

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

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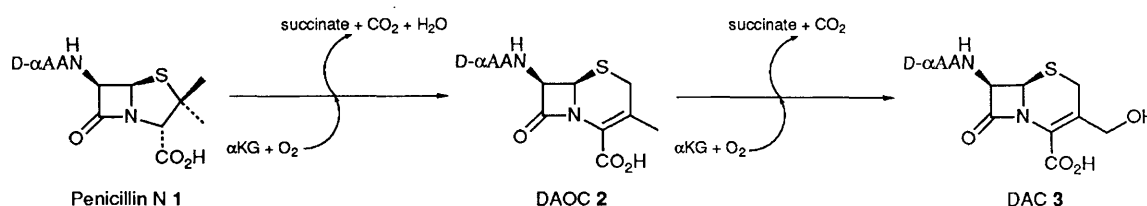
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Cyclopropyl substituted cephalosporin analogues were prepared and evaluated as substrates for the  $\text{Fe}^{\text{II}}$ / $\alpha$ -ketoglutarate dependent oxygenase, DAOC–DAC synthase; the first example of catalytic product formation from a cyclopropyl ring cleavage pathway by an  $\alpha$ -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).<sup>1</sup> In *Cephalosporium acremonium* these transformations are mediated by a single bifunctional enzyme,<sup>2</sup> whereas in *Streptomyces clavuligerus*, the ring-expansion and hydroxylation steps are largely catalysed by separate enzymes.<sup>3</sup> All three enzymes are  $\text{Fe}^{\text{II}}$  and  $\alpha$ -ketoglutarate dependent oxygenases which have been cloned and expressed in *Escherichia coli*.<sup>4–6</sup> Using labelled substrates, DAOC–DAC synthase from *C. acremonium* has been shown to catalyse the stoichiometric conversion of substrate (either **1** or **2**) and  $\alpha$ -ketoglutarate with the concomitant production of  $\text{CO}_2$  and succinate, the hydroxy group of **3** having been shown to be partially derived from dioxygen.<sup>7,8</sup> In addition, these experiments revealed the production of a minor 'shunt product' from

the ring-expansion process; the  $3\beta$ -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.<sup>9,10</sup> 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.<sup>11</sup>

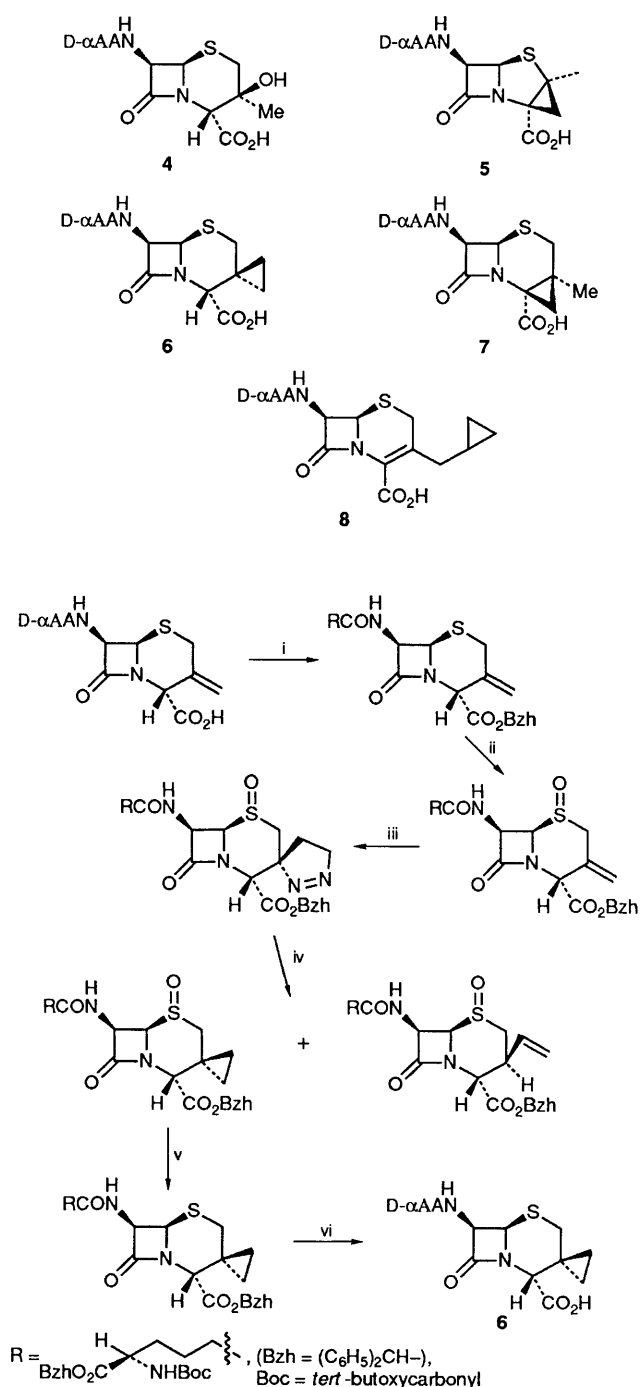
Mechanistic proposals for the  $\alpha$ -ketoglutarate dependent oxygenases<sup>12</sup> have been largely based upon analogies with the haem  $\text{Fe}^{\text{II}}$  dependent P-450 cytochromes.<sup>13</sup> It is proposed that an enzyme bound ferryl-complex ( $\text{Fe}^{\text{IV}}=\text{O}$ ) is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



