Cephalosporin C Biosynthesis; a Branched Pathway Sensitive to a Kinetic Isotope Effect

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Incubation of [3-^{2}H] penicillin N with preparations of deacetoxycephalosporin C/deacetylcephalosporin C synthetase activity from *Cephalosporium acremonium* CO 728 gave, along with the normal product deacetoxycephalosporin C, another β-lactam metabolite, namely 7β-[(*R*)-5-amino-5-carboxypentanoyl]-3β-hydroxy-3α-methyl[4-^{2}H)-cepham-4α-carboxylic acid. This material arises as a result of a deuterium isotope effect on a branched pathway in the enzymic mechanism. The 3β-hydroxy group in this substance arises from molecular oxygen.

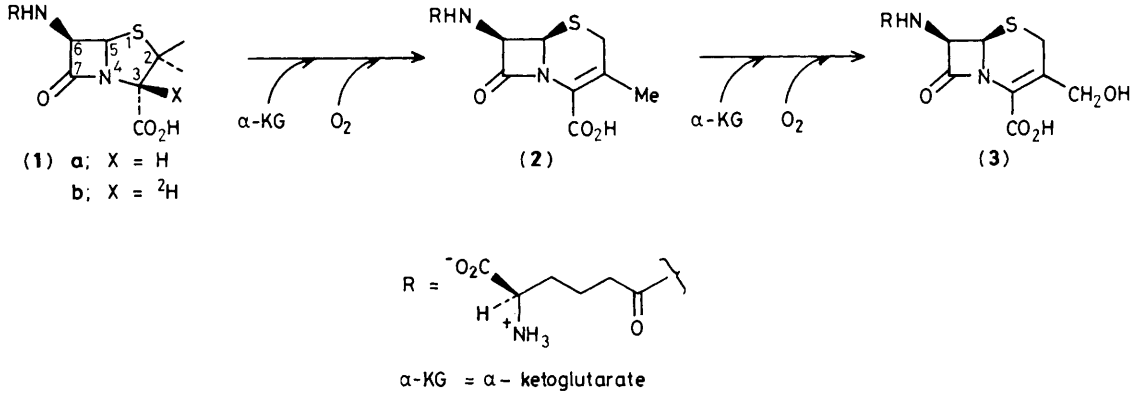
Although the conversion of penicillin N (**1**) into deacetoxycephalosporin C (DAOC) (**2**) and deacetylcephalosporin C (DAC) (**3**) by a cell-free extract from *Cephalosporium acremonium* was first rigorously proven in 1980,[1] no intermediates in this process have been subsequently revealed (Scheme 1). Both sequential steps consume dioxygen and α-ketoglutarate, and the latter is converted into succinate and carbon dioxide. The isolation of 7β-[(*R*)-5-amino-5-carboxypentanoyl]-3β-hydroxy-3α-methylcepham-4α-carboxylic acid (**4a**)† from a filtered broth of *C. acremonium* led to early mechanistic speculation. The involvement of an episulpho-

† We have previously referred to the (*R*)-5-amino-5-carboxypentanoyl group as δ-(D-α-aminoadipoyl).

