Studies on the Mechanism of Deacetoxy–Deacetylcephalosporin C Synthase using Cyclopropyl Substituted Cephalosporin Probes

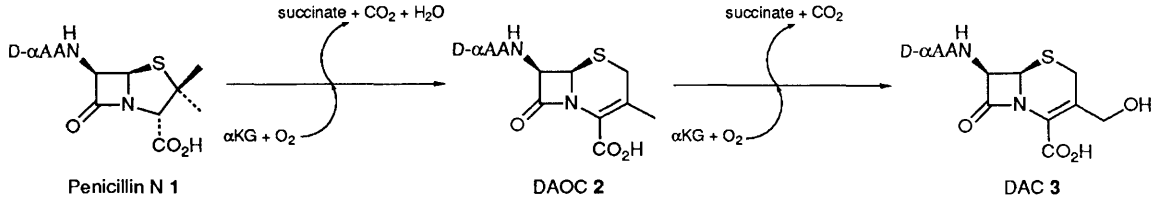
Jack E. Baldwin,* Robert M. Adlington, Nicholas P. Crouch, John W. Keeping, Simon W. Leppard, Janos Pitlik, Christopher J. Schofield, Wendy J. Sobey and Mark E. Wood

The Dyson Perrins Laboratory and the Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, UK

Cyclopropyl substituted cephalosporin analogues were prepared and evaluated as substrates for the Fe^{II}/α-ketoglutarate dependent oxygenase, DAOC–DAC synthase; the first example of catalytic product formation from a cyclopropyl ring cleavage pathway by an α-ketoglutarate dependent oxygenase is reported.

The key steps in the biosynthesis of the cephalosporins are the oxidative ring-expansion of penicillin N **1** to deacetoxycephalosporin C (DAOC, **2**) and the subsequent hydroxylation of the latter to deacetylcephalosporin C (DAC, **3**) (Scheme 1).[1] In *Cephalosporium acremonium* these transformations are mediated by a single bifunctional enzyme,[2] whereas in *Streptomyces clavuligerus*, the ring-expansion and hydroxylation steps are largely catalysed by separate enzymes.[3] All three enzymes are Fe^{II} and α-ketoglutarate dependent oxygenases which have been cloned and expressed in *Escherichia coli*.[4–6] Using labelled substrates, DAOC–DAC synthase from *C. acremonium* has been shown to catalyse the stoichiometric conversion of substrate (either **1** or **2**) and α-ketoglutarate with the concomitant production of CO_2 and succinate, the hydroxy group of **3** having been shown to be partially derived from dioxygen.[7,8] In addition, these experiments revealed the production of a minor 'shunt product' from the ring-expansion process; the 3β-hydroxy cepham **4**. Using valines stereospecifically labelled with H, D and T in either the *pro-R* or *pro-S* methyl groups, in intact cell experiments, incorporation of the *pro-R* methyl group into the dihydrothiazine ring of cephalosporin C was shown to occur with total loss of stereochemistry whereas the subsequent hydroxylation of DAOC **2** to DAC **3** proceeded with retention of stereochemistry.[9,10] This randomisation of stereochemistry in the ring-expansion process may be rationalised by the intermediacy of a methylene radicaloid species and we have demonstrated the chemical feasibility of a ring-expansion process involving such an intermediate.[11]

