 750

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