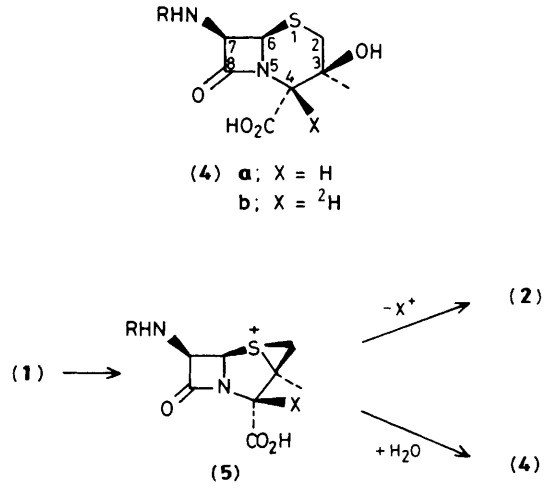
Table 1. Incubation experiments

Expt.	Conditions		DAC(3)[a] Products (*m/z*; relative intensity) (6)(*MH*)$^+$						Fragment (7)						3β-hydroxycepham (**4b**)[b] (**4**)(*MH*)$^+$					
1	(**1b**); $H_2{}^{16}O$; $^{18}O_2$	*m/z*	355	356	357	358	359	360	155	156	157	158	159	160	376	377	378	379	380	381
		Found (%)	4	100	25	72	27	22	4	100	14	70	10	6	29	80	53	100	38	8
2	(**1b**); $H_2{}^{16}O$; $^{18}O_2$	*m/z*							155	156	157	158	159	160	376	377	378	379	380	381
		Found (%)							4	100	13	54	7	6		69	19	100	23	15
		m/z													375	376	377	378	379	380
		Found (%)[c]													7	100	22	10	1	
		Calc. (%)[d]														100	18	7	1	

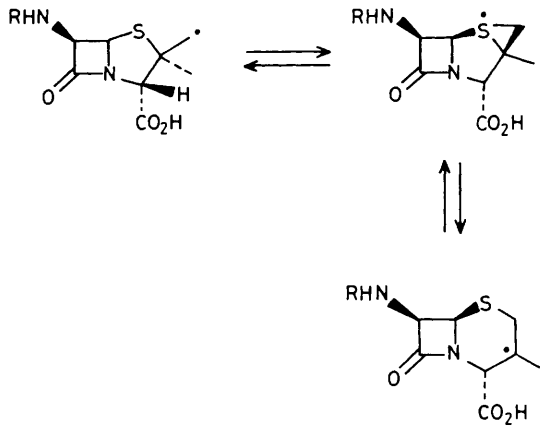
[a] DAC (**3**) was treated with formic acid to give the lactone (**6**) prior to mass spectral analysis. Samples were then run under positive ion thermospray h.p.l.c. mass spectrometry conditions, using a reverse phase octadecylsilane column with 0.05M-ammonium acetate containing 1% acetonitrile adjusted to pH 5 with formic acid as eluant. [b] Samples evaporated on stage from water, pre-protonated with 5% methanolic oxalic acid, then run under positive argon fast atom bombardment using glycerol as matrix. [c] For synthetic sample of (**4a**). [d] Calculated for $C_{14}H_{22}N_3O_7S$



Scheme 1



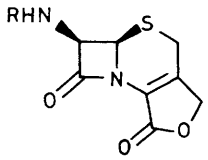
Scheme 2



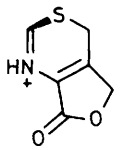
Scheme 3

nium ion (**5**) was suggested, which could directly collapse *via* proton loss to (**2**), or alternatively be intercepted by water to give (**4a**), (Scheme 2).[2] More recently, chemical modelling of the ring-expansion step has led to the suggestion that an equilibrating free radical (Scheme 3) could equally well explain this reaction,[3] but clearly heterolytic trapping by water of such a radical to give (**4a**) would be unreasonable. We now report the results of using [3-^{2}H]penicillin N (**1b**) on the course of the enzymic reaction.

Initially the product composition of an incubation of penicillin N (**1a**) with partially purified DAOC/DAC synthe-



(6)