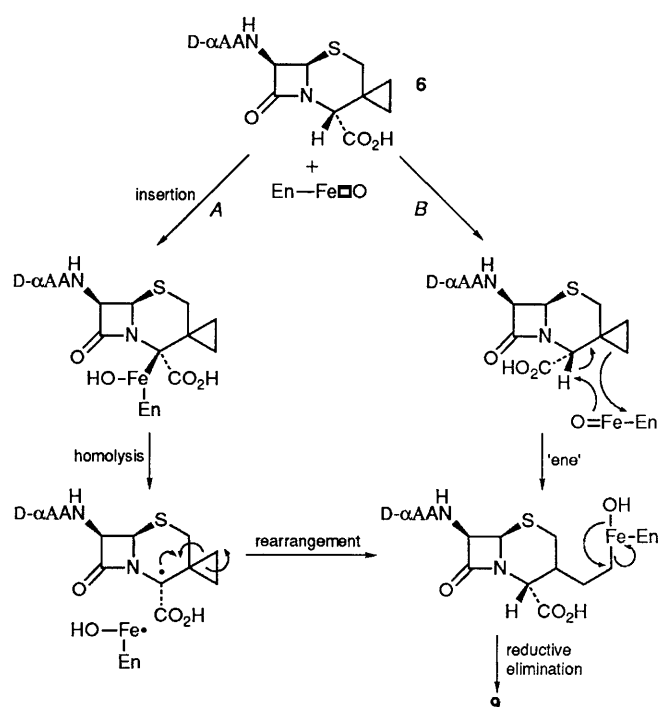
**Scheme 1**  $\alpha\text{KG}$  =  $\alpha$ -ketoglutarate ( $\alpha$ -oxoglutarate); D- $\alpha\text{AA}$  = D- $\delta$ -( $\alpha$ -aminoadipoyl) [(5S)-5-amino-5-carboxypentanoyl]

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

Incubation conditions	(MH) <sup>+</sup>						
1. H <sub>2</sub> <sup>16</sup> O/ <sup>16</sup> O <sub>2</sub> (air), product <b>9a</b>	<i>m/z</i>	388	389	390	391		
	(%)	100	20	17	5		
2. H <sub>2</sub> <sup>16</sup> O/ <sup>18</sup> O <sub>2</sub> , product <b>9b</b>	<i>m/z</i>	388	389	390	391	392	393
	(%)	56	12	100	22	8	5