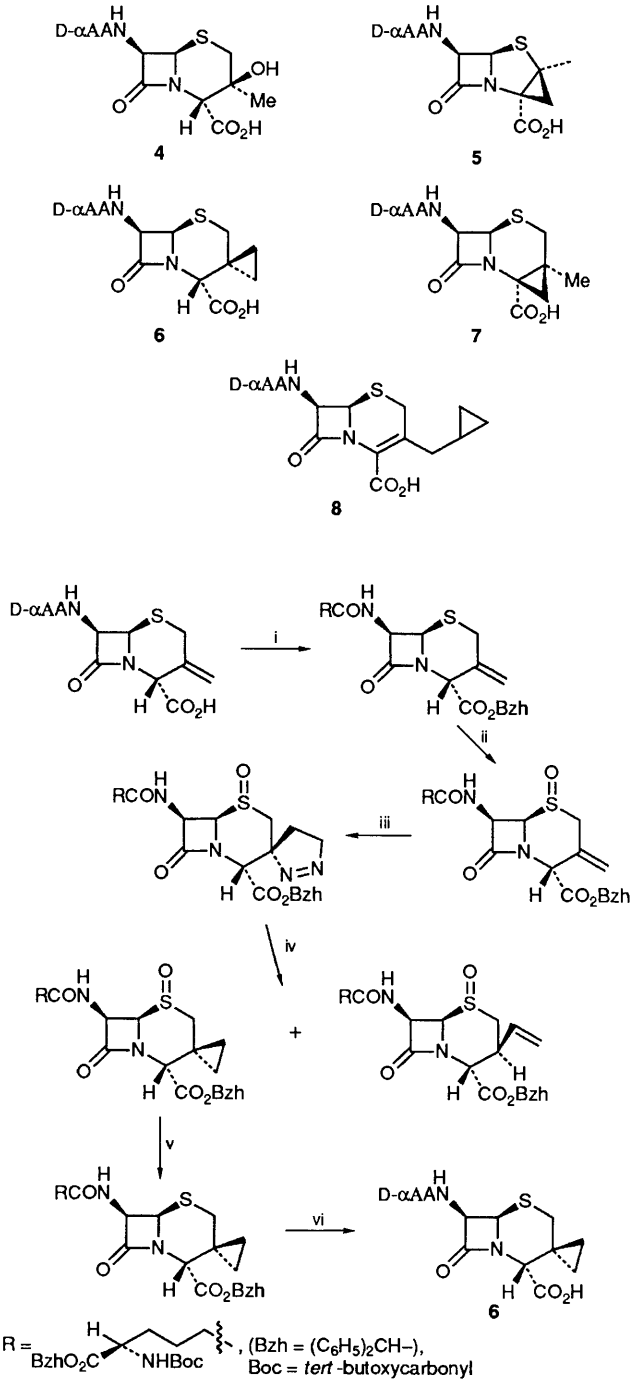
Mechanistic proposals for the α-ketoglutarate dependent oxygenases[12] have been largely based upon analogies with the haem Fe^{II} dependent P-450 cytochromes.[13] It is proposed that an enzyme bound ferryl-complex (Fe^{IV}=O) is capable of abstracting hydrogen to give an iron complex and a carbon



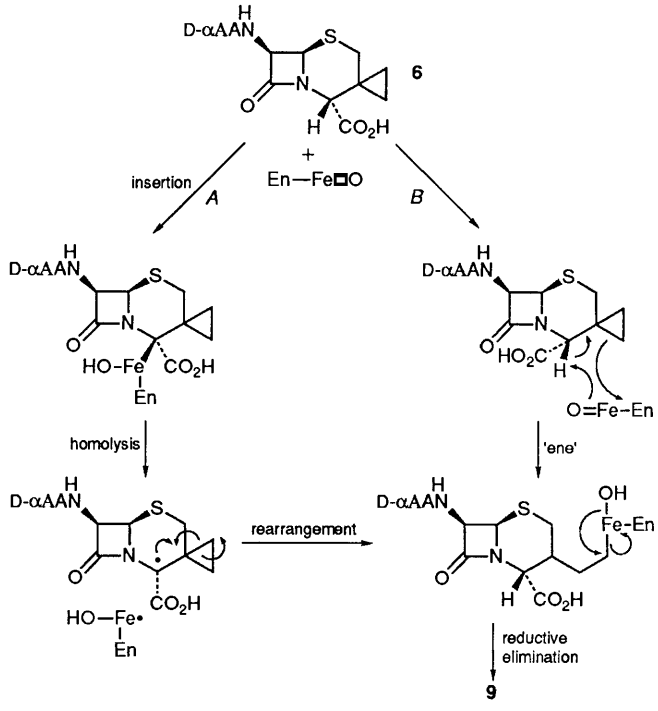
Scheme 1 αKG = α-ketoglutarate (α-oxoglutarate); D-αAA = D-δ-(α-aminoadipoyl) [(5*S*)-5-amino-5-carboxypentanoyl]

Table 1 Electrospray mass spectral data for **9a** and **9b**

Incubation conditions	(MH)$^+$						
1. $H_2{}^{16}O/{}^{16}O_2$ (air), product **9a**	*m/z*	388	389	390	391		
	(%)	100	20	17	5		
2. $H_2{}^{16}O/{}^{18}O_2$, product **9b**	*m/z*	388	389	390	391	392	393
	(%)	56	12	100	22	8	5



Scheme 2 *Reagents and conditions*: i, Di-*tert*-butyl dicarbonate then Ph_2CN_2; ii, *m*-chloroperbenzoic acid; iii, CH_2N_2, 5 °C; iv, xylene, heat; v, MeCOCl, KI, 0 °C; vi, CF_3CO_2H, anisole, room temperature