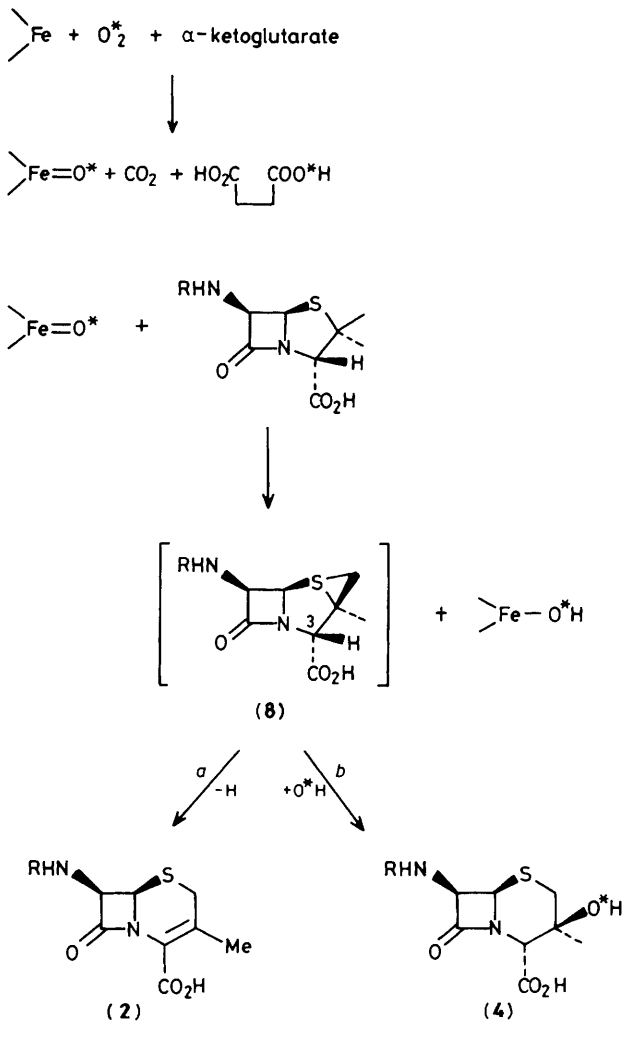


(7)

tase from *C. acremonium* CO 728‡ and standard co-factors§ was examined by 500 MHz n.m.r. and shown to contain three products [(**2**) : (**3**) : (**4a**) 40 : 20 : 1]. Repeating the incubation with [3-^{2}H]penicillin N (**1b**)¶ gave the same three products *but in substantially different ratio*, [(**2**) : (**3**) : (**4b**) 40 : 25 : 35].

The structure of (**4b**), purified by h.p.l.c. [reverse phase octadecylsilane column; 25 mM-NH_4HCO_3 as eluant], was determined from its spectral data (consistent with literature values)[2] and by chemical synthesis[5] of (**4a**). For (**4b**) δ_H (500 MHz; D_2O, ref. sodium 3-trimethylsilyl [2,2,3,3-$^2H_4$] propanoate) 1.38(3H, s, Me), 1.65—1.80 and 1.85—1.95(4H, 2 × m, $(CH_2)_2CH_2CO$), 2.42(2H, *ca.* t, *J* 7.5 Hz, CH_2CO), 2.64 and 3.54(2H, ABq, *J* 14 Hz, 2-H), 3.6—3.7(1H, m, $CH[CH_2]_3$), and 5.29 and 5.44 (2 × 1H, 2 × d, *J* 4 Hz, 6-H, 7-H); *m/z* (positive argon fast atom bombardment) 377 (MH^+); no antibacterial activity towards *Staphylococcus aureus* N.C.T.C. 6571 or *Escherichia coli ESS* at a concentration of 100 μg ml^{-1} (sample size 100 μl). The hydroxycepham (**4b**) was shown *not* to be a substrate for cephem formation with DAOC/DAC synthetase in separate experiments.

Secondly, we examined the origin of the 3β-hydroxy function of (**4**). Thus incubation of (**1b**) under a closed atmosphere of $^{18}O_2$ gas (99%) gave both labelled 3β-hydroxycepham (**4b**) and DAC (**3**), which were purified by h.p.l.c. Lactonisation of (**3**) to (**6**) (formic acid) gave a sample suitable for mass spectral analysis. This technique revealed ^{18}O incorporation into both (**3**)[6] and the 3β-hydroxycepham (**4b**)** (Table 1). A similar analysis of the co-produced DAOC (**2**) from the $^{18}O_2$ experiment revealed *no* label incorporation.



Scheme 4

These experiments require that the conversion of penicillin N (**1a**) into (**2**) proceeds *via* a branched pathway through an intermediate which provides (**2**) as well as (**4**).†† With unlabelled penicillin N (**1a**) the ratio (**2**) + (**3**) : (**4**) is 60 : 1, so that (**4**) is a minor product of the ring-expansion step, explaining its low concentration relative to cephalosporin C in normal fermentations.[2] However the operation of a deuterium isotope effect on the breakage of the C(3)–H bond (penicillin numbering) substantially shifts the above ratio to *ca.* 2 : 1. Additionally, the hydroxy-containing product (**4**) is formed by the specific incorporation of oxygen from *dioxygen*. All these facts may be accommodated by a mechanism (Scheme 4), in which a bridged species such as (**8**), *either* the cation *or* the radical, can decompose by loss of hydrogen at C-3 to the 'normal' product (**2**) (path *a*) or by interception of the bridged species by a specific hydroxy group derived from the α-ketoglutarate–penicillin coupled reduction of dioxygen, which produced (**8**) (path *b*). Those processes emanating from the bridged cation would be heterolytic whereas the bridged radical would proceed through homolytic reactions.