**Scheme 2** Reagents and conditions: i, Di-*tert*-butyl dicarbonate then Ph<sub>2</sub>CN<sub>2</sub>; ii, *m*-chloroperbenzoic acid; iii, CH<sub>2</sub>N<sub>2</sub>, 5 °C; iv, xylene, heat; v, MeCOCl, KI, 0 °C; vi, CF<sub>3</sub>CO<sub>2</sub>H, anisole, room temperature

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

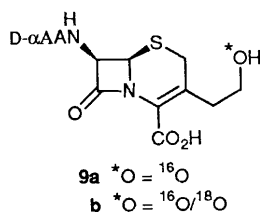
**Scheme 3**

desaturative process (as in penicillin N **1** to DAOC **2**).<sup>12</sup> 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<sup>14</sup> and isopenicillin N synthase.<sup>1</sup> Recent analogous approaches on the α-ketoglutarate dependent oxygenase, γ-butyrobetaine hydroxylase,<sup>15,16</sup> 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.<sup>15</sup> Previously, we have shown that the β-cyclopropyl penicillin **5** is a potent, reversible inhibitor of the ring-expansion of penicillin N **1**.<sup>2</sup> 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<sub>2</sub>N<sub>2</sub>, Pd(OAc)<sub>2</sub>], which was prepared by standard methodology.<sup>17</sup> 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.<sup>5,6</sup>

‡ Full details will appear elsewhere.



using the dipolar cycloaddition of diazomethane followed by thermal decomposition was utilised (*e.g.* Scheme 2).<sup>18</sup>

In the cases of **7** and **8**, it was disappointing to find that, under our assay conditions, we were unable to detect any new  $\beta$ -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  $^1\text{H}$  500 MHz NMR spectrum and mass spectrum. A repeat of the incubation under an atmosphere of  $^{18}\text{O}_2$  gas, followed by mass spectral analysis, indicated a *ca.* 60% level of incorporation of  $^{18}\text{O}$  into the product **9b** consistent with results previously obtained for molecular oxygen incorporation into DAC **3** and the  $3\beta$ -hydroxy cepham **4**.<sup>7,8</sup> For **9b**  $\delta_{\text{H}}$  (500 MHz;  $\text{D}_2\text{O}$ , ref. HOD  $\delta$  4.63) 1.51–1.67 and 1.67–1.83 [ $2 \times 2\text{H}$ ,  $2 \times \text{m}$ ,  $(\text{CH}_2)_2\text{CH}_2\text{CO}$ ], 2.27 [ $2\text{H}$ , *ca.* t,  $J$  7 Hz,  $(\text{CH}_2)_2\text{CH}_2\text{CO}$ ], 2.40–2.49 ( $2\text{H}$ , m,  $\text{CH}_2\text{CH}_2\text{OH}$ ), 3.21 and 3.51 ( $2\text{H}$ , ABq,  $J_{\text{AB}}$  18 Hz, S- $\text{CH}_2$  of dihydrothiazine ring), 3.53–3.61 [ $3\text{H}$ , complex,  $\text{CH}_2\text{CH}_2\text{OH}$  and  $\text{CH}(\text{CH}_2)_3$ ], 4.97 and 5.46 ( $2\text{H}$ , ABq,  $J_{\text{AB}}$  5 Hz, 6-*H*, 7-*H*), electrospray mass spectral data (see Table 1).

Compound **9** may arise by an ( $\text{Fe}^{\text{IV}}=\text{O}$ ) insertion–homolysis process to form a radicaloid species, or its equivalent, at C-2 of **6** (Scheme 3A) as has been proposed in the case of similar ring openings catalysed by cytochrome-containing enzymes.<sup>14</sup> Alternatively, an ‘ene’ type reaction may occur as indicated in Scheme 3B. 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,  $\alpha$ -ketoglutarate,  $\text{Fe}^{\text{II}}$  dependent oxygenase enzyme, DAOC–DAC synthase.

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