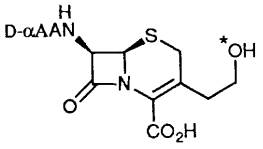
Scheme 3

radical, which can either recombine to give hydroxylated products (as in penicillin N **1** to the 3β-hydroxy cepham **4**, and DAOC **2** to DAC **3**) or rearrange and undergo elimination in a desaturative process (as in penicillin N **1** to DAOC **2**).[12] Analogues of substrates containing a cyclopropyl ring have been used successfully to probe the lifetime and mechanism of decomposition of transient radicals produced in reactions catalysed by the P-450 cytochromes[14] and isopenicillin N synthase.[1] Recent analogous approaches on the α-ketoglutarate dependent oxygenase, γ-butyrobetaine hydroxylase,[15,16] however, did not result in the isolation of any products resulting from turnover of the cyclopropanated analogues, although one apparently mechanism-based inhibitor was reported.[15] Previously, we have shown that the β-cyclopropyl penicillin **5** is a potent, reversible inhibitor of the ring-expansion of penicillin N **1**.[2] Herein, we report initial results of the incubation of further cyclopropyl containing analogues with DAOC–DAC synthase† and the first report of the formation of a product derived from the ring-opening of a cyclopropyl substrate in a reaction catalysed by this class of oxygenase.

Three further cyclopropyl containing substrates, **6**, **7** and **8**, were selected and synthesised‡ as mechanistic probes. Substrate **8** was synthesised *via* direct cyclopropanation of the appropriate alkene [CH_2N_2, $Pd(OAc)_2$], which was prepared by standard methodology.[17] For **6** and **7**, however, this was not synthetically viable and previously developed methodology

† The enzyme used in this study was recombinant material prepared essentially as previously described.[5,6]

‡ Full details will appear elsewhere.



9a $^{*}O = {}^{16}O$
b $^{*}O = {}^{16}O/{}^{18}O$

using the dipolar cycloaddition of diazomethane followed by thermal decomposition was utilised (*e.g.* Scheme 2).[18]

In the cases of **7** and **8**, it was disappointing to find that, under our assay conditions, we were unable to detect any new β-lactam products from their incubations with DAOC–DAC synthase, neither was there any evidence for time dependent inactivation of the enzyme relative to control experiments. Substrate **6**, however, was converted by DAOC–DAC synthase to a new product, which was isolated by reverse-phase HPLC (octadecylsilane, ammonium hydrogencarbonate) and identified as the alcohol **9a** on the basis of its ^{1}H 500 MHz NMR spectrum and mass spectrum. A repeat of the incubation under an atmosphere of $^{18}O_2$ gas, followed by mass spectral analysis, indicated a *ca.* 60% level of incorporation of ^{18}O into the product **9b** consistent with results previously obtained for molecular oxygen incorporation into DAC **3** and the 3β-hydroxy cepham **4**.[7,8] For **9b** δ_H (500 MHz; D_2O, ref. HOD δ 4.63) 1.51–1.67 and 1.67–1.83 [2 × 2H, 2 × m, $(CH_2)_2CH_2CO$], 2.27 [2H, *ca.* t, *J* 7 Hz, $(CH_2)_2CH_2CO$], 2.40–2.49 (2H, m, CH_2CH_2OH), 3.21 and 3.51 (2H, ABq, J_{AB} 18 Hz, S–CH_2 of dihydrothiazine ring), 3.53–3.61 [3H, complex, CH_2CH_2OH and $CH(CH_2)_3$], 4.97 and 5.46 (2H, ABq, J_{AB} 5 Hz, 6-*H*, 7-*H*), electrospray mass spectral data (see Table 1).

Compound **9** may arise by an (Fe^{IV}=O) insertion–homolysis process to form a radicaloid species, or its equivalent, at C-2 of **6** (Scheme 3*A*) as has been proposed in the case of similar ring openings catalysed by cytochrome-containing enzymes.[14] Alternatively, an 'ene' type reaction may occur as indicated in Scheme 3*B*. One possible way to distinguish between these two processes is a detailed examination of the stereospecificity of the cyclopropyl ring-opening and such studies are in progress.

In summary, we have demonstrated the first example of catalytic product formation from a cyclopropyl containing cephalosporin analogue by a non-haem, α-ketoglutarate, Fe^{II} dependent oxygenase enzyme, DAOC–DAC synthase.

We thank the SERC and Eli Lilly and Co., Indianapolis, for financial support of this work.

Received, 15th February 1991; Com. 1/00730K

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