‡ This preparation was shown to contain both DAOC synthetase and DAC synthetase activities by its ability, in separate experiments to convert both (**1a**) into (**2**) and (**2**) into (**3**); see 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.

§ Partially purified DAOC/DAC synthetase (2 ml; *ca.* 0.5 International Units) in Tris–HCl buffer (pH 7.4; 50 mM) was pre-incubated for 5 min at 27 °C and 250 rev. min^{-1} with 200 μl of co-factor solution prepared from α-ketoglutarate (14.6 mg), L-ascorbate (17.6 mg), dithiothreitol (30.8 mg), iron (II) sulphate (1.4 mg), and ammonium sulphate (1.32 g) in distilled water (10 ml). The substrate (**1a**)/(**1b**) (1 mg) in Tris–HCl (pH 7.4; 1.8 ml; 50 mM) was added and the pH adjusted to 7.4 (NaOH). The resulting solution was incubated at 27 °C, and 250 rev. min^{-1} for 2 h, after which the protein was precipitated by the addition of acetone to 70% v/v. After centrifugation (10 000 rev. min^{-1}; 2 min; 0 °C) the supernatant was evaporated to dryness and the residue dissolved in D_2O (0.5 ml) and examined by n.m.r. (500 MHz; D_2O; *H*OD suppressed).

¶ Prepared from [(*R*)-5-amino-5-carboxypentanoyl]-L-cysteinyl-D-[2-^{2}H] valine by enzymic synthesis with isopenicillin N synthetase; see ref. 4. The level of deuteriation of C-3 was estimated to be > 98% by ^{1}H 500 MHz n.m.r. and mass spectral analysis.

** Less than a quantitative incorporation of ^{18}O was expected as complete degassing of the enzyme solution (prepared in normal air) could not be achieved without extensive enzymic degradation.

†† Mixtures of (**1a**) and (**1b**) are converted into (**2**), (**3**), and (**4**) with *no* isotopic enrichment in the pool of either (**1a**) or (**1b**). Thus the isotope effect responsible for the changing ratio of (**2**) + (**3**) to (**4**) must occur subsequent to an irreversible step on a single enzyme, at a branching point in the reaction (see following paper).

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References

1 J. E. Baldwin, P. D. Singh, M. Yoshida, Y. Sawada, and A. L. Demain, *Biochem. J.*, 1980, **186**, 889.
2 R. D. Miller, L. L. Huckstep, J. P. McDermott, S. W. Queener, S. Kukolja, D. O. Spry, T. K. Elzey, S. M. Lawrence, and N. Neuss. *J. Antibiotics*, 1981, **34**, 984.
3 J. E. Baldwin, R. M. Adlington, T. W. Kang, E. Lee, and C. J. Schofield, *J. Chem. Soc., Chem. Commun.*, 1987, 104. Note that a bridged radical has also been postulated to explain the photolytic ring closures of *seco*-penicillins; *cf.* E. M. Gordon and C. M. Cimarusti, *Tetrahedron Lett.*, 1977, 3425.
4 J. E. Baldwin, E. P. Abraham, R. M. Adlington, G. A. Bahadur, B. Chakravarti, B. P. Domayne-Hayman, L. D. Field, S. L. Flitsch, G. S. Jayatilake, A. Spakovskis, H.-H. Ting, N. J. Turner, R. L. White, and J. J. Usher, *J. Chem. Soc., Chem. Commun.*, 1984, 1225.
5 Synthesised by methods analogous to those described; see D. O. Spry, R. D. Miller, L. L. Huckstep, N. Neuss, and S. Kukolja, *J. Antibiotics*, 1981, **34**, 1078.
6 The role of molecular oxygen as the source of the C-3 methylene-linked hydroxy group of (**3**) has been observed in intact cell experiments: C. M. Stevens, E. P. Abraham, F.-C. Huang, and C. J. Sih, reported at the annual meeting of the Federation of American Society for Experimental Biology and Medicine (American Society for Biological Chemistry), April 1975.