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

Jack E. Baldwin,* Robert M. Adlington, Robin T. Aplin, Nicholas P. Crouch, Graham Knight, and Christopher J. Schofield

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

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).

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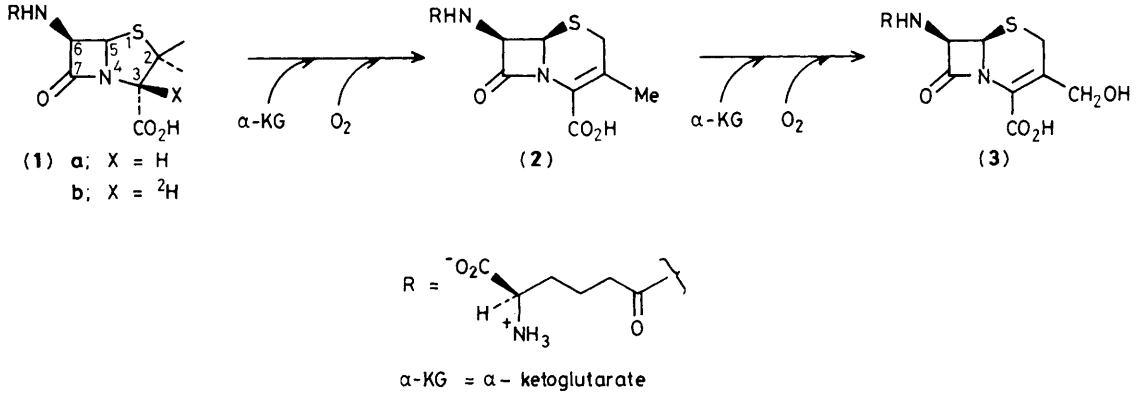
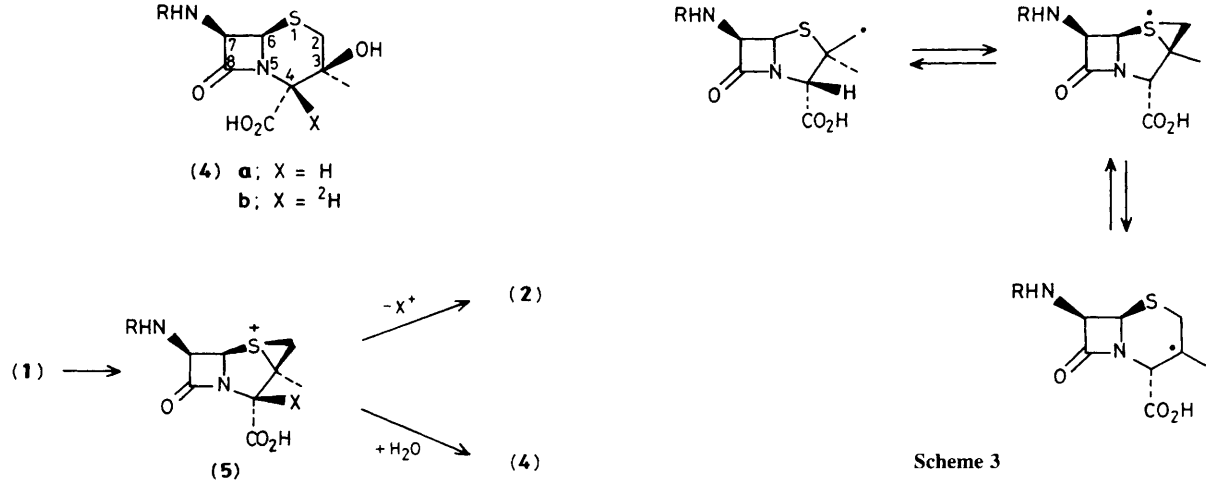
Table 1. Incubation experiments

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-

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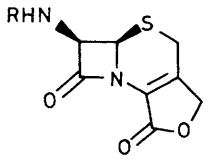
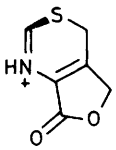


(6)

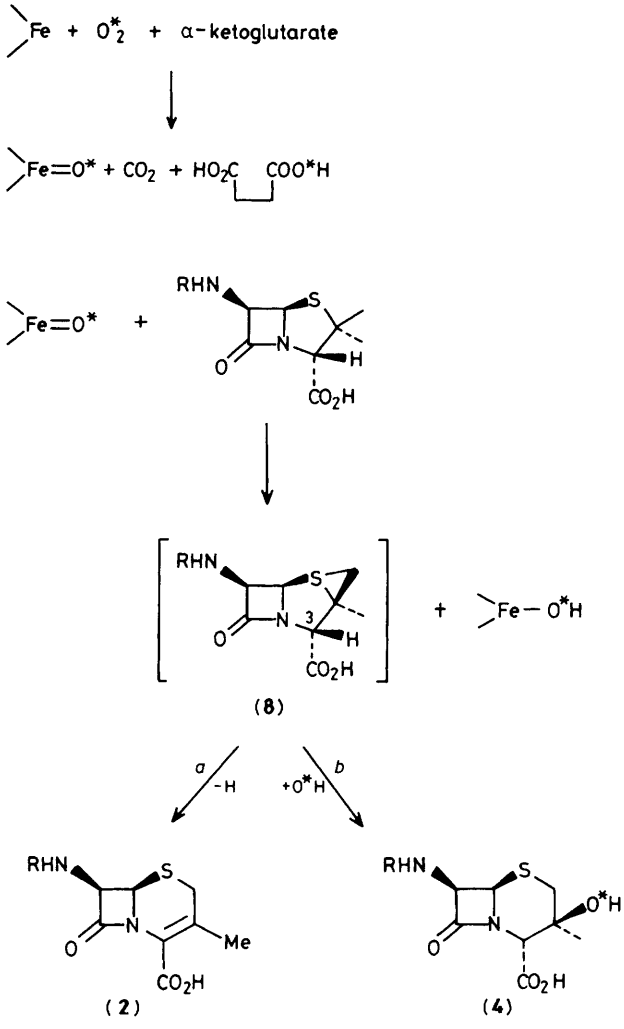


(7)